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Differentiation of disaccharide isomers *via* diagnostic-ion-weighted spectral library searching using data from tandem MS analysis of chloride adducts

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Rare sugars, comprising over 50 known saccharide isomers, have broad significance in food science, pharmaceuticals, and biomedical applications. However, the inherent structural complexity (due to isomerization) and low natural abundance of these saccharides pose substantial analytical challenges to their characterization. Herein, we developed a spectral library searching platform capable of distinguishing disaccharide isomers based on chloride-adducted (M + Cl^-) tandem MS (MS/MS) data. A set of unique diagnostic fragment ions was generated from each of the 15 disaccharide isomers, allowing the development of a spectral similarity algorithm that incorporates diagnostic-ion weighting to improve isomer differentiation. The resulting diagnostic-ion-weighted scores enabled discrimination among stereoisomers, linkage isomers, and compositional isomers. As a direct infusion MS/MS method, we tested spectral library searching for binary mixtures using both a traditional algorithm without diagnostic-ion weighting and our method based on diagnostic-ion weighting. With our platform incorporating diagnostic-ion weighting, false-positive assignments were reduced from 74 to 21, and true negatives increased from 555 to 608. Finally, we applied the method to analyze complex mixtures such as apple juice, Coca-Cola, and Rhinegeist beer. In all cases, the known disaccharide isomers were confirmed by our diagnostic-ion-weighted library-searching workflow. Collectively, these results suggest that negative-ion MS/MS data derived from conventional collision-induced dissociation can be a powerful method for saccharide isomer characterization, especially when paired with automated spectral library searching algorithms.

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Introduction

The current study aims to introduce an improved similarity-scoring algorithm based on direct-infusion tandem MS (MS/MS) analysis of disaccharide isomers ionized through chloride (Cl^-) adduction in the negative-ion mode. An improvement is observed because negative-ion mode MS/MS analyses of the selected rare sugars generated a set of diagnostic fragment ions, which are included in the algorithm in the form of a diagnostic-ion weighted computational strategy, resulting in superior prediction. The significance of the study is related to the fact that rare sugars, typically composed of low-abundance monosaccharides and their derivatives, have attracted increasing attention due to their distinct structures and enhanced biological functions.[1–3] With reduced sweetness and digestion, many rare sugars have proved to be promising alternatives to conventional sugars (*e.g.*, sucrose), with demonstrated benefits in mitigating obesity, type 2 diabetes, cardiovascular disease, and dental decay.[4–7] Although naturally present in low abundance, the use of industrial enzymatic and microbial processes has greatly reduced the production cost of rare sugars, making them more accessible for research and for application in food and medicine.[2,8] The purpose of the current study is to develop a direct-infusion MS method that can be used to effectively characterize small quantities of target rare sugars, especially during the initial synthetic method development, where it might not be necessary to prepare milligram-scale quantities of material for structure confirmation.

Despite the growing evidence demonstrating rare sugars to be important for human health, accurate characterization of individual rare sugars remains challenging. For example, rare disaccharides are structural isomers of sucrose differing only in linkage positions and stereochemical arrangements, and occur in low natural abundance.[8,9] Conventionally, nuclear magnetic resonance (NMR) has been used to distinguish such

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isomers,[10,11] but NMR requires high sample purity in large quantities and prolonged data acquisition times, making it less compatible with the high-throughput analysis required for the systematic synthesis of the rare sugars. Spectroscopic techniques, such as infrared and ultraviolet-visible spectroscopy, are rapid but generally lack the resolution and sensitivity needed for carbohydrate isomer differentiation.[12] In contrast, mass spectrometry (MS) offers high sensitivity, minimal sample preparation, and strong tolerance for complex mixtures. Nevertheless, MS alone often fails to resolve saccharide isomers due to identical precursor masses. Front-end separation techniques, such as high-performance liquid chromatography (HPLC), capillary electrophoresis, and ion mobility spectrometry, can aid with identification, but they also increase analytical complexity in terms of instrumentation requirements.[13–18] Gas-phase ion activation strategies, especially collision-induced dissociation (CID), are widely employed for isomer differentiation and are a standard feature on most MS platforms.[19,20] However, CID MS/MS is typically based on positive-ion mode analysis of sodium adducts, which yields a limited set of fragments, providing incomplete structural information for isomer differentiation.[21–23]

Unlike positive-ion mode sodium adducts, the negative-ion mode analysis of halide adducts of disaccharides has been shown to generate more abundant fragment ions based on both glycosidic and crossed-ring cleavages, yielding more detailed MS/MS spectra.[24–29] Previous reports demonstrated that chloride adducts $[M + Cl]^-$ generated by direct-infusion nano-electrospray ionization (nESI) produced diagnostic fragment ions under CID MS/MS, allowing the differentiation of five rare sugars, which were structural isomers of sucrose, without front-end separation.[3] This finding motivated the development of a spectral library searching approach,[30] which integrates the full MS/MS spectral features with diagnostic fragment ions derived from chloride adducts of disaccharides. The library-searching platform automatically identifies the disaccharide isomer, reducing challenges associated with manual interpretation.[31–34] The in-house spectral library was constructed using MS/MS spectra of fifteen (15) closely related disaccharide standards (Fig. 1) analyzed as chloride adducts $[M + Cl]^-$ in the negative-ion mode. These disaccharides were selected to represent a broad range of linkage, positional, and stereo/configurational isomers, providing extensive structural diversity for evaluating the accuracy and predictive power of our method. Within this framework, based on spectral library identification, we have developed a normalized dot-product scoring algorithm that integrates MS/MS spectral similarity while applying weights to targeted diagnostic fragment ions to enhance disaccharide characterization.[35–38] Cosine-similarity scoring algorithms have been widely applied in mass spectral library searching; however, most of the published works have focused on MS/MS data derived from the positive-ion mode analysis of either sodium adducts or protonated species.[31,39] The use of MS/MS data derived from the negative-ion mode

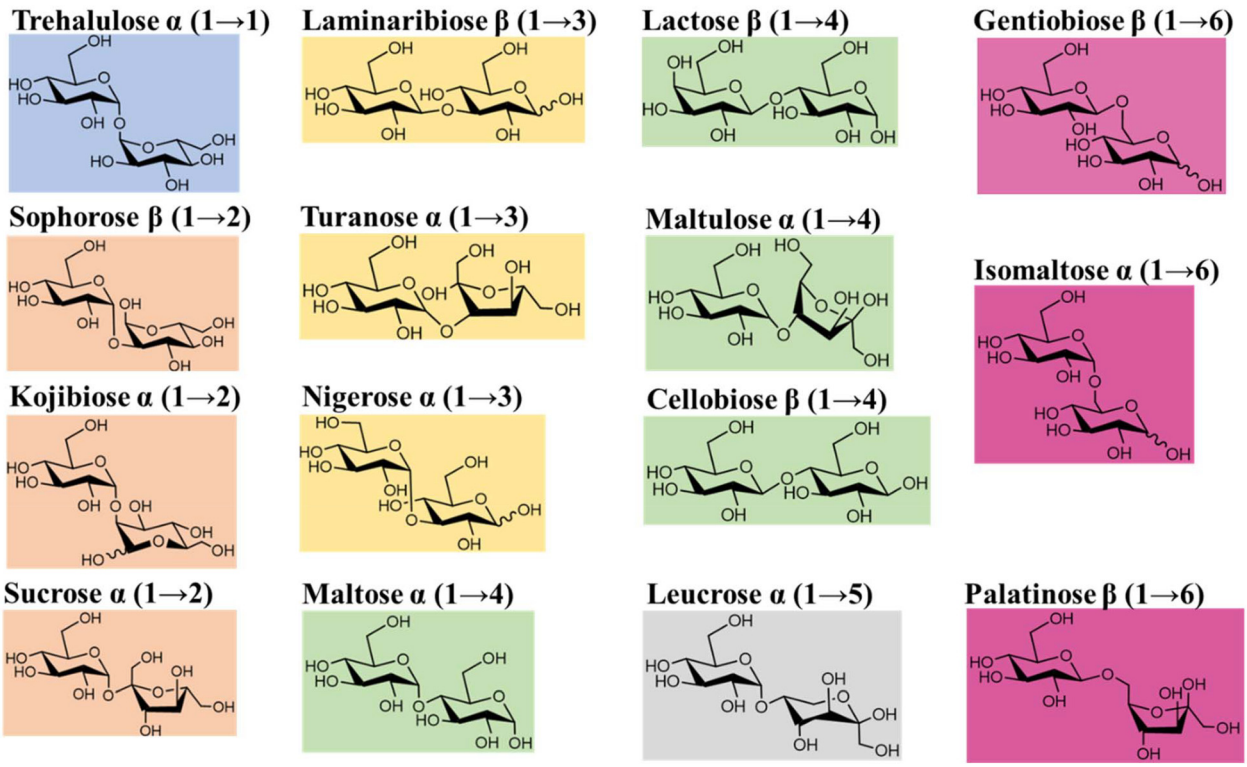

Fig. 1 Chemical structures of the disaccharide isomers studied, which include compositional, linkage, and configurational isomers. The molecular weight of disaccharide isomers is 342 Da.

analysis of chloride adducts for mass-spectral library searching is novel. Additionally, although our previous publication identified diagnostic fragment ions, isomer characterizations were done manually and were based only on a single diagnostic ion. With an increased number of isomers (*i.e.*, 15 disaccharide isomers in this study), a single diagnostic ion is insufficient for isomer differentiation. Therefore, we had to identify a new set of diagnostic ions that collectively form the distinguishing feature of a specific disaccharide isomer. Therefore, the cosine-similarity scoring algorithm described herein allows automatic isomer identification by evaluating the entire MS/MS spectrum, incorporating specific values of mass-to-charge (m/z) ratios and their relative intensities into the similarity calculation.

To validate the platform, we analyzed standard disaccharides in pure form and in binary mixtures using direct infusion nESI MS/MS. In each case, the workflow demonstrated high selectivity and accuracy in saccharide isomer identification. Further, the method was applied to analyze disaccharides in complex matrices, including apple extract, beer, and Coca-Cola samples. These samples contain known but diverse carbohydrate compositions with multiple disaccharide isomers, providing an effective means to evaluate the selectivity, sensitivity, and robustness of the method. Moreover, the spectral library can be continuously expanded with additional saccharide standards, enhancing its scalability and utility for future studies.[39]

Experimental section

Library and software development

Each MS/MS data file was originally acquired in Thermo Fisher's Xcalibur 2.2 SP1 software, raw format, using a Velos Pro ion trap mass spectrometer. The data were subsequently converted to mzML format using MZmine.[40] The mzML file contained 54 scans collected over one minute, with each scan containing a mass-to-charge (m/z) array and its corresponding signal intensity. The average m/z array and signal intensity across all scans were computed to generate a representative dataset, which was subsequently uploaded to MySQL (version 8.0.43) platform. The MySQL library also included annotated diagnostic fragment ion information for each disaccharide isomer (Fig. S1). An in-house Python code was established to compare the MS/MS spectra from the unknown sample against reference spectra stored in the MySQL database. The detailed code can be found in the GitHub repository (https://github.com/firstvx/Improved-Disaccharide-Isomer-Discrimination-via-Diagnostic-Ion-Weighted-Cl–Adduct-MS-MS/tree/main). This workflow utilized a spectral-similarity algorithm to generate numerical score values and produced mirror plots for compound annotations. A schematic overview of the entire workflow is presented in Fig. 2.

The numerical value is calculated based on a dot-product scoring algorithm that integrates MS/MS spectral similarity while applying weights to targeted diagnostic ions (Scheme 1).[35–38] Spectra that closely match library references and contain high-intensity diagnostic ions received elevated diagnostic-ion-weighted (S_D) scores, whereas spectra with poor matches or missing diagnostic ions yielded lower scores. This approach effectively addresses isomers that generate nearly indistinguishable fragmentation patterns but display subtle variations in relative ion intensities. The S_D algorithm quantifies spectral similarity on a normalized scale from 0 to 1, where a value of 0 indicates orthogonal (dissimilar) spectra and 1 indicates perfect alignment.[41–44] Such scoring provides an interpretable numerical value that improves confidence, reproducibility, and throughput in saccharide isomer identification.[45]

Noncontact nano-electrospray ionization setup

For each disaccharide, a 10 mM stock solution was prepared by dissolving 34.23 mg of the analyte in Milli-Q water. Then, 40 μM working solutions were prepared by appropriate dilution. For NH_4Cl, we first prepared a 1.0 M stock solution in water, which was subsequently diluted to 2 mM for analytical use. The final disaccharide samples were prepared at a concentration of 20 μM with 1 mM NH_4Cl in water, by mixing equal volumes of the 40 μM disaccharide working solution and the 2 mM NH_4Cl solution. For nano-electrospray ionization (nESI) MS analysis (Fig. 2A), 10 μL of the sample was loaded into a pulled glass capillary that was created in-house from a disposable borosilicate glass (I.D. 1.17 mm; O.D. 1.5 mm) using a micropipette puller (Model P-97, Sutter Instrument Co., Novato, CA, USA). The ionization process occurred *via* a noncontact nESI method, which eliminated the physical contact of the nESI metal (Ag) electrode with the analyte solution. The non-contact nESI approach reduced potential analyte oxidation and Joule heating, which can damage the glass tip, and improved analytical sensitivity.[46,47] A spray voltage of −1.5 kV was applied to the silver electrode to generate charged nanodroplets *via* an electrostatic induction mechanism.[48–50] The droplets underwent further solvent evaporation, assisted by a heated inlet capillary, at a temperature of 250 °C. The nESI glass capillary was positioned 5 mm in front of the mass spectrometer inlet. The charged nanodroplets contained the sugar–chloride adducts, which were directly infused into the mass spectrometer for analysis.

Mass spectrometry

All mass spectra were collected using a Thermo Fisher Scientific Velos Pro ion trap (San Jose, CA, USA). The MS parameters were set as follows, based on the previously reported parameters: three microscans and an ion injection time of 100 ms, with data acquired in centroid mode.[3] The precursor ion mass was selected at m/z 377 for chloride adducts in negative-ion mode. The isolation window was set at 1.0, with the CID setting at a collision energy of 30%, according to the manufacturer's instructions. Helium was used as the collisional gas for CID. All data were processed and analyzed by using the Xcalibur 2.2 SP1 software (Thermo Fisher Scientific).

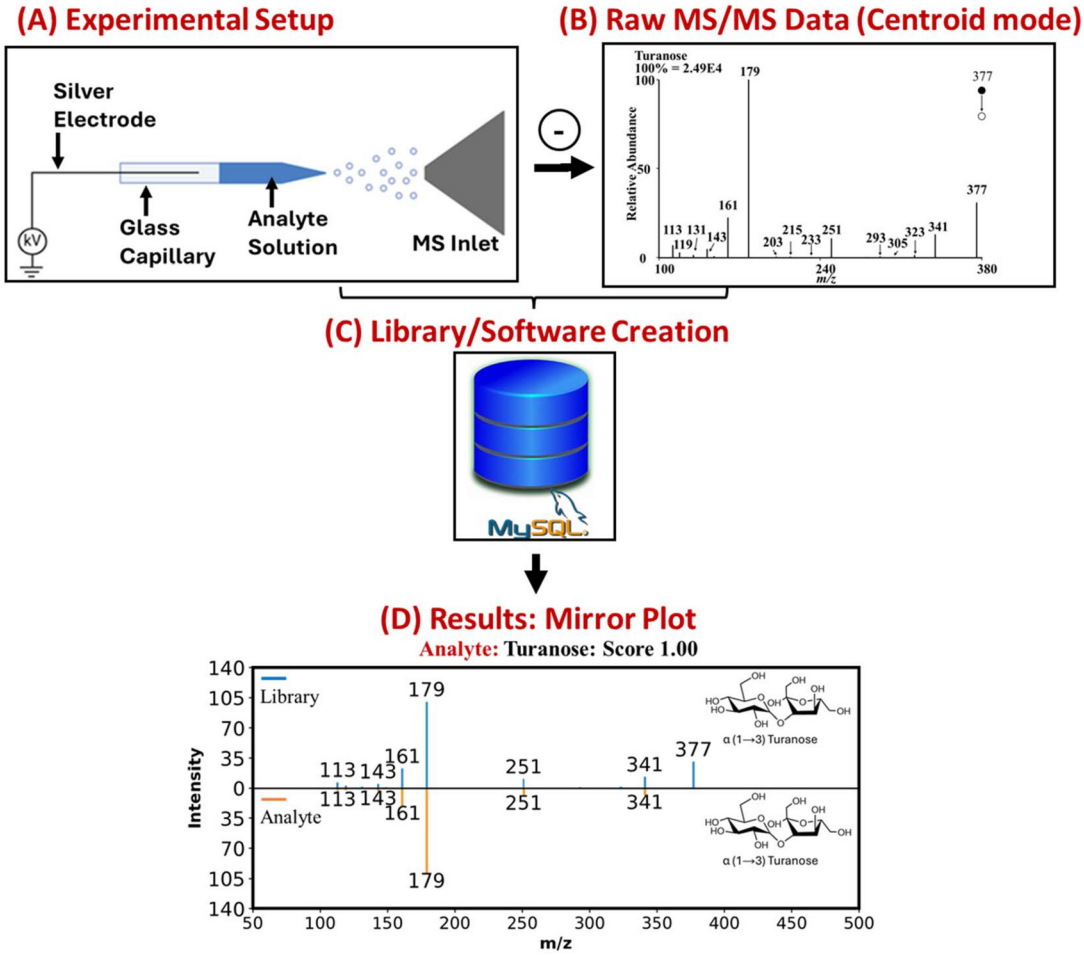

Fig. 2 Workflow of disaccharide identification based on (A) direct infusion nESI MS/MS analysis using −1.5 kV spray voltage, with (B) MS/MS data recorded in centroid mode. (C) MS/MS spectra were stored using the MySQL platform, with the search algorithm written using Python. (D) Mirror plot illustrating the output of the library searching.

$$Score = \left(\frac{A*B}{\|A\|*\|B\|}\right)(1+q) = \left(\frac{\sum_i a_i*b_i}{\sqrt{\sum_i a_i^2}\sqrt{\sum_i b_i^2}}\right)(1+q)$$

$q = 0.1$ Diagnostic ion match for top 28 most intense ions

$q = -0.3$ No diagnostic ion match for top 28 most intense ions

Scheme 1 Dot-product scoring algorithm integrating MS/MS spectral similarity with targeted diagnostic ions. A represents the library spectrum, whereas B represents the experimental spectrum. a_i and b_i denote the normalized intensities of the fragment ion m/z_i in the experimental and library spectra, respectively.

Chemicals and reagents

Sucrose (≥99.5%), turanose (≥98%), maltulose (≥99.3%), nigerose (≥90%), leucrose (≥98%), lactose (≥99%), laminaribiose (≥95%), palatinose (≥98%), kojibiose (≥98%), and sophorose (≥98%) were purchased from Sigma-Aldrich (St Louis, MO). Trehalulose (≥98%) was obtained from Biosynth International, Inc. (San Diego, CA). Maltose (≥98%), isomaltose (≥97%), and gentiobiose (≥96%) were purchased from TCI. Cellobiose (≥99%) was purchased from MP Biomedicals. HPLC-grade acetonitrile and ammonium chloride were purchased from Acros Organics (New Jersey, USA) and Sigma-Aldrich (St Louis, MO), respectively. All sugar powder and ammonium chloride were dissolved in Milli-Q grade water produced in-house. The sugar reagents were first premixed with ammonium chloride and subsequently diluted to yield a 20 μM concentration of sugar and 1 mM ammonium chloride.

Sample preparation

Apple samples (Royal Gala and Granny Smith) were peeled to remove the outer skin. Approximately 1 g of apple flesh was weighed and homogenized using a mortar and pestle. Ten milliliters of Milli-Q water were added to obtain a 100 mg mL^{-1} slurry, which was then transferred to an Eppendorf tube (20 mL) and centrifuged at 3000 rpm for 30 min. Approximately 4 mL of the resulting supernatant was transferred to Microsep™ Advance 3 k MW cutoff Centrifugal Filter

(Pall Corp., Ann Arbor, MI) and centrifuged at 3000 rpm for 1 h. The filtrate was collected and diluted to a final concentration of 300 μg mL^{-1} in MilliQ water for analysis.

The beer sample (Rhinegeist IPA) was opened and allowed to stand for 1 h for degassing. Four milliliters of beer were then transferred to molecular-weight-cutoff filters. The filtrate was collected and diluted 10-fold prior to analysis. A similar approach was used for Coca-Cola samples, where the filtrate was diluted 2000-fold prior to analysis.

Results and discussion

Tandem MS analysis of chloride adducts in negative-ion mode

In negative-ion mode, saccharides formed adducts with the chloride anion (Cl^-) to generate stable chloride adducts.[51–53] The characteristic chloride isotope ratio of 3 : 1 for $^{35}Cl : ^{37}Cl$ assisted in identifying the saccharide-related peak in full MS (Fig. S2). When the sugar–chloride adducts were subjected to CID, distinct sets of fragment ions were observed for each disaccharide isomer. In contrast to sugar–sodium adducts detected in positive-ion mode, which produced a limited number of fragments, chloride adducts produce cleavages at both glycosidic bonds and cross-ring positions, yielding a more abundant and diverse set of fragment ions.[3] A schematic illustration of all potential fragment ions in negative-ion mode (Cl^- adduct) MS/MS is shown in Fig. 3, with each isomer following a unique fragmentation pathway characterized by different combinations of common neutral losses, including HCl, H_2O, CO_2, and $C_nH_{2n}O_n$.[3,54]

The actual MS/MS spectra collected from each of the 15 disaccharide isomers are shown in Fig. S3, corroborating the unified fragmentation pathway described in Fig. 3. In our previous study analyzing five isomers, a single fragment ion was sufficient to serve as a diagnostic ion for differentiating the isomers. However, as the library was expanded to 15 disaccharide isomers, reliance on a single diagnostic fragment ion became insufficient for accurate discrimination. Although in general, isomeric molecules share identical molecular formulas and often yield similar fragmentation patterns, differences in peak intensities can still be observed among the 15 disaccharide isomers.[32,55] In the current work, distinct diagnostic fragment ions were established for each disaccharide isomer, selected from the top 28 most intense ions (Table 1). The selected diagnostic ions exhibited distinctly higher relative intensity in a given disaccharide compared to other isomers, and (2) the specific ion(s) were present in the target isomer and absent (or present at significantly lower intensity) in most other isomers. A candidate isomer that satisfies all selected diagnostic fragment ions and exhibits strong spectral matching is assigned a higher diagnostic-ion-weighted score; otherwise, the SD score is reduced. These unique sets of fragment ions for each disaccharide provide great distinction between each isomer, which improves isomer characterization compared to utilizing only MS/MS spectra.

The mirror plot output

After constructing the library, which included MS/MS spectra and diagnostic ions for each isomer, we used kojibiose as a representative (unknown) analyte to evaluate the performance of the library-searching platform. The diagnostic-ion-weighted

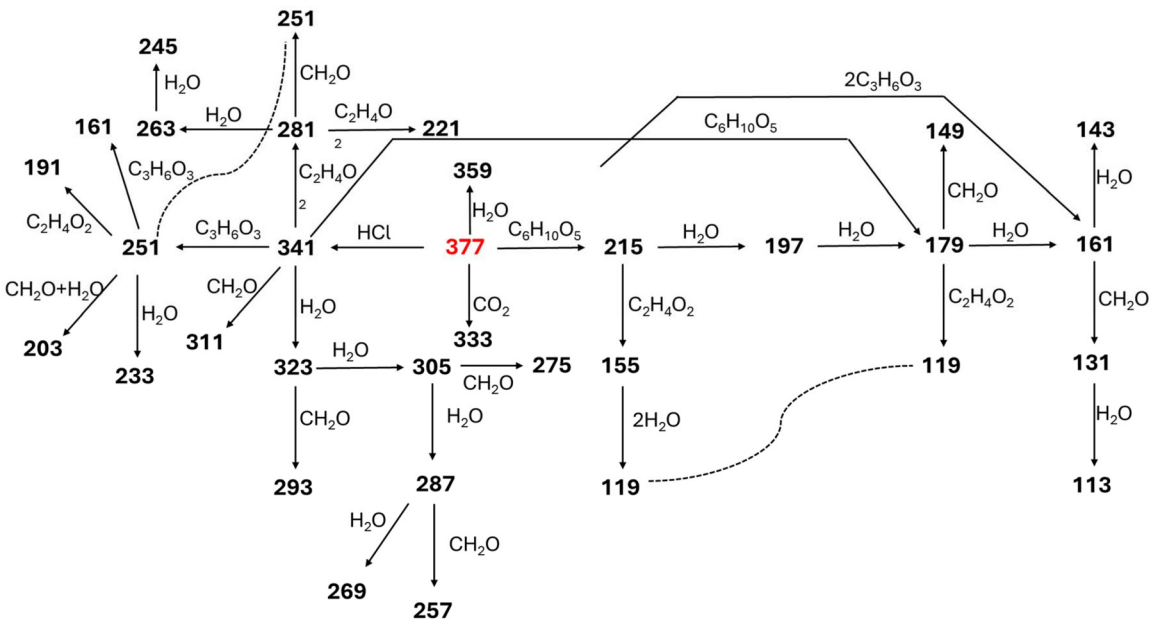

Fig. 3 Overview of the fragmentation pattern of chloride-adducted disaccharides $[M + Cl]^-$. All chloride-adducted disaccharide isomers were isolated at the same precursor mass of *m/z* 377 using a unit-mass isolation width. However, different disaccharide isomers may undergo distinct fragmentation pathways.

Table 1 Each disaccharide isomer exhibits distinct diagnostic ions, with the selected ions falling among the top 28 most intense MS/MS fragment ions

#	Disaccharide (MW 342 Da)	Diagnostic fragment Ions from $[M + Cl]^-$ (precursor ion *m/z* 377)
1	Trehalulose	221/251/269/287/281
2	Sophorose	263/221/359/311/323/245
3	Kojibiose	263/221/359/257/311/323
4	Sucrose	197/215
5	Laminaribiose	191/215/221/233/251
6	Turanose	203/215/233/251/269/293
7	Nigerose	191/215/203/221/233/251
8	Maltose	185/323/221/263/293/275
9	Lactose	115/221/263/293/317/323
10	Maltulose	115/185/221/251/263/293/295
11	Cellobiose	115/215/263/221/245
12	Leucrose	215/233/251/293/311
13	Gentiobiose	221/251/293/311/317
14	Isomaltose	221/251/269/293/311/305/317
15	Palatinose	221/251/269/287/293/305/311

scores between kojibiose and the other 14 remaining isomers were computed, the corresponding score values were generated, and the results were visualized in a mirror plot for comparison. Fig. 4 illustrates the comparisons of the spectrum derived from kojibiose α(1→2) with stereoisomer sophorose β(1→2), linkage isomer isomaltose α(1→6), and compositional isomer sucrose α(1→2). As expected, the correct matching isomer [kojibiose α(1→2)] produced the highest similarity score value 1 (Fig. 4A), reflecting an excellent match due to the identical fragmentation pattern and the presence of diagnostic ions. The stereo/configurational isomer sophorose β (1→2) differs only in the α and β orientation of the C1→C2 linkage, yet a diagnostic-ion-weighted score of 0.52 (Fig. 4B) was obtained, showing significant differences in the MS/MS data recorded for the two isomers. Likewise, the linkage isomer isomaltose α(1→6) produced an even lower score of 0.17 (Fig. 4C), indicating high selectivity between the two isomers. Finally, sucrose α(1→2), which is a constitutional isomer comprising glucose and fructose rather than the two glucose molecules in kojibiose, produced a much lower score of 0.03 (Fig. 4D). Collectively, these results highlight the strong isomer-differentiating ability of our platform, utilizing the diagnostic-ion-weighted algorithm.

Two-dimensional heat map output: pure standards

To emphasize the generality of the observed improved performance for the diagnostic-ion-weighted scoring algorithm, we established a two-dimensional (2D) heat map comparing scores with and without the influence of diagnostic fragment ions (Fig. 5). The heat maps show the comparison of each pure disaccharide standard against the MS/MS spectra stored in the library. In Fig. 5, the rows represent reference library compounds, while the columns represent unknown compounds. Each score was tested using triplicate (independent analysis) measurements to ensure consistency (Fig. S4). The average score indicates the similarity between the MS/MS spectra of the unknown and reference spectra. Higher score values (shown in maroon) indicate stronger spectral matches, while blue colors show dissimilarity between the unknown sample and reference spectra. The presence of diagonal maroon color patterns along the same rows and columns, corresponding to identical isomeric compounds, demonstrates strong characterization performance. Using a positive decision threshold of

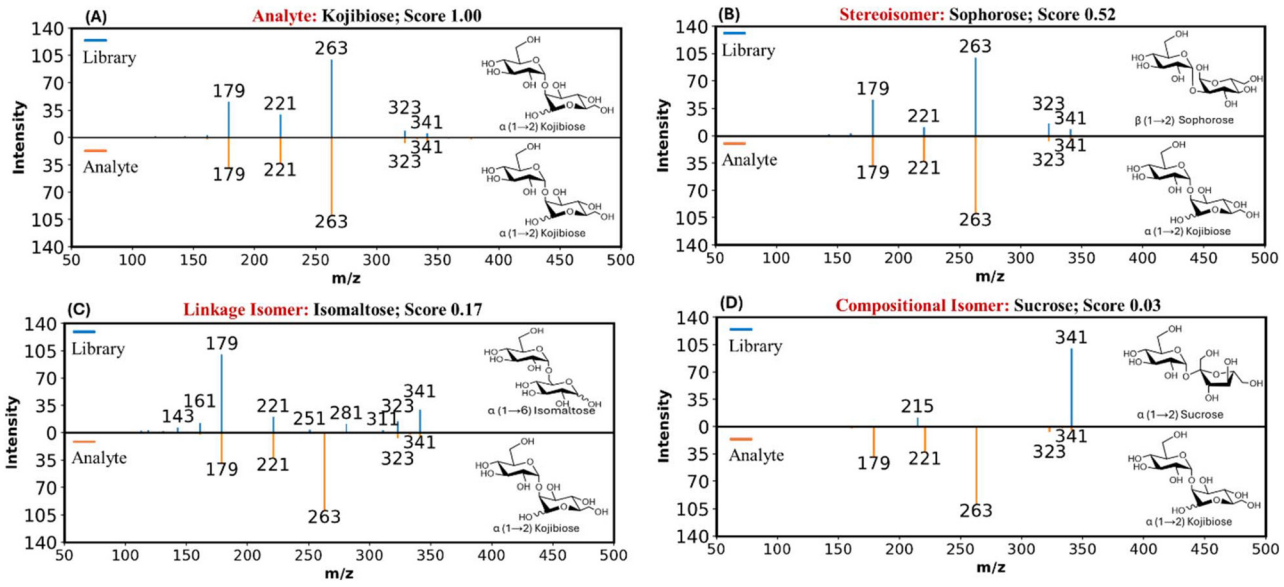

Fig. 4 The MS/MS spectrum of the analyte kojibiose is compared with related isomers in the database in the form of mirror plots. (A) Experimental data for kojibiose matches exactly (score 1.0) with previous data stored for kojibiose. Mirror plots comparing experimental data for kojibiose with previously stored data for (B) stereoisomer sophorose, (C) linkage isomer isomaltose, and (D) compositional isomer sucrose, with limited scores of 0.52, 0.17, and 0.03, respectively.

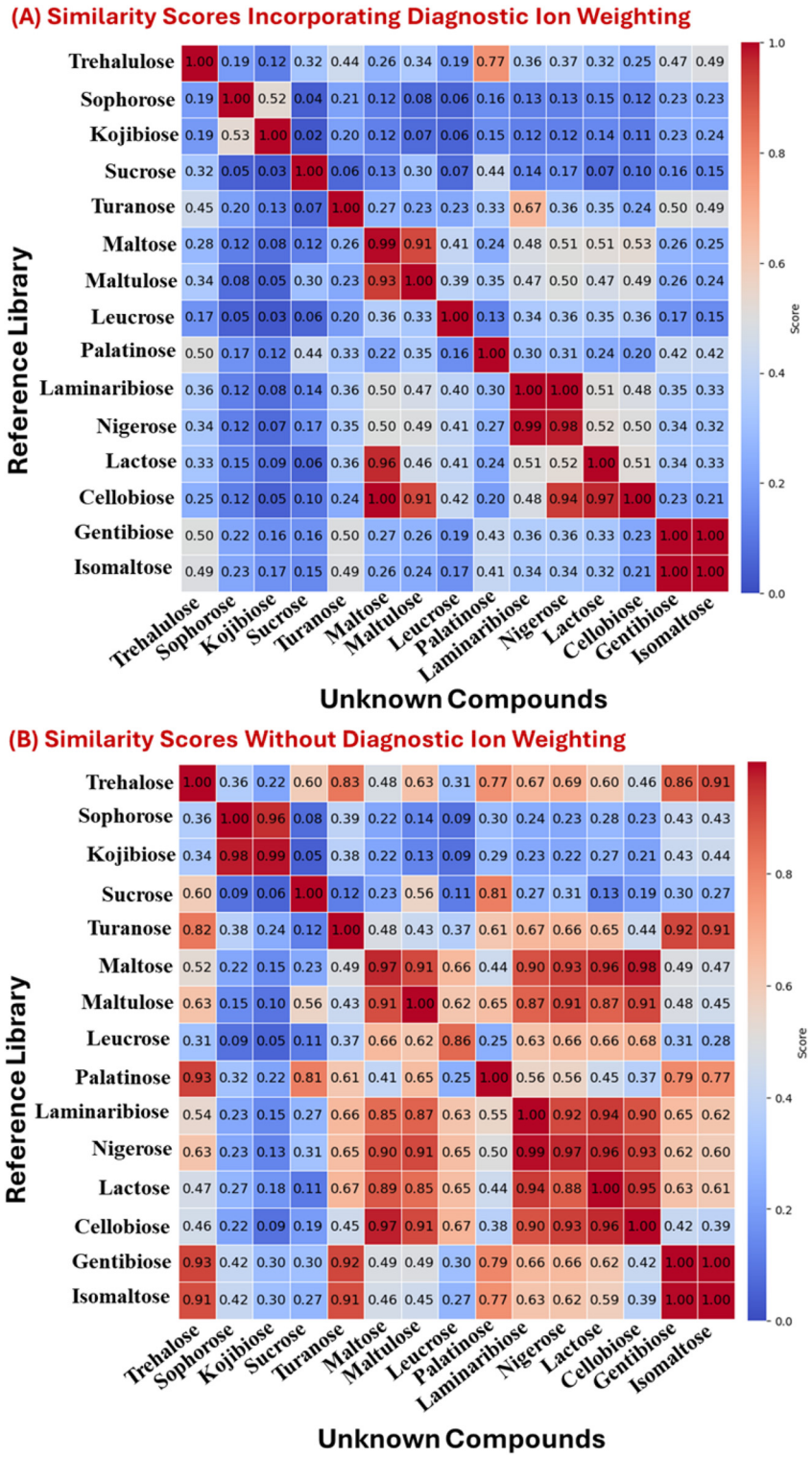

Fig. 5 Heat maps show similarity scores based on computational algorithm (A) with diagnostic-ion weighting and (B) without diagnostic-ion weighting.

approximately 0.9, scores computed with diagnostic-ion weighting (Fig. 5A) achieved clear separation between matching and non-matching isomers with minimal false positives. The 0.9 threshold was empirically established based on observations that consistently yielded true-positive identifications, where the similarity scores were close to 1.0. Allowing for 10% error, the 0.9 threshold enables confident identification. The high performance observed under this condition is due to the

integration of overall MS/MS spectral similarity and diagnostic-ion weighting. In contrast, when diagnostic fragment ions are excluded (Fig. 5B), applying the same threshold of 0.9 identifies several true positives but also results in a substantial number of false-positive assignments. This comparison underscores the importance of diagnostic-ion information, which is available in negative-ion mode with chloride-adducted analysis.

As an example of the high selectivity displayed in this work, consider maltose α(1→4), which is a configurational isomer to cellobiose β(1→4), a linkage isomer to isomaltose α(1→6), and a compositional isomer to sucrose α(1→2). In the dot-product analysis without diagnostic-ion weighting (Fig. 5B), the stereo/configurational isomer cellobiose was identified as a false positive. Additionally, using a threshold score >0.90, we observed several false positives, including nigerose and lactose. However, the score with the diagnostic-ion algorithm improved performance by enhancing the score for the correct match while reducing the scores for unmatched isomers, thereby increasing both selectivity and confidence in identification. Again, we considered turanose and trehalulose, which produced several false positives (*i.e.*, isomaltose, gentiobiose, and palatinose) when they were characterized using the algorithm without diagnostic-ion weighting. These false-positive outcomes were eliminated for both analytes when diagnostic-ion weighting was employed. Although the diagnostic-ion-weighted algorithm significantly improved disaccharide characterization, certain limitations remain in distinguishing configurational isomers. For example, the score for cellobiose similarity was comparable to scores derived for maltose, lactose, and nigerose (Fig. 5A). Likewise, gentiobiose and isomaltose were indistinguishable, even with diagnostic-ion weighting. After careful consideration, we observed that among the 15 disaccharides tested, several isomers had identical linkages but differed in composition and stereochemistry. These isomers included kojibiose, sophorose, laminaribiose, nigerose, maltose, cellobiose, gentiobiose, and isomaltose. Many of these disaccharides fragmented through a common intermediate (Fig. S5), resulting in highly similar MS/MS spectra, which posed a particular challenge for accurate isomeric differentiation using MS/MS alone. Nonetheless, the results are relevant because the purpose of developing the direct infusion nESI MS/MS method is to provide opportunities to rapidly characterize minute quantities of isomers synthesized during the initial stages of the (synthetic) method development. In such circumstances, we expect analytical sensitivity and speed of analysis to be more important than structural sensitivity since the analyte structure is known.

Two-dimensional heat map output: binary mixture

The pure standard analysis yielded promising results for disaccharide characterization. Since most rare sugars are synthesized from an isomerization process, it was necessary to evaluate the ability of the diagnostic-ion-weighted algorithm to identify the correct isomers in binary mixture systems. We expect such a condition to exist for incomplete conversion, assuming 100% specificity (*e.g.*, for an enzymatic synthetic method) with no byproducts. Thus, we prepared 53 different binary mixtures by pairing two isomers at a time using a set of 12 randomly selected rare and regular sugars: gentiobiose, isomaltose, kojibiose, lactose, leucrose, maltose, maltulose, palatinose, sophorose, sucrose, trehalulose, and turanose. The mixtures were analyzed using the non-contact nESI MS/MS platform and the results were subjected to library searching with and without diagnostic-ion weighting. The results of these analyses are summarized in Fig. S6 and S7. Here, the scores were normalized by dividing by the maximum score value. Then, four different colors were used to represent the four potential outcomes of the algorithm: (1) green for true-positive results (score >0.9), (2) red for false-positive results, (3) blue for true-negative results, and (4) yellow for false-negative results. Green indicates correctly predicted isomers with diagnostic-ion-weighted scores exceeding the 0.9 threshold (true positives). Red boxes represent incorrectly predicted isomers with diagnostic-ion-weighted scores exceeding the threshold (false positives). Yellow corresponds to missed predictions in which a compound should be present, but the diagnostic-ion-weighted score falls below the threshold (false negatives). Blue denotes true negatives, indicating isomers that are correctly identified as absent in the binary mixture.

For example, when the mixture containing trehalulose and sophorose was analyzed, the algorithm with diagnostic-ion weighting correctly identified trehalulose as present (entry #1, Fig. S6). However, the diagnostic-ion-weighted score for sophorose was not above 0.9, so it was assigned as a false-negative prediction. However, without diagnostic-ion weighting, sophorose was correctly identified (entry #1, Fig. S7). Trehalulose did not show a score above 0.9, indicating false-negative results. Besides sophorose, kojibiose also registered a score >0.9 without diagnostic-ion weighting, which resulted in a false-positive result. Such a false positive is not surprising given that sophorose β(1→2) and kojibiose α(1→2) are stereoisomers. No such false positive was observed when the algorithm with diagnostic-ion weighting was used. For the analysis of the laminaribiose and nigerose mixture (entry #37, Fig. S6), the two isomers were correctly identified, with no false negatives or false positives. When the same MS/MS data were analyzed without diagnostic-ion weighting (entry #37, Fig. S7), five isomers were ranked with scores >0.9. Among the five, laminaribiose and nigerose were correctly identified as true positives, making the remaining three isomers (lactose, maltose, and maltulose) false-positive predictions. Among the 53 different binary mixtures analyzed, at least one isomer in the mixture was correctly identified, except for entry #53 (*i.e.*, isomaltose and palatinose mixture), where both analytes were missed when employing diagnostic-ion weighting. Overall, compared to the conventional scoring method, the inclusion of diagnostic-ion weighting registered 55 true positives as opposed to 67 for the conventional method. With diagnostic-ion weighting, false-positive predictions were reduced from 74 to 21, and true negatives increased from 555 to 608. Overall, the data show that the scoring method incorporating diagnos-

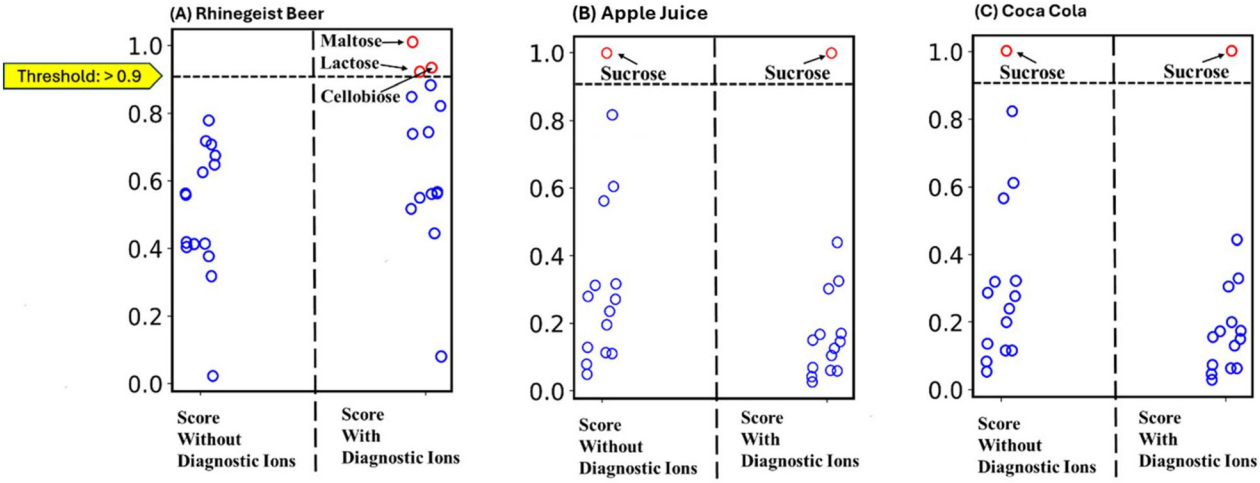

Fig. 6 Prediction results obtained from our diagnostic-ion-weighted scoring algorithm for disaccharide isomer identification from complex samples: (A) Rhinegeist beer, (B) apple juice, and (C) Coca-Cola. Results are compared with computations done without diagnostic-ion weighting for each sample. On applying a threshold score of 0.9, maltose, lactose, and cellobiose were identified in the Rhinegeist beer sample, while only sucrose was identified in both apple juice and Coca-Cola.

tic ions significantly improved the performance of isomer characterization in binary mixtures. We expect true-positive predictions to improve significantly by employing front-end chromatographic separation, a research direction currently being pursued in our laboratory.

Analysis of complex mixtures

To evaluate the suitability of the diagnostic-ion-weighted algorithm for real-world applications, we analyzed three sample matrices containing known disaccharides. These samples included Mexican Coca-Cola and apple juice, both of which contain sucrose as the primary disaccharide, as well as Rhinegeist beer, which contains maltose and lactose.[56,57] After minimal preparation (see the Experimental section), MS/MS spectra (Fig. S8) were recorded for these complex samples using the direct infusion non-contact nESI MS/MS platform. The spectra were uploaded to the spectral library-searching workflow, and scores were computed with and without diagnostic-ion weighting. The results from this work are summarized in Fig. 6, in which the algorithm accurately identified sucrose as present in both Coca-Cola and apple juice samples, with diagnostic-ion-weighted scores approaching 1.0 (Fig. 6B and C). Consistent results were also obtained from the beer sample (Fig. 6A), where diagnostic-ion-weighted scores of 1.0, 0.94, and 0.93 were recorded, with spectra matching those stored in our database for maltose, cellobiose, and lactose, respectively. Although cellobiose is typically not reported as present in Rhinegeist beer, its presence was confirmed in our ongoing HPLC studies. While the conventional algorithm, without diagnostic-ion weighting, correctly predicted sucrose to be present in both apple juice and Coca-Cola, this method failed to identify any of the disaccharides in the beer sample. Such results reinforce and validate the applicability of the diagnostic-ion-weighted spectral library searching approach for real-world saccharide isomer discrimination.

Conclusion

This study demonstrates a significant improvement in the characterization of disaccharide isomers. The method is applicable to both rare sugars and common sugars (*e.g.*, sucrose). By incorporating diagnostic fragment ions derived from negative-ion mode chloride-adduct MS/MS spectra, the proposed method successfully discriminates among configurational, linkage, and compositional isomers. The library-searching framework is robust and allows the high-throughput differentiation of isomers. Validation with pure standards showed that correct isomer assignments consistently yielded the highest diagnostic-ion-weighted scores, confirming improved specificity and analytical performance. Similar results were observed for binary mixture analyses, in which the algorithm reduced false positives while maintaining true positives. Finally, the application of the platform toward real sample complex mixtures enabled the accurate and reproducible identification of disaccharide isomers, demonstrating the reliability, reproducibility, and practical utility of the method in heterogeneous matrices.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data that support the findings of this study are available in the supplementary information (SI) of this article. The

Supporting Information includes details on the construction and user interface of the MySQL 8.0.43 spectral library platform, full MS characterization of chloride-adducted disaccharides in negative-ion mode, and tandem MS spectra of all the 15 chloride-adducted disaccharide isomers. Additional information is provided on CID fragmentation patterns, reproducibility evaluated by RSD analysis, structural comparisons among isomers with different compositions, configurations, and linkage positions. Binary mixture analysis with and without diagnostic-ion-weighted scoring is included, as well as application of the method to real samples including apple juice, Coca-Cola, and beer.

Supplementary information is available. See DOI: https://doi.org/10.1039/d6an00268d.

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