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Resolution and quantification of carbohydrates by enantioselective comprehensive two-dimensional gas chromatography

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ABSTRACT

Carbohydrates, in particular the D-enantiomers of ribose, 2-deoxyribose, and glucose, are essential to life's informational biopolymers (RNA/DNA) and for supplying energy to living cells through glycolysis. Considered to be potential biosignatures in the search of past or present life, our capacity to detect and quantify these essential sugars is crucial for future space missions to the Moon, Mars or Titan as well as for sample-return missions. However, the enantioselective analysis of carbohydrates is challenging and both research and routine applications, are lacking efficient methods that combine highly sensitive and reproducible detection with baseline enantioselective resolution and reliable enantiomeric excess (*ee*) measurements. Here, we present four different derivatization strategies in combination with multidimensional gas chromatography coupled to a reflectron time-of-flight mass spectrometer (GC×GC–TOF-MS) for the enantioselective resolution of C3 to C6 carbohydrates potentially suitable for sample-return analyses. Full mass spectral interpretation and calibration curves for one single-step (cyclic boronate derivatives) and three two-step derivatization protocols (aldononitrile–acetate, hemiacetalization–trifluoroacetylation, and hemiacetalization–permethylation) are presented for concentrations ranging from 1 to 50 pmol μL⁻¹ with correlation coefficients $R^2 > 0.94$. We compared several analytical parameters including reproducibility, sensitivity (LOD and LOQ), overall separation, chiral resolution (R_S), mass spectrum selectivity, stability during long term storage, and reliability of *ee* measurements to guide the application-dependent selection of optimal separation and quantification performance.

1. Introduction

Monosaccharides are simple carbohydrates or polyols that are key building blocks of all known forms of life. Their polymerization gives access to various complex structures playing crucial biological roles. The most important monosaccharides for information storage and regulation are ribose and 2-deoxyribose, constituent monomers of single-stranded RNA and double-helical DNA, respectively, while the most abundant and central monosaccharide in terms of nutrition and cell fuel in higher organisms is glucose. Glucose is, moreover, the exclusive component of cellulose, a polysaccharide which is abundantly present in bacterial and plant cells. Monosaccharides, with the exception of dihydroxyacetone, are optically active compounds due to the presence of at least one asymmetric carbon atom. Consequently, each monosaccharide exists in two enantiomeric forms, D (dextrorotatory or right) and L (levorotatory or left) with the D-stereoisomer being the dominant form in biological systems.

Due to their biological implications, the enantioselective analysis of sugars plays a significant role in various fields including *i*) pharmaceuticals to determine purity and stereochemistry of new drugs [1]; *ii*) the food industry to ensure the quality and authenticity of food products as sugar enantiomers affect taste, texture, and stability [2]; *iii*) environmental studies to monitor the presence and distribution of sugars in environmental samples such as soil and water [3]; and *iv*) biotechnology, where sugar enantiomers play a role in cellular processes, including protein and DNA synthesis, cell signaling, and gene regulation [4]. Moreover, monosaccharides are increasingly considered as crucial target molecules in the search for the origin of life, because understanding the presence and distribution of sugar enantiomers in different environments can provide insights into the evolution of life and the conditions necessary for life to exist. In this regard, latest analyses of meteorites [5,6], simulated interstellar ices [7,8] or early Earth geochemical settings [9] provided evidence that simple sugars, including ribose and glucose, form readily under primordial conditions,

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suggesting that they could have been present on the early Earth and could have played a role in the origin of life. Future space missions in the search for extraterrestrial life may target sugar molecules as bio-signatures for extinct or present life on Mars and Saturn's moon Titan [10–12]. However, hunting for traces of monosaccharides of biological origin is challenging because analytical methods require the combination of highly sensitive and reproducible detection with baseline enantioselective resolution and accurate enantiomeric excess (*ee*) measurements, all of which are severely compromised by the capability of sugars to produce multiple stereoisomers in solution.

Gas chromatography coupled to mass spectrometry is considered as one of the best methods for enantioselective analysis of low molar mass molecules in complex mixtures thanks to the high resolution power permitted by GC capillary columns and the selectivity of the generated mass spectra [13]. However, polar compounds such as sugars or amino acids require a derivatization procedure to replace their active (polar) hydrogen atoms to decrease their boiling point and increase their thermal stability prior GC analyses. This step is critical because it is directly linked to the GC sensitivity of the obtained derivatives, but also to the enantioselective resolution of chiral compounds. In general, two strategies can be applied for the enantioselective analysis of sugar molecules by GC-MS [14]. The *direct* approach preserves all original stereogenic centers during the derivatization step and requires subsequent analysis on adequate chiral stationary phases. Typically, derivatized cyclodextrins such as octakis-(2,6-di-*O*-pentyl-3-*O*-butyryl)- γ -cyclodextrin (Lipodex E) or heptakis(2,3,6-*O*-trimethyl)- β -cyclodextrin bonded to poly(dimethylsiloxane) (Chirasil-Dex) are employed due to their large enantioselectivities for different classes of chiral compounds [15]. The alternative *indirect* approach, is the *offline* transformation of nonvolatile sugar molecules with an enantiomerically pure chiral auxiliary into volatile diastereomers that are analyzable on achiral GC columns [16]. Monosaccharides present an additional analytical challenge as they exhibit another type of stereoisomerism due to intramolecular cyclization. Both, five-carbon (C5, pentoses) and C6 sugars (hexoses) can adopt up to four different cyclic forms by intramolecular reactions between one alcohol group and the terminal aldehyde group, leading to the formation of either 5-membered rings, known as α - and β -furanose, or 6-membered rings, known as α - and β -pyranose. These cyclic forms are generally in equilibrium with their linear (open) form in solution. For instance, ribose can exhibit up to ten different stereoisomers in solution, comprising five *D*- and five *L*-enantiomers. This characteristic considerably minimizes the intensity of each sugar signal, increases chromatographic complexity, and consequently increases the risk of coelution.

Several methods for the analysis of sugars by GC and LC have been reported, yet few of them report on sensitivity, chiral resolution, or the preferential formation of one major isomeric form [17–21]. For example, the often-employed reduction of the aldehyde moiety into an alcohol prevents the cyclization and the formation of several isomers of higher sugars, but has the drawback to form either *meso* isomers, e.g. ribitol and xylitol, or to convert two different pentoses, such as arabinose and lyxose, into the same sugar alcohol, arabinitol. This results in the loss of any information on the original parent sugar and its initial *ee* [22]. The nitration/acetylation of monosaccharides, also called aldono-nitrile acetate (ANA) method, is a highly selective and sensitive derivatization procedure that locks any aldose in its linear form [23,24]. However, no enantioseparation has been reported for C4 to C6 sugars with this method.

Regarding the resolution of C3 to C6 sugar enantiomers, several effective methods have been described so far. Among the most efficient derivatization protocols are (i) the pertrifluoroacetylation (per-TFA method) of all free OH groups [25,26], and (ii) the hemiacetalization of the anomeric OH with either isopropanol (iPrOH) or (iii) enantiopure *S*-(+)-butan-2-ol followed by trifluoroacetylation of the remaining OH groups (iPrOH-TFAA or *S*)-2-BuOH-TFAA), also known as C1/OH methods [27].

The aim of this article is to extend the spectrum of monosaccharides

that can be enantioseparated and reliably quantified, covering C3 glyceraldehyde up to C6 sugars. The importance of our work lies in the lack of a high-resolution chromatographic approach capable of combining enantiomeric resolution of a wide range of monosaccharides with accurate quantification of small enantiomeric excess of these molecules. This investigation is vital for the analyses of natural samples, which are often characterized by interfering compounds or matrices with large variability. For this purpose, we used the numerous advantages of enantioselective comprehensive two-dimensional gas chromatography combined with a reflectron time-of-flight mass spectrometer (GC \times GC-TOF-MS), such as improved noise reduction, resolution, and reproducibility [28]. An approach that has already been demonstrated to be successful for the reliable enantioselective analysis of several proteinogenic and non-proteinogenic amino acids in trace amounts by Pepino et al. [29].

We report on the enantioselective separation of C3 to C6 sugars while highlighting the advantages and disadvantages of four different derivatization approaches including the (i) ANA, (ii) *S*)-2-BuOH-TFAA, (iii) *S*)-2-BuOH-MeI, and (iv) methylboronic acid (MBA) method adapted from Van Dongen et al. [30]. In general, all sugar derivatives resulting from both tested C1/OH methods were successfully resolved on chiral GC columns using derivatives of Chirasil-Dex β -cyclodextrin stationary phases. Notably, the C1/OH derivatization methods demonstrated the advantage of favoring one stereoisomer of the sugars' cyclic forms leading to improved sensitivity and resolution compared to the reported per-TFA method. In contrast, C5- to C6-ANA derivatives could not be resolved; although this method showed excellent enantioseparation of the smallest C3 sugar, glyceraldehyde. Finally, the application of methyl boronic acid derivatization proved particularly valuable for achieving sensitive and reliable enantioselective analysis of C3 to C5 sugars.

2. Materials and methods

2.1. Chemicals and reagents

Single enantiopure standards were purchased in the highest available analytical grade and enantiopurity from the following commercial suppliers: *L*-lyxose (99%) and *L*-ribose (99%) from Alfa Aesar, *L*-xylose (>99%) from Lancaster Synthesis, and *L*-arabinose (98%), *D*-arabinose (>98%), *D*-lyxose (99%), *D*-xylose (>99%), *D*-ribose (>99%), 2-deoxy-*D*-ribose (>99%), 2-deoxy-*L*-ribose (>97%), *D*-erythrose (75%), *D*-glyceraldehyde (>98%), *D*-glucose (>99.5%), *L*-glucose (>99%), *D*-galactose (>99%), *L*-galactose (>99%), *D*-mannose (>99%), *D*-allose (98%), *D*-ribulose (>98%), *L*-ribulose (>97%) as well as racemic glyceraldehyde (>90%) from Sigma Aldrich. For standard solutions of sugars, single sugar stereoisomers were weighed, dissolved in Milli-Q® water (Direct 8; 18.2 M Ω cm at 25 °C, <2 ppb total organic carbon) and mixed, except for glyceraldehyde for which the racemic standard was commercially available. Serial dilutions from 5×10^{-5} to 10^{-8} M were prepared for the generation of calibration curves, the calculation of *ee*, and the determination of the limits of detection (LOD) and quantification (LOQ). Solutions of single enantiomers with concentrations of 10^{-4} M were prepared to correctly assign the elution order of each stereoisomer. All derivatization reagents, including trifluoroacetic anhydride (TFAA) (>99%), *S*-(+)-2-butanol (*S*)-2-BuOH (>99%), 4-(dimethylamino)pyridine (>99%), hydroxylamine hydrochloride (99.99%), pyridine (>99.5%), sodium hydroxide (>98%), methyl iodide and methylboronic acid (97%), as well as the solvents, including ethyl acetate (>99.5%), dimethyl sulfoxide (99%), 2-propanol (99.9%), and dichloromethane (99.9%), and the internal standards methyl laurate (97%) and methyl myristate (>99.5%), were purchased from Sigma Aldrich and used without further purification. All glassware used was cleaned with ethanol and Milli-Q® water, wrapped in aluminum foil, and heated at 500 °C for 5 h.

2.2. Derivatization protocols

Aldononitrile acetate (ANA). The standard solutions were dried under a gentle flow of nitrogen and transformed into aldononitrile derivatives by adding 75 μL of the derivatization reagent hydroxylamine hydrochloride (32 g L^{-1}) and 4-(dimethylamino)pyridine (40 g L^{-1}) in pyridine/methanol (4:1). After addition, the mixture was vigorously stirred and kept at 90 $^{\circ}\text{C}$ for 60 min. For the acetylation step, 75 μL of acetic anhydride were added and the reacting mixture was kept at 90 $^{\circ}\text{C}$ for 60 min after being stirred. After addition of 200 μL dichloromethane (CH_2Cl_2) to the reaction mixture, the organic phase was twice washed with $2 \times 200 \mu\text{L}$ 1 M HCl and $3 \times 200 \mu\text{L}$ Milli-Q® water to remove excess derivatization reagents and potential by-products. The organic phase was transferred into another vial and the aqueous phase extracted with $2 \times 200 \mu\text{L}$ CH_2Cl_2 for improved recovery of sugar derivatives. The organic phase was evaporated under nitrogen, and the samples were dissolved in 50 μL of ethyl acetate containing methyl myristate as internal standard at a concentration of 10^{-6} M.

C1/S-(+)-2-butanol hemiacetalization–trifluoroacetylation ((S)-2-BuOH-TFAA). 50 μL of S-(+)-2-butanol/acetyl chloride (20:1) were added to the dried standard solution and kept at 60 $^{\circ}\text{C}$ for 45 min after being vigorously stirred. After drying under a gentle flow of nitrogen, 50 μL TFAA were added to the mixture, stirred, and kept at room temperature for 60 min. The samples were again dried under a flow of nitrogen and dissolved in 50 μL of ethyl acetate containing the internal standard at a concentration of 10^{-6} M. For this derivatization, methyl laurate was used instead of methyl myristate to prevent coelution with the sugar derivatives, particularly D-ribose.

C1/S-(+)-2-butanol hemiacetalization–permethylation ((S)-2-BuOH-MeI). 50 μL of S-(+)-2-butanol/acetyl chloride (20:1) were added to the dried standard solution and kept at 60 $^{\circ}\text{C}$ for 45 min after being vigorously stirred. After drying the solution of the first derivatization step under a gentle flow of nitrogen, 200 μL of DMSO and ~ 30 mg crushed NaOH were added and the mixture was vigorously stirred. $3 \times 10 \mu\text{L}$ CH_3I were added while stirring the mixture for 1 min after each addition. The reaction was quenched with 200 μL dichloromethane, neutralized with $2 \times 300 \mu\text{L}$ 0.2 M HCl and then washed with $3 \times 200 \mu\text{L}$ Milli-Q® water. The organic phase was transferred into another vial and the aqueous phase extracted with $2 \times 200 \mu\text{L}$ CH_2Cl_2 . The organic phase was evaporated under a nitrogen stream before dissolving the derivatives in 50 μL ethyl acetate containing methyl myristate as internal standard at a concentration of 10^{-6} M.

Methylboronic acid (MBA). The standard solutions were dried under a gentle flow of nitrogen and transformed into methylboronate ester derivatives by adding 50 μL of 10 g L^{-1} methylboronic acid in pyridine to the dried samples that were kept at 60 $^{\circ}\text{C}$ for 30 min. The samples were then dried under nitrogen until approximately 2–3 μL of the solvent remained, to avoid the loss of the most volatile derivatives. Finally, the MBA derivatives were dissolved in 50 μL of ethyl acetate containing methyl myristate as internal standard at a concentration of 10^{-6} M. Limited tests were conducted to explore the addition of a secondary acetylation step using 50 μL of TFAA, following the protocol described above, after drying the MBA derivatization to 2–3 μL (MBA-TFAA).

2.3. GC×GC–TOF-MS instrumentation

The enantioselective multidimensional analysis was carried out by a GC×GC Pegasus BT 4D instrument coupled to a reflectron time-of-flight mass spectrometer (LECO, St Joseph, Michigan, USA) and equipped with a dual-stage thermal jet modulator. The MS system operated at a storage rate of 150 Hz, with a 50–580 amu mass range, a detector voltage of 1500 V, and a solvent delay of 15 min. Ion source and injector temperatures were set to 230 $^{\circ}\text{C}$ and the temperature of the transfer line to 240 $^{\circ}\text{C}$. The column setup included an Agilent J&W CP-Chirasil-Dex CB column (25 m \times 0.25 mm, 0.12 μm film thickness; Agilent, Santa Clara, USA) as the first dimension, connected via a SilTite μ -Union connector

(SGE, Restek), to a DB-Wax in the second dimension (1.5 m \times 0.1 mm, 0.1 μm film thickness; Agilent). Note that for the MBA and MBA-TFAA methods, the first-dimension column had an extended length of 29.9 m. Alternatively, Lipodex A, Lipodex E, and Chirasil-I-Val have been used as primary capillary columns for screening optimal resolution (Table S1). Modulation was performed using cryomodulation with a total 2nd dimension run time of 5 s. For all four derivatization protocols, the secondary oven and the modulator used temperature offsets of 20 $^{\circ}\text{C}$ and 35 $^{\circ}\text{C}$, respectively. Hydrogen was used as a carrier gas. All samples were injected in splitless mode to maximize analyte transfer to the column, using 1 μL of the derivatized solutions for GC×GC–TOF-MS analyses. An Agilent single taper, Ultra-Inert liner with a 4 mm internal diameter in contact with an Ultra-Inert gold inlet seal was used to minimize adverse interactions and improve peak shape and reproducibility. Data were processed using the LECO Corp. ChromaTOF™ software.

2.4. Instrumentation and optimal separation conditions

To achieve optimal separation performance, several GC×GC parameters were adjusted according to the investigated sugar derivatives, including the oven program and flow conditions. The initial temperature of the primary oven was always >10 $^{\circ}\text{C}$ below the boiling point of the sample solvent to avoid peak broadening of the early-eluting analytes. All sugar-ANA derivatives were injected at a constant hydrogen flow of 1 mL min^{-1} . The temperature of the primary oven was held at 40 $^{\circ}\text{C}$ for 1 min, then increased to 190 $^{\circ}\text{C}$ at a rate of 5 $^{\circ}\text{C min}^{-1}$, and finally held for 10 min.

For the (S)-2-BuOH-TFAA method, all samples were injected at a constant hydrogen flow of 1 mL min^{-1} . The temperature of the primary oven was held at 40 $^{\circ}\text{C}$ for 1 min, 40–90 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C min}^{-1}$, and held for 10 min, 90–170 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C min}^{-1}$, and finally 170–190 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C min}^{-1}$ (10 min final isotherm).

For the (S)-2-BuOH-MeI method, all samples were injected at a constant hydrogen flow of 1.2 mL min^{-1} . The temperature of the primary oven was held at 40 $^{\circ}\text{C}$ for 1 min, 40–90 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C min}^{-1}$, 90–115 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C min}^{-1}$ and held for 10 min, 115–165 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C min}^{-1}$, and finally 165–190 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C min}^{-1}$ (10 min final isotherm).

For the MBA and MBA-TFAA derivatizations, all samples were injected at a constant hydrogen flow of 1.2 mL min^{-1} . The temperature of the primary column was held at 55 $^{\circ}\text{C}$ for 1 min, 40–90 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C min}^{-1}$ and held for 8 min, 90–180 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C min}^{-1}$, and finally 180–190 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C min}^{-1}$ (10 min final isotherm).

2.5. Method validation

All samples used to produce calibration curves were derivatized in triplicates for each concentration and injected three times ($n = 9$) to allow for accurate peak area calculations with reliable statistical error bars. For statistical evaluation, a linear regression was performed.

The LOD is a critical parameter for assessing the suitability of a methodology for the analysis of sugars in extraterrestrial samples, where the need to confirm their presence at extremely low concentrations with confidence is paramount. Traditionally, the LOD has been calculated as a multiple of the average background noise originating from a reagent blank. This approach is, however, highly dependent on the chromatogram region chosen to measure the background noise, which is notably low for MS-based chromatographic techniques, and also excludes the measurement of the analyte itself [31,32]. Therefore, a multi-injection approach for LOD determination was applied here. This method determines the amount of an analyte that causes a sufficiently strong signal so that 99% of trials with that amount will produce a detectable signal [33], expressed as: $t_{\alpha} \times s_n$, where t_{α} corresponds to the one-sided Student's t -distribution value associated with a 99% confidence interval (α) for $n-1$ measurements (degrees of freedom), and s_n represents the standard deviation of the analyte response for the n samples in

concentration units. The concentrations employed for LOD measurements for each sugar and derivatization method in this study are detailed in Table S2.

3. Results and discussion

3.1. Resolution and structural information of the mass spectra

3.1.1. Aldonitrile acetate method (ANA)

Introduced in the 1960s [34] and subsequently employed for the separation of neutral, alcohol, and amine monosaccharides [23,35], the aldonitrile acetate method is a convenient two-step derivatization for aldoses. This method locks them in their linear form, reducing the number of isomers formed and simplifying the resulting chromatogram. The first step converts the terminal aldehyde group into a nitrile and the remaining alcohols are esterified with acetic anhydride (Fig. 1A). However, in this study, individual 5- and 6-carbon carbohydrates could not be resolved as aldonitrile acetates on any of the four chiral stationary phases tested (Table S1). Using this derivatization method, only glyceraldehyde and erythrose were successfully resolved (Fig. 2). No mass fragment corresponding to the potential ketoxime-derivative formed by ribulose was detected. The elution order of glyceraldehyde enantiomers was determined from separate injections of single enantiomers. On the CP-Chirasil-Dex CB column, on which all further tests were carried out, the D-enantiomer of glyceraldehyde elutes first.

The mass spectrum of glyceraldehyde and erythrose aldonitrile acetates as well as the interpretation of the most characteristic fragments are shown in the Supplementary Information (Figs. S1–2) and summarized in Table 1. The molecular ion peaks, m/z 171 and m/z 243, are absent in the mass spectra. For glyceraldehyde, the predominant peak at m/z 73 corresponds to the fragment ion $[\text{CH}_3\text{COOCH}_2]^+$ and results from a homolytic σ -bond cleavage. The second major fragment at m/z 141 results from the loss of 30 amu corresponding to the neutral elimination of formaldehyde (CH_2O) combined with a rearrangement of the cyano radical CN^\cdot binding to a $\text{CH}_3\text{CO}^\cdot$ moiety. Loss of CN^\cdot and $\text{CH}_3\text{COO}^\cdot$ from the molecular ion, leads to the formation of the resonance-stabilized ion at m/z 86; alternatively, the rearrangement of two $\text{CH}_3\text{CO}^\cdot$ radicals with the elimination of neutral $\text{O}(\text{CH}_2)_2\text{OCN}$ can lead to the resonance-stabilized ion $[\text{CH}_3\text{COCOCOCH}_3]^+$ at m/z 86. Fragment ions with m/z 112 and m/z 99 result from the loss of $\text{CH}_3\text{COO}^\cdot$ and a rearrangement leading to the loss of the CH_3COOCH moiety, respectively. For erythrose, the m/z 145 fragment corresponds to the fragment ion $[\text{CH}_3\text{COOCH}_2\text{CHOOCC}_3\text{H}_7]^+$ which results from a homolytic σ -bond cleavage while the predominant peak at m/z 103 results from the elimination of ketene $\text{CH}_2=\text{CO}^\cdot$ from m/z 145. For the sake of

comprehensiveness, the major mass fragments of the larger C5 and C6 sugars are provided in Table 1 and in accordance with previously reported mass spectral data [35].

3.1.2. C1/S-(+)-2-butanol hemiacetalization–trifluoroacetylation method ((S)-2-BuOH-TFAA)

Introduced in 2019 by Cooper et al. [27], this derivatization method has been proposed for the enantioseparation of C3 to C6 aldoses. The first step of the derivatization is the acetalization of sugars by the addition of enantiopure (S)-2-BuOH under acidic conditions followed by a classic alcohol derivatization using TFAA to form fluorinated esters (Fig. 1B). Compared to the per-TFA method [26], the introduction of (S)-2-BuOH on C1 to form an acetal is crucial and considerably promotes the preferential formation of only one pyranose form, while the abundance of other isomeric forms drastically decreases. Presumably, the β -pyranose isomer is favored, as it reduces the steric hindrance imposed by the adjacent OH-functionalized bulky groups. However, xylose, arabinose, glucose, galactose, and allose yielded two pairs of peaks, corresponding to both α/β -pyranose isomers (Fig. 3). For each sugar, the L-enantiomer elutes before the D-enantiomer using the CP-Chirasil-Dex CB column in the first dimension, except for glucose, galactose, and one of the two isomers of xylose and arabinose.

The mass spectra of the sugars are undoubtedly distinguishable depending on the molecular mass, which is one of the advantages of this method. However, no distinct differences were detected in the mass spectra among sugars with the same mass or between α/β anomeric forms. The characteristic mass spectra and the associated fragmentation of the C3 to C6 sugars are shown in the Supplementary Information with the example of glyceraldehyde, erythrose, ribose, 2-deoxyribose, ribulose, and glucose (Figs. S3–8). The C5 aldopentoses, arabinose, lyxose and xylose, and the C6 aldohexoses, galactose, mannose and allose, exhibit identical fragmentation pattern to ribose and glucose, respectively. Generally, the ions at m/z 57 and 69 are omnipresent in the mass spectra of this method, simply resulting from the derivatization reagents, (S)-2-BuOH and TFAA. The homolytic cleavage of the O-butyl moiety leads to the heaviest of the major fragment ions in erythrose, 2-deoxyribose and the other C5 aldoses, with m/z 295, 309, and 421, respectively. The fragment ion with m/z 295 is the most intense in the mass spectrum of erythrose followed by the characteristic fragments with m/z 153 and 181 from $[\text{TFAO-C}_3\text{H}_4\text{O}]^+$ and $[\text{TFAO-C}_3\text{H}_4]^+$. The most intense ions in the mass spectra of the C5 sugars, with m/z 278 and 193, correspond to the $[\text{TFAO-C}_4\text{H}_5\text{-OTFA}]^+$ and $[\text{TFAO-C}_5\text{H}_4\text{O}]^+$. For 2-deoxyribose, the dominant fragments are attributed to the pyrylium ion $[\text{C}_5\text{H}_5\text{O}]^+$ at m/z 81 and $[\text{TFAO-C}_5\text{H}_6\text{O}]^+$ at m/z 195. Whereas the fragment ions with m/z 319 and 265 in the mass spectra of glucose,

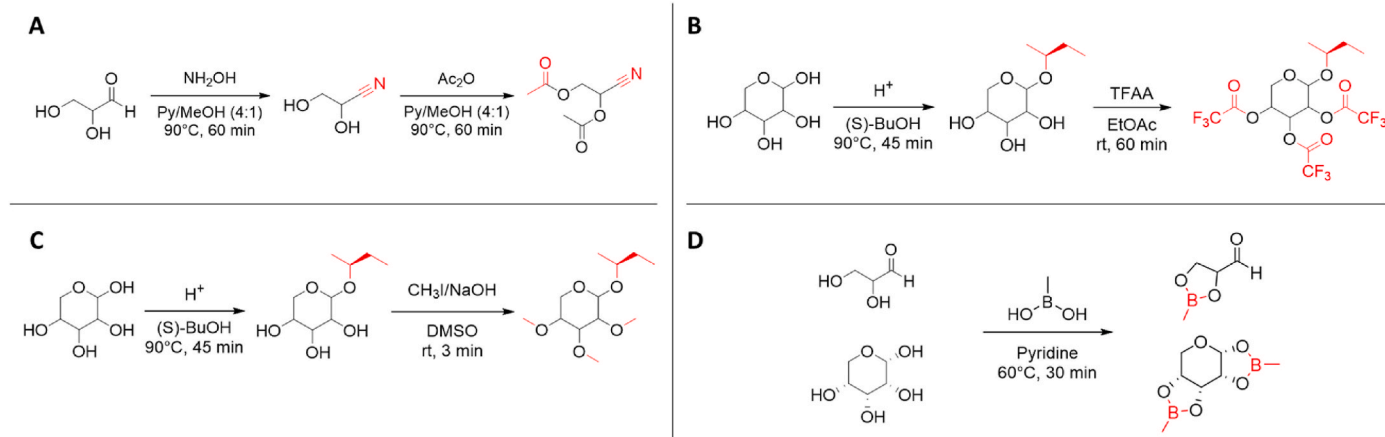


Fig. 1. Sugar derivatization protocols. (A) Two-step derivatization of glyceraldehyde into aldonitrile acetate. (B) Derivatization of a C5 sugar by C1/S-(+)-2-butanol hemiacetalization followed by trifluoroacetylation. (C) Derivatization of a C5 sugar by C1/S-(+)-2-butanol hemiacetalization followed by permethylation. (D) Glyceraldehyde (up) and D-ribose (bottom) derivatization with methyl boronic acid in pyridine.

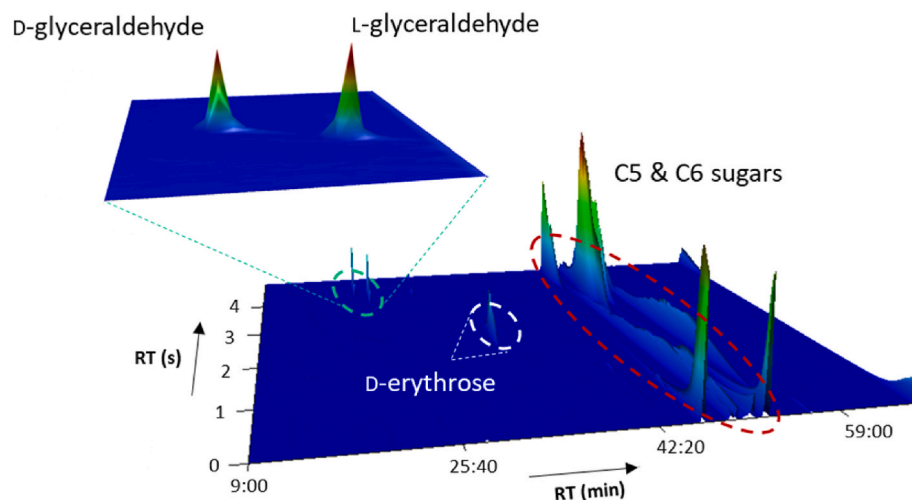


Fig. 2. Enantioselective two-dimensional gas chromatogram of a standard sugar mixture as aldnonitrile acetate derivatives. Mass-to-charge ratios m/z 103, 141, and 145 are displayed.

Table 1

Most prominent fragment ions m/z of C3 to C6 sugars according to the derivatization method.

Compounds	Main fragments, m/z (most intense ion peak in bold)			
	ANA	(S)-2-BuOH-TFAA	(S)-2-BuOH-MeI	MBA
Glyceraldehyde	57, 73, 86, 99, 112, 141	140, 126 , 100	58, 72, 87	84, 85, 112, 113
Erythrose	103 , 141, 145, 170	153, 169, 181, 198, 295	71 , 87, 102, 131	84, 85 , 112, 113, 141
2-Deoxyribose	103, 145	81 , 99, 195, 309	55, 73, 89	85, 97 , 111
Lyxose	103, 115, 145 , 242	97, 193 , 265, 278, 421	88, 101 , 117, 143, 175	84, 97 , 110, 113, 139
Xylose	103, 115, 145 , 242	97, 193 , 265, 278, 421	88, 101 , 117, 143, 175	84, 97 , 110, 113, 139
Arabinose	103, 115, 145 , 242	97, 193 , 265, 278, 421	88, 101 , 117, 143, 175	84 , 97, 110
Ribose	103, 115, 145 , 242	97, 193 , 265, 278, 421	88, 101 , 117, 143, 175	84 , 97, 110
Ribulose	–	105, 285, 383 , 398	87, 101 , 115, 143, 175	97 , 98, 101, 126, 168
Allose	103, 115, 145 , 187, 212	265, 319 , 404, 547	71, 88 , 101, 117, 187	85 , 97, 113, 139, 183
Glucose	103, 115, 145 , 187, 212	265, 319 , 404, 547	71, 88 , 101, 117, 187	84 , 85, 97, 113, 141, 225
Galactose	103, 115, 145 , 187, 212	265, 319 , 404, 547	71, 88 , 101, 117, 187	85, 97 , 98, 113, 141, 197, 225
Mannose	103, 115, 145 , 187, 212	265, 319 , 404, 547	71, 88 , 101, 117, 187	84 , 85, 97, 113, 141, 225

galactose, mannose and allose are assigned to $[\text{TFAO}-\text{C}_5\text{H}_3\text{O}-\text{CH}_2\text{-OTFA}]^+$ and $[\text{TFAO}-\text{C}_3\text{H}_3-\text{OTFA}]^+$, respectively. Cooper et al. reported a fragmentation pattern with m/z 177 and 337 as the prominent ions for allose, suggesting that it may result from the incomplete derivatization at the anomeric carbon [27]. However, in our study, this fragmentation was observed only in an additional, albeit low-intensity peak (Fig. S9), while the principal signals exhibited the same fragmentation pattern of

the other fully derivatized C6 sugars. The absence of peaks resulting from the cleavage of the exocyclic C5 group, $\text{CH}_2\text{-OTFA}$, in all spectra confirm that this derivatization favors the pyranose form over the furanose form.

3.1.3. C1/S-(+)-2-butanol hemiacetalization–permethylation method (S)-2-BuOH-MeI

This method is an adapted combination of the *hemiacetalization* method reported by Cooper et al. [27] and the *Ciucanu permethylation* method using sodium hydroxide and methyl iodide in DMSO [36–38]. As hemiacetalization with (S)-2-butanol of the anomeric carbon has a significant impact on the number of isomers and their abundance, it was kept as the first step for this derivatization protocol (Fig. 1C). The conversion of each hydroxyl group (-OH) into a methyl ether (-OCH₃) group was used as the second derivatization step in order to enhance the accuracy and precision of isotopic measurements. This is particularly useful for carbon isotope analyses (¹³C/¹²C ratio) since it minimizes the bias introduced by additional ¹²C-functional groups from the derivatization agent.

The major drawback of this method is its very poor detectability for glyceraldehyde, 2-deoxyribose, and ribulose. However, D-erythrose (C4), all the other C5 aldoses and the C6 glucose, galactose, mannose, and allose were appropriately detected and clearly resolved by increasing number of carbon atoms (Fig. 4). All C5 sugars and glucose were baseline enantio-separated. One of the two isomers of galactose was also enantio-separated but the L-enantiomer co-elutes with another isomer of galactose that is not resolved. Like the (S)-2-BuOH-TFAA method, xylose, arabinose, glucose, galactose, and allose yield two intense isomers.

Unlike the (S)-2-BuOH-TFAA method, the mass spectra of most C5 and all C6 sugars exhibit the same major mass fragments. By contrast, the mass spectrum of glyceraldehyde and erythrose show more pronounced differences. The loss of the O-methyl moiety from the per-O-methyl glyceraldehyde derivative gives the fragment m/z 87, while the most intense signal at m/z 72 corresponds to $[\text{CHO}-\text{CH}=\text{O}-\text{CH}_3]^+$ ion (Fig. S10). For erythrose, the loss of the O-butyl moiety leads to the fragment ion with m/z 131 which, after the elimination of formaldehyde (CH_2O), gives m/z 101 (Fig. S11). The most intense fragment at m/z 71 corresponds to $[\text{CH}_2-\text{CH}-\text{CHO}-\text{CH}_3]^+$ resulting from the loss of CH_3O - and the neutral molecule $\text{C}_5\text{H}_{10}\text{O}_2$. The m/z 87 signal is assigned to $[\text{CH}_3\text{O}-\text{C}_2\text{HO}-\text{CH}_3]^+$ and the fragment at m/z 102 can be attributed to either $[\text{CH}_3\text{O}-\text{C}_3\text{H}_4-\text{OCH}_3]^+$ or $[\text{HCOO}-\text{C}_4\text{H}_9]^+$. 2-Deoxyribose exhibits a distinctive fragmentation pattern, with major fragments at m/z 73 and 89, that can be attributed to the O-butyl moiety and

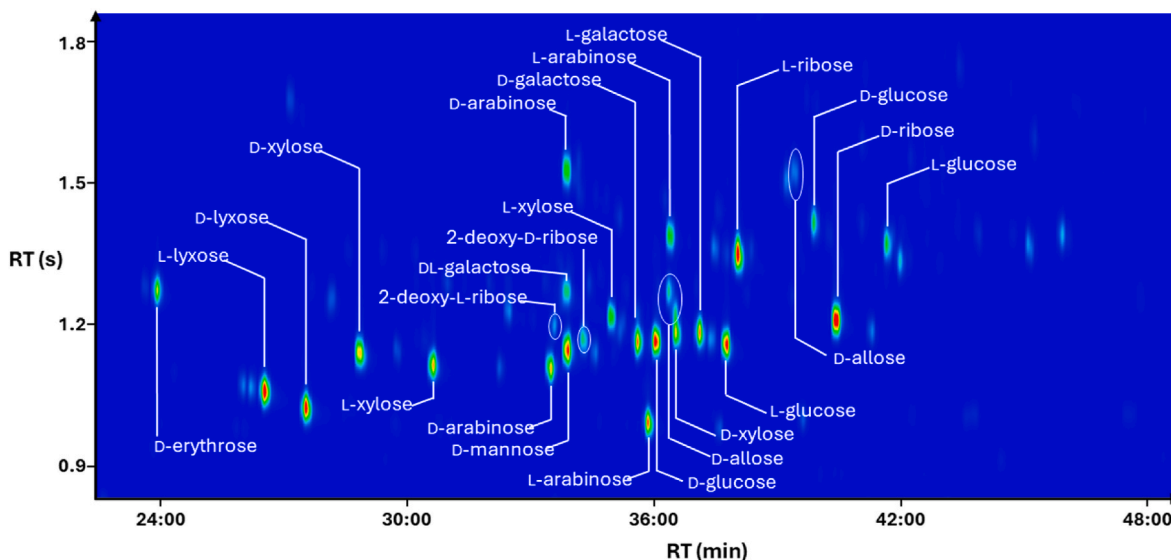


Fig. 3. Enantioselective two-dimensional gas chromatogram of a standard sugar mixture derivatized with the (S)-2-BuOH-TFAA method. Mass-to-charge ratios m/z 195, 278, 295, and 319 are displayed.

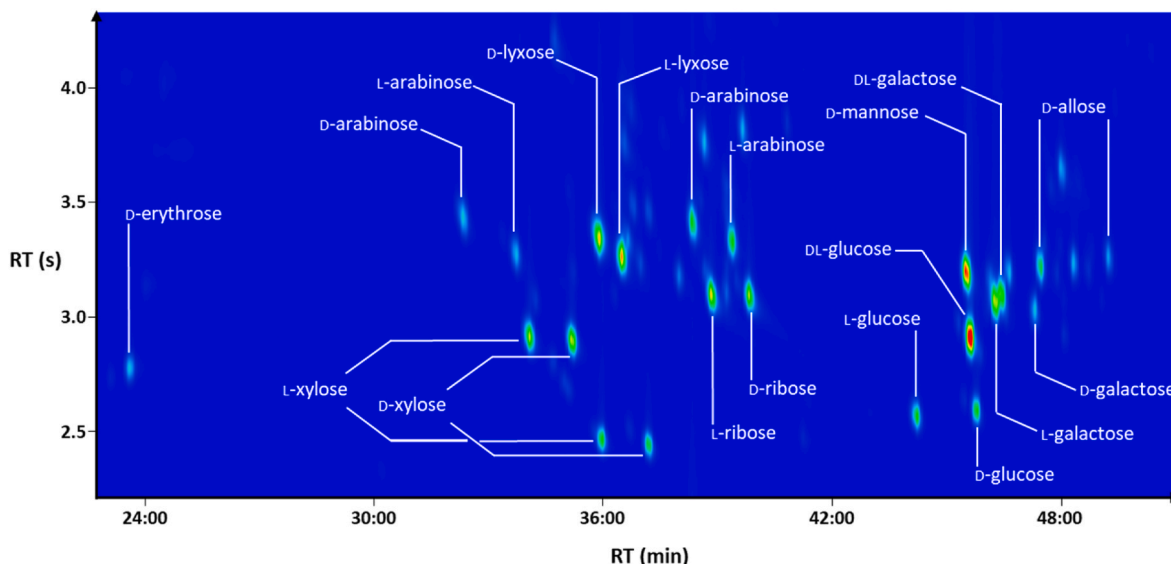


Fig. 4. Enantioselective two-dimensional gas chromatogram of a standard sugar mixture derivatized with the (S)-2-BuOH-MeI method. Mass-to-charge ratios m/z 88, 101, and 102 are displayed.

$[\text{CH}_3\text{O}-\text{CH}_2-\text{CH}=\text{OCH}_3]^+$ ion, respectively (Fig. S12).

Regarding the other C5 aldoses and C6 sugars, although sharing three major fragments (m/z 88, 101, and 117), they can be readily distinguished based on differences in the intensity of these fragments, with m/z 101 being the most intense fragment for C5 aldoses (Fig. S13), while the m/z 88 fragment predominates for C6 sugars (Fig. S14). Their heaviest fragment ions (m/z 175 and 187) also constitute a distinctive difference between the two groups. The loss of the *O*-butyl moiety in C5 sugars results in the fragment ion with m/z 175 and the signals at m/z 88, 101, and 117 correspond to $[\text{CH}_3\text{O}-\text{CH}=\text{CH}-\text{OCH}_3]^+$, $[\text{CH}_3\text{O}-\text{C}_3\text{H}_3=\text{OCH}_3]^+$, and $[\text{CHO}-\text{CH}(\text{OCH}_3)-\text{CH}=\text{OCH}_3]^+$, respectively. Furthermore, the m/z 101/88 ratio allows to differentiate ribose and arabinose from xylose and lyxose. Additionally, in contrast to xylose, the pair of arabinose signals with the lowest retention time displays a slight deviation from the typical fragmentation pattern, with m/z 87 and 115 predominating over m/z 88 and 117, respectively (Fig. S15). A very similar pattern is observed for the C5 ketose, ribulose

(Fig. S16). Since there were no mass fragments resulting from the loss of the exocyclic C5 group (CH_2-OME) and, consequently, the presence of the furanose form was ruled out, this pattern may be attributed to the presence of α - and β -pyranose isomers. The C6 sugars, glucose, galactose, mannose, and allose, share the fragment at m/z 187 corresponding to the neutral elimination of 2-butanol and methanol. The ions at m/z 88 and 101 are the same as for the C5 sugars. In contrast, the fragment ion at m/z 117 results from the H-rearrangement combined with ring cleavage to give $[\text{CH}_3\text{O}-\text{CH}_2-\text{CO}-\text{CHO}-\text{CH}_3]^+$ (Fig. S14). The m/z 101/88 ratio also allows here to distinguish glucose and mannose from galactose and allose.

3.1.4. Methylboronic acid method (MBA)

The study by van Dongen et al. [30] applied the MBA method to C5 and C6 sugars, without intending to achieve enantioselective separation. The authors noted that for some sugars, a secondary silylation step was required to convert any remaining unfunctionalized alcohol groups. This

was necessary because these groups could not react with methyl boronic acid due to their unique positioning or distance from an adjacent hydroxyl group. In our study, we decided to prioritize the first step, which involved the reaction between hydroxyl groups and methyl boronic acid (Fig. 1D), as only the C6 sugars in our standard mixture require the second step. Although C6 sugars can be detected by the MBA derivatization alone (Fig. S27), their detectability is rather low, with expected LODs around 10^{-5} M. Furthermore, long-tailing peaks characterize the C6-MBA derivatives probably due to the interaction of the underivatized hydroxyl group with the stationary phase [39]. Initial trials involving trifluoroacetylation as a secondary step (MBA-TFAA) to improve the detectability of the C6 sugars were effective but resulted in a 95% reduction in yields of glyceraldehyde and peak area losses ranging from 51% to 77% for most C5 sugars (Fig. 5, Table S3). This is likely due to the displacement of cyclic-boronate groups by TFA, a process already reported for trimethylsilyl groups [30]. Erythrose and 2-deoxyribose, for which an -OH group remained underivatized after the primary MBA step, were exceptions to this negative impact of TFA. In these cases, we observed an increase in detectability of 218% for erythrose and 30% for one of the two signals of 2-deoxyribose (Table S3), although enantio-separation was lost for the latter (Fig. 5B). Thus, despite the mentioned disadvantages that may limit its applicability for trace analyses, the MBA-TFAA methodology can be valuable for applications involving relatively high concentrations of C3 and C5 sugars.

The MBA method allowed us to detect and resolve glyceraldehyde, erythrose, the four C5 aldoses, 2-deoxyribose, and to improve

significantly the detectability of the C5 ketose ribulose, compared with the (S)-2-BuOH-TFAA method (Fig. 5A). Overall, MBA has the advantages of favoring the formation of one isomeric form per sugar and of adding only one carbon per two OH groups. This feature makes it particularly suitable for isotopic studies, similar to the (S)-2-BuOH-Mel method.

The mass spectra of the sugar-MBA derivatives exhibit similar fragments. Glyceraldehyde (Fig. S17) and erythrose (Fig. S18–19) display a fragment at m/z 113, resulting from the loss of an H-radical for glyceraldehyde and the loss of an H-radical and neutral CH_2O for erythrose. They also exhibit a signal at m/z 85, as the result of the loss of $\text{CHO}\cdot$ for glyceraldehyde and the loss of CH_3BO_2 and H \cdot for erythrose. Three peaks were observed for D-erythrose, arising from two possible derivatization sites at the C1/C2 and C2/C3 positions (zoomed area in Fig. 5A). The two most intense peaks correspond to the α and β isomers, sharing the same mass spectra and a major fragment at m/z 97, which results from the loss of CO_2H_2 . Ribose, lyxose, arabinose, and xylose share the same fragments, although the intensities differ. The fragment at m/z 84, attributed to $\text{CH}_3\text{-BO}_2\text{C}_2\text{H}_4$, is most intense for ribose and arabinose, for which the derivatization favors the pyranose form (Fig. S20). In contrast, for xylose and lyxose, where the furanose form is preferred, the mass fragment at m/z 97, corresponding to the loss of $\text{C}_3\text{H}_6\text{BO}_3$, predominates (Fig. S21). The presence of the fragment ion at m/z 139 also constitutes a distinctive difference between the two groups. Moreover, the m/z 84/97 ratio allows for the distinction between ribose and arabinose, as well as between lyxose and xylose. The ion at m/z 110

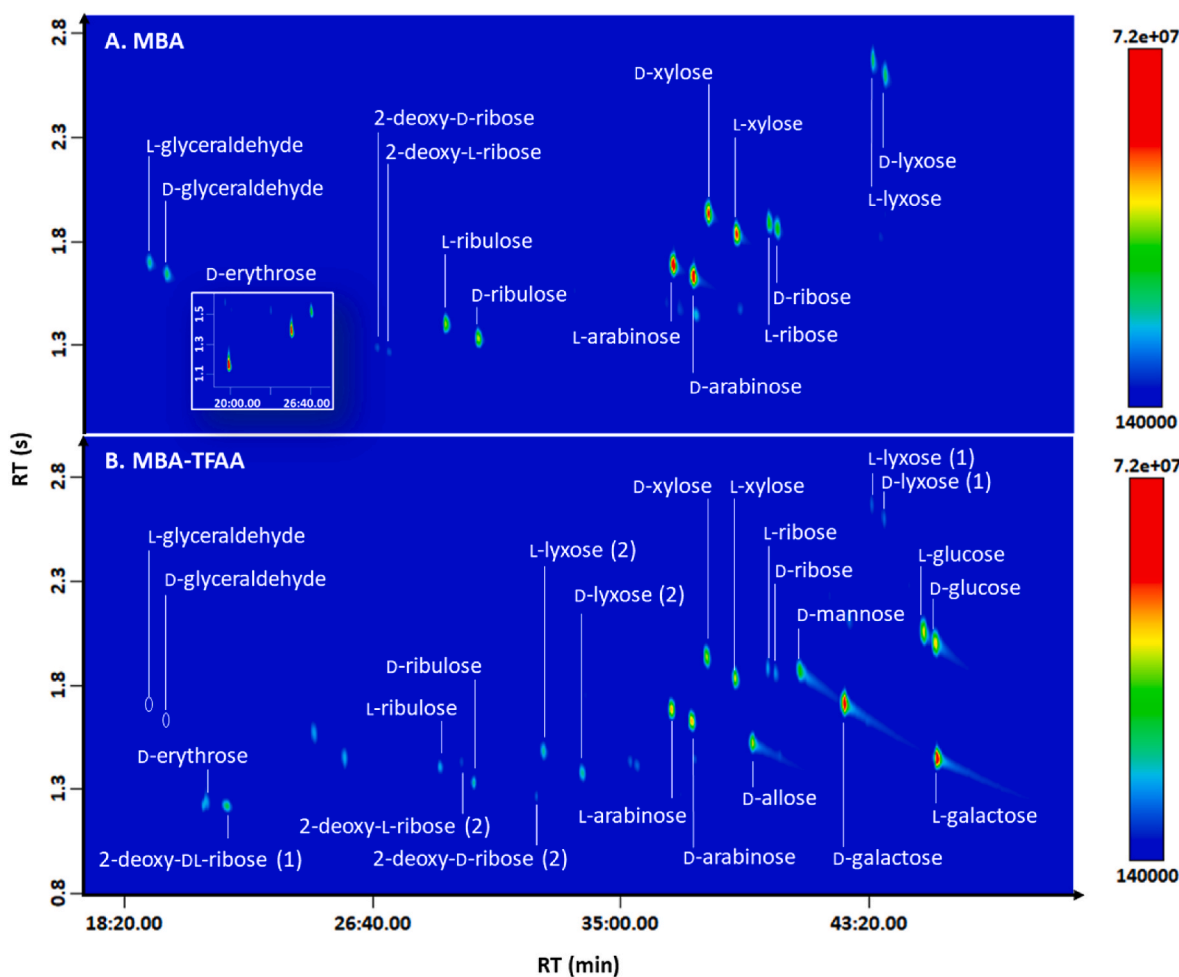


Fig. 5. Two-dimensional gas chromatograms of a standard sugar mixture derivatized using the MBA method (A) and the MBA-TFAA method (B). Mass-to-charge ratios m/z 84, 85, 97, 113, and 140 are displayed. In panel A, only m/z 85 and 97 are shown in the zoomed area to enhance visualization of the D-erythrose peaks.

results from the loss of neutral CH_2O and CH_3BO_2 . 2-Deoxyribose is most likely preserved in the furanose form, and thus the most intense signal at m/z 97 can be explained by the elimination of $\text{C}_2\text{H}_5\text{O}_2$ (Fig. S22). Ribulose was also detected in the furanose form, displaying a distinctive fragmentation pattern with the same m/z 97 fragment as ribose and arabinose, but with additional intense fragments at m/z 98 and at m/z 168, the latter being absent in the spectra of the other C5 sugars (Fig. S23).

Concerning the C6 sugars (Figs. S24–26), we detected the usual fragments generated from the MBA derivatization at m/z 84, 85, 97, and 113. Additionally, there were notably heavy fragments at m/z 225 for glucose, mannose, and galactose, resulting from the loss of two H-radicals and at m/z 183 for allose. These distinctive fragments allowed for differentiation between C6 and C5 sugars. In each case, a specific isomer was favored due to the required geometric configuration for an efficient reaction. For galactose- and allose-MBA derivatives, a second intense peak with a unique fragmentation pattern and an ion fragment at m/z 252 was observed (Fig. S27). This unusually high molecular mass fragment may result from the derivatization of the remaining $-\text{OH}$ group with MBA, which could also account for the prolonged peak-tailing. The MBA method failed to differentiate between glucose and mannose and did not resolve glucose enantiomers (Fig. S27). However, these limitations can be overcome by introducing the trifluoroacetylation step (Fig. 5B).

3.2. Reproducibility, enantioseparation, sensitivity, and derivatization stability

3.2.1. Aldononitrile acetate method (ANA)

The ANA method has three significant advantages for the enantioselective analysis of glyceraldehyde. First, the entire protocol is highly reproducible as shown by the calibration curves that were realized with sample concentrations of 10^{-4} M, 5×10^{-5} M, 10^{-5} M, and 5×10^{-6} M (Fig. S28). Secondly, D- and L-glyceraldehyde are baseline resolved with $R_s = 2.5$ (Fig. 2, Table 2), and both enantiomers are well separated from the larger sugars.

The LOD and LOQ values are 670 and 2010 fmol, respectively (Table 2). The ANA method is therefore suitable for quantifying glyceraldehyde enantiomers with confidence but with limitations for trace analyses.

3.2.2. C1/S-(+)-2-butanol hemiacetalization–trifluoroacetylation method ((S)-2-BuOH-TFAA)

The calibration curves of ribose, 2-deoxyribose, and glucose shown in Figs. S30–32, illustrate the high reproducibility of the (S)-2-BuOH-TFAA derivatization protocol for these C5 and C6 sugars. In the case of erythrose, the error bars are slightly larger (Fig. S29) suggesting lower

reproducibility of C4 and smaller sugars, likely due to their higher volatility and subsequent loss during the drying steps.

The overall resolution is a key advantage of this method as well as the excellent enantioseparation of all C5 sugars, glucose and galactose with resolution values equal to or greater than 3 (Table 2). The importance of the second chromatographic dimension in GC×GC is exemplified by the capacity to avoid coelution of sugars with identical or similar retention time in the first dimension. Moreover, a *third-dimensional* separation of partially co-eluting analytes is accomplished by the mass spectrometer due to its capability to deconvolute the mass spectra, as demonstrated for certain C5 sugars that co-elute with C6 sugars (Fig. 3). An additional strength of this derivatization lies in the achievable LOD values. These values range between 20 and 50 fmol for all C4 to C6 sugars, except for the challenging 2-deoxyribose, for which the LOD is one order of magnitude higher and for the C5 ketose ribulose, which was not detected at the concentration employed for LOD measurements (Table 2). All these positive features make this method extremely efficient and reliable for the analysis of samples with rather low sugar quantities such as non-terrestrial samples, including meteorites, samples returned from asteroids, or simulated interstellar ices. Nonetheless, it is important to acknowledge two significant limitations associated with this methodology. Firstly, the high LOD of glyceraldehyde renders this derivatization method unsuitable for trace-level analyses of this specific sugar. Secondly, the good reproducibility achieved for the quantification of ribose, deoxyribose, and glucose does not readily extend to the quantification of other C5 and C6 sugars due to the possibility of diastereomer formation from enantiomers, which cannot be excluded with this method [27].

3.2.3. C1/S-(+)-2-butanol hemiacetalization–permethylation method ((S)-2-BuOH-MeI)

As indicated by the calibration curves with concentrations ranging from 10^{-6} M to 5×10^{-5} M (Figs. S33–35), the whole derivatization procedure is reliable and reproducible. Compared with the (S)-2-BuOH-TFAA method, the LOD of D-erythrose is higher, likely due to the higher volatility of the (S)-2-BuOH-MeI derivative (Figs. 3–4). All C5 sugars and glucose are well enantio-separated with a minimum resolution R_s of 1.8 for lyxose. The crucial role of applying two-dimensional GC for the overall resolution of a complex sugar mixture is again evident. Some of the most obvious examples include the resolution of ribose and arabinose, xylose and lyxose or glucose and mannose (Fig. 4).

The generation of two equally intense signals for xylose and arabinose does not negatively impact their LOD and LOQ values, which are close to those of ribose and lyxose. In general, the determined LOD values are in the range of 42 fmol (allose) and 93 fmol (xylose), with only erythrose presenting an LOD value of 507 fmol (Table 2). Except for

Table 2
Chiral resolution (R_s), limit of detection (LOD) and quantification (LOQ) in [fmol].

Compound	ANA			(S)-2-BuOH-TFAA			(S)-2-BuOH-MeI			MBA		
	R_s	LOD	LOQ	R_s	LOD	LOQ	R_s	LOD	LOQ	R_s	LOD	LOQ
glyceraldehyde	2.5	670	2010	NE	ND	ND	1.5	ND	ND	1.8	4	12
erythrose	U	ND	ND	U	26	78	U	507	1521	U	41	123
2-deoxyribose	NR	–	–	3	634	1901	1.8	ND	ND	1.5	46	138
lyxose	NR	–	–	3	36	108	1.8	89	267	1.8	23	69
xylose	NR	–	–	5.3	30	91	4.9	93	279	4.0	3	9
arabinose	NR	–	–	7	50	150	3.8	102	306	2.8	3	9
ribose	NR	–	–	7.3	32	95	3	73	219	1.5	13	39
ribulose	ND	–	–	5.6	ND	ND	4.3	ND	ND	3.0	3	9
glucose	NR	–	–	5.3	23	70	3.6	77	231	NE	ND	ND
galactose	NR	–	–	4.5	21	63	NE	46	138	0.8	ND	ND
mannose	NR	–	–	U	20	60	U	63	189	U	ND	ND
allose	NR	–	–	U	49	148	U	42	126	U	ND	ND

Chiral resolution R_s calculated as $R_s = |t_{R(D)} - t_{R(L)}| / [w(D) + w(L)]$, where t_R is the retention time and w the full peak width at base in the 1st dimension. LOD values were determined based on the most intense signal corresponding to the D-enantiomer for compounds detected at concentrations equal to or below 10^{-6} M. LOQ values were calculated as three times the LOD values. ND = not detected at the concentration used for LOD determination (see Table S2). NE = not enantio-separated. NR = not resolved. U = unknown because only one enantiomer was available.

erythrose, these LOD values are of the same order of magnitude as the ones found for the (S)-2-BuOH-TFAA method.

3.2.4. Methylboronic acid method (MBA)

This method does not allow the detection of trace amounts of C6 sugars without a second derivatization step. However, all C5 sugars, 2-deoxyribose, erythrose, ribulose, and glyceraldehyde show excellent detectability values (Table 2). Furthermore, the whole derivatization procedure exhibits a high degree of reproducibility, even for the most volatile compounds, i.e. glyceraldehyde and erythrose, as proven by the calibration curves and the related error bars (Figs. S36–39).

The enantioselective resolution of this method is strongly isomer dependent. Arabinose, xylose, and ribulose display the best enantioseparation while ribose, lyxose, glyceraldehyde, and 2-deoxyribose display the minimal required resolution (Table 2). Overall, enantioseparation is achieved for all C3 to C5 sugars with a minimum R_S of 1.5 up to 4.0. Besides, all eluting sugar derivatives are well spread over the entire 2D-separation space preventing any coelution among individual sugar molecules (Fig. 5A).

The detectability of the MBA method is also strongly dependent on the size and stereochemistry of the individual sugar. Arabinose, xylose, ribulose, and glyceraldehyde result in the most intense chromatographic signals, followed by ribose and lyxose, in agreement with the lower reaction yields reported by van Dongen et al. for these two monosaccharides [30]. Erythrose and 2-deoxyribose exhibit less intense signals, likely due to the presence of an underivatized hydroxyl group (Figs. S16–17 and S20). This is illustrated by the determined LOD values, with LODs of 3–4 fmol for the most intense sugar-MBA derivatives and 41 and 46 fmol for erythrose and 2-deoxyribose, respectively (Table 2). These values are in the same order of magnitude for erythrose and lyxose as the LODs of the (S)-BuOH-TFAA method but were significantly improved for all the other sugars, especially for the C5 ketose, ribulose, which failed to be detected by the other methods at trace concentrations. In summary, the overall strength of this analytical method is the simultaneous detection and enantioseparation of glyceraldehyde, all C5 sugars and 2-deoxyribose at trace-level concentrations, which is of great advantage for a large range of applications, including the analyses of meteorites and ice analog samples.

To evaluate the potential for storing sugar derivatives without significant degradation, stability studies were conducted for the four derivatization methods. A sugar standard with a concentration of 5×10^{-5} M was derivatized and analyzed at different time intervals (1 day, 3 days, 7 days, and 10 days) for each method. After each injection, samples were stored at 4 °C. The results, presented in Table 3, show that the ANA and MBA methods had overall recoveries ranging from 92% to 100% and 81% to 100%, respectively. The (S)-2-BuOH-MeI derivatives were similarly stable over time, with only a 30% loss observed for the more volatile erythrose after 10 days. In contrast, the (S)-2-BuOH-TFAA derivatives showed considerable losses after only three days, indicating their low stability. These findings suggest that the ANA, MBA, and (S)-2-BuOH-MeI derivatives are relatively stable and suitable for reliable analysis over an extended period in the laboratory, while caution should be exercised with (S)-2-BuOH-TFAA derivatives due to their low stability.

Table 3

Stability of derivatized sugars after $t_x = 1, 3, 7,$ and 10 days. Numbers represent the percentage of non-degraded sugars using the GC×GC peak areas at t_x divided by the corresponding GC×GC peak areas at t_0 .

Compounds	ANA				(S)-2-BuOH-TFAA				(S)-2-BuOH-MeI				MBA			
	1d	3d	7d	10d	1d	3d	7d	10d	1d	3d	7d	10d	1d	3d	7d	10d
Glyceraldehyde	100	100	100	92	–	–	–	–	–	–	–	–	100	91	81	83
Erythrose	–	–	–	–	92	78	73	62	95	84	86	71	100	95	96	96
2-Deoxyribose	–	–	–	–	100	91	82	81	–	–	–	–	100	100	100	97
Ribose	–	–	–	–	100	76	62	49	100	96	95	93	100	95	86	86
Glucose	–	–	–	–	96	65	43	26	100	96	97	98	–	–	–	–

3.3. Assessing enantiomeric excess with confidence

The chiral resolution of D- and L-glyceraldehyde-ANA derivatives is highly effective, with a resolution of 2.5 (as indicated in Table 2), allowing for accurate calculations of small enantiomeric excesses (*ees*). To assess this parameter, a racemic glyceraldehyde solution was spiked with D-glyceraldehyde to produce a 5% ee_D solution. To ensure reliable *ee* calculations, three samples of the racemic solution and three samples of the 5% ee_D solution were derivatized and analyzed at the same concentration (10^{-5} M). The results, presented in Table 4 and Fig. S40, show an ee_D of $-1.00 \pm 0.33\%$ for the racemic solution and $3.19 \pm 0.20\%$ for the D-glyceraldehyde spiked solution, resulting in a relative ee_D difference of $4.19 \pm 0.39\%$. It should be noted that the 5% ee_D value is purely theoretical because the racemic glyceraldehyde and the D-glyceraldehyde standards may have different levels of purity.

The potential of the (S)-2-BuOH-TFAA method for detecting a relative *ee* difference of 5% was assessed by analyzing racemic and 5% ee_D sugar mixtures, each sample injected three times. Ribose, 2-deoxyribose, and glucose were used for the calculations (Table 4 and Fig. S41). None of the three sugars showed the relative 5% ee_D difference ($6.48 \pm 0.47\%$ for ribose, $3.89 \pm 0.67\%$ for deoxyribose, and $4.48 \pm 1.46\%$ for glucose), possibly due to coelution, instrumental artefacts and/or the purity of standards. The method allows excellent resolution of ribose enantiomers, with R_S equal to 7.3. Very good enantioresolution is achieved also for two pairs of enantiomers of two different isomers of glucose, however, the drawback is almost equal split of the signal between the two isomers leading to overall reduced S/N ratios (Fig. 3), which is very likely associated with the relatively high error bars of the *ee* values (Table 4).

We conducted the same *ee* test using the (S)-2-BuOH-MeI method. We ran identical mixtures (racemic and 5% ee_D) at the same concentration (5×10^{-6} M) and determined the experimental *ee* for ribose and glucose while 2-deoxyribose was not detected at this concentration. The expected relative 5% ee_D difference was retrieved for both sugars ($5.03 \pm 0.17\%$ for ribose and $4.95 \pm 0.15\%$ for glucose), with remarkably low errors (Table 4, Fig. S42). The primary benefit of this method is therefore the high precision of the relative *ee* measurement in combination with the minimized bias for isotopic measurements – often conducted for extraterrestrial sample analyses – due to limited additional ^{12}C -functional groups introduced by the derivatization agent.

Regarding the MBA method, we examined the potential for detecting relative ee_D difference of 5% by injecting racemic and 5% ee_D sugar solutions at a concentration of 5×10^{-6} M. Unfortunately, similar to the (S)-2-BuOH-TFAA method, it was not feasible to confidently measure the enantiomers of 2-deoxyribose and to evaluate the *ee* at this concentration. In fact, the apparent relative 5% ee_D difference could not be retrieved and the standard deviation displays a significant uncertainty in the value ($2.11 \pm 0.77\%$, Table 4, Fig. S43), which was caused by relatively low signal-to-noise levels and coelutions with other peaks. However, the cases of glyceraldehyde and ribose demonstrate more favorable outcomes, with calculated *ees* that are quite consistent, as indicated by the errors, and a relative 5% ee_D difference was retrieved for both sugars ($4.95 \pm 0.52\%$ for glyceraldehyde and $5.15 \pm 0.32\%$ for ribose). The primary advantage of the MBA method is that it is the only

Table 4

Calculation of ee_D of a racemic and a 5% ee_D solution of glyceraldehyde, ribose, 2-deoxyribose, and glucose. The calculated ee_D for each solution is the average of 9 injections (3 samples injected 3 times). The error represents the sample standard deviation for the total of 9 injections.

		Calculated ee_D (%)			
		Glyceraldehyde	Ribose	2-Deoxyribose	Glucose
ANA	Racemic (10^{-5} M)	-1.00 ± 0.33	–	–	–
	5% ee_D (10^{-5} M)	3.19 ± 0.20	–	–	–
(S)-2-BuOH-TFAA	Racemic (5×10^{-6} M)	–	-5.41 ± 0.38	-2.08 ± 0.49	1.64 ± 0.88
	5% ee_D (5×10^{-6} M)	–	1.07 ± 0.27	1.81 ± 0.45	6.12 ± 1.16
(S)-2-BuOH-MeI	Racemic (5×10^{-6} M)	–	-7.89 ± 0.09	–	0.04 ± 0.09
	5% ee_D (5×10^{-6} M)	–	-2.86 ± 0.14	–	4.99 ± 0.12
MBA	Racemic (5×10^{-6} M)	2.87 ± 0.32	2.62 ± 0.18	1.99 ± 0.41	–
	5% ee_D (5×10^{-6} M)	7.82 ± 0.41	7.77 ± 0.27	4.10 ± 0.65	–

ee_D is calculated as $ee_d = [\text{Area}(D) - \text{Area}(L)] / [\text{Area}(D) + \text{Area}(L)]$.

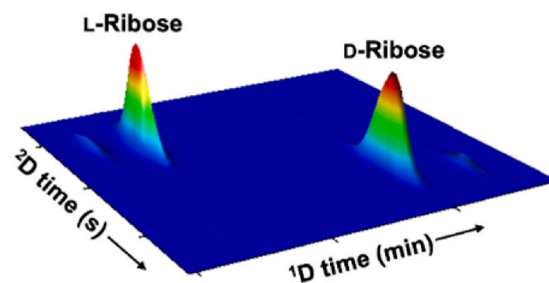
one capable of simultaneously quantifying small relative ees of ribose and glyceraldehyde at low concentrations while also enantioselectively separating and measuring the ee values of 2-deoxyribose, albeit with lower accuracy and precision than for the other sugars.

Table 4 well illustrates that using different derivatization strategies for the same sample may result in very different measured absolute ee values. The most striking discrepancies between the absolute ee values are apparent for ribose, where the ee may differ by $\sim 10.5\%$ ((S)-2-BuOH-MeI vs. MBA derivatization). A closer look at the GC \times GC chromatograms in Figs. 3–5 (Fig. 6) reveals that while the (S)-2-BuOH-TFAA method assures excellent baseline enantioselective separation and separation of ribose enantiomers from other compounds, this is not the case for the other two reported derivatizations (Table 4). For (S)-2-BuOH-MeI, the L-enantiomer has a slight coelution with an earlier eluting compound, which could, at least partly, explain slightly lower measured ee_D as opposed to the (S)-2-BuOH-TFAA method. For the MBA derivatization, the two enantiomers are not baseline separated and hence the tail of the earlier eluting L-enantiomer clearly contributes to the signal of the D-enantiomer, artificially enhancing the measured ee_D values of ribose. Finally, despite the excellent resolution, the absolute ee value measured for the (S)-2-BuOH-TFAA method may be distorted due to different reactivities of diastereomers formed in the first derivatization step. Such procedural and/or instrumental artefacts become critical especially for enantioselective analyses of extraterrestrial samples, where we aim for the highest accuracy in determining the absolute ee values as we lack corresponding racemic references. These are often simulated by mineral matrix analogs or extraterrestrial samples spiked with racemic standards. However, one must be aware that the real ee of a racemic standard is likely to differ from 0%. Moreover, the matrix of extraterrestrial samples is expected to be inhomogeneous within and between different parent bodies, and therefore there may be a significant difference in the matrix effects between the sample of interest and the corresponding reference. Thus, investigating and minimizing procedural and/or instrumental artefacts is of key importance for enhancing the accuracy of the absolute ee determination.

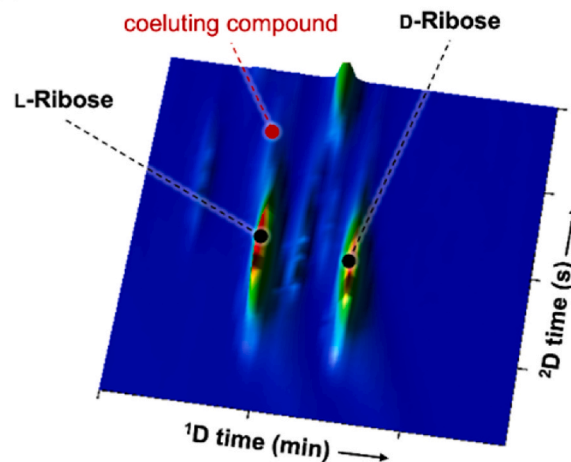
4. Conclusion

The qualitative and quantitative gas chromatographic analysis capable of resolving all aldose enantiomers ranging from three to six carbon atoms is a challenging undertaking, but derivatization can largely simplify chromatographic separation. The four methods examined in this study did not meet all requirements, including high sensitivity, selectivity, and baseline resolution of all C3 to C6 monosaccharides, as well as accurate and reliable determination of small enantiomeric excesses. Instead, the one- and two-step derivatization approaches complement each other, providing different derivatization strategies depending on the specific application. For instance, the ANA and MBA methods can detect the enantiomers of glyceraldehyde at trace concentrations, with the ANA method affording an excellent enantioselective separation. The MBA method provides about 100-times lower

A. (S)-2-BuOH-TFAA



B. (S)-2-BuOH-MeI



C. MBA

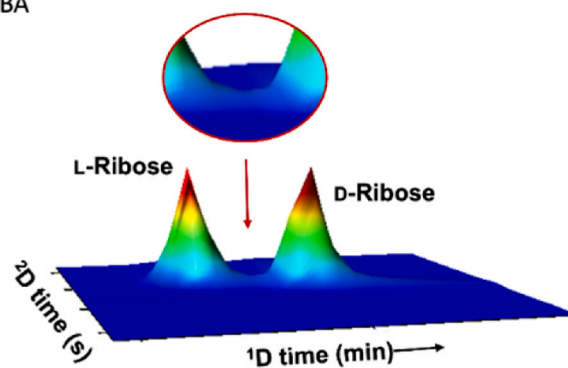


Fig. 6. Comparison of enantioselective two-dimensional gas chromatographic resolution of ribose depending on the derivatization. (A) (S)-2-BuOH-TFAA, (B) (S)-2-BuOH-MeI, and (C) MBA derivatives of D- and L-enantiomers of ribose. The insert in (C) shows the tail of the L-ribose peak contributing to the signal of D-ribose.

limit of detection, thus allowing reliable determination of relative *ee* even at very low concentrations. In addition, the MBA method enables enantioseparation of higher homologues of monosaccharides and surpasses the other methods in terms of detectability for C5 sugars. Concerning C4 to C6 sugars, the (S)-2-BuOH-TFAA method outperforms the (S)-2-BuOH-MeI and MBA methods in terms of overall resolution, enantioseparation, and, notably in the detectability of C4 and C6 sugars. However, the determination of *ee* is less precise compared to the two other methods, which are more confident in small *ee* quantitation, except for 2-deoxyribose. Lastly, the MBA method has the advantage of selectively converting monosaccharides into high yields and enantio-separating glyceraldehyde, C5 sugars, 2-deoxyribose, ribulose, and even C6 sugars after the second derivatization step. The chiral resolution R_S is lower than for the (S)-2-BuOH-TFAA and (S)-2-BuOH-MeI methods, although the quantitation of small relative *ees* is reliable, except for 2-deoxyribose.

A robust analytical method for the quantification of small *ees* of 2-deoxyribose is still missing and future studies should particularly focus on this aldose sugar. In summary, we believe that this study can provide guidance for selecting the appropriate derivatization method based on the primary goals of the analytical study and the targeted sugar compounds.

CRedit authorship contribution statement

Adrien D. Garcia: Investigation, Methodology, Validation, Writing – original draft. **Vanessa Leyva:** Investigation, Validation, Writing – original draft, Writing – review & editing. **Jana Bocková:** Investigation, Validation, Writing – review & editing. **Raphaël L. Pepino:** Investigation. **Cornelia Meinert:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Cornelia Meinert reports financial support was provided by European Research Council. Jana Bocková reports financial support was provided by French Space Agency. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.talanta.2024.125728>.

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