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Evaluation of different derivatisation approaches for gas chromatographic–mass spectrometric analysis of carbohydrates in complex matrices of biological and synthetic origin

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ABSTRACT

Gas chromatographic analysis of complex carbohydrate mixtures requires highly effective and reliable derivatisation strategies for successful separation, identification, and quantitation of all constituents. Different single-step (per-trimethylsilylation, isopropylidenation) and two-step approaches (ethoximation–trimethylsilylation, ethoximation–trifluoroacetylation, benzoximation–trimethylsilylation, benzoximation–trifluoroacetylation) have been comprehensively studied with regard to chromatographic characteristics, informational value of mass spectra, ease of peak assignment, robustness toward matrix effects, and quantitation using a set of reference compounds that comprise eight monosaccharides (C_5–C_6), glycolaldehyde, and dihydroxyacetone. It has been shown that isopropylidenation and the two oximation-trifluoroacetylation approaches are least suitable for complex carbohydrate matrices. Whereas the former is limited to compounds that contain vicinal dihydroxy moieties in *cis* configuration, the latter two methods are sensitive to traces of trifluoroacetic acid which strongly supports decomposition of ketohexoses. It has been demonstrated for two "real" carbohydrate-rich matrices of biological and synthetic origin, respectively, that two-step ethoximation–trimethylsilylation is superior to other approaches due to the low number of peaks obtained per carbohydrate, good peak separation performance, structural information of mass spectra, low limits of detection and quantitation, minor relative standard deviations, and low sensitivity toward matrix effects.

1. Introduction

Analysis of complex carbohydrate mixtures is still a challenging issue with regard to separation and identification of all constituents and reliable quantitation of target compounds. Natural, carbohydrate-rich samples are ubiquitous and include plant extracts, body fluids, or hydrolysates of polysaccharides (e.g., biomass processing) and glycoproteins (*O*- and *N*-glycans etc.). Quantitation of the sugar composition might be required for product or process control, structure elucidation, or understanding of metabolic processes. Deviations in the natural sugar fingerprint of healthy grapevine parts (leaves, roots), for example, are expected to provide immediate information about stress situations caused by drought stress, enhanced UV radiation, fungal attack, or parasites.

On the other hand, carbohydrates play a considerable role in technical applications where they are used as nonionic surfactants (alkyl polyglucosides), natural adhesives, feedstock for bioplastics (e.g., polyurethane, polylactide, polyalkanoate), acid stabilizers in food technology, or as a source for bioethanol, organic solvents, or fine chemicals such as hydroxymethylfurfural and 2,5-dimethylfurane [1]. The huge global sugar consumption, the increasing number of carbohydrate-based industrial applications, and the awakening awareness of the necessity of utilizing alternative carbohydrate sources are some of the reasons that recently fortified efforts to establish synthetic approaches to low-molecular carbohydrates. The reaction of formaldehyde with calcium hydroxide, known as formose reaction, is regarded as a promising synthetic approach in this respect even though the complexity of the reaction mixtures would render the isolation of individual compounds a challenging task.

Regardless of their biological or synthetic origin, both of the sample matrices described above are characterized by a complex carbohydrate composition with constituents that vary significantly in molecular weight and polarity [2,3]. Depending on their origin, analysis of the sugar pattern is interfered by further constituents that add to the complexity of the matrix, and may interfere with analysis and derivatisation [4].

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Capillary gas chromatography (GC) is a powerful separation technique that complements high performance liquid chromatography and capillary electrophoresis. In particular, the high reproducibility, lower sensitivity of the separation column toward matrix effects, and the availability of comprehensive mass spectra libraries render GC in combination with mass spectroscopy (MS) a powerful technique for quantitative analysis of complex mixtures. However, unlike HPLC, polar, hydrophilic, low-volatile, or thermally sensitive compounds like carbohydrates are not suited to direct analysis by GC/MS and require preceding derivatisation [5]. Effective derivatisation strategies have been developed to increase the signal intensity and stability of derivatives, to improve the compound-specific fragmentation pattern, and to allow for simultaneous analysis of both lower and higher molecular compounds in one run.

High-performance GC/MS analysis requires derivatisation strategies that allow for simultaneous analysis of a wide range of structurally different targeted and non-targeted compounds, sufficient chromatographic resolution, and high informational value of the obtained mass spectra. The latter are both required for unambiguous peak assignment and quantitation, respectively, and can be controlled by choosing a suitable derivatisation reagent [6]. Silylation, methylation, acetylation, and trifluoroacetylation (TFA) are single-step derivatising techniques that are widely employed in the analysis of polyalcohols and non-reducing sugars [7]. The analysis of reducing sugars is more challenging due to the variety of isomers that co-exist in aqueous solutions with instable hemiacetals being usually the dominating species [8]. Derivatisation of an aldohexose, for example, can theoretically afford five tautomers: α- and β-pyranose, α- and β-furanose, and the respective open-chain aldohexose [9]. Two- or multi-step derivatisation procedures have been developed for GC/MS analysis of carbohydrate-rich samples to reduce the number of isomer-derived peaks and to improve both chromatographic resolution and informational value of mass spectra, which is of particular use for complex matrices [10].

Two-step derivatisation approaches commonly convert first the carbonyl group into a specific derivative that no longer supports interconversion of the different tautomers described above [6]. This can be accomplished either by reduction, which affords the respective primary alcohols (alditols), or by oximation using (substituted) hydroxylamine.

In the second step, the remaining “un-protected” primary and secondary hydroxyl groups are derivatised such as by (trifluoro) acetylation (alditol acetates, oxime acetates), which leaves the originally polar carbohydrates lipophilic compounds with a considerably higher volatility. However, even though two-step derivatisation approaches have commonly the great advantage that only one or two derivatives for each sugar is formed, their applicability suffers sometimes from certain weaknesses such as the large number of manual processing steps needed or the fact one and the same derivative can be formed from different sugars as reported for alditol acetates [10].

Unlike reduction, oximation quantitatively converts reducing carbohydrates that preferably form cyclic hemiacetals into the corresponding open-chain aldose derivatives.

During this process, the anomeric carbon atom is converted via prochiral aldehyde groups into a stereo isomeric center that can form the corresponding *syn*- and *anti*-isomers. The benefit of oximation from the analytical point of view is that the number of derivatisation products obtained from reducing carbohydrates can be reduced from five to two peaks [8,11], and even down to one single peak for aldoses if the latter are dehydrated and subsequently converted into the respective aldonitrile acetates [10]. In general, oximation simultaneously improves the chromatographic resolution; it disperses and, therefore, separates peaks originating from reducing sugars and those derived from alditols or lactones [8] and protects α-keto acids from decarboxylation [5].

The conversion of reducing sugars into oxime derivatives has been recently advanced using *O*-alkylated or *O*-aralkyl substituted hydroxylamines, such as *O*-methylhydroxylamine[8], *O*-ethylhydroxylamine [4,12] and *O*-benzylhydroxylamine [8], commonly applied as the respective hydrochloride, instead of the unsubstituted oximation reagent [13]. This measure has been confirmed as affording a higher oximation performance due to both higher nucleophilicity of the amine and higher informational value of the respective mass spectra [14].

Cu(II)-catalyzed formation of cyclic *O*-isopropylidene derivatives [15] under acidic conditions using acetone as derivatising agent is another technique that has been successfully applied to carbohydrate analysis. The low mass increment of 20 g mol^{-1} introduced per hydroxyl group (96 g mol^{-1} for trifluoroacetylation, 72 g mol^{-1} for trimethylsilylation) is probably the major advantage of this derivatisation approach [5].

The aim of the current study was to evaluate different derivatisation approaches with regard to their performance for GC/MS analysis of carbohydrate-rich, complex matrices. The evaluation criteria were derivatisation efficiency, chromatographic characteristics (number of isomers formed per compound, resolution etc.), analytical sensitivity (limit of detection), informational value of mass spectra and hence reliability of peak assignment, reproducibility of quantification results, and robustness toward matrix effects. Besides common single-step derivatisation techniques, such as per-trimethylsilylation and *O*-isopropylidenation, two-step approaches comprising sequential oximation (with *O*-ethyl- or *O*-benzylhydroxylamine) followed by trifluoroacetylation or trimethylsilylation were also studied. The different derivatisation approaches were compared using a set of eight monosaccharides (C_5 to C_6), glycolaldehyde (GA), and dihydroxyacetone (DHA). Even though the latter two compounds are not members of the monosaccharide family in the strict sense due to the lacking stereo center, these di- (GA) and trioses (DHA) were included in the study as they are involved in many metabolic processes. The different derivatisation methods were also applied to “real” carbohydrate-rich samples of biological and synthetic origin to verify the results obtained for the set of reference compounds described above. Furthermore, their robustness toward matrix effects was investigated based on recovery rates, which were determined by a standard addition approach.

2. Materials and methods

2.1. Chemicals and reagents

All reference compounds (Table 1), the internal standard methyl β-D-galactopyranoside, calcium hydroxide, anhydrous pyridine, 36% aqueous formaldehyde stabilized with CH_3OH, anhydrous $CuSO_4$, molecular sieve 3 Å, anhydrous sodium carbonate, dichloromethane (DCM), ethyl acetate, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), *N*-methyl-bis(trifluoroacetamide) (MBTFA), *O*-ethylhydroxylamine hydrochloride, *O*-benzylhydroxylamine hydrochloride and 4-(dimethylamino)pyridine (DMAP) were purchased from Sigma–Aldrich (Sigma–Aldrich Handels GmbH, Vienna, Austria). D-glucose-$^{13}C_6$, 99 atom% $^{13}C_6$ (^{13}C-glucose) were supplied by Isotec, Miamisburg, OH, USA. Methanol, sulfuric acid, hydrochloric acid, and anhydrous acetone were obtained from Carl Roth (Graz, Austria). Acetone used for preparation of *O*-isopropylidene derivatives was further dried over freshly vacuum-dried molecular sieve 3 Å in an argon atmosphere for two days. Sodium hydroxide was obtained from Merck (Vienna, Austria). All standards, chemicals