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Ethoximation-silylation approach for mono- and disaccharide analysis and characterization of their identification parameters by GC/MS

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The qualitative and quantitative analysis of complex carbohydrate mixtures is a challenging problem. When tackled by GC/MS, close retention times and largely similar mass spectra with no specific features complicate unambiguous identification, especially of monosaccharides. An optimized pre-capillary ethoximation-silylation GC/MS method for determination of monosaccharides and disaccharides was applied to a wide range of analytes (46 compounds). The two-step derivatization resulted in a pair of *syn* and *anti* peaks with specific retention and intensity ratio. The resulting dataset of mass spectra was subjected to a PCA-based pattern recognition. An oxime peak identifier (OPI) of the carbohydrate analytes, based on the combination of an internal standard and the corresponding *syn*/*anti* peak ratios, increased the reliability of the identification of reducing carbohydrates. Finally, the introduced EtOx-TMS derivatization method was applied to four different carbohydrate matrices (agave sirup, maple sirup, palm sugar, and honey).

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1. Introduction

Carbohydrate analysis is diverse, multifaceted, and encountered in a large number of applications. One important area is the identification and quantification of low-molecular weight carbohydrates, especially monosaccharides and small oligosaccharides, in different matrices. Examples where this type of analysis is needed are the evaluation of stress responses for plant breeding [1], analysis of complex mixtures from different biorefinery scenarios for production of biofuels and biomaterials [2], and food quality monitoring [3].

Analysis of mono- and disaccharide constituents in complex mixtures is commonly performed by MS-hyphenated gas chromatography (GC/MS) following an appropriate pre-capillary derivatization [4]. Gas chromatography today is an affordable and widespread technique and is superior to capillary electrophoresis and high performance liquid chromatography due to its relatively high resolution and sensitivity [5]. Its main limitation arises from the similar molecular weights of the carbohydrate analytes, and determination beyond trisaccharides is usually not feasible. All carbohydrates, before analysis by GC/MS, require a suitable derivatization to convert them into volatile derivatives since they naturally exhibit high polarity, pronounced hydrophilicity with a strong tendency to hydrogen bonding, and near -zero volatility [6]. The derivatization strategies aim at enhancing signal intensity and compound stability, increasing the information content of the mass spectra, and improving quantification [7].

A wide range of derivatization strategies is available for GC/MS carbohydrate analysis, and the type of pre-capillary derivatization is the main factor that distinguishes the different approaches. Silylation and trifluoroacetylation reactions are single-step derivatization methods that are widely employed in analysis of polyalcohols and non-reducing sugars [8,9]. Nearly all functional groups are present in the relevant analytes and most of them are problematic in gas chromatographic analysis in one way or another due to their polarity and hydrogen bonding capacity. These groups, which include hydroxyl, amine, amide, phosphate and thiol groups can be converted into their trialkylsilyl derivates by displacement of the active proton [10,11]. The reagent N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), introduced by Stalling et al. [12], has the ability to react with all common protic sites and has become a quasi-standard derivatization reagent. The high volatility of the derivatization by-products, namely trimethylsilyl trifluoroacetamide and trifluoroacetamide, is an additional advantage over alternative silylation reagents [11]. Trimethylsilyl chloride (TMS-Cl) has been added to BSTFA as a catalyst to increase the silyl donor strength [6]. When used on its own as reagent, TMS-Cl is often combined with pyridine, which acts as a basic auxiliary and HCl trap, as it does with other trialkylsilyl halides [13]. The advantages of pyridine are its catalytic capability [14] and the increased stability of the silylated products in its presence [15]. A mixture of 4-(dimethylamino)pyridine (DMAP) and pyridine, when used at ambient temperatures, has been shown to minimize side reactions during

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silylation and acylation, and thus reduce the complexity of the chromatograms [16].

The analysis of mixtures of reducing monosaccharides in solution is generally hindered by their structural similarity and the existence of up to five isomers (α- and β-pyranosides, α- and β-furanosides, and the open-chain form) per monosaccharide [17,18]. The eight hexoses, for instance, may form up to 40 of these configurational isomers, each of which might appear as a separate peak in the chromatograms. After silylation, these isomer peaks increase the risk of peak overlapping, especially in complex mixtures, although a well-separated peak from one of the isomers can be used for quantification and might also improve the possibility of compound identification [19,20].

Oximation of carbonyl functionalities eliminates the occurrence of furanosidic and pyranosidic isomers, and thus significantly reduces chromatogram complexity. The sensitivity of the sugar analysis is boosted because of the increasing signal intensity with decreasing amounts of isomers and peaks [21,22]. At the same time, the formed oximes also result in only two peaks corresponding to the *syn* (*E*) and *anti* (*Z*) forms of the oxime. Non-reducing carbohydrates (e.g., sucrose, trehalose, raffinose) show only one peak, since they cannot form the corresponding oxime due to the missing aldehyde (hemiacetal) function [23].

Combining silylation (for rendering the compound volatile) and oximation (for reducing the numbers of isomers, and thus, peaks) consequently requires a two-step derivatization strategy: (1) conversion into oximes with hydroxylamine or alkoxyamines, prior to (2) trialkylsilylation with an appropriate silylating agent. Oximation is always carried out as the first derivatization step. An additional advantage of an oximation step is the protection of α-keto acids against decarboxylation [24]. In previous work, reducing carbohydrates were analyzed as their oxime [25], O-methyloxime [18], O-ethyloxime [8], and O-benzyloxime trimethylsilyl derivatives [18].

The distinction of monosaccharides by MS-hyphenated chromatographic techniques, based on retention times and mass spectra, is rather difficult and attempts have revealed the general difficulties in this type of carbohydrate analysis [26]. The derivatized compounds of similar molecular weight show only small differences in retention time, which results in partial peak overlapping or even complete coelution. The mass spectra of these structural isomers are quite similar and differences are mainly related to fragment intensity. This similar fragmentation pattern of carbohydrate isomers largely hampers the use of databases for identification purposes.

In a previous study of carbohydrate derivatization with oximation reagents followed by trimethylsilylation, we applied ethoximation followed by trimethylsilylation derivatization to complex matrices of biological (grapevine leaf) and synthetic (formose mixture) origins. This method showed advantages compared to other derivatization approaches due to low limits of detection and quantitation, minor relative standard deviations, and low sensitivity toward matrix effects [26]. Peak assignment was supported by NMR and provided information on the peak ratios: 2-deoxyhexoses and ketohexoses formed almost equal ratios of the *syn* and *anti* peak, while aldopentoses and aldohexoses were solely reported as the *syn* form [27,28]. However, complex matrices may easily contain more sugars than are available as reference standards and *syn*/*anti* peak ratios might be influenced by matrix effects.

All these difficulties point to the great need for a standardized and robust analytical method that overcomes at least some of the current limitations in qualitative and quantitative mono- and disaccharide analysis, especially regarding the problems of similar mass spectra and close retention times. Obligatory methodological requirements are high derivatization efficiency, reproducibility, and stability of products, low number of by-products, and a constant ratio of *syn*/*anti* forms of the oximes. Other important, but less pressing, demands are easy handling, acceptable analysis times, and low overall costs. Today's requirements also include compatibility with automated derivatization robots and the possibility for simultaneously analysis of a wide range of other organic (non-carbohydrate) compounds (e.g., in metabolome analysis).

In this study, we communicate a two-step derivatization method that begins with an initial formation of O-ethyloximes, followed by trimethylsilylation, for optimized GC/MS analysis of mono- and disaccharides in complex matrices. Derivatization conditions and derivatization efficiency were optimized. The chromatographic and mass spectra characteristics for 46 carbohydrates ranging from carbohydrate-related C2- and C3-bodies to monosaccharides (tetroses to heptoses) and up to disaccharides and one trisaccharide. Of particular interest were the separation and identification characteristics of derivatized carbohydrates with similar structures and identical masses. An oxime peak identifier (OPI) to improve carbohydrate identification is presented, which was elaborated based on the combination of an internal standard and the retention of the corresponding *syn*/*anti* peaks of the reducing carbohydrates.

2. Material and methods

2.1. Chemicals and reagents

The reference compounds (Supplemental Table S1) included 1,3-dihydroxyacetone dimer, glycolaldehyde dimer, methylglyoxal solution (ca. 40% in H_2O), D-(+)-glyceraldehyde, D-(−)-threose, L-(+)-erythrulose, D-(−)-erythrose, D-(+)-xylose, D-(−)-lyxose, D-ribulose, D-psicose, D-(−)-tagatose, D-allose, D-(+)-mannose, L-(+)-gulose, D-(+)-galactose, D-(+)-talose, D-altrose, D-(+)-glucose, D-apiose solution, D-(−)-arabinose, D-(−)-fructose, D-(−)-ribose, L-sorbose, 2-deoxy-D-ribose, 2-deoxy-D-glucose, D-(+)-fucose, L-(+)-rhamnose, D-(+)-digitoxose, D-glucoheptose, D-(+)-trehalose (glucose-α,α'-(1→1)-glucose), sucrose (glucose-α-(1→2)-fructose), D-(+)-turanose (glucose-α(1→3)-fructose), D-(+)-maltose monohydrate (glucose-α-(1→4)-glucose), D-(+)-cellobiose (glucose-β-(1→4)-glucose), D-lactose monohydrate (galactose-β-(1→4)-glucose), lactulose (galactose-β-(1→4)-fructose), xylobiose (xylose-β(1→4)-xylose), maltulose monohydrate (glucose-α(1→4)-fructose), leucrose (fructose-(1→5)-glucose), β-gentiobiose (glucose-β-(1→6)-glucose), D-(+)-melibiose (galactose-(1→6)-glucose), palatinose hydrate (glucose-(1→6)-fructose), D-(+)-raffinose, the internal standard methyl α-D-galactopyranoside, C7–C40 saturated alkane mixture, anhydrous pyridine, ethyl acetate, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), O-ethylhydroxylamine hydrochloride, 4-(dimethylamino)pyridine (DMAP), dimethyl formamide (DMF), dimethyl sulfoxide (DMSO) and trimethylsilyl chloride (TMS-Cl); all were purchased from Sigma-Aldrich-Fluka (Sigma-Aldrich, Schnelldorf, Germany). All standards, chemicals, and reagents were of GC grade and used without further purification. L-*glycero*-D-Mannoheptose and D-sedoheptulose were kindly provided by P. Kosma, Department of Chemistry, BOKU, Vienna, Austria.

2.2. Carbohydrate samples

Different carbohydrate-containing samples of biological origin were analyzed with the EtOx-TMS method, focusing on fructose, glucose, and sucrose content. Orange honey from Spain (Allos GmbH, Drebber, Germany), buckwheat honey (Rainbauer, St. Magdalena, Austria), honeydew honey from EU and non-EU countries (Honigmayr, Tenneck, Austria), agave sirup from Mexico (Allos GmbH, Drebber, Germany), maple sirup (grade C) from Canada (Dennree GmbH, Töpen, Germany), and palm sugar (Gula Java Brut/Amanprana, coconut blossom sugar) from Indonesia (Noble House, Brasschaat, Belgium) were purchased from local supermarkets.

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