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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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ABSTRACT

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).

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 co-elution. 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.

2.3. Optimized method

2.3.1. Derivatization: O-ethyloximation (EtOx)/trimethylsilylation (TMS)

Different volumes (0.5 μl–1000 μl) of carbohydrate model solutions (0.4 mg/ml in MeOH/H_2O, 80:20, v/v) and the honey sample were lyophilized. The lyophilized model mixtures (containing 0.2 μg–400 μg per carbohydrate) and the honey sample (2.33 mg) were dissolved in 200 μl of anhydrous pyridine containing 40 mg/ml O-ethylhydroxylamine hydrochloride and 1 mg/ml methyl α-D-galactopyranoside (internal standard, IS) and were heated at 70 °C for 1 h. Subsequently, 200 μl of a solution of 1.5 mg/ml DMAP in pyridine was added to the mixture, followed by 200 μl of BSTFA (containing 10% TMCS). The mixture was heated at 70 °C for 2 h. The derivatized samples were kept at −20 °C until analysis.

2.3.2. GC/MS analysis

GC/MS analysis was performed on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass selective detector. Column: HP-5 MS (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA); carrier gas: helium, injector: 280 °C; column flow: 0.9 ml/min; purge flow: 32.4 ml/min, 0.6 min; oven program: 50 °C (2 min), 5 °C/min, 280 °C (20 min); MS: EI mode, 70 eV, source pressure: 1.13×10^{-7} Pa, source temperature: 230 °C. Scan range was set from 50 to 950 Da. The derivatized samples were diluted with ethyl acetate (600 μl) and filtered prior to injection. Aliquots of 0.2 μl were introduced into the splitless injector with an Agilent GC Sampler 120.

2.4. Peak identification and quantification

Peak assignment and quantification was accomplished with MSD Chemstation E.2.01.1177 (Agilent Technologies, USA). Peaks were assigned by comparing their retention times and mass spectra with those of respective reference compounds. Calibration curves were based on peak areas obtained for up to ten concentration levels.

2.5. Method validation

The optimized method was validated for all reference compounds with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), and precision. Regression coefficients were determined as acceptance criterion for linearity. The linearity of the calibration curve, based on peak area, was evaluated with respect to concentration, corrected by an internal standard. The LOD and LOQ were calculated according to the 3σ- and 10σ-criteria, i.e., the three- and tenfold standard deviation of the noise quantified by single point calibration (8.33 μg/ml), according to DIN 32465:2008–11 [29]. The stability of the GC/MS performance (retention time, peak response and peak geometry) was confirmed by repetitive analysis of identical standards.

2.6. Statistical analysis

Multivariate data analysis was performed with Origin 8.6 software (OriginLab, Northampton, MA, USA). Principal component analysis (PCA), an unsupervised clustering method, was used to compare GC/MS originated mass spectra of different carbohydrates to uncover data structures that account for a large percentage of the total variance and to create new hypothetical constructs that may be employed to predict or classify observations into groups. Each mass spectrum was collected as a set of raw intensities and normalized during calculation. PCA was applied using the eleven mass fragments with the largest fragment intensity. The calculated eigenvalues greater than one were used as criteria to determine the number of components.

3. Results and discussion

3.1. Method optimization

The chromatographic and mass spectrometric characteristics of the ethoxime-TMS derivatives of 46 carbohydrates and carbohydrate-related compounds were analyzed. The substances were selected in a way that comprised all compounds relevant in biorefinery analytics, plant hydrolysates, and plant metabolom analysis.

The derivatization procedure and GC/MS conditions were optimized using a solution that contained mono-, di- and trisaccharides in water/methanol solution (80:20, v/v) at a concentration of 100 μg/ml each. The effects of different solvents (pyridine, N,N-dimethylformamide, dimethyl sulfoxide) on the efficiency of ethoximation and silylation were studied. The success of the ethoximation reaction was investigated with regards to the concentration of O-ethylhydroxylamine hydrochloride (20–60 mg/ml solvent) and the time of the derivatization (30–120 min). Further parameters studied for optimization included the volume ratio between solvent and silylation reagent (1:1–2:3, v/v), silylation temperature (room temperature, 50 °C, 70 °C), and silylation time (30–240 min). The effect of catalysts on the derivatization efficiency was examined at various concentrations of DMAP (0–1.5 mg/ml) and TMS-Cl (0–10%). Derivatized samples were either diluted with ethyl acetate or dried with nitrogen gas and re-dissolved in ethyl acetate prior to GC/MS analysis. Optimization of the GC/MS program mainly involved the heating rate (4–12 °C min^{-1}) and the column flow (0.6–1.3 ml min^{-1}).

We verified that an oximation time period of 1 h at 70 °C was sufficient to complete the conversion of saccharidic carbonyl groups into oximes, as no non-ethoximated TMS-derivatives were observed. The optimization of the silylation procedure showed no significant peak area increase between 1–2 h of derivatization, so that 1 h appeared to be a sufficiently long reaction time. However, to ensure complete derivatization of compounds in complex matrices, silylation time was set to 2 h at 70 °C. The high volatility of derivatization by-products and the possibility for injection of the reaction mixture directly into GC without harming the GC columns led to a preference for use of BSTFA over other silylation agents [11].

The overall two-step derivatization strategy is straightforward and facile. The combination of oximation and trimethylsilylation can be carried out in the same vial and allows injection of the reaction mixture into GC/MS without any additional sample manipulation.

Different techniques were applied to enhance method sensitivity, such as stripping the solvent in a stream of nitrogen and removing excess BSTFA by rotavaporation. However, these attempts to reduce the volume of the reaction mixture led to precipitation of sugar phosphates, so they were not subsequently performed so that the range of application was not limited. Pyridine, DMF, and DMSO were tested as solvents to optimize the efficiency of ethoximation and silylation. Eventually, pyridine was chosen as the most promising solvent for the combination of both ethoximation and silylation. The advantage of pyridine is its catalytic effect, in addition to its action as a solvent for derivatized compounds. The amount of the derivatization mixture used must be sufficient to cover the complete sample during the ethoximation process; see experimental section. The volumes applied should be as small as possible to maintain good method sensitivity, but at the same time enough solvent must be present to avoid precipitation of excess O-ethylhydroxylamine hydrochloride.

The derivatization catalysts TMCS and DMAP were used to further increase the silylation efficiency. The combination of DMAP and TMCS with BSTFA was used for the first time in the present

study. Both reagents, on their own, bring about an increase in silylation efficiency, but their combination had a distinct synergistic effect. Since the two catalysts induced formation of by-products during the oximation reaction, they were added afterward, in the silylation step, together with BSTFA as the actual reagent. The addition of the two derivatization catalysts to the silylation mixture had no negative influence on the previously formed oximation products.

Optimum chromatographic peak resolution was obtained with a heating rate of 5 °C min^{-1} and a gas flow of 0.9 ml min^{-1}. Even in cases of structurally closely related carbohydrates (e.g., members of the aldohexose series), a qualitative differentiation was easily possible. No complete overlapping of the *syn* and *anti* peak of a carbohydrate with the peaks of other carbohydrates was observed under the conditions investigated.

3.2. Method validation

In most cases, biological samples (and their hydrolysates) contain larger amounts of glucose, fructose, and sucrose, while other carbohydrates are present in smaller amounts. Therefore, a broad concentration range was covered for calibration curves, in order to allow quantification of carbohydrates with largely different concentrations in one run.

Aliquots (0.2 μl) of the derivatization mixture (1.2 ml), containing carbohydrate concentrations from 0.16 μg/ml μg to 333 μg/ml, were injected in splitless mode. Linearity of the calibration plots was satisfactory for most metabolites, with regression coefficients better than 0.99. The high linearity of peak area-based calibration curves confirmed that quantitative analysis was possible by means of the single ethoxime-TMS isomer peaks (either *syn* or *anti*). Quantification limits were below 1 pg and up to 70 pg on-column; only methylglyoxal, glucoheptose, and raffinose show higher values (see Supplemental Table S1). No MS detector saturation or non-linearity of the calibration curves was observed, not even at the highest concentrations tested. Calibration equations and response factors are given in Supplemental Table S1 and examples are shown in Supplemental Fig. S2. As the *syn*/*anti* ratio increased, so did the sensitivity for the respective carbohydrate, so that carbohydrates with only one eluting peak, as well as those with very prominent *syn* peaks, showed LODs well below 1 pg on-column (Supplemental Table S1).

3.3. GC-separation characteristics

3.3.1. Peak separation

The challenge of carbohydrate analysis is the good baseline separation of isomers of high structural similarity. The chromatographic behavior of 46 carbohydrates was examined in the present study. The carbohydrates were grouped according to their number of carbon atoms (e.g., tetroses, pentoses, hexoses, etc.), the availability and position of a carbonyl group (e.g., non-reducing sugars, ketoses, and aldoses) and stereoisomers within one class (e.g., aldohexoses: glucose, mannose, gulose, etc.), see Fig. 1 and Supplemental Fig. S1a and b. A temperature increase of 5 °C/min in the GC oven program resulted in a good chromatographic resolution of all carbohydrates investigated. High molecular weight derivatives, such as the trisaccharide raffinose, show high elution times (62.11 min) and resulted in a relatively low detector response. For less complex mixtures, a higher temperature increase of the oven program is feasible to increase the sample throughput. Corresponding to theory, only one peak can be observed for the non-reducing carbohydrates, such as methyl-galactopyranoside, sucrose, trehalose, and raffinose, as no oximation occurs and thus no *syn*/*anti* oximes are formed. In addition, the reducing carbohydrate apiose, the aldopentoses arabinose and ribose, the ketohexoses sorbose and fructose, and the disaccharide lactulose only generated one peak, due to insufficient separation of the *syn* and *anti* oxime peaks. Even though both isomers occurred, the deconvolution of *syn* and *anti* peak was not possible due to the equal fragmentation patterns. Dihydroxyacetone does not produce *syn* and *anti* forms upon oximation, and hence only one peak was observed.

Apart from these exceptions, all other mono- and disaccharides showed the typical occurrence of two peaks (*syn*/*anti*) of ethoxime-TMS derivatives (Fig. 1; Supplemental Fig. S1a and b). Several co-eluting *anti* peaks were observed, such as those of galactose and glucose, as well as mannose and gulose, while their *syn* peaks could neatly be separated (Supplemental Fig. S1a). The optimized conditions provided at least one well-separated peak for each carbohydrate. However, with increasing number and concentration of carbohydrates in the mixtures, especially in the case of hexoses and disaccharides, overlapping of peak tails was evident (e.g., in the mixture of all 46 saccharides). Even though the replacement of hydroxylamine [30,31] by O-ethyl hydroxylamine did not afford a significant improvement of chromatographic

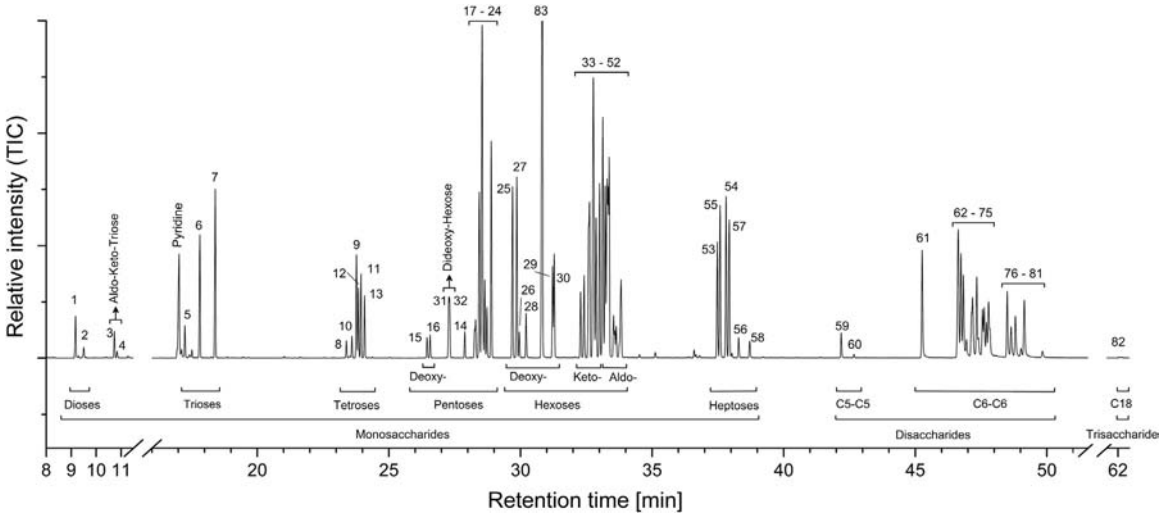

Fig. 1. Gas chromatogram with MS detection of a mixture of 46 carbohydrates and carbohydrate-derived compounds converted to ethoxime-trimethylsilyl (EtOx-TMS) derivatives according to the optimized analytical procedure (carbohydrate concentration 42.5 μg/ml; internal standard concentration 166.6 μg/ml; injected volume 0.2 μl). A higher resolution of EtOx-TMS derivatized pentoses, hexoses and disaccharides is given in Supplemental Fig. S1a and b. Description of peak assignment is given in Supplemental Table S1 (supplementary part).

resolution, the ethyl substituent is considered to increase the nucleophilicity of the derivatisation reagent, impede follow-up reactions that could occur in complex matrices, and simplify mass spectrometric fragmentation. For highly complex sugar mixtures, we recommend applying a calibration based on peak height instead of peak area, to avoid integration errors caused by peak tailing and peak overlapping.

3.3.2. Structural conformation and chromatographic retention

Obvious retention time differences were evident between the different groups of carbohydrates, ranging from tetroses to disaccharides (Figs. 1 and 2). However, the individual isomers within one carbohydrate family, with their equal molecular weights and similar structures, also showed small differences in their retention times. The relationship between molecular weight and retention time (Supplemental Fig. S3) of the 46 carbohydrate analytes showed a very good correlation ($R^2=0.989$).

Besides the molecular weight, the conformation of the oxime derivatives is an important factor influencing the chromatographic retention of the isomers [32]. Snyder [28] concluded from NMR spectroscopy that the acyclic oximes of aldopentoses adopt different geometries: the derivatives of arabinose and lyxose have a planar “zigzag” arrangement, whereas those of ribose and xylose show a “bent” or “sickle” conformation. We can assume that these types of differences are also responsible for differences in the retention time within the groups of pentoses, hexoses, or heptoses. Close retention times of stereoisomers (Fig. 1; Supplemental Fig. S1a and b) complicate the identification process, and yet they became the crucial factor and often the only means of distinguishing different carbohydrates [23].

Different from the Kovats retention index for isothermal elution conditions that is based on non-linear increments added to the retention times of consecutive peaks [33], a constant increment is added to the retention times of respective precedent peaks for the linear retention index (LRI) or programmed-temperature retention index (PTRI) which is commonly used in temperature-programmed gas chromatography. The calculation of LRI can be accomplished according to the non-logarithmic equation proposed by Van Den Dool and Kratz [34,35].

The retention data, obtained for compounds found in an EtOx-TMS derivatized Spanish orange honey sample (Fig. 3) and the corresponding compounds in the carbohydrate standard solution, were used to calculate the values of relative retention (rG; Eq. (1)) and the linear retention index (LRI). Although complex biological matrices are known to cause retention time drifts, which can negatively affect the rG precision [36], the rG values in this study showed the most stable values between the standard solution and the honey sample (Table 1). The LRI showed stable values with low variability. The advantage of both indices is that the calculation is based on one peak of the compound and can be applied to non-reducing carbohydrates or when the corresponding oxime peak coelutes.

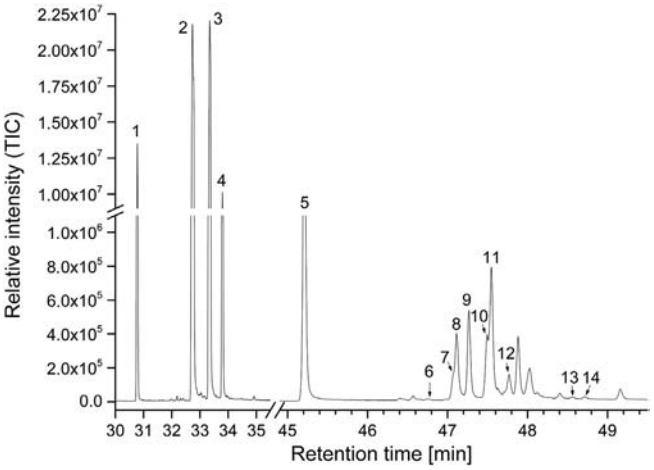

Fig. 3. Chromatogram of an EtOx-TMS derivatized honey sample (Orange/Spain). For peak description, see Table 1.

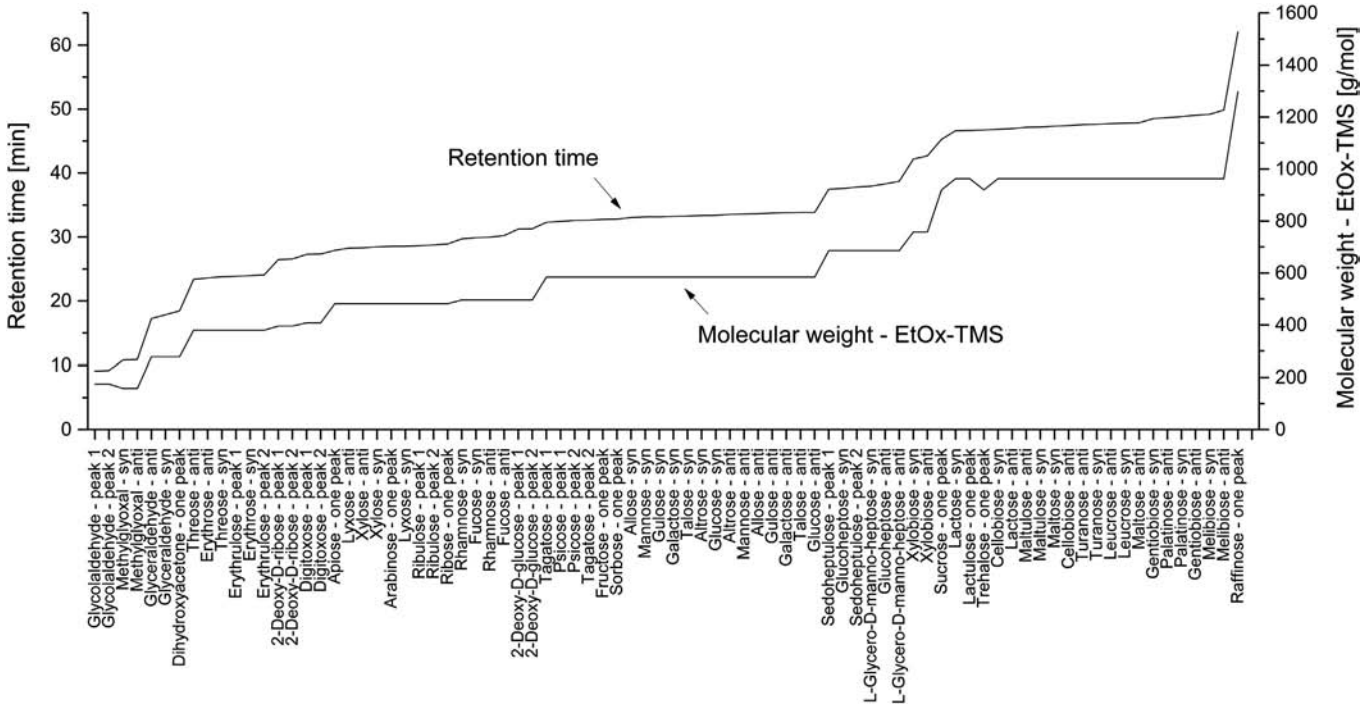

Fig. 2. Course of retention time (left *y*-axis) and corresponding molecular weight (right *y*-axis) of 46 carbohydrate analytes converted to ethoxime-trimethylsilyl (EtOx-TMS) derivatives.

Table 1
Overview of calculated retention values between a carbohydrate standard compound solution and a honey sample (Orange/Spain).

No	Compound	Standard compound solution			Orange honey		
		R.T. [min]	rG	LRI	R.T. [min]	rG	LRI
1	Methyl-galactopyranoside	30.770	1.000	1858.67	30.781	1.000	1859.21
2	Fructose	32.706	1.063	1956.63	32.734	1.063	1958.08
3	Glucose (*syn*)	33.306	1.082	1987.61	33.348	1.083	1989.78
4	Glucose (*anti*)	33.762	1.097	2011.67	33.799	1.098	2013.67
5	Sucrose	45.197	1.469	2711.14	45.210	1.469	2712.07
6	Cellobiose (*syn*)	46.759	1.520	2822.78	46.758	1.519	2822.70
7	Maltulose (*anti*)	47.076	1.530	2846.07	47.074	1.529	2845.92
8	Maltulose (*syn*)	47.107	1.531	2848.35	47.114	1.531	2848.86
9	Maltose (*syn*)	47.263	1.536	2859.81	47.268	1.536	2860.18
10	Turanose (*anti*)	47.484	1.543	2876.05	47.498	1.543	2877.08
11	Turanose (*syn*)	47.546	1.545	2880.60	47.548	1.545	2880.75
12	Maltose (*anti*)	47.757	1.552	2896.11	47.770	1.552	2897.06
13	Palatinose (*anti*)	48.561	1.578	2953.26	48.559	1.578	2953.12
14	Palatinose (*syn*)	48.726	1.584	2964.96	48.718	1.583	2964.40

R.T.=Retention time.
rG=Relative retention=R.T. compound/R.T. internal standard (α-methyl-galactopyranoside).
LRI=Linear retention index according to Van Den Dool and Kratz [34].

3.3.3. Retention time shift of the syn/anti oxime peak pairs

The elution order of the *syn* and *anti* oxime peaks is directly associated with the structural properties of the carbohydrate, such as the position of carbonyl group (keto/aldo), molecular weight, and type of carbohydrate subunits in disaccharides. The present study showed an elution order of the *syn* peak appearing before the corresponding *anti* peak for aldohexoses and aldoheptoses. The aldotrioses, aldotetroses, and aldopentoses showed the opposite chromatographic behavior, with the *syn* peak following the *anti* peak (Fig. 4).

The greatest time shift between *syn* and *anti* peaks was observed for aldoheptoses. Fructose-containing disaccharides (maltulose, turanose, leucrose, and palatinose) showed a relatively small time shift and the *anti* peak eluted before the *syn* peak, similar to the two oxime peaks of ketohexoses. All other investigated disaccharides showed a *syn* peak that eluted before the *anti* peak, as in the aldohexose group (Fig. 4).

The data in Fig. 4 show a coherent effect of the position of the glycosidic linkage on the elution order. We observed a *syn* peak elution order of 1,4-linked disaccharides (xylobiose, lactose, cellobiose, maltulose, and maltose), before 1,3- (turanose), 1,5- (leucrose) and 1,6-linked disaccharides (gentiobiose, palatinose, and melibiose). Garcia-Raso et al. [37] proposed that the glycosidic bond affects the retention time via its effect on the overall molecular shape of the disaccharide, suggesting that the higher retention of the 1,6-disacharides is due to their greater conformational flexibility. Their data on monosaccharides also indicated that more planar forms translate into higher retention times [37]. These results fully agreed with our findings on the retention characteristics of the ethoxime derivatives.

Close retention times of the *syn* peaks and a similar *syn*/*anti* peak time shift were observed for the following carbohydrates: allose/gulose, galactose/talose, and turanose/leucrose, which complicated the identification of these sugars. In any case, the elution order of *syn* and *anti* peaks and their time shifts were characteristic parameters of the individual carbohydrates that were used for their unambiguous identification.

3.3.4. Syn/anti peak ratios

The different carbohydrate groups showed characteristic *syn*/*anti* peak ratios after ethoximation (Fig. 5). The silylation procedure maintained the ratio of the two oxime peaks [27]. The mass spectra of *syn* and *anti* forms of the oximes were generally quite similar and reliable structural information could not be easily derived. The peaks were identified in previous studies based on ^{1}H NMR data in pyridine-d5 [27] and D_2O [28]. The major oxime peak formed by aldoses corresponded to the *syn* form, the smaller peak to the *anti* peak. Due to both the lack in reference data for peak assignment of the *syn*- and *anti*-isomers of oximated ketoses and 2-deoxysugars and the fact that the oxime isomer pairs were found to have similar peak areas, the peak caused by the first eluting isomer is now consistently termed as "peak 1" and that of the later eluting respective isomer as "peak 2". We calculated the *syn*/*anti* peak ratios of ethoxime-trimethylsilyl (EtOx-TMS) derivatized saccharides (cf. Supplemental Table S1) at a concentration level of 83.3 μg/ml for each carbohydrate (Fig. 5). In general, aldoses (C3–C6) showed a high *syn*/*anti* peak ratio from 3.93 (galactose) to 9.34 (mannose). Ketoses (C4–C6) had low oxime peak ratios, ranging from 0.54 (tagatose) to 1.38 (ribulose), if the peak area of the first eluting isomer is divided by the peak area of the second eluting isomer. Using the same calculation procedure, small oxime peak ratios were also observed for 2-deoxysugars, whereas the 6-deoxysugars (fucose and rhamnose) showed values similar to aldoses. Despite serious attempts, separation of the *syn*/*anti* peak pair of the two ethoxime-TMS isomers of fructose was unfortunately not possible, so the peak ratio for that monosaccharide is unity. Disaccharides containing galactose, glucose, or xylose (lactose 4.76, xylobiose 4.93, cellobiose 5.75, gentiobiose 7.35, maltose 7.39, and melibiose 7.69) yielded high *syn*/*anti* peak ratios compared to fructose-containing disaccharides, which gave values in the range of ketoses (palatinose 1.32, turanose 1.38, and leucrose 1.71).

The observed *syn*/*anti* peak ratios of monosaccharides were in agreement with the values of the smaller range of O-methyloxime-TMS derivatized carbohydrates studied by Snyder [28] and Funcke and Sonntag [27]. We observed similar *syn*/*anti* peak ratios for monosaccharides and disaccharides that contain the same monosaccharide as the reducing sugar. Hence, we can assume that the reducing end of disaccharides plays an important role in determining the *syn*/*anti* peak ratio of the EtOx-TMS-disaccharides, and thereby, their chromatographic behavior.

3.4. Characteristics of mass spectra

The mass spectra of 83 ethoxime-TMS and TMS (sucrose, trehalose, and raffinose) derivatives were investigated with regard to their fragmentation patterns and structure-related mass ions. The spectra showed a nearly equal distribution of mass fragments between the corresponding *syn* and *anti* peaks as well as similarities between different groups of carbohydrates. The only difference between two

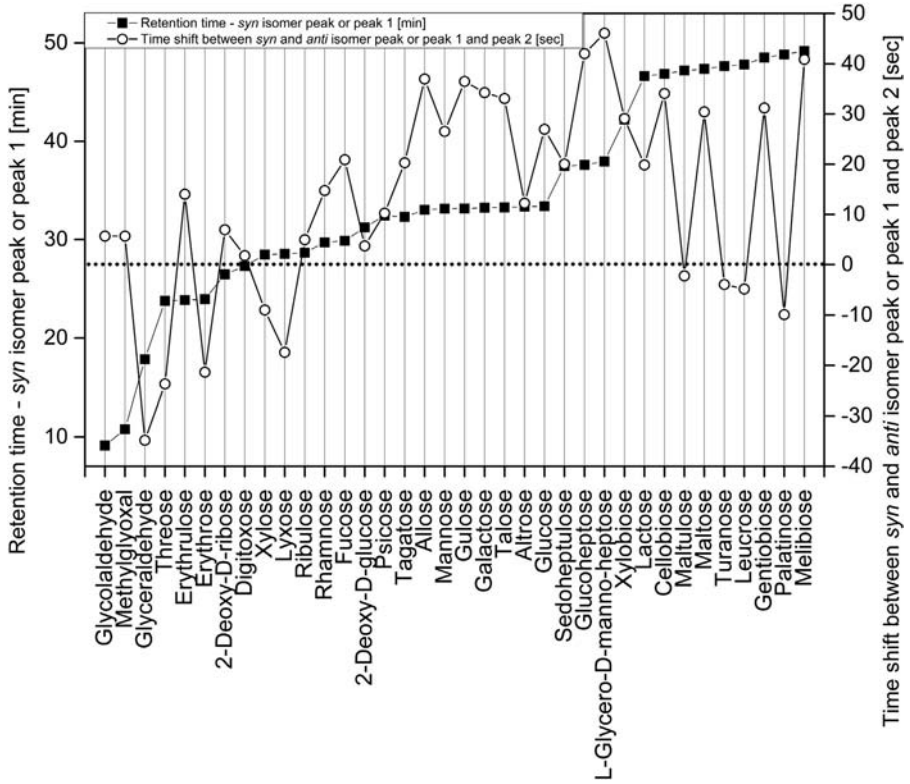

Fig. 4. Retention time of *syn* peaks (▪) and retention time shift (○) between the *syn* peak and corresponding *anti* peak or peak 1 and peak 2 of different ethoxime-trimethylsilyl (EtOx-TMS) derivatized carbohydrates. A positive retention time shift corresponds to the appearance of the *anti* peak after the corresponding *syn* peak. The first eluting isomer of oximated ketoses and 2-deoxysugars has been termed as "peak 1" and that of the later eluting respective isomer as "peak 2".

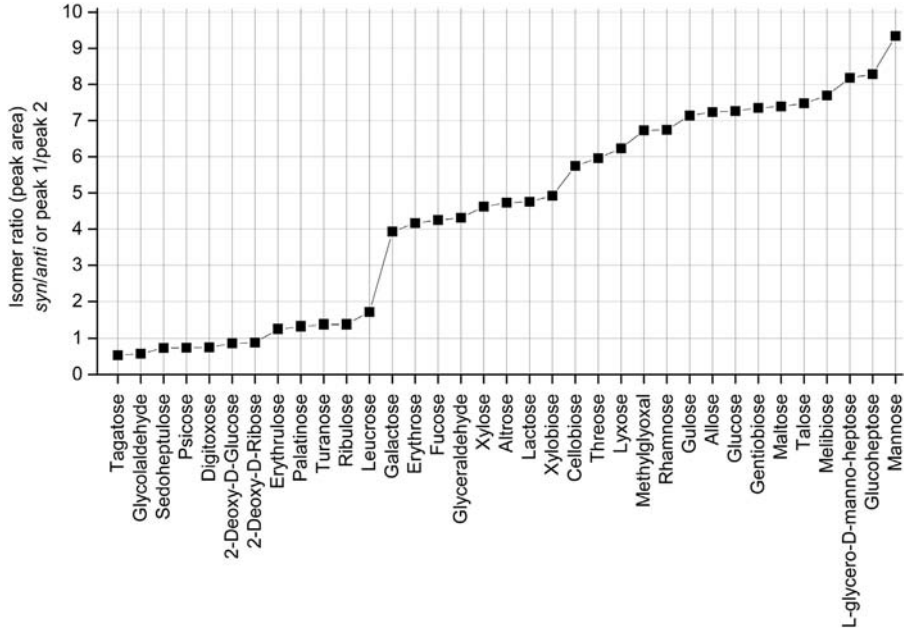

Fig. 5. Isomer ratios (based on peak area) between the *syn* peaks and corresponding *anti* peaks or peak 1 and peak 2 of different ethoxime-trimethylsilyl (EtOx-TMS) derivatized carbohydrates. The first eluting isomer of oximated ketoses and 2-deoxysugars has been termed as "peak 1" and that of the later eluting respective isomer as "peak 2".

corresponding oxime peaks was recognized as a lower overall intensity of fragments of the *anti* isomer compared to *syn* isomer spectra, especially evident for aldohexoses and aldopentoses. MacLeod et al. [38] also observed these differences in methoxime-TMS derivatized keto sugars, where both isomers showed very low intensities of the molecular ion [M] or the [M - CH_3] ion.

The ethoxime-carrying moiety in the mass spectra of the EtOx-TMS derivatized aldohexose D-glucose, selected as example (Supplemental Fig. S4a), appeared as the *m/z* 174 ion (C_2H_5ON=C-CH-OTMS). This fragment contained the former reducing end of glucose. The spectrum of non-oximated TMS-glucose is different from that of EtOx-TMS-glucose; although it contains some

common fragments to both derivatives (*m/z* 73, 103, 147, and 217) it also shows typical fragments as *m/z* 191, 204 (205 as isotopic contribution) and 435, whereas *m/z* 319 is absent or very low. The mass spectrum of EtOx-TMS D-fructose (Supplemental Fig. S4b), a 2-ketohexose, did not yield the *m/z* 174 ion; instead, two ions *m/z* 378 and *m/z* 277 occurred, which represented a C_4- and C_3-part with ethoxime moiety, respectively. Once more, all other fragment ions were independent of the oxime functionality and are similar to the non-ethoximated TMS-derivatives. Laine and Sweeley [39] concluded that the increased stability of bonds directly adjacent to the oxime carbon of ketohexoses caused this difference when compared to aldohexoses. They support their proposal further by the missing *m/z* 319 ion in spectra of the derivatized ketohexoses. Ethoximation gave rise to key fragment peaks in the spectra, which differed between different carbohydrate types (cf. the two examples in Supplemental Fig. S4a and b), and thus introduce a good tool for compound distinction.

Although the mass spectra of EtOx-TMS derivatized stereoisomeric carbohydrates are congruent with regard to the fragment ion pattern, distinguishing between carbohydrate groups may be possible based on fragment intensities (e.g., different intensities of mass ions or characteristic mass ion ratios). Generally, aldoses and ketoses of the respective carbohydrate groups showed opposite intensity behavior of certain mass ions. Aldotetroses showed high *m/z* 117, 174, 204 and low *m/z* 103, while ketotetroses fragmented into high *m/z* 103 and low *m/z* 117, 174, 204. Aldopentoses and ketohexoses were characterized by high intensities of *m/z* 103, 217, 307 and low intensity of *m/z* 204, and the opposite behavior was observed for ketopentoses (high *m/z* 204; low *m/z* 103) and aldohexoses (high *m/z* 174, 204, 319; low *m/z* 103, 217). 6-Deoxyhexoses (rhamnose: 6-deoxy-L-mannose; fucose: 6-deoxy-L-galactose) showed *m/z* 117 as a major ion. This ion was also observed in the mass spectra of the dideoxyhexose digitoxose, but not in 2-deoxy-D-glucose, a decrease of *m/z* 204 and *m/z* 319 was observed here.

Previous studies have shown that the mass spectra of oxime-TMS derivatized disaccharides contain fragments of a cyclic monosaccharide-TMS part that includes the glycosidic oxygen and fragments resulting from an open-chain TMS-monosaccharide with the oxime group [31]. In the present study, we observed an effect of monomer composition of the disaccharide on the fragment intensity patterns. Fructose-containing disaccharides (maltulose, palatinose, turanose, leucrose, lactulose, and sucrose) produced a very low *m/z* 174 compared to disaccharides without fructose. No specific mass fragmentation was observed according to the position of the glycosidic linkage, just intensity differences. This is consistent with previous studies [31,40], suggesting a relationship between mass fragmentation and the structural characteristics of 1,2- or 1,6-linked disaccharides, estimated from the relative intensity of fragment ions, but without giving more detailed information.

Based on the sample mass fragmentation and ion intensity data in the current study, we attempted a pattern recognition of carbohydrate mass fragments using principal component analysis (PCA), an unsupervised exploratory data analysis technique. Where no prior knowledge about the data was introduced during statistical analysis, further sample information was used for interpreting PCA analysis [41]. The principal components (PCs) were used to discover and interpret the dependencies that exist among the variables and to examine relationships that may exist among the spectra of individual carbohydrates [42]. Discrimination analysis techniques were not carried out because they require an assignment of the spectra into groups before analysis. This introduces user bias into the data analysis through emphasizing the differences between assigned groups instead of the differences between mass fragment intensities [41].

PCA was performed on the relative intensities of the eleven highest fragment ions of each mass spectrum of the EtOx-TMS derivatives of 32 monosaccharides, 13 disaccharides, and one trisaccharide and afforded one overall score plot for a total of 83 saccharides (Fig. 6). The score dataset was clustered into different groups associated with different types and sizes of carbohydrates. The first two components combined account for 52.84% of the total variance. The 2D PCA score plot was used for objective comparison of the carbohydrate mass spectra profiles.

Principal component 1 (PC1) separated the clusters of monosaccharides, disaccharides, the trisaccharide raffinose, and methyl-galactopyranoside (internal standard), capturing 28.09% of the total variance. Principal component 2 (PC2) reflected 24.75% of the total variance and separated the clusters of deoxysugars, trioses, aldohexoses, and ketopentoses from the cluster of aldopentoses and ketohexoses, implying high similarity between the members of each group, which was also described above. The score plot of PC1 and PC2 demonstrates an easy differentiation between three groups of disaccharides, containing either (1) xylose–xylose; (2) glucose–glucose and glucose–galactose or (3) glucose–fructose and galactose–fructose. The PCA method revealed that the relative abundances of eleven fragment ions only (*m/z* 73, 103, 129, 147, 174, 204, 205, 217, 307, 319 and 361) were sufficient for a reliable classification.

3.5. Oxime peak identifier (OPI)

Based on our findings, the existence of two corresponding oxime peaks and their characteristic time shift between the *syn* and *anti* peak (Fig. 4) of the EtOx-TMS derivatized carbohydrates can be used as direct and reliable parameters to distinguish between carbohydrates with similar mass spectra. Therefore, we have introduced an oxime peak identifier (OPI). The oxime peak identifier (OPI) represents a relative retention, based on one of the two EtOx-TMS peaks (*syn/anti*), while the other peak is used as dynamic standard in combination with a static internal standard. The OPI value is calculated according to Eq. (2). This novel approach offers significantly more positive match identifications. The equation of the OPI is similar to the calculation of a relative retention [43]. The principle of the positive identification of a particular carbohydrate is the existence of a second peak at a definite position, while the static internal standard acts as anchor point to set the unambiguous relation to other carbohydrates.

$$rG = RT_{target}/RT_{IStd} \quad (1)$$

$$OPI = 100 * (RT_{target} - RT_{corresponding\ oxime\ peak})/(RT_{high} - RT_{low}) \quad (2)$$

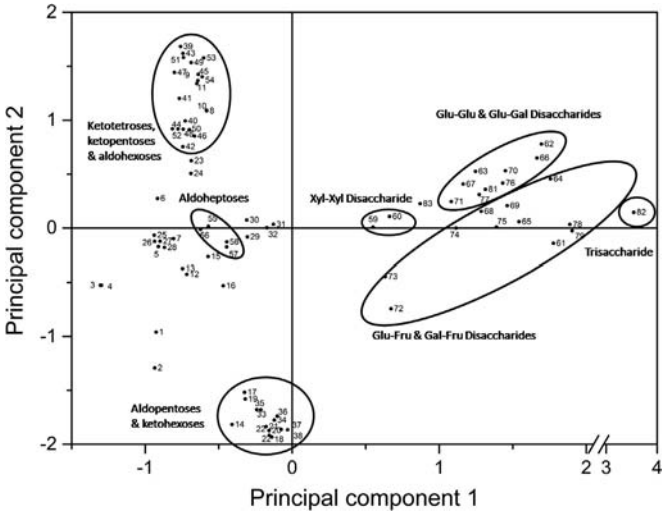

Fig. 6. PCA score plot of the eleven fragment ions (*m/z* 73, 103, 129, 147, 174, 204, 205, 217, 307, 319 and 361) with the largest intensity from the mass spectra of 46 EtOx-TMS derivatized carbohydrates. Each number in the score plot represents one specific ethoxime-TMS derivative as listed in Supplemental Table S1.

rG: relative retention, OPI: oxime peak identifier, RT_{target}: retention time of an oxime isomer peak, RT_{lStd}: retention time of the internal standard, $RT_{corresponding\ oxime\ peak}$: retention time of the corresponding oxime peak isomer, RT_{high}: retention time of the compound with the latest elution, either the internal standard or the corresponding oxime peak isomer, RT_{low}: retention time of the compound with the earliest elution, either the internal standard or the corresponding oxime peak isomer.

The OPI of the carbohydrate derivatives (Fig. 7, right) shows an increasing value for the *syn* peak, while the value of the *anti* peak decreases. For carbohydrates eluting before glycolaldehyde ($OPI_{syn} < 1$, $OPI_{anti} > 1$), the *syn* peak elutes before the *anti* peak. In contrast, for carbohydrates from maltulose on ($OPI_{syn} > 1$, $OPI_{anti} < 1$), the *anti* peak elutes before the *syn* peak. The conventional rG-plot (Fig. 7, left) also starts with glycolaldehyde at an rG value of 0.29576 and the rG value increases with increasing elution order of the carbohydrate. rG values smaller than unity (up to fucose) indicate a retention below the internal standard (methyl α-D-galactopyranoside), while rG values greater than 1 (starting from 2-deoxy-D-glucose) come from analytes eluting after the internal standard.

The graph of conventional rGs (Fig. 7, left) shows several plateaus in the group of tetroses, pentoses and hexoses, which indicated "regions" where the distinction between individual carbohydrates is very difficult, if not impossible, due to missing retention time differences between stereoisomers and their similar mass spectra. Compared to the rG, the graph of the novel OPIs (Fig. 7, right) also shows plateaus, but most of the carbohydrates within one plateau are coming from different carbohydrate groups and can be distinguished from the mass spectra and the *syn*/*anti* peak characteristics. Compared to rG, the OPI shows a better differentiation of individual carbohydrate as well as a higher resolution (Supplemental Table S2). In contrast to the rG, which only considers the elution order, the OPI combines the elution order and the characteristic time shift between *syn* and *anti* peaks, which ensures positive match identification of all tested carbohydrates. The OPI value accounts for the positive assignment of two peaks, and the calculation of the corresponding peak values can validate the positive identification.

3.6. Application to carbohydrate matrices

Four different carbohydrate-containing matrices were lyophilized to remove water residues and subsequently analyzed with the introduced EtOx-TMS derivatization method. The sample matrices were compared for their fructose, glucose, and sucrose contents on a dry mass basis (Fig. 8).

The honey group, consisting of buckwheat, orange, and honeydew honeys, showed similar fructose/glucose/sucrose-ratios. The fructose content within the group ranged from 52.21% to 54.31%, followed by glucose, ranging from 44.99% to 47.19%. Slightly higher amounts of glucose were observed in the honeydew honey compared to the other two honeys, while orange honey showed the highest sucrose amount within the honey group (2.1%). Maple sirup and palm sugar were characterized by high sucrose amounts (91.97% and 85.93% respectively) and low fructose and glucose contents. In contrast, agave sirup showed the highest amount of fructose (93.2%) among all analyzed samples.

4. Conclusion

Gas chromatography, hyphenated to mass spectrometry, is an appropriate method for analyzing carbohydrates; however, the existence of ubiquitous mass ions and very similar mass spectra limit the use of mass spectroscopy for carbohydrate identification. Differentiations between ketoses and aldoses, as well as molecular weight differences, are useful but insufficient for positive compound identification by mass spectrometry. A distinction between

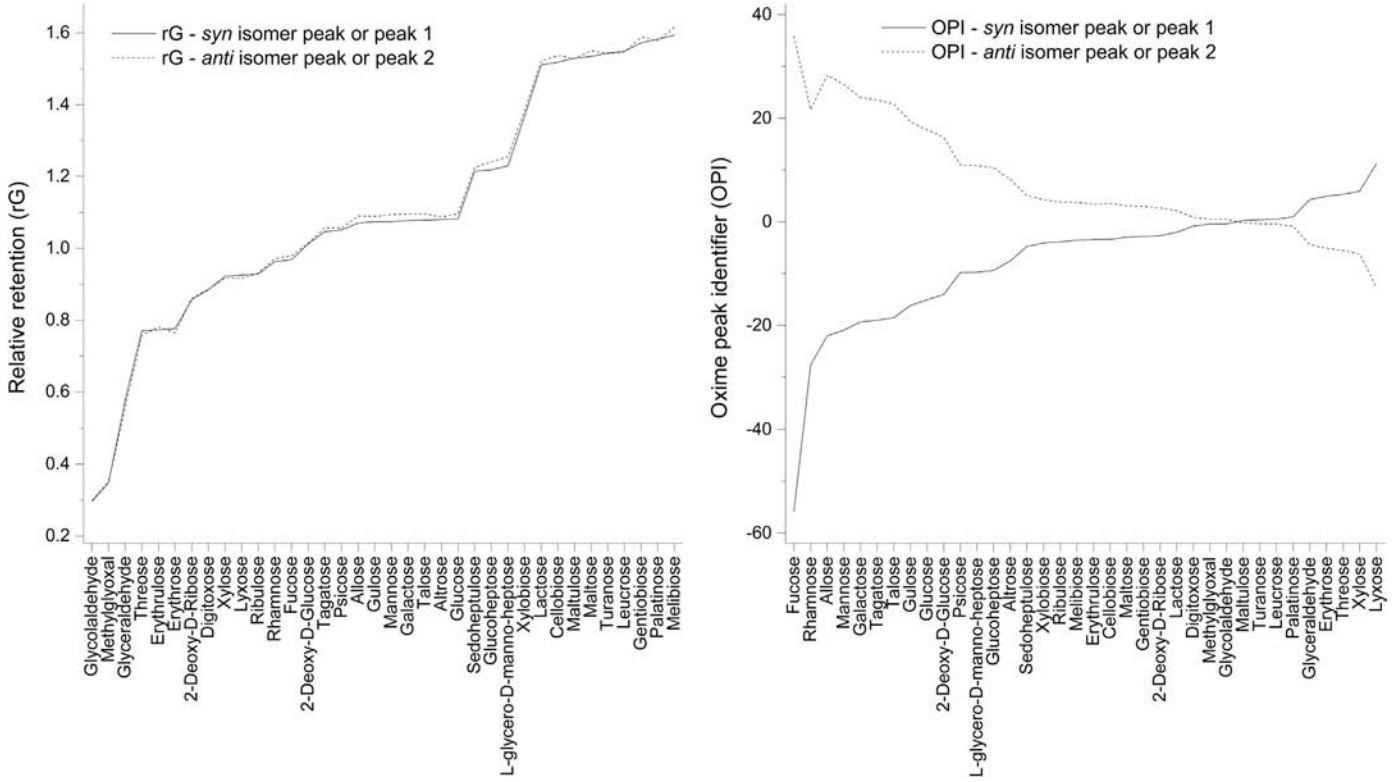

Fig. 7. Comparison of the relative retention (rG, based on one static internal standard) and the oxime peak identifier (OPI, based on one static internal standard and the corresponding *syn* or *anti* peak/peak 1 or peak 2 of the carbohydrate) for ethoxime-trimethylsilyl (EtOx-TMS) derivatized carbohydrates. The first eluting isomer of oximated ketoses and 2-deoxysugars has been termed as "peak 1" and that of the later eluting respective isomer as "peak 2".

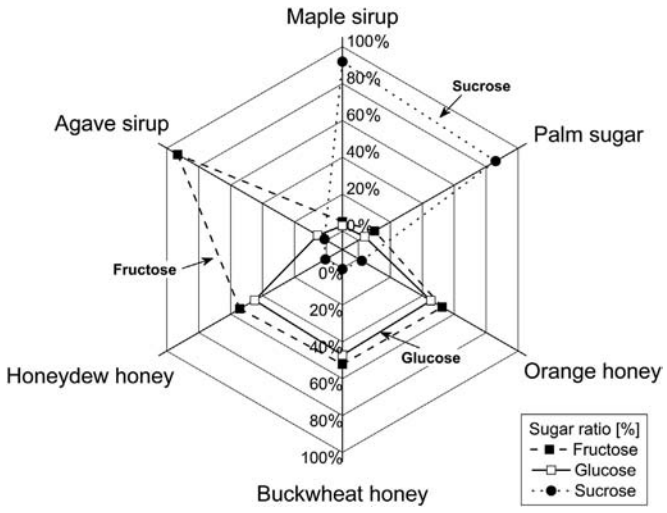

Fig. 8. Analysis of fructose/glucose/sucrose-ratio (%) in dry mass of different sugar matrices: agave sirup, maple sirup, palm sugar, and honeys (buckwheat, orange, and honeydew).

isomeric carbohydrates is only possible using retention times and chromatographic behavior as discrimination parameters, but as this is not generally successful, different derivatizations are usually performed to improve their chromatographic behavior.

The combination of oximation followed by silylation for carbohydrate analysis is a particularly powerful approach for carbohydrate analysis, its full potential has not yet been exploited. We have aimed at advancing the ethoxime-trimethylsilyl derivatization method with regard to both practical aspects and data interpretation. This method is probably the best one amongst all related procedures that were hitherto assayed by the authors. Together with the proposed oxime peak identifier (OPI), this method overcomes several pressing problems in GC/MS carbohydrate analysis and allows for a reliable and straightforward identification of a large range of analytes.

The chromatographic and mass spectra characteristics of 46 different carbohydrates were used to evaluate possible unambiguous identification criteria. Specific parameters for the individual carbohydrates were the area ratio of *syn* and *anti* oxime peaks, their elution order, and the retention time shift between the two peaks. Two of these parameters were incorporated into the oxime peak identifier (OPI), which allows reliable identification of the carbohydrates. In addition, in contrast to conventional retention indices, the OPI is based on the second peak of the *syn*/*anti* pair as dynamic standard. In the case of disaccharides, the *anti* peak order and *syn*/*anti* peak area were influenced by the monosaccharide moieties, while the *syn* peak elution was effected by the position of the glycosidic linkage. We are confident that the present EtOx-TMS derivatization with GC/MS analysis and the developed oxime peak identifier evaluation can help to enhance positive carbohydrate identification, even in highly complex biological samples. Our hope is that this approach will become widely accepted in different areas of carbohydrate analysis and quantification.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.052.

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