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Development of an advanced derivatization protocol for the unambiguous identification of monosaccharides in complex mixtures by gas and liquid chromatography

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The separation and analysis of complex monosaccharide mixtures is highly challenging and requires typically carefully selected derivatization procedures to avoid changes in the sample composition. Here we present in a comparative study several single- and two-step derivatization approaches for LC and GC separations using a set of reference compounds ranging from C1 building block such as formaldehyde to C6 monosaccharides. Separation conditions have been optimized resulting in the simultaneous separation of 15 unbranched aldoses. By parallel derivatization using hydroxylamine hydrochloride (HACl)/ *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and *O*-ethylhydroxylamine hydrochloride (EtOx)/ *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and comparative GC measurements we developed a protocol for the unambiguous identification and separation of aldoses, ketoses, alditols and aldonic acids, which commonly occur in complex sugar mixtures as reaction by-products or decomposition products. In particular this procedure helps to deconvolute overlapping analytes and facilitates quantification. Additionally, the method presented here has been investigated in regard to storage life, detection limits, quantification and MS analysis. The broad applicability of this method to different sample matrices is shown for the analysis of food samples and complex aldol reaction mixtures in the formose reaction, which is of great relevance in the context of the origin of life.

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1. Introduction

The separation of complex monosaccharide mixtures poses a challenging task due to the structural similarity and the number of the stereoisomers. This complexity is one of the major obstacles in the quantitative and time-resolved analysis of formose reaction with formaldehyde, which results in a mixture ranging from the C1 building block formaldehyde up to hexoses and even longer-chain products [1–3], cannot be easily achieved. The number of species is not only defined by the number of possible aldoses and hexoses, but also by the highly dynamic occurrence of an open-chain, furanose and pyranose form, the latter forming two anomers each.

This analytical task has been targeted by a variety of chromatographic methods in the past, but remains still challenging because of the high complexity and the risk to change sample composition by the derivatization conditions [4,5]. For LC separation, due to the lack of a chromophore unit, detection is mostly limited to refractive index and evaporative light scattering detectors, which are not as common as UV detectors and often suffer from disadvantages such as low stability or sensitivity. An alternative is charged aerosol detection (CAD), which is nonlinear but shows good sensitivity in the detection of sugars [6]. Analysis of underivatized sugars is possible by HPLC-MS and HPLC-MS/MS, however derivatization offers advantages in the detection of small molecules [7]. GC analysis of native carbohydrates is hampered by decomposition, therefore derivatization of the hydroxyl groups is mandatory to improve the volatility and stability. Therefore, different derivatization approaches have been published, including hydrazone formation [8,9], reductive alkylation [10] and conversion to pyrazolones [11–14] for LC. For GC silylation [15], trifluoroacetylation [16,17], both also in combination with prior oximation [18,19], as well as isopropylidene derivatives [20] have been established. Derivatization involving the carbonyl group is generally preferred to reduce the maximum number of chromatographic peaks by avoiding the formation of cyclic hemiacetals of the monosaccha-

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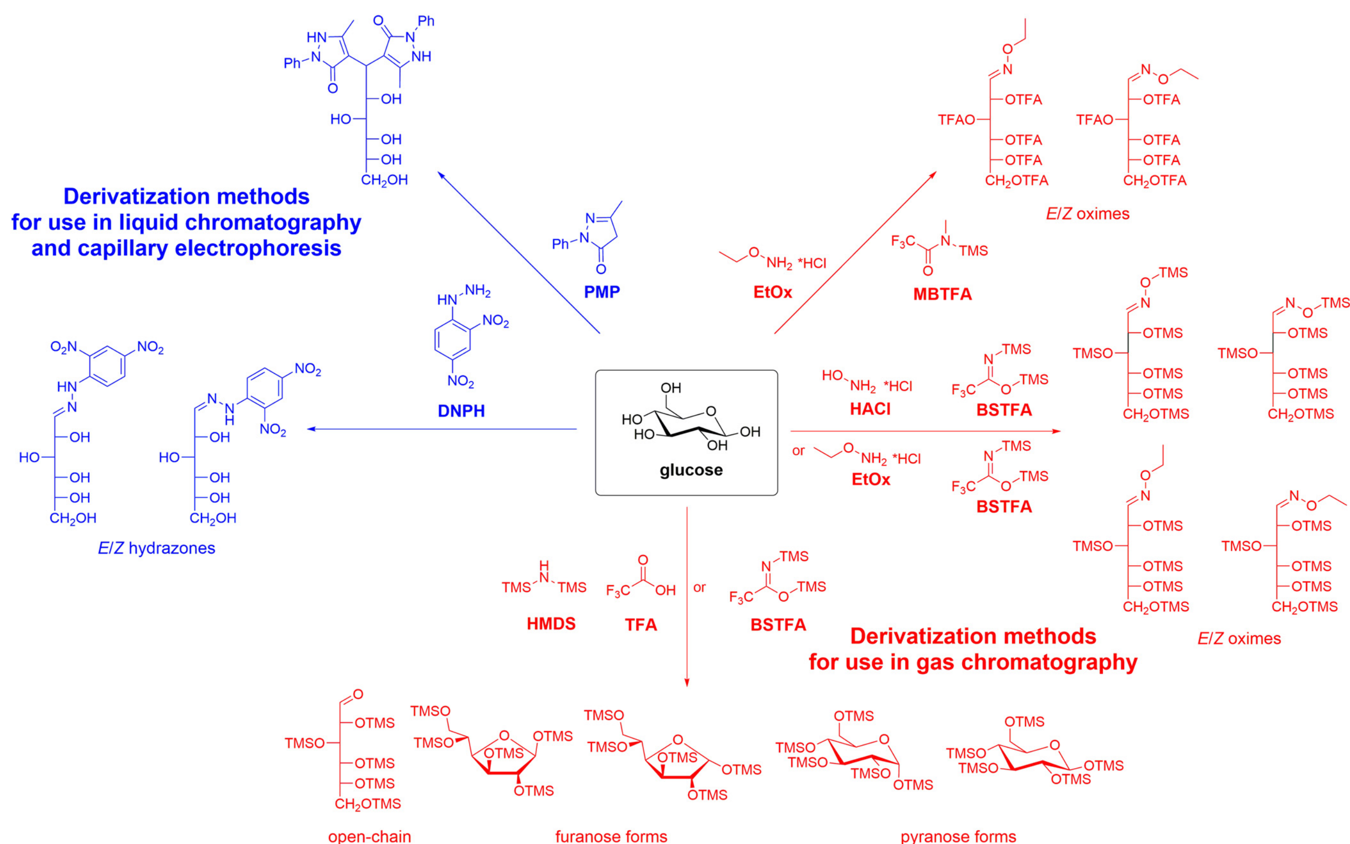

Fig. 1. Derivatization approaches for monosaccharides investigated in this work.

rides, which results in additional isomers. On the other hand, this is problematic, because these methods can also lead to loss of analytical information in the case of reduction, as aldose and ketose can form the same product.

In this study, we present a comparison of different derivatization approaches for monosaccharide separation *by* HPLC and GC (Fig. 1) and aim to develop a protocol that allows separation of all aldoses and distinguish between aldoses and ketoses as well as other similar substance classes such as polyalcohols and sugar acids. This is in particular of great importance in the quantitative analysis of complex sugar mixtures that are formed in the formose reaction [21].

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), Carbosynth Limited (Berkshire, United Kingdom) and Apollo Scientific (Cheshire, United Kingdom) and used without further purification. High purity water was obtained from a VWR Puranity PU 15 (Darmstadt, Germany). Food samples were obtained from local grocery stores. Aqueous monosaccharides and food samples were lyophilized prior to derivatization.

2.2. Derivatization procedures

2.2.1. 1-Phenyl-3-methyl-5-pyrazolone

Monosaccharides were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) employing the procedures described by Sun [14] and McRae [13].

2.2.2. 2,4-Dinitrophenylhydrazine

A saturated solution of 2,4-dinitrophenylhydrazine (DNPH) in acetonitrile was prepared, stored at 5 °C and used for a maximum of one week. A sample of either 2 mg of monosaccharide or 5 μL of formalin (37% solution in H_2O) was dissolved in 200 μL of deionized water. 600 μL of the stock solution, 195 μL of acetonitrile and 5 μL of an aqueous solution of hydrochloric acid (2 M) were added and the reaction allowed to proceed at room temperature for 30 min. An aliquot of 1 μL was directly injected to the HPLC.

2.2.3. Silylation

Monosaccharides (2 mg) were dissolved in 200 μL pyridine and 120 μL *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) or 100 μL hexamethyldisilazane (HMDS) and 10 μL trifluoroacetic acid (TFA) were added. The reaction mixture was heated at 70 °C for 30 min on a rocking shaker and 0.1 μL was directly injected to the GC.

2.2.4. Oximation/Silylation

A stock solution of 40 mg/mL of either *O*-ethylhydroxylamine hydrochloride (EtOx) or hydroxylamine hydrochloride (HACl) with 50 mM of the internal standard (IS) phenyl-β-D-glucopyranoside was prepared and used for a maximum period of one week. Monosaccharides (2 mg) were dissolved in 200 μL pyridine and 200 μL of the stock solution was added. The mixture was heated at 70 °C for 30 min on a rocking shaker. After cooling to room temperature, 120 μL *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added, the mixture was vortexed and heated again at 70 °C for 30 min. An aliquot of 0.1 μL was directly injected into the GC.

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