Journal of Chromatography A, 1350 (2014) 44–50

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast carbohydrate analysis via liquid chromatography coupled with ultra violet and electrospray ionization ion trap detection in 96-well format

Broder Rühmann, Jochen Schmid, Volker Sieber*

Chemistry of Biogenic Resources, Technische Universität München, Germany

ARTICLE INFO

Article history:
Received 14 February 2014
Received in revised form 5 May 2014
Accepted 6 May 2014
Available online 14 May 2014

Keywords:
Carbohydrates
1-Phenyl-3-methyl-5-pyrazolone-derivatization
LC–UV-ESI-MS/MS
96-Well high throughput format
Micro scale hydrolysis
Carbohydrate polymers

ABSTRACT

A fast carbohydrate screening platform processible in 96-well format is described. The method is suitable for the determination of various carbohydrates out of complex mixtures as obtained by acidic hydrolysis of carbohydrates polymers. The chromatographic conditions for an efficient separation (12 min) and the derivatization process with 1-phenyl-3-methyl-5-pyrazolone (PMP) were optimized for high resolution separation and simultaneous determination of deoxy-, amino-, anhydro-sugars as well as hexoses, pentoses, dimers, uronic acids and degradation products like furfural and hydroxymethylfurfural (HMF). The potential to quantify with UV- and MS-detector in the same range has been demonstrated for 20 different compounds. Finally, the matrix effects of the hydrolysis were positively evaluated. The micro scale hydrolysis and PMP-derivatization without any extraction or drying steps, both in 96-well format, result in a fast and intuitive sample preparation. In combination with a fast liquid chromatography coupled to UV and electrospray ionization ion trap detection (LC–UV-ESI-MS/MS) for the qualification and quantification of various sugars, dimers and degradation products, this method shows great performance in carbohydrate analysis.

1. Introduction

Carbohydrates are the most abundant biological molecules, but still the analysis of their complex mixtures represents a challenge. Many analytical methods for identification and quantification of carbohydrates have already been developed. Becker et al. [1] give an overview of existing methods by evaluating different derivatization approaches for gas chromatographic–mass spectrometric (GC–MS) analysis in complex matrices. However, the existing techniques are still facing numerous problems. Carbohydrates are non-volatile and have to be silylated first or made more volatile with other described derivatizations to be analyzed via GC or GC–MS. By a one-step derivatization aldohexoses can generate up to five tautomers: α- and β-pyranose, α- and β-furanose and the respective open chain aldose. For this reason, Becker et al. concluded that sequential ethoximation and trimethylsilylation (EO-TMS) provide the highest GC–MS performance; however this derivatization needs several steps of chemical treatment and is not applicable for a high throughput measurement.

Additionally, there also exist various HPLC methods, all with their own strengths and weaknesses for the different applications. For example, anion exchange chromatography (HPAEC) with pulsed amperometric detection is an effective method for carbohydrate analysis. However, the high pH value of the eluent results in epimerization and degradation [2,3] of the different carbohydrates. Furthermore, the eluent of HPAEC mostly is not directly compatible with MS detection. The eluent has to be desalted before entering the MS detector and the ionization efficiency of underivatized carbohydrates is not very high.

Another carbohydrates determination technique represents ion exclusion chromatography (IEC), which is often connected with refraction index or evaporative light scattering detector. But, the IEC-columns (lead or calcium form) often show problems in peak separation and cannot be used to separate acidic and basic carbohydrates [4]. Isocratic elution with water results in long chromatographic runs for these columns and a low ionization efficiency for MS analysis [5].

Further methods would be for example hydrophilic interaction liquid chromatography (HILIC) [6], capillary electrophoresis (CE) [7] and various other techniques for special applications and matrices, all of them having their benefits and disadvantages.

The intention was to establish a high throughput screening platform for analyzing the composition of different carbohydrate

* Corresponding author. Tel.: +49 9421 187300; fax: +49 9421187310.
E-mail address: Sieber@tum.de (V. Sieber).

http://dx.doi.org/10.1016/j.chroma.2014.05.014
0021-9673/

polymers and plant material. Therefore, a fast analyzing method for a wide variety of carbohydrates, in a 96-well format with an excellent opportunity for quantification and qualification via mass spectra is required. Unfortunately, none of the existing methods described above could provide all of those parameters. For this reason, an adapted method based on 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization coupled with a liquid chromatography UV and electrospray ionization ion trap technique (LC–UV-ESI-MS/MS) was developed.

Honda et al. [8] first reported the use of (PMP) labeling for analysis of monosaccharide components from glycoproteins. Compared to benzylation or hydrazine derivatives the open ring aldehyde of the reducing sugar reacts with two PMP-molecules to a single *bis*-PMP carbohydrate derivative. The strong absorbance at 245 nm, the excellent ionization (positive ESI) and fragmentation efficiency for MS/MS detection, results in an easily quantifiable method [9]. Different applications were established based on the first report of Honda et al., such as the pre-column derivatization with PMP for the simultaneous determination of neutral, acidic and basic carbohydrates [10]. Additionally, oligosaccharide characterization and quantification using PMP-derivatization and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF) were reported [11]. However, in all publications dealing with PMP-labeling, sample preparation was performed with multiple steps of drying or extraction to remove the unreacted PMP reagent. Accordingly the PMP method as described so far is not capable for high throughput measurements. Therefore, an optimized PMP-derivatization without any drying and extraction steps was developed. This simplification of the process reduces the workflow to a minimum and saves the amount of reagents needed.

Additionally, the chromatographic conditions were optimized and the total run time was reduced to 12 min. The validation of the method was performed with 18 different carbohydrates and the respective degradation products furfural and hydroxymethylfurfural (HMF). Elution of all eight possible glucose dimers and seven hexoses to evaluate the performance of the method were determined. The opportunity to quantify each peak with UV- and MS-detector and qualify the peaks by mass analysis makes the method highly reliable. Especially, unknown or overlapping peaks can be classified by their different masses. This benefit allows the determination of various carbohydrates from complex mixtures e.g. as obtained by acidic hydrolysis of polymeric carbohydrates. Furthermore, the influence of the hydrolysis matrix on the derivatization process was evaluated. By proving the robustness and reliability of the optimized method, it is not only considered to be a real high throughput method, but also having the potential for carbohydrate analysis of various hydrolyzed polymers such as polysaccharides, microbial exopolysaccharides or plant material.

2. Experimental

2.1. Chemicals and reagents

The following carbohydrate standards were purchased from Sigma–Aldrich (Germany): D-(−)-ribose, L-(−)-gulose, D-(+)-xylose, D -(+)-cellobiose, L-(+)-rhamnose, D (+) altrose, D-(+)-galacturonic acid, D-(−)-deoxy-ribose and N-acetyl-D-(+)-galactosamine; from Carl Roth GmbH (Germany): D-(+)-galactosamine, 2-deoxy-D-glucose, N-acetyl-D-(+)-glucosamine, D-(+)-glucose, L-(−)-fucose, L-(+)-arabinose, D-(+)-gentiobiose and D-(+)-lactose; from Carbosynth Limited (England): D-(+)-kojibiose, D-(+)-sophorose, D-(+)-nigerose and D-(+)-isomaltose; from Serva (Germany): D-(+)-mannose and D-(+)-galactose; from Alfa Aesar GmgH Co KG (Germany): D-(+)-allose and D-(+)-talose; from Megazyme International (Ireland): D-(+)-laminaribiose; from Molekula GmbH (Germany): D-(+)-glucuronic acid and from Merck KGaA (Germany): D-(+)-glucosamine. All other chemicals were, unless otherwise stated, purchased in analytical grade from Sigma–Aldrich (Germany), Merck KGaA (Germany) and Carl Roth GmbH (Germany)

2.2. Optimization of PMP-derivatization for HPLC-UV-ESI-MS analysis

For the adaption of the original method toward a high throughput measurement, the following optimization steps of the derivatization process were necessary. Each sample was measured in duplicate.

2.2.1. Optimization of PMP-concentration

50 μL of differently concentrated (0.2, 0.1, 0.075, 0.05, 0.025 M) methanolic-PMP solutions were added to 25 μL of standard (50 mg/L) and 25 μL of ammonium hydroxide solution (1.6%) in 1.5 mL reaction tubes which were vortexed and pulse centrifuged. After incubation at 70 °C for 60 min (water bath), 25 μL of acetic acid (0.5 M) and 875 μL of water were added. Finally, the samples were filtrated (0.2 μm, Supor, Pall Corporation), transferred into HPLC-vial and measured via HPLC–UV-ESI-MS.

2.2.2. Determination of optimum ammonium hydroxide concentration

50 μL of 0.1 M methanolic-PMP solution were added to 25 μL of standard (50 mg/L) and 25 μL of various ammonium hydroxide concentrations (6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1 or 0.01%). The 1.5 mL reaction tubes were vortexed and pulsed centrifuged. After incubation at 70 °C for 60 min (water bath) 25 μL of acetic acid (2.0, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125 M, or 3.125 mM) and 875 μL of water were added. The samples were filtrated (0.2 μm, Supor, Pall Corporation), transferred into HPLC-vial and measured via HPLC–UV-ESI-MS.

2.2.3. Optimization of incubation time

50 μL of 0.1 M methanolic-PMP solution were added to 25 μL of standard (50 mg/L) and 25 μL of ammonium hydroxide solution (1.6%). The 1.5 mL reaction tubes were mixed on a vortex and pulsed centrifuged. After incubation for 30, 60, 90, 120, and 150 min at 70 °C (water bath), 25 μL of acetic acid (0.5 M) and 875 μL of water were added. The 0.2 μm filtrated samples were transferred into HPLC-vial and measured.

2.3. Optimized 96-well method

75 μL of derivatization reagent (0.1 M methanolic-PMP-solution: 0.4% ammonium hydroxide solution 2:1) were added to 25 μL of sample in a 96-well-PCR micro titer plate (Brand 781350, Germany). The plate was sealed with a TPE cap mat (Brand 781405, Germany), mixed well and centrifuged at 2000 × *g* for 2 min at 20 °C. After incubation (100 min at 70 °C) in a PCR-cycler (Biorad My-cycler, USA) and a following automated cool down to 20 °C, an aliquot of 20 μL was transferred to a fresh 96-well micro titer plate (Greiner) and mixed with 130 μL of 19.23 M acetic acid. The samples were then transferred into a 96-well filter plate (0.2 μm Supor, Pall Corporation) and centrifuged at 2500 × *g* for 5 min at 20 °C. Finally, the plate was sealed with a 96-well silicon cap mat (Whatmann 7704-0105, USA).

2.4. HPLC analysis of monosaccharide-PMP-derivative

The HPLC system (Ultimate 3000RS, Dionex) was composed of a degasser (SRD 3400), a pump module (HPG 3400RS), an