Food Chemistry 215 (2017) 347–353

Contents lists available at ScienceDirect

Food Chemistry

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

Development, optimization and validation of an HPLC-ELSD method for the analysis of enzymatically generated lactulose and saccharide by-products

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A R T I C L E   I N F O

Article history:
Received 13 May 2016
Received in revised form 13 July 2016
Accepted 31 July 2016
Available online 1 August 2016

Keywords:
HPLC-ELSD
Complex saccharide solution
Enzymatically generated lactulose
Optimization
Validation

A B S T R A C T

The aim of this study was to develop an HPLC-ELSD method for the quantification of lactulose in complex sugar solutions. Lactulose is a well-known prebiotic and supports the alleviation of digestive disorders. The enzymatic generation of lactulose requires fructose as nucleophilic acceptor. By-products such as glucose and galactose are generated. Four amino-modified silica-columns were tested and compared. The most suitable column based on peak resolution was used to optimize the method. Furthermore, sample preparation was optimized for the recovery of analytes. During the validation step, the following parameters were determined (e.g. for lactulose): recovery (106 ± 7%), precision (98%), correctness (99%), limit of detection (3.9 mg/L), limit of quantification (13.4 mg/L) and linearity (0.993). The validated method was applied to samples from an enzymatic process for the production of lactulose at the laboratory scale. A final lactulose concentration of 6.7 ± 0.4 g/L was determined.

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1. Introduction

Only a few methods have been established for the quantification of saccharides in complex food matrices. Many involve reacting the saccharides with strong acids or enzymes whereby either the saccharide itself reacts to a colored agent or a colored by-product is generated. These colored products can be detected photometrically. The change in the absorbance of light at a certain wavelength correlates with the concentration of the colored product and therefore, with the concentration of saccharide in the starting solution (Adhikari, Sahai, & Mathur, 1991; Amine, Moscone, Bernardo, Marconi, & Palleschi, 2000; Zhang, Yang et al., 2010). While these methods are fast and have a high sample throughput (Marconi et al., 2004; Zhang, Wang, Yang, & Jiang, 2010), they tend to be disturbed by the presence of other saccharides which react in a similar or identical way (Adachi, 1965; Zhang, Yang et al., 2010). Hence, a different method is required for the detection of saccharides in a complex saccharide solution. A 'complex saccharide solution' refers to a solution of different mono-, di- and/or oligosaccharides with similar structures and partial the same molecular weight.

High performance liquid chromatography (HPLC) is known to be able to separate complex saccharide solutions (Chávez-Servın, Castellote, & López-Sabater, 2004; Schuster-Wolff-Bühring, Michel, & Hinrichs, 2011; Zhang, Yang et al., 2010). Mainly two types of columns are used for the detection and quantification of saccharides in complex food matrices by HPLC: ion-exchange resins and amino-modified silica-phases (Zhang, Yang et al., 2010). While the former have the advantages of a very good separation of monosaccharides and high stability, the latter are more selective for higher polymerized saccharides and have a more diverse applicability. This is due to the order of elution. Oligosaccharides eluate faster than monosaccharides on ion-exchange resins while the monosaccharides eluate first on amino-modified columns. The earlier the analyte elutes, the less it interacts with the selective column material. Therefore, a coelution with another analyte is more likely (Indyk, Edwards, & Woollard, 1996; Schuster-Wolff-Bühring et al., 2011).

Complex saccharide solutions are mainly generated when saccharides are hydrolyzed, isomerized or polymerized. One example of a saccharide generated in such a process is lactulose. Lactulose (4-O-β-D-galactopyranoyl-D-fructose) is an artificial, nondigestible disaccharide consisting of fructose and galactose, connected over a $\beta 1 \rightarrow 4$ glycosidic bond (Wang, Yang, Hua, Zhao, & Zhang, 2013). It was discovered in heated milk in 1930 by Montgomery and Hudson (Montgomery & Hudson, 1930). Lactulose is formed from lactose during the heating process of milk (Manzi & Pizzoferrato, 2011). For this reason, lactulose can be used as a heat indicator, especially to distinguish between sterilized and

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http://dx.doi.org/10.1016/j.foodchem.2016.07.184
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UHT-treated milk. The boundary is 0.6 g/L (Cataldi, Angelotti, & Bufo, 1999; Lan et al., 2010).

Currently, the Lobry de Bruyn-Alberda van Ekenstein transformation is used for the technical production of lactulose from lactose. The reaction needs an alkali agent and temperatures above 70 °C and a yield of up to 40% can be achieved (Nagasawa, Tomita, Tamura, Obayashi, & Mizota, 1974). The yield can be increased up to 80% by using complexing agents as the generation of by-products, especially galactose, decreases (Zokaee, Kaghazchi, Zare, & Soleimani, 2002). The complexing agent and the by-products are then separated via a cost-intensive chromatographic process. For the food industry, this complex and cost intensive production is not an option. Therefore, an alternative was searched and found in a side reaction of most β-galactosidases, mostly from *Aspergillus* or *Kluveromyces* (Wang et al., 2013). The main reaction of β-galactosidases is well-known. It catalyzes the hydrolysis of lactose to glucose and galactose. The glucose is hereby cut off while the galactosyl group remains bound to the enzyme. A nucleophilic acceptor is needed to release this group, e.g. water. Other compounds, like fructose can also be used as nucleophilic acceptor. In this case, lactulose is generated (Mayer et al., 2004; Wallenfels, 1951). However, the solution contains low amounts of lactulose and high amounts of monosaccharides (fructose, glucose and galactose) so that a purification or fractionation is needed. Advantages of this process are the natural production of food grade lactulose and the utilization of whey which is a by-product of cheese production (Nath et al., 2016).

Lactulose is mainly used in the medical industry for different applications (Schumann, 2002) as it is not hydrolyzed during the passage of stomach and reaches the colon. Gut bacteria can metabolize the disaccharide, produce short chain fatty acids and lower the pH value in the colon (Aït-Aissa & Aïder, 2014; Blei & Córdoba, 2001). Furthermore, lactulose is an osmotically active laxative that can be used for the treatment of constipation. The short chain fatty acids generated increase the osmotic pressure in the colon, which increases the stool volume. In addition, the lower pH value stimulates the vermicular movement (Aït-Aissa and Aïder, 2014).

Lactulose promotes the proliferation of Lactobacilli and Bifidobacteria. These microorganisms support the immune system and enhance the digestion and absorption of nutrients (Gibson & Roberfroid, 1995; Olano & Corzo, 2009; Petuely, 1957). For this prebiotic effect, a daily intake of about 3 g lactulose is needed (Förster-Fromme et al., 2011).

The objective of this work was to develop a method for the detection of lactulose and other saccharide by-products in complex saccharide solutions. An example for a complex saccharide solution is the output of the enzymatic production of lactulose, which contains fructose, galactose, glucose, lactulose and lactose. The challenge hereby was to find a column which offers a high resolution for mono- as well as for disaccharides. Amino-modified silica-columns were chosen since the main analyte (lactulose) should be clearly separated from other substances. Furthermore, it was intended to optimize the extraction of the analytes from a complex food matrix during the sample preparation and to validate the resulting method. Finally, the method was applied to samples from an enzymatic process for the production of lactulose, in order to verify its suitability for the intended purpose.

2. Experimental

2.1. Material for standards and model solutions

The reagents used for preparing standards were fructose (CAS 57-48-7), glucose (CAS 50-99-7), galactose (CAS 59-23-4), lactose-monohydrate (CAS 10039-26-6) (Carl Roth, Cologne, Germany), lactulose (CAS 4618-18-2) (Sigma-Aldrich, Munich, Germany), HPLC-grade Acetonitrile (Honeywell, Seelze, Germany) and double distilled water (Aq_{DD}: <0,55 µS/cm, Purelab Classic, ELGA LabWater, Celle, Germany). Each saccharide was dissolved in Aq_{DD} in a volumetric flask at the desired concentration. Calibrated ranges are found in Table 4. The calibration was performed on five levels for each saccharide. The solvent of the standards for the column Unison was 80% ACN and 20% Aq_{DD} in order to achieve better peak shapes.

A solution was prepared from ultrafiltration permeate powder (Bayolan PT, BMI, Landshut, Germany) to simulate a complex food matrix. According to the certificate of analysis, the Bayolan PT powder contained protein (2.9%) and minerals (4.0%) in addition to lactose-monohydrate (89%). The fat content (<0.5%) was negligible. The initial concentration of lactose desired was adjusted by varying the amount of Bayolan PT dissolved in distilled water (Aq_D). Other saccharides (fructose, galactose, glucose and lactulose) were added in desired concentrations, corresponding to the first, third and fifth calibration level. All model solutions were heated (70 °C, 10 min) prior to sample pretreatment to prevent microbiological contamination of the HPLC columns.

2.2. HPLC-system

HPLC-analysis was carried out using an Agilent 1100 series unit consisting of a vacuum degasser, quaternary pump, auto sampler and column compartment with oven (Agilent Technologies, Santa Clara, USA). Analytes were detected by a low temperature evaporative light scattering detector (LT-ELSD: Sedex 80, Sedere, Alfortville, France). ELSD-conditions were optimized to detect low-levels of lactulose without increasing the baseline noise too much. The following parameters were applied: temperature 40 °C, Gain 8, Offset 0 mV, Sampling time 10 Hz, Filter 10 s.

2.3. Stationary phases

Four commercially available amino-modified silica-columns from different companies were tested (Table 1). Each column was used under conditions recommended by the manufacturer. In the case that all tested saccharides were separated, the method for the corresponding column was optimized. For the column Unison, a suitable precolumn (Unison UK Amino 3 µm, 5 × 2.0 mm, Imtakt Corporation, Japan) was used after optimization.

2.4. Optimization of an HPLC-method

The separation of analytes is characterized by three parameters. The retention coefficient k measures the time t_R an analyte takes to pass the column compared to the delay time t_D.

$$k = \frac{t_R - t_D}{t_D} \quad (1)$$

The resolution R is the parameter for the separation quality of two adjacent peaks. A value of 2.0 is considered optimal (Shabir, 2003). The resolution is calculated by the retention time t_R and the peak width w. An indexed 1 refers to the faster eluting analyte and 2 to the slower eluting analyte.

$$R = \frac{2(t_{R2} - t_{R1})}{(w_1 - w_2)} \quad (2)$$

The selectivity α compares the retention coefficients of two adjacent peaks. For a baseline-separated analysis, the selectivity of all analytes should be bigger than one.

$$\alpha = \frac{k_2}{k_1} \quad (3)$$

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