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Journal of Chromatography A, 1118 (2006) 281–284
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Thermal stability of glucose and other sugar aldoses in normal phase high performance liquid chromatography

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Received 10 March 2006; received in revised form 26 April 2006; accepted 28 April 2006

Abstract

Analysis of glucose and other simple sugars are often performed by use of normal phase HPLC methods with acetonitrile as major eluent. The present results clearly show that column temperature plays an important role with respect to chromatographic performance and detection limits of glucose when using a specific carbohydrate column. A change in column temperature from 25 to 45 °C reduced the detection of glucose (with ELSD) by more than 41%, whereas the detection of other sugar aldoses (galactose, xylose and rhamnose) were suppressed even more. By increase of column temperature to 70 °C the detector signal of glucose was found to be less than 2% compared to that obtained at 20 °C. Neither fructose nor sucrose showed similar correlation between column temperature and detection. The rate of decreased response is not dependent on sample concentration or the ELSD settings. The results express the importance of accurate temperature control in the analysis of sugar aldoses, and also values low column temperatures for samples with low concentrations of sugar aldoses in order to improve detection.

Keywords: Aldoses; Glucose; Ketoses; Fructose; Sugars; HPLC; Normal phase; Column temperature; Evaporative light scattering detection (ELSD)

1. Introduction

The analysis of glucose and other sugars are among the most widespread chemical analyses that are performed within the industries of food, forage, biomass, pulp and paper, among others. Several methods for sample preparation and analysis exist, and the protocols have been revised as methods and new generations of instruments have emerged. During the last years HPLC has strengthened its position as an analytical technique during the development and improvement of specially designed carbohydrate columns together with more suitable detection techniques as mass spectrometry (LCMS), evaporative light scattering (ELSD) and charged aerosol (CAD) [1].

The most widely used HPLC methods for sugar analysis use cyano- or amino-bonded silica columns [2]. More recently developed carbohydrate columns are those based on hybrid particles, which combine the efficiencies of silica-based materials with the pH-resistance more common to polymer packing materials. The normal phase chromatographic methods require an organic solvent, often acetonitrile, as the major eluent in combination with water and other solvents for modification of the elution profile (e.g. methanol).

In industrial laboratories large analytical series of similar samples are the rule rather than the exception. Time of analysis for each sample is therefore of great importance, and a lot of effort is used to develop rapid chromatographic methods. A well-known way of reducing the retention times (but not the retention volume) of a specific HPLC method is to raise its flow rate. This often produces similar chromatograms though the separation factors are reduced. Many silica based HPLC columns can be operated up to 330 bar (5000 psi) whereas other column particles have a lower max pressure. In order to reduce the back pressure generated by a higher flow rate, a raise in column temperature will change the viscosity of the solvents in use and thereby also generate a lower back pressure [3]. Although this will in general give lower retention times, care should be taken with respect to the thermal stability of each solute that is subject of analysis.

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doi:10.1016/j.chroma.2006.04.087

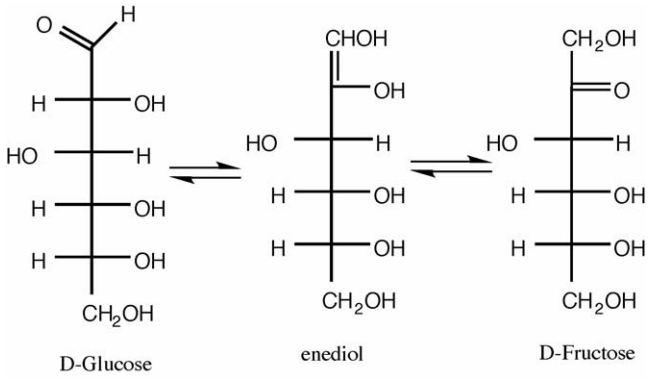

Fig. 1. Keto-enol tautomeric transformations which is a start reaction in the decomposition of glucose.

It is well known from the paper, pulp and food industries that carbohydrates are modified or decomposed as a result of elevated temperature and pressure [4]. It has been demonstrated that glucose in water decompose under subcritical conditions (<374 °C/220 bar) to a variety of intermediate products such as fructose, glyceraldehydes, dihydroxyacetone, pyruvaldehyd, glycolaldehyd, 5-hydroxymethylfurfural (5-HMF) and furfural [5,6]. An interesting finding suggested that fructose is formed from glucose in hydrothermal experiments at 240 °C at low pressure, whereas glucose is not formed from fructose [7]. In chemical terms this can be explained by a series of keto-enol tautomeric transformations called Loby de Bruyn-Alberda van Ekenstein (LBAE) (Fig. 1) [8]. Water itself suppresses keto-enol tautomerism by stabilisation of the aldehyd-group through H-bonding from water. The isomerization and similar starting routes of decomposition of reducing sugars (aldoses) in particular, seem thus to be a question of temperature, pressure and solvent.

Some molecular changes on glucose during HPLC-analysis have been reported previously. It has been demonstrated that mutarotation of some sugars occur at 25–45 °C [9]. The α- and β-anomers were detected as two distinctive peaks at 25 °C, but the peak area of the α-anomer was suppressed with elevation in column temperature followed by an increased peak area of the β-anomer. Despite the changes in the chromatograms, the total peak area was found to be constant at all temperature levels (25–75 °C). Separation was achieved on a Ca^{2+}-form Aminex column with water as eluent. Another paper reports on a method developed with a carbamoyl-silica HILIC column and 82% acetonitrile (pH 5.5) as eluent and with column temperature at 60 °C. The detection limits were reported to be three times lower compared to those obtained at column temperature 40 °C. The method, however, did not separate glucose, mannose and galactose but eluted these compounds as one broad peak, and fructose was not included in that work [10].

In the present communication we demonstrate the impact of HPLC column temperature on the detection of glucose and other sugar aldoses (reducing sugars) on normal phase HPLC analysis using a specially designed carbohydrate column from Alltech (Prevail Carbohydrate).

2. Materials and methods

D-Glucose (glc), D-galactose (gal), D-xylose (xyl), D-fructose (fru), L-rhamnose (rha) and sucrose (sucr) together with acetonitrile (HPLC grade) were obtained from Sigma (Oslo, Norway). Two standard solutions were prepared: 0.40 mg/ml each of fru, glc and sucr (Solution 1), and 0.40 mg/ml each of rha, xyl and gal (Solution 2). In addition a series of fru, glc and sucr at different concentrations was prepared. All analytical samples were diluted with destilled water and filtered through 0.45 μm Nylon filters prior to HPLC-analysis.

An Agilent 1100 HPLC system with a diode array detector was used together with an Alltech 800 evaporative light scattering detector (ELSD). Nitrogen was used as the ELSD nebulizer gas (2.0 bar), tube temperature was set to 65 °C and the gain was set to 8. UV-detection was made at 230 ± 4 nm in order to observe possible degradation products. Separation was primarily achieved on a Prevail Carbohydrate ES column (250 mm × 4.6 mm, 5 μm, Alltech). The column oven temperature was within the range 15–70 °C in the different experiments. The solvent gradient consisted of a linear increase in the amount of water in acetonitrile (%H_2O): 0–10 min (25–36%), 10–11 min (36–25%), 11–13 min (25%). The gradient was modified from that described by Alltech for sugar analysis on the specific Prevail column. The recommended maximum pressure for this column is 147 bar (2200 psi), and the experiments were conducted in accordance with this upper limit. The flow rate was set to 1.0 ml/min, and injections of 10 μl were made.

Comparable analyses were performed on a Kromasil NH_2-column (250 mm × 4.6 mm, 5 μm, Supelco). The gradient of increasing amount of water in acetonitrile was (%H_2O): 0–14 min (10–35%), 14–16 min (35–10%), 16–18 min (10%), and flow rate was set to 0.8 ml/min. In order to detect possible effects on the chromatograms produced by an initial absence of water, the gradient based on the amino-column was slightly changed to: 0–2 min (0%), 2–14 min (0–35%), 14–16 min (35–0%), 16–18 min (0%). The flow was adjusted to 1.0 ml/min.

3. Results and discussion

The present HPLC-method was intended to be used as a rapid way of determining the content of fructose, glucose and sucrose in various fruits and vegetables. A Prevail Carbohydrate ES column was used due to good separation and peak performance. These columns are packed with a rugged, hydrophilic polymeric gel that is claimed to give high efficiency and stability, good reproducibility, and long column lifetime. The columns are designed to predominantly analyze mono- and oligosaccharides by normal phase liquid chromatography within the temperature range 4–50 °C. The approval of the method uncovered however a column temperature effect on the peak detection of glucose (Fig. 2). Initially, the column temperature was set to 25 °C, and similar amounts of fructose, glucose and sucrose were injected on the column. The peak area of glucose was, however, smaller than those of both fructose and sucrose. A lowering of the temperature increased this peak area rendering the other

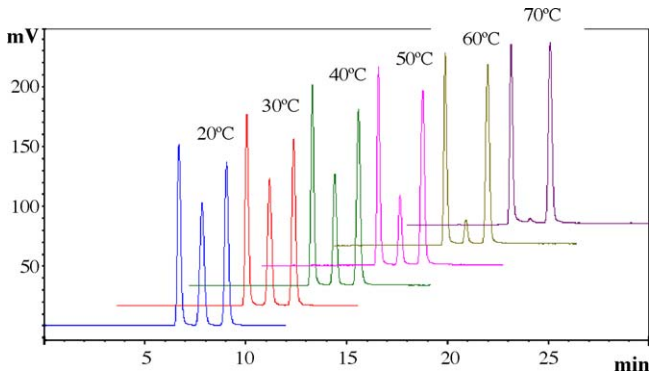

Fig. 2. HPLC chromatograms of fructose, glucose and sucrose, respectively (each 55.6 μg) at different column temperatures. The peak corresponding to glucose gradually disappears with increasing temperature according to the equation: area = (1.831 − 0.032 × temp)/(1 − 0.013 × temp) (R^2 = 0.996). The chromatograms were developed on a Prevail Carbohydrate column (Alltech).

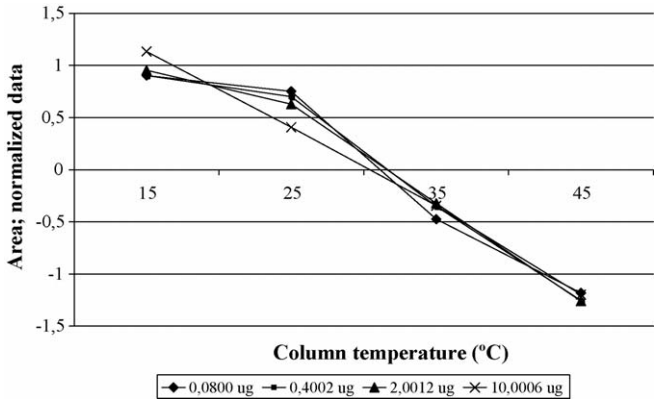

Fig. 3. Function of mass load (glucose) and column temperature on chromatogram peak area.

two unmoved, whereas an increase of column temperature to 70 °C (by steps of 5 °C) suppressed the signal of glucose by >98% from that obtained at 20 °C. A systematic screening of the two standard solutions within a narrow down temperature area (15–45 °C) revealed a similar pattern to the previous observations. The appearance of glucose was reduced by 41% by an increase in column temperature from 25 to 45 °C. The effect seemed to be more distinct at temperatures above 25 °C compared to those below (Table 1). In this case, a slight reduction in the detection of fructose and sucrose was observed as well. For Solution 2 the three peaks decreased with elevation in temperatures (Table 1). The column temperature effect (from 15 to 45 °C) was found to be even more pronounced for these compounds than for glucose: galactose (87% reduction), xylose (94% reduction) and rhamnose (82% reduction). The signal to noise ratios of the aldoses was also calculated. These clearly show that an increase in column temperature from 35 to 45 °C make both galactose and xylose to fall below the limit of detection (LOD). The observations clearly show that the sugar aldoses (reducing sugars) are strongly affected by column temperature under the specific HPLC conditions. As fructose has been described as the first step in hydrothermal degradation of glucose (Fig. 1), pure glucose was eluted under the same conditions as the standard sugar solution. However, no top appeared in the region of fructose in the chromatogram as the top of glucose disappeared. Neither did any other top appear in the chromatogram even if the gradient was extended and the column washed with pure water. None of the decomposition products of glucose that have been described in literature could be detected [5,6]. This means that the mechanisms that have been used to explain the decomposition of glucose in water either at high temperatures or at high pressure cannot be used to interpret the present observations. Inspections were made to see whether the phenomenon of reduced peak amplitude of glucose was connected to sample concentration. A series of four different mass loads of glucose was performed on HPLC (Fig. 3). The results reveal similar drops in detection for the samples with different glucose concentrations. In other words, the relative response factor for glucose is not changed as a function of sample loads.

Table 1
Retention and detection of common sugars (4 μg each) by HPLC-ELSD at different temperatures on a Prevail Carbohydrate ES column

	Glc[a]	Gal	Rha	Xyl	Fru[a]	Sucr[a]
t_R (min)						
15 °C	7.82	7.79	5.47	6.38	6.74	9.01
25 °C	7.56	7.41	5.19	6.10	6.45	8.79
35 °C	7.12	6.98	4.90	5.80	6.04	8.28
45 °C	6.71	6.53	4.66	5.49	5.68	7.80
Area (mV s)						
15 °C	174	117	147	141	190	190
25 °C	176	65	116	95	209	204
35 °C	115	21	72	41	169	167
45 °C	73	3	26	8	166	170
S/N (ASTM)						
15 °C	142	102	198	145	161	175
25 °C	169	65	171	119	253	198
35 °C	112	18	122	62	166	137
45 °C	53	1	37	10	119	106

[a] Mean values, n = 4.

The reduced stability of the sugar aldoses as obtained from the Prevail column was compared to similar analysis on a traditional carbohydrate column; amino-bonded silica. A series of analyses were performed on Solution 1 at column temperatures from 15 to 45 °C (Fig. 4, upper). The systematic decrease of glucose detection as column temperature raised was not observed on this column. Instead, a slight increase in detection was obtained from 15 to 35 °C, with a certain decrease at 45 °C. This chromatographic method consisted of minimum 10% water in acetonitrile. A slight modification of this (see Section 2) was done in order to remove water from the start conditions of the gradient. The results revealed a decrease in the detection of both fructose and glucose as column temperature increased from 15 to 75 °C but no similar effect was detected on sucrose (Fig. 4, lower). It should thus be recognized that water is an important stabilizer for some sugars at temperature and pressure conditions typically used within HPLC analysis.

ELSD have reached popularity as detectors for HPLC analysis of carbohydrates. Two advantages of this technology are the lack of baseline drift as compared to the refractive index detector (RI), and the enhanced sensitivity compared to more

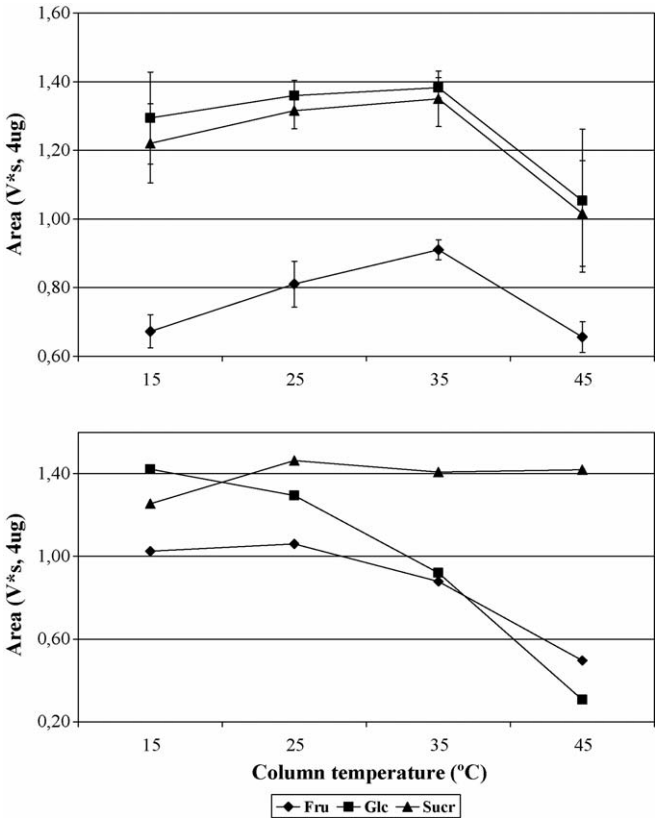

Fig. 4. Upper: detection vs. column temperature by separation of fructose, glucose and sucrose (4 μg each) on a Kromasil NH_2-column ($n = 4$). Lower: similar analysis but with absence of water as initial part of the method (see Section 2).

traditional detection techniques [10]. The ELSD nebulizer gas was in these experiments set to 65 °C, as is an all-round recommendation from the manufacturer. As part of validation of the present HPLC-ELSD method, the nebulizer temperature setting was adjusted from 50 to 80 °C. No deviations in the analytical results were found between the different settings.

4. Conclusion

The detection of aldoses by ELSD and UV (230 nm) decreases with elevated column temperature in normal phase chromatography on an Alltech Prevail Carbohydrate column with acetonitrile and water as solvents. The reaction is strongly temperature dependent, and the first step in the reactions is rate-limiting, as no fructose or other detectable products are recorded. The rate of decomposition is not dependent on sample concentration. Similar temperature effects have to the best of our knowledge not been reported in connection to the most typical HPLC methods that analyze on glucose or other sugars.

The results imply that temperature control is essential in the analysis of sugar aldoses. It is of great importance to calibrate the methods at exactly the same temperatures as used in analysis. In order to reduce LOD, a reduction in column temperature is recommended. It does not make sense to discuss LOD (or limit of quantification, LOQ) without connecting the method to a specific temperature. Moreover, the temperature range from 20 to 30 °C covers the term that often is found in literature when speaking of chromatographic conditions; ambient temperature. This term cannot be used in the measurement of glucose or other reducing sugars without a more exact temperature control. In cases with no column oven it is important to keep the temperature conditions stable throughout the series of analysis, and repeated calibrations are necessary.

Acknowledgement

This work has been funded by the Research Council of Norway.

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