

Application of conventional HPLC RI technique for sugar analysis in hydrolysed hay

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Abstract. To determine the potential biofuel yield and necessary technological parameters a known concentration and type of fermentable sugars should be produced during chemical or biological extraction from lignocellulose. The most popular method for sugar interpretation and quantification is liquid chromatography (HPLC) using refractive index (RI) detector. The aim of this research was to show the applicability of the high–performance liquid chromatography using refractive index (HPLC RI) technique for sugar interpretation in hydrolysed hay and possible solutions for optimisation of this method. Analysis of hydrolysed hay with standard additive showed low recovery of sugar concentrations and inconsistencies with dinitrosalicylic acid (DNS) method, which was mostly due to low separation of peaks of these sugars on the chromatograms. As result HPLC RI method was useful for qualitative analysis of sugars only, not for its quantification.

Key words: lignocellulosic biomass, sugar determination, DNS method, HPLC RI.

INTRODUCTION

Agricultural biomass is one of the most sustainable sources for biofuel production (Chandra et al., 2012). The first stage in biofuel production is chemical or biological extraction of sugars from recalcitrant lignocellulose (Hahn-Hägerdal et al., 2006). Different types of sugars can be extracted in solution through the process of hydrolysis depending on source material: monosaccharides, oligosaccharides, polysaccharides, neutral sugars, acidic sugars, amino sugars, sugar alcohols, and their various isomers (Solomons, 1980). To determine the potential biofuel yield, most appropriate product type and technological parameters, all produced sugars should be accurately quantified and interpreted.

Various methods can be used for sugar determination and quantification depending on the sugar source and technological process: (i) the total sugar content in cotton fibres by spectrophotometric method (ISO, 2014); (ii) the reference method for the determination of lactose content in milk (ISO, 2007); (iii) dinitrosalicylic acid (DNS) method to determine total isolated reducing sugar concentration in solution. These methods are relatively simple, fast and with good precision, however, most of them do not offer any discrimination among sugar types (pentose and hexose sugars). Quantification of specific sugar concentrations in any solution can be performed

with high-performance liquid chromatography (HPLC) (Solomons, 1980; Shaw, 1988; Kelebek et al., 2009; Nefolar et al., 2010; De Goeij, 2013).

To perform sugar HPLC analyses different detection systems can be used: (i) UV-visible detection; (ii) fluorescence detection; (iii) electrochemical detection or (iv) evaporative light scattering detectors (Solomons, 1980; Baranenko et al., 2014). Some of these detectors are better for determination and description of sugars found in regions near 190 to 195 nm, other for components that emit fluorescence. However, it is known that generally sugars are not fluorescent, thus, the selectivity of the previous mentioned detection systems is not very high and the equipment is difficult to operate. The most popular method for sugar interpretation is liquid chromatography (HPLC) using refractive index (RI) detector due to the simplicity of analysis, lower costs of equipment and reagents when compared with other techniques (Ellefson, 2003; De Goeij and detector must be found (De Goeij, 2013).

The aim of this research was to study the applicability of the high-performance liquid chromatography using refractive index (HPLC RI) technique for sugar interpretation in hydrolysed hay and possible solutions for the optimisation of this method.

MATERIALS AND METHODS

Sugar production from lignocellulosic biomass

Hay or straw biomass was grounded by grinder (Retsch GM200) to obtain fractions < 0.5 cm. Further the biomass was diluted in 0.05 M sodium citrate buffer (3% w/v, pH 5.5) and boiled for 5 min to eliminate any indigenous microorganisms.

For enzymatic hydrolysis, laboratory prepared enzyme (0.2 FPU ml⁻¹, Mezule et al., 2015) or 1% v/v cellulolytic enzyme mixture (Viscozyme L, Sigma Aldrich) was added to the diluted substrates and incubated on an orbital at 30 °C. Samples for sugar analyses were collected after dilution with buffer, after boiling, after 6, 24 and 48 h of incubation. At least 2 samples from each test were collected for sugar measurements.

Sugar standards for HPLC RI analysis

Standard stock solutions of individual sugars (L-(+)-Arabinose, D-(+)-Xylose, D-(-)-Fructose, D-(+)-Mannose, D-(+)-Galactose and D-(+)-Glucose (Sigma Aldrich, Germany)) in methanol (MeOH, Sigma Aldrich) and water (1:1) were prepared in separate volumetric flasks. Working solutions were prepared by diluting the stock solutions with the same solvent to contain (0.5–50 mg ml⁻¹ final concentration). Linearity was established by triplicate injections of different concentrations of the standards obtained by dilution in water of the mixture containing the standards. Calibration curves were obtained by plotting peak area versus amount injected.

Determination of total sugar concentration by DNS method

Sugar concentration with dinitrosalicylic acid (DNS) method was performed according to Ghose (1987). In brief, all samples were centrifuged (6,600 g, 5 min). Then 0.1 ml of the supernatant was mixed with 0.1 ml of 0.05 M sodium citrate buffer (mono-sodium citrate pure, AppliChem) and 0.6 ml of DNS (3,5-dinitrosalicylic acid, Sigma Aldrich). For blank control, distilled water was used instead of the sample. Then all samples were boiled for 5 min and transferred to cold water. Further 4 ml of distilled

water was added. Absorption was measured with spectrophotometer at 540 nm (Camspec M501, UK). To obtain absolute concentrations, a standard curve against glucose was constructed.

Sugar determination with HPLC RI

Determination and quantification of specific reducing sugars: arabinose, xylose, fructose, mannose, galactose and glucose was performed by HPLC RI (Perkin Elmer, USA) using COL-AMINO 150 x 4.6 mm column (Perkin Elmer, USA). The analysis was performed at 35°C with a flow rate of 1 ml min⁻¹ using isocratic elution with 75% acetonitrile (AcN):25% water (H₂O) mixture as a mobile phase. All samples were centrifuged at 4,000 rpm for 10 min (Nuve, Turkey) and the supernatant was filtered through a 0.22 µm PES membrane (Sartorius Stedim Biotech, Germany). Then the filtrate was diluted 10 times before direct injection into the HPLC.

To determine the effect of hydrolysate chemical composition and pre-treatment process on the results, blank samples were used as background. To optimise the sugar separation process the following procedures were checked: changes of oven temperature (from 25 °C to 50 °C), changes of eluent and sample flow rate (from 0.5 to 1 ml min⁻¹) and eluent AcN/H₂O composition (50:50; 75:25; 90:10).

Each sample was tested by triplicate injections. The blank and control solutions were analysed with each series of sample in order to verify the accuracy of the obtained results. Accuracy of each test was calculated by considering the recovery obtained for each compound at 10 mg l⁻¹ concentration level in control solution (Table 1) and the mean value was calculated (coefficient of variation CV < 10%). HPLC results (retention time and resolution) of each specific reducing sugar in samples were compared with the retention time and resolution of sugar standards.

Statistical data analysis

General MS Excel statistical data analysis was used for data processing. To determine if the data sets are significantly different or not, t-test analyses (MS Excel 2007) were performed for two tailed distributions. Probabilities of ≤ 0.05 were considered as significant. HPLC Flextar programme Chromera Manager 3.4.0.5712 was used for chromatogram data processing.

RESULTS AND DISCUSSION

In this study, the dinitrosalicylic acid (DNS) method was used to determine total reducing sugar concentration and the HPLC RI method for identification and quantification of specific reducing sugars isolated from hydrolysed hay. The HPLC system used in this study was equipped with gradient pump, column oven, RI detector and conventional amino column (COL-AMINO 150 x 4.6 mm).

The results obtained for the method validation are shown in Table 1. Linearity and range of application of the method were measured by determining the calibration curve by the linear models of L-(+)-Arabinose, D-(+)-Xylose, D-(-)-Fructose, D-(+)-Mannose, D-(+)-Galactose and D-(+)-Glucose respectively.

Table 1. Linear regression parameters obtained from the sugar standard calibration curves and control tests

Sugar	Regression Equation	R ²	MDL, mg l ⁻¹	% RSD (RT)
D-(+)-Glucose	y = 42.7944x - 9.2866	0.9999	0.28	0.36
D-(+)-Xylose	y = 29.9900x - 4.7208	0.9998	0.19	0.23
D-(-)-Fructose	y = 46.2561x + 0.2385	0.9999	0.04	0.24
D-(+)-Mannose	y = 22.9531x - 4.1942	0.9999	0.31	0.11
L-(+)-Arabinose	y = 22.6182x - 1.7041	0.9995	0.21	0.26
D-(+)-Galactose	y = 26.7419x + 9.3517	0.9997	0.35	0.33

The minimal detection limit (MDL) was obtained by the dilution of standards to evaluate the minimal concentration of sugars that can be determined with-in this study. Retention time relative standard deviation (RSD RT) was calculated by HPLC Flextar programme Chromera Manager 3.4.0.5712, which was used for chromatogram data processing by using the retention time of individual sugars during the calibration injections. The obtained detection limits, regression coefficients (R²) and RSD RT indicated that the system is technically suitable for hay analyses.

Analysis of individual sugar standard solutions in water showed that retention time (RT) of arabinose was 4.7 min, xylose-4.0 min, fructose-4.7 min, mannose-5.3 min, galactose-5.5 min and glucose-5.4 min. It was possible to perform calibration by using single sugar standard solution or with a mixture of multiple sugars with different RT: mix 1-xylose, glucose and fructose (Fig. 1), mix 2-mannose, arabinose and galactose.

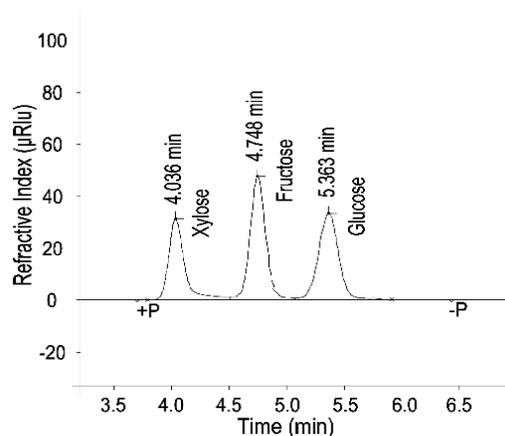


Figure 1. Chromatogram of sugars during calibration of HPLC.

The analysis of hydrolysed hay showed that all sugars (arabinose, xylose, fructose, mannose, galactose and glucose) were found in the enzymatically-hydrolysed hay samples (Fig. 2), but not identified by HPLC programme. The results showed that there is a low separation of peaks of these sugars on the chromatograms. Moreover, the corresponding samples differed from DNS method (28-96%), which always gave lower results. At the same time DNS method presented lower STD values and no difference ($p < 0.05$) among sample repetitions and, similarly as reported before, produced higher

reducing sugar yields from hay biomass than from straw (Mezule et al., 2015). Further evaluation of the HPLC results showed that there is a relatively high concentration of fructose (0–24.4%; MEDIAN 16.9%), mannose (0–48.2%; MEDIAN 31.5%) and galactose (0–33.2%; MEDIAN 0%), which are usually regarded as minor lignocellulosic sugar components (Yabushita, 2016).

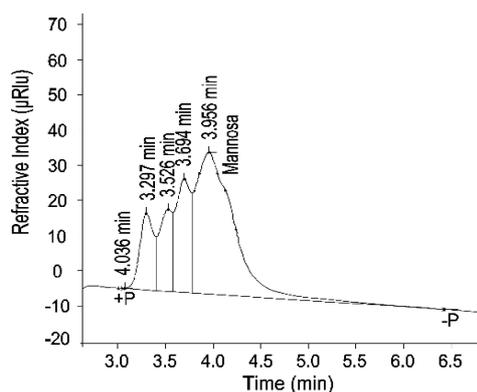


Figure 2. Chromatogram of sugars in hydrolysed hay solution.

To further determine and evaluate the differences in results obtained with both methods, a set of samples were spiked with either glucose or fructose standards. To overcome any changes in sugar standard quality, the spiking was performed after 24 h of hydrolysis and the added volume had a minor effect on overall sample volume (< 1%). The results with DNS method showed that the difference between the spiked samples and unspiked samples and standard concentration does not exceed 5% for both glucose and fructose. At the same time the HPLC data showed that both sugar concentrations in the spiked samples are more than two times higher. Moreover, again high concentrations of fructose (13% v/v) and mannose (34% v/v) were observed in unspiked hydrolysed samples. At the same time glucose yielded only around 22% from all sugars. Low correlation with dinitrosalicylic acid (DNS) method can be explained by low separation of the peaks of these sugars on the chromatograms (Fig. 2).

Different techniques can be used for improving various chromatographic parameters: peak shapes, detection, sensitivity and retention time. Some studies showed that optimization of mobile phase and oven temperature is crucial (Sluiter et al., 2006; Nelofar et al., 2010; Zielinski et al., 2013; Correia et al., 2014). To optimise the sugar separation process the following procedures were performed in this study: changes of oven temperature (25, 30, 35, 40 and 50 °C), changes of RI detector temperature (25 and 35 °C), changes of eluent and sample flow rate (0.5 and 1 ml min⁻¹) and eluent AcN/H₂O composition (50:50; 75:25 and 90:10). Representative results are shown in Table 2.

Table 2. Comparison of RT depending on the oven temperature, RI detector temperature and flow rate

Sugar	RT, min					
	Flow 1 ml min ⁻¹ ; Oven 25 °C; RI 25 °C Injection 5µl	Flow 1 ml min ⁻¹ ; Oven 25 °C; RI 35 °C Injection 5µl	Flow 1 ml min ⁻¹ ; Oven 25 °C; RI 35 °C Injection 20µl	Flow 1 ml min ⁻¹ ; Oven 35 °C; RI 35 °C Injection 5µl	Flow 1 ml min ⁻¹ ; Oven 35 °C; RI 35 °C Injection 20µl	Flow 0.5 ml min ⁻¹ ; Oven 35 °C; RI 35 °C Injection 5µl
arabinose	4.717	3.963	3.970	3.637	3.610	5.468
xylose	4.543	3.643	3.701	3.393	3.351	5.445
fructose	5.131	4.202	4.257	3.840	3.797	5.881
mannose	5.047	4.392	4.404	4.046	4.004	6.111
galactose	6.282	4.287	4.299	4.168	4.107	6.282
glucose	5.732	4.556	4.659	4.183	4.156	6.243

Changes of RT of the sugars in chromatographic column were possible by changing various parameters (oven temperature, flow rate). The results showed that RT increased by 2 min with the decrease of sample flow rates. Injection volume had no effect on the RT, but it can improve detection of low sugar concentrations in samples due to the increase in peak area.

Differences of RT were 0.6–1.2 min in chromatograms at 25 °C and 35 °C temperature of RI detector. RT decreased by 0.1–0.3 min with the increase of oven temperature by 5 °C. Thus, changes in temperature had low effect on the separation of sugars in hydrolysed hay. Changes in eluent AcN/H₂O compositions (50:50; 75:25 and 90:10) had no effect on the chromatogram quality. RT of arabinose and xylose, as well as, fructose and mannose was still too similar.

Some studies (Sluiter et al., 2008; Correia et al., 2014) showed that detector temperature must be close to column or oven temperature. Other showed better result when difference of temperature between detector and column was very high—column temperature 80 °C and detector temperature 50 °C (Zielinski et al., 2013). Other authors showed that additional chemical reagents are better for optimisation of separation process of sugars in column. Depending on the chromatographic column characteristic sulphuric acid (5–12 mM), ammonium hydroxide (0.04%) or addition of MeOH to solvent solution or changes of eluent AcN/H₂O composition can be used (Hernandez et al., 1998; Sluiter et al., 2008; De Goeji, 2013; Correia et al., 2014). The results of this study showed that for the identification of sugars by HPLC RI in hydrolysed hay it is necessary to examine other HPLC columns and chemical reagents to improve the separation.

CONCLUSIONS

Standard HPLC RI protocol and conventional amino column (COL-AMINO 150 x 4.6 mm) were used for experiments. No specific enhancements were tested in this study. The following general conclusions can be drawn from this research: (i) all sugars (arabinose, xylose, fructose, mannose, galactose and glucose) were found in hydrolysed hay samples by HPLC RI method with amino column (COL-AMINO 150 x 4.6 mm); (ii) analysis of hydrolysed hay showed that there is a low separation of peaks of these sugars on the chromatograms and there is no correlation of sugar concentrations determined with dinitrosalicylic acid (DNS) method; (iii) changes of oven temperature,

flow rate, temperature of RI detector and changes in eluent AcN/H₂O compositions had no effect on the chromatogram quality.

To quantitatively identify sugars with HPLC RI in hydrolysed hay it is necessary to examine other available HPLC columns and chemical reagents to improve the separation.

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