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Selective ketopentose analysis in concentrate carbohydrate syrups by HPLC

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Abstract—A new high performance liquid chromatographic (HPLC) method is described for the selective determination of small quantities of ketoses obtained by the action of immobilized isomerases on wheat bran hydrolysates, in the concentrated syrups of the corresponding glucose, arabinose, and xylose. This method uses MilliQ water instead of dilute sulfuric acid as a mobile phase on an Aminex HPX-87H column. Excellent discrimination between xylulose and ribulose was achieved. Selective detection of ketoses was made possible by the much higher UV absorbance at 210 nm. The sensitivity limit is 0.5 g/L for D-xylulose and L-ribulose. The response is linear up to a 20 g/L ketose concentration regardless of the presence of less than 50 g/L of D-xylose or L-arabinose.

Keywords: Xylulose; Ribulose; Pentose; Wheat bran; Isomerization; Biofuels

1. Introduction

Industrial wheat bran accounts for approximately 20% of the grain weight.[1] It can be hydrolyzed by different techniques to a mixture of hexoses and pentoses, including, mannose, galactose and the major constituents glucose, xylose and arabinose.[2] The increasing interest in biofuels in the last 10 years has seen the emergence of numerous studies dealing with the use of pentoses in ethanol production. All of these processes require the isomerization of aldoses into the corresponding ketoses. In order to prevent back-up mechanisms, it is often necessary to carry out the fermentation simultaneously or to include the isomerase activity in the genetic construction as described in the *Zymomonas mobilis* or *Saccharomyces cerevisiae* strains.[3–5] Different protocols such as the carbazole test, originally described by Dische and Borenfreunds,[6] or Kulka's resorcinol method,[7,8] can be used for the detection of these isomers but only in media containing a single carbohydrate and its corresponding isomer. Various analytical methods including HPLC, gas chromatography, pulsed amperometric ion exchange chromatography HPAEC, or IR spectroscopy have been described for the determination of these carbohydrates but these methods are not well-adapted to complex biological mixtures.[9–11] Proper sample preparation is undoubtedly the primary difficulty encountered.[12] None of the previously described methods allow the determination of small quantities of ketose isomers such as ribulose or xylulose simultaneously in the presence of large quantities of the corresponding aldoses.

In this study we wish to report the results of a HPLC protocol using UV detection to selectively analyze the ketoses in complex carbohydrate mixtures. In pentoses, two major types of primary structures are found: the hydroxyaldehydic one (aldoses) and the hydroxyketonic one (ketoses). In pentuloses, the ring closing pyranose form is rendered impossible. Using NMR analysis, Köpper and Freimund reported that the major isomeric form

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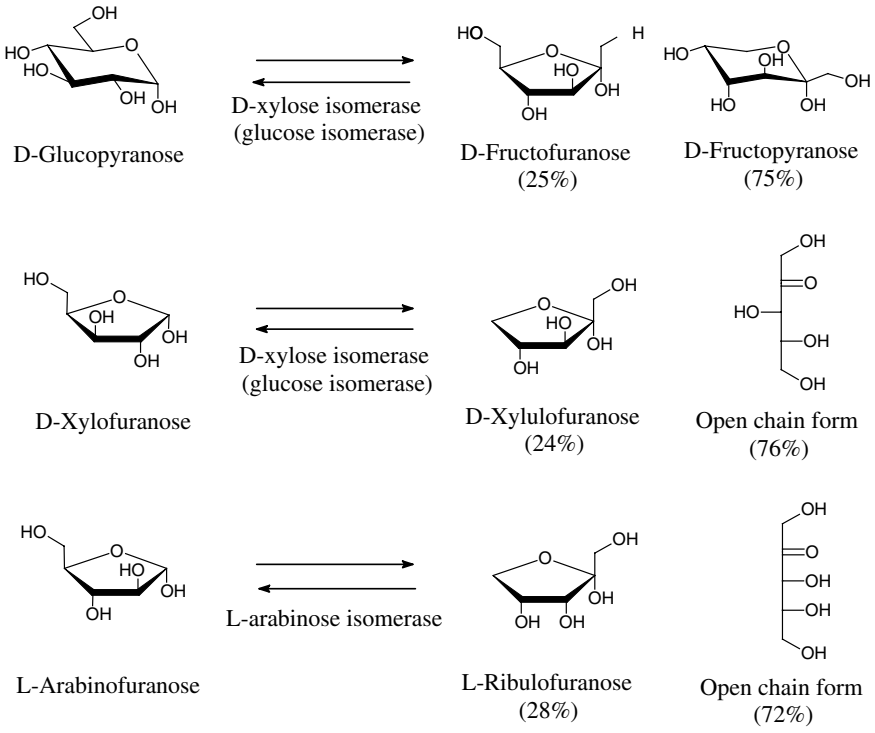

Figure 1. Configurational representations and ratio in water at 25 °C of the major forms of uloses obtained by isomerase action.[14]

under which both ribulose and xylulose were found in aqueous solution was the ketone containing open-chain form, the remainder being the mixture of the two anomeric cyclic furanoses (Fig. 1).[13] The presence of this free ketone function was the key in carrying out the selective UV detection of ribulose and xylulose.

2. Experimental

2.1. Apparatus

The chromatographic analysis was carried out in a high performance liquid chromatograph (Beckman System Gold, Fullerton, California) (Programmable Solvent Module 126 (Pump), Autosampler 502 equipped with a 20 μL loop, Analogue Interface Module 406 and the Gold 4.0 controller software). The chromatographic separation was achieved with different 300×10 mm chromatographic columns.

The columns tested were Aminex HPX-87H, HPX-42A (BioRad Lab., Richmond, CA, USA) and Metacarb 87P (Varian, Les Ulis, France). For the mobile phase, MilliQ water was used. The flow rate was set to 0.6 mL/min and the separation temperature was regulated at 65 °C. Supelcosil® column (Supelco, Bellefonte, PA) was tested using a mobile phase consisting of 75% acetonitrile in water. The working conditions were flow rate of 0.6 mL/min and the separation temperature regulated at 85 °C.

2.2. Detection

Two detectors: a variable wavelength ultraviolet Beckmann Programmable Solvent Detector 166 and a Iota 2 refractive index detector (Precision Instruments, Marseille, France) were used for the simultaneous detection of the hexo- and keto-carbohydrate compounds. Peaks were measured at 190–220 nm, respectively. Chromatographic peaks were identified by comparing retention times and spectra with known standards.

2.3. Reagents and standards

L-Arabinose, D-glucose, D-fructose, D-xylose, D-xylulose, D-ribulose, acetonitrile (HPLC grade) and sulfuric acid (99.999%) were high purity chemicals from Sigma Chemical Co and were used without further purification. MilliQ Water (with a specific resistance higher than 18.2 Ω) used in the chromatographic system was filtered through a 0.22 μm porosity cellulose acetate membrane filter from Sartorius (Goettingen, Germany) and was subsequently degassed prior to use.

2.4. Sample preparation

Highly concentrated samples were prepared by diluting with MilliQ water, and filtering the resulting solution over a Microcon Centrifugal Filter Devices (pore size 10 kDa) from Amicon (Millipore Corporation, Bedford, USA).

3. Results and discussion

The tested columns in Table 1 were those already used for the analysis of ketose isomers after the isomerization of simple aldoses,[15,16] or for the determination of the metabolites during fermentation.[17,18] The analysis of mixtures of arabinose and ribulose on the Aminex HPX-87P column has been reported by Pastinen et al.[16] However, under our conditions we found that ribulose standard could not be detected using this column. De Muynck et al. suggested that ribulose was degraded at the column temperature.[19] On Aminex HPX-42A and Supelcosil® columns all sugar were not separated. Thus, for the remainder of the study we preferred to use the Aminex HPX-87H column in which each sugar could be separated.

Several sulfuric acid concentrations (ranging from 1 to 5 mM) were tested as mobile phases. The presence and concentration of sulfuric acid had no influence on both retention times and the separation quality of the two analyzed ketoses using RI detection (data not shown). However, when sulfuric acid was used as the mobile phase, UV detection of the ketoses was impossible because of the high sulfuric acid absorption interference between 210 and 254 nm (Fig. 2).[20]

Different wavelengths ranging from 190 and 220 nm were tested (Fig. 3). The results show that the detection of ketoses such as ribulose and xylulose at 220 nm is too low. At lower wavelengths the sensitivity of the detection and thus the peak area increases. However, the interference with the aldoses absorption increases significantly from 205 to 190 nm; 210 nm giving the best ketose/aldose selectivity.

The effect of the temperature was tested in the range of 25–85 °C. The results show that the increase in temperature causes changes in the retention time of ketoses. However, this effect is different for each ketose. We observed a reduction in the retention time for ribulose and

Table 1. Retention time (min) of the standards detected by RI on various columns at a 0.6 mL/min flow rate with the respective mobile phases

Column	Mobile phase	Carbohydrate					
		Arabinose	Glucose	Xylose	Ribulose	Fructose	Xylulose
Aminex-87H	5 mM H_2SO_4	11.33	9.62	10.47	11.43	10.23	10.88
Aminex-87P	H_2O	17.57	13.89	15.05	ND[a]	19.16	18.10
Aminex-42A	H_2O	18.14	16.44	16.45	17.93	17.40	16.84
Supelcosil®	75% CH_3CN	15.91	18.29	14.74	13.36	16.61	13.45

[a] ND: no detection.

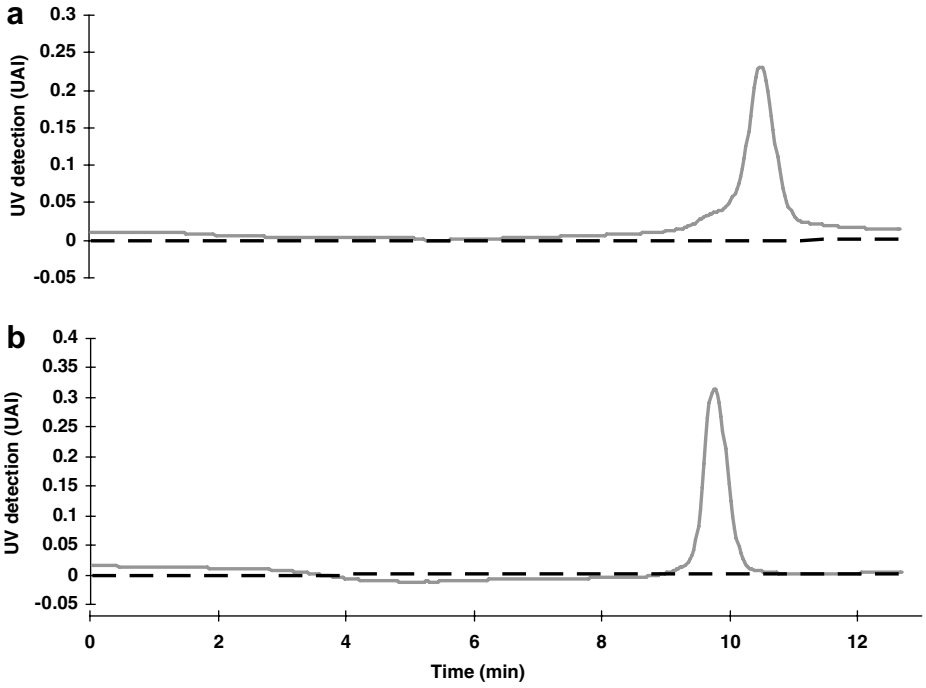

Figure 2. Comparative detection of ribulose (a) and xylulose (b) at 5 g/L concentration on Aminex HPX 87H column with MilliQ water (grey line) and 5 mM H_2SO_4 (dotted line) mobile phase using UV detection at 210 nm at 65 °C.

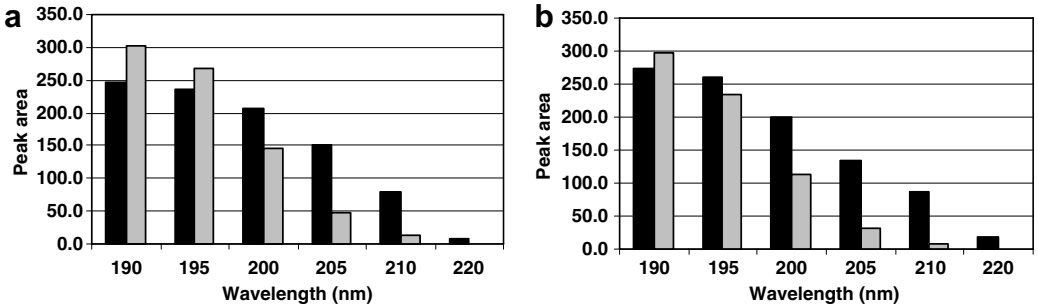

Figure 3. Comparison of peak areas obtained by detection of the pentoses and their corresponding ketoses at different wavelength: (a) ribulose (■) and arabinose (■) and (b) xylulose (■) and xylose (■) ($n = 4$ for each sugar and concentration).

an increase for xylulose (Fig. 4b). Moreover, the temperature has an effect on both the xylulose and ribulose peak area. The observed effect corresponds to an increase of the ulose peak area from 25 to 45 °C and a decrease at temperatures higher than 45 °C (Fig. 4a). In the following experiments the temperature was kept at 45 °C in order to obtain the optimal ketose detection and to appreciably increase the interval between the peaks.

A linear relationship between the sugar concentrations using UV detections was observed (Fig. 5). Higher concentrations than 15 g/L resulted in peak tailing and deformation (data not shown). The correlation coefficients of the calibration graphs were 0.9995 for ribulose and 0.9997 for xylulose. Moreover, the detection limits for ketoses was 0.2 g/L for xylulose and 0.4 g/L for ribulose.

Using UV detection at 210 nm, we found that the resolution was consistent and stable regardless of running times or sample numbers (Table 2). In spite of the fact that aldoses are mostly under the pyranose form there is an equilibrium between the different forms including the opened chain form which might absorb UV.

In order to determine the aldo-pentose concentrations above which there is interference with the ketose determination, the specific absorbance at 210 nm of both ald-

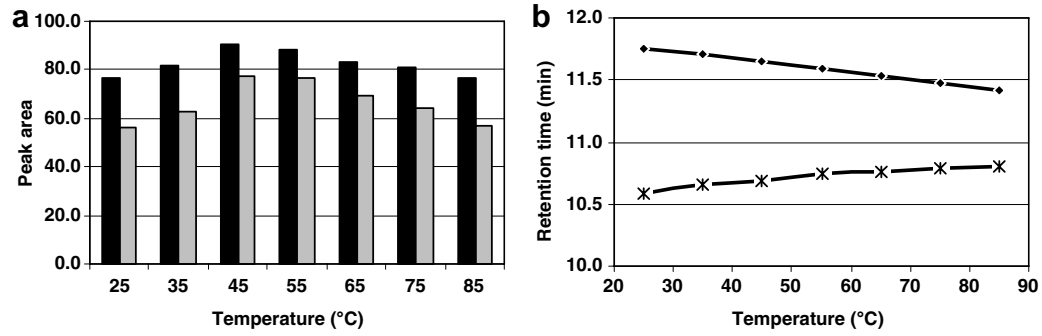

Figure 4. Influence of the temperature on the peak area and the retention times of the ketose using UV detection at 210 nm. (a) Peak area of 2.5 g/L ribulose (■) and 2.5 g/L xylulose (■). (b) Retention time of ribulose (∗) and xylulose (◆) ($n = 4$ for each sugar and concentration).

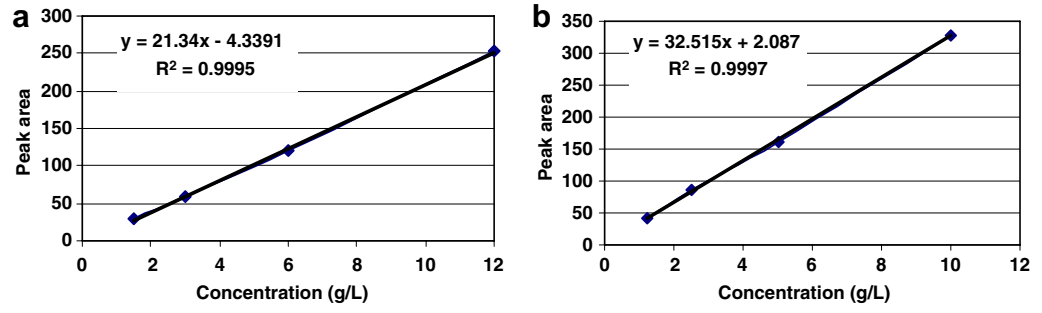

Figure 5. Linearity in UV detection of ribulose (a) and xylulose (b) between 0.5 and 12 or 10 g/L, respectively ($n = 4$ for each sugar and concentration).

Table 2. Variability of the retention times

Component	Retention time[a]			
	Mean (min)		RSD[b] (%)	
	UV detection	RI detection	UV detection	RI detection
Glucose	ND[c]	9.33		1.28
Xylose	ND	10.83		0.56
Xylulose	10.79	11.35	0.69	0.72
Arabinose	ND	11.33		0.85
Ribulose	11.29	11.98	0.32	0.31

[a] Calculated on the basis of four analyses of 1, 2, 5 and 10 g/L standard solutions.
[b] RSD: relative standard deviation.
[c] ND: non detected.

opentoses was measured at various concentrations (Fig. 6). The results indicate that under a 50 g/L aldose concentration the interference is negligible especially in the case of arabinose. However, in the case of xylose, it was still possible to distinguish two distinct peaks between xylose and 2.5 g/L xylulose even at 50 g/L xylose.

This method was applied to the detection of the presence of low ketose concentrations in a mixture containing high concentrations of aldo-hexoses and aldo-pentoses. The carbohydrate components in wheat straw syrups were determined by RI detection for aldoses and by UV detection for ketose using the previously described method (Fig. 7). The recoveries of ribulose and xylulose were 93.1% and 96.3% with RSD of 3.1% and 2.95%, respectively. The results show that using UV for detection and quantification of ketoses is reliable and well adapted to the analysis of low ketose concentrations in the presence of high quantities of other carbohydrates.

4. Conclusion

The method described in this paper presents a rapid, accurate and specific technique using UV detection at 210 nm to measure low ketose concentrations in complex syrups which contain high concentrations of other carbohydrates such as glucose and pentoses. Currently used methods only allow the determination of one ketose in a simple medium containing the corresponding aldose. In this study, small quantities of ketoses

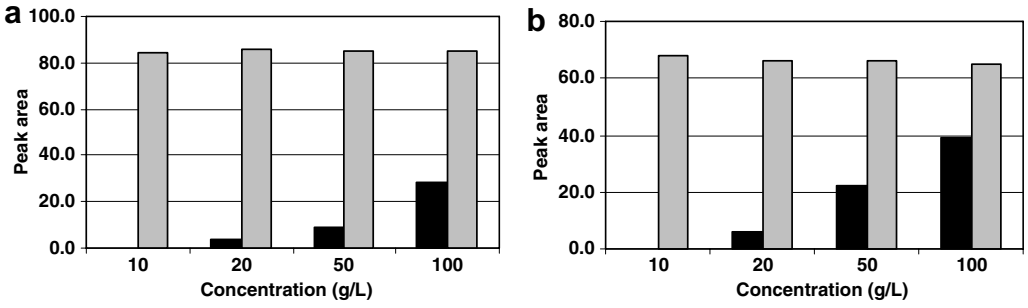

Figure 6. Influence of the aldose concentration (up to 100 g/L) on the peak area of the corresponding ketose (2.5 g/L) using UV detection at 210 nm. (a) Arabinose (■) and ribulose (■) (b) xylose (■) and xylulose (■) ($n = 4$ for each sugar and concentration).

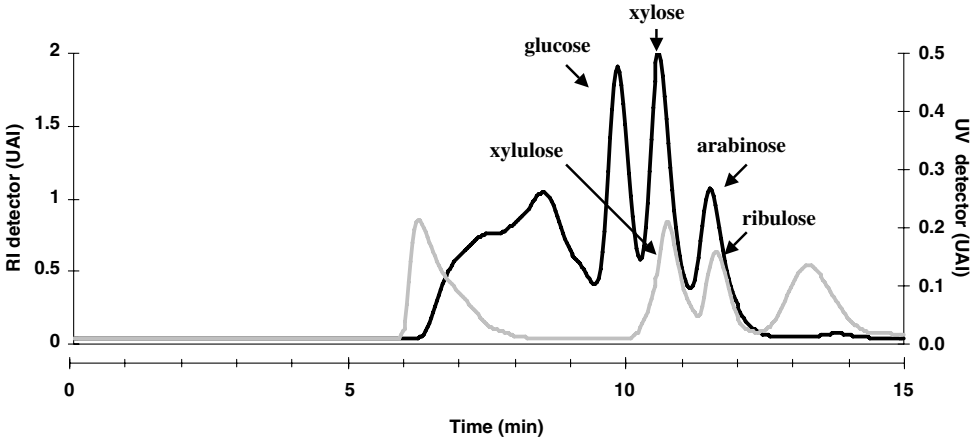

Figure 7. Separation of arabinose, xylose and glucose from wheat bran syrup with a final concentration of 100 g/L and addition of ribulose and xylulose to a concentration of 2 g/L on an Aminex HPX 87H column at 45 °C with water at a flow rate of 0.6 mL/min. RI detection with a sensitivity 32 (▬) and UV detection at 210 nm (▬).

obtained in the processes of pentose isomerization and used in a simultaneous fermentation process were determined in the broth. Both aldoses and ketoses were detected and analyzed simultaneously in the same experimental run. This method allowed us to measure both the isomerization level and the concentration of each individual component in the mixture.

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