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# HPLC DETERMINATION OF XYLULOSE FORMED BY ENZYMATIC XYLOSE ISOMERIZATION IN LIGNOCELLULOSE HYDROLYSATES

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## Summary

In the concentration range appropriate for enzymatic xylose isomerization, xylulose was measured in a lignocellulose hydrolysate using HPLC with two hydrogen loaded ion exchange columns in series. Spent sulphite liquour (SSL) was used as a model for lignocellulose hydrolysates. In buffer the separation took 22 minutes and in SSL the analysis time was 47 minutes due to the presence of ethanol. The enzymatic isomerization of xylose to xylulose was followed directly in SSL, providing a method for the direct determination of xylose isomerase activity in lignocellulose hydrolysates.

## Introduction

The xylose fraction in lignocellulose hydrolysates can be fermented to ethanol using the combination of xylose isomerase (commercially known as glucose isomerase) and bakers yeast *Saccharomyces cerevisiae*(Wang et al., 1980; Jeffries, 1981; Lindén and Hahn-Hägerdal, 1989). The technical and economical optimization of this process concept demands direct analysis methods for activity and stability of the enzyme xylose isomerase. It is therefore essential to be able to measure the isomerization product xylulose directly in the fermentation substrate.

Indirect methods have been described (Gong et al.,1980; Lindén and Hahn-Hägerdal,1989). A sample of enzyme is withdrawn from the reaction solution and the activity is measured in buffer, which means that reversible inhibition is not accounted for. Furthermore, it is mostly the glucose isomerase activity that is measured. Colourimetric methods (Dische and Borenfreund, 1951; Yamanaka 1967), enzymatic methods (Callens et al., 1986), and HPLC methods (Kabel et al., 1983; Roman et al., 1985; Tewari et al.,1985; Pronk et al., 1988) to quantify xylulose in the presence of xylose have been reported. These methods work in buffer solutions with only xylose and xylulose present but in a complex technical substrate like a lignocellulose hydrolysate there are severe interferences from other compounds. A liquid chromatography system has been described for the determination of xylose isomerase activity in sugar cane bagasse (du Toit, 1984 and 1986). The drawback with this method is that it takes seven hours for analysis.

The present communication describes a HPLC method that separates xylulose without large interference from the compounds commonly present in lignocellulose hydrolysates, summarized in Table 1.

**Table 1**

| Compound | SSL (g/l) | SSL hexose fermented (g/l) |
|---|---|---|
| glucose | 3.1 | 0 |
| xylose | 6.7 | 3.9 |
| galactose | 3.2 | 2.4 |
| mannose | 11 | 0 |
| arabinose | 1.4 | 0.9 |
| xylitol | 0 | 0.7 |
| glycerol | 0 | 2.1 |
| ethanol | 0 | 6.7 |
| furfurals<br>phenolics<br>aromatic acids<br>and aldehydes<br>sulphite<br>lignosulfonates<br>ions from hydrolysis<br>and neutralisation | NOT DETERMINED IN THIS STUDY | |

## Materials and Methods

### Substrate

Hexose fermented (*S. cerevisiae*) spent sulphite liquor (SSL), sodium based, was supplied by MODO, Örnsköldsvik, Sweden. For sugar content see Table 1. The high amount of yeast added, 300 g wet weight per liter SSL, causes a dilution, since the volume increases with a factor of 1.37. The pH was adjusted to 6.0 with 10 M KOH before and after fermentation. After fermentation the yeast was removed by centrifugation, the supernatant was filtered (0.22 µm; Millipore membrane filter) and used as a substrate for xylos e isomerization.

### Isomerization

SSL treated as above was incubated on a rotary shaker,30°C. To 5 ml of SSL, 250 mg of Maxazyme GI-Immob (a generous gift from GIST Brocades, Delft, The Netherlands) was added in a sealed glass flask. Samples were withdrawn at intervalls, immidiately diluted with carrier liquid, 0.005 M $H_2SO_4$, and frozen until analysis.

### Analysis

The samples were analysed on a Schimadzu HPLC (LC 6A) system equipped with two hydrogen loaded ion exchange columns (Bio-Rad Aminex HPX-87H) in series. The mobile phase consisted of 0.005 M $H_2SO_4$ with a flowrate of 0.5 ml/min at 40°C. A Schimadzu Rid 6A refractive index detector was used for detection. For the xylulose (Sigma) standards, SSL was diluted to the same concentration as in the isomerization samples. Both for isomerization samples and standards the integrator was programmed to process the peaks apppearing after xylose as peaks on a tail.

## Results and Discussion

Fermentation products may influence the xylose isomerase activity. The substrate used in the present investigation was therefore a SSL in which the hexose fraction had been fermented with bakers yeast, *S. cerevisiae*, prior to enzymatic isomerization of xylose.

The equilibrium of the isomerization of xylose to xylulose is at most shifted 15 to 20 % towards xylulose at 30° C (Tewari et al., 1985). Therefore to reach the appropriate concentration range both for xylose and xylulose the separation of the sugars was optimized for an initial concentration of 6 g xylose /l (Table 1) in buffer and SSL (Figure 1). Despite the fact that SSL contains numerous components which are detected by refractive index the xylulose peak is quite well separated between the xylose and the arabinose peaks with the two hydrogen loaded ion exchange columns in series.

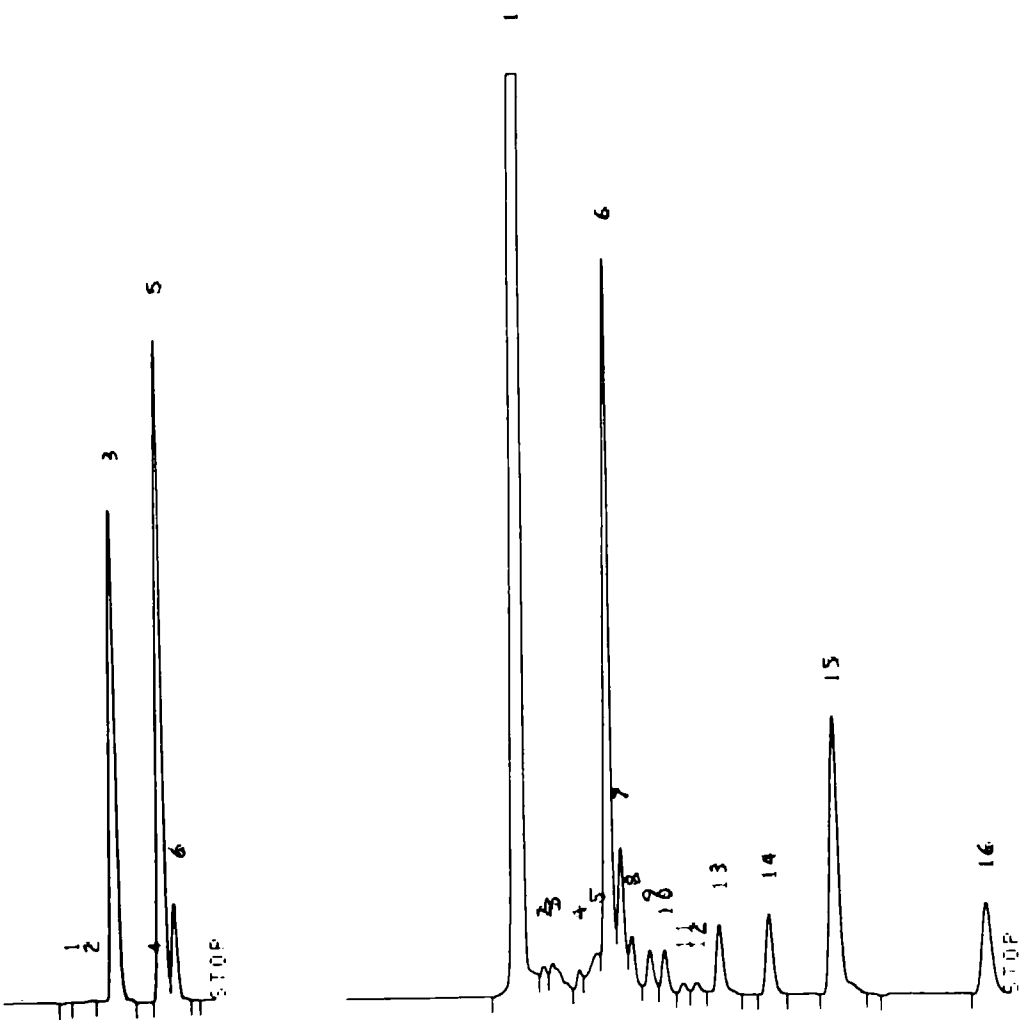
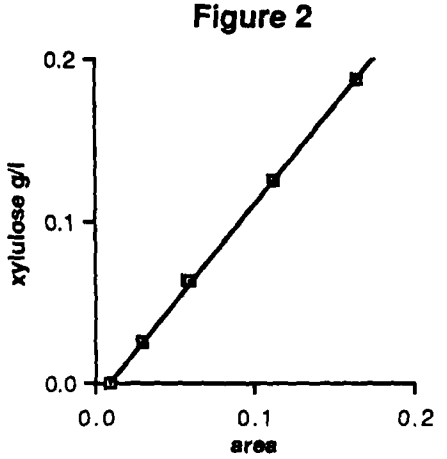



Figure 1 **a.** *Chromatogram of isomerized xylose in buffer. Peak 3: buffer, peak 5: xylose, peak 6: xylulose.* **b.** *Chromatogram of isomerized xylose in prefermented SSL. Peak 1: salts, peak 6: xylose, galactose, mannose, peak 7: xylulose, peak 8: arabinose. Peak 16 is ethanol and elutes at 47 min. For separation conditions see Materials and Methods.*

Next, known amounts of xylulose were added to SSL and the concentration measured with the system described above (Figure 2). There is a linear relation between the peak areas and the concentration range up to at least 0.2 g xylulose/l. Finally, the isomerization was followed to equilibrium (Figure 3). The equilibrium concentration of xylulose under these conditions estimated from (Figure. 3), is 18 %.



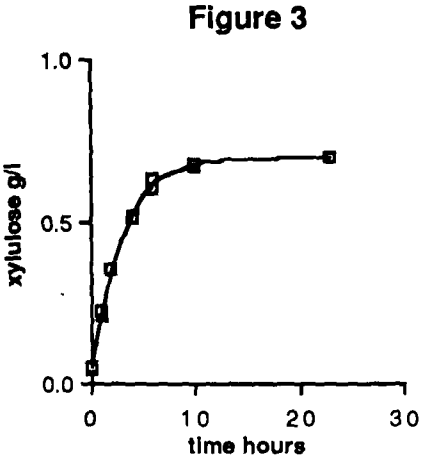




Figure 2 *Standard curve for xylulose in SSL. For analysis conditions see Materials and Methods.*
Figure 3 *Isomerization of xylose, 3.9 g/l, in SSL* (Table 1). *For experimental conditions see Materials and Methods.*

Thus with two HPX-87H columns in series it is possible to follow the enzymatic isomerization of xylose in a lignocellulose hydrolysate such as SSL. With one HPX-87H column the separation of the sugars is not sufficient. Other possible columns such as HPX-87C and HPX-87P were also investigated (data not shown). With HPX-87C xylulose and arabinose coeluted and with HPX-87P xylulose, mannose and ethanol coeluted. The two HPX-87H columns in series have two additional advantages: (i) the same system can be used both for following the isomerization and the fermentation in lignocellulose hydrolysates because cellobiose, glucose, arabinose, xylulose, (xylose, galactose and mannose coelutes), xylitol, glycerol, acetic acid and ethanol are separated; (ii) when using a precolumn with the hydrogen columns, samples do not need desalting as is necessary with the metal ion columns.

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