Application Note

Determination of xylitol in microbial fermentation broth

Category	Food
Matrix	Fermentation broth
Method	HPLC
Keywords	monosaccharides, xylitol, fermentation, Eurokat H, Eurokat Na, Eurokat Ca, AZURA RID 2.1L
Analytes	arabinose, glycerol, mannitol, xylitol, xylose
ID	VFD0149N_A_E

Summary

This application note describes the analysis of a fermentation broth with Eurokat columns and the usage of the new KNAUER AZURA RID 2.1L. Different Eurokat columns were tested revealing that the Eurokat Ca is the best column for the analysis of monosaccharides and sugar alcohol containing samples. In contrast to the toxic Eurokat Pb, the Eurokat Ca column can directly be upscaled for purification purposes.

Introduction

The worldwide demand for sustainable renewable energy sources is increasing as a reaction of the depletion of fossil fuel reserves. The first generation of bio refinery utilizes C6 rich carbon sources such as sugar cane. The newer, second generation bio refinery is using biomass with lower contents of C6 sugars such as wheat straw. Furthermore, this generation aims to use and valorize the whole product and waste stream. The used biomass is often rich in xylose which is normally not used as a carbon source by microorganisms for ethanol production.

It was shown that xylose can be converted to xylitol by different yeast and bacteria species[1,2]. Xylitol is a five-carbon sugar alcohol occurring in nature mostly in low concentrations. Xylitol has found its application in the food and pharmaceutical industry for example as an artificial sweetener in chewing gum and tooth paste. Since the extraction of xylitol from natural sources is too unproductive it is mainly produced by chemical conversion of xylose to xylitol. Using microorganisms to convert xylose to xylitol followed by a simple purification process presents an economical and environmentally-friendly alternative[3].

The exact determination of the fermentation broth before purification is an important step of the purification process. Sugar and sugar alcohols can be easily detected by refractory index detection[4]. Polymer based columns coupled with metallic cations are often used for such analyses.

Here, a sample of fermentation broth provided by the project partner Vogelbusch

Biocommodities GmbH, Vienna was analyzed with three different KNAUER Eurokat columns. Detection was conducted with the AZURA RID 2.1L. The results revealed a good separation with the Eurokat Ca column and high xylitol content in the sample.

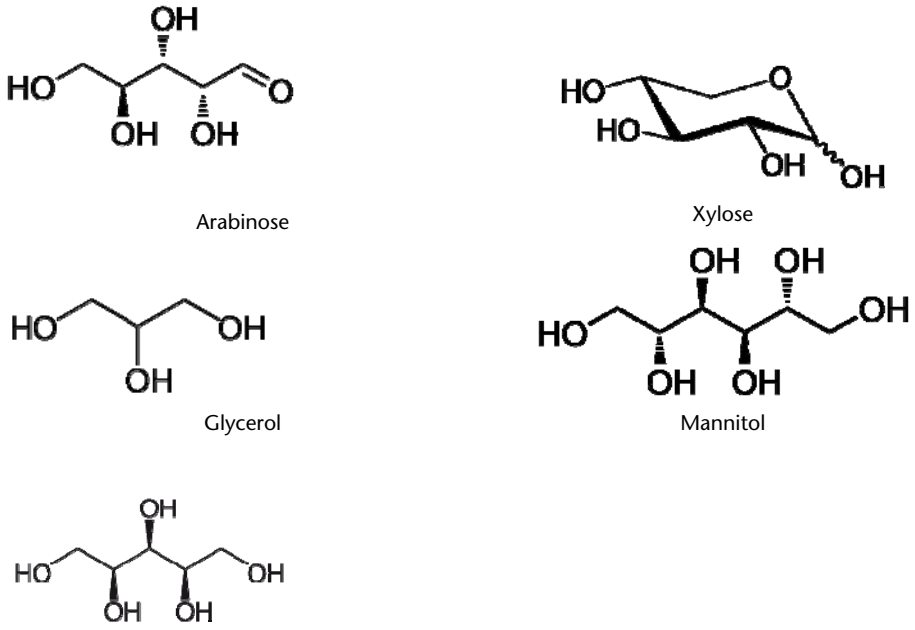

Fig. 1
Chemical structures of identified sugars and sugar alcohols

Experimental sample preparation

Standard solutions of arabinose, xylose, glycerol, mannitol and xylitol (2 mg/ml each) were prepared in H_2O_{dd} and passed through a 0.45 µm filter. The fermentation broth was centrifuged, filtrated and diluted 1:10. For all experiments 20 µl of the samples were injected.

For calibration, a stock solution with 15 mg/ml of all five sugars and sugar alcohols was prepared and six dilutions were prepared. Final concentrations were: 15 mg/ml; 7.5 mg/ml; 5.0 mg/ml; 2.5 mg/ml; 1.25 mg/ml; 0.625 mg/ml; 0.313 mg/ml.

Method parameters

Column	Eurokat H	Eurokat Na	Eurokat Ca
Dimension	30x8+300x8mm	30x8+300x8mm	30x8+300x8mm
Particle size	10µm	10µm	10µm
Eluent A	H_2Odd + 5 mM H_2SO_4	H_2Odd	H_2Odd
Flow rate	0.8ml/min	0.5ml/min	0.5ml/min
Injection volume	20 µl	20 µl	20 µl
Column temperature	60°C	75°C	75°C
Detection	RI	RI	RI
Run time	12 min	18 min	30 min

Results

The fermentation broth from the conversion of xylose rich hemicellulose sample to xylitol by yeast provided by Vogelbusch Biocomidities was analyzed by HPLC. For the analysis, three different Eurokat columns, specialized for saccharide, alcohol and acid analysis, were used.
A first set of experiments revealed that a 1:10 dilution of the original sample is best suitable working solution. All columns were used with pre – columns (30x8 mm) to prevent column contamination. Previous analysis by Vogelbusch showed a good separation profile for the sample with a polymer based Pb-column. Columns that contain lead however, are not adequate as they should not be used for purification purposes.
The separation profiles of the fermentation sample obtained with Eurokat H, Na and Ca revealed that Eurokat Na showed the weakest and Eurokat Ca the best separation behavior for the analyzed sugars and sugar alcohols (Fig. 2). The Eurokat H column revealed a good separation of the five compounds and the run was significantly faster than on Eurokat Ca. Unfortunately, xylitol (Nr. 1) was not baseline separated from the other compounds by the Eurokat H column. The run on the Eurokat Ca was longest of the tested columns. Moreover, the peaks attained with the Eurokat Ca were significantly broader then on the two other columns, resulting in lower intensities. However, the Eurokat Ca columns showed nearly baseline separation for the five main substances.
The main task was to find a column that clearly separates xylitol and was applicable for purification. Thus, the Eurokat Ca fulfilled these criteria the best and was therefore used for further analysis of the sample (Tab.1).

Tab. 1
Comparison of column properties.

Column	**Pb**	**Eurokat H**	**Eurokat Na**	**Eurokat Ca**
Separation behavior of tested compounds	good	good	weak	good
Baseline separation of xylitol?	yes	no	yes	yes
Speed	fast	fast	slow	slow
Suitable for purification?	no	yes	yes	yes

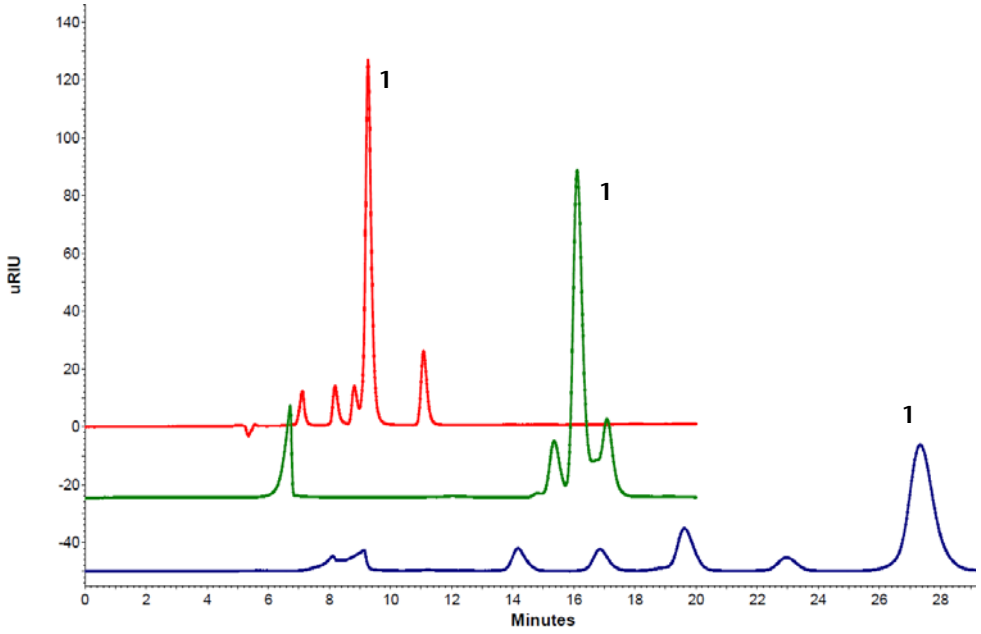

Fig. 2

Separation of fermentation broth (1:10 dilution) on three different Eurokat columns: **red** - H; **green** - Na, **blue** – Ca; **1** – xylitol peak; all columns with pre column 30x8 mm + 300x8mm

The analytical results provided by Vogelbusch gave some indication which substances might be found in the fermentation broth. Using standards of various sugars and sugar alcohols, five main compounds were detected: arabinose, xylose, glycerol, mannitol and xylitol (Fig. 3).

Calibration curves of a mix with the above mentioned compounds were used for the measurement of the concentrations (Fig. 5). All five compounds showed a good linearity over the range of 0.3 – 15 mg/ml.

The Chromatograms revealed a baseline separation of all five substances in the mixture as exemplary shown for the 5 mg/ml mix (Fig. 4).

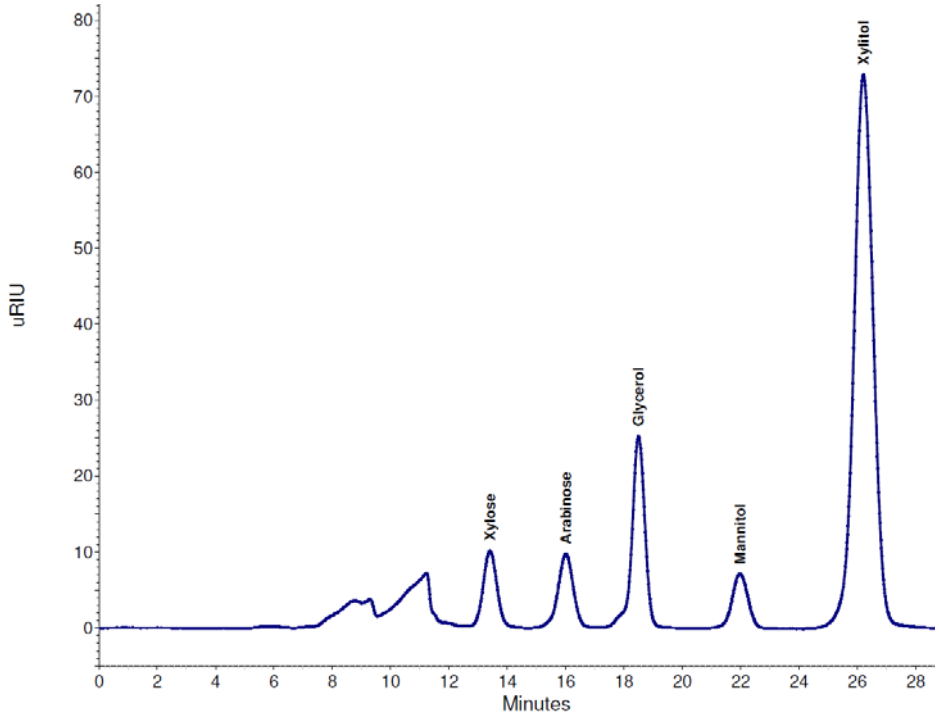

Fig. 3

Separation of fermentation broth (1:10) dilution on Eurokat Ca 300x8 mm w/o pre column, indication of identified peaks

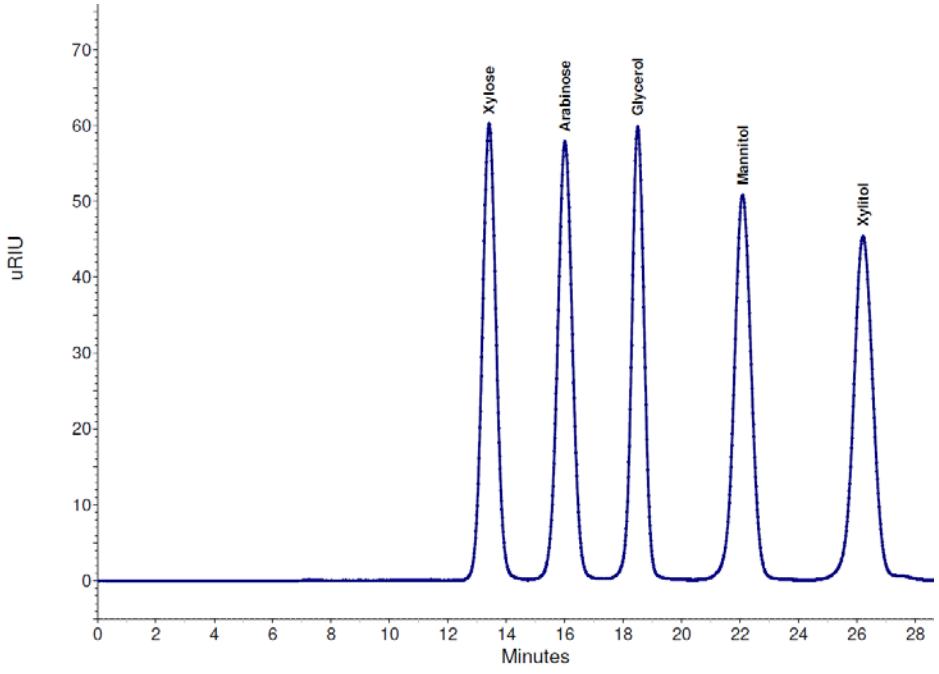

Fig. 4

Separation of the standard mix for calibration, 5 mg/ml each, Eurokat Ca 300x8mm

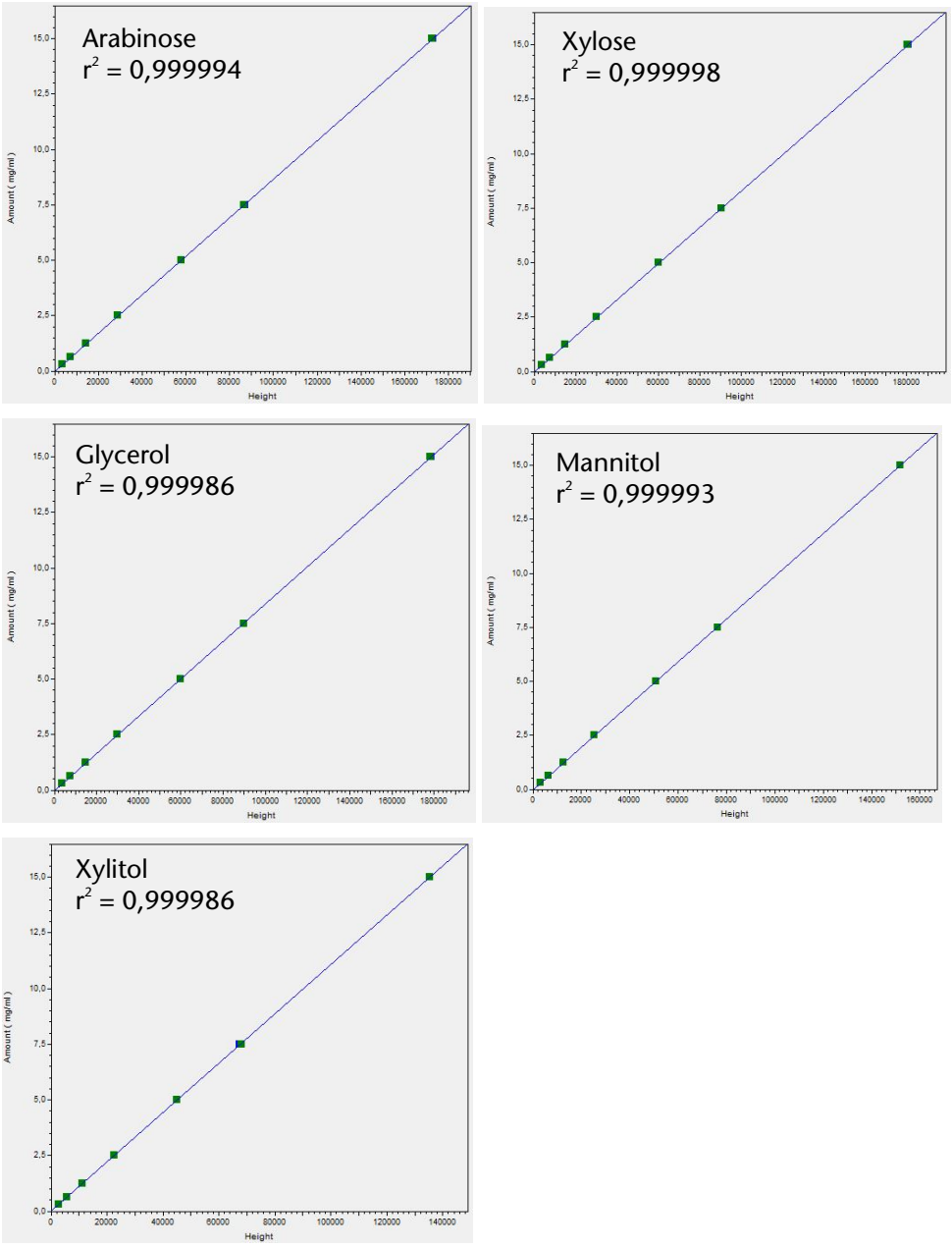

Fig. 5

Calibration graphs for all five tested sugars and sugar alcohols with indication of linearity r^2

The measurements revealed that xylitol had the highest concentration with 80 mg/ml among the detected sugars/sugar alcohols (Tab. 2). This also reflects the observations from the chromatograms as the xylitol peak was significantly dominant in height and area (Fig. 3). Glycerol had the second highest concentration of 21 mg/ml, whereas the three other compounds had similar concentrations of approximately 8 mg/ml.

Tab. 2

Calculated LOQ (limit of quantification) and measured concentration in fermentation broth

Sugars/ Sugar alcohols	LOQ in µg/ml	Concentration in fermentation broth in mg/ml
Xylose	19.8	8.2
Arabinose	13.4	8.3
Glycerol	12.8	21.0
Mannitol	24.7	7.0
Xylitol	35.0	80.6

Conclusion

A sample from microbial conversion of xylose to xylitol in a fermented was analyzed with Eurokat H, Eurokat Na and Eurokat Ca columns. The latter showed the best separation profile even though it had the longest run time.

A more detailed analysis detected xylose, arabinose, glycerol, mannitol and xylitol in the sample. Determination of the concentrations of these substances showed a ten - fold higher concentration of xylitol compared to xylose in the sample. For glycerol second highest concentration was detected.

It can be concluded that the Eurokat Ca is the optimal column for the detection of sugar and sugar alcohols in fermentation broth. The results also showed that the conversion of xylose to xylitol was successful. The application of a Eurokat Ca column allows up-scaling of the process in order to purify xylitol in higher quantities.

Physical properties of recommended column

Eurokat Ca is applicable for the separation of sugar mono- and dimers.

Stationary phase	Sulfonated cross-linked styrene-divinylbenzene co-polymer in calcium form
Particle size	10 µm
Form	spherical
Cross linkage %	6
Dimensions	300x8 mm
Order number	30GX360EKN

Recommended instrumentation

The analysis of saccharides requires a HPLC system equipped with degasser, autosampler, column oven and a refractive index detector. Other configurations are also available. Please contact KNAUER to configure a system that fits your needs.

Description	Order No.
AZURA P 6.1L Quaternary HPLC pump, stainless steel 10ml	APH34EA
AZURA RID 2.1L	ADD31
AZURA CT2.1 Column Thermostat	A05852
Autosampler 3950	A50070
AZURA Eluent Tray E 2.1L	AZC00
OpenLab CDS EZChrom Edition	A2600-1

References

1. Tamburini E.; Costa S., Gabriella Marchetti M., Pedrini P. *Optimized production of xylitol from xylose using a hyper-acidophilic Candida tropicalis*. Biomolecules 5: 1979-1989 (2015)
2. Hernandez-Perez A.F., Vaz de Arruda p., Gracas de Almeidan Felipe M.d. *Sugarcane straw as a feedstock of xylitol production by Candida guilliermondii FTI 20037*. Brazilian Journal of microbiology 47: 489-496 (2016)
3. Chen X., Jian Z.-H., Chen S., Qin W. *Microbial and bioconversion production of d-xylitol and ist detection and application.* Int. J. Biol. Sci. 6(7): 834-844 (2010)
4. Correira D. M., Dias L. G., et al *Dietary sugars analysis: quantification of fructooligossacharides during fermentation by HPLC-RI method.* Frontiers in nutrition 1: article 11 (2014)

Author

Dr. Yannick Krauke, LC LAB Application Specialist/Project Manager

Contact information

KNAUER
Wissenschaftliche Geräte GmbH
Hegauer Weg 38
14163 Berlin, Germany

Tel: +49 (0)30 / 809727-0
Fax: +49 (0)30 / 8015010
E-Mail: info@knauer.net
Internet: www.knauer.net

This project has received funding from the European Union's Seventh Framework Program for research, technological development and demonstration under grant agreement no FP7-KBBE-2013-7-613802; http://www.valorplus.eu/