



## A HPLC tool for process monitoring: rare sugar D- psicose and D- fructose contents during the production through an enzymatic path

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### ABSTRACT

D-Psicose/allulose, a rare sugar, is an essential raw material in the pharmaceutical and food industries. It is scantily found in nature and to meet its demand in industries, D-Psicose is generated enzymatically using D-fructose as a substrate. In these conversions, it is important to monitor D-Psicose, in order to control the process impurities to optimize the reaction time and reduce the process cost. The available analytical methods have their limitations in quantifying D-psicose and D-fructose mixtures. Hence there is a need for the development of a routine, sensitive, quick and precise analytical method for D-psicose production on-line monitoring of reaction mixer. In the present work, a simplified reverse phase HPLC technique is developed and validated for the quick reaction monitoring of D-psicose from D-fructose, during enzymatic conversions procedures. The analysis is conducted at different concentrations ranging from 0.05 % to 0.5 % of the standard solutions of the D-psicose and D-fructose, by using water and Acetonitrile (at a ratio of 20:80) as eluent with a flow rate of 1.0 mL/min on isocratic HPLC-RID system with an aminopropyl silane stationary phase [ZORBAX SIL 4.6 x 150 mm, 5 μm particle size column (USP-L8)]. The applicability of this method is illustrated in reaction monitoring, where D-fructose (substrate) is converted to D-psicose (product) in the presence of the enzyme: D-Tagotose 3- epimerase. Separation of D-psicose and D-fructose is achieved within 8 minutes with a resolution  $\geq 4$  which is the key advantage for reaction monitoring and linearity is established with regression of  $\geq 0.99$ . Additionally, the current method uses a simple mobile phase, without any buffers. It can be used routinely for reaction monitoring.



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### INTRODUCTION

D-psicose, a rare sugar, is turning to be a potential pharmaceutical raw material for many industrial applications viz; as additive for pharma products (Li *et al.*, 2013), food processing and in the beverages production, etc. (Zeng *et al.*, 2013; Smythe *et al.*, 2017) Due to its properties such as low calories and low energy content. D-psicose (D-allulose/ D-ribose-2-hexulose/ C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>) is a hexoketose monosaccharide sweetener with low-calorific value and is a C-3 epimer of D-fructose (Bosshart *et al.*, 2016). It is rarely found in nature (Yagi and Matsuo, 2009)

and it is one of the rare sugars with a potential advantage of using as a precursor and sweetener for pharmaceutical use. It has 0.3 % energy and 70 % relative sweetness of the table sugar (Chung *et al.*, 2012; Chattopadhyay *et al.*, 2014) and is a perfect sucrose alternate for food products. It exhibits significant physiological functions, such as neuro-protective effect, reactive oxygen species scavenging activity (Sun *et al.*, 2008) and blood glucose suppressive effect (Hayashi *et al.*, 2010). It also produces high-quality flavor during food processing and enhances gelling performance (Mu *et al.*, 2012).

### Current existing methods

D-psicose can be enzymatically generated from a cheaper substrate, D-fructose. Because of the position of the thermodynamic equilibrium (Wagner *et al.*, 2015), the enzymatic reactions yield an epimeric mixture of a substrate (D-fructose) and product (D-psicose) (Takeshita *et al.*, 2000; Lee *et al.*, 2006). It is important to monitor the conversion of substrate to the product in the reaction in order to control the process to avoid the process impurities and to optimize the reaction time and also to reduce the process cost (Pazourek, 2019; Kuligowski *et al.*, 2012). Therefore, the development of a routine, sensitive, quick and precise method for the determination of D-psicose content in a reaction mixer is required for the mass production of D-psicose. Currently, there are many methods that are reported in the literature. Chromatographic separation is one of the techniques of choice; it includes High pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), Thin Layer Chromatography (Kim *et al.*, 2008), GC (Gas chromatography) and other HPLC methods with different detectors and different column chemistries. Recently electrophoresis method is also reported using Capillary Electrophoresis (CE) (Surapureddi *et al.*, 2019).

The major disadvantage with HPAEC-PAD analysis is the co-elution of D-psicose and D-fructose, where both the sugars elute as a merged peak. Hence, the technique is not selective enough and also do not serve the purpose. Additionally, the high pH of the mobile phase in the method results in the partial conversion of D-fructose to D-psicose. This makes it difficult and ineffective method for reaction monitoring (Oshima *et al.*, 2006).

HPLC with RID (Refractive index detector) and ELSD (Electro light scattering detectors) methods reported for D-psicose analysis suffers from long run time of 20 to 30 min (Morimoto *et al.*, 2006; Men *et al.*, 2014; Qi *et al.*, 2017) Only short methods allow the researcher to decide on the process devel-

opment, especially during the enzymatic reactions. Moreover, this method is based on the size and ion interactions, which make the technique less preferred ones, as both the D-psicose and D-fructose have a same molecular size (as they are isomers).

GC method with FID is one of the sensitive methods reported for sugar analysis. Though this method is sensitive enough to quantify the sugars, there are a couple of major disadvantages. Long preparation time (5 to 6 hours) due to the derivatization of the sugars makes the method not a good choice for.

HPLC (2 AB labeling Fluorescence-based method) is one of the sensitive methods reported in the literature (Hasehira *et al.*, 2010) where the sugars are labeled with fluorescence dye. The common disadvantages of the method are similar to the GC method, where the labeling step is cumbersome and time-consuming. Fluorescence labeling in the crude sample is again challenging to recovery.

One of the recently reported electrophoresis (Surapureddi *et al.*, 2019) method using Capillary electrophoresis has taken care of most of the above-discussed disadvantages. In the CE method that is reported, UV detection is used for the identification and quantification of rare sugars along with the process impurities and the run time is 20 min. There is always a growing demand for cost-effective, better analytical methods with further less run time, especially for in-process sample analysis. Moreover, the method should be economical enough, with simple instrumentation.

This investigation pertains to the separation of D-psicose from D-fructose by HPLC with RID, using a novel stationary phase, Amino-propyl Silane bonded to ZORBAX SIL, which is not reported till date for monitoring of D-psicose in the presence of D-fructose in the enzymatic conversion reactions. The current method reduces the run time by 2.5 fold in comparison with the most recent method (Surapureddi *et al.*, 2019). The current isocratic separation method on an amino-propyl silane phase bonded column make it possible to resolve D-psicose and D-fructose from other potential by-products within 8 minutes, using acetonitrile and water (80:20) as a mobile phase and with simple instrumentation of HPLC with RI detector (Tihomirova *et al.*, 2016). The method is illustrated by applying to quick real-time monitoring of the reaction mixture.

### MATERIALS AND METHODS

The Analytical Reagent (AR) grade chemicals were used. Acetonitrile (Merck), D-psicose, D-glucose and

D-fructose were acquired from Sigma. Milli-Q/ Purified water was employed for the mobile phase and other standard solution preparations.

HPLC System with RI detector (Shimadzu – Prominence i / Nexera X2) was purchased from Shimadzu. The column Amino-propyl Silane phase bonded to ZORBAX SIL 4.6 x 150 mm, 5  $\mu$ m particle size column (USP-L8) was purchased from Zorbax.

Of the various stationary phases tested from the viewpoint of the chemical structure of the analytes, Amino-propyl Silane was found to be sensitive towards these sugars. Hence in this work, the said stationary phase was used.

Various solvents and their blends were tested for isocratic elution for the said separation. It was observed that the blend of acetonitrile and water in the ratio 80:20 without buffer was found to be optimum. As the content of water was increased above 20%, the resolutions of the sugars were decreased and the column material was getting deteriorated due to the hydrolysis of the silica, though the run time was reduced.

The flow rate of the mobile phase was optimized and it was found to be 1.0 mL/min. When the flow rate was increased by more than 1.0 mL/min, the resolution of peaks was not satisfactory, though the run time was reduced. Moreover, it was creating back pressure that was causing the destruction of the column material and thereby affecting the column lifetime. When the flow rate was reduced to less than 1 mL/min, though the resolution was good, it was increasing the run time beyond reasonable time limits of the experiment.

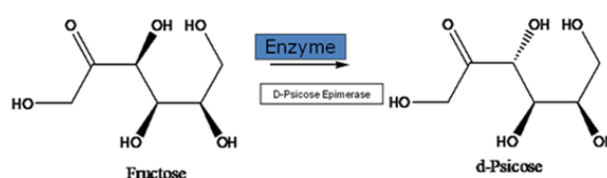
Hence, the optimal conditions for the successful resolution of the three sugars were found to be: isocratic elution with the mobile phase having composition: Acetonitrile and water (80:20% v/v); flow-rate 1.0 mL/min; column compartment temperature: 35°C; injection volume: 20  $\mu$ L; RID flow cell temperature: 40°C. The said experimental conditions were used in this investigation for monitoring the conversion of D-fructose to D-psicose.

## RESULTS AND DISCUSSION

In the present investigation, D-glucose is also included in the mixture with D-fructose and D-psicose as in most of the enzymatic conversions after the lapse of a certain time of Chromatographic elution. The former may appear as an impurity.

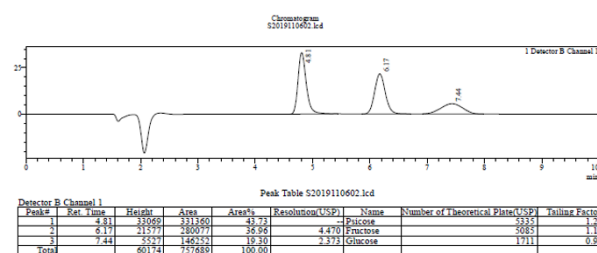
The conversion of Fructose (substrate) to D-Psicose (product) is presented in Figure 1.

### The separation method



**Figure 1: Schematic representation of reaction, where D-fructose is converted to D-psicose in the presence of Epimerase enzyme**

Separation of mixtures of D-psicose, D-fructose and D-Glucose were carried by an HPLC method at the optimum conditions as was described in Sec 2. Resolution and the peak separation are represented in Figure 2.



**Figure 2: Schematic representation of Separation, where D-psicose peak is obtained at 4.81 min, D-fructose peak is obtained at 6.17 min and the D-glucose peak is obtained at 7.44 min**

The resolution was found to be > 4.4 between D-psicose–D-fructose and > 2.3 between D-fructose–D-glucose, when the mixture of the three pure sugars was analyzed.

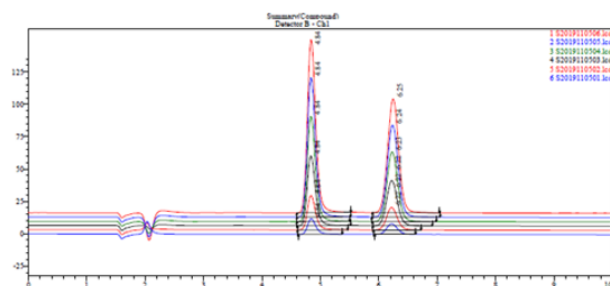
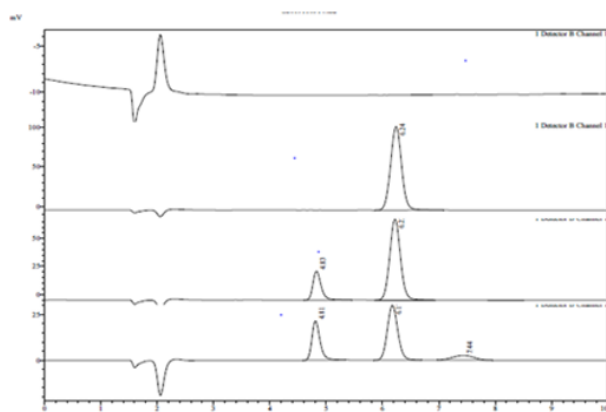
The present developed method has different chemistry, involving polarity and ion exchange nature, which is unique for reaction monitoring. The amino column has -NH<sub>2</sub> group in the terminal of the carbon chain and the lone pair available on Nitrogen provides sufficient binding affinity towards the three sugars. Due to the structural differences between these three sugars, the molecular interactions (mainly hydrogen bonding) between the -NH<sub>2</sub> groups of the Colum metrical and three sugars varies (Dashnau *et al.*, 2005; Hewetson *et al.*, 2014). D-psicose with just two hydroxyl groups structurally oriented towards the NH<sub>2</sub> has the least affinity towards the column when compared with the fructose, which has three hydroxyl groups available for the interactions. As a consequence of this, D-psicose is eluted faster than the D-fructose. In the case of D-glucose, the presence of an additional-CHO group imparts more binding nature towards the column and hence it is retained more time in the Column than the rest of the two sugars.

**Table 1: Method validation in terms of linearity, range, the limit of detection, the limit of quantification, reproducibility, retention times, resolution and recovery of sugars**

Analyte	Linear Range (%)	Correlation coefficient $r^2$	LOD/LOQ	%RSD Day 1(n=6)	%RSD Day 2(n=6)	Rt's	Resolution	Recovery
D- psicose	0.05-0.5	0.9993	0.007 / 0.02	1.85	2.14	16.78	4.4	96 to 98
D- fructose	0.05-0.5	0.9998	0.006 / 0.018	1.38	1.95	19.09		

**Table 2: Applications**

S.No.	Monitoring of D-psicose in enzymatic conversion from D-fructose	Sample collected at times:	% of D-psicose conversion
1	0 hrs	0	From 100mM D-fructose
2	48.0 hrs	8.0 mM	
3	60.0hrs	20.0 mM	

**Figure 3: Overlay of different concentrations of D-psicose and D-fructose. D-psicose peak is obtained at 4.4 min and the D-fructose peak is obtained at 6.2 min****Figure 4: Reaction monitoring for 48 hours. D-psicose peak is obtained at 4.41 min, D-fructose peak is obtained at 6.2 min and the D-glucose peak is obtained at 7.4 min**

So far, existing HPLC methods available in literature used different chemistries, which are based on Size or ion exchange or in combination, which is one of the major reasons for extended run time. Column chemistry used in the current method reduces the run time to less than 8 min.

The current method is the first of its kind HPLC based method with a simple mobile phase without any salts in the current study makes it an adaptable method for mass analysis. Other existing methods either use salt buffers or derivatization techniques or tag the molecules with fluorescent dyes. The use of a simple mobile phase will also help in sample recovery, which is not possible with all the reported methods. Though the method published by (Surapureddi *et al.*, 2019) using CE is sensitive and uses simple buffers, sample recovery is not possible, as the CE instrument does not have the option for sample collection. Additionally, the current method reduces the run time from 18 min to less than 8 min in comparison with the CE method. The current method can also be optimized to ELSD to further increase the sensitivity.

### Validation

To find out the range of the method, different concentrations (ranging from 0.05% to 0.5%) of D-fructose and D-psicose were subjected for the analyses using the present developed method. Linearity was established with regression of 0.99 for both D-psicose and D-fructose within the worked out concentration range. Overlay of different concentration data is represented in Figure 3. The method repro-

ducibility was established with a variation of less than 5% at 0.5% concentrations for both the sugars when injected multiple times (n=6). Reproducibility, LOD, LOQ, recovery and the regression data are presented in Table 1.

### Application of the method to monitor D-psicose formation from D-fructose

To exhibit the applicability of the present developed technique, we carried out the conversion reaction using a modified (Itoh *et al.*, 1995) method. Purified D-Tagatose Isomerase was immobilized onto Chito pearl beads. The immobilized beads were poured into a glass column. Substrate solution (Fructose) was passed through the column. Eluted product was collected at different time points (data was represented only at 0 min, 48 hrs and 60 hrs). The reaction was carried out at 30°C. Samples that were collected were subjected to the analysis using the current method. Reaction monitoring was performed till 60 hours, where the increasing concentration of D-psicose was maximal at 48 hours. Further, processing of reaction to 60 hours resulted in the formation of Glucose. Reaction monitoring data is represented in Figure 4 and Table 2.

### CONCLUSION

An accurate, simple, fast and precise technique based on HPLC with RID has been developed with less run-time by employing the mobile phase of acetonitrile and water in the ratio 80:20% (v/v). All the published methods till date have many disadvantages viz, longer run time, derivitization or tagging of the molecule of interest, usage of salt-based buffers and the usage of high-end equipment, where the technical expertise is the bottleneck. The current method has overcome all the shortfalls of the existing methods, where the analysis can be completed within 8 min, a sample can be recovered easily and there is no requirement for complex instrumentation and no processing of the sample. The current method can be an ideal choice for the identification and quantification of D-psicose, D-fructose and D-glucose. The method can be the best choice for in-process sample analysis, where reaction monitoring within a short time helps the researches to optimize process parameters effectively.

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### REFERENCES

- Bosshart, A., Wagner, N., Lei, L., Panke, S., Bechtold, M. 2016. Highly efficient production of rare sugars D-Psicose and L-Tagatose by two engineered. *Tagatose Epimerases Biotechnology and Bioengineering*, 113:349–358.
- Chattopadhyay, S., Raychaudhuri, U., Chakraborty, R. 2014. Artificial sweeteners - a review. *Journal of Food Science and Technology*, 51(4):611–621.
- Chung, M.-Y., Oh, D.-K., Lee, K. W. 2012. Hypoglycemic health benefits of d -Psicose. *Journal of Agricultural and Food Chemistry*, 60:863–869.
- Dashnau, J. L., Sharp, K. A., Vanderkooi, J. M. 2005. Carbohydrate Intramolecular Hydrogen Bonding Cooperativity and Its Effect on Water Structure. *The Journal of Physical Chemistry B*, 109(50):24152–24159.
- Hasehira, K., Nakakita, S. I., Miyanishi, N., Sumiyoshi, W., Hayashi, S., Takegawa, K., Hirabayashi, J. 2010. A comprehensive HPLC analytical system for the identification and quantification of hexoses that employs 2-aminobenzamide coupling. *Journal of Biochemistry*, 147(4):501–509.
- Hayashi, N., Iida, T., Yamada, T., Okuma, K., Takehara, I., Yamamoto, T., Tokuda, M. 2010. Study on the Postprandial Blood Glucose Suppression Effect of D-psicose in Borderline Diabetes and the Safety of Long-Term Ingestion by Normal Human Subjects. *Bioscience, Biotechnology, and Biochemistry*, 74(3):510–519.
- Hewetson, M., Aaltonen, K., Tulamo, R. M., Sankari, S. 2014. Development and validation of gas chromatography-flame ionization detection method for quantifying sucrose in equine serum. *Journal of Veterinary Diagnostic Investigation*, 26(2):232–239.
- Itoh, H., Sato, T., Izumori, K. 1995. Preparation of d-psicose from d-fructose by immobilized d-tagatose 3-epimerase. *Journal of Fermentation and Bioengineering*, 80(1):101–103.
- Kim, N.-H., Kim, H.-J., Kang, D.-I., Jeong, K.-W., Lee, J.-K., Kim, Y., Oh, D.-K. 2008. Conversion Shift of D-Fructose to D-Psicose for Enzyme-Catalyzed Epimerization by Addition of Borate. *Applied and Environmental Microbiology*, 74(10):3008–3013.
- Kuligowski, J., Cascant, M., Garrigues, S., Guardia, M. 2012. An infrared spectroscopic tool for process monitoring: Sugar contents during the production of a depilatory formulation. *Talanta*, 99:660–667.
- Lee, Kim, D.-K. O. H.-J., Hyun, E.-K., Kim, Y.-S., Yong-Joo 2006. Characterization of an Agrobacterium tumefaciens D-Psicose 3-Epimerase That Converts

- D-Fructose to D-Psicose. *Applied and Environmental Microbiology*, 72(2):981-985.
- Li, Z., Gao, Y., Nakanishi, H., Gao, X., Cai, L. 2013. Biosynthesis of rare hexoses using microorganisms and related enzymes. *Beilstein Journal of Organic Chemistry*, 9:2434-2445.
- Men, Y., Zhu, Y., Zeng, Y., Izumori, K., Sun, Y., Ma, Y. 2014. Co-expression of d-glucose isomerase and d-psicose 3-epimerase: Development of efficient one-step production of d-psicose. *Enzyme and Microbial Technology*, pages 64-65.
- Morimoto, K., Park, C. S., Ozaki, M., Takeshita, K., Shimonishi, T., Granström, T. B., Izumori, K. 2006. Large scale production of D-allose from D-psicose using continuous bioreactor and separation system. *Enzyme and Microbial Technology*, 38:855-859.
- Mu, W., Zhang, Y., Feng, B., Jiang, L., Zhou 2012. Recent advances on applications and biotechnological production of d-psicose. *Applied Microbiology and Biotechnology*, 94(6):1461-1467.
- Oshima, H., Kimura, I., Izumori, K. 2006. Psicose Contents in Various Food Products and its Origin. *Food Science and Technology Research*, 12(2):137-143.
- Pazourek, J. 2019. Rapid HPLC method for monitoring of lactulose production with a high yield. *Carbohydrate Research*, 484:107773-107773.
- Qi, Z., Zhu, Z., Wang, J.-W., Li, S., Guo, Q., Xu, P., Lu, F., Qin, H.-M. 2017. Biochemical analysis and the preliminary crystallographic characterization of d-tagatose 3-epimerase from *Rhodobacter sphaeroides*. *Microbial Cell Factories*, 16(1):193-193.
- Smythe, E., Fletcher, J. N., Kolberg, L. W. 2017. D-Allulose as a flavoring substance with flavor modifying properties. *Glob. J. Food Sci. Technol*, 5(4):258-261.
- Sun, Y., Hayakawa, S., Ogawa, M., Fukada, K., Izumori, K. 2008. Influence of a Rare Sugar, D-Psicose, on the Physicochemical and Functional Properties of an Aerated Food System Containing Egg Albumen. *Journal of Agricultural and Food Chemistry*, 56(12):4789-4796.
- Surapureddi, S. R. K., Kunta, R., Kumar, G. S. S., Sappidi, S. R., Dadke, S. 2019. A sensitive and high throughput method for the analysis of d-psicose by capillary electrophoresis. *Food Chemistry*, 281:36-40.
- Takeshita, K., Suga, A., Takada, G., Izumori, K. 2000. Mass production of d-psicose from d-fructose by a continuous bioreactor system using immobilized d-tagatose 3-epimerase. *Journal of Bioscience and Bioengineering*, 90(4):453-455.
- Tihomirova, K., Dalecka, B., Mezule, L. 2016. Application of conventional HPLC RI technique for sugar analysis in hydrolysed hay. 14:1713-1719.
- Wagner, N., Bosshart, A., Failmezger, J., Bechtold, M., Panke, S. 2015. A Separation-Integrated Cascade Reaction to Overcome Thermodynamic Limitations in Rare-Sugar Synthesis. *Angewandte Chemie International Edition*, 54(14):4182-4186.
- Yagi, K., Matsuo, T. 2009. The Study on Long-Term Toxicity of D-Psicose in Rats. *Journal of Clinical Biochemistry and Nutrition*, 45(3):271-277.
- Zeng, Y., Zhang, H., Guan, Y., Zhang, L., Sun, Y. 2013. Comparative study on the effects of d-psicose and d-fructose in the Maillard reaction with  $\beta$ -lactoglobulin. *Food Science and Biotechnology*, 22(2):341-346.