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Application News

High Performance Liquid Chromatograph Nexera™ Reducing Sugar Analysis System

Simultaneous Analysis of Constituent Sugars and Glucuronic Acid in TEMPO-Oxidized Cellulose Nanofiber

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User Benefits

- This method enables simultaneous analysis of the constituent sugars and glucuronic acid in TEMPO-oxidized cellulose nanofiber.
- High sensitivity and selectivity analysis of saccharides is possible by using the post-column fluorescence derivatization method.

■ Introduction

Cellulose nanofiber (CNF) is produced by mechanically treating wood pulp (fiber diameter: 20 to 30 µm, fiber length: 0.5 to 3 mm). However, because strong hydrogen bonds form between the CNF microfibrils in wood pulp, various problems arise if only mechanical treatment is used, as nanoization to the smallest crystal size (fiber diameter: 3 to 4 nm) is insufficient, damage to the CNF is excessive, and a large amount of energy is required in nanoization.

A group led by Special Research Professor Akira Isogai of the University of Tokyo has reported that nanofibers can be obtained by TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical) mediated oxidation of cellulose and is promoting mass production by this approach. The Application News 01-00023-EN introduced an example of constituent sugar analysis in various CNF samples using a Shimadzu Nexera reducing sugar analysis system. This article introduces a simultaneous analysis of the constituent sugars and glucuronic acid in TEMPO-oxidized cellulose nanofiber.

■ TEMPO-Oxidized Cellulose Nanofiber

One technique for reducing the energy required in nanoization with a view to mass production of CNF is introduction of an ionic functional group on the crystal surface of the cellulose in wood pulp before mechanical treatment. Because an osmotic pressure effect of water and electrostatic repulsion effect between the CNF microfibrils can be obtained by introducing an ionic functional group, nanoization is possible by simple mechanical treatment in water.

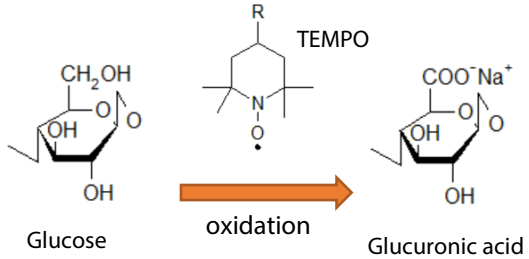

Fig. 1 TEMPO-Oxidized CNF

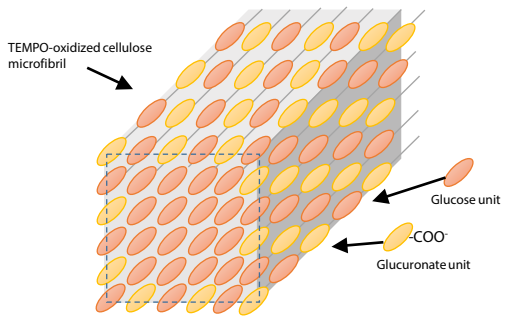

Fig. 2 Structural Model of TEMPO-Oxidized CNF

In TEMPO-oxidized CNF, the hydroxyl groups are partially carboxylated by TEMPO catalyst (Fig. 1) [1].

Only in the exposed part of the cellulose microfibril surface, the hydroxyl group at the C6 position of glucose is converted to a sodium salt of the carboxyl group by TEMPO catalyzed oxidation, and as a result, CNF can be obtained uniformly (Fig. 2) [2].

The amount of the introduced carboxyl group is considered to be critical for obtaining homogenous CNF. Although the amount of the carboxyl group can be measured by conductometric titration and NMR [3] [4], in this article, we investigated the simultaneous analysis of constituent sugars and glucuronic acid.

■ Sample Preparation

Table 1 shows a list of the samples used in the analyses, and Fig. 3 shows the sample preparation protocol.

Table 1 List of Samples

No.	Sample
1	Tempo oxidized CNF-1
2	Tempo oxidized CNF-2
3	Tempo oxidized cellulose

1. Freeze drying

All samples are lyophilized.

2. Primary hydrolysis

Transfer about 0.03 g of the sample to 25 mL of the test tube. 300 µL of 72 % sulfuric acid is added to the test tube. Immerse the test tube in a 30 °C water bath for about 1 hour. Mix with a vortex mixer or glass rod every 15 minutes to completely dissolve the sample.

3. Secondary hydrolysis

Add 8.4 mL of ultrapure water to the test tube, and mix well. The test tube is heated in an autoclave for 60 minutes at 120 °C.

4. Neutralization

Filter through glass fiber filter paper, and dilute with 10 mL of ultrapure water. While checking the pH with a pH test paper, a saturated aqueous solution of barium hydroxide*1 is added, neutralized*2, and sulfuric acid is salted out.

5. Filtration and dilution

Filter through a membrane filter with a pore size of 0.2 µm. Dilute with acetonitrile*3.

*1 Dissolve 8 g of barium hydroxide octahydrate in 100 mL of ultrapure water.
*2,*3 The ratio and magnification vary depending on the sample. See Table 2.

Fig. 3 Sample Preparation Protocol

Since the neutralization conditions and the concentrations of the saccharides differ depending on the sample, the conditions for each sample were set as shown in Table 2. In addition, acetonitrile was used as the diluent solvent, considering the effect of the sample solvent on the peak shape.

Table 2 Neutralization Conditions and Dilution Ratios Used in Sample Preparation

Sample	Sample : Saturated barium hydroxide solution (v:v)	pH	Dilution ratio
1	600 : 300	3	4
2	600 : 300	5	4
3	500 : 500	3	8

■ Analytical Conditions

Table 3 shows the analytical conditions used in the analysis of the hydrolyzed samples. The column used here was Asahipak NH2P-50, which is a polymer-based amino group-modified column. Reducing sugars are normally adsorbed to the packing material of the column due to the formation of the Schiff base. This may result in insufficient peak intensity. Therefore, phosphoric acid-added mobile phases were used to suppress the formation of Schiff's base. Fig. 4 shows the chromatogram acquired by analyzing the standard solution using these conditions.

Table 3 Analytical Conditions

System	: Nexera Reducing Sugar Analysis System
<Separation>	
Column	: Asahipak NH2P-50 4E (250 mm × 4.6 mm I.D., 5 µm)
Guard column	: Asahipak NH2P-50G 4A (10 mm × 4.6 mm I.D., 5 µm)
Mobile phase A	: Water / 85 % Phosphoric acid = 1000 : 3
Mobile phase B	: Acetonitrile / 85 % Phosphoric acid = 1000: 3
Flow rate	: 0.8 mL/min
Time program	: B Conc. 90 % (0 min) - 87 % (30 min) - 80 % (40 min) - 75 % (55.01 - 65 min) - 90 % (65.01 - 100 min)
Gradient mixer capacity	: 1.7 mL
Column temp.	: 35 ˚C
Injection vol.	: 10 µL
Vial	: SHIMADZU LabTotal™ for LC 1.5 mL, Glass*4
<Post-column reaction>	
Reaction reagent	: Mixed aqueous solution of 5 g/L arginine, 0.4 mol/L borate and 0.2 mol/L potassium hydroxide
Flow rate	: 0.5 mL/min
Reaction temp.	: 150 ˚C
Detection	: Ex. 320 nm, Em. 430 nm (RF-20AXS)
Cell temp.	: 25 ˚C
Reaction coil	: SUS tubing, 8 m × 0.5 mm I.D.

*4 P/N: 227-34001-01

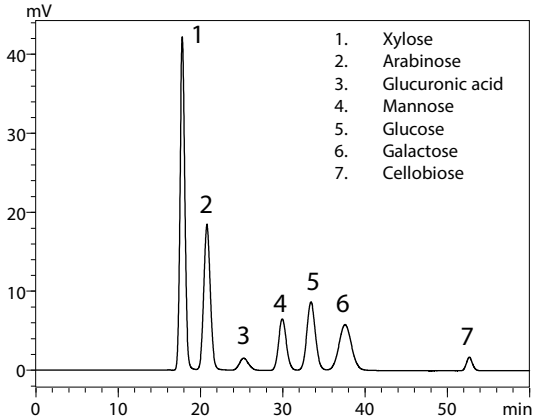

Fig. 4 Chromatogram of Standard Solution (500 µmol/L each)

■ Linearity of Calibration Curves

Fig. 5 shows the calibration curves prepared based on the results of the analyses of the standard solution. The ranges of the calibration curves were from 10 to 1000 µmol/L. Great linearities were confirmed for all saccharides and glucuronic acid, as the contribution ratio $r^2 = 0.9999$ or greater.

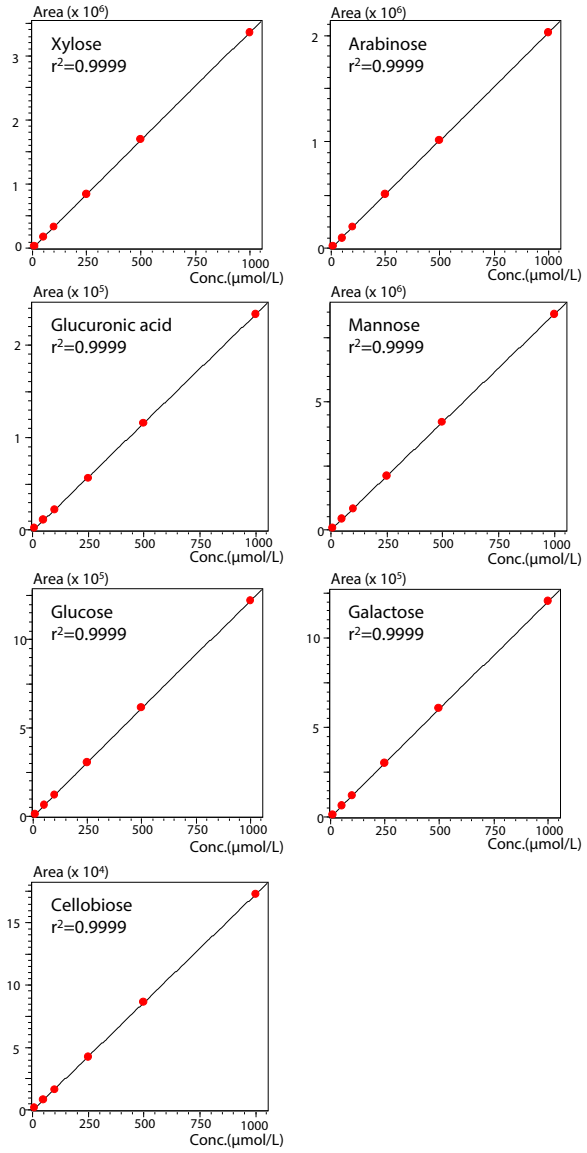

Fig. 5 Calibration Curves of Compounds

■ Constituent Sugar Analysis of TEMPO-Oxidized CNF Samples

The samples were prepared in accordance with the protocol in Fig. 3 and analyzed with the Nexera reducing sugar analysis system. Figs. 6 to 8 show the respective chromatograms. Saccharides and glucuronic acid can be detected with high sensitivity and selectivity by using the post-column derivatization reaction.

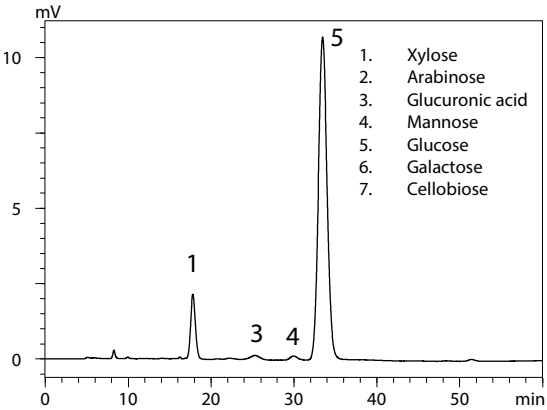

Fig. 6 Chromatogram of Sample No. 1

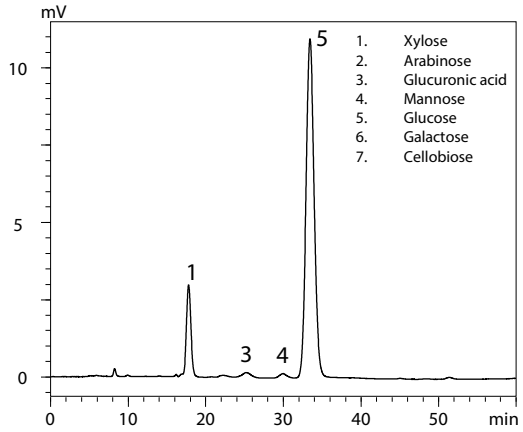

Fig. 7 Chromatogram of Sample No. 2

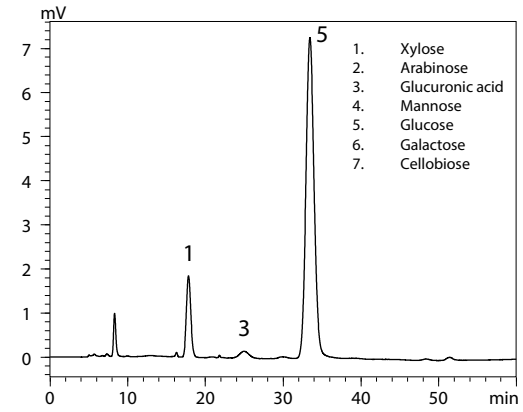

Fig. 8 Chromatogram of Sample No. 3

■ Ratios of Constituent Sugars

Based on the quantitative values of the detected saccharides and glucuronic acid, spike-and-recovery test, correction for excessive decomposition, and calculation of polysaccharide were carried out in the same manner as in the Application News 01-00023-EN. Fig. 9 shows the ratios of constituent sugars and glucuronic acid in the samples when the integrated value is defined as 100. The glucuronic acid produced by TEMPO catalyst was successfully detected.

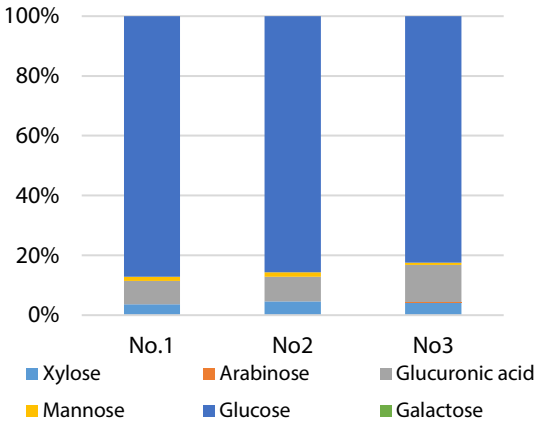

Fig. 9 Ratios of Constituent Sugars and Glucuronic Acid in CNF Samples

■ Precautions on Completion of Analysis

Under the analytical conditions in this article, an organic solvent is used as the mobile phase and an aqueous solution containing a high concentration of salt is used as the reaction reagent. If the delivery of mobile phase and reaction reagent is stopped immediately after the analysis, it may cause trouble, including clogging of the piping by salt precipitation. The procedure for completion of this analysis is described below.

1. After the analysis is completed, turn off the heater of the chemical reaction box. Continue to supply the mobile phase and the reaction reagent at the same flow rates as in the analysis until the temperature in the reaction box decreases to 100 ˚C or less.
2. After the temperature in the chemical reaction box decreased, change the flow rates of both the mobile phase and the reaction reagent to 0.2 mL/min and cool to room temperature.
3. After cooling the column, promptly remove the column and replace the mobile phase and reaction liquid with ultrapure water. Supply ultrapure water at a flow rate of 1 mL/min for approximately 15 minutes to wash the entire flow line.
4. If the instrument will not be used for an extended period (1 month or longer), replace the liquid in the entire flow line, except for the column, with methanol or ethanol.

■ Summary

It was possible to determine the ratios of the constituent sugars in TEMPO-oxidized CNF samples by analyzing the saccharides and glucuronic acid in the samples after hydrolysis treatment using the Shimadzu Nexera reducing sugar analysis system. Simultaneous analysis of constituent sugars and glucuronic acid in TEMPO oxidized CNF is possible.

<Acknowledgment>
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<References>
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(4) Saito, T., & Isogai, A.(2004). TEMPO-mediated oxidation of native cellulose. The Effect of Oxidation Conditions on Chemical and Crystal Structures of the Water-Insoluble Fractions, Biomacromolecules, 5, 1983-1989

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