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Application News

High Performance Liquid Chromatograph Nexera™ Reducing Sugar Analysis System

Constituent Sugar Analysis of Cellulose Nanofibers

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User Benefits

- This method enables simultaneous analysis of the main constituent sugars of cellulose nanofiber (CNF).
- High sensitivity and selectivity analysis of saccharides is possible by using the post-column fluorescence derivatization method.

■ Introduction

Cellulose nanofiber (hereinafter, CNF) is a technology which effectively utilizes biomass with high efficiency, and thus is one approach toward building a sustainable recycling society system.

Plant cell walls consist of fibers called cellulose microfibrils, in which cellulose molecule chains are bound together in a regular structure. The substance obtained by physically or chemically defibrillating cellulose microfibrils is called CNF. In recent years, bacterial cellulose nanofibers (BCNF) has also been produced by using bacteria to synthesize cellulose from biomass-derived glucose.

Because the saccharides that make up the complete CNF differ depending on the type of biomass used as the material of the CNF, it is necessary to determine the type and ratio of the saccharides when commercializing a CNF product. Here, various types of CNF were hydrolyzed, and the types and ratios of their constituent sugars were determined with a Nexera reducing sugar analysis system.

■ Nexera Reducing Sugar Analysis System

Fig. 1 shows the flow path diagram of the Nexera reducing sugar analysis system. In this system, saccharides are separated with a column, then detected by a fluorescence detector due to post-column derivatization. The fluorescence detection method takes advantage of the fact that saccharides form strongly fluorescent derivatives when thermally reacted with arginine in the presence of boric acid. The derivatized saccharides can be detected with high sensitivity using a fluorescence detector (RF-20AXS). This system enables the analysis of saccharides at low concentrations, which was difficult with differential refractive index detectors.

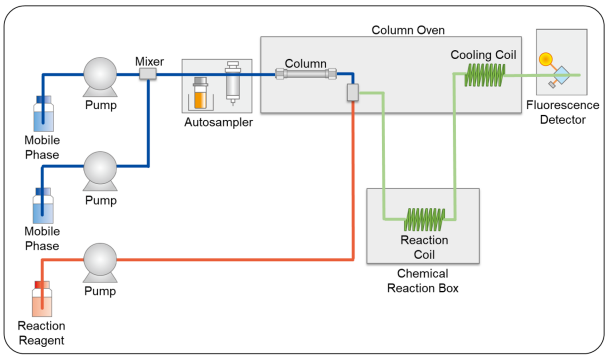

Fig. 1 Flow Path Diagram of Nexera Reducing Sugar Analysis System

■ Sample Preparation

Table 1 shows a list of samples used in the analyses, and Fig. 2 shows the sample preparation protocol.

Table 1 List of Samples

No.	Sample
1	CNF derived from broadleaf tree pulp-1
2	CNF derived from broadleaf tree pulp-2
3	CNF derived from broadleaf tree pulp-3
4	Carboxymethyl cellulose
5	CNF derived from coniferous tree pulp
6	Fermented nanocellulose (Hydrophilic type)
7	Fermented nanocellulose (Amphipathic type)
8	Commercially available cotton

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 ultrapure water*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. 2 Sample Preparation Protocol

Because the neutralization conditions and concentrations of saccharides differ depending on the type of sample, the ratio of the saturated barium hydroxide solution and the sample dilution ratio with water were changed for each sample. Table 2 shows the conditions used with each sample.

Table 2 Neutralization Conditions and Dilution Ratios Used in Sample Preparation

Sample	Sample : Saturated barium hydroxide solution (v:v)	pH	Dilution ratio
1	150 : 450	4	8
2	600 : 300	3	8
3	600 : 300	7	8
4	600 : 300	3	4
5	500 : 250	4	8
6	600 : 300	4	4
7	600 : 300	3	4
8	600 : 400	3	8

■ Analytical Conditions

Table 3 shows the analytical conditions used for the hydrolyzed samples. Because saccharides have the property of forming anionic complexes with boric acid, the saccharides were separated with a Shim-pack™ ISA-09 anion exchange column using borate buffer solutions as the mobile phase. Fig. 3 shows the chromatogram of the standard solution.

Table 3 Analytical Conditions

System	:Nexera Reducing Sugar Analysis System
<Separation>	
Column	:Shim-pack ISA-09 (250 mm × 4.0 mm I.D.)*4
Guard column	:Shim-pack ISA-09 (G) (50 mm × 4.0 mm I.D.) *5
Mobile phase A	:0.1 mmol/L Potassium borate buffer solution (pH 8)
Mobile phase B	:0.4 mmol/L Potassium borate buffer solution (pH 9)
Flow rate	:0.6 mL/min
Time program	:B Conc. 0 % (0 min) - 100 % (50 min) - 100 % (65 min) - 0 % (65.01-90 min)
Gradient mixer capacity	:1.7 mL
Column temp.	:65 ˚C
Injection vol.	:10 µL
Vial	:SHIMADZU LabTotal™ for LC 1.5 mL, Glass*6
<Post-column reaction>	
Reaction reagent	:10 g/L Arginine and 30 g/L boric acid aqueous solution
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, 10 m × 0.8 mm I.D.

*4 P/N: 228-59512-41, *5 P/N: S228-59512-42, *6 P/N: 227-34001-01

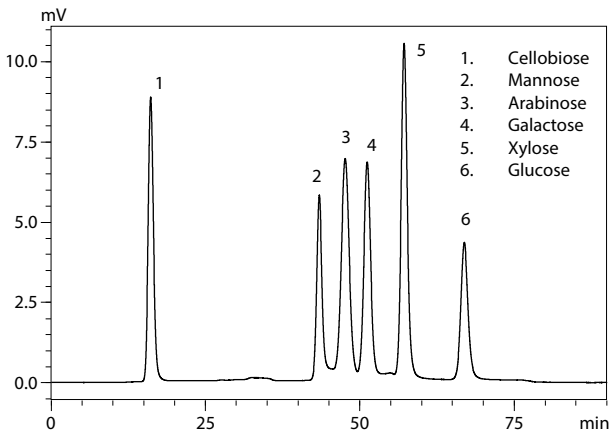

Fig. 3 Chromatogram of Standard Solution (500 µmol/L each)

■ Linearity of Calibration Curves

Fig. 4 shows the calibration curves prepared based on the results of the analysis of the standard solution. Table 4 shows the ranges of the calibration curves. Good linearities were confirmed for all saccharides, as the contribution ratio $r^2 = 0.999$ or greater.

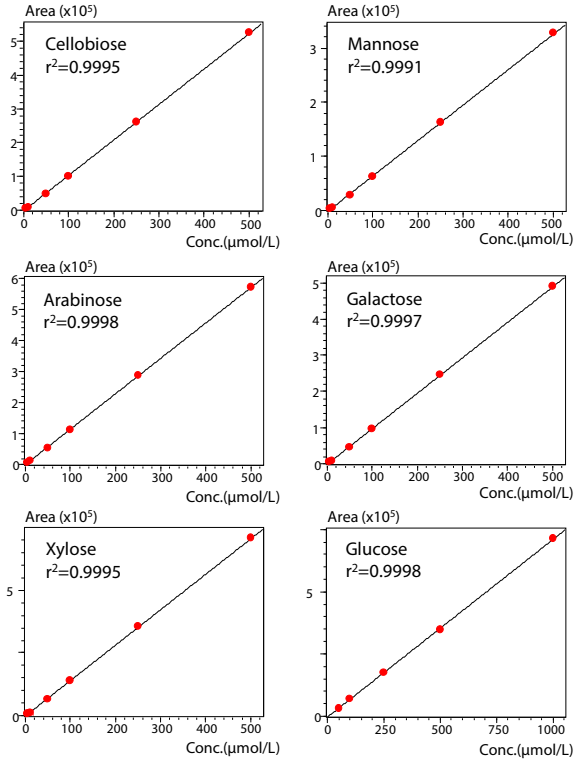

Fig. 4 Calibration Curves of Compounds

Table 4 Calibration Curve Ranges of Compounds

Compound	Calibration curve range (µmol/L)
Cellobiose	5-500
Mannose	5-500
Arabinose	5-500
Galactose	5-500
Xylose	5-500
Glucose	50-1000

■ Repeatability of Standard Solution

Table 5 shows the relative standard deviation (%RSD) of the retention time and peak area for 6 repeated analyses of a standard solution containing 500 µmol/L of each compound. Good repeatability was obtained for all compounds, showing that the system performance is stable.

Table 5 Repeatability of Retention Time and Peak Area of Compounds (n = 6)

Compound	Retention time (%RSD)	Peak area (%RSD)
Cellobiose	0.04	0.10
Mannose	0.02	0.17
Arabinose	0.01	0.13
Galactose	0.01	0.17
Xylose	0.01	0.12
Glucose	0.01	0.13

■ Constituent Sugars Analysis of CNF Samples

The samples listed in Table 1 were prepared in accordance with the protocol shown in Fig. 2, and were then analyzed with the Nexera reducing sugar analysis system. Fig. 5 shows the chromatographs for each CNF sample. Saccharides can be detected with high sensitivity and selectivity by using the post-column derivatization reaction method.

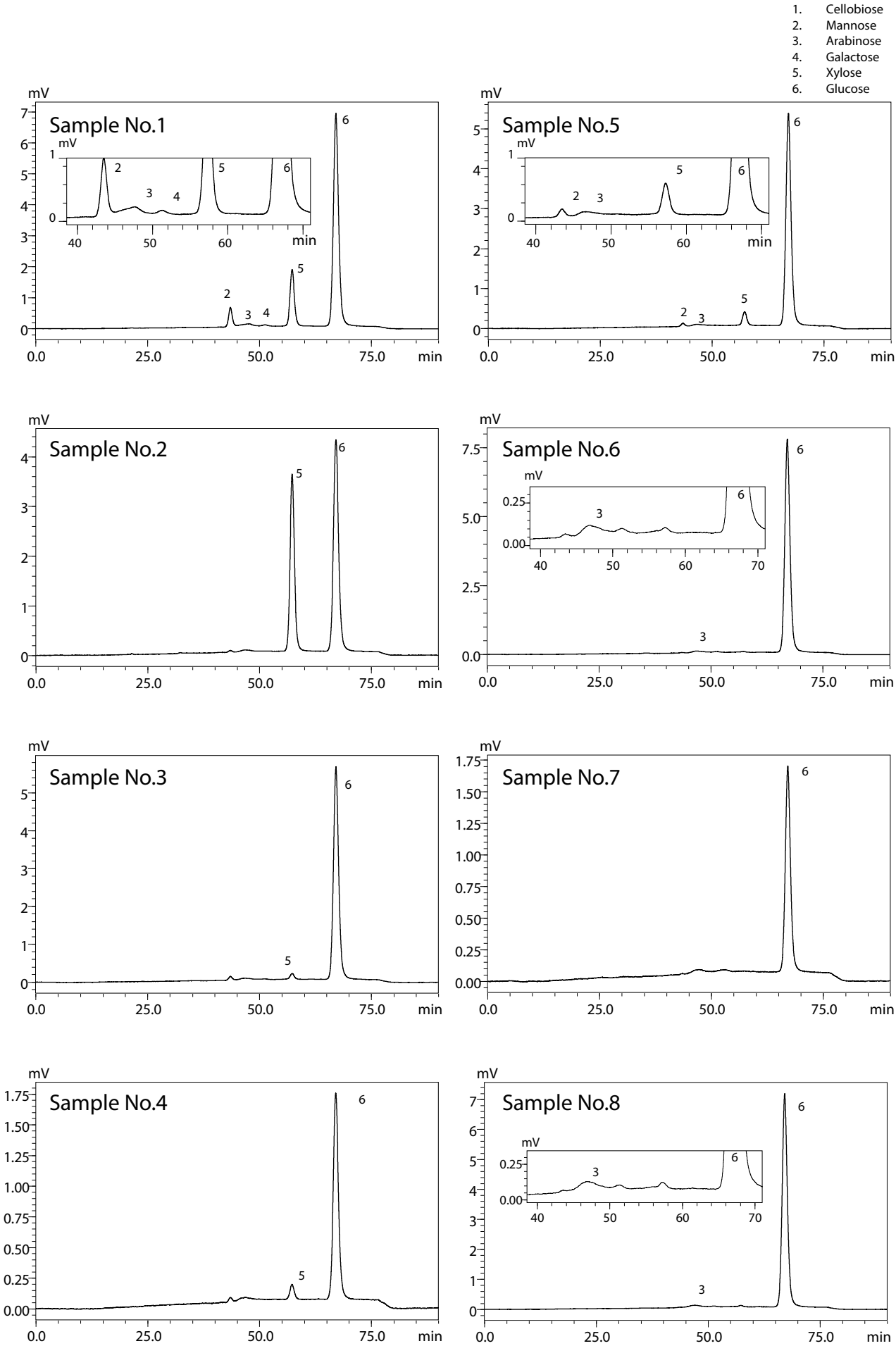

Fig. 5 Chromatograms of CNF Samples

■ Spike-and-Recovery Test, Correction for Excessive Decomposition, and Calculation of Polysaccharide

Because the saccharides in the samples may decompose due to excessive decomposition during hydrolysis, it is necessary to correct by performing a spike-and-recovery tests of the monosaccharides. For this test, 8.4 mL of a mixed standard solution of the monosaccharides (each saccharide: 500 µmol/L) was taken in a 25 mL test tube, and 300 µL of 72 % sulfuric acid was added with a pipette. Two test solutions were prepared. One (solution a) was decomposed by heating in an autoclave for 1 hour at 120 ˚C simultaneously with hydrolysis. After cooling, water was added to the autoclaved solution until the total volume reaches 10 mL. The other (solution b) was simply adjusted to 10 mL with water without being heated in the autoclave. Both solutions were diluted 4 times, after which quantitative analyses of solutions a and b were performed, and the recovery rate of each monosaccharide before/after hydrolysis was obtained. Correction for excessive decomposition was carried out by multiplying the monosaccharide concentration of the sample before correction by the reciprocal of the recovery rate. Table 6 shows the recovery rates and the excessive decomposition correction factors for each monosaccharide. The calculation of polysaccharide was carried out by multiplying the corrected monosaccharide concentration by the scale factor of monosaccharide that is 0.9 for a hexose and 0.88 for a pentose.*7

Table 6 Recovery Rates and Excessive Decomposition Correction Factors for Monosaccharides

Compound	Recovery rate (%)	Correction factor for the excessive decomposition
Xylose	87	1.15
Arabinose	92	1.08
Mannose	94	1.07
Glucose	95	1.06
Galactose	92	1.08

*7 In this analysis, the samples were hydrolyzed in sample preparation, and a quantitative analysis of the monosaccharides was performed. Scale factor is the coefficient that takes into account dehydration condensation.

■ Ratio of Constituent Sugars

Spike-and-recovery test, correction for excessive decomposition, and calculation of polysaccharide were carried out using the quantitative values of the detected saccharides. Fig. 6 shows the composition ratio of each saccharide when the integrated value is defined as 100.

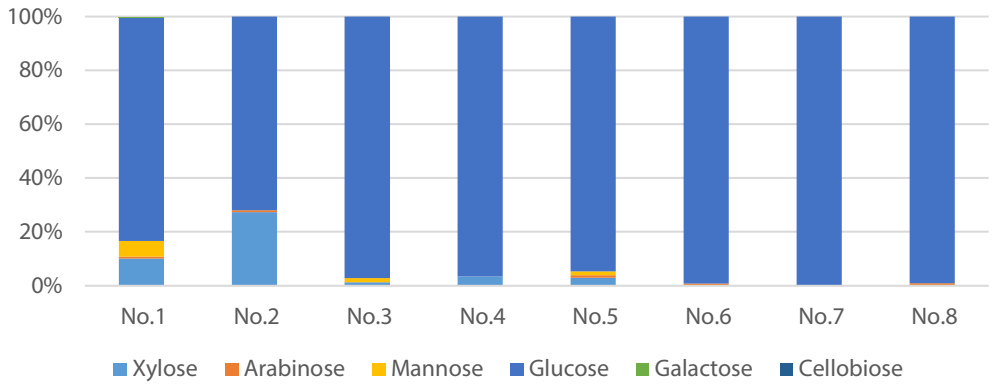

Fig. 6 Ratios of Constituent Sugars of CNF Samples

Differences in the types and amounts of constituent sugars could be seen in the CNFs derived from different materials. As shown in Fig. 7, cellulose is a polysaccharide which is polymerized by glycosidic linkage of glucose, whereas the main components of hemicellulose are the insoluble dietary fibers xylan, which is formed by polymerization of xylose or other saccharides, and mannan, which is formed by polymerization of glucose and mannose. It is known that hemicellulose is distributed in large amounts in broadleaf trees.

Analysis of Binfi-s (Sugino Machine Limited, Toyama, Japan) produced from wood pulp showed that, a large amount of xylose tends to be detected in addition to glucose. However, BCNFs such as fermented nanocellulose, which are formed by bacteria from the glucose in molasses or agricultural raw materials, consisted of 90 % or more glucose, and did not contain xylose, which has been confirmed to originate from wood pulp.

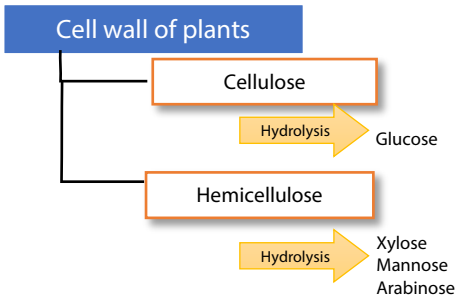

Fig. 7 Cellulose and Hemicellulose

■ Summary

It was possible to determine the ratios of the constituent sugars in various CNF samples by analyzing the saccharides in the samples after hydrolysis treatment using the Nexera reducing sugar analysis system.

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