

Analysis of monosaccharide composition of *Cyclocarya paliurus* polysaccharide with anion exchange chromatography

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ABSTRACT

A simple, rapid and sensitive analytical method for the determination of monosaccharide composition in *Cyclocarya paliurus* polysaccharide was developed and validated. This method was based on hydrolysis of the polysaccharides followed by high-performance anion-exchange chromatography analysis. The effects of sodium hydroxide concentration and column temperature on retention and separation of the monosaccharide were investigated with Carbo PAC™ PA10 analytical column. The established method was validated and the results showed that this method had good linearity (R^2 , 0.9987–0.9999), adequate accuracy (98.53–102.13% recovery), high precision (relative standard deviation <3.8%) and sensitive detection limits (2.57–7.86 nM), with a simple preparation of the samples (no need to derivatize the samples) and short run time (20 min). The results showed that the *C. paliurus* polysaccharide consisted of rhamnose, arabinose, galactose, glucose, mannose and xylose in the molar ratio of 1.00:1.85:3.26:3.12:0.85:0.29.

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1. Introduction

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*), also named as sweet tea tree, is a well-known edible and medicinal plant, grown on cloudy and foggy highlands in Southern China (Xie, Li, Nie, Wang, & Lee, 2006). The leaves of *C. paliurus* have been commonly used as a traditional Chinese medicine in the treatment of hyperglycemia, hyperlipidemia and diabetes mellitus for thousands of years in China (Kurihara, Asami, Shibata, Fukami, & Tanaka, 2003; Xie et al., 2006, 2010a). In recent years, great advances have been made in the biological studies of *C. paliurus* polysaccharide, an active component in the leaves of *C. paliurus*. Our previous studies have revealed that *C. paliurus* polysaccharide had a variety of bioactivities, such as hypoglycemic (Xie et al., 2006), antioxidant (Xie et al., 2010b), antimicrobial (Xie et al., 2012), anticancer (Xie et al., 2013a) and immunomodulatory activities (Huang, Nie, Xie, Han, & Xie, 2009).

Previous studies have shown that polysaccharide possessed higher physiological activity, which is closely related to the level of monosaccharide compositions (Zhang, Cui, Cheung, & Wang, 2007). In particular, the monosaccharide composition analysis of polysaccharides is the most important step for the further discovery of its physicochemical properties, structure and structure–bioactivity relationship. Furthermore, monosaccharide composition of polysaccharides is an essential parameter for the evaluation of

quality standard of polysaccharide materials and basic information on the polysaccharides (Wang, Zhao, Wang, Wang, & Mei, 2005). Hence, the determination of monosaccharide composition has been a common practice in the food industry for many years, and the quantification of monosaccharide composition in polysaccharides is of special interest. Various techniques have been utilized to analyze the monosaccharide composition of polysaccharides, including high performance liquid chromatography (HPLC) with fluorometric or UV detection (Dai et al., 2010; Kakita, Kamishima, Komiya, & Kato, 2002; Lv et al., 2009; Wang et al., 2005), gas chromatography (GC) with flame ionization detection (Chen, Xie, Wang, Nie, & Li, 2009; Xie et al., 2010a), gas chromatography–mass spectrometry (GC–MS) (Guadalupe, Martínez-Pinilla, Garrido, Carrillo, & Ayestarán, 2012; Kenne & Stromberg, 1990), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) (Arnous & Meyer, 2009; Yokota, Mori, Yamaguchi, Kaniwa, & Saisho, 1999), thin-layer chromatography (TLC) (Bischel, Austin, Kemeny, Hubble, & Lear, 1966) and capillary electrophoresis (CE) with fluorometric detection (Guttman, 1997). However, the treatments possess inherent limitations. HPLC method with a refractive index detector has been tried for analyzing monosaccharide compounds, but it has problems such as poor sensitivity and long analysis time. Detection without derivatization by measurement of the refractive index or absorption in the UV region at 190–210 nm is restricted to the mol to nmol range (Racaiyte, Kiessig, & Kálmán, 2005). The method of pre- or post-column derivatization and UV or fluorometric detection by HPLC has been widely used in sugar analysis. However, the method still requires time-consuming clean-up procedures to separate carbohydrates.

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GC method has good sensitivity, but derivatization chemistry is tedious and it may result in very complex separation patterns due to stereochemical isomeric reaction products of the monosaccharides (Lottspeich & Zorbas, 1998). TLC method using ninhydrin and anisaldehyde reagent could be used to separate monosaccharides, but this method is only applied to the qualitative detection of monosaccharide compounds, not to quantitative analysis.

High pH anion-exchange chromatography (HPAEC) with pulsed-ampereometric detection (PAD) is one of the useful techniques for monosaccharide determination. Once the hydrolysis of the polysaccharides is complete, the monosaccharides can be separated underivatized by HPAEC and detected by PAD. It gives high resolution of all common monosaccharides in less than 30 min, and has the advantage of not requiring pre-column derivatization of monosaccharide (Yokota et al., 1999). Furthermore, PAD is sensitive and is most commonly used with anion-exchange chromatography for monosaccharide and oligosaccharide determination (Holmbom, 1999). The method of HPAEC-PAD has been applied to a variety of routine monitoring and research applications including analysis of oligo- and polysaccharides samples (Corradini, Cavazza, & Bignardi, 2012). For example, Caseiro et al. (2007) reported the analysis of primary sugars, sugar alcohols and anhydrosugars in atmospheric aerosols by HPAEC-PAD. Jeong, Yoon, and Hong (2007) developed a new non-derivatization analytical method for the determination of galactose in the diagnosis of galactosemia by HPAEC-PAD. Zhang, Khan, Nunez, Chess, and Szabo (2012) also developed a HPAEC-PAD method to separate and simultaneously determine levels of neutral, amino and acidic sugars. To our knowledge, however, the suitability of HPAEC-PAD for direct quantification of monosaccharide released from *C. paliurus* polysaccharides has not been reported.

The aim of the present study was to develop a simple and rapid HPAEC-PAD method for the quantification of the monosaccharide composition without sample derivatization. The newly developed HPAEC-PAD method was successfully applied to analyze the composition of monosaccharides released from *C. paliurus* polysaccharides. The results of linearity, precision, reproducibility and accuracy showed that this method can also be used for the quantification of monosaccharide composition in other plant polysaccharides.

2. Materials and methods

2.1. Materials and reagents

The plants of *C. paliurus* were cultivated in Xiushui County, Jiangxi Province, China. A voucher specimen of leaves was deposited at the State Key Laboratory of Food Science and Technology, Nanchang University, China. The materials were thoroughly washed with tap water, air-dried at room temperature and finely powdered.

Standard monosaccharide references (arabinose, rhamnose, fucose, fructose, xylose, galactose, glucose and mannose) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals and solvents used were of analytical grade or HPLC-grade unless otherwise specified.

2.2. Preparation of *C. paliurus* polysaccharide

The water-soluble polysaccharide was extracted from the leaves of *C. paliurus* according to Xie, Shen, Nie, Li, and Xie (2011) with some modifications. Briefly, the pretreated samples were extracted three times in 80 °C hot water. The aqueous extract was concentrated to 20% of the original volume under reduced pressure in a rotary evaporator, and proteins were removed with Sevag reagent.

After removal of the Sevag reagent, the solution was decolorized with 30% H₂O₂, and then dialyzed for 36 h in tap water and 12 h in ultra pure water (molecular weight cut-off 14 kDa) before concentration under vacuum evaporator at 55 °C. In order to obtain the crude polysaccharide, the extract was precipitated with three times volume of 95% ethanol at 4 °C for overnight and the precipitate was centrifuged at 8400 × g for 15 min in a model Sigma 3K3D high speed freezing centrifuge (Sigma Company, German). The precipitate was dissolved in ultra-pure water, frozen and freeze-dried, then the polysaccharide was obtained.

2.3. Hydrolysis of polysaccharides

The *C. paliurus* polysaccharide sample (20 mg) was placed in 5 mL of 2 M H₂SO₄ in a sealed tube (10 mL). The sealed tube was kept at 110 °C for 8 h. The resulting reaction solution was cooled to room temperature. The hydrolyzates were obtained by filtration and centrifugation at 4000 × g for 10 min after being neutralized to pH 6.0 by adding BaCO₃. The sample solution was then added with 1.0 mL ultra-pure water.

2.4. Preparation of monosaccharide standard solutions

Stock standard solutions (2.0 mM) were prepared by dissolving each standard monosaccharide in a mixture of water solution. Working monosaccharide standard solutions for generating the calibration curve were further obtained by appropriate dilution of the stock standard solutions with ultra-pure water. The sample solutions were filtered through a 0.45 μm syringe filter, and were degassed using an ultrasonic bath for 2 min prior to use. All the solutions were stored at 4 °C until being used.

2.5. Chromatographic analysis

The neutral monosaccharide compositions of *C. paliurus* polysaccharides were analyzed by HPAEC after acid hydrolysis. HPAEC was performed on a Dionex ICS-2500 system, coupled with PAD, and equipped with a Carbo PAC™ PA10 column (2.0 mm × 250 mm). The hydrolysates (25 mL) were filtered through a 0.45 μm filter before being injected into the column. Various concentrations of NaOH were used to analyze the sugars. The waveform of PAD was the Dionex default program for carbohydrates. Twenty-five microliters of this solution were used for the ionic chromatography analysis by HPAEC-PAD with Dionex ICS-2500 system, eluted with a 12.5 mM NaOH. The flow rate was adjusted to 0.25 mL/min and the column temperature was set at 30 °C. The retention time of each monosaccharide standard in the mixtures with different methods was confirmed by the analysis of each monosaccharide.

2.6. Validation study

Linearity, reproducibility, limit of detection (LOD), accuracy and precision for the analytical methodology were evaluated. Linearity was determined by calibration curves established with various concentrations (1, 2, 3, 5 and 10 μM) of mixture standard solutions. The peak areas of each analyte were plotted against the concentrations, and linear regression was performed on the resulting curves using the minimum least squares method. The LOD was determined by at least three injections at or below the low level standard yielding a signal-to-noise ratio of at least 3:1. Intra- and inter-day variations were chosen to determine the precision of the developed assay and expressed by relative standard deviation (RSD). The intra-day precision was performed with the interval of 4 h in the same day, and the inter-day precision was performed over 2 days. The recovery was used to evaluate the accuracy of the method. Various amounts of

each analyte were added to a variety of samples, and the resulting spiked samples were subjected to the entire analytical sequence. Injection repeatability was determined as the RSD for triplicate injections of the sample solution.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Although anion-exchange chromatography has been used extensively to analyze acidic carbohydrates and glycopeptides, it has not been commonly used for analysis of neutral sugars. However, examination of the pK_a values of the neutral monosaccharides showed that carbohydrates were in fact weak acids (Corradini et al., 2012). At high pH values, they were at least partially ionized, and thus can be separated by anion exchange mechanisms (Caseiro et al., 2007). Furthermore, anion exchange chromatography on high pH-resistant polymeric-based strong anion exchange columns specifically tailored for carbohydrate analysis enabled selective elution of carbohydrates, where the most important parameter influencing the separations was the number of hydroxyl groups (Corradini et al., 2012). Rocklin and Pohl (1983) have described the effects of NaOH on the retention behavior of sugars, and it was generally observed that selectivity changes as NaOH concentration was varied. Hence, the effects of NaOH concentrations (2.5, 5.0, 7.5, 10.0, 12.5 and 25.0 mM) on the separation of monosaccharide were investigated. NaOH concentrations between 2.5 and 25.0 mM were tested on the CarboPac PA-10 column. As too low, a hydroxide concentration was difficult to control, resulting in variable retention times (Caseiro et al., 2007). Increasing NaOH concentration, however, remarkably increased the retention time of all analytes (Fig. 1). For high NaOH concentrations, we noted a large peak including galactose, glucose and mannose (Fig. 1a). It was also found that these components (galactose, glucose, mannose, xylose and fructose) were not well separated, and rhamnose and arabinose almost shared the same peak at 25.0 mM NaOH (Fig. 1a). For low NaOH concentration (5.0 mM), all analytes were eluted as excessively broad peaks, and the retention time was very long. These results were in agreement with previous studies (Cheng & Kaplan, 2003; Kerhervé, Charrière, & Gadel, 1995). The best resolution and highest separation efficiency were achieved at 12.5 mM NaOH (Fig. 1b).

Temperature was reported to influence the resolution of neutral sugars (Panagiotopoulos, Sempéré, Lafont, & Kerhervé, 2001). Therefore, the effects of temperature on separation of the analytes were also studied. As expected, the mobility of all the analytes increased as temperature was raised from 20 to 40 °C. When temperature was higher than 30 °C, the baseline gradually worsened due to the higher current. The results showed that the best separation was achieved at 30 °C. Therefore, the column temperature was set at 30 °C.

3.2. Validation of the HPAEC analysis method

3.2.1. Linearity and limits of detection

To determine the linearity of the HPAEC-PAD method, the detection linearity was verified by the analysis of seven data points ($n = 3$) of different concentrations of mixture monosaccharide standards (mannose, fucose, rhamnose, glucose, fructose, xylose, galactose and arabinose), and the linear regression parameters of the calibration curves are shown in Table 1.

The linearity of the plot peak areas (x) for each monosaccharide vs concentration of monosaccharide references (Y) was investigated; the results were expressed as the values of square of the correlation coefficient (R^2 , Table 1). All eight calibration curves of monosaccharides, mannose, fucose, rhamnose, glucose, fructose,

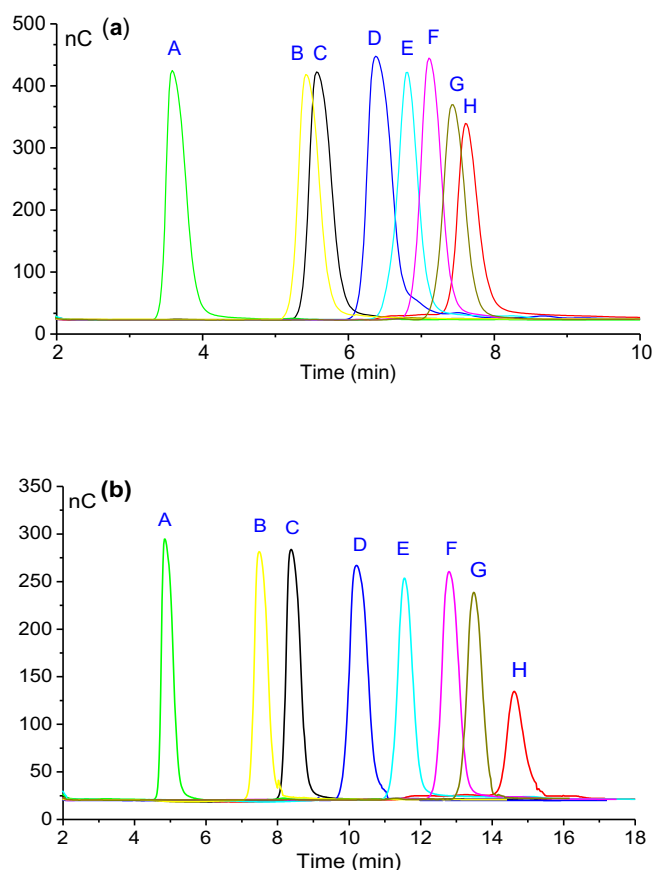


Fig. 1. HPAEC-PAD chromatogram profiles of standard monosaccharide mixture solution. (a) NaOH concentration of 25.0 mM, (b) NaOH concentration of 12.5 mM. Peak identity: A, Fucose; B, Rhamnose; C, Arabinose; D, Galactose; E, Glucose; F, Mannose; G, Xylose; H, Fructose.

xylose, galactose and arabinose exhibited good linearity with excellent correlation coefficients. For all the monosaccharide standards analyzed, the R^2 values of the calibration curves were at least 0.9987, suggesting a good linearity within the concentration range tested. The calibration curves for all monosaccharide standards were linear from 1 to 10 μ M.

The limit of detection (LOD) was calculated as the lowest concentration level that is statistically different from a blank ($LOD = 3SD/m$; m is the slope of the addition graph, and SD is the within-run standard deviation of single blank determination) (Xie et al., 2013b). Results (Table 1) showed that the LOD values of the eight monosaccharide standards by HPAEC-PAD method under the optimum conditions described above were in the range from 2.57 to 7.86 nM, indicating that the sensitivity of the method was satisfactory.

3.2.2. Precision and reproducibility

The method precision was determined by measuring repeatability (intra-day variability) and intermediate precision (inter-day variability) of retention time and peak area for each monosaccharide tested. The precision of method was calculated as the coefficient of variation for three successive injections of each monosaccharide and the results are summarized in Table 2. The results showed that the intra-day reproducibility values were less than 1.49% for the retention time and 2.05% for the peak area, and the inter-day coefficient of variation values were less than 2.36% for the retention time and 3.79% for the peak area. The results showed excellent reproducibility of the absolute migration times,

Table 1

The linearity and limits of detection (LOD) of HPAEC-PAD method in different monosaccharide references.

Monosaccharide reference	Retention time (min)	Regression equation ^a	Correlation coefficient (R^2)	Detection limit ^b (LOD, nM)
Fucose	4.584	$Y = 0.4709x - 0.2205$	0.9991	6.87
Rhamnose	7.521	$Y = 0.5818x - 0.1072$	0.9994	7.12
Arabinose	8.125	$Y = 0.4943x - 0.1545$	0.9987	2.57
Galactose	9.792	$Y = 0.3852x - 0.0588$	0.9992	5.33
Glucose	10.709	$Y = 0.4574x - 0.0688$	0.9994	4.42
Mannose	12.084	$Y = 0.3875x - 0.0732$	0.9994	2.97
Xylose	12.487	$Y = 0.4769x - 0.1452$	0.9996	3.05
Fructose	13.813	$Y = 0.7955x + 0.0561$	0.9999	7.86

^a Y and x stand for the concentration (μM) and the peak area of monosaccharide references, respectively.^b The detection limits correspond to concentrations giving a signal-to-noise ratio of 3.**Table 2**

Precision of the retention time and peak area of the tested analytes with HPAEC-PAD method.

Monosaccharide	Intra-day precision (RSD%, $n = 5$)		Inter-day precision (RSD%, $n = 3$)	
	Retention time	Peak area	Retention time	Peak area
Fucose	0.79	2.02	1.92	2.87
Rhamnose	0.98	1.65	2.36	2.12
Arabinose	1.24	2.05	1.64	3.75
Galactose	1.34	1.21	2.17	3.11
Glucose	1.26	1.37	2.01	2.34
Mannose	1.25	1.32	2.12	2.79
Xylose	0.92	1.48	1.98	3.03
Fructose	1.49	1.89	1.54	3.46

and acceptable reproducibility of the absolute peak area, indicating that the method precision was satisfactory.

3.2.3. Accuracy

In order to evaluate the accuracy of the proposed method, the standard adding method was adopted to determine the recovery rate of each monosaccharide standard. The average recovery rate and relative standard deviation (RSD) of each monosaccharide were determined for each experiment. Three replicate experiments were performed under the chosen conditions. The results showed that the recoveries of all the eight monosaccharides ranged from 98.53% to 102.13%, and the RSD values were less than 3% (Table 3). Considering the results of the recovery test, the method was deemed to be accurate.

Results of the method validation showed that the method has acceptable selectivity, linearity, precision, stability and accuracy. The benefit of the method is that it does not require labeling of the monosaccharides for the detection. The method is fast since only sample hydrolysis and dilution are required in the sample preparation and is also eco-friendly for its non-consumption of organic solvents as compared with other analytical techniques.

3.3. Application in experimental samples

In this study, the proposed method was applied to determine the monosaccharide composition of polysaccharide in *C. paliurus* samples. Fig. 2 shows the HPAEC-PAD chromatogram profiles of fucose, rhamnose, arabinose, glucose, xylose, mannose, fructose and galactose standards (Fig. 2a), and those in *C. paliurus* polysaccharide sample (Fig. 2b). As shown in Fig. 2b, the component monosaccharides released from *C. paliurus* polysaccharide, could be perfectly separated from each other. By comparing retention times of unknown peaks with reference sugar standards, six monosaccharides including rhamnose, arabinose, galactose, glucose, mannose and xylose were identified, with the retention times being 7.52, 8.13, 9.79, 10.71, 12.08 and 13.81 min, respectively, and their molar ratio being 1.00:1.85:3.26:3.12:0.85:0.29. It is clear that the predominant monosaccharides in the *C. paliurus* polysaccharide were glucose and galactose, with up to 31.42%

and 30.11% of total identified monosaccharides, respectively. The monosaccharide content of *C. paliurus* polysaccharide, measured by HPAEC-PAD was in good agreement with the relative concentration ratios of 1.00:9.67:9.65:4.96:3.29:2.70 for xylose, arabinose, glucose, galactose, rhamnose and mannose reported by Xie et al. (2010b). The difference in molar ratio of monosaccharides in *C.*

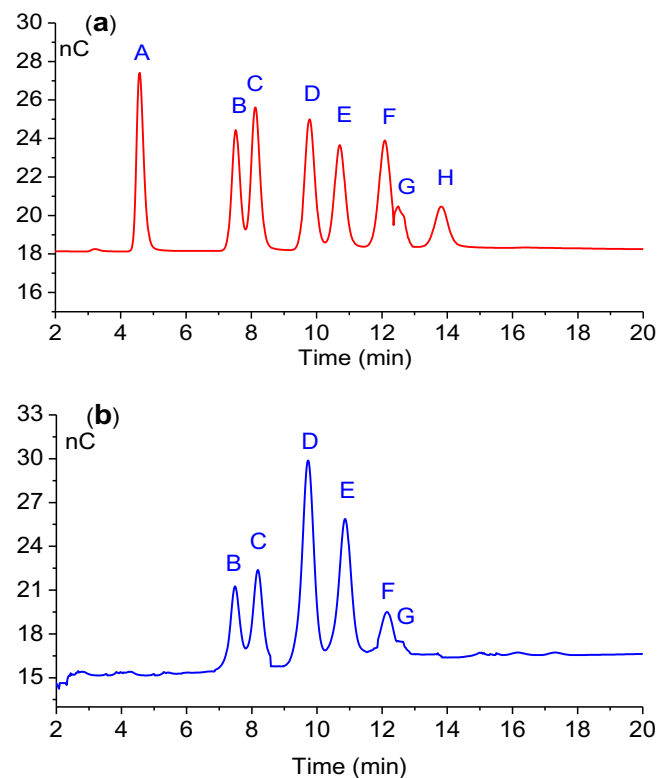


Fig. 2. HPAEC-PAD chromatogram profiles of standard monosaccharide mixture solution (a), and the monosaccharides from *Cyclocarya paliurus* polysaccharide sample (b). Peak identity: A, Fucose; B, Rhamnose; C, Arabinose; D, Galactose; E, Glucose; F, Mannose; G, Xylose; H, Fructose.

Table 3Recoveries of the tested analytes with HPAEC-PAD method ($n = 3$).

Monosaccharide	Content (μM)	Added value (μM)	Detected value (μM)	Recovery ^a (%)	RSD (%)
Fucose	n.d. ^b	3.00	3.01	100.33	2.62
Rhamnose	1.44	3.00	4.42	99.55	0.96
Arabinose	2.66	3.00	5.67	100.18	1.14
Galactose	4.70	3.00	7.73	100.39	1.92
Glucose	4.50	3.00	7.39	98.53	2.36
Mannose	1.22	3.00	4.31	102.13	1.85
Xylose	0.42	3.00	3.39	99.12	1.61
Fructose	n.d. ^b	3.00	3.04	101.33	2.27

^a Data are means of three experiments.^b n.d.: not detected in the sample.

paliurus polysaccharide sample might be caused by variation in the analytical conditions employed.

In summary, the results have demonstrated that the proposed HPAEC-PAD method provided an excellent example for the determination of molar ratios among monosaccharides of *C. paliurus* polysaccharide. There are several methods currently used for the separation of carbohydrates. Compared with other instrumental techniques, HPAEC-PAD has many advantages, including rapid analysis, high sensibility, high accuracy, no need of sample derivatization and simultaneous determination of monosaccharide and oligosaccharides.

4. Conclusions

In this study, a HPAEC-PAD method has been established for the quantitative analyses of monosaccharide composition in the *C. paliurus* polysaccharide. The method is sensitive, simple and reliable. The great benefit of the method is the non-required labeling of the analytes for the detection, which greatly simplified the sample clean-up process and shortened the total analytical time. The linear range was 1–10 μM for each monosaccharide, and the LOD value was 2.57–7.86 nM. The analytical recovery rates were between 98.53% and 102.13%, and the RSD values were less than 3.8%. The average recovery rates obtained by the standard addition method ranged from 94.34% to 105.69%. Results showed that the intra-day reproducibility were less than 1.49% for the retention time and 2.05% for the peak area, and the inter-day coefficient of variation values were less than 2.36% for the retention time and 3.79% for the peak area. These results indicated that the established method was particularly suitable for determining the component monosaccharide in the *C. paliurus* polysaccharide, and could also be applied to the simultaneous determination of monosaccharide composition in other polysaccharides.

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