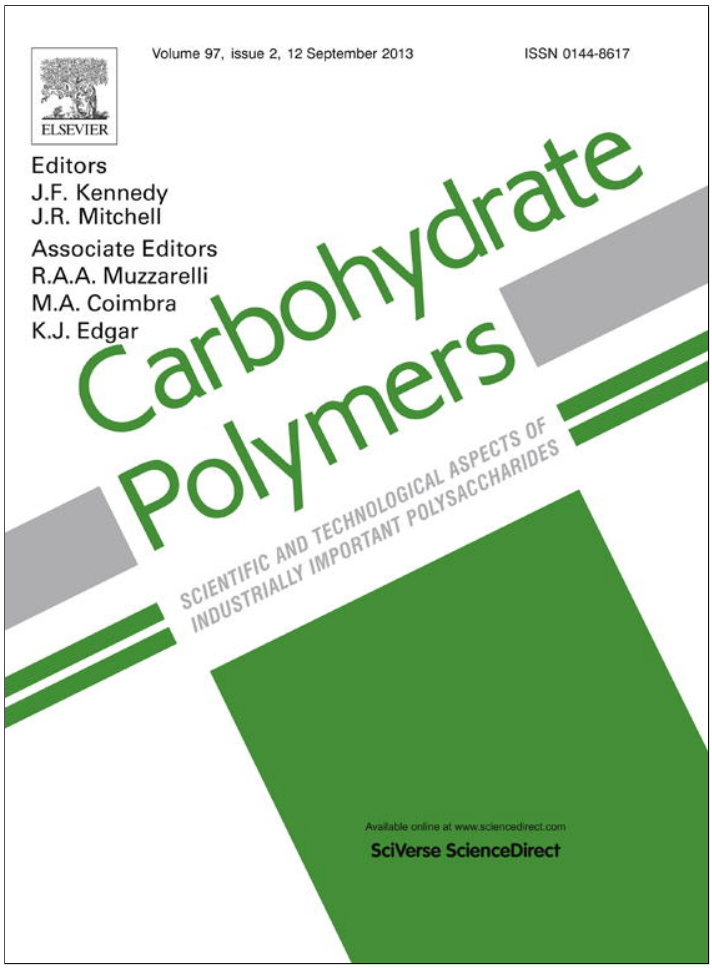

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Determination of the carbohydrates from *Notopterygium forbesii* Boiss by HPLC with fluorescence detection

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ABSTRACT

A sensitive pre-column derivatization method was developed for analysis of carbohydrates by HPLC with fluorescence detection. The introduction of 2-(12-benzo[b]acridin-5(12H)-yl)-acetohydrazide (BAAH) with excellent fluorescence property into the molecules of monosaccharides greatly enhanced the HPLC sensitivity of the analytes. Meanwhile, derivatization with BAAH also greatly increased the hydrophobicity of the monosaccharides and made them elute at increased retention times. The monosaccharides with similar properties therefore could be completely separated due to the increased interaction between the analytes and the column. Component monosaccharides of the polysaccharides obtained from the roots, stems and leaves of *Notopterygium forbesii* Boiss (NF) were analyzed by the developed method. The results indicated that the polysaccharides of NF were mainly composed of D-galactose and D-glucose. This is the first systematic study of the sugar composition of the polysaccharides of NF. It will be helpful for the quality control of NF.

1. Introduction

Carbohydrates are among the most abundant biological molecules in nature (Harvey, 2011; Ruiz-Matute, Hernández-Hernández, Rodríguez-Sánchez, Sanz, & Martínez-Castro, 2011). They provide the main energy source to maintain the life of all creatures. Besides, sugars are raw materials for organisms to produce other compounds. Polysaccharides in traditional Chinese medical herbs have attracted great attention in recent decades because they show a lot of functions such as antioxidation, anticancer, antibacterial, antiviral, and so on (Bhandari, Kumar, Singh, & Kaul, 2008; Chen, Zhang, Wu, & Ye, 2005; Ghazarian, Idoni, & Oppenheimer, 2011; Sheng et al., 2008; Shukla & Tiwari, 2012). *Notopterygium forbesii* Boiss (NF), a plant which has been used for centuries as a traditional Chinese medicine because of its diaphoretic, anti-inflammatory and analgesic properties (Liu et al., 2009; Qiu et al., 2007; Tang, Wang, Zhang, & Halliwell, 2008), is well known as *Qianghuo* in Chinese. The roots of *Qianghuo* have been traditionally used for the treatment of common cold, headache, and rheumatism in folk. In China, at least 13 prescriptions used *Qianghuo* as ingredients (Tang et al., 2008). NF is also among the five Chinese herbs which are used in a formula to treat heroin addiction in Hong Kong (Ma, Xu, Liu-Chen, & Lee, 2008). There were many researches focused on the analysis of volatile oil of NF, but little attention was paid to the analysis of the component monosaccharides of the polysaccharides of NF. The only research was carried out by Fan, Sun and Qin (1986) who confirmed the existence of three monosaccharides in the root polysaccharide of *Notopterygium incisum*, a plant possessing similar property with NF, with no research of their content.

Monosaccharides are compounds with similar property and high hydrophilicity. Therefore, the analysis of monosaccharides is challenging. The colorimetric methods can provide the total amount of carbohydrates but it is nonspecific. Because of the lack of effective chromophores or fluorophores in the structure of monosaccharides, the application of high performance liquid chromatography (HPLC) method is greatly limited. Specialized carbohydrate columns in combination with refractive index detector (RID) was often used in direct HPLC analysis of monosaccharides. However, the RID was not as common as ultraviolet (UV) or fluorescence (FL) detector for most of the researchers and the sensitivity was usually not satisfying (Sun et al., 2008). Besides, since monosaccharides are nonvolatile, direct gas chromatography (GC) method is also not applicable. Thus, most of the methods applied to the analysis of monosaccharides are GC or HPLC methods in combination with derivatization (Bernárdez, De la Montaña Miguélez,

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& Queijeiro, 2004; Dai et al., 2010; Lv et al., 2009; Soria, Sanz, & Villamiel, 2009). An often used HPLC method for the analysis of monosaccharides was carried out by derivatizing the analytes with labeling reagents containing chromophore moieties. Therefore, the derivatives of the analytes could be detected by UV detector (Dai et al., 2010; Lv et al., 2009; Sheng et al., 2008). In recent years, HPLC with FL detection plays an increasing important role in sugar analysis because of the higher sensitivity it provides (Han, Lv, Jiang, & Wang, 2007; Kakita, Kamishima, & Inouye, 2006; Lin, Xu, Feng, & Shen, 2005; Matsumoto, Hosoyama, Higashi, & Toida, 2012; Zhang, Wang, Zhang, Zhou, & Huang, 2011). However, most of them are post-column derivatization method, which is not accessible by many laboratories.

In this paper, we report a sensitive pre-column derivatization HPLC method for the determination of monosaccharides. 2-(12-benzo[b]acridin-5(12H)-yl)-acetohydrazide (BAAH) with excellent fluorescence property was introduced into the molecules of monosaccharides to enhance the HPLC sensitivity. Meanwhile, the introduction of strong hydrophobic BAAH moiety into the hydrophilic sugar molecules also greatly increased the retention of the analytes on a reversed phase column. Therefore, isomers with similar properties could be separated. The proposed method was successfully applied to the analysis of the polysaccharides in the roots, stems and leaves of NF. The content of the component monosaccharides of the polysaccharides in NF was reported for the first time.

2. Materials and methods

2.1. Instruments

The HPLC analysis was performed using an Agilent 1290 series HPLC system, equipped with an on-line-degasser, a binary pump, an autosampler and a thermostated column compartment. A fluorescence detector (model G1321B, Agilent, USA) was adjusted at wavelengths of 280 and 510 nm for excitation and emission. Chromatographic separation was achieved on a SB C18 column (2.1 × 50 mm, 1.8 μm i.d., Agilent, USA). Solvent A was 30 mM ammonium formate in water and B was acetonitrile. The flow rate was constant at 0.2 mL min^{-1} and the column temperature was kept at 30 °C. The gradient condition of mobile phase was as follows: 15–18% B from 0 to 20 min; 18–22% B from 20 to 28 min; 22–90% B from 28 to 30 min and then hold for 2 min. The column was equilibrated with the initial mobile phase for 5 min before the next injection. The injection volume was 5 μL.

2.2. Reagents and chemicals

D-Galactose (Gal), D-glucose (Glc), D-Mannose (Man), D-xylose (Xyl), L-rhamnose (Rha) and L-arabinose (Ara) were purchased from Sinopharm Group Chemical Reagent Co. Ltd., China. Acetonitrile, methanol and ethanol were of HPLC grade (Sigma–Aldrich, USA). Chloroform, trifluoroacetic acid (TFA) and *n*-butyl alcohol were purchased from Yuwang Company, China. Pure distilled water was purchased from Watsons (Guangzhou, China). All other reagents used were also of analytical grade unless otherwise stated. BAAH was synthesized in authors' laboratory as described in our previous study (Xie et al., 2012).

2.3. Samples

NF 01 was collected from Tongde, Qinghai province. NF 02 was collected from Weiyuan, Gansu province. NF 03 was purchased from Sichuan province. According to the procedure described in our previous study (Zhang, You, Zhou, Li, & Suo, 2012), the leaves, roots and stems of NF samples were dehydrated, milled and passed through a 0.25 mm sieve prior to analysis.

2.4. Sample extraction

The prepared sample (10.0 g) was extracted by ultrasonication at 60 °C with 200 mL of water. The extracted solution was centrifuged and the supernatant was then concentrated and mixed with three times volume of ethanol. The solution was kept at 4 °C overnight, then the sample was centrifuged and the precipitate was redissolved. The proteins were removed by Sevag reagent (chloroform:*n*-butanol = 4:1, v/v) for 20 min by vortexing and the procedure was repeated 5–6 times until the two layers were clear. Finally, the polysaccharides were precipitated with three times volume of ethanol and kept at 4 °C overnight. The precipitates were evaporated to dryness for later analysis.

2.5. Hydrolysis of the NF polysaccharide

Ten milligrams of polysaccharide sample was dissolved in 2 mL of 2 M TFA in a 3-mL ampoule. The ampoule was sealed under nitrogen atmosphere and kept for 8 h in an oven at 100 °C to hydrolyze the polysaccharide into component monosaccharides. After being cooled to room temperature, 100 μL of the supernatant was evaporated to dryness under a gentle nitrogen gas flow. It was then ready for the following experiments.

2.6. Derivatization procedure

To a solution containing a standard monosaccharide mixture or dried sample in a 2-mL vial, 100 μL derivatization reagent solution, 10 μL glacial acetic acid and 100 μL acetonitrile were added. The vial was sealed and allowed to react in a water bath at 75 °C overnight. The derivatization procedure is shown in Fig. 1. After the reaction was completed, the mixture was cooled to room temperature, then an appropriate volume of acetonitrile solution was added to dilute the derivatization solution to 1.0 mL. The diluted solution was syringe filtered using a 0.22 μm nylon filter and injected directly for HPLC analysis (5 μL).

3. Results and discussion

3.1. Optimization of derivatization conditions

The concentration of derivatizing reagent should be adequate enough to guarantee the sufficient reaction of the analytes. In this study, the influence of BAAH concentration on derivatization was studied carefully. The results indicated that constant fluorescence intensity was achieved with the addition of an eight-fold molar reagent excess to total molar monosaccharides. Further increasing the excess of reagent beyond this level had no significant effect on the yields. For the convenience of operation, 2.0×10^{-3} mol L^{-1} BAAH was applied throughout the experiment since most of the monosaccharides in real samples were far below this level after dilution.

Glacial acetic acid served as catalyst and therefore played a vital important role in the derivatization between BAAH and monosaccharides. The influence of glacial acetic acid volume on derivatization was studied carefully. As shown in Fig. 2, the signal intensity increased dramatically when the glacial acetic acid volume was increased from 2 to 5 μL, and the highest fluorescence intensity was achieved when the volume of glacial acetic acid was 10 μL. Further increasing the amount of glacial acetic acid lead to decreased signal intensity. That is probably due to the hydrolysis of the derivatives at strong acidic condition.

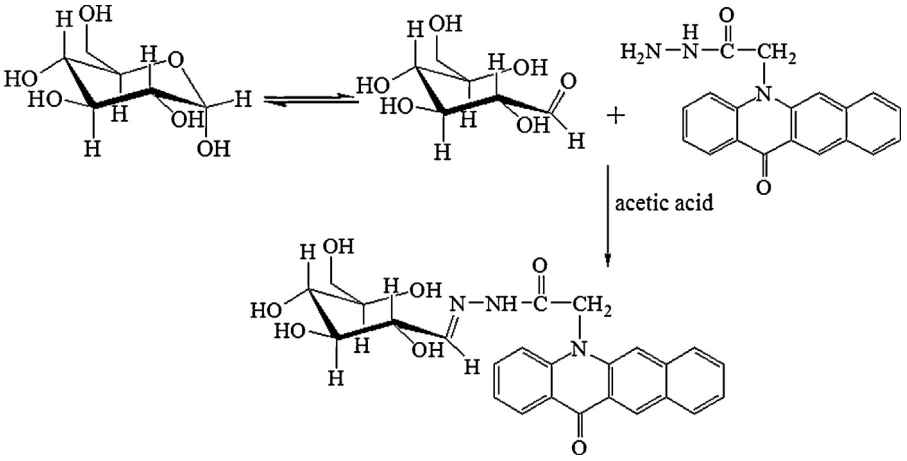

Fig. 1. Derivatization scheme of BAAH with monosaccharides.

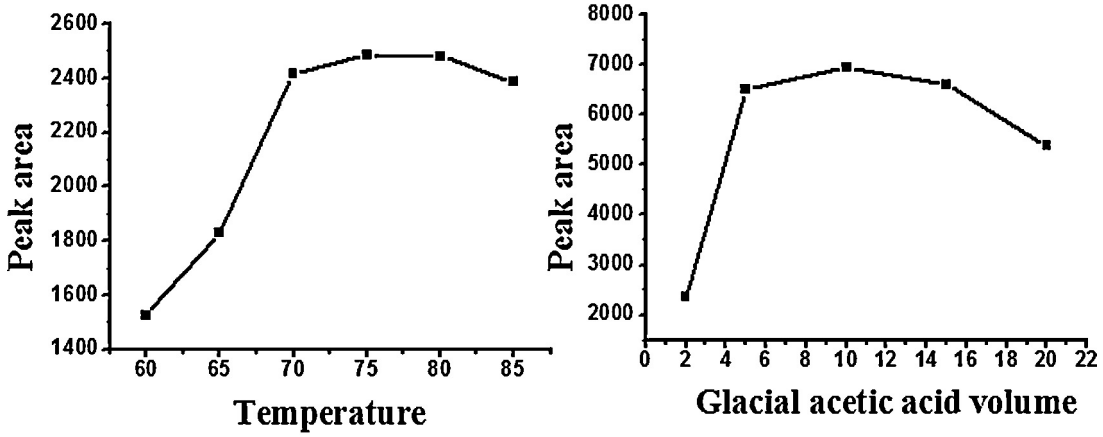

Fig. 2. Effect of reaction temperature and glacial acetic acid volume on fluorescence intensity.

Derivatization temperature played an important role in derivatization. The signal intensity increased obviously when the temperature was increased from 60 to 70 °C, and it was constant in the temperature range of 70–80 °C. Therefore, 75 °C was applied for derivatization. As to the influence of reaction time, the derivatization was not sufficient when the reaction time was less than 6 h. Longer reaction time led to higher signal intensity, but the increase was not obvious after 6 h. For the convenience of operation, derivatization was carried out overnight since longer reaction time is beneficial for the reaction. Therefore, derivatization at 75 °C overnight with BAAH concentration of 2.0×10^{-3} mol L^{-1} was applied in later experiments.

3.2. Chromatographic separation

Containing several hydroxyl groups, monosaccharides are quite hydrophilic and thereby less retained by the reversed phase column. They usually elute at early retention times in HPLC analysis. Besides, Man, Gal and Glc are isomers, which greatly increased the difficulty in separation. In this study, the introduction of BAAH into the molecules of monosaccharides increased the hydrophobicity of the analytes and made them elute at increased retention times. Thus, the interaction between the analytes and the column was greatly increased. Therefore, monosaccharides with similar property could be separated on the reversed column. As shown in Fig. 3, the six monosaccharides were completely separated within 30 min. The excess labeling reagents eluted after the elution of monosaccharides and had no influence on the detection.

3.3. Method valuation

The method was validated in terms of linearity, limits of detection (LOD), precision and accuracy. Linearity data was generated by plotting peak areas versus concentrations in the range of 20–400 ng mL^{-1}. Samples with higher concentrations were diluted to meet this standard. The correlation coefficients were found to be >0.997, indicating excellent linearity of the analytes. As shown in Table 1, instrument LODs calculated at a signal-to-noise ratio (*S*/*N*) of 3 were in the range of 10–12 μg L^{-1} (55.6–79.9 nmol L^{-1}), much lower than the LODs of μmol L^{-1} obtained by UV detector (Lv et al., 2009).

Precision of the method was evaluated by repeatability and reproducibility. Repeatability was determined by running

Table 1
Linearity, LOD and precision.

Component	*R*	LOD (μg L^{-1})	Repeatability (RSD%, *n* = 5)	Reproducibility (RSD%, *n* = 5)
Gal	0.998	10	3.5	5.8
Glc	0.999	10	4.6	7.6
Man	0.996	12	4.2	7.0
Ara	0.996	12	4.8	7.2
Xyl	0.997	10	3.6	6.4
Rha	0.997	12	3.7	5.6

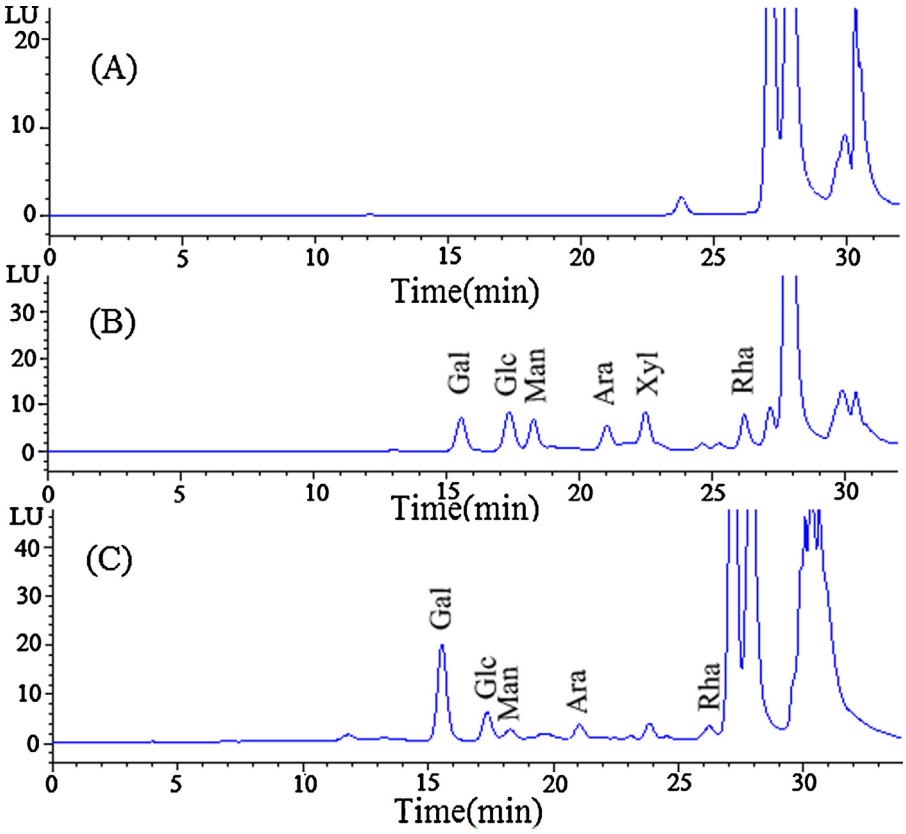

Fig. 3. The HPLC chromatograms of monosaccharide derivatives from (A) blank sample, (B) standard solution and (C) a NF root sample.

hydrolyzed samples with spiked standards at a level of 10 mg g^{-1} with five replicate, and reproducibility was analyzed by running hydrolyzed samples spiked at 10 mg g^{-1} with three replicates on three different analysis cycles by different analysts. Each sample experienced the whole derivatization and HPLC analysis procedures. Results for repeatability and reproducibility revealed good precision of the method with mean relative standard deviations (RSDs) of less than 7.6% for all compounds. Accuracy of the method was measured by analyzing hydrolyzed samples spiked with 5, 10 and 50 mg g^{-1} of monosaccharides. For the convenience of comparison, spiked levels were expressed as the concentrations in solid polysaccharide. All analyses were carried out in triplicate. The results indicated that the recoveries of monosaccharides were in the range of 90–95% (Table 2).

The stability of BAAH and sample derivatives was also tested. Anhydrous acetonitrile solution of BAAH could be stored at 4 °C for two weeks without obvious decrease in derivatization yields for the target compounds compared to those newly prepared BAAH solution. The stabilities of the corresponding derivatives were also investigated. Standard solution of 100 μg L^{-1} and NF root sample

Table 2
Recoveries and RSDs of target compounds in NF (n = 3).

Analyte	Spiked level (mg g^{-1})	Content in sample (mg g^{-1})	Determined level (mg g^{-1})	Recovery (%)	RSD (%)
Gal	5.0	40.6	45.3	94.0	4.7
	10		50.1	95.0	4.1
	50		87.4	93.6	3.6
Glc	5.0	24.9	29.5	92.0	4.5
	10		34.3	94.0	3.2
	50		71.6	93.4	4.1
Man	5.0	6.09	10.6	90.2	5.1
	10		15.3	92.1	4.3
	50		52.5	92.8	4.5
Ara	5.0	11.2	15.8	91.6	4.4
	10		20.5	93.0	3.9
	50		57.3	92.2	3.5
Xyl	5.0	–[a]	4.54	90.8	4.8
	10		9.24	92.4	4.3
	50		46.6	93.3	4.5
Rha	5.0	8.68	13.3	92.4	4.4
	10		18.0	93.2	4.2
	50		55.4	93.4	3.7

[a] Not detected.

Table 3
Monosaccharide content of polysaccharides in NF.

Samples		Monosaccharide content (mg g^{-1})					
		Gal	Glc	Man	Ara	Xyl	Rha
NF01 (Qinghai)	Leaves	85.24	20.45	12.64	14.43	–[a]	9.868
	Stems	33.03	17.80	5.517	4.382	–	6.278
	Roots	65.26	189.5	17.92	5.957	–	7.557
NF02 (Gansu)	Roots	70.45	79.88	5.287	1.896	–	13.34
NF03 (Sichuan)	Roots	40.55	24.93	6.092	11.23	–	8.684

[a] Not detected.

spiked at 10 mg g^{-1} for each monosaccharide were derivatized by BAAH. These solutions were repeatedly analyzed by HPLC after being placed at room temperature for 0.5, 1, 2, 3, 4 and 5 days, respectively. The corresponding derivatives were stable with peak area deviations (RSDs) of less than 4.5%.

3.4. Analysis of the polysaccharides of NF

The proposed method was applied to the analysis of component monosaccharides of the polysaccharides obtained from the roots, stems and leaves of NF. Fig. 3 shows a representative chromatogram of monosaccharide standard solution and a chromatogram of the monosaccharides in the root polysaccharide of NF. The sugar compositions of the polysaccharides obtained from different parts of NF are listed in Table 3. The results showed that the NF polysaccharides were heteropolysaccharides. The main neutral monosaccharides of NF polysaccharides were Gal, Glc, Man, Rha and Ara, among which Gal, Man and Ara were reported for the first time. The contents of Gal and Glc were higher than those of other monosaccharides. The highest content of Gal was found in the polysaccharides obtained from the leaves of NF, while the highest content of Glc was found in root polysaccharide. The polysaccharide in NF root from Qinghai province had higher content of the target monosaccharides, but its total amount of crude polysaccharide was less than those from Sichuan province. Besides, the components of the polysaccharides obtained from different places also showed some differences. For the root polysaccharides of Sichuan province, the Gal content was higher than those of other monosaccharides, while for the root polysaccharides obtained from Qinghai or Gansu province, the Glc content was the highest among the monosaccharides studied in this study. These differences may come from the different altitudes and latitudes that NF was cultivated. It is noticeable that the stems and leaves of NF also contain some amounts of polysaccharides though they are seldom used for clinical purpose.

4. Conclusion

A new sensitive HPLC method with fluorescence detection was applied to the analysis of polysaccharides in NF. The HPLC sensitivity was greatly enhanced due to the introduction of BAAH with excellent fluorescence property into the analyte molecules. Meanwhile, the introduction of high hydrophobic BAAH moiety into the monosaccharide molecules also greatly enhanced the retention of the analytes on a reversed phase column. Therefore, monosaccharides with similar property could be separated. The proposed method was successfully applied to the analysis of the component monosaccharides of the polysaccharides in the roots, stems and leaves of NF. The results indicated Gal and Glc were the main neutral monosaccharides of NF. To the best of our knowledge, this is the first systematic analysis of the component monosaccharides of the polysaccharides from NF. It will be helpful for the safety assessment and quality control of NF. This research is a preliminary study of NF, and more kinds of monosaccharides will be included in our later study.

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