

# Simultaneous Quantification of Uronic Acid, Amino Sugar, and Neutral Sugar in the Acidic Polysaccharides Extracted from the Roots of *Angelica sinensis* (Oliv.) Diels by HPLC

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**Abstract** High-performance liquid chromatography (HPLC) is routinely used to analyze monosaccharide composition. This is the first report describing the simultaneous determination of uronic acid, amino sugar, and neutral sugar in the polysaccharide from *Angelica sinensis* using pre-column derivatization. We were able to analyze the monosaccharide composition of acidic *Angelica* polysaccharides (AAPS) using this method. Ten different monosaccharides, namely D-mannose, D-glucosamine, L-rhamnose, D-galactosamine, D-glucuronic acid, D-galacturonic acid, D-glucose, D-galactose, L-arabinose, and L-fucose were made into pre-column derivatives to serve as the standard for AAPS analysis. Samples were separated on a Hypersil BDS C<sub>18</sub> column (4.6 mm × 250 mm, i.d.) and detected by UV detection at 250 nm wavelength. The mobile phase was composed of aqueous ammonium acetate buffer (100 mM, pH 5.0), acetonitrile, and tetrahydrofuran in the ratio of 81:17:2. Analytes were performed at 25 °C with a flow rate of 1.0 ml/min. Our study found that AAPS is composed of D-mannose, D-glucosamine, L-rhamnose, D-galactosamine, D-glucuronic acid, D-galacturonic acid, and D-glucose in a molar ratio of 1.5:14.1:1.7:1.6:2.0:2.3:1.

**Keywords** *Angelica sinensis* · High-performance liquid chromatography · Pre-column derivatization · Monosaccharide

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## Introduction

In recent years, herbs are continuing to be recognized for their medicinal value—possessing therapeutic qualities with minimal side effects (Ajayi et al. 2013). The root of *Angelica sinensis* (Oliv.) Diels is a widely used herb in traditional Chinese medicine. Dating back from around 2000 years, it has predominantly been used in the treatment of gynecological conditions; in recent times, it continues to be used for the treatment of several diseases (Sarker and Nahar 2004). However, the bioactive constituents in *A. sinensis* are difficult to identify. Analytical methods have identified more than 70 different kinds of chemical components, such as essential oils, amino acids, organic acids, carbohydrates, vitamins, and phenolic compounds, to name a few (Chao and Lin 2011; Wong et al. 2008). Recently, the polysaccharide has been recognized as an important component of this herb (Cao et al. 2006a; Cao et al. 2006b; Cao et al. 2010; Sun et al. 2010; Zhao et al. 2012). Several researchers have demonstrated various bioactivities of the polysaccharide (Jin et al. 2012), including hematopoiesis, immunomodulation, anti-tumor, antioxidant, anti-ulcer, and radioprotective activities, indicating that *A. sinensis* polysaccharide possesses important research and development value.

Therefore, much attention has been paid to the structure elucidation of the polysaccharides exhibiting biological activities. Several studies in the recent times have analyzed the monosaccharide composition of various polysaccharides using several methods, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), and high-performance liquid chromatography–mass spectrometry (LC–MS) due to the basic status of analysis on monosaccharide composition (Jiang et al. 2009; Zhao et al. 2012). GC, the most common method to analyze monosaccharide composition, requires the samples to be converted into their alditol

acetate derivatives, which is a time-consuming and complicated process (Sevcik et al. 2011). Moreover, the LC–MS method for monosaccharide composition analysis is limited by the loss of sensitivity owing to the low ionization efficiency of monosaccharide (Wu et al. 2014). Several recent studies have reported that HPLC, coupled with pre-column derivatization, where the derivative is easy to generate, can effectively analyze the monosaccharide composition with high separation efficiency and sensitivity. 1-phenyl-3-methyl-5-pyrazolone (PMP) is used to react with the reducing saccharide without an acid catalyst (Honda et al. 1989). However, the conditions in the referred studies were only used for the analysis of the neutral sugars and uronic acid. Whether amino sugars can be separated under the same conditions or not is underway in the study.

In this paper, we prepared PMP pre-column derivatives of acidic *Angelica* polysaccharides (AAPS) and firstly added tetrahydrofuran into the HPLC elution to separate the uronic acid, amino sugar, and neutral sugars from the acidic polysaccharide extracted from *A. sinensis*. Using this HPLC method, our analysis of the monosaccharide composition yielded good results, in terms of both number and types of monosaccharides identified.

## Materials and Methods

### Chemicals and Reagents

The roots of *A. sinensis*(Oliv.) Diels were collected from Minxian county, Gansu province, China in the year 2012. The root was identified according to the identification standard of the Pharmacopeia of the People's Republic of China. The herbs were air-dried in the shade and stored in a well-closed vessel for use.

HPLC-grade methanol and acetonitrile were purchased from Duksan pure chemicals (Korea). The column of Hypersil BDS C<sub>18</sub> column (4.6 mm i.d. × 250 mm, 5 μm) was purchased from Elite (Dalian, China).

1-phenyl-3-methyl-5-pyrazolone (PMP), D-glucose (Glc), D-(+)-galactose (Gal), L-rhamnose (Rha), L-arabinose (Ara), D-(+)-galacturonic acid (GalUA), D-mannose (Man), D-(+)-glucosamine (GlcN), L-(–)-fucose (Fuc), D-glucuronic acid (GluUA), D-galactosamine (GalN), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents were of analytical reagent grade.

### Extraction and Purification of Acidic Polysaccharide from *Angelica sinensis*

We cut the herbs (200 g) into manageable-sized pieces and used the method of reflux extraction with 95 % EtOH (1 L) for

three times (2 h per time) to remove the liposoluble substance. The residue was air-dried, and then extracted with 1600 ml of 1.0 M NaOH for 2 times every 1 h. The supernatant was collected after filtering through gauze and subsequently centrifuged to remove water-insoluble materials. The aqueous extract was concentrated at 55 °C in vacuum to remove excess water and placed in the refrigerator at 4 °C overnight to remove water-insoluble materials. Next, four times the volume of 100 % ethanol was added to the pre-cooling aqueous extract and stayed at 4 °C again for 24 h to obtain the precipitation. We place the water-dissolved precipitation in –20 °C, then thawed and centrifuged. After the freeze–thaw process was repeated ten times; the supernatant was dialyzed against distilled water for 48 h. Lastly, we used the method of freeze-drying to obtain a brown product, which was the crude *Angelica* acidic polysaccharide (AAPS, 9.92 g).

Using D-glucose as the standard, phenol-sulfuric acid method was employed for the measurement of the content of carbohydrates (Wu et al. 2014). The extraction efficiency was calculated by following formula:  $X (\%) = (C \times D / M) \times 100$ , where  $X$  was the sugar content every 100 g,  $C$  was the sugar content calculated in the standard curve,  $D$  was the dilution ratio, and  $M$  was the herb weight. The extraction efficiency of AAPS was 4.66 %.

### Hydrolysis of AAPS

The complete acid hydrolysis method was referred to Honda (Honda et al. 1989). The acidic polysaccharide (5.0 mg) was infiltrated with 2.5 ml of 2 M TFA in an ampoule. The ampoule was sealed under nitrogen atmosphere and kept in an oil bath at 110 °C for 6 h. After being cooled to room temperature, the reaction mixture was removed of any remaining TFA by rotary evaporation at 50 °C. Then, 2 M NaOH was added to neutralize the mixture and obtained a pH of 7.0. The completely hydrolyzed sample solution was ready for the subsequent experiments.

### Preparation of PMP Derivatives

The hydrolysis product of AAPS and nine kinds of standard monosaccharide (5.0 mg) were separately added with 1 ml of distilled water. Fuc as an internal standard was added to each sample before the derivatization. Then, 400 μl of each solution was removed to a centrifugal tube in which 400 μl of 0.5 M methanol solution of PMP and 200 μl of 0.3 M NaOH were added before. After vigorous mixing, each mixture was allowed to react in a water bath at 70 °C for 30 min. Once the mixtures cooled to room temperature, 200 μl of 0.3 M HCl was added to neutralize the solution to about pH 7.0. Then, 2 ml of distilled water and 4 ml of chloroform was added into each centrifugal tube in order to perform a rotary extraction. The organic phase (under aqueous layer) was carefully

abandoned to remove the excess reagents, and the extraction process was repeated three times. Lastly, the aqueous layer was filtered through a 0.45- $\mu\text{m}$  microporous filtering film and diluted with distilled water before HPLC analysis.

### HPLC Analysis

The analysis of PMP-labeled monosaccharides was carried out on a Shimadzu LC-2010A HT system (Shimadzu, Kyoto, Japan) equipped with a built-in UV–Vis detector. The PMP-labeled monosaccharides were analyzed using a Hypersil BDS  $C_{18}$  column (4.6 mm i.d.  $\times$  250 mm, 5  $\mu\text{m}$ , Elite, China), and the mobile phase was composed of acetate buffer solution (100 mM, pH 5.0), acetonitrile, and tetrahydrofuran in the ratios of 81:17:2 (v/v/v). The flow rate was 1.0 ml/min at room temperature, and the detection was performed at 250 nm.

### Data Analysis

A known amount of Fuc was placed in the samples before hydrolysis or derivatization. The principle of data analysis is explained below. The correction factor ( $f_i/s$ ) was calculated from the following:

$f_i/s = (W_i/W_s)(A_i/A_s)$ , wherein  $A_i$  and  $A_s$  are representatives of the integration value of peak area for a component monosaccharide in tested sample and Fuc, respectively.  $W_i$  and  $W_s$  are representatives of the values of weights for a component monosaccharide in tested sample and Fuc, respectively.

The molar ratio value is calculated as follows:

$R_{i/s} = f_{i/s} \times (A_i/A_s)/M$ . In the equation,  $A_i/A_s$  is the ratio value of peak area for the component monosaccharide in tested samples and Fuc, and  $M$  is the molecular weight of the monosaccharide.

## Results and Discussion

### Optimization of Extraction Conditions

Earlier published works have used water for the extraction of *A. sinensis* polysaccharide. Although the direct use of water is sufficient to extract neutral polysaccharide, acidic polysaccharide cannot be efficiently extracted by the same. Therefore, in our study, a solution of NaOH was used for the extraction of acidic polysaccharide.

The extraction process is influenced by the amount of NaOH liquor used, extraction time, the number of extractions, and the NaOH concentration used for extraction. Hence, our study adopted an orthogonal test of four factors at three levels to obtain the optimal conditions for extraction. To elaborate, these four factors included the consumption of NaOH liquor (A: 6, 8, and 10 times the volume), extraction time (B: 1, 2,

and 3 h), the number of extractions (C: once, twice, and three times), and the NaOH concentration (D: 0.5, 1.0, and 1.5 M). The polysaccharide contents of different conditions were measured using phenol–sulfuric acid method (Wu et al. 2014). Finally, the sample preparation method was obtained by using eight times the amount of NaOH liquor, a two-time extraction process every 1 h, and the use of 1.0 M NaOH. Using this method, the extraction efficiency of polysaccharide from *A. sinensis* can achieve 4.66 %.

It is widely believed that the yield of polysaccharide increases with the increase in decoction time and the amount of alkaline water used. However, our orthogonal test result showed that this might not be true, because the chemical structure of the polysaccharide could be influenced by the extraction condition. First, the amount of alkaline water was an important factor, because the stereochemical structure of polysaccharide can be hampered by alkali. Second, long decoction time could adversely affect the temperature-sensitive polysaccharide. Therefore, only with proper concentration of alkaline water and optimal decoction time could we obtain the best yield—0.4962 g (4.96 g from 100 g herb).

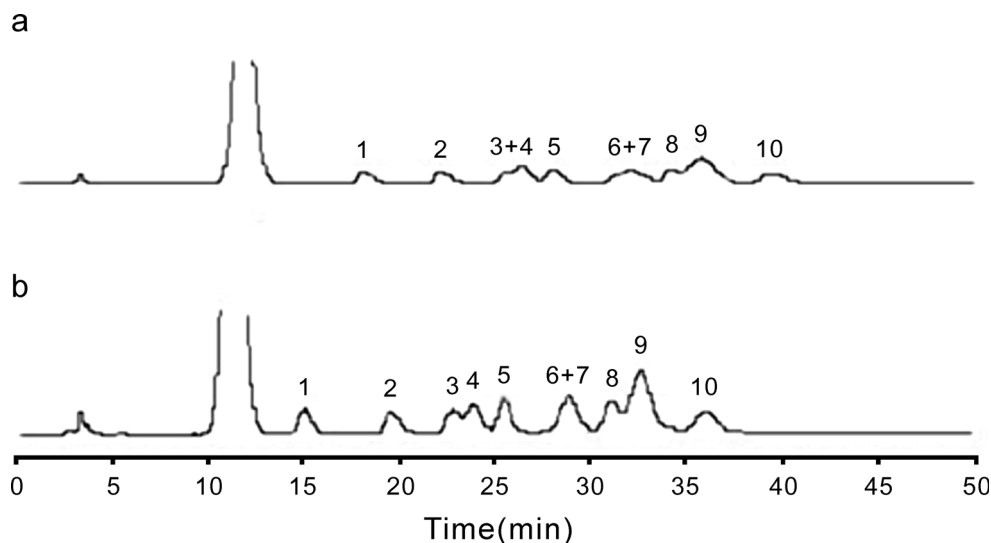
### Optimization of Chromatographic Conditions

This study aimed to develop a methodology that would enable fast, effective separation as well as a simultaneous quantification of uronic acid, amino sugar, and neutral sugars in the acidic polysaccharide obtained from *A. sinensis*. The HPLC analytical conditions for the ten standard monosaccharides derivatized with PMP were optimized by changing pH values, acetonitrile concentration, and tetrahydrofuran concentration of the mobile phase. This enabled us to obtain the optimal analysis conditions for the monosaccharide composition analysis of AAPS.

The HPLC separation was based on previously published works (Zhang et al. 2003), and the experimental condition for the separation of PMP derivatives was set as 100 mM ammonium acetate buffer (pH 5.5)/acetonitrile (78:22, v/v). However, this condition is effective only for the analysis of neutral polysaccharide and some types of acidic polysaccharide that are only composed of glycosyl and some uronic acid monomers. For others in which there are amino monosaccharide monomers, this HPLC separation method is not the most effective. In this study, we converted amino monosaccharides into PMP derivatives to serve as standards and investigated optimal experimental conditions. We used the same HPLC separation method as mentioned in the previously study (Zhang et al. 2003). However, ten PMP derivatives of standard monosaccharides were not separated well.

The effects of pH on the separation of the ten PMP derivatives of standard monosaccharides were shown in Fig. 1. It

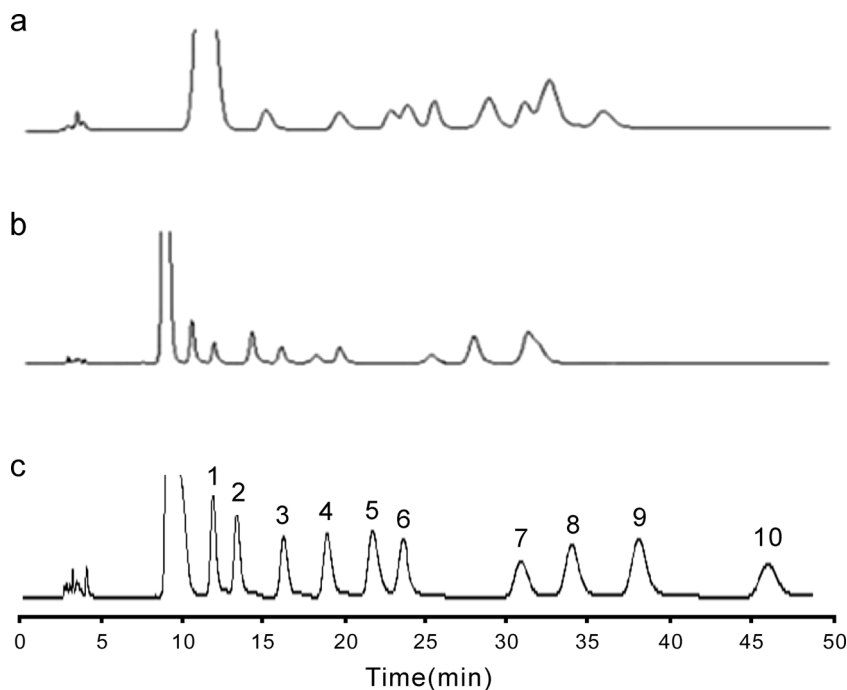
**Fig. 1** The effect of the pH on the separation of ten PMP derivatives. **a** 22 % acetonitrile, pH=5.5; **b** 22 % acetonitrile; pH=5.0. 1 Man, 2 GlcN, 3 Rha, 4 GalN, 5 GlcUA, 6 GalUA, 7 Glc, 8 Gal, 9 Ara, and 10 Fuc



was found that, under our experimental conditions, a decrease of pH in the 100 mM ammonium acetate buffer could accelerate the elution of all the derivatives and optimize the peak shape as well. Therefore, the variation of pH could significantly influence the separation. For example, the ten PMP derivatives were not separated well at pH 5.5, and only seven peaks could be seen; however, at pH 5.0, nine peaks could be seen. We also changed the pH to 4.5, but the separation effect was similar to that of pH 5.0. So, we conducted the experiment at pH 5.0 and began to change the mobile phase in order to get the optimal separation condition.

The effects of mobile phase on the separation parameters of the ten standard monosaccharides derivatized with PMP at pH 5.0 of ammonium acetate buffer were shown in Fig. 2. It was found that a decrease in acetonitrile could prolong the elution of all the derivatives and optimize the peak shape as well. For example, PMP derivatives were not separated well at first (Fig. 2a), but after the acetonitrile decreased to 17 %, eight peaks were separated completely (Fig. 2b). However, because two peaks were still not separated, we added 2 % THF into ammonium acetate buffer. As a result, all ten peaks separated completely as shown in Fig. 2c.

**Fig. 2** The effect of the organic phase content on the separation of ten PMP derivatives. **a** 22 % acetonitrile, pH=5.0; **b** 17 % acetonitrile, pH=5.0; **c** 17 % acetonitrile, 2 % tetrahydrofuran, pH=5.0. 1 Man, 2 GlcN, 3 Rha, 4 GalN, 5 GlcUA, 6 GalUA, 7 Glc, 8 Gal, 9 Ara, and 10 Fuc



As shown in Figs. 1 and 2, the separation of PMP derivatives was dramatically influenced by pH, the concentration, and type of organic phase.

#### HPLC Method Validation

For the method validation, a system suitability test (SST) and validation parameters were measured. The method of validation was conducted on the polysaccharide samples of *A. sinensis*, including the determinations of linearity, precision, accuracy, and the limits of detection and quantification. The validation results were within the criteria range of Reviewer Guidance, Validation of Chromatographic Methods (FDA Center for Drug Evaluation and Research, November 1994). Therefore, the method is suitable for the analysis of monosaccharide composition in the AAPS samples.

#### System Suitability Test (SST)

SST includes the injection of ten standard solutions of the same concentration, the calculation of the theoretical plate number, the symmetry factor, the relative standard deviation of the retention time ( $t_R$ ), and peak area ( $A$ ). The result of SST is shown in Table 1. The relative standard deviation (RSD) of repeatability of  $t_R$  and area were all below 1 %, the theoretical plates ( $N$ ) > 1500, the resolution ( $R_{ij}$ ) > 1.5, and the symmetry factor ( $T$ ) was between 0.8 and 1.5. According to a relevant report (Havlíková et al. 2013), the data mentioned above indicated that the SST was achieved in the investigated range.

#### Linearity

The measurement of linearity was conducted as follows. The standard solutions of six different concentrations (0, 5, 10, 15, 30, 50 mg/l, respectively) were injected to HPLC system, separately. Each solution (ten kinds of solutions) was injected for three times. The result is shown in Table 2. The  $R$  values were in the range of 0.9990 to 0.9998, which indicated the good linearity.

**Table 1** Method validation

Parameter(SST)	Man	GlcN	Rha	GalN	GlcUA	GalUA	Glc	Gal	Ara	Fuc
Repeatability $t_R$	0.84	0.18	0.49	0.89	0.63	0.43	0.35	0.53	0.31	0.37
Repeatability areas	0.69	0.20	0.66	0.69	0.69	0.66	0.66	0.58	0.65	0.50
Theoretical plates	12,668	14,146	13,462	14,679	10,343	14,717	15,479	17,688	11,900	16,884
Resolution	4.20	3.26	2.06	4.10	2.67	11.7	5.30	10.51	6.83	9.10
Symmetry factor	1.09	1.09	1.45	1.48	1.21	1.33	0.99	0.93	1.44	1.29

**Table 2** Standard curve of monosaccharides and the linearity

Peak	Sugar	Standard curve	$R$ ( $n=6$ )
1	Man	$Y=0.057X+0.005$	0.9990
2	GlcN	$Y=0.743X-0.048$	0.9992
3	Rha	$Y=0.800X-0.039$	0.9996
4	GalN	$Y=0.106X+0.002$	0.9998
5	GlcUA	$Y=0.283X-0.016$	0.9990
6	GalUA	$Y=0.459X-0.037$	0.9994
7	Glc	$Y=1.029X-0.060$	0.9993
8	Gal	$Y=1.061X-0.053$	0.9996
9	Ara	$Y=0.470X+0.034$	0.9992
10	Fuc	$Y=1.031X-0.077$	0.9991

#### Precision

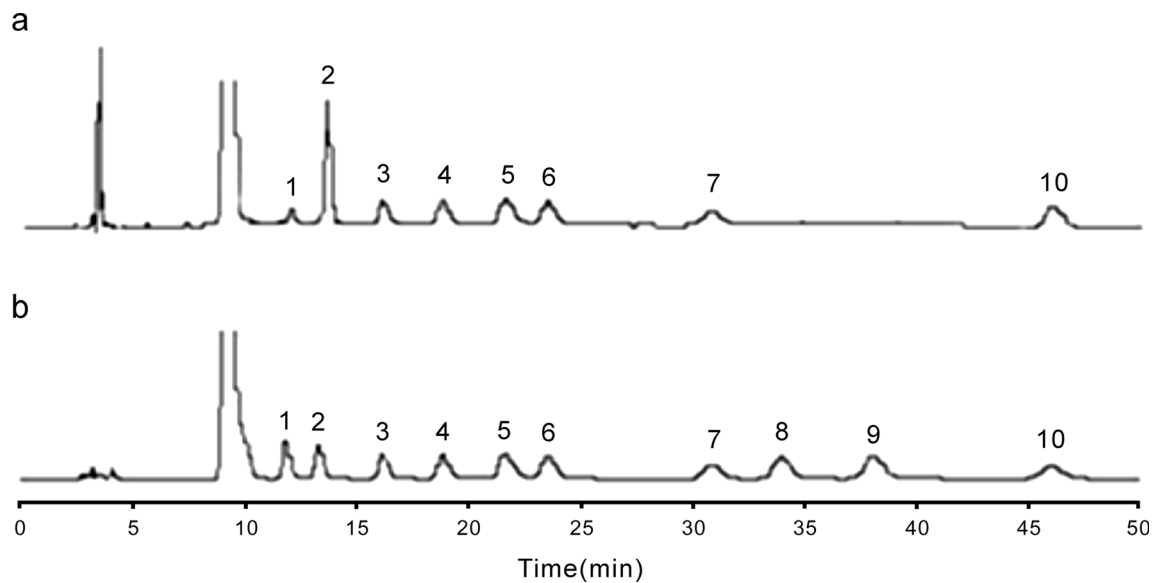
The precision was conducted by injecting ten standard solutions to the system (each solution was injected for six times), and then the HPLC parameters were analyzed. The results indicated that the RSD value was less than 2.0 %.

#### Accuracy

The accuracy was checked using the AAPS sample added along with a solution of the mixture of ten standard PMP derivatives at three different concentrations. Every sample was injected three times. The changed sample solutions and unchanged standard solution were compared for recovery evaluation. All the calculated recovery values were between 98.02 and 103.17 %. The recovery results proved that a satisfactory extraction and derivatization method were obtained.

#### Limits of Detection and Quantification

The limit of detection (LOD) was determined as the sample concentration that produced a peak with a height three times the level of the baseline noise, and the limit of quantification (LOQ) was evaluated as the concentration equal to ten times the value of the signal-to-noise. The LODs of ten monosaccharides (Man, GlcN, Rha, GalN, GlcUA, GalUA, Glc, Gal,



**Fig. 3** The HPLC chromatograms of Angelica acidic polysaccharide and standard monosaccharides. **a** The HPLC chromatogram of Angelica acidic polysaccharide added with Fuc and **b** the HPLC

chromatogram of ten standard monosaccharides. 1 Man, 2 GlcN, 3 Rha, 4 GalN, 5 GlcUA, 6 GalUA, 7 Glc, 8 Gal, 9 Ara, and 10 Fuc

Ara, and Fuc) were 0.097, 0.52, 0.19, 1.47, 0.55, 0.97, 1.53, 1.55, 0.79, and 1.43 mg/l, respectively. The LOQs of ten monosaccharides (Man, GlcN, Rha, GalN, GlcUA, GalUA, Glc, Gal, Ara, and Fuc) were 0.32, 1.73, 0.65, 4.91, 1.84, 3.22, 5.10, 5.18, 2.65, and 4.78 mg/l, respectively.

#### Monosaccharide Composition Analysis of AAPS Sample

Monosaccharide composition analysis of AAPS was carried out according to the method described in the experimental section. Briefly, AAPS was hydrolyzed with 2 M TFA for 6 h, and then the mixture was directly neutralized and derivatized using PMP. Fuc was used as the internal standard. The reaction mixture was analyzed by HPLC, as described earlier. The monosaccharide composition analysis of AAPS is shown in Fig. 3a. The presence of Man, GlcN, Rha, GalN, GlcUA, GalUA, and Glc was confirmed by comparing with the results of ten PMP derivatives of standard monosaccharides (Fig. 3b). The apparent peak height of GlcN in the chromatogram demonstrated that AAPS was mainly composed of GlcN. At the same time, the peaks as for Man, Rha, GalN, GlcUA, GalUA, and Glc were smaller than the major peak but were also observable, thereby proving the presence of Man, Rha, GalN, GlcUA, GalUA, and Glc in AAPS.

The result of monosaccharide composition of AAPS is shown in Fig. 3. The data shows that AAPS is composed of seven monosaccharides: Man, GlcN, Rha, GalN, GlcUA, GalUA, and Glc at the concentrations of 67.5, 581.8, 57.6, 59.1, 91.4, 96.0, and 39.7  $\mu\text{g}/\text{mg}$ , respectively (the molar ratio is 1.5:14.1:1.7:1.6:2.0:2.3:1). With this method, the within-day and day-to-day precisions of the composition determinations were 1.07–1.46 and 3.01–3.53 % (RSD), respectively.

Therefore, this method was successfully applied to the monosaccharide composition analysis of AAPS.

Monosaccharide composition analysis of polysaccharides has long been conducted by GC. However, GC has many disadvantages, such as a complicated derivatization procedure, low resolution of sugar, and ignorance of uronic acid. However, these disadvantages were overcome by the HPLC method developed in our experiment. Besides, our method was also capable of conducting a simultaneous quantification of uronic acid, amino sugar, and neutral sugars in the acidic polysaccharides. The PMP pre-column derivatization method is simple, rapid, and convenient and suitable for HPLC analysis of acidic Angelica polysaccharides. This method could provide reference for other studies on polysaccharides.

#### Conclusions

This study describes an accurate and reliable HPLC method to determine monosaccharide components in AAPS. Furthermore, this is the first report for the simultaneous determination of uronic acid, amino sugar, and neutral sugars in *A. sinensis* using reverse-phase HPLC. According to our findings, AAPS is composed of Man, GlcH, Rha, GalH, GlcUA, GalUA, and Glc in the molar ratio of 1.5:14.1:1.7:1.6:2.0:2.3:1.

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**Conflict of Interest** Wei-Yan Li, Ping Li, Xiao-Qiang Li, Hai Huang, Hanwei Yan, Ya Zhang and Wei Cao declare that they have no conflict of interest.

**Compliance with Ethics Requirements** This article does not contain any studies with human or animal subjects.

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