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Analytical Methods

Separation and quantification of component monosaccharides of the tea polysaccharides from *Gynostemma pentaphyllum* by HPLC with indirect UV detection

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (HPLC) method is described for the simultaneous determination of aldoses and uronic acids. The separation was carried out on a RP-C_{18} column (4.6 mm i.d. × 250 mm, 5 μm, Venusil, USA) using precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) and UV detection at 250 nm, and the 10 PMP derivatives of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, arabinose and fucose were baseline separated within 40 min. Furthermore, the described method was applied to the quantitative analysis of component monosaccharides in the water-soluble polysaccharides extracted from *Gynostemma pentaphyllum* Makino tea and the result showed that the tea polysaccharide was a typical heteropolysaccharide and consisted of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose in the molar contents of 16.3, 10.3, 47.1, 5.6, 24.0, 128.4, 25.0, 101.4 and 71.1 μM, respectively. Quantitative recoveries of the component monosaccharides in the tea polysaccharide were in the range of 94.6–108.0% and the RSD values were lower than 4.9%. The results demonstrated that the proposed HPLC method was precise and practical for the analysis of the *G. pentaphyllum* tea polysaccharide.

1. Introduction

Tea has been used as the second most consumed beverage for thousands of years in the world next to water. The ingestion of herbal teas is a common occurrence in many countries by ancient medicinal cultures and is popularly consumed in unfermented (green tea), semifermented (oolong tea) and fermented (black or red) forms (Zhu, Hackman, Ensunsa, Holt, & Keen, 2002). In South America, herbal teas frequently consist of pure coca leaf or coca leaf mixed with herbs and in Asian countries, the consumption of green tea is especially popular due to its association with human health benefits, resulting in the inclusion of green tea extracts as common botanical ingredients in dietary supplements and functional foods (Chen, Zhang, Qu, & Xie, 2007).

Gynostemma pentaphyllum Makino, a perennial liana herb belonging to the *Cucurbitaceae*, is a well-known edible and medicinal plant and is distributed wild in China (particularly south of the Qinling Mountains and Yangtze River), Japan and many other Asian countries. *G. pentaphyllum* is known as 'Jiao-Gu-Lan' in China and as 'Cha-Satun' in Thailand, and as "Amachazuru" in Japan. *G. pentaphyllum* has been clinically used for depressing cholesterol levels, regulating blood pressure, strengthening the immune system, treating chronic bronchitis and gastritis, and reducing inflammation in China (Aktan, Henness, Roufogalis, & Ammit, 2003; Circosta, De Pasquale, & Occhiuto, 2005; Cour, Molgaard, Yi, & La-Cour, 1995; Cui, Eneroth, & Bruhn, 1999; Huang et al., 2005; Lin, Huang, & Lin, 2000), and has been described as having minimal toxicity (Attawish et al., 2004). For this reason, it is claimed that drinking tea made of *G. pentaphyllum* could regularly promote good health and lessen the severity of many disorders. Therefore, the *G. pentaphyllum* green tea is presently promoted in China and is sold in Europe as an herbal tea which is "advantageous to one's health and beauty" (Takemoto, Arihara, Nakajima, & Okuhira, 1983; Rujjanawate, Kanjanapothi, & Amornlerdpison, 2004). Because of the similarity in bioactive components to the expensive ginseng root, cheap *G. pentaphyllum* was named as "second ginseng" and recently has attracted much interest as a potential new medicinal plant and hence the cultures of *G. pentaphyllum* or their extracts for health care have been put into production on a large scale (Cui et al., 1999).

In recent years, many herbs used in popular medicine have been reported to contain polysaccharides with a great variety of biological activities and the water-soluble tea polysaccharides are also demonstrated to be one of the main bioactive constituents of *G. pentaphyllum* green tea except for a series of dammarane-type saponins (Cipriani et al., 2006; Wang & Luo, 2007; Wang, Luo, & Ena, 2007). For these reasons, great interest arose on the reliable analytical methods of the tea polysaccharides, which can be used for the authentication and routine quality control of commercial

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herbal teas derived from *G. pentaphyllum* (Chen, Zhang, & Xie, 2004). This will be helpful for evaluating the possibility for human consumption and exploring the new functional products with tea polysaccharide due to its pharmacological importance and application in the food industry.

However, the lack of chromophores or fluorophores in the structure of monosaccharides limits the modes of detection. Refractive index detection and other related methods do not often meet the demands of modern trace level analysis with regard to sensitivity and/or selectivity (Wang & Fang, 2004). Therefore, the derivatization of monosaccharides is indispensable to obtain highly sensitive detection (Honda et al., 1989). The reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) is one of the popular labels that react with reducing carbohydrate under mild condition, requiring no acid catalyst and causing no desialylation and isomerization (Daotian & Roger, 1995; Honda, Suzuki, & Taga, 2003; Zhang, Xu, Zhang, Zhang, & Zhang, 2003).

The present paper is specifically concerned with the simultaneous separation of the 10 monosaccharides (aldoses and uronic acids) possibly found in natural herbs using precolumn PMP derivatization HPLC and UV detection at 250 nm. Furthermore, the developed HPLC method was applied to the quantitative analysis of component monosaccharides in the water-soluble crude polysaccharides extracted from *G. pentaphyllum* green tea.

2. Materials and methods

2.1. Materials and reagents

The green tea of *G. pentaphyllum* was purchased from Pingli country Fiveleaf Gynostemma Institute, Shaanxi province, China. D-Mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-glucose, D-xylose, D-galactose, L-arabinose, and D-fucose were obtained from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). 1-Phenyl-3-methyl-5-pyrazolone (PMP), purchased from Beijing Reagent Plant (Beijing, China), was re-crystallized twice from chromatographic grade methanol before use. Triethylamine was from Xi'an Reagent Plant (Xi'an, China). HPLC grade acetonitrile and methanol were purchased from TEDIA (USA). All other chemicals were of the highest grade available.

2.2. Extraction of the tea polysaccharide from G. pentaphyllum

The tea polysaccharide was isolated from *G. pentaphyllum* green tea by hot-water extraction and ethanol precipitation. The dried *G. pentaphyllum* tea (200 g) were defatted with 95% alcohol and then extracted with distilled water (1:10, w/v) at 80 °C for 3 h. The water extracts were collected and the residues were extracted again for three cycles. The combined extracts were pooled, concentrated to 30% of the original volume under a reduced pressure and then centrifuged at 2000 rpm for 15 min. The supernatant was collected and 3 volume of 95% alcohol was added slowly by stirring to precipitate the polysaccharide, and then kept at 4 °C overnight and finally, the polysaccharide pellets were obtained by centrifugation at 4000 rpm for 15 min and repeatedly washed sequentially with possibly less amounts of ethanol, acetone and ether, respectively. The refined polysaccharide pellets were completely dissolved in appropriate volume of distilled water and intensively dialyzed for two days against distilled water (cut-off M_w 8000 Da). The retentate portion was concentrated, deproteinated with Sevag reagent ($CHCl_3$:BuOH = 4:1, v/v) for 30 min under the magnetic force stirring and the procedure is repeated two times (Navarini, Gilli, & Gombac, 1999). Finally, the extracts were centrifuged to remove insoluble material and the supernatant was lyophilized in the freeze–dry apparatus (FD-1, Henan Yuhua Instrument Co., China) to give the crude tea polysaccharides (about 1.8 g) with a brown fluffy shape.

2.3. Hydrolysis of the tea polysaccharide

20 mg of polysaccharide sample was dissolved in 2 ml of 3 M TFA in an ampoule (5 ml). The ampoule was sealed under a nitrogen atmosphere and kept in boiling water bath to hydrolyze the polysaccharide into component monosaccharides for 8 h. After being cooled to room temperature, the reaction mixture was centrifugated at 1000 rpm for 5 min. The supernatant was collected and dried under a reduced pressure. The hydrolyzed and dried sample solutions are added with 1 ml distilled water and then ready for the following experiments.

2.4. Preparation of standard solution

Stock standard solutions (2.0 mM) were prepared by dissolving each standard monosaccharide in a mixture of water solution containing 10% methanol. Working standard solutions were further obtained by appropriate dilution of the stock standard solutions with deionized water. The sample solutions were filtered through a 0.22 μm syringe filter and were degassed using an ultrasonic bath for 2 min prior to use. All the solutions prepared were stored in the dark at 4 °C until being used.

2.5. Derivatization procedure

PMP derivatization of monosaccharides was carried out as described previously with proper modification (Daotian & Roger, 1995; Honda et al.,1989, 2003; Zhang et al., 2003). Briefly, 10 standard monosaccharides or the hydrolyzed samples of the tea polysaccharide were dissolved in 0.3 M aqueous NaOH (50 μl) and a 0.5 M methanol solution (50 μl) of PMP was added to each. Fucose as an internal standard was added to each sample before the derivatization. Since PMP was neutralized with NaOH, the resultant solution was almost neutral. Each mixture was allowed to react for 60 min at 70 °C, then cooled to room temperature and neutralized with 50 μl of 0.3 M HCl. The resulting solution was extracted with chloroform (1 ml) and the process was repeated three times; then the aqueous layer was filtered through a 0.45 μm membrane.

2.6. HPLC equipment and conditions

The analysis of PMP-labeled monosaccharides was carried out on a Shimadzu LC-2010A HPLC system equipped with a quaternary gradient pump unit, an UV–Vis detector (190–700 nm), an autosampler (0.1–100 μl) and the column oven (273–333 K) was controlled by Shimadzu Class-VP 6.1 chromatography workstation. The analytical column used was a RP-C_{18} column (4.6 mm *i.d.* × 250 mm, 5 μm, Venusil, USA). The wavelength for UV detection was 250 nm. Elution was carried out at a flow rate of 1.0 ml/min at 35 °C. The mobile phase A consisted of acetonitrile and the mobile phase B was 0.045% KH_2PO_4–0.05% triethylamine buffer (pH 7.0) using a gradient elution of 90–89–86% B by a linear decrease from 0–15–40 min. The injection volume was 20 μl.

3. Results

3.1. HPLC separation of PMP-monosaccharide derivatives

To improve the accuracy for sugar composition analysis of the polysaccharide from *G. pentaphyllum* green tea, the separation behavior of PMP-labeled derivatives of the 10 reductive

monosaccharides possibly existed in plant polysaccharides was investigated by using commonly reversed-phase C_{18} column and UV detection was 250 nm. In this study, it was found that it was not possible to separate galactose from xylose, nor arabinose from xylose on the C_{18} columns with only phosphate–acetonitrile mobile phases under any of the conditions tested. As triethylamine was chose as modifier, a significant improvement in the resolution among the tested monosaccharides was observed. To deeply study the separation behavior of the PMP derivatives in the C_{18} column, different proportional mixtures of phosphate buffer (different ionic strengths and pH), acetonitrile and triethylamine as mobile phases were optimized at immobile 35 °C. As can be seen in Fig. 1A, the baseline separation among the 10 monosaccharides labeled with PMP was achieved at a gradient mode beginning with solvent A/solvent B (10:90) until 15 min, and then to gradually decrease the elution strength by adjusting solvent B (15–40 min, 86% B). The peaks were identified in the order of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, arabinose and fucose by comparing the retention time of the unknown peaks with that of the standards under the same conditions. As a result, triethylamine as modifier for the separation of carbohydrates was shown to have a good elution profile of PMP-derivatized monosaccharides by HPLC with indirect UV detection.

3.2. Validation

The HPLC method was validated in terms of linearity, reproducibility, limit of detection (LOD) and precision. The linearity was verified by the analysis of six points ($n = 5$) in the range of 5–150 μM of standard sugars (mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose), and the linear regression parameters of the calibration curves were shown in Table 1. As a consequence, the good linearity (correlation coefficient $r > 0.9994$) between Y (peak area ratio of the analytes with internal standard) and X (concentration of the standards) was achieved in the tested range. Furthermore, LOD of each tested analyte was obtained by injecting 10 μl of gradational dilutions of a standard mixture derivatized as mentioned above in the derivatization procedure, followed by the comparison of peak height with baseline noise level and a signal-to-noise ratio (S/N) of 3 assigned the detection limit. The results showed that the LOD of the monosaccharides was in the range from 0.8 to 1.6 μM (Table 1), indicating that the sensitivity of the method was satisfactory.

Moreover, method precision was also determined by measuring repeatability (intra-day variability) and intermediate precision (inter-day variability) of retention time and peak area for each tested monosaccharide. The precision of method was calculated as the coefficient of variation (CV) for five successive injections of each tested monosaccharide at the concentration of 20 μM and the results were summarized in Table 2. The results showed that the intra-day reproducibility (CV values) were less than 1.03% for the migration time and 2.75% for the peak areas (A), and the inter-day CV values were less than 2.35% for the migration time and 4.48% for the peak areas (A), indicating that the method precision was satisfactory.

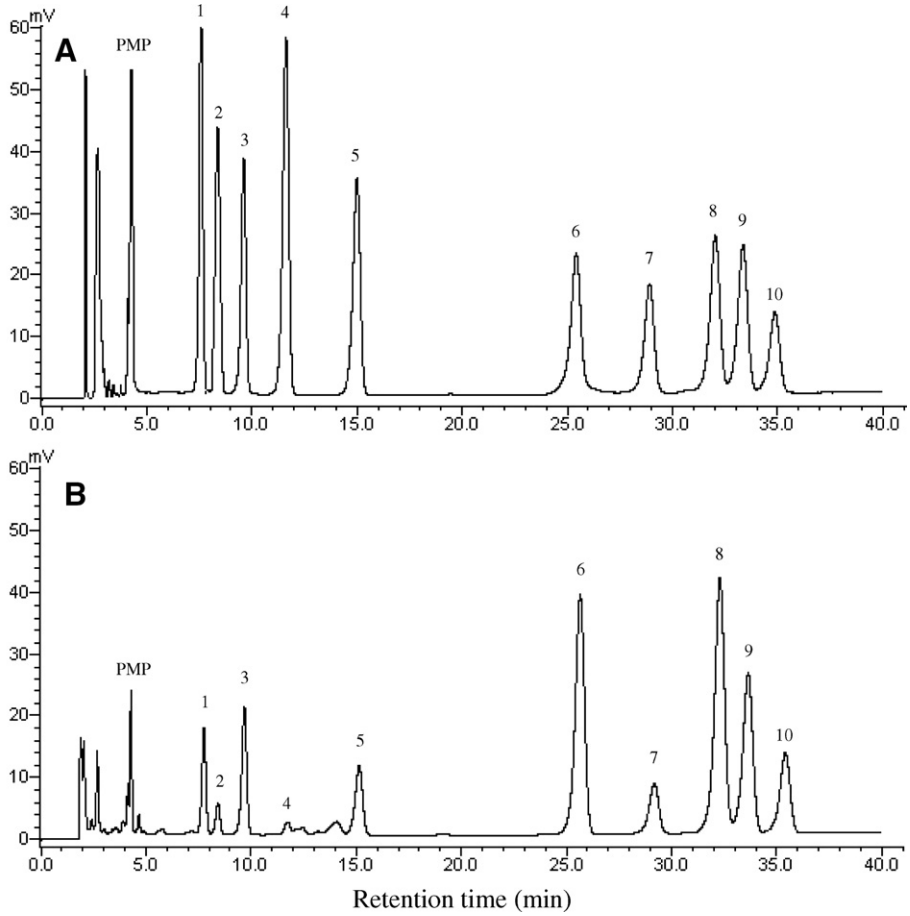

Fig. 1. The HPLC chromatograms of PMP derivatives of 10 standard monosaccharides (A) and component monosaccharides released from *G. pentaphyllum* tea polysaccharides (B). The polysaccharide was hydrolyzed with TFA at 100 °C for 8 h and then was labeled with PMP. The HPLC analysis was carried out as described in the experimental section. Peaks: 1. mannose; 2. ribose; 3. rhamnose; 4. glucuronic acid; 5. galacturonic acid; 6. glucose; 7. xylose; 8. galactose; 9. arabinose; 10. Fucose (internal standard).

Table 1
Retention time, limit of detection (LOD) and regression analysis of the proposed HPLC method

Carbohydrates	t_m (min)[a]	Regression equation, $Y = a + bX$[b]		Correlation coefficient	Detection limit (μM)[c]
		a	*b*		
Mannose	7.762	+0.0307	0.1048	0.9999	0.9
Ribose	8.426	+0.0084	0.0644	0.9999	1.5
Rhamnose	9.683	−0.0116	0.0526	0.9998	1.6
Glucuronic acid	11.734	−0.0412	0.1144	0.9996	0.8
Galacturonic acid	14.06	+0.0074	0.0903	0.9994	1.0
Glucose	25.658	−0.0318	0.0606	0.9998	1.5
Xylose	29.181	−0.0123	0.0534	0.9999	1.4
Galactose	32.302	−0.0069	0.0858	0.9998	1.1
Arabinose	33.646	+0.0078	0.0771	0.9996	1.3

[a] t_m: Retention time; the mobile phase A consisted of acetonitrile and the mobile phase B was 0.045% KH_2PO_4–0.05% triethylamine buffer (pH 7.0) using a gradient elution of 90–89–86% B by a linear increase from 0–15–40 min. The injection volume was 20 μl.
[b] The *Y* and *X* are peak area ratio of the analytes to internal standard (fucose) and concentration of the analytes (5–150 μM), respectively.
[c] 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 analytes in the present method

Carbohydrates	Intra-day precision (CV%, $n = 5$)		Inter-day precision (CV%, $n = 5$)	
	Retention time	Peak area	Retention time	Peak area
Mannose	0.35	0.61	0.82	1.55
Ribose	0.44	0.98	0.97	1.23
Rhamnose	0.84	1.16	0.77	1.81
Glucuronic acid	0.51	0.88	0.71	1.95
Galacturonic acid	0.89	1.61	1.21	2.01
Glucose	0.67	1.98	1.47	2.44
Xylose	0.97	1.76	2.03	3.17
Galactose	1.03	2.75	2.11	3.04
Arabinose	1.02	2.64	2.35	4.48
Fucose	0.91	1.99	2.29	3.11

3.3. Analysis of the tea polysaccharide from G. pentaphyllum

This experiment was designed to develop a rapid, repeatable and accurate analysis method for the quantification of the component carbohydrates in the tea polysaccharide from *G. pentaphyllum*. In order to evaluate the applicability of the proposed method, the isolated tea polysaccharide was hydrolyzed with TFA, dried and PMP-labeled as described in the experimental section and finally, the released monosaccharide derivatives were analyzed by the described HPLC method under the optimized conditions using fucose as internal standard. Fig. 1B shows a typical chromatogram of the tea polysaccharide sample and the detected contents were listed in Table 3. As can be seen, the PMP derivatives of the component monosaccharides released from the tea polysaccharide sample could be still baseline separated and the component monosaccharides could be identified by comparing with the chromatogram of the mixture of standard monosaccharides (Fig. 1A). The results showed that the tea polysaccharide was a typical heteropolysaccharide and was composed of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose and arabinose in the molar contents of 16.3, 10.3, 47.1, 5.6, 24.0, 128.4, 25.0, 101.4 and 71.1 μM, respectively (Table 3), and their corresponding mole percentages were 3.8%, 2.4%, 11.0%, 1.3%, 5.6%, 29.9%, 5.8%, 23.6% and 16.6% (mol%), respectively. It was clear that the predominantly composition monosaccharides in the tea polysaccharide were neutral glucose and galactose up to 53.5% (mol%) of total carbohydrates, and 6.9% of total carbohydrates were uronic acids.

Furthermore, recovery experiments were performed in order to investigate the accuracy of the method. Known amounts of each monosaccharide solute were added to the sample detected, and the resulting spiked sample was subjected to the entire analytical sequence. Each solute was spiked at a close concentration with the sample and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate. The results show that the recoveries of all the nine monosaccharides ranged between 94.6% and 108.0% and the RSD values fell within 1.8–4.9%. Such results further demonstrated that this method is precise and

Table 3
Determination of the component monosaccharides in the sample of *G. pentaphyllum* tea polysaccharide and their recovery analysis ($n = 3$)

Component	Content in sample (μM)	Spiked amount (μM)	Found amount (μM)	Recovery (%)	RSD (%)
Mannose	16.3	25.0	41.2	99.6	2.4
Ribose	10.3	25.0	35.9	102.2	3.4
Rhamnose	47.1	50.0	97.4	100.6	1.8
Glucuronic acid	5.6	5.0	11.0	108.0	4.9
Galacturonic acid	24.0	25.0	48.5	98.1	3.0
Glucose	128.4	50.0	175.7	94.6	4.7
Xylose	25.0	25.0	51.1	104.4	3.8
Galactose	101.4	50.0	154.1	105.4	3.6
Arabinose	71.1	50.0	120.7	99.2	2.9

practical for the analysis of the tea polysaccharide from *G. pentaphyllum*.

4. Discussion

HPLC is an important technique for the qualitative and quantitative analysis of carbohydrates. Although methods for the separation of the neutral sugars and uronic acids in samples have been examined by a number of researchers, only 7–8 monosaccharides were usually baseline separated, which significantly affected the analytical accuracy (Cataldi et al., 2000; Wang & Fang, 2004; Yang, Zhao, Wang, Wang, & Mei, 2005). In recent years, the 10 monosaccharides mentioned above were successfully separated by high pH anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) although the analysis was accomplished within 100 min (Currie & Perry, 2006). However, in this study the good baseline separation of the 10 monosaccharide derivatives was achieved within 40 min. As shown in Fig. 1A, the shape of the peaks was very sharp and it was clear that a substantial improvement in the separation of neutral sugar and uronic acid derivatives was obtained by the conventional HPLC method, and thus, the analytical cost is lower and the HPLC analysis can be carried out in common laboratory.

It is well-known that a series of dammarane-type saponins have for a long time been considered as the pharmacologically active ingredients of the *G. pentaphyllum* plant used as a tea for treatment of various diseases and symptoms, but they cannot account for all the effects mentioned above. In recent years, more and more polysaccharides isolated from various traditional medicinal plants have been regarded as an important class of biological response modifiers to exhibit a variety of biological activities, such as antitumour, immunostimulant, anticancer, anticomplementary, anti-inflammatory, anticoagulant hypoglycaemic and antiviral activities (Jeon, Han, Ahn, & Kim, 1999; Lee & Jeon, 2003; Tzianabos, 2000). Accordingly, an understanding of the basic composition properties of polysaccharides could contribute to our understanding of their practical applications. Therefore, the investigation on basic sugar composition of the tea polysaccharide was particularly necessary to better find their functional properties for the wide application in food and pharmaceutical industries.

In summary, a HPLC method with indirect UV detection was optimized and validated for quantification of carbohydrates in the tea polysaccharide from *G. pentaphyllum*. The proposed HPLC method provides a rapid, repeatable, accurate and economic alternative for the separation of the natural monosaccharides. The proposed method is particularly suitable for determining the component monosaccharides in the tea polysaccharide and can also be applied to routine analysis of monosaccharides in real-life samples, such as other plant polysaccharides, fruit juices, wines, brandies, etc. Such information would facilitate the use of the *G. pentaphyllum* in food, pharmaceutical and other technical applications, which would contribute to the sustainable use of *G. pentaphyllum* agricultural resource.

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