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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