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Research Article

Rapid Determination of the Monosaccharide Composition and Contents in Tea Polysaccharides from Yingshuang Green Tea by Pre-Column Derivatization HPLC

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A pre-column derivatization high-performance liquid chromatography (HPLC) method was developed and optimized to characterize and quantify the monosaccharides present in tea polysaccharides (TPS) isolated from Yingshuang green tea. TPS sample was hydrolyzed with trifluoroacetic acid, subjected to pre-column derivatization using 1-phenyl-3-methyl-5-pyrazolone (PMP), and separated on an Agilent TC-C_{18} column (4.6 mm × 250 mm, 5 μm) with UV detection at 250 nm. A mixture of ten PMP derivatives of standard monosaccharides (mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, arabinose, and fucose) could be baseline separated within 20 min. Moreover, quantitative analysis of the component monosaccharides in Yingshuang green tea TPS was achieved, indicating the TPS consisted of mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, and arabinose in the molar contents of 0.72, 0.78, 0.89, 0.13, 0.15, 0.36, 0.39, 0.36, 0.36, and 0.38 μM, respectively. Recovery efficiency for component monosaccharides from TPS ranged from 93.6 to 102.4% with RSD values lower than 2.5%. In conclusion, pre-column derivatization HPLC provides a rapid, reproducible, accurate, and quantitative method for analysis of the monosaccharide composition and contents in TPS, which may help to further explore the relationship between TPS monosaccharides isolated from different tea varieties and their biological activity.

1. Introduction

Tea, coffee, and cocoa are the three major nonalcoholic beverages consumed throughout the world. Crude tea is commonly used to treat diabetes mellitus in Chinese and Japanese traditional medicine, and TPS have been identified as the pharmacologically active ingredients in crude tea. Recent pharmacological studies indicated TPS may help to prevent and treat cardiovascular disease [1], possess antioxidative [2], anticancer [3], anticoagulative, and antiatherosclerotic activities [4, 5], exert hypoglycemic and bradycardic effects [6, 7], and enhance nonspecific immunological function [8]. Therefore, the components of TPS may have the potential to treat a variety of conditions.

TPS are a range of complex glycoproteins, and their biological activity is related to their primary structure. TPS composed of arabinose, ribose, and galactose demonstrated the highest ability to reduce bloody fat [9], whereas TPS are composed primarily of galactose and glucose demonstrated hypoglycemic activity [10]. In addition, our laboratory reported that TPS isolated from different tea varieties had varied antioxidant capacities, with TPS from Yingshuang green tea showing the highest antioxidant activity [11, 12]. Therefore, it is vitally important to characterize the monosaccharide and polysaccharide compositions of TPS isolated from different tea varieties in order to investigate the relationship between the structure and composition of TPS and their antioxidant capacity and biological activities in more detail.

As analytical methods for sugars have developed, the main chromatographic methods currently used to analyze monosaccharides are gas chromatography (GC) [13], thin-layer chromatography (TLC) [14], and HPLC [15]. However, each of these techniques possesses a number of disadvantages. As monosaccharides are nonvolatile, monosaccharide molecules need to be converted into volatile derivatives with high thermal stability via a complicated process in order

to be analyzed by GC. TLC is subject to the unresolved issues of poor reproducibility and poor color rendering for microconstituents. HPLC with a carbohydrate analysis column or NH2 column are commonly used for monosaccharide analysis; however, both techniques are expensive. Additionally, derivative isomers of monosaccharides can be generated during HPLC, which produces other peaks and affects the separation and quantitative analysis of sugars. Moreover, as monosaccharides do not absorb ultraviolet light, pre- or post-column derivatization is required in order to improve the separation selectivity and detection sensitivity of HPLC. The PMP pre-column derivation HPLC method uses mild reaction conditions, does not result in stereoisomerism products and has a high detection sensitivity, and is used more widely than other derivation methods [16, 17].

In recent years, pre-column derivatization HPLC has been used to successfully separate mixtures of ten monosaccharides (Rha, Ara, Xyl, Man, Glc, Gal, Fuc, Rib, GlcA, and GalA); this analysis can be accomplished within 40 to 30 min [18, 19], though all of reported methods are time consuming and inaccurate. In order to develop a more rapid and accurate method for analysis of the monosaccharides present in TPS, in this study we developed and optimized a pre-column PMP derivatization HPLC method using a Diode Array Detector (DAD) at 250 nm to simultaneously separate ten monosaccharides (aldoses and uronic acids). Moreover, the pre-column PMP derivatization HPLC method was validated by performing quantitative analysis of the composition and concentrations of monosaccharides in TPS isolated from Yingshuang green tea.

2. Materials and Methods

2.1. Materials and Reagents. Yingshuang green tea was purchased from the Tea Research Institute, Chinese Academy of Agricultural Sciences, Zhejiang province, China; *D*-mannose, *D*-ribose, *L*-rhamnose, *D*-glucose, *D*-xylose, *D*-galactose, *L*-arabinose, *D*-glucuronic acid, *D*-galacturonic acid, and *D*-fucose were obtained from Sigma (St. Louis, MO, USA); trifluoroacetic acid (TFA) was purchased from Sinopharm (Shanghai, China); PMP was obtained from Merck (Darmstadt, Germany) and recrystallized *three times* from chromatographic-grade methanol before use. HPLC-grade methanol and acetonitrile were purchased from TEDIA (Fair Lawn, NJ, USA); triethylamine was purchased from Sinopharm. All other chemicals were of analytical purity. All aqueous solutions were prepared using freshly prepared double-distilled water.

2.2. Extraction of TPS from Yingshuang Green Tea. Powdered Yingshuang green tea (100 g) was extracted with distilled water (1 : 20, w/v) at 55°C for 2 h. The extract was centrifuged at 4000 rpm for 15 min to remove contaminants, and the supernatant was precipitated with three volumes of 95% ethanol, centrifuged at 4000 rpm for 15 min, and washed thrice with petroleum ether, acetone, and pure ethanol. The polysaccharide pellets were dissolved in an appropriate volume of distilled water and intensively dialyzed for three days against distilled water (Mw cut-off 8000 Da). Proteins were removed by repeating the Sevag method six times [20, 21] and lyophilized as TP (1.6 g) using a freeze-dry apparatus (HO10-0035, Labogene Co., Denmark).

2.3. Hydrolysis of TPS. The TPS sample (10 mg) was dissolved in 1 mL of 3 M trifluoroacetic acid in a 5 mL ampoule, incubated at 130°C for 2 h, the cooled reaction mixture was centrifuged at 2000 rpm for 5 min and evaporated to dryness under reduced pressure to remove TFA, and the hydrolyzed and dried samples were redissolved in 1 mL of distilled water for the following experiments.

2.4. Derivatization of Hydrolyzed TPS with PMP. The hydrolyzed TPS sample was labeled by adding 30 μL of NaOH (0.3 M) and 20 μL of PMP solution (0.5 M in methanol). Fucose was added as an internal standard to each sample before derivatization. The mixtures were incubated at 70°C for 60 min, cooled to room temperature, and neutralized with 30 μL of HCl (0.3 M), 1 mL of trichloromethane was added, and, after vigorous shaking and layering, the organic phase was carefully removed and discarded. The aqueous layer was passed through a 0.45 μm syringe filter before HPLC analysis. Standard solutions of the ten monosaccharides (Rha, Ara, Xyl, Man, Glc, Gal, Fuc, Rib, GlcA, and GalA; 0.1 μM) were also treated as described above.

2.5. PMP-HPLC-DAD Analysis. The PMP-labeled monosaccharides were analyzed using an Agilent 1260 HPLC system (Waldbronn, Germany) consisting of a G1311C Quaternary pump, G1329B autosampler (0.1–100 μL), G1316A column oven (273–333 K), and G1315D-DAD detector (190–950 nm). The analytical column was a TC-C_{18} column (4.6 mm × 250 mm, 5 μm; Agilent). The injection volume was 20 μL with an eluant flow rate of 1.0 mL/min at 35°C. Mobile phase A was 100% acetonitrile and mobile phase B was a mixture of distilled water and acetonitrile (90 : 10, v/v) with 0.045% KH_2PO_4-0.05% triethylamine buffer (pH 7.5); gradient elution was performed at 94-94-88-88% B with linear decreases at 0-4-5-20 min. The UV detection wavelength was 245 nm.

3. Results

3.1. HPLC Separation of Monosaccharide PMP Derivatives. There have been numerous reports on the separation behavior of PMP-labeled monosaccharides derivatives; however, many of these reports [18, 19] were subsequently shown to be unsatisfactory. For example, intermethod and interexperimental variation occur due to the use of different solvents for different solvents and chemicals; the baseline fluctuates widely due to the presence of impurities; rapid and accurate determination cannot be achieved because of the long testing time; the peaks are not sharp enough to enable precise quantitative analysis; and the complexity of the preparation for both eluents can result in poor reproducibility. In this study, distilled water was the only solvent used in addition to dissolved PMP; before derivatization, three methanol-ether-acetone extraction and centrifugation steps were performed to remove fat-soluble impurities and pigments from the

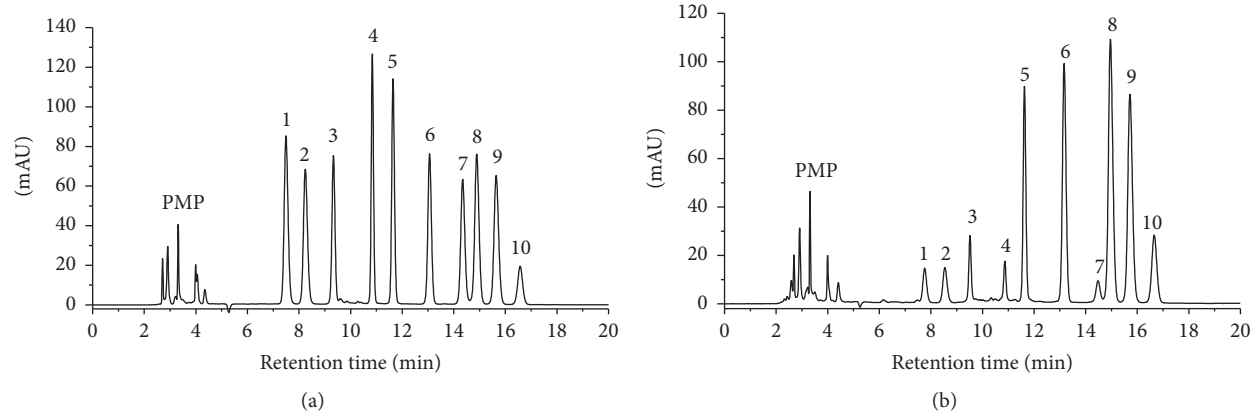

Figure 1: Chromatograms for analysis of (a) the synthetic mixture of ten standard monosaccharides and (b) the component monosaccharides present in Yingshuang green tea TPS using the pre-column PMP derivatization HPLC method. Peaks: 1: mannose; 2: ribose; 3: rhamnose; 4: glucuronic acid; 5: galacturonic acid; 6: glucose; 7: xylose; 8: galactose; 9: arabinose; 10: fucose.

Table 1: Retention time, regression analysis, and LOD for the pre-column PMP derivatization HPLC method.

Carbohydrates	Time (min)	Regression equation, $Y = a + bX$[a]		Correlation coefficient	Detection limit (μM)[b]
		a	b		
Mannose	6.673	−0.0496	2593.1	0.9994	0.09
Ribose	7.292	−0.0455	2268.8	0.9994	0.10
Rhamnose	8.244	−0.0287	1875.1	0.9993	0.19
Glucuronic acid	10.771	−0.0957	1997.6	0.9991	0.30
Galacturonic acid	11.672	−0.1579	2174.9	0.9983	0.20
Glucose	12.478	+0.0139	2097.8	0.9991	0.10
Xylose	13.678	−0.0259	2577.2	0.9980	0.07
Galactose	14.386	+0.0153	2480.6	0.9994	0.09
Arabinose	15.12	+0.0211	2436.6	0.9994	0.10

a: Y and X are the peak area ratio of the analytes to the internal standard (fucose) and concentration of the analytes (0.2–2 μM), respectively.
b: LOD was defined as the value equal to a signal-to-noise ratio of three.

TPS, thus reducing the baseline fluctuations and protecting the columns; and only eluant B needs to be prepared, thus reducing interexperimental errors. As shown in Figure 1(a), a clear HPLC chromatogram with sharp peaks for each of the ten monosaccharides present in the synthetic mixture was obtained as a result of optimizing the experimental process. Though continually improving the protocol, good baseline separation of the ten monosaccharide derivatives could be achieved within 20 min.

3.2. Validation. Linearity, limit of detection (LOD), reproducibility, and precision were assessed to validate the pre-column PMP derivatization HPLC method. Linearity was assessed by analysis of eight standard sugars (mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, and arabinose; $n = 5$). The linear regression parameters of the resulting calibration curves are shown in Table 1.

Good linearity (correlation coefficient, $r > 0.998$) between the peak area ratio of the analytes relative to the internal standard (Y) and the concentration of the standards (X) was achieved over the range of 0.2~2 μmol/L. The LOD for each monosaccharide was determined by analyzing 10 μL aliquots of serial dilutions of PMP-derivatized monosaccharide standards prepared using the modified derivatization method. The LOD was defined as the concentration equal to the peak height of a signal-to-noise ratio (S/N) of three (baseline noise level). The LOD of the individual monosaccharides ranged from 0.09 to 0.2 μM (Table 1), indicating that the optimized method is more accurate than previously reported methods.

Furthermore, the precision of the method was assessed by measuring repeatability (intraday variability) and intermediate precision (interday variability) in terms of the retention time and peak area for each monosaccharide standard. The coefficient of variation (CV) for five successive injections of each monosaccharide standard solution (1 μM) is presented in Table 2.

The intraday CV values were lower than 0.89% for the migration time and 3.75% for the peak areas (A), and the interday CV values were lower than 1.52% for the migration time and 4.48% for the peak areas (A), demonstrating that the precision of the method was similar to previous methods.

3.3. Analysis of TPS from Yingshuang Green Tea. This study was designed to develop a rapid, reproducible, and accurate

Table 2: Intraday and interday precision of the pre-column PMP derivatization HPLC method in terms of the retention time and peak areas of the monosaccharide standards.

Carbohydrates	Intraday precision (CV%, $n = 5$)		Interday precision (CV%, $n = 5$)	
	Retention time	Peak area	Retention time	Peak area
Mannose	0.72	0.08	1.52	0.46
Ribose	0.78	0.09	1.47	0.37
Rhamnose	0.89	0.08	0.93	0.43
Glucuronic acid	0.13	1.93	0.46	2.59
Galacturonic acid	0.15	2.75	0.51	4.48
Glucose	0.36	0.08	0.97	0.48
Xylose	0.39	0.68	0.84	1.53
Galactose	0.36	0.66	0.88	1.62
Arabinose	0.36	0.44	0.98	1.07
Fucose	0.38	0.11	1.12	0.39

Table 3: Determination of the component monosaccharides in Yingshuang green tea TPS and recovery analysis ($n = 3$).

Carbohydrates	Content in sample (μM)	Spiked amount (μM)	Found amount (μM)	Recovery (%)	RSD (%)
Mannose	0.28	0.50	0.76	95.2	0.7
Ribose	0.31	0.50	0.78	93.6	0.4
Rhamnose	0.44	0.50	0.93	97.7	0.5
Glucuronic acid	0.28	0.50	0.75	94.7	0.8
Galacturonic acid	0.78	0.50	1.24	91.9	1.0
Glucose	2.00	0.50	2.51	102.4	1.2
Xylose	0.20	0.50	0.70	99.3	2.5
Galactose	1.93	0.50	2.43	98.7	0.2
Arabinose	1.82	0.50	2.30	95.9	0.4

analysis method for the quantification of the composition and contents of monosaccharide in TPS isolated from Yingshuang green tea.

The chromatogram for the crude TPS isolated from Yingshuang green tea is shown in Figure 1(b). Each monosaccharide peak was distinct from the baseline and separated from the other peaks, and by comparison with chromatogram of the synthetic mixture of the ten monosaccharide standards, the component monosaccharides present in Yingshuang green tea TPS could be identified. The monosaccharide composition of Yingshuang green tea TPS is summarized in Table 3.

The main monosaccharides present in the crude TPS isolated from Yingshuang green tea were mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, glucose, xylose, galactose, and arabinose, with contents of 0.72, 0.78, 0.89, 0.13, 0.15, 0.36, 0.39, 0.36, 0.36, and 0.38 μM, respectively. Neutral glucose, galactose, and arabinose were the predominant monosaccharides in Yingshuang green tea TPS, representing up to 72.1% (mol%) of total monosaccharides; uronic acids represented 13.0% of the total carbohydrates in Yingshuang green tea TPS.

Moreover, recovery experiments were performed to assess the accuracy of the method. Known amounts of each monosaccharide standard were individually added to Yingshaung green tea TPS and the samples were measured in triplicate. The recoveries of each of the nine monosaccharides ranged from 93.6% and 102.4%, and the RSD values were between 0.2% and 2.5%; these results indicated that the pre-column PMP derivatization HPLC method had a satisfactory sensitivity for the analysis of the monosaccharides present in TPS isolated from Yingshuang green tea.

4. Discussion

HPLC represents an effective, quantitative, and comprehensive analysis technique for analysis of carbohydrates. In recent years, methods for the separation of the neutral sugars and uronic acids in polysaccharides have been developed by a number of researchers, but only seven to eight monosaccharides can be successfully separated using these methods, which significantly limits their analytical accuracy [22, 23]. Pre-column derivatization HPLC has been used to successfully separate mixtures of ten monosaccharides mentioned above, but the analysis was carried out for more than 30 min [18, 19]. However in this study, by optimizing previously developed methods to devise a pre-column PMP derivatization HPLC method, good baseline separation of ten monosaccharide derivatives could be obtained within as little as 20 min. Separation of neutral sugars and uronic acids could be achieved with very sharp peaks obtained for each monosaccharide. Furthermore, the recoveries of the component monosaccharides and repeatability of the method described in this study are superior to the method

devised by Lv's et al. [18]; therefore, our method has a higher accuracy. We propose that method described in this study will be applicable and feasible for monosaccharide composition analysis in food and medicinal plants too as well as general laboratories.

In conclusion, the pre-column PMP derivatization HPLC method described in this paper represents a rapid, accurate, reproducible, cost-effective, and quantitative alternative for the separation of the naturally occurring monosaccharides in TPS isolated from Yingshuang green tea. This method could be applied to analyze the monosaccharide composition and contents in TPS isolated from other tea varieties and may help to explore the relationship between the structure of TPS and their biological activities.

Competing Interests

The authors declare that they have no competing interests.

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