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DETERMINATION OF TEA POLYSACCHARIDES IN *CAMELLIA SINENSIS* BY A MODIFIED PHENOL-SULFURIC ACID METHOD

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Abstract - A direct procedure for the determination of total polysaccharides (TPS) in *Camellia sinensis* was set up based on the modified phenol-sulfuric acid method. The monosaccharide composition of TPS was analyzed by GC. Based on the results of GC, model monosaccharide mixtures were made which provided an adequate standard for this procedure. Through single-factor and orthogonal ($L_9 3^4$) experiments, the experimental conditions such as the volume of phenol, the volume of concentrated sulfuric acid, the reaction time, and the incubation temperature, were optimized. The highest sensitivity of absorbance was obtained when the volume of concentrated sulfuric acid, the volume of phenol (6%), and the incubation temperature were 2.5 ml, 0.2 ml, and 50°C, respectively. Under optimum conditions, the prepared samples were determined satisfactorily, with the recovery from 100.2% to 103.7%, and a relative standard deviation (RSD) of 2.1%. Overall, the modified method is easily operated, rapid, sensitive and accurate. A similar procedure can be applied to the determination of other plant polysaccharides as well.

Keywords: Tea polysaccharides, phenol-sulfuric, determination

UDC 663.95:582.688

INTRODUCTION

Green tea (*Camellia sinensis*) is the second most consumed beverage in the world and has caused great interest among researchers (Ghosh et al., 2009; Lee et al., 2009; Stalmach et al., 2009; Wang et al., 2009). The polysaccharides from tea have been shown to have a variety of bioactivities, such as immunostimulation (Monobe et al., 2008), hypoglycemic (Chen et al., 2003; Zhou et al., 2007), and anti-bacterial activities (Lee et al., 2006). The polysaccharides from tea are a type of heteropolysaccharide (Haixia Chen et al., 2005; Haixia Chen et al., 2008; Yuanfeng Wang et al., 2009), containing neutral sugars and uronic acid. The quantitative determination of tea polysaccharides has important effects on the evaluation of its quality monitoring, extraction, preparation, purification and so on. However, there are few reports about the quantitative determination of tea polysaccharides. Several methods, including the colorimetric method, the pre-labeling method (J. Dawes et al., 1989; A.N. De Belder et al., 1973), and high-performance liquid chromatography (Hirotaka Kakita et al., 2002; Lina Liang et al., 2006), have been used to determine the polysaccharide. The pre-labeling method and HPLC are often not suitable to measure the total carbohydrates because they are time-consuming, procedurally complex, it is hard to get a labeling substance, there are serious interferences and so on. Obviously, compared with these methods, the colorimetric method is rapid, simple, and economic. The phenol-sulfuric method (Dubois et al., 1956) is a classical colorimetric method for determining total polysaccharides. Recently a micro-plate format version of this method (M. Monsigny et al., 1988; Tatsuy Masuko et al., 2005) has been reported. More recently still, the use of a model mixture of the component monosaccharides as standard to establish the calibration curves has been proposed by Saha et al.., (1994), and Gabriela Cuesta et al. (2003). In order to improve the accuracy, multivariate calibration was used to replace the conventional univariate calibration for modification of the phenol-sulfuric acid method (Mauro Mecozzi, 2005).

The tea polysaccharide is a heteropolysaccharide with molecular weights from several thousand to several hundred thousand. Given the lack of simple and more specific colorimetric reactions for this complex carbohydrate, the phenol-sulfuric acid method remains a useful approach. When we planned to use this method for our approach, we found that direct measuring of the polysaccharides with tea leaves as sample was significantly affected by other components, and the results had poor reproducibility. Herein, we have improved the method's accuracy and reproducibility through a modified strategy and optimization of the experimental conditions, and have thereby allowed its application for the determination of the polysaccharides present in tea from *Camellia sinensis*.

MATERIALS AND METHODS

Materials and reagents

Tea leaves were purchased from the Hebei province of China. D-ribose (Rib), L-rhamnose (Rha), D-arabinose (Ara), L-fucose (Fuc), D-xylose (Xyl), D-mannose (Man), D-glucose (Glc), D-galactose (Gal), D-galacturonic acid (GalA), polyphenol, L-Glu, glycin, vitamin C, caffeine, theanine, EGCG and bovine serum albumin (BSA) were purchased from Sigma (MO, USA). m-Hydroxyl biphenyl was purchased from Fluka Co. (MO, USA). All other reagents and solvents were of analytical grade and used without further purification unless otherwise noted. All aqueous solutions were prepared using newly double-distilled water.

Analytical methods

Preparation of polysaccharides from tea leaves

The dry ground tea leaves (250 g) were extracted with 2 L distilled water at 90°C in a water bath for 2 h. After filtering, the residue was extracted again with 2.5 L distilled water for another 2 h. Then the extracts were centrifuged to remove contaminants. The supernatant was concentrated via rotary evaporation and precipitated with 95% ethanol. The precipitate was dissolved in water and dialyzed to remove small molecules. The dialyzed solution was freeze-dried to yield the sample, named tea polysaccharide powder (TPP).

Analysis of monosaccharide composition

TPP was hydrolyzed in 4 mol/L trifluoroacetic acid (TFA) for 6 h at 120°C in a sealed glass tube. The residual acid was removed under vacuum and then the hydrolyzates and monosaccharides were converted to acetylated aldononitrile derivatives according to conventional protocols (Macías-Rodríguez et al., 2002) and analyzed by an Agilent GC 900A incubation with a 123-0732 DB1701 column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector. The elution was realized by a temperature gradient: the oven conditions included an initial temperature of 180°C and an initial time of 5 min that was raised to 250°C with a slope of 3°C/min. The vaporizer temperature was 260°C and the detector temperature was 260°C. A 1 μL aliquot was injected for each run.

Phenol-sulfuric acid method

The 40 μg/ml monosaccharide mixtures solution was made according to the mass percent ratios of several monosaccharide, obtained from the results of GC analysis. The monosaccharide mixture solution (MMS) was used as a standard solution for the calibration curves. To a 20 ml test tube, solutions were added in the following order: 1.2 ml MMS, an appropriate amount of phenol (6%), and an appropriate amount of concentrated sulfuric acid.

Volume of 6% phenol. To 1.2 ml of MMS in a test tube (20 ml) was added 0-1.5 ml of 6% phenol, and immediately vortexed after mixing with 2.5ml of concentrated sulfuric acid. After standing for 20 min at room temperature, the absorbance of the sample solution was measured at 480 nm against the blank (prepared by double-distilled water for the sample solution).

Volume of concentrated sulfuric acid. To 1.2 ml of MMS in a test tube (20 ml) 0.2 ml of 6% phenol was added rapidly. Immediately thereafter, 0.5-4ml of concentrated sulfuric acid was added and vortexed for at least 30 s. After standing for 20 min at room temperature, it was measured at 480 nm.

Reaction time. In brief, 1.2 ml of MMS was rapidly vortexed, mixed with 2.5 ml of concentrated sulfuric acid before adding 0.2ml of 6% phenol from a glass dispenser. After standing for 10-50 min at room temperature, the absorbance of the sample solution was measured at 480 nm.

Incubation temperature. All the analysis procedures followed the same steps, but the reaction time was set at 20 min and incubation temperatures ranged from 10 – 80°C. After recovere to room temperature, $A_{480\ nm}$ was measured.

$L_9 3^4$ orthogonal. In order to obtain the best sensitivity of absorbance, the orthogonal was designed in this protocol. The colorimetry was conducted by adding 0.1, 0.2 and 0.3 ml of 6% phenol and 2.0, 2.5 and 3.0 ml of concentrated sulfuric acid, and the incubation temperatures for reaction were 50, 60 and 70 °C. The best analysis conditions were found through the orthogonal experiment, and the samples (TPP) were determined at the optimized condition.

RESULTS AND DISCUSSION

Composition of tea leaves

The primary ingredients in TPP are shown in Table 1, and each of them was used in an appropriate method for quantifying their content in tea leaves. These primary ingredients would probably interfere with the phenol-sulfuric acid method for determination of carbohydrates in TPP.

Table 1. The contents of primary ingredients in tea leave

Ingredients	Methods	Content/%
Soluble proteins	Coomassie Brilliant Blue G-250	3.00
caffeine	HPLC	2.08
crude fat	Soxlhet extraction	2.37
tea polyphenol	ferrous tartrate method	20.91
water-soluble ash	SN/T0925-2000 (CHINA)	3.56
water-insoluble ash	SN/T0925-2000 (CHINA)	1.98
acid-insoluble ash	SN/T 0921-2000 (CHINA)	0.24
crude fiber	GB/T8310-2002(CHINA)	10.46

Monosaccharide composition of TPS

The GC profiles of monosaccharide composition in TPS are shown in Fig. 1. The monosaccharide compositions of the polysaccharides were determined by GC using following the monosaccharides as standard: Rhamnose, D-ribose, L-fucose, L-arabinose, D-xylose, D-mannose, D-glucose, D-galactose, inositol. Gas chromatogram of TPS showed that monosaccharides of TPS consisted of Rhamnose, D-ribose, L-fucose, L-arabinose, D-xylose, D-mannose, D-glucose, and D-galactose with a molar ratio of 1.8:3.8:8.7:1.0:1.8:1.8:5.2:7.6, and the contents of L-arabinose and D-galactose were very higher.

Optimization of reaction conditions

To survey the optimal reaction conditions, we first prepared the standard solution, which was based on the molar ratio of TPS. In the equivalent environment, MMS and D-glucose were significant different in absorbance (Fig. 2). The maximum absorption wavelengths of TPP and MMS were both at 480 nm, but the D-glucose was at 490 nm. From the experimental data of MMS and TPS, it can be seen that the difference in the slopes is about 45%. This is because the maximum absorption wavelength varies considerably with the monosaccharide (Dubois et al., 1956; Susumu Honda et al., 1981). The use of a model mixture of the component monosaccharides to construct the calibration curve, as proposed by Saha et al., (1994) and demonstrated by Gabriela Cuesta et al., (2003), represents a practical and economic alternative to the use of the natural polysaccharide. So we

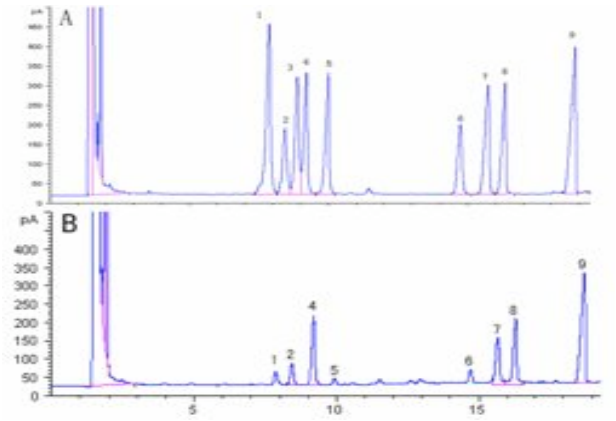

Fig. 1. GC profiles of monosaccharides of tea polysaccharides: (A) Gas chromatogram of standards: (1) Rhamnose, (2) D-ribose, (3) L-fucose, (4) L-arabinose, (5) D-xylose, (6) D-mannose, (7) D-glucose, (8) D-galactose, (9) inositol; (B) Gas chromatogram of TPS.

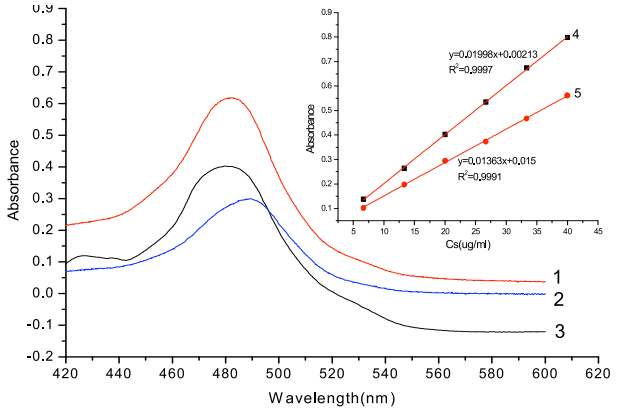

Fig. 2. Absorption spectra of systems and calibration curves: 1, TPS; 2, MMS; 3, D-glucose; 4, Calibration curve of MMS; 5, Calibration curve of D-glucose. Conditions: 1, TPS, 50 μg/ml; 2, MMS, 20 μg/ml; 3, D-glucose, 20 μg/ml; calibration curve of MMS and TPS were measured at 480 nm and 490 nm, respectively.

constructed a standard curve with the MMS, which could show the monosaccharide compositions of TPS, and this resulted in an improvement in the accuracy of the measurements (Gabriela Cuesta et al., 2003). On the basis of the MMS, we considered four factors, including the amount of 6% phenol, the amount of concentrated sulfuric acid, the reaction time and the incubation temperatures. After the optimal conditions were ascertained, we used the orthogonal experiment to further optimize the analysis conditions.

We selected the sample size of 1.2 ml to allow for the addition of sufficient amounts of phenol solution and sulfuric acid. It appeared that the maximal absorbance was obtained when 0.2 ml of 6% phenol and 2.5 ml of concentrated sulfuric acid were added in rapid succession to 1.2ml of MMS (Fig. 3.A, B). From Fig. 3 it can be seen that the time had hardly any influence on the absorption, but that the incubation temperature was an important factor which effected the reaction. From Fig. 4B, it can be seen that the maximal absorbance was obtained when the incubation temperature was set at 60°C. And the experimental data indicated that it also had a good reproducibility and linearity at this temperature.

Since various parameters potentially affect the determination process, the optimization of experimental conditions is a critical step in the development of a determination method. On the basis of the single-factor test, an orthogonal L_9 (3^4) test design in the measurement mode was used for further optimizing the determination conditions. The total evaluation index was used for analysis by statistical method. The results of the orthogonal test and the extreme difference analysis are presented in Table 2. The analysis of variance was performed by the statistical software SPSS 12.0 and the result is listed in Table 2. The results of experiments presented in Table 2 indicated that the maximal absorbance was 0.534. However, we cannot select the best determination conditions only based on the outcomes given in Table 2, and a further orthogonal analysis was warranted. Thus, the *K*, and *R* values were calculated and listed in Table 2. As seen from Table 2, we can find that the effect factor on the determination conditions decreases in the order: A > B > C, which is according to the *R* values. The volume of concentrated sulfuric acid was found to be the most important determinant of the measurement. In a word, maximum sensitivity was obtained when the volume of 6% phenol, volume of concentrated sulfuric acid, incubation temperature were 0.2 ml,

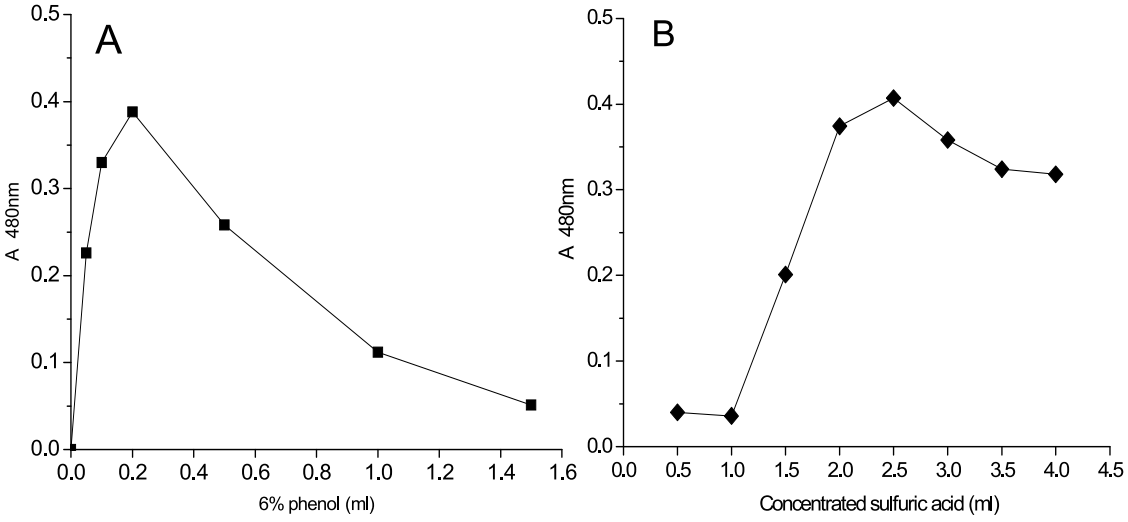

Fig. 3. (A) Influence of the volume of 6% phenol on the absorbance at 480 nm. (B) Influence of the volume of concentrated sulfuric acid on the absorbance at 480 nm.

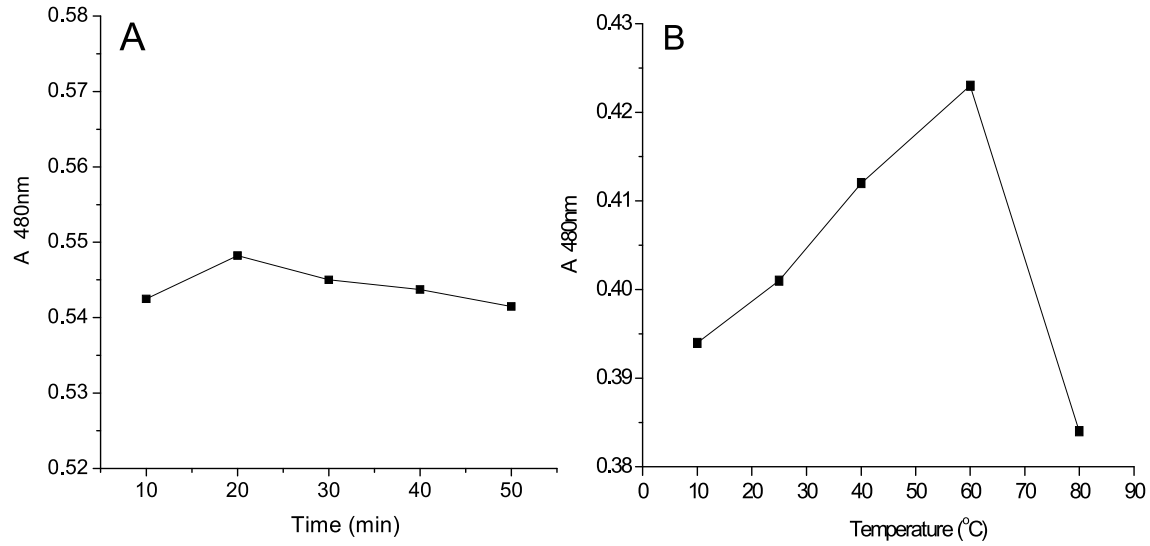

Fig. 4. Effect of time (A) and environment temperature (B) on the absorbance at 480 nm. Conditions: MMS, 20μg/ml; 6% phenol, 0.2ml; concentrated sulfuric acid, 2.5ml.

2.5 ml and 50°C, respectively, and the phenol was added before the concentrated sulfuric acid.

Interferences

Under optimal conditions the influence of coexisting substances, such as amino acids, proteins, polyphenol, vitamin and metal ions, was tested and the results are shown in Table 3. It can be seen that protein, amino acids, caffeine and most metal ions hardly interfered with the assay and that a very high concentrations (at a tolerance level of 10%) were allowed. Polyphenol was the main interfering material, which is due to the fact that its structure also contains polyhydroxyl, which is similar to polysaccharides. Therefore, some special treatment was required before the sample was analyzed. In order to decrease the interference, the sample was dialyzed se-

Table 2. Analysis of L_9 (3^4) test results

No.	sulfuric acid (ml)	6% phenol (ml)	temperature (°C)	$A_{480\ nm}$
1	2	0.1	50	0.488
2	2	0.2	60	0.423
3	2	0.3	70	0.334
4	2.5	0.1	60	0.449
5	2.5	0.2	70	0.534
6	2.5	0.3	50	0.479
7	3	0.1	70	0.435
8	3	0.2	50	0.513
9	3	0.3	60	0.481
K1	0.415	0.457	0.49	
K2	0.484	0.49	0.451	
K3	0.476	0.428	0.434	
R	0.069	0.062	0.056	

R refers to the result of extreme analysis

veral days before being analyzed. Through the dialysis, most of the salt, monosaccharide oligosaccharide, polyphenol and some other components, can be removed from the tea polysaccharides sample. This procedure can observably increase the accuracy of a sample measure.

Calibration curves and analysis of samples

Calibration graphs for the determination of TPS were constructed. Under the optimal conditions, the *A* value was proportional to the concentration of MMS with a good linear relationship (Figure 2.).

According to the general procedure described above, the polysaccharide (active components) contents in the polysaccharides sample (TPP) were determined using the modified method. MMS was used to construct a standard curve. The standard addition method was used to determine the recovery of MMS and the results are presented in Table 4. The relative standard deviation (RSD) was 4.23% and the recovery yields ranged from100.90 to 103.65%,

Table 3. Influence of coexisting substances

Coexisting substance	Coexisting concentration (μg ml^{-1})	Change of absorbance (%)	Coexisting substance	Coexisting concentration (μg ml^{-1})	Change of absorbance (%)
K^+, Cl^-	500	4.44	vitamin C	10	3.64
Ca^{2+}, Cl^-	850	5.02	Caffeine	3000	4.86
L-Glu	500	1.21	Theanine	5200	5.01
Gly	2000	3.08	EGC	30	4.95
BSA	20	0.39	EGCG	45	4.81

Table 4. Recovery results of proposed method.

Sample (μg)	TPS, found (μg)	Average (μg)	MMS, added (μg)	MMS, found(μg)	Recovery (S.D, %)	RSD%
20	8.87	8.75	20	21.94	103.65	1.29
20	8.83		20	21.53	101.90	
20	8.74		20	20.18	100.90	
20	8.66		20	20.03	100.15	
20	8.71		20	21.94	103.15	
20	8.68		20	20.43	102.15	

indicating that the modified method has good sensitivity and validity in the direct determination of polysaccharides from the TPP. The reproducibility was low when tea leaves were directly used. This is because the measured value was strongly affected by other components in the TPP.

CONCLUSION

The modified phenol-sulfuric acid method can be conveniently applied to the quantitative determination of polysaccharides in tea polysaccharides powder (TPP). This can be achieved by means of three procedures: (1) a series of purification procedures to prepare the tea polysaccharides powder, (2) using a model mixture as the standard, made up of the individual sugars of the polysaccharide in the same molar ratio as in the natural substance, and (3) optimum conditions, with a 0.2ml volume of 6% phenol and a 2.5ml volume of concentrated sulfuric acid added to a 1.2 ml system in sequence, with an incubation temperature of 50°C. This procedure achieves a good sensitivity, allows for rapid analysis, has good linearity over a wide range, and good reproducibility. With all these merits, a similar approach should be considered for wider application in the analysis of other complex polysaccharides from plant origin.

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