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Quantification of Uronic Acids in Tea Polysaccharide Conjugates and Their Antioxidant Properties

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A technique of high-performance liquid chromatography (HPLC) was described for the measurement of total uronic acids in tea polysaccharide conjugates. This method was applied to polysaccharide conjugate extracts obtained from green tea after most of the components that produce interference were removed. The preliminary extraction process was according to the procedure of isolation of polysaccharide conjugates. The uronic acid content of different polysaccharide conjugate fractions was quantified by HPLC on a Sugar-Pak I column with a 1.0×10^{-4} mol L^{-1} calcium disodium ethylenediaminetetraacetic acid solution as the mobile phase and refractive index detection. The validation study showed high recoveries (>97.0%) and low coefficients of variance (<3.0%). The minimum detectable limit concentration of uronic acid was 10 μg mL^{-1}. The analysis of a standard range of galacturonic acid concentrations (100−4000 μg mL^{-1}) yielded linear results. The use of the method on different polysaccharide conjugate fraction samples confirmed its effectiveness. With the high content of uronic acids in polysaccharide conjugates, the stronger reactive oxygen species scavenging activities were found.

KEYWORDS: HPLC; uronic acid; tea polysaccharide conjugates; antioxidant properties

INTRODUCTION

Polysaccharides and their conjugates from varieties of sources such as animals, plant cell walls, and fungal cells, possess marked immunological properties; antitumor, antiviral, and antiinfective effects; antioxidant, antimutagenic, and hematopoitic activities, etc. (*1*). Many of the polysaccharide conjugates are acid complex carbohydrates, which are composed of uronic acids. The uronic acid residues can alter the characteristics and modify the solubility of associated polysaccharide conjugates. Therefore, they are correlated to the activities of polysaccharide conjugates. So, it is important to get an accurate method to assay the content of uronic acids for the quantititative and structural analysis of complex carbohydrates.

For the quantitative determination of the uronic acid content, the first method was applied by using carbazole in concentrated sulfuric acid. Other spectrophotometric methods such as sulfamate/*m*-hydroxydiphenyl have been used in biochemistry research to quantify uronic acids in biological samples (*2*). The sensitivity of the reaction was approximately 1 μg for glucuronic acid and 2 μg for complex polysaccharides, according to the conventional assay with a linear function of glucuronic acid concentration between 1 and 100 μg (*3*). Because of the sensitivity, uronic acids in some samples need to be separated and preconcentrated. Some adaptation methods, such as the microtiter plate assay for the determination of uronic acid (*4*), were also developed. Fourier transform infrared (FT-IR) spectroscopy was also used to analyze uronic acid in pectic samples. The regression model obtained from olive data had a correlation of 0.989 and a root-mean-square error of prediction of 2.01% by the method of FT-IR spectroscopy (*5*). Additionally, gas chromatography (GC) and high-performance liquid chromatography (HPLC) have also been used to measure uronic acids in natural samples such as food, plankton, plant tissues, animal tissues, etc. HPLC with pulsed amperometric detection (PAD) is a common method for the quantification of saccharides, amino sugars, and uronic acids by applying HPAEC with gradient elution. Determination limits are in the range of 100−500 μg L^{-1} (*6*). By means of the ion pair reversed phase HPLC method, the concentration detection limits of *p*-aminobenzoic acid-derivatized uronic acids range between 20 and 30 μg L^{-1} [$(1-2) \times 10^{-7}$ M] for fluorescence detection and between 30 and 75 μg L^{-1} for UV detection. A good linearity was achieved in the concentration range from 50 μg L^{-1} to 100 mg L^{-1} (r^2 > 0.99) (*7*). By the method of GC and HPLC, the samples commonly require pretreatment such as reducing or derivatizing of the uronic acid.

Tea consumption benefits health in a wide rage of aspects. Lower grade green tea has traditionally been used to cure diabetics in East Asia, especially in China and Japan (*8*). It was also found that tea polysaccharide conjugates are one of the main components related to hypoglycemic activity (*9*, *10*). The polysaccharide conjugates from green tea have also been reported to possess immunological, antiradiation, antiblood coagulation, anticancer, anti-HIV, and hypoglycemic activities,

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etc. Great advances have been made in chemical and hypoglycemic studies of tea polysaccharides (*11*−*13*). Tea polysaccharide conjugates were found to be mainly composed of uronic acids, especially the galacturonic acid (*14*). The present paper reports an uronic acid assay method in polysaccharide conjugates by HPLC with galacturonic acid as the standard and evaluates the relationship between uronic acids content and antioxidant properties. The novel and essential feature of the method is that there is no pretreatment procedure of the samples. Hence, this assay is convenient to apply routinely if large numbers of samples must be analyzed.

MATERIALS AND METHODS

Chemicals and Reagents. Calcium disodium ethylenediaminetetraacetic acid (EDTA) was purchased from Fluka (Buchs, SG1, Switzerland). Galacturonic acid, nitro blue tetrazolium (NBT), and 2-deoxy-ribose were purchased from Sigma (St. Louis, MO). Diethylaminoethyl cellulose (DEAE cellulose) was purchased from Shanghai Chemical Co. (Shanghai, China). Fresh deionized and redistilled water prepared in our lab was used.

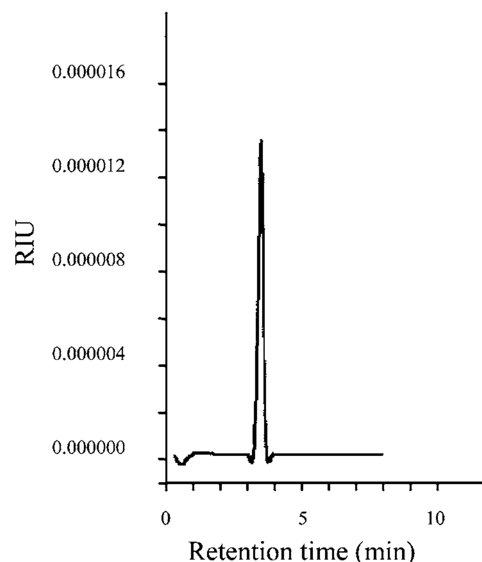
Apparatus. Chromatographic analysis was performed on a model SCL-6A liquid chromatograph (Shimadzu, Japan) equipped with a model RID-6A refrective index detector (RID) connected to a C-R6A data processor. Separations were obtained on a Sugar-Pak I analytical column (300 mm × 6.5 mm i.d., Waters).

Chromatographic Conditions. The mobile phase was a calcium disodium EDTA water solution (1.0×10^{-4} mol L^{-1}) at a flow rate of 0.6 mL min^{-1}. The column and cell temperatures were accurately set at 90 °C, and the measuring range of IR was set at 32 AUFS.

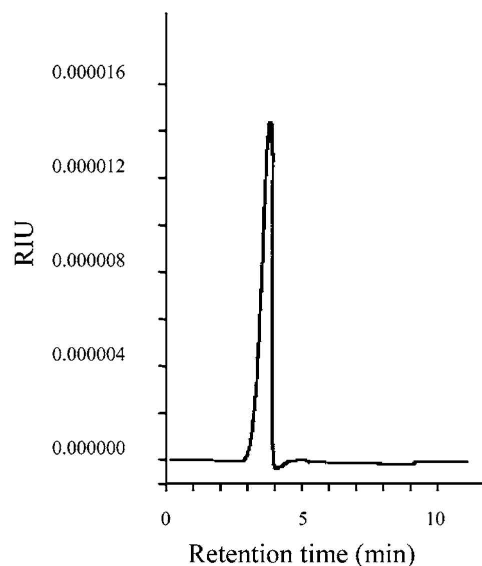
Standard Solution Preparation. *A* 4000 μg mL^{-1} amount of galacturonic acid in water was prepared as the basic standard solution. Different standard solutions were used at the concentrations of 100, 400, 800, 1600, and 4000 μg mL^{-1}, respectively.

Sample Preparation. Tea polysaccharide conjugates were extracted from the green tea, and impurities were removed by the decolor and deprotein procedures as follows. Low grade green tea powders (100 g) were mixed with 500 mL of 80% (v/v) ethanol and shaken at 30 °C for 24 h to remove most of the polyphynols and monosaccharide. After the mixture was filtered, the residues were dried in air and then extracted with hot water (70 °C) three times (1:20, w/v). The tea extract was concentrated in a rotary evaporator under reduced pressure, precipitated by 95% (v/v) ethanol at 4 °C for 24 h, and then centrifuged (10 min, 5000*g*). The precipitate was vacuum freeze-dried, and 2.9 g of crude tea polysaccharide conjugates was obtained. Crude tea polysaccharide conjugates were dissolved in water and separated through a polyamide adsorption resin column (60 cm × 3.0 cm, i.d.) with water at a flow rate of 0.4 mL min^{-1}. The amount of saccharide per tube was determined by using the sulfuric acid−phenol method (*15*), and the protein that eluted was determined automatically at 280 nm (*16*). The sugar part of the high molecular weight was gathered and precipitated with 95% (v/v) ethanol and then lyophilized. A DEAE cellulose column (50 cm × 2.5 cm, i.d.) was used to give three acid polysaccharide conjugate fractions, TPC-1, TPC-2, and TPC-3, with the gradient NaCl (from 0.1 to 1.0 mol L^{-1}) solution as elution. The tea polysaccharide conjugate fractions were dissolved in redistilled water and filtered through a 0.45 μm polycarbonate filter. Then, a 20 μL volume of the sample filtrate was introduced into the HPLC system.

Assay for the Scavenging Effects on Hydroxyl Radicals. The deoxyribose method for determining the rate of reaction of hydroxyl radical with antioxidant was performed as described by Halliwell et al. (*17*). Reaction mixtures in a final volume of 1.0 mL contained 167 μg mL^{-1} of the tea polysaccharide conjugates, 60 mM deoxyribose, KH_2PO_4−KOH 20 mM buffer (pH 7.4), 100 μM $FeCl_3$, 100 μM EDTA, 1 mM H_2O_2, and 100 μM ascorbate acid. Solutions of $FeCl_3$ and ascorbate acid were made up immediately before use. After the mixtures were incubated at 37 °C for 1 h, the color was developed by adding 1 mL of 1.0% thiobarbituric acid (w/v) and 1.0 mL of 25% (v/v) HCl, which was then heated in a boiling water bath for 15 min. The absorbance of the resulting solution was measured spectrophotometricly at 532 nm.



a Galacturonic acid standard



b Tea polysaccharide conjugate TPC-3

Figure 1. (**a**) Typical chromatogram for galacturonic acid at the concentration of 200 μg mL^{-1}; retention time = 3.878. (**b**) Typical chromatogram for tea polysaccharide conjugate TPC-3 at the concentration of 500 μg mL^{-1}; retention time = 3.578.

Assay for the Scavenging Effects on Superoxide Radicals. The assay was performed by using the method of photoreduction of NBT (*18*) but with some modifications. Reaction mixtures contained, in a final volume of 3.0 mL, the following regents at final concentrations of 13 mM methionine, 10 mM riboflavin, 75 μM NBT, 100 mM EDTA, 0.05 M phosphate buffer (pH 7.8), and 167 μg mL^{-1} of tea polysaccharide conjugates. The color was developed by illumination of the mixtures at 3000 Lx for 30 min, and then, the absorbance was measured at 560 nm.

Statistical Treatment of Data. The results were analyzed statistically using Student's *t*-test, an analysis of variance, and regression analysis. The values were considered to be significantly different when the *P* value was less than 0.05.

RESULTS AND DISCUSSION

Chromatographic Conditions Evaluated. Figure 1 is a chromatogram of galacturonic acid standard at a concentration of 200 μg mL^{-1} and a typical chromatogram of the tea

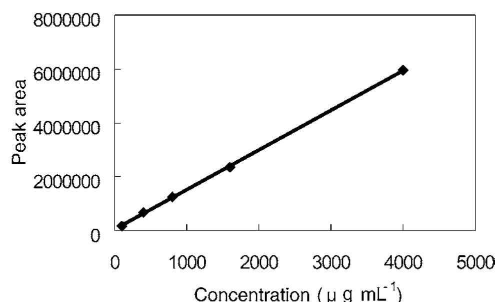


Figure 2. Calibration curves of D-galacturonic acid analyzed by HPLC.

Table 1. Recovery and Precision Studies of HPLC Method for Quantifying Uronic Acid in the Polysaccharide Conjugate Samples

amount added (μg)	amount detected (μg) 1	2	3	average (mg)	recovery (%)	CV (%)
1000	1030	990	1040	1020	102.0	2.6
2000	1910	1970	1940	1940	97.1	1.5
4000	4040	3970	4070	4026	100.6	1.3

polysaccharide conjugate sample TPC-3 at the chromatography conditions. It was shown that the uronic acid in tea polysaccharide conjugates could be well-separated and detected. There was only one uronic acid peak in tea polysaccharide conjugates, which has the same retention time as the standard galacturonic acid. The results sugguested that only galacturonic acid existed in tea polysaccharide conjugates, which was in accordance with ref *14*.

Linearity of Standard Solutions. Accurately, different concentrations of each standard solution were analyzed three times. The response for the analysis was linear over a range of six point concentrations. A good correlation was found between the integrated peak area (A) and the content of galacturonic acid in each solution (C, μg mL^{-1}), as confirmed by the value of r by linear regression. The regression equation was $A = 1472C + 56783$. The correlation coefficient was $R^2 = 0.9997$. The linear range was from 100 to 4000 μg mL^{-1}. The results indicated that a good linearity was achieved.

Sensitivity Studies. The minimum detectable limit determined at two times the signal-to-noise ratio was 200 ng. So, the minimum detectable limit concentration of uronic acid was 10 μg mL^{-1} if the injection volume was 20 μL. The detetion limit of the HPLC-RID method was less than that of the spectrophotometric method with the sensitivity of about 1 μg, but it was larger than that of HPLC-PAD, HPLC with fluorescence detection, GC-FID, or GC-MS, whose sensitivity was less than 0.2 nmol (*19*). The novel and essential feature of the HPLC-RID method was that the uronic acid samples required no derivatization.

Recovery and Precision Studies. Different volumes of standard solution were added to the tea polysaccharides samples. The amount added was analyzed three times (**Table 1**) and gave the results of precision and recovery of the sample analysis. The recovery of uronic acid was from 97.1 to 102.0%, and coefficients of variance (CV) were less than 3.0%.

Uronic Acid Analysis in Tea Polysaccharide Conjugates. The HPLC-RID method for determination of uronic acid was applied to determine the uronic acid content of different tea polysaccharide conjugate fractions. As seen in **Table 2**, the uronic acid content evaluated in TPC-1, TPC-2, and TPC-3 was increased gradually, which correlated with the increasing ion-exchanging ability. The fraction eluted with a high ion strength solution possessed a high uronic acid content. The results also showed that the components of polysaccharide conjugate fractions would differ from each other according to the separation method even though they came from the same material.

Table 2. Uronic Acids Content of Different Tea Polysaccharide Conjugate Fractions ($n = 3$, Mean ± SD)

tea polysaccharide conjugates	uronic acid (%)
TPC-1	30.0 ± 0.53
TPC-2	47.6 ± 0.20
TPC-3	51.8 ± 0.45

Table 3. Scavenging Effects of Different Tea Polysaccharide Conjugate Fractions on Hydroxyl Radicals and Superoxide Radicals ($n = 3$, Mean ± SD)

tea polysaccharide conjugates	•OH inhibition (%)	•O_2^- inhibition (%)
TPC-1	45.9 ± 0.98	36.2 ± 0.80
TPC-2	49.2 ± 0.71	38.5 ± 1.51
TPC-3	58.0 ± 1.35	55.9 ± 1.91

Antioxidant Properties Evaluation of Different Tea Polysaccharide Conjugate Fractions. As shown from **Table 3**, different tea polysaccharide conjugate fractions were all found to have the ability to scavenge hydroxyl radicals and superoxide radicals at the same concentration of 167 μg mL^{-1}. The scavenging effects increased with the increasing uronic acids content in different tea polysaccharide conjugate fractions. Results showed that the uronic acids content had a direct relationship with the radical scavenging effects of tea polysaccharide conjugates.

In conclusion, a method of HPLC-RID for determination of uronic acid in tea polysaccharide conjugates was described. The uronic acid of polysaccharide conjugates, which was purified according to the polysaccharides isolation procedure, could be analyzed by HPLC directly. The method gave a good recovery and CV. The technique has the advantage of rapid analysis and reducing disturbance. The method may applied to other acid polysaccharides and its conjugates. Antioxidant evaluation of the different tea polysaccharide conjugate fractions showed that the uronic acid content affected the antioxidant properties directly.

LITERATURE CITED

(1) Chang, R. Bioactive polysaccharides from traditional Chinese medicine herbs as anticancer adjuvants. *J. Altern. Complement Med.* **2002**, *8*, 559−565.
(2) Blumenkrantz, N.; Asboe, H. G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484−489.
(3) Bitter, T.; Muir, H. M. A modified uronic acid carbazole reaction. *Anal. Biochem.* **1962**, *4*, 330−334.
(4) Bianca, M. H.; Weeren, P. R.; Matthijs, L. C.; Lambert, M. G.; Barneveld, A.; Chris, H. A. Microtiter plate assay for the determination of uronic acids. *Anal. Biochem.* **1998**, *257*, 107−111
(5) Manuel, A. C.; Antonio, B.; Mario, B.; Douglas, N. R.; Ivonne, D. Multivariate analysis of uronic acid and neutral sugars in whole pectic samples by FT-IR spectroscopy. *Carbohydr. Polym.* **1998**, *37*, 241−248.
(6) Garleb, K. A.; Bourquin, L. D.; Fahey, G. C. Galacturonate in pectic substances from fruits an vegetables: comparison of anion exchange HPLC with pulsed amperometric detection to standard colorimetric procedure. *J. Food Sci.* **1991**, *56*, 423−426.

(7) Meyer, A.; Raba, C.; Fische, K. Ion-pair RP-HPLC determination of sugars, amino sugars, and uronic acids after derivatization with p-aminobenzoic acid. *Anal. Chem.* **2001**, *73*, 2377−2382.

(8) Isiguki, K.; Takakuwa, T.; Takeo, T. Anti-diabetes mellitus effect of water-soluble tea polysaccharide. In *Proceedings of International Symposium on Tea Science, 1991*; Tea Science Society of Japan: Shizuoka, Japan, 1992; pp 240−241.

(9) Chen, H. X.; Xie, B. J. Studying progress in the pharmacological bioactivities of tea polysaccharides. *J. Chin. Med. Mater.* **2001**, *24*, 65−67.

(10) Chen, H. X.; Xie, B. J. The preventive and curative effect on diabetic mice of tea polysaccharides. *Acta Nutr. Sin.* **2002**, *24*, 85−86.

(11) Wang, D. F.; Li, J.; Wang, C. H.; Zhao, G. W.; Jin,Y.; Cheng, D. D.; Ye, S. Study on the component and immune activity of polysaccharides from tea. *J. Tea Sci.* **2000**, *20*, 45−50.

(12) Tadakazu, T.; Tomoki, U.; Hitoshi, K. The chemical properties and functional effects of polysaccharides dissolved in green tea infusion. *Nippon Shokuhin Kagaku Kogaku Kaishi* **1998**, *45*, 270−272.

(13) Wang, D. F.; Wang, C. H.; Li, J.; Zhao, G. W. Components and activity of polysaccharides from coarse tea. *J. Agric. Food Chem.* **2001**, *49*, 507−510.

(14) Wang, D. F.; Xie, X. F.; Wang, S. L.; Zheng, J.; Yan, J.; Yan, H. D.; Wang, Z. N. Composition and the physical chemical characteristics of tea polysaccharide. *J. Tea Sci.* **1996**, *16*, 1−8.

(15) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

(16) Chinpal, K.; Frederick, L. C.; Page, W. F. D.; James, M. Purification and characterization of a doxorubicin-inhibited NADH-quinone (NADH-ferricyanide) reductase from rat liver plasma membranes. *J. Biol. Chem.* **2002**, *277*, 16441−16447.

(17) Halliwell, B.; Gutteridge, J. M. C.; Aruoma, O. I. The deoxyribose method: A simple "test-tube" assy for determination of rate constants for reactions of hydroxyl radicals. *Anal. Biochem.* **1987**, *165*, 215−219.

(18) Stewar, R. C.; Beewley, J. D. Lipid peroxidation associated with associated with accelerated aging of soybean areas. *Plant Physiol.* **1980**, *65*, 245−248.

(19) Chaplin, M. F.; Kennedy, J. F. *Carbohydrate Analysis: A Practical Approach*; Oxford University Press: London, 1994; pp 17−34.

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