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Validation of Isocratic Eluting and Stepwise Flow Rate Gradient for HPLC Determination of Catechins, Gallic Acid and Caffeine in Tea

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ABSTRACT: An easy, rapid and precise high performance liquid chromatographic determination for catechins, gallic acid and caffeine in tea with an isocratic elution and stepwise flow rate gradient was developed. The chromatographic separation system consisted of a C18 reverse phase column, and an isocratic elution system of 16:1:3 (0.1% *ortho* phosphoric acid-methanol-acetonitrile). The validation of this method was confirmed from specificity, linearity, precision, accuracy, limit of detection and limit of quantification. The method exhibited good linearity and accuracy and has been used to analyze commercial tea products. The results showed high amounts of (+) epigallocatechin gallate, caffeine and (+) catechin in these products, ranging from 206±13 to 9,630±56, 137±13 to 4,870±130 and <1.29 to 1,790±7 mg/100g dried weight, respectively. The amounts of gallic acid, (-) gallocatechin gallate and (-) epicatechin gallate found were14.9±0.3 to 104±0.4, <14.2 to 399±67, 122±8 to 3,020±45 mg/100g dried weight, respectively.

KEYWORDS: HPLC, stepwise flow rate gradient, green tea, catechin, gallic acid, caffeine, antioxidant.

INTRODUCTION

Tea beverage can be made from the young shoots and apical bud leaves of *Camellia sinensis*. In manufacturing, green tea is non-fermented, oolong tea is partially fermented and black tea is a completely fermented, which has an influence on the amount of polyphenolic compounds. This effect is caused by the polyphenol oxidase enzyme, which changes the catechins into theaflavins and thearubigins. Therefore, the more intense color of fermented tea exhibits lower amounts of catechins[1, 2]. Catechins are a group of polyphenolic compounds that are widely distributed in many plants, fruits and vegetables[2]. In tea beverages, several catechins have been reported, including (-) epigallocatechin gallate (EGCg), (-) epicatechin gallate (ECg), (-) epigallocatechin (EGC) and (+) catechin. These compounds effectively scavenge reactive oxygen species (ROS), such as superoxide free radical, hydroxyl radicals, and also prevent Cu^{2+}-mediated low-density lipoprotein oxidation[3]. ROS cause oxidative stress and pathogenesis in many chronic diseases, such as arteriosclerosis, ischemia-reperfusion injury, cancer, stroke and neurodegenerative disorders[3] Previous reports have shown that catechins have diverse pharmacological activities, such as anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-arteriosclerosis and anti-bacterial effects[4-5]. This study aims to improve the easy and rapid isocratic HPLC method for determination the amount of phenolic compound in selected tea brands in the market.

MATERIALS AND METHODS

Chemicals

Acetonitrile (HPLC grade, Fisher Scientific, UK), *ortho* phosphoric acid (analytical grade, BHD, England), ultrapure water from Milli-Q system (Millipore, Bedford, USA) and methanol (analytical grade) were used for the mobile phase preparation. Gallic acid (reagent grade, Sigma-Aldrich Chemie GmbH, Germany), caffeine anhydrous (HPLC grade, Fluka Chemie GmbH, Switzerland), (+) catechin hydrate, (-) epigallocatechin gallate (EGCg), (-) gallocatechin gallate (GCg) and (-)-epicatechin gallate (ECg) (HPLC grade, Sigma-Aldrich Chemie GmbH, Germany) were used as received.

Standard and Sample Preparations

Stock standard solutions were prepared in a mixture of acetonitrile and deionized water (1:1, v/v). Gallic acid stock solution was prepared at a concentration of

0.15 mg/ml and all other standards were prepared at a concentration of 0.1 mg/ml.

Commercial instant teas were purchased from abroad as well as from domestic markets, *i.e.* Japanese green teas (Brands #1, #2, #3 and #4), Chinese teas (Brands #5, #6 and #7), European Ceylon tea (Brands #8 and #9), Thai green tea (Brand #10) and Thai green tea extract capsule (Brand #11). Tea infusion was prepared by a modified method of Sharma et al. (2004)[1]. Briefly, 0.2 g of dried tea was weighed, tapped into a 50-ml volumetric flask and warmed at 100 °C in a hot bath for 5 minutes. The dried tea was infused for a total of 5 minutes with 30 ml 80 °C boiled water, by adding 10 ml of boiled water at a time. The tea infusion was adjusted to 50 ml with 80 °C boiled water, then immediately centrifuged at 3,000 rpm for 5 minutes and filtered through a 0.45 mm poly-vinylidene fluoride (PVDF) membrane (Millex®-HV 13, Sigma-Aldrich Chemie GmbH). The tea filtrate was diluted with acetonitrile in a ratio of 1:1. The final concentrations of tea infusions were 2.0 mg dried weight per milliliter of solvent. For the green tea capsule, the powder extract was dissolved in a mixture of acetonitrile-deionized water (1:1). The final concentration of the green tea powder extract was 2.5 mg/ml.

Chromatographic Analytical Conditions

The chromatographic system consisted of an Agilent series 1100 HPLC with a pumping system model G1310A, a manual injection model G1328B and a variable wavelength detector model G1314A. Absorption was detected at 280 nm. A C18 reverse phase column (Phenomenex 10 ODS-2), 4.6 mm I.D. × 250 mm with 5 µm particle size was used. Reverse phase HPLC was used and was carried out using an isocratic mobile phase. The mobile phase consisted of 0.1% *ortho*-phosphoric acid (%v/v; pH 4.2), methanol and acetonitrile (16:1:3). The injection volume was 20 µl. The stepwise flow rate was as follows: 1.2 ml/min (10 min), 1.4 ml/min (10 min) and 1.0 ml/min (5 min).

Validation Parameters

The stock gallic acid solution was diluted to concentrations of 0.6, 0.3, 0.15, 0.075, 0.0375 and 0.01875 mg/ml. Stock solutions of catechin, caffeine, EGCg, GCg and ECg were diluted to concentrations of 40, 20, 10, 5, 2.5 and 1.25 mg/ml. The solutions were filtered through a 0.45 µm PVDF membrane prior to injection. Each solution was repeated in six replications and the calibration curves were constructed by linear regression. The retention time of the standard solution was identified. The percentage relative standard deviation (%RSD) of retention time was calculated to confirm the specificity of the peaks. The linearity of the calibration curves were determined for the gallic acid, catechin, caffeine, GCg, EGCg and ECg standard solutions. The peak area versus concentration was plotted. The slope and the regression coefficient of the calibration curves were calculated by linear regression. Evaluation of the method repeatability (intra-day precision) and reproducibility (inter-day precision) were performed. The intra-day precision was determined at six concentrations in six replications. The inter-day precision was determined in triplicate at six concentrations and conducted for three days. Percentage relative standard deviation (%RSD) values were calculated for intra-day and inter-day precision.

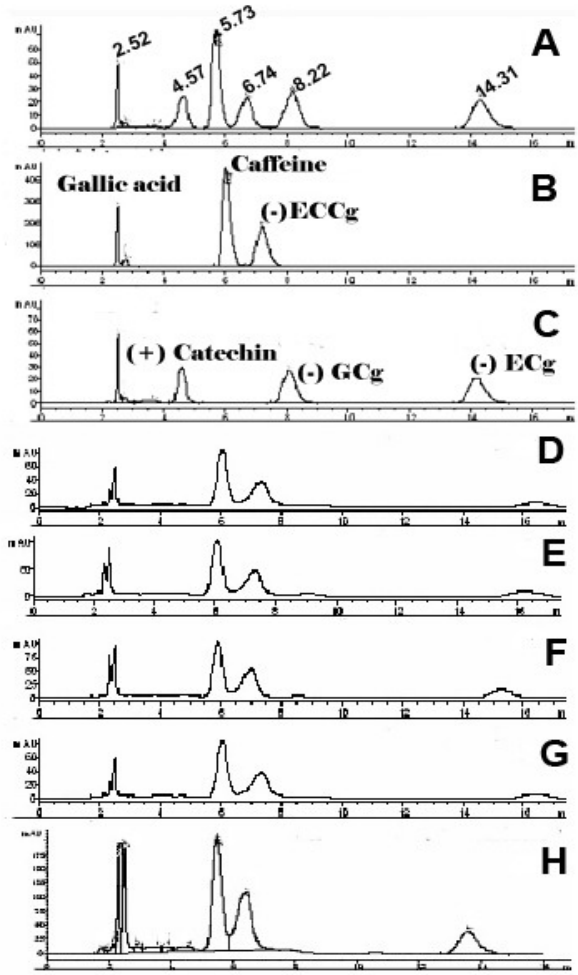

Fig 1. Chromatographic profiles of standards and representative tea samples.
(A) six standards mixture: 0.60 mg/ml gallic acid standard and 40.0 mg/ml of other standards; (B) 300 mg/ml gallic acid, caffeine and epigallocatechin gallate standards mixture; (C) mixture of 0.60 mg/mlgallic acid standard and 40.0 mg/ml catechin, gallocatechin gallate and epigallocatechin gallate standards; (D) Japanese tea Brand #3; (E) Chinese tea Brand #6; (F) European Ceylon tea Brand #9; (G) Thai green tea Brand #10; and (H) Thai green tea extract capsule Brand #11.

Table 1. Linearity and retention time of six phenolic standard peaks.

Standard peak	LOD(ng)	LOQ(ng)	Linearity			Retention time (min)	
			slope	y-intercept	r^2	Intra-day	Inter-day
Gallic acid	1.1	2.9	24.43	-1.22	0.999	2.5 ; 0.3	2.5 ; 0.5
Catechin	2.6	7.9	0.71	0.74	0.999	4.7 ; 0.8	4.7 ; 1.5
Caffeine	1.0	3.0	1.80	12.66	0.999	5.8 ; 1.8	5.7 ; 1.7
EGCg	1.8	5.4	0.73	12.31	0.994	6.9 ; 2.5	6.8 ; 2.2
GCg	1.6	4.9	1.10	12.53	1.000	8.3 ; 1.6	8.3 ; 2.6
ECg	5.0	15.2	1.10	3.01	1.000	14.6 ; 1.2	14.6 ; 2.4

Note: Linearity was from 6 determinations expressed as slope, y-interception and the correlation coefficients (r^2). The retention time was expressed as mean; percentage of relative standard deviation (%RSD).

The variability of the HPLC response was studied during the intra-day precision and expressed as the percentage of coefficient variation (%CV). The standard deviations (SD) of the response and slopes of the concentration curves of the calibration curves were used to estimate the limit of detection (LOD) and limit of quantification (LOQ) with the following formulas: LOD = 3.3 σ/S and LOQ = 10 σ/S, where σ is the residual standard deviation of the regression line and S is the slope of the standard curve[6].

Results and Discussion

Optimization of Analytical Method

Six phenolic compounds including gallic acid, caffeine, catechin, EGCg, GCg and ECg were determined in 10 brands of dried tea products from Japan, China, Europe and Thailand and a capsule of green tea extract from Thailand. In previous reports[7-9], an isocratic system of 0.1% H_3PO_4-acetonitrile (90:10, %v/v) showed low resolution for separation of the caffeine peak from the catechin peak and the EGCg peak from the EC peak [8]. Since the presence of acid in the mobile phase is essential for separation of closely related structures [7-9], this current study modified the mobile phase to be 0.1% *ortho* H_3PO_4-acetonitrile (85:15, %v/v) with a flow rate 1.0 ml/min. The peaks were clearly separated, except the peak of EGC, which was not separated from catechin peak. The condition of 0.1% *ortho* H_3PO_4-acetonitrile (80:20, %v/v) did not separate the peak of catechin from the caffeine and EGCg peaks. Therefore, an isocratic system of 0.1% H_3PO_4-methanol-acetonitrile (80:5:15, %v/v) with a stepwise flow rate gradient was used. Under this condition, ECg was completely eluted within 15-20 min. Therefore, for a better resolution of all phenolic components, a gradient elution program is needed.

Validation of Analytical Method

The HPLC chromatogram profile shows distinct separation of the six phenolic compounds (Fig 1A-1C). The peak specificity was shown by %RSD of 0.51-2.60. The results indicate good linearity with correlation coefficients (r^2) between 0.994 and 1.000 (Table 1). In general, repeatability or intra-day precision exhibited low %RSD, between 0.9-5.7, for most concentrations of the standard solutions. However, at 50 ng of GCg and 25 ng ECg the %RSD was relatively high at 17.0% and 13.1%, respectively. The reproducibility or inter-day precisions demonstrated low %RSD, except at 800 ng EGCg (41.8%), 50 ng GCg (25.2%) and 25 ng ECg (13.9%). From these results, the proper concentration for determination of EGCg, GCg and ECg is in the range of 50-100 ng, 100-800 ng and 100-800 ng, respectively. This optimized method revealed good accuracy (95.2-108.5%) (Table 2). The values of LOD and LOQ are shown in Table 1 and demonstrate the good sensitivity of this method.

Sample Determinations

As shown in (Fig 1D-1H) and Table 3, the amount of the six phenolic compounds varied among different brands of tea. According to our study, EGCg was the major compound found. The order of compounds found in the tea samples from highest to lowest content was EGCg, caffeine, catechin, ECg, GCg and gallic acid. The green tea capsule was found to contain higher amounts of phenolic compounds than the other tea samples, except that GCg was found in larger quantity in Chinese tea. The EGCg found in tea samples ranged from 206±13 to 9,630±56mg%. It is important to note that caffeine was found at considerable amounts, between 137±13 to 4,870±130 mg%. Catechin ranged from <1.29 to 1,790±7.1 mg %. The levels of ECg and GCg were 122±7.6 to 3,020±45 and <14.2 to 399±67 mg%, respectively. The gallic acid content in tea samples was 14.9±0.3 to 104±0.4 mg%.

Conclusion

A high-performance liquid chromatographic method was developed and can be applied to routine quantitative analysis of the phenolic compounds in tea with better resolution than previous reports, especially

Table 2. Precision and accuracy of the HPLC chromatographic system.

Standard peak	Concentration (ng)	Intra-day precision (Repeatability)(n = 6)	Inter-day precision (Reproducibility)(n = 3)	Accuracy (n = 6)
Gallic acid	600	1.2	1.5	100.5 ; 1.7
	12	1.2	2.6	100.0 ; 1.2
	1.5	3.8	6.4	98.4 ; 3.8
Catechin	800	1.4	2.1	100.2 ; 1.4
	100	1.3	3.0	101.4 ; 1.3
	25	3.8	6.2	106.6 ; 3.6
Caffeine	800	1.2	1.7	101.0 ; 1.1
	100	0.9	3.0	101.7 ; 0.8
	50	2.0	3.3	108.5 ; 2.7
EGCg	800	1.2	41.8	100.5 ; 3.1
	100	0.9	10.3	98.9 ; 6.1
	50	2.0	5.6	102.2 ; 2.1
GCg	800	2.2	4.3	100.1 ; 2.1
	100	5.7	11.7	100.1 ; 5.0
	50	17.0	25.2	95.2 ; 12.9
ECg	800	1.6	4.2	100.0 ; 1.6
	100	4.7	9.1	100.8 ; 4.6
	25	13.1	13.9	100.0 ; 11.7

Note: Intra-day and inter-day precision were expressed as relative standard deviation (%RSD). Accuracy was expressed as percentage of mean recovery and percentage of coefficient variation (%CV). Numbers of analytical replications are represented as n.

at low concentrations of phenolic compounds. The validation results confirm a promising and acceptable method to analyze other tea products. The determination of six bioactive compounds from the tea samples demonstrated that EGCg, catechin and caffeine were the major components found in each brand of tea.

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Table 3. Levels of polyphenolic compounds in tea samples.

Manufacturing sources	Brand	Gallic acid	Caffeine	Catechin	EGCg	GCg	ECg
Japanese green tea	#1	24.9±3.0	1780±38	298±15	4,200±98	173±5.5	482±12
	#2	23.8±1.2	969±20	234±48	2,050±89	78.8±3.3	338±2.7
	#3	19.1±1.2	1,320±5.1	99.7±3.7	2,550±36	144±22	378±2.5
	#4	45.2±1.6	1,650±35	643±35	4,530±21	298±44	1,250±31
Chinese tea	#5	14.9±0.3	1,180±18	<1.29	1,900±11	< 14.2	167±2.9
	#6	34.6±0.2	1,810±23	446±6.0	3,490±18	327±11	685±6.2
	#7	37.1±0.7	1,630±29	535±23	3,530±97	319±89	1,008±20
European ceylon tea	#8	49.5±2.8	137±13	213±18	4,070±87	399±67	122±7.6
	#9	55.6±0.9	369±27	1,790±7.1	538±27	< 14.2	148±9.0
Thai green tea	#10	22.8±1.1	490±14	227±2.6	206±13	< 14.2	254±8.9
	#11	104±0.4	4,870±130	949±93	9,630±56	< 14.2	3,020±45

Note: Data were expressed as mean ± standard deviations of 3 determinations in terms of mg/100 g dried weight (mg %).

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