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Original Article

An improved HPLC method for simultaneous determination of phenolic compounds, purine alkaloids and theanine in Camellia species

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ABSTRACT

A simple and fast HPLC analysis method for phenolic compounds, purine alkaloids and theanine in various Camellia species was developed. Using an amide-C16 column, catechins, gallic acid, caffeine, theobromine, theacrine, theophyline and theanine could be rapidly separated within 45 min with a gradient elution system. Excellent linearity was observed for all 14 compounds in the range studied, with correlation coefficients between 0.9994 and 0.9999. Limit of detection and limit of quantification of the 14 compounds varied from 0.0001 to 0.072 ng/μL and 0.0004 to 0.24 ng/μL, respectively. Four kinds of Camellia species were analyzed using this method. In traditional cultivated tea trees, *Camellia sinensis* and *Camellia assamica*, the main purine alkaloid was caffeine and main phenolic compound was (−)-epigallocatechin gallate (EGCG). The main purine alkaloids in *Camellia ptilophylla* and *C. assamica* var. *kucha* were theobromine and theacrine; their contents were 4.001 ± 0.1184% dry weight and 2.116 ± 0.0270% dry weight, respectively. The main phenolic compounds in *C. ptilophylla* and *C. assamica* var. *kucha* were (−)-gallocatechin gallate (GCG) and EGCG respectively. The content of theanine in the four Camellia species samples ranged from 0.136 ± 0.0026% to 1.485 ± 0.0491% dry weight.

1. Introduction

Tea is the most widely consumed beverage in the world and has become an important agricultural product. Recently, many epidemiologic and preclinical studies suggest that drinking tea may reduce the risks of cancer and cardiovascular disease (Yang et al., 2002; Kris-Etherton and Keen, 2002). Moreover, other beneficial effects also have been reported, such as anti-oxidation, anti-inflammation and anti-obesity (Yang and Landau, 2000; Sueoka et al., 2001). These beneficial effects have been partly attributed to purine alkaloids and mainly to phenolic compounds. The major tea purine alkaloids include caffeine, theobromine, theophyline and theacrine (Axel et al., 1996; Ye et al., 1997, 1999; Zheng et al., 2002; Ashihara et al., 1998). The major tea phenolic compounds are gallic acid and eight naturally occurring tea catechins, including (+)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-catechin gallate (CG), (−)-gallocatechin gallate (GCG), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG). Theanine is the characteristic and main amino acid in tea. Theanine was shown to down-regulate cerebral function, reduce blood pressure and inhibit the excitatory effects of caffeine (Terashima et al., 1999; Yokogoshi et al., 1995; Kakuda et al., 2000).

The amount of tea catechins, gallic acid, purine alkaloids and theanine varies according to the tea variety, climate and cultivation. For example, the major purine alkaloid is caffeine in traditional cultivated tea plants *Camellia sinensis* and *Camellia assamica*, but they have less theobromine and theophyline in their buds and young leaves (Axel et al., 1996). *Camellia ptilophylla* and *C. assamica* var. *kucha*, two new Camellia species, mainly contain theobromine and theacrine, respectively (Ye et al., 1997, 1999; Zheng et al., 2002; Ashihara et al., 1998). Therefore, it is important to establish a simple and reliable analytical method for the simultaneous determination of the levels of these compounds in tea samples in order to develop high-quality tea products.

Much work has focused on the development of a separation of tea catechins by HPLC (Lee and Ong, 2000; Bronner and Beecher, 1998; Yao et al., 2004; Goto et al., 1996; Nishitani and Sagesaka, 2004; Sharma et al., 2005; Dalluge et al., 1998; Neilson et al., 2006; Friedman et al., 2006). Recently, an improved HPLC analysis system could simultaneously determine the contents of tea catechins, caffeine and gallic acid in teas (Wang et al., 2000, 2003; Zuo et al., 2002; Mizukami et al., 2007). Yang et al. (2007) had developed a method for simultaneous analysis of seven catechins and three purine alkaloids including caffeine, theobromine and theacrine in *C. sinensis*, *C. ptilophylla* and *C. assamica* var.

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kucha by HPLC. Although their method seems suitable for the routine analysis of many samples, there exists no study that has been carried out concerning a simultaneous analysis of catechins, purine alkaloids, gallic acid or theanine in tea. The published separation methods in the literature were unsuitable for the separation of other purine alkaloids like theacrine, gallic acid and theanine because the retention times of the alkaloids and catechins were similar and the retention time of each published method using a C18 column was too short to analyze gallic acid and theanine.

In the present paper, a new and simple HPLC method using an amide-C16 column was demonstrated to rapidly and simultaneously determine 14 important compounds including eight catechins, four purine alkaloids, gallic acid and theanine in *C. sinensis*, *C. assamica*, *C. ptilophylla* and *C. assamica* var. *kucha*. This method was successfully validated and it was used to simultaneously determine 14 important tea compounds.

2. Materials and methods

2.1. HPLC instrumentation, column and condition

The HPLC system consisted of two waters 515 LC pumps for high-pressure gradient elution, a waters 2487 UV detector (Waters, USA) and a HT-230A column heater (Heng'ao, Tianjing, China). The analytical column used was Discovery RP-Amide C_{16} column (4.6 mm × 150 mm, 5 μm) (Supelco, USA) equipped with a Discovery RP-Amide C_{16} guard column (4 mm × 20 mm, 5 μm) and was operated at 30, 35 and 40 °C. Two-gradient elution system was used, mobile phase A contained ortho-phosphoric acid (85%) and water (0.05:99.95, V:V); mobile phase B was acetonitrile (ACN). The gradient was as follows: 0–4 min, 2% B; 4–21 min, linear gradient from 2% B to 9% B; 21–32 min, linear gradient from 9% B to 23% B; 32–45 min, 23% B. Post-run time was 10 min. Elution was performed at a solvent flow rate of 0.8 mL/min. The sample injection volume was 20 μL. Peak purity testing and detection at 210 and 280 nm was accomplished using a Waters 2996 photodiode array detector (Waters, USA). Data analysis was performed using Yihai data analysis software (Yihai, Guangzhou, China).

2.2. Standards and other chemicals

C, EC, GC, EGC, CG, ECG, GCG and EGCG were purchased from Sigma Chemical Company (St. Louis, MO, USA). Caffeine was purchased from Wako Pure Chemical Industries Ltd. Company (Osaka, Japan). Theophylline, theobromine, theanine and gallic acid were purchased from Fluka Chemical Company (St. Louis, MO, USA).

Theacrine was isolated from tea leaves of *C. assamica* var. *kucha*. Tea sample of *C. assamica* var. *kucha* (20 g) was extracted with 600 mL water at 90 °C for an hour. The filtrate was treated with Pb(OH)Ac, the precipitate was discarded, and then the filtrate was concentrated in vacuo to obtain extract powder (8 g). The powder was subjected to chromatography over silica gel with petroleum ether and ethyl acetate (20:80) and fractions were further purified with preparative HPLC on a YMC-Pack ODS-A column (10 mm × 250 mm, 5 μm, YMC Co., Ltd., Japan) with methanol–H_2O (85:15). The purified compound was identified by comparing its physicochemical data with those described in the literature (Ye et al., 1999).

HPLC-grade ACN was purchased from Merck Company (Darmstadt, Germany). HPLC-grade water (18 mΩ) prepared using a Millipore Milli-Q purification system (Millipore Corporation, Bedford, MA, USA) was used for the mobile phases and to prepare all solutions.

2.3. Preparation of standards and tea samples

The standard aqueous solution containing of gallic acid 0.102 μg/μL, GC 0.05 μg/μL, CG 0.05 μg/μL, EGC 0.05 μg/μL, ECG 0.05 μg/μL, EGCG 0.196 μg/μL, EC 0.05 μg/μL, C 0.054 μg/μL, GCG 0.1 μg/μL, theobromine 0.05 μg/μL, theacrine 0.05 μg/μL, theophylline 0.001 μg/μL, caffeine 0.0503 μg/μL and theanine 0.05 μg/μL, respectively, was prepared and used in all method development.

Green tea (Longjing Tea), made from the leaves of *C. sinensis* plant and pu'er tea, made from *C. assamica* was purchased at a local market and used for quantitative analysis. Samples of *C. ptilophylla* and *C. assamica* var. *kucha* were obtained from Sun Yat-Sen University. After collecting, one apical bud and two adjacent leaves were steamed for 5 min and then dried under 80 °C.

In all, 0.1 g of the four dried tea samples was extracted at 90 °C for 30 min in 50 mL water and shaken once every 10 min. The filtrates were diluted to 100 mL. The diluted solutions were then filtered again through a 0.45 μm nylon filter and analyzed directly by HPLC.

2.4. Method evaluation

Calibration curves of 14 compounds were constructed using six different standard concentrations over the concentration ranges

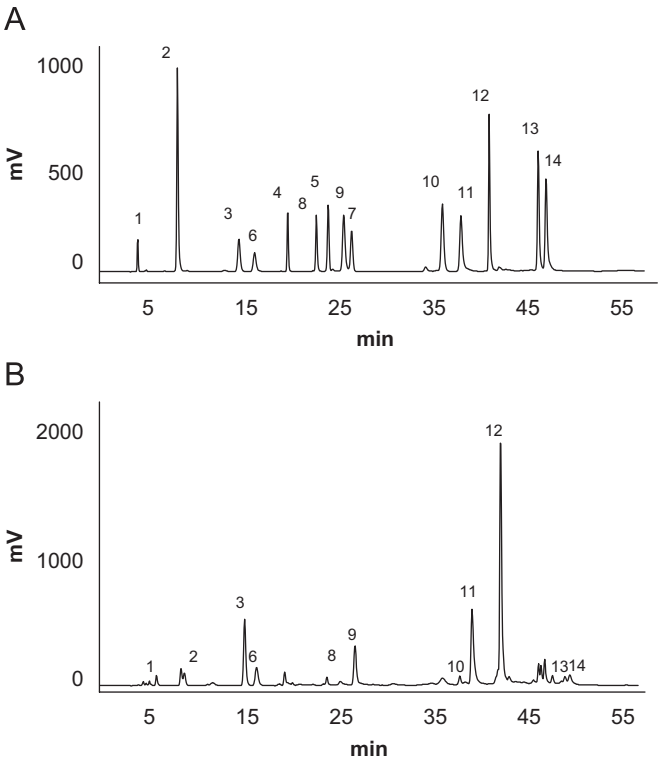

Fig. 1. The chromatogram of standards and tea samples using acetonitrile–water–ortho-phosphoric acid solvent system on a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 μm). Conditions: mobile phase A: ortho-phosphoric acid–water (0.05:99.95, V:V); mobile phase B: acetonitrile. The gradient was as follows: 0–12 min, 5% B; 12–30 min, linear gradient from 5% B to 7% B; 30–40 min, linear gradient from 7% B to 20% B; 40–50 min, 20% B. Flow rate 0.8 mL/min. UV detection at 210 nm. (A) Standard; (B) sample of *C. ptilophylla*. Peak identification: (1) theanine, (2) gallic acid, (3) theobromine, (4) theophylline, (5) theacrine, (6) GC, (7) caffeine, (8) EGC, (9) catechin, (10) EC, (11) EGCG, (12) GCG, (13) ECG, (14) CG.

expected in various tea samples. Limit of detection (LOD), limit of quantification (LOQ) and repeatability were evaluated using standard compounds. The recovery tests were carried out by adding known amounts of the standard sample at low, medium and high levels to a preparation of green tea extract solution. To confirm the repeatability of the method, a preparation of green tea extract solution was analyzed repeatedly six times. The stability was evaluated by the analysis of green tea extract solution stored in a refrigerator at 2 h intervals over 10 h. Tea made from *C. ptilophylla* was used for optimization of the analytical method.

3. Results and discussion

3.1. Development of the analytical method

Initial efforts to develop a separation using a C18 column and a methanol-based mobile phase were unsuccessful because the 14 compounds could not separate efficiently. The ACN–water–ortho-phosphoric acid solvent system was attempted and then optimized. The result demonstrated that the experimental conditions separated the 14 standards well (Fig. 1A). When the ACN–water–ortho-phosphoric acid solvent system was used for the analysis of *C. ptilophylla*, it was found that the gallic acid peak combined with an unknown component (Fig. 1B). The gradient elution system for the C18 column could not be further optimized.

We subsequently shifted our efforts toward evaluating different columns. Amide-C16 column is a palmitamidopropylsilane-bonded silica column; the polar amide group gives less retention and different selectivity than a C18. The column has a thick hydration layer including amide group; so multiple interaction (hydrophobic interaction and hydrogen bonding) contributes to the solute separation. The amide-C16 column was used to extend the retention time of gallic acid in order to separate it from the unknown component in three kinds of tea plants, and the optimized solvent system could separate all 14 standards and was used for the analysis of 14 components in all three types of samples (Fig. 2A and B). The column used was only 150 mm long in order to shorten analysis time. The final conditions could be used to analyze the tea samples within 45 min.

For column temperature, as the temperature increased, the retention time of compounds decreased, otherwise the magnitude of the change varied for each compound. Catechin and caffeine could not be separated at 40 °C, and EGC could not be separated from another component in the sample of *C. ptilophylla* at 30 °C. Optimum chromatographic separation of all standards and tea samples could be obtained at 35 °C.

A wavelength of 210 or 280 nm was chosen for detection of catechins and caffeine in tea samples (Goto et al., 1996; Nishitani and Sagesaka, 2004; Wang et al., 2003). At the wavelength of 280 nm, caffeine and each catechin showed strong absorption but theanine showed no absorption. Therefore, 210 nm was used as the detection wavelength at which all 14 compounds showed good absorption.

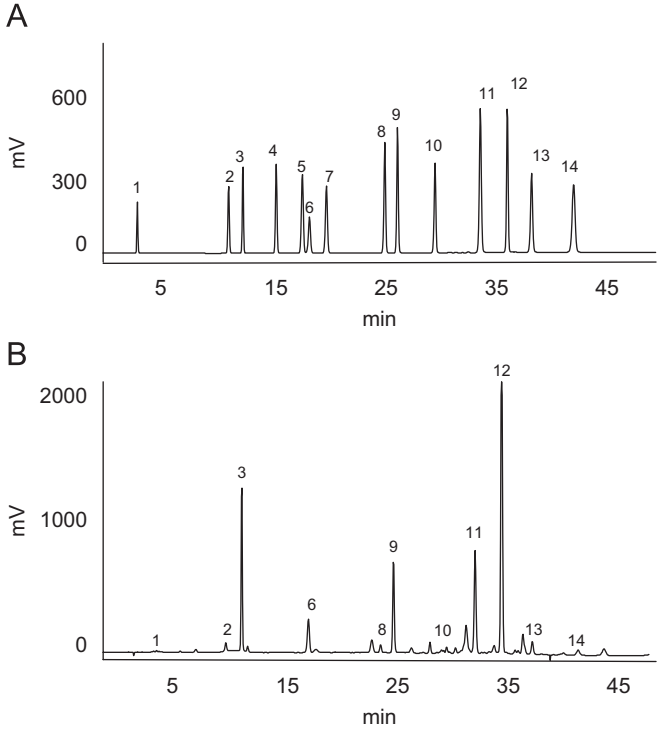

Fig. 2. The chromatogram of standards and tea samples using acetonitrile–water–ortho-phosphoric acid solvent system on a Discovery RP-Amide C16 column (4.6 mm × 150 mm, 5 μm). (A) Standards, (B) sample of *C. ptilophylla*. Peak identification: (1) theanine, (2) gallic acid, (3) theobromine, (4) theophylline, (5) theacrine, (6) GC, (7) caffeine, (8) EGC, (9) catechin, (10) EC, (11) EGCG, (12) GCG, (13) ECG, (14) CG.

Table 1
The linearity, correlation coefficient (R), limit of detection (LOD) and limit of quantification (LOQ) of the compounds studied

Compound	Retention time	Linearity (μg)	R^a	LOD[b] (ng)	LOQ[b] (ng)
Theanine	3.06	0.01–1	0.9998	1.9	6.3
Gallic acid	11.32	0.2–2.04	0.9997	1.5	4.9
Theobromine	12.60	0.01–1	0.9998	0.3	0.9
Theophylline	15.63	0.01–0.02	0.9995	0.8	2.6
Theacrine	17.99	0.1–1	0.9999	0.7	2.3
GC	18.63	0.01–1	0.9999	0.8	2.6
Caffeine	20.17	0.001–1.06	0.9994	0.2	0.7
EGC	25.45	0.01–1	0.9998	1.3	4.3
Catechin	26.59	0.01–1.08	0.9996	1.6	5.3
EC	29.99	0.01–1	0.9997	2.8	9.2
EGCG	34.08	0.2–3.92	0.9996	1.2	3.9
GCG	36.53	0.2–2	0.9994	1.5	4.9
ECG	38.71	0.01–1	0.9996	1.4	4.6
CG	42.52	0.01–1	0.9998	1.6	5.3

[a] R was the correlation coefficient of each calibration curve, which was determined by six calibration points with three measurements.
[b] LOD and LOQ were estimated by successively diluting the standard solutions, considering a signal-to-noise ratio of 3 and 10, respectively.

Table 2
Validation results of the analytical method using a green tea extract solution

Compound	Recovery (%)	Accuracy RSD (%)	Repeatability RSD (%)	Stability RSD (%)
Theanine	99.44	0.83	2.51	4.83
Theobromine	95.45	0.81	2.81	1.04
GC	85.56	0.49	2.67	4.01
EGC	101.06	0.62	2.26	2.01
C	98.81	0.91	1.61	3.83
Caffeine	99.04	0.59	0.98	0.87
EC	95.15	0.89	1.06	4.30
EGCG	92.56	0.46	2.61	0.79
GCG	103.86	0.76	2.56	4.53
ECG	103.04	0.78	2.81	4.51
CG	101.41	0.92	1.37	2.74
Theophylline	–	0.89	–	–
Theacrine	–	0.75	–	–

–, not determined.

3.2. Quantitative analysis

An excellent linearity was observed for all 14 compounds in the range studied, with correlation coefficients between 0.9994 and 0.9999. LOD and LOQ of 14 compounds varied from 0.0001 to 0.072 ng/μL and from 0.0004 to 0.24 ng/μL, respectively (Table 1).

Recovery, accuracy, repeatability and stability were evaluated using a green tea extract solution (Table 2). The recoveries of 14 compounds were in the range of 85–104%. The RSD of all measurements were less than 1.0%. The stability result showed that the sample was stable in the refrigerator for over 10 h. These results confirmed the validity of the analytical method.

3.3. Analysis of tea samples

Fig. 3 showed the HPLC profile of extracts from three kinds of plants. The main purine alkaloid in green tea and pu'er made from *C. sinensis* and *C. assamica* was caffeine, at 2.985 ± 0.0008% and 3.664 ± 0.0432%, respectively. The main purine alkaloid was theobromine in *C. ptilophylla* and at 4.001 ± 0.1184%, while it was theacrine in *C. assamica* var. *kucha* at 2.116 ± 0.0270%. The main phenolic compound in *C. sinensis*, *C. assamica* and *C. assamica* var. *kucha* was EGCG and in *C. ptilophylla* it was GCG. The contents of theanine ranged from 0.136 ± 0.0026% to 1.485 ± 0.0491%, and it was found to be the highest in pu'er. Table 3 shows the contents of the 14 compounds studied in the three kinds of plants.

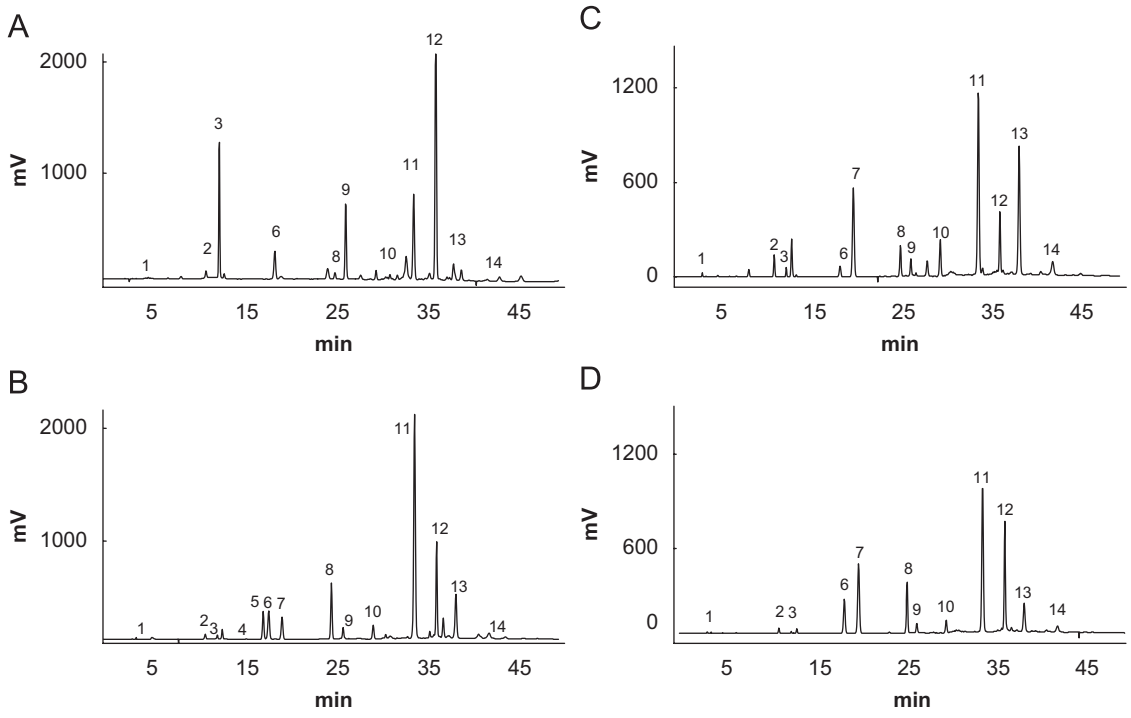

Fig. 3. The chromatogram of samples made from three kinds of plant. Detection was carried out with UV at 210 nm. (A) *C. ptilophylla*, (B) *C. assamica* var. *kucha*, (C) pu'er tea. (D) green tea. Peak identification: (1) theanine, (2) gallic acid, (3) theobromine, (4) theophylline, (5) theacrine, (6) GC, (7) caffeine, (8) EGC, (9) catechin, (10) EC, (11) EGCG, (12) GCG, (13) ECG, (14) CG.

Table 3
Contents of the 14 studied compounds in tea samples

Compound	Content (% w/w)			
	C. sinensis (green tea)	*C. assamica* (pu'er tea)	*C. ptilophylla*	C. assamica var. *kucha*
Theanine	0.69 ± 0.04[a]	1.48 ± 0.04	0.13 ± 0.01	0.42 ± 0.01
Gallic acid	0.13 ± 0.01	0.59 ± 0.01	0.29 ± 0.01	0.08 ± 0.01
Theobromine	0.01 ± 0.01	0.24 ± 0.01	4.00 ± 0.12	0.80 ± 0.01
Theophylline	nd[b]	nd	nd	0.006 ± 0.01
Theacrine	nd	nd	nd	2.11 ± 0.03
GC	1.61 ± 0.02	0.50 ± 0.01	2.72 ± 0.01	1.63 ± 0.01
Caffeine	2.98 ± 0.01	3.66 ± 0.04	nd	0.79 ± 0.02
EGC	1.04 ± 0.01	0.59 ± 0.03	0.22 ± 0.07	1.29 ± 0.01
Catechin	0.10 ± 0.01	0.24 ± 0.01	2.59 ± 0.13	0.20 ± 0.03
EC	0.22 ± 0.01	0.77 ± 0.01	0.29 ± 0.02	0.28 ± 0.01
EGCG	3.60 ± 0.13	4.58 ± 0.07	2.29 ± 0.02	7.28 ± 0.58
GCG	2.74 ± 0.05	1.43 ± 0.17	7.60 ± 0.59	3.69 ± 0.64
ECG	0.69 ± 0.10	3.83 ± 0.02	0.50 ± 0.02	1.51 ± 0.15
CG	0.14 ± 0.01	0.35 ± 0.01	0.11 ± 0.01	0.23 ± 0.06

[a] Data were expressed as mean ± standard deviation of three measurements.
[b] nd = not detected.

4. Conclusions

An improved HPLC method using an amide-C16 column was established to rapidly and simultaneously determine 14 important compounds including eight catechins, four purine alkaloids, gallic acid and theanine in *C. sinensis*, *C. assamica*, *C. ptilophylla* and *C. assamica* var. *kucha*. This method showed a successful validation and the 14 important compounds can be determined simultaneously by this method. The present system had several advantages over previously described methods including improved chromatographic efficiency, and complete separation of more components which determine the beneficial health effects and flavor in Camellia species.

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