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Food Chemistry 100 (2007) 1132–1136
Food Chemistry
www.elsevier.com/locate/foodchem

Simultaneous analysis of purine alkaloids and catechins in *Camellia sinensis*, *Camellia ptilophylla* and *Camellia assamica* var. *kucha* by HPLC

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Received 13 September 2005; received in revised form 4 November 2005; accepted 17 November 2005

Abstract

Simultaneous analysis of purine alkaloids and catechins in tea from dry leaves of *Camellia sinensis*, *Camellia ptilophylla* and *Camellia assamica* var. *kucha* by a reversed-phase high-performance liquid chromatography (RP-HPLC) method. This HPLC method had been proved to be appropriate for the identification and quantification of purine alkaloids and catechins, and exhibited good correlation coefficients, detection levels and recovery rates. Caffeine, theobromine, theacrine, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-epicatechin gallate, (−)-gallocatechin gallate and (−)-gallocatechin were identified and quantified in the three species of genus *Camellia* Sect. *Thea*. There was 2.72% caffeine and 0.26% theobromine in *C. sinensis*, 4.85% theobromine in *C. ptilophylla*, and 1.58% theacrine, 0.94% caffeine and 0.45% theobromine in *C. assamica* var. *kucha.* Theacrine in *C. sinensis* and *C. ptilophylla*, and caffeine in *C. ptilophylla* were not detected. These data highlight differences in the relative proportions of purine alkaloids in the three species of *Camellia* Sect. *Thea*. In addition, different catechins were identified and quantified. The highest content of catechin in dry leaves was (−)-epigallocatechin gallate (EGCG) 3.51%, (−)-gallocatechin gallate (GCG) 9.88% and (−)-epigallocatechin gallate (EGCG) 6.78% in *C. sinensis*, *C. ptilophylla* and *C. assamica* var. *kucha*, respectively.

Keywords: Alkaloids; Catechins; Genus *Camellia* Sect. *Thea*; Tea; Dry leaves

1. Introduction

There are 32 species and 4 variations in *Camellia* Sect. *Thea* (Chang & Ren, 1998). Tea from *Camellia sinensis* and *Camellia assamica* is a popular beverage due to the presence of bioactive substances such as alkaloids and catechins (Fernandez, Lopez, Pablos, Gonzalez, & Martin, 2003; Robb & Brown, 2001; Wang, Provan, & Helliwell, 2003).

The stimulating effect of tea is attributed to caffeine (Luque-Perez, Rýos, & Valcarcel, 1999; Yang, Chung, & Yang, 2000). Epidemiological studies suggest that the consumption of tea may help to prevent cancer in humans because that tea leaves contain an abundance of catechins. All catechins have been shown to have antioxidant activity, and are postulated to have antimutagenic and anticarcinogenic properties (Ho, Chen, Shi, Zhang, & Rosen, 1992; Jankun, Selman, & Swiercz, 1997; Yang & Wang, 1993).

It was reported that the plants of Sect. *Thea* contain purine alkaloids (caffeine, theobromine, theophylline, theacrine, adenine, xanthine, hypoxanthine and paraxanthine) and a small amount of pyrimidine alkaloids (Fernandez et al., 2003). Caffeine is the major alkaloid present (about 2–5%) in dry leaves of *C. sinensis* and *C. assamica*, which

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doi:10.1016/j.foodchem.2005.11.021

also contains small amounts of theobromine and theophylline (Axel, Tharcisse, & Günter, 1996). Theobromine is the major alkaloid present (about 4–6%) in *Camellia ptilophylla*, which contains no caffeine or a small amount of it and a small amount of theophylline (Ye, Lin, Zhou, Chen, & Li, 1997). Theacrine is the major alkaloid present (about 1.3–3.4%) in *C. assamica* var. *kucha*, which also contains some caffeine and a small amount of theobromine and theophylline (Ye, Lin, Su, Song, & Chang, 1999).

The leaves of *C. sinensis* and *C. assamica* contain about eight catechins: (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-catechin gallate (CG), (−)-gallocatechin gallate (GCG) and (−)-gallocatechin (GC). Of these catechins, EGCG is the major one (about 3–13%) in dry leaves of the two tea plants (Delrio et al., 2004; Zhu et al., 2004). Meanwhile the type and amount of catechins present in leaves of *C. ptilophylla* and *C. assamica* var. *kucha* are unknown.

Simple or simultaneous analysis of alkaloids and catechins by HPLC had been conducted in tea leaves of various varieties belonging to *C. sinensis* and *C. assamica* (Delrio et al., 2004; Goto, Yoshida, Kiso, & Nagashima, 1996; Nakakuki, Horie, Yamauchi, & Kohata, 1999; Watanabe, Nishiyama, Yamaoto, Nagai, & Terabe, 1998; Sano et al., 2001; Yao et al., 2004). However there is little information on the simultaneous analysis of purine alkaloids and catechins in *C. ptilophylla* and *C. assamica* var. *kucha*.

The objective of this study was to determine purine alkaloids and catechins in tea from three typical tea plants with different purine alkaloid and catechin patterns by a rapid and simultaneous HPLC method. The three typical tea plants tested were *C. sinensis*, *C. ptilophylla* and *C. assamica* var. *kucha*.

2. Materials and methods

2.1. Apparatus and chemicals

The HPLC system consisted of Dionex P680 LPG pumps with a two pump gradient system, a Dionex ASI-100 autosampler, a Dionex PDA-100 photodiode array detector and a TCC-100 thermostat column oven (Dionex, Sunyvale, CA, USA). The analytical column was a Cat. No.25396-96 Mightysil RP-18 150–4.6 mm (5 μm) from Kanto chemical Co. Inc. (Tokyo, Japan). All operations and data processing were controlled by Chromeleon® (Version 6.50) software for peak identification and integration.

Caffeine (Caff), theobromine (Tb) and (+)-catechin (C) were obtained from Wako Pure Chemical Industries Company (Osaka, Japan). Theacrine (Tc) was prepared in our laboratory by HPLC. (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), gallic acid (GA), (−)-epicatechin gallate (ECG), (−)-catechin gallate (CG), (−)-gallocatechin gallate (GCG) and (−)-gallocatechin (GC) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Tedia Company, Inc. (Fairfield, OH, USA). Ultrapure water was prepared using a Millipore Milli-Q purification system (Millipore Corp, Bedford, MA). Other reagents used were of analytical grade.

2.2. Preparation of standard solutions

Standard solutions of Tb, GC, EGC, Tc, C, Caff, EC, EGCG, GCG and ECG were prepared by dissolving them in a small volume of 5% (v/v) acetonitrile containing 0.05% (v/v) phosphoric acid (85%), to generate stock concentrations of 2.184, 0.983, 0.996, 0.970, 0.729, 1.084, 0.398, 2.407, 0.983 and 0.930 mg/ml, respectively.

2.3. Preparation of sample solutions

Fresh samples consisting of one apical bud and two adjoining leaves from April to July 2004, were hand-plucked, respectively, from *C. sinensis*, *C. ptilophylla* and *C. assamica* var. *kucha* growing in the tea specimen garden in Sun Yat-Sen University, Guangzhou, China. After collection, fresh samples were steamed for 5 min and then placed in an oven at 80 °C to dry. Dry samples were stored in a dryer for use. Precise dry ground sample (0.50 g) was put into a 50 ml conical flask with a cap. Alkaloids and catechins were extracted with 40 ml boiling water for 30 min in a thermostated bath set at 90 °C and shaken once every 20 min. The extract was filtered through Whatman No. 1 paper. One milliliter of the filtrate was then diluted to 4 ml with ultrapure water. Sample solution was finally filtered through a 0.45 μm filter. The filtrate was directly injected for HPLC analysis without further treatment.

2.4. Chromatographic conditions

The HPLC analysis was performed on an analytical cartridge system, using a mobile phase of either 5% (v/v) acetonitrile (solvent A) or 50% (v/v) acetonitrile (solvent B) containing 0.05% (v/v) phosphoric acid (85%) (Goto et al., 1996). The gradient was programmed as follows. Solvent A maintained at 90% and solvent B maintained at 10% within the first 7 min. B increased linearly from 10% to 15%

Table 1
Limit of detection (LOD), limit of quantification (LOQ), linear range and correlation coefficient (*R*) of analysis

Compound	Linear range (μg)	*R*	LOD (ng)	LOQ (ng)
Tb	0.0218–2.184	0.9911	16.1181	46.2241
GC	0.0098–0.983	0.9984	15.4556	45.0132
EGC	0.0096–0.996	0.9842	26.128	62.1556
Tc	0.0097–0.97	0.9999	22.4433	60.3583
C	0.0073–0.729	0.9879	14.1898	40.1185
Caff	0.0108–1.084	0.9998	29.7966	85.1144
EC	0.0040–0.398	0.9999	20.8814	59.7008
EGCG	0.0241–2.4074	0.9989	27.4379	70.5481
GCG	0.0096–0.983	0.9992	23.2258	62.4935
ECG	0.0093–0.93	1	8.3457	22.2316

during 7–10 min, and this condition maintained for 2 min. B increased linearly from 15% to 70% during 12–20 min and this condition maintained for 2 min. The flow rate was 1.0 ml/min and 10 μl was injected. The column temperature set at 40 °C and the monitored wavelength was 231 nm.

2.5. Standard curves and limit of detection

The standard curves were made using seven different concentrations. Three replicate injections were made, and peak areas were plotted against the corresponding concentrations to generate the standard curves using linear regressions. The

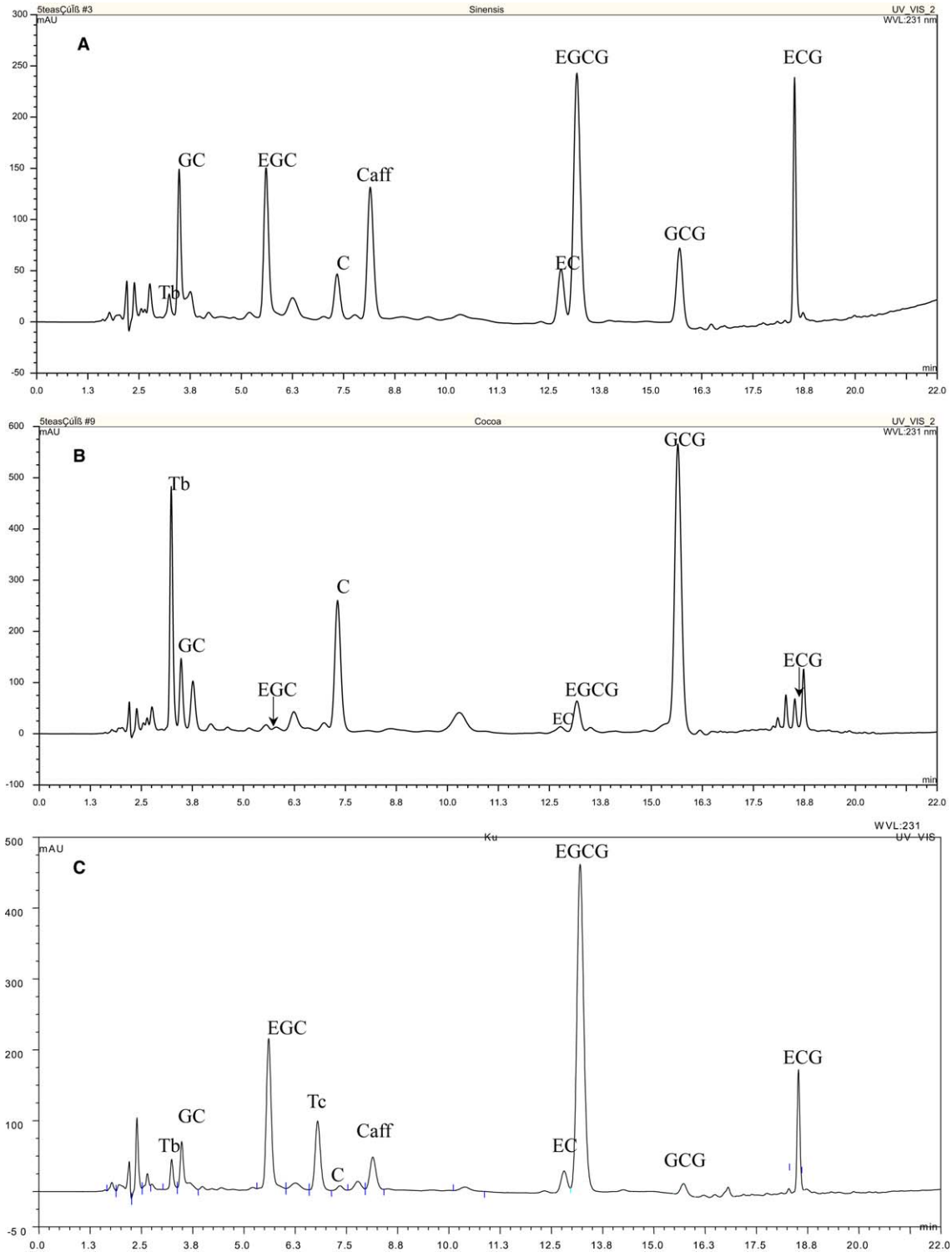

Fig. 1. Chromatograms of alkaloids and catechins in fresh young shoots of tea [*Camellia sinensis* (**A**), *Camellia ptilophylla* (**B**) and *Camellia assamica* var. *kucha* (**C**)].

limit of detection (LOD) was determined as the mass given a signal that equaled to three times of noise ($S/N = 3$). The limit of quantification (LOQ) was taken as the mass resulting in 5% or less relative standard deviation (RSD) upon quantification of the peak area. The results were summarized in Table 1.

2.6. Recovery test

Two known standards (Caff and EGCG) at three levels of about 1, 2 and 3 mg were mixed and added to sample of *C. Sinensis* (each level replicated 3 times). The extraction was conducted according to the preparation of sample solution. The recovery were calculated by comparing the determined standards to those added.

$$(\text{content})\ \% = \text{concentration}\ (\mu\text{g/ml}) \times \frac{40\ \text{ml} \times 4}{1\ \text{ml} \times 0.50\ \text{g} \times 10^5} \times 100 = \text{concentration} \times 0.032$$

3. Results and discussion

3.1. Reliability

In this study, gradient elution programs were changed little based on the gradient set by Goto et al. (1996). It showed short separation time and well shaped chromatograms (Fig. 1).

Reproducibility and precision were good. Correlation coefficients, LOD and LOQ of 10 compounds are given in Table 1. Good correlations existed for contents of three purine alkaloids and seven catechins. The recovery of Caff and EGCG were 93.27–95.04% at low level about 1 mg, 93.36–94.58% at mid-level of about 2 mg, and 96.54–97.83% at high level about 3 mg. All these results suggested that the HPLC was reliable.

3.2. Sample analysis

Samples of three species of Sect. *Thea* were analyzed for individual purine alkaloids and catechins (Table 2). The concentrations (μg/ml) of three purine alkaloids and seven catechins were calculated from the peak area of each component and its corresponding calibration curve, and their contents based on dry leaves were determined from the following expressions:

The contents of purine alkaloids were 2.73% Caff and 0.27% Tb in *C. sinensis* (Fig. 1A), 4.85% Tb in *C. ptilophylla* (Fig. 1B), 1.58% Tc, 0.94% Caff and 0.45% Tb in *C. assamica* var. *kucha* (Fig. 1C). Tc in *C. sinensis*, and Caff and Tc in *C. ptilophylla* were not detected.

The contents of catechins present >2% were 3.51% EGCG and 2.02% EGC in *C. sinensis* (Fig. 1A), 9.88% GCG and 2.23% C in *C. ptilophylla* (Fig. 1B), 6.78% EGCG and 2.95% EGC in *C. assamica* var. *kucha* (Fig. 1C). The contents of other catechins such as GC, EC, ECG, etc. were lower than 2% in samples examined.

Both the total purine alkaloids and the total catechins in *C. ptilophylla* were higher than that in the other two. The results were in agreement with the previous reports on the content of purine alkaloids and catechins in *C. sinensis* and *C. assamica* (Bonoli, Pelillo, Toschi, & Lercker, 2003; Delrio et al., 2004; Lee & Ong, 2000; Zeeb, Nelson, Albert, & Dalluge, 2000; Zhu et al., 2004).

Table 2
Relative proportion of each analyzed compounds in dry leaves of samples (%, w/w)

Compounds	Rotation time (min)	*C. sinensis*	*C. ptilophylla*	*C. assamica* Var. *kucha*
Tb	3.242	0.27 ± 0.01	4.85 ± 0.001	0.45 ± 0.004
GC	3.483	1.54 ± 0.03	1.17 ± 0.04	0.95 ± 0.06
EGC	5.608	2.02 ± 0.01	0.14 ± 0.002	2.95 ± 0.003
Tc	6.800	ND	ND	1.58 ± 0.006
C	7.375	0.46 ± 0.005	2.23 ± 0.009	0.15 ± 0.003
Caff	8.142	2.72 ± 0.001	ND	0.94 ± 0.02
EC	12.808	0.71 ± 0.003	0.20 ± 0.003	0.38 ± 0.016
EGCG	13.200	3.51 ± 0.003	0. 90 ± 0.001	6.78 ± 0.001
GCG	15.725	1.33 ± 0.002	9.88 ± 0.01	0.35 ± 0.005
ECG	18.525	1.24 ± 0.004	0.27 ± 0.003	0.90 ± 0.004
Total purine alkaloids		2.99	4.85	2.97
Total catechins		10.81	14.79	12.46

ND, not detected. All values were the averages and standard deviations of three replicate determinations.

Acknowledgement

Appreciation is expressed to the Laboratory of Medicine Research Institute, Jinan University, Guangzhou, for support of HPLC system.

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