ELSEVIER

Journal of Chromatography A, 881 (2000) 439–447

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Comparative analysis of tea catechins and theaflavins by high-performance liquid chromatography and capillary electrophoresis

Bee-Lan Lee, Choon-Nam Ong*

Department of Community, Occupational and Family Medicine, National University of Singapore, Singapore 117597, Singapore

Abstract

This paper describes the simultaneous determination of catechins and theaflavins in green and black teas, using reversed-phase high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The tea polyphenols analyzed included (+)-catechin, catechin gallate, (−)-epicatechin, epicatechin-3-gallate, epigallocatechin, epigallocatechin-3-gallate, theaflavin, theaflavin-3-monogallate, theaflavin-3′-monogallate and theaflavin-3,3′-gallate. These polyphenols together with six other tea ingredients such as caffeine, adenine, theophylline, quercetin, gallic acid and caffeic acid were separated within 27 min by HPLC and in less than 10 min by CE. The optimal analytical conditions of both chromatographic methods were investigated for the convenience and reliability for routine analysis. Both HPLC and CE were found to be reliable and compatible. The reproducibility of the within-day assay using both methods was generally >90%. The day-to-day variation of retention time was <5% for HPLC, while the variation of migration time for CE was <2%. The analysis time of CE was three-times faster, however it is five-times less sensitive than HPLC, which has detection limits of 0.05 μg/ml and 0.5 μg/ml for catechins and theaflavins, respectively.

Keywords: Tea; Food analysis; Catechins; Theaflavins; Flavonoids; Polyphenols

1. Introduction

Tea is a popular beverage. Green tea (non-fermented) is more popular among the Chinese and Japanese, whereas black tea (fermented) is preferred in India, Southeast Asia, Africa and Europe. Recent epidemiological studies suggest that the consumption of tea may help to prevent cancers in humans. This is because tea leaf contains abundant groups of polyphenols. (−)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol component in green tea, and this and other catechins have been shown to have antioxidant activity, and are postulated to have antimutagenic and anticarcinogenic properties [1–3]. Among black tea polyphenols, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-gallate are generally considered to be the more effective components for the inhibition of carcinogenesis [4,5]. A recent study also reported theaflavin-3,3′-gallate to be a better inhibitor of tyrosine receptor kinase than green tea polyphenol EGCG [6]. Thus, the increasing interest in tea polyphenols and their anticarcinogenic effects have led to many analytical developments for their determination. In the literature, several methods have been established based upon reversed-phase high-performance liquid chromatography (HPLC) for catechins analysis in green tea [7–10], in food [11] and in human bio-

*Corresponding author. Tel.: +65-8744-982; fax: +65-7791-489.

E-mail address: cofongcn@nus.edu.sg (C.-N. Ong)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00215-6

logical samples [12–15]; as well as analyses using capillary electrophoresis (CE) were reported [16–21]. In contrast, fewer analytical methods were established for analysis of theaflavins (TFs) in black tea [22,23]. Furthermore, neither HPLC nor CE methods have been developed for simultaneous analysis of catechins and TFs in green or black tea.

This paper describes the direct analysis of six types of catechins, four types of TFs and other major components of tea by HPLC and CE. The optimum conditions for both analytical methods were investigated for best resolution and highest sensitivity of detection. The proposed procedures have been evaluated with various types of popular and commercially available tea produced from China, Japan and India. The results showed that both the HPLC and CE methods were compatible and promising.

2. Experimental

2.1. Reagents and chemicals

Standards of the analytes: (+)-catechin (C), catechin gallate (CG), (−)-epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), caffeine, adenine (A), theophylline (T), gallic acid (GA) and caffeic acid (CA) were purchased from Sigma (St. Louis, MO, USA). The TF mixture containing theaflavin, theaflavin-3-monogallate theaflavin-3′-monogallate and theaflavin-3,3′-gallate was a gift from Dr. S. Wiseman of Unilever Nutrition Centre (The Netherlands). β-Cyclodextrin (β-CD) and trifluoroacetic acid were also purchased from Sigma. Boric acid, potassium dihydrogenphosphate, absolute alcohol and acetonitrile (HPLC-grade) were obtained from Merck (Darmstadt, Germany). Distilled and deionized water was used for the preparation of all samples and solutions.

2.2. Standard preparation

The main stock solutions (2 mg/ml) of caffeine, Q and TFs were prepared by dissolving 2 mg of the respective compound in 0.2 ml of ethanol then followed by 0.8 ml of water. The main stock solutions of the other tea ingredients were prepared individually from 2 mg of the respective compound dissolved in 1 ml of 20% (v/v) acetonitrile. Working standards for calibration were prepared with concentrations ranging from 0.05 to 500 μg/ml of analytes.

2.3. Sample preparation

Various types of tea samples were randomly selected and purchased in Singapore. There were six types of common Chinese tea including Long-jing, Pu-erh, Jasmine, Chrysanthemum, Iron Buddha and Oolong tea, one type of Japanese green tea and one type of Ceylon tea. As the catechins were photo sensitive, the samples preparation was carried out under a dim lighting working environment. The sample was prepared by extracting 0.1 g of the tea leaf with 10 ml of boiling water and incubating at 90°C for 30 min including shaking for 30 s. This condition was similar to an actual brewing condition for a cup of tea. An aliquot of 0.2 ml of the tea extract was mixed with 0.4 ml of 20% (v/v) acetonitrile in an amber centrifuge tube. After centrifugation at 15 000 *g* for 2 min, the samples was transferred into two vials, one for HPLC and the other for CE analysis.

2.4. Chromatography

The HPLC system used consisted of a Hewlett-Packard (HP) Model 1050 quaternary pumping system (Palo Alto, CA, USA), with a Gilson Model 231-401 autoinjector (Villiers-le-Bel, France), a Waters photodiode array detection (DAD) system (Model 996) was used for peak purity determination and Millennium 2010 software for peak identification and integration (Milford, MA, USA). The chromatographic separation was performed on a guard and analytical cartridge system (PartiSphere 5 C_{18}, 5 μm, 110 mm×4.6 mm I.D.) (Whatman), with column temperature set at 32°C. A Whatman Solvent IFD disposable filter device was used for in-line filtration and degassing of the mobile phase. Sixteen types of tea ingredients were detected with UV detection at 205 nm. The two mobile phases used for gradient HPLC elution were (A) 5% (v/v) acetonitrile containing 0.035% (v/v) trifluoroacetic acid; and (B) 50% (v/v) acetonitrile containing 0.025% (v/v)

trifluoroacetic acid. The flow-rate was set at 1.0 ml/min. The gradient elution profile started with A–B (90:10), B was gradually increased to 20% at 10 min, to 40% at 16 min, to 50% at 20 min and back to 40% from 25 to 27 min. The column was than re-equilibrated with the initial conditions for 3 min before the next injection. The injection volume was 10 μl.

A Hewlett-Packard ^{3D}CE system was also used for the determination of polyphenols in green and black teas. The capillary cartridge contained an extended light path capillary (40 cm×50 μm I.D.). A buffer solution was freshly prepared by mixing 800 μl of 500 m*M* boric acid (pH 7.2), 200 μl of 100 m*M* potassium dihydrogenphosphate (pH 4.5), 450 μl of 20 m*M* β-CD and 550 μl of acetonitrile in a 2-ml centrifuge tube. After centrifugation at 15 000 *g* for 2 min, each 0.5-ml aliquot of this solution was transferred into three sample vials, one was used for column preconditioning and the other two as inlet and outlet vials. Every batch of this solution could be used for three electrophoretic analyses without changes in separation efficiency. Prior to sample introduction, the capillary column was preconditioned with the buffer solution for 3 min. The sample introduction was carried out by pressurized injection of buffer, sample and buffer into the capillary column, each at 2.5 kPa for 3 s subsequently. Electrophoresis was carried out for 10 min at 25 kV with the column oven temperature set at 30°C. UV absorbance detection using DAD was set at 205 nm. Peak height measurement was carried out using the HP ^{3D}CE ChemStation software.

3. Results and discussion

3.1. Chromatographic performance and sensitivity

Dalluge et al. have reported that the presence of trifluoroacetic acid is essential for high resolution and efficient chromatography of catechins in tea [9]. Although the most sensitive HPLC method for catechins analysis was reported by Nakagawa and Miyazawa using chemiluminescence detection, with a detection limit of 1 pg/ml [12], this level of sensitivity is in fact not required for the analysis of major tea polyphenols. It has been shown earlier that a detection sensitivity of 0.5–1 μg/ml of different catechins in green tea could be obtained with UV absorbance at 280 nm [16]. On the other hand, it was reported that most HPLC methods monitored TFs at 375 nm [7]. In the present study it was observed that with the use of acetonitrile instead of methanol as the eluent, a much higher UV absorbance could be detected at 205 nm instead of 275 or 375 nm. Thus, in the proposed study two mobile phases used for the gradient separation consisted of different compositions of acetonitrile and trifluoroacetic acid. The UV spectra of all tea polyphenols were reviewed using DAD. Fig. 1 shows chromatograms of an injection of a standards mixture (200 μg/ml caffeine; 500 μg/ml TFs; 100 μg/ml EGCG and Q; and 50 μg/ml of the rest of analytes) extracted from the three respective wavelengths. The results demonstrate that a much higher sensitivity is obtained at UV 205 nm. Using the proposed HPLC conditions, a low concentration of aqueous standard containing 0.2 μg/ml caffeine; 0.5 μg/ml TFs; 0.1 μg/ml of EGC, EGCG and Q; and 0.05 μg/ml for the rest of analytes could be separated within 27 min, as shown in Fig. 2a. The detection limits for most analytes were around 0.05 μg/ml and 0.5 μg/ml for TFs (signal-to-noise ratio>3). The total analysis time was 30 min per injection, which included a 3 min allowance for column equilibration prior to the next injection.

To date, only a few CE methods have been introduced for the analysis of catechins and TFs in tea samples. This may be due to the fact that analyses using CE are generally more complex and conditions are more difficult to optimize for the complete separation of numerous catechins and other tea components. Although the use of borate–phosphate–sodium dodecyl sulfate buffer system in CE has been reported to offer much better resolution of catechins [20,21], our preliminary investigation suggest that they do not allow for efficient detection of theaflavins. In addition, the consistency and reproducibility of the CE method are usually much more difficult to achieve. It has been reported that CE determination of polyphenols present in green tea could be achieved in about 20 min with 150 m*M* boric acid as buffer (pH 8.5) [18]. However, the application of boric acid alone in buffer solution was found to be inadequate for the complete separation of all tea polyphenols. Increase of electrophoretic volt-

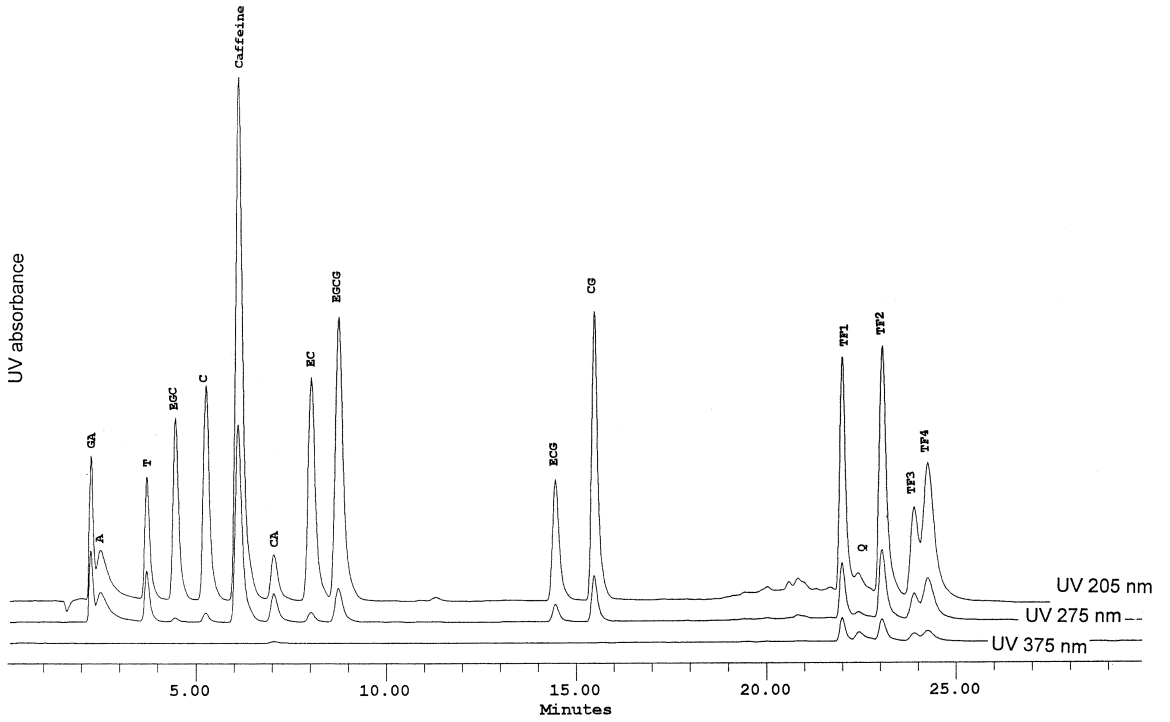

Fig. 1. Chromatograms of a standards mixture (200 μg/ml caffeine; 500 μg/ml TFs; 100 μg/ml EGCG and Q; and 50 μg/ml of the rest of analytes) monitored at UV 205, 275 and 375 nm.

age or temperature did not show much improvement on the resolution. On the other hand, it was noted that the components of the running buffer play an important role. In the present study, we carried out a rather extensive investigation on the effect of pH and buffer concentration for the separation of various tea compounds. It was observed that in order to achieve an efficient separation of TFs, it was essential to maintain the boric acid concentration around 150 m*M* to 200 m*M*. Furthermore, the addition of 20–25% (v/v) acetonitrile was found useful in reducing the solute–wall interactions and thus enhanced the efficiency of the separation. However, the concentrations of boric acid and acetonitrile for electrophoretic separation of TFs did not appear to affect the separation of catechins in a similar manner. To alter the selectivity, addition of a chiral selector, β-CD, was necessary. Thus, the ratio of β-CD, boric and acetonitrile in the running buffer was critical; changed in either factor could affect the separation of catechins from TFs. Owing to the high ionic strength of boric acid, 10 m*M* of potassium dihydrogenphosphate was also added to maintain the buffer conductivity and minimize catechins and caffeine peak distortions. Based on the present investigation the optimum conditions for highest resolution were obtained with a running buffer solution containing 200 m*M* boric acid (pH 7.2), 10 m*M* potassium dihydrogenphosphate (pH 4.2), 4.5 m*M* of β-CD and 27.5% (v/v) of acetonitrile. Compared to the earlier studies [16–21] the efficiency of the present CE method was much higher. The complete separation of 16 tea ingredients can be achieved within 10 min (Fig. 3). However, the lowest detection limits of tea polyphenols when analyzed using CE were about five-times lower than using HPLC method.

3.2. Analysis using HPLC and CE

The chromatograms of a green tea and a black tea extract are shown in Fig. 2c and d, respectively. The electropherograms of the same samples as determined by CE method are shown in Fig. 3c and d, respectively. It can be seen that the separation

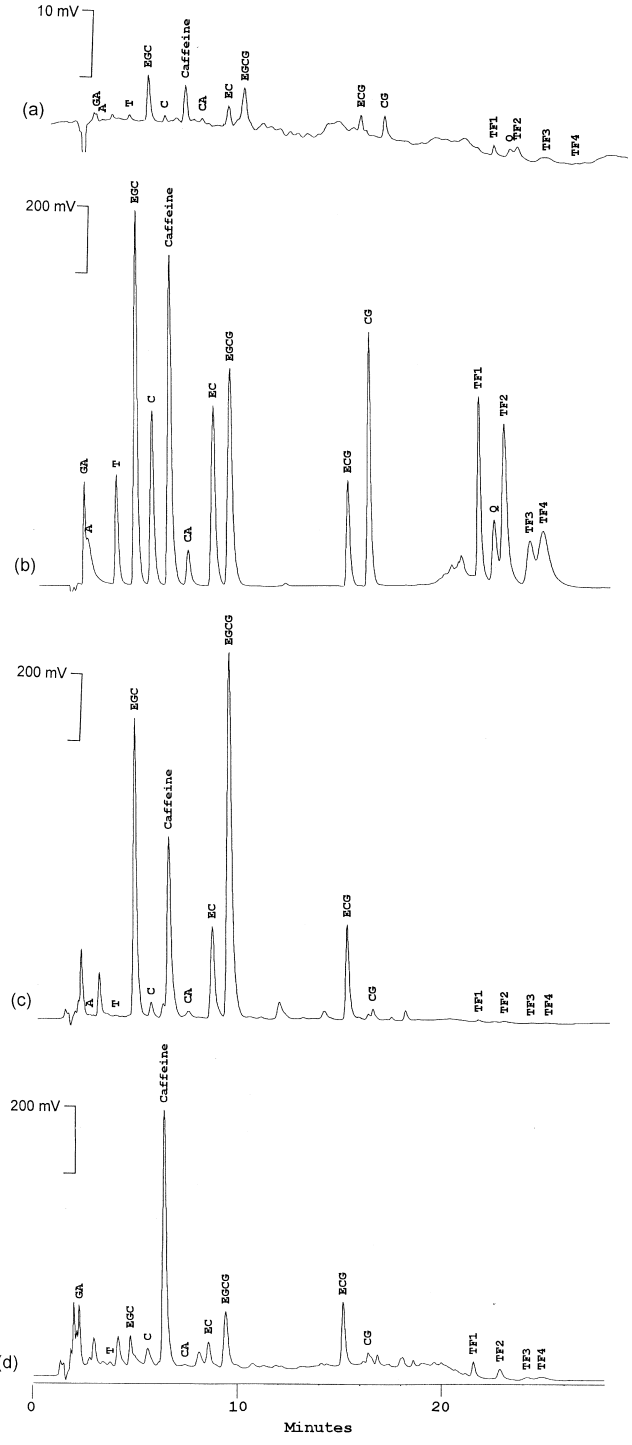

Fig. 2. Chromatograms of (a) a low concentration of pure standards containing 0.2 μg/ml caffeine, 0.1 μg/ml EGC, EGCG and Q; 0.5 μg/ml TFs and 0.05 μg/ml for the rest of tea constituents; (b) a 1000-fold higher concentration of standards mixture; (c) a green tea sample and (d) a black tea analyzed by the present HPLC method with UV absorbance detection at 205 nm.

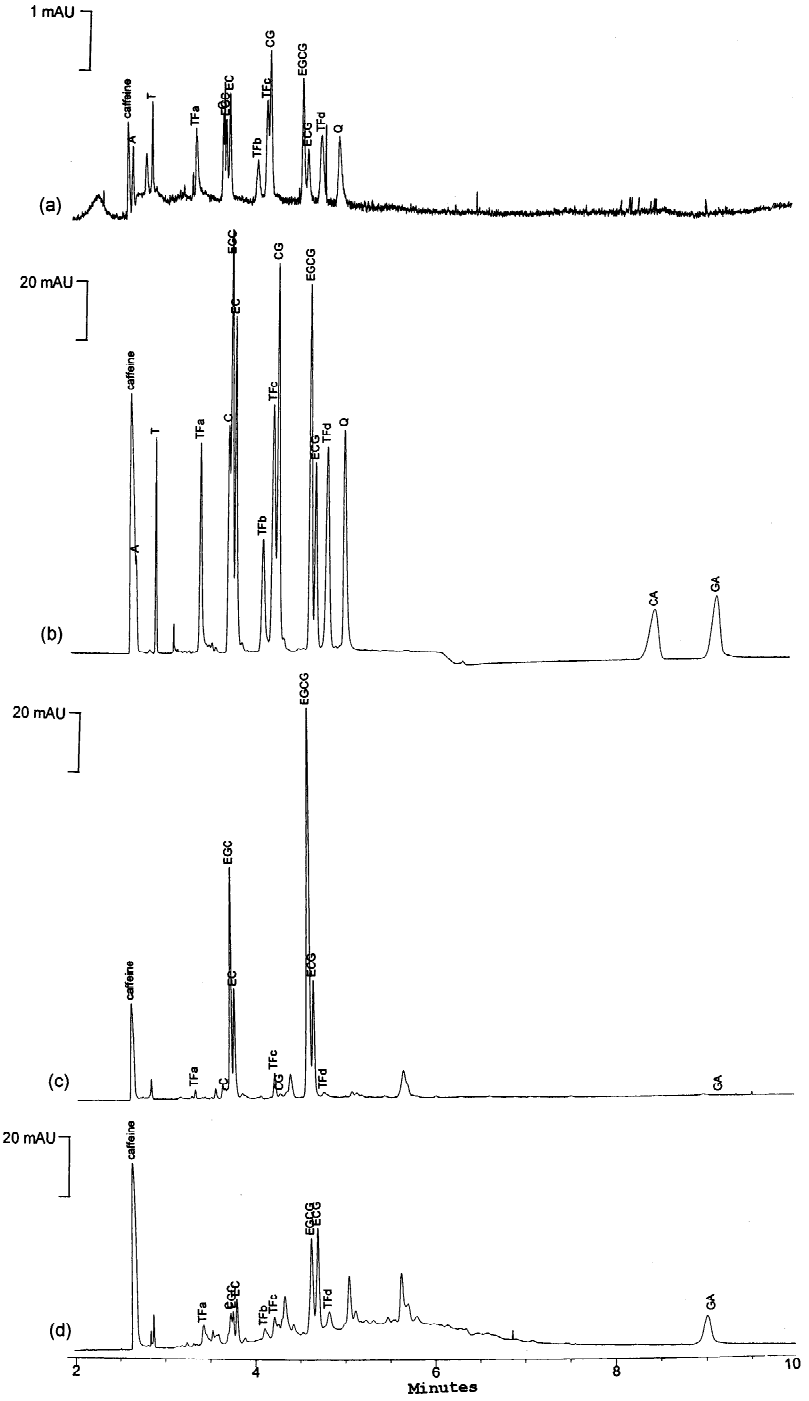

Fig. 3. Electropherograms of (a) a 10-fold higher concentration standards as in Fig. 2a; (b), (c) and (d) were the same samples as in Fig. 2b, c and d, respectively; analyzed by the proposed CE technique with UV absorbance detection at 205 nm.

profiles of tea polyphenols by CE (Fig. 3) are different from that of HPLC (Fig. 2). However, the analytes of interest were found to have identical retention times as that of the standards (Fig. 2a and b; Fig. 3a and b), suggesting that both methods provide equal chromatographic efficiency for the catechins and TFs. Using the HPLC, the retention times of all analytes were reproducible. The relative standard deviations (RSDs) of <3% for within-day assay and <5% for between-days analysis. For the CE method, the RSDs of migration time for within-day and between-days analysis were <1% and 2%, respectively.

The elution of TFs is generally more complex as they tend to be retained in the C_{18} column and are organic modifier dependent. Simultaneous HPLC analysis of catechins and TFs could only be achieved with gradient elution. Using the proposed method the total analysis time was 30 min, including 3 min for column re-equilibration. This is considered short, as earlier methods only allow for individual polyphenol analysis [7–10,22,23].

In terms of the speed of separation, CE is obviously much faster than HPLC, especially for TFs. Total analysis time for tea catechins and theaflavins could be achieved with 6 min. Although the proposed HPLC methods are more sensitive, the CE method is considered more convenient for routine analysis. However, the use of CE has a tangible benefit of substantial saving on the consumption of mobile phase as compared to HPLC. In long term, CE method is considered as more time and cost effective than HPLC.

3.3. Sample preparation and matrix interference

To date, HPLC method available for TFs analysis is scarce [22,23]. This may due to the difficulty in obtaining the individual standards of TFs or the lack of sophisticated equipment for identification. In our present study, a standard mixture known to contain theaflavin, theaflavin-3-monogallate, theaflavin-3′-monogallate, theaflavin-3,3′-gallate was well separated (Figs. 2 and 3). However, they could not be confirmed by using UV detection alone. These four peaks detected were labeled as TF1, TF2, TF3 and TF4 in the chromatograms as shown in Fig. 2, and TFa, TFb, TFc and TFd in the electropherograms of Fig. 3. The calibration was done by total peak height of the four compounds vs. the known series concentrations of standard mixture. As shown in Fig. 2 the analytes were free from the interference of other tea components. Although under the present CE condition, C was found not completely separated from EGC, however the concentration of C in tea samples is known to be of lower concentration, thus peak height quantification of both peaks by using the HP ChemStation software was not crucial (see Table 1 for linearity and day-to-day variation data). Peak purity assessment was conducted for every peak using DAD for both HPLC and CE. No evidence of coelution of other compound was found during the

Table 1
Linearity and day-to-day variation ($n=3$)[a]

Compound	Concentration	HPLC				CE			
	(μg/ml)	Mean		RSD (%)		Mean		RSD (%)	
		LR	r	LR	r	LR	r	LR	r
C	0.05–50	$y=0.04+0.073x$	0.999	21.9	0.12	$y=0.93+0.697x$	0.992	17.6	0.90
CG	0.05–50	$y=0.06+0.052x$	0.998	17.1	0.14	$y=0.45+0.449x$	0.992	5.7	0.68
EC	0.05–50	$y=0.05+0.068x$	0.999	17.0	0.07	$y=0.24+0.492x$	0.994	12.1	0.90
ECG	0.05–50	$y=0.04+0.127x$	0.998	17.6	0.14	$y=0.23+0.849x$	0.991	12.8	0.44
EGC	0.05–100	$y=0.04+0.113x$	0.999	21.9	0.14	$y=0.06+1.014x$	0.994	12.1	0.86
EGCG	0.05–100	$y=0.04+0.117x$	0.999	15.9	0.06	$y=0.68+0.939x$	0.990	8.6	0.88
TFs	0.5–100	$y=0.04+0.446x$	0.999	15.8	0.04	$y=-2.03+1.936x$	0.995	32.2	1.61

[a] Note: LR=Linear regression; y=concentration (μg/ml); x=peak height (mV) for HPLC or peak height (mAU) for CE; r=coefficient of correlation; RSD=relative standard deviation.

determination, suggesting that direct injection of tea sample provide desire results by both methods.

3.4. Reliability and quantification

Using the present HPLC method, we analyzed eight commercially available tea samples and the results of tea catechins and TFs are shown in Table 2. The results are in good agreement with the fact that green tea generally has higher concentrations of catechins such as EC, ECG, EGC and EGCG and only a minute amount of TFs. In contrast, black tea is processed after fermentation the EC and other catechins have been oxidized to form TFs. It was noted that there was a 5% drop of EGCG concentration and 10% for TFs if samples were allow to remain at room temperature for 3 h and at 4°C for 24 h.

The proposed CE method was also validated with the same black and green tea extracts. The values obtained for various catechins were closed to those obtained from HPLC (Table 2). The coefficients of correlation (r) for analysis using HPLC and CE generally exceeded 0.97. It was however noted that the TFs values were generally higher when analyzed using CE than HPLC. This could probably attributed to the instability of the TFs, as longer time is needed for HPLC analysis, in addition to the fact that CE tends to be also less sensitive. Nevertheless, the overall data showed that the r for TFs analyzed using HPLC and CE generally exceeded 0.96 (Table 2), indicate that both methods are also in good agreement.

The green and black teas samples of Figs. 2 and 3 were analyzed three times a day and on three consecutive days, with samples kept at 4°C at all time. The reproducibility of within-day assay for both HPLC and CE methods were >90% and >75% for between-days analyses, respectively.

In summary, in the present study we investigated the optimum analytical conditions for catechins and TFs determination using HPLC and CE. The findings show that both methods were sensitive enough to detect all known polyphenols in green and black teas. These methods allow direct injection of diluted tea extract without further sample pretreatment. Both analytical conditions described here have been optimized to offer the most rapid, reliable and effective approach for routine analysis. The very small volumes of reagents required for CE analysis greatly minimizes the problems associated with solvent disposal by HPLC. Thus, the CE method is considered an alternate useful and time effective tool for tea analysis.

Acknowledgements

We thank Ms. Sylvia Cha for her technical assistance. This project was partially supported by the National Medical Research Council grant No. NMRC/0336/1999 and the Centre for Environment

Table 2
Comparison of HPLC and CE for the determination of major tea polyphenols (mg/g tea leaves)

Sample	EC		ECG		EGC		EGCG		TFs	
	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE	HPLC	CE
Japanese green tea	6.06	7.27	5.34	6.13	36.53	35.59	18.10	23.12	0.88	1.79
Long-jing	5.27	4.90	9.97	8.98	28.07	21.06	35.46	32.93	1.50	5.56
Jasmine	6.06	6.93	12.66	13.79	23.46	24.56	29.83	30.96	1.81	3.65
Chrysanthemum	8.59	9.91	12.58	13.21	18.62	19.92	16.85	19.11	1.03	4.32
Pu-erh	0.49	0.33	0.07	0.11	0.60	0.12	0.30	0.12	1.03	0.68
Iron Buddha	4.27	4.85	3.35	3.28	30.61	29.41	11.82	12.24	0.66	2.02
Oolong tea	1.75	1.88	3.58	3.07	7.70	3.57	8.99	7.36	0.66	3.63
Ceylon tea	1.41	2.19	6.82	8.92	2.84	3.23	5.52	9.18	10.70	17.28
y – HPLC; x – CE	$y=0.12+0.899x$		$y=0.22+0.915x$		$y=1.53+0.991x$		$y=-1.01+1.00x$		$y=-0.78+0.629x$	
r – coefficient of correlation	0.988		0.981		0.977		0.977		0.965	

and Occupational Health Research, National University of Singapore.

References

[1] C.T. Ho, Q. Chen, H. Shi, K.Q. Zhang, R.T. Rosen, Prev. Med. 21 (1992) 520.
[2] C.S. Yang, Z.Y. Wang, J. Natl. Cancer Inst. 85 (1993) 1038.
[3] J. Jankun, S.H. Selman, R. Swiercz, Nature 387 (1997) 561.
[4] M.A. Lea, Q. Xiao, A.K. Sadhukhan, S. Cottle, Z.Y. Wang, C.S. Yang, Cancer Lett. 68 (1993) 231.
[5] Y.P. Lu, Y.R. Lou, J.G. Xie, P. Yen, M.T. Huang, A.H. Conney, Carcinogenesis 18 (1997) 2163.
[6] Y.C. Liang, Y.C. Chen, Y.L. Lin, S.Y. Lin-Shiau, C.T. Ho, J.K. Lin, Carcinogenesis 20 (1999) 733.
[7] A. Finger, S. Kuhr, U.H. Engelhardt, J. Chromatogr. 624 (1992) 293–315.
[8] S. Khokhar, D. Venema, P.C.H. Hollman, M. Dekker, W. Jongen, Cancer Lett. 11 (1997) 171.
[9] J.J. Dalluge, B.C. Nelson, J.B. Thomas, L.C. Sander, J. Chromatogr. A 793 (1998) 265.
[10] P.C.H. Hollman, D.P. Venema, S. Khokhar, I.C.W. Arts, Oxidants Antioxidants 299 (1999) 202.
[11] U. Justesen, P. Knuthsen, T. Leth, Cancer Lett. 114 (1997) 165.
[12] K. Nakagawa, T. Miyazawa, Anal. Biochem. 248 (1997) 41.
[13] G. Maiani, M. Serafini, M. Salucci, E. Azzini, A. Ferro-Luzzi, J. Chromatogr. B 692 (1997) 311.
[14] P.H. Gamache, I.N. Acworth, Biol. Med. 217 (1998) 274.
[15] J.J. Dalluge, B.C. Nelson, J.B. Thomas, M.J. Welch, L.C. Sander, Rapid Commun. Mass Spectrom. 11 (1997) 1753.
[16] J. Harms, G. Schwedt, J. Fresenius, J. Anal. Chem. 350 (1994) 93.
[17] H. Horie, T. Mukai, K. Kohata, J. Chromatogr. A 758 (1997) 279.
[18] L. Arce, A. Rios, M. Valcarcel, J. Chromatogr. A 827 (1998) 113.
[19] B.C. Nelson, J.B. Thomas, S.A. Wise, J.J. Dalluge, J. Microcol. Sep. 10 (1998) 671.
[20] P.J. Larger, A.D. Jones, C. Dacombe, J. Chromatogr. A 799 (1998) 309.
[21] M.B. Barroso, G. van de Werken, J. High Resolut. Chromatogr 22 (1999) 225.
[22] C.M. Temple, M.N. Clifford, J. Sci. Food Agric. 74 (1997) 536.
[23] C.W. Chen, Y.W. Chang, L.S. Hwang, J. Food Drug Anal. 6 (1998) 713.