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Short communication

Simultaneous analysis of tea catechins, caffeine, gallic acid, theanine and ascorbic acid by micellar electrokinetic capillary chromatography

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Abstract

A micellar electrokinetic capillary chromatography (MEKC) method for the simultaneous analysis of five tea catechins, theanine, caffeine, gallic acid and ascorbic acid has been developed. The catechins are (−)-epicatechin, (+)-catechin, (−)-epigallocatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate. *p*-Nitrophenol serves as both reference and internal standard. All the components are separated within 13 min with a 57 cm uncoated fused-silica column. On-column detection was carried out at 200 nm. This method has been used to measure these compounds in fresh tea leaves and tea liquor. The limit of detection for all analytes ranged from 1 to 20 μg/ml. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Catechins are the most abundant polyphenols in the leaves of tea (*Camellia sinensis*). They may constitute up to 30% of the dry mass of tea leaves. The major catechins in tea are (+)-catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg) and (−)-epigallocatechin gallate (EGCg). The total catechin content is highest in young leaves and decreases significantly with leaf aging [1]. A positive correlation has been found between the quality of made black tea and the catechin composition in fresh leaves of Kenyan cultivars [2]. Caffeine (3–4%, w/w), theanine (1–2%, w/w) and gallic acid also contribute to the quality of green and black tea. Some of the gallated catechins are de-esterified during the manufacturing process to yield free gallic acid in the made tea. Bottled iced teas are treated with tannin acylhydrolases (tannases) to hydrolyze gallated theaflavins and prevent complex formation with caffeine. These complexes aggregate at low temperatures, forming a sediment known as 'cream'. Gallic acid is produced during the hydrolysis reaction and influences the pH and color of the tea. Bottled ice tea is fortified with ascorbic acid for preservation and nutrition.

Several studies indicate that catechins and polyphenols in general, from fruits, vegetables, tea and red wine, have prophylactic properties that are beneficial to the health of humans. A lower relative

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risk for stomach [3], esophageal [4] and lung cancer [5] has been shown with increased tea consumption. Increased tea consumption has also been associated with a decreased incidence of stroke [6], lower atherogenic index [7] and improved liver function [8].

Gradient reversed-phase HPLC is the most frequently used method for analysis of catechins [9,10]. Capillary zone electrophoresis (CZE) has been used to analyze compounds in tea samples [11,12]. Several micellar electrokinetic chromatographic (MEKC) methods have been developed for the separation of caffeine, theanine, ascorbic acid and several tea catechins [13–18]. The resolution of the analytes was much better with MEKC than with CZE. The MEKC methods utilize borate or boric acid as a selector for polyphenols with vicinal hydroxyl groups in conjunction with hydrophobic interactions with the surfactant to achieve separation of the analytes. The structures of the five major catechins, caffeine, gallic acid, ascorbic acid and theanine are shown in Fig. 1. None of the above MEKC methods separates this set of nine compounds. Here we report a MEKC method that achieves the separation of the

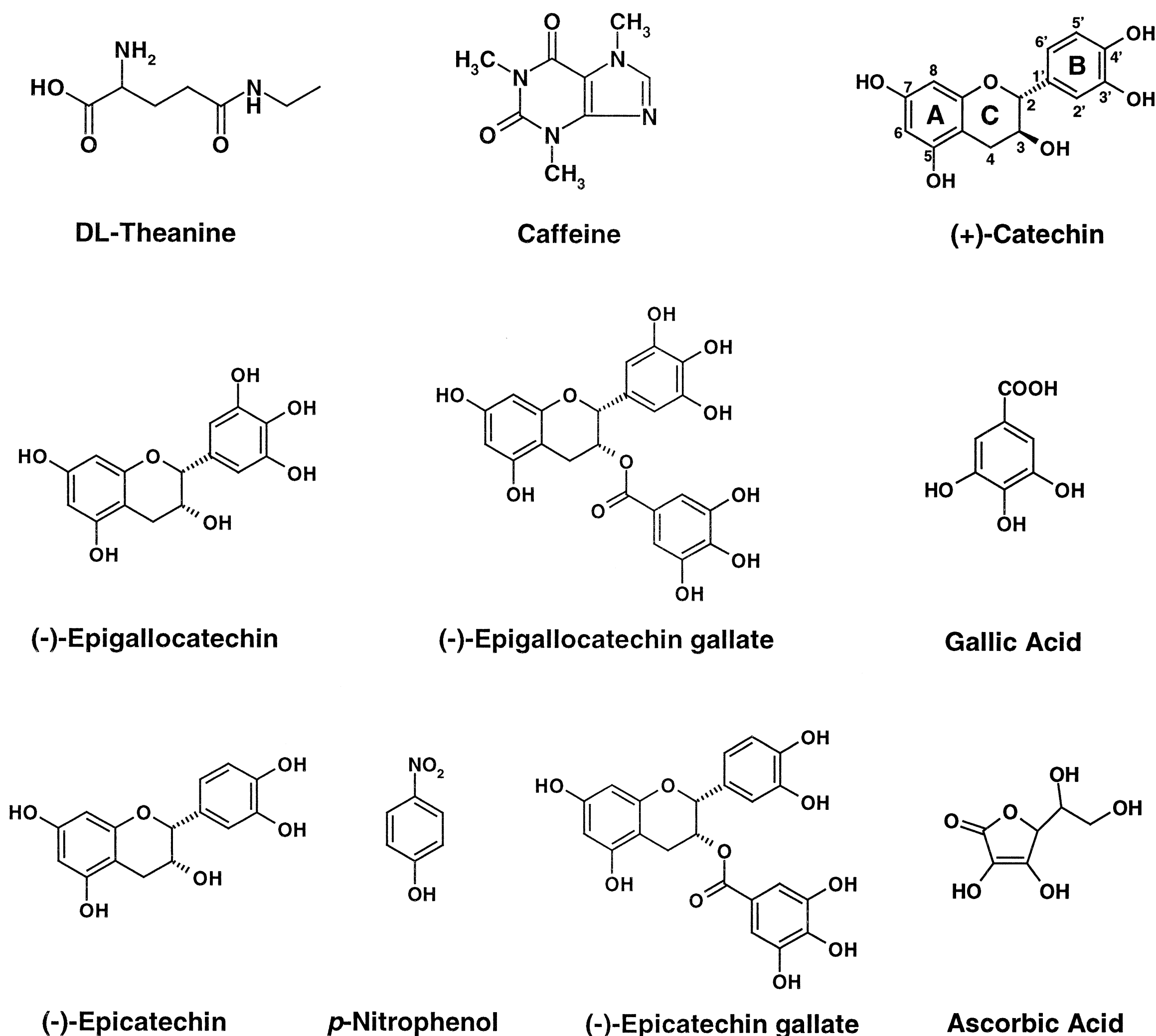

Fig. 1. Chemical structures of the major tea compounds and the pNP internal standard.

tea catechins without using borate or boric acid as a selector in the running buffer. This method provides the additional separation and quantification of gallic acid. A univariate optimization method was followed to determine the optimal buffer pH, surfactant concentration, organic solvent concentration, phosphate concentration and applied voltage.

2. Conditions

2.1. Instrumentation

Electrophoresis was carried out using the Beckman P/ACE 2100 (Beckman Instruments, Fullerton, CA, USA) with on-column detection. An uncoated fused-silica capillary column from Beckman with an internal diameter of 50 μm and total length of 57 cm was used. The effective separation length was 50 cm. The operating temperature was 25°C. Detection was effected by measurement of UV absorbance at 200 nm.

2.2. Reagents

The five catechin standards C, EC, EGC, ECg and EGCg as well as theanine were gifts from Mitsui Norin (Tokyo, Japan). The gallic acid and ascorbic acid were obtained from Sigma (St. Louis, MO, USA). Caffeine and sodium dodecylsulphate (SDS) were obtained from E. Merck (Darmstadt, Germany). The SDS was of analytical grade suitable for electrophoresis. All buffer salts were of analytical grade. All solutions were prepared with distilled water that was deionized with a Milli-Q system (Millipore, Bedford, MA, USA). *p*-Nitrophenol (pNP) was obtained from BDH (Poole, UK).

2.3. Analytical conditions

Dried tea leaf and black tea samples were analyzed with a running buffer of 100 m*M* SDS, 25 m*M* phosphate, 6% (v/v) methanol pH 7.0. Bottled tea samples were analyzed with the running buffer containing 5% (v/v) methanol. All samples were injected pneumatically (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for 2 s and were analyzed with an applied voltage of 14 kV.

At the beginning of each day the capillary was regenerated by rinsing for 5 min with water, 10 min with 0.1 *M* HCl, 5 min with water, 5 min with 1.0 *M* NaOH, 5 min with water, 10 min with 0.1 *M* NaOH and finally 2 min with water. The column was equilibrated by repeating five analyses of the standards after the regeneration. Before each analysis the capillary was rinsed with running buffer for 2 min. After each analysis the capillary was rinsed for 30 s with water, 2 min with 0.1 *M* NaOH and 2 min water. The capillary was stored in water overnight.

2.4. Preparation of samples and standard

Tea liquors were prepared from made black tea samples and dried fresh tea leaves as 1% (w/v) tea solutions. The water extracts of dried tea leaves and black tea leaves were made by shaking them for 10 min in boiling hot water in a thermal flask. The extracts were then filtered through Schleicher & Schüll No. 595 filter paper (Germany) to remove particulate matter. Black tea samples were diluted to a concentration of 0.5% (w/v) before analysis. For the analysis of dried fresh tea leaf samples, a dilution of 0.5% (w/v) was used for catechin and theanine determination, while a 0.1% (w/v) dilution was used for the determination of all remaining compounds. All samples were spiked with the internal standard to give a final concentration of 0.1 mg/ml pNP. pNP was added to commercial bottled ice tea at a concentration of 0.1 mg/ml before analysis. A separate standard solution was prepared for each analyte. All standards were dissolved in water. EC and ECg were prepared at 0.5 mg/ml, theanine and ascorbic acid at 10 mg/ml, pNP at 5 mg/ml and all the other analytes at 1 mg/ml.

2.5. Method validation

Precision tests were performed to determine both intra-day and inter-day variation in migration times. Calculations are based on the analysis of dried fresh tea leaf samples. The statistical evaluation was carried out from data from 32 consecutive runs for the intra-day precision test and data from 9 days with a minimum of 10 runs per day for the inter-day precision test. Repetitive runs were carried out with dried fresh leaf extracts, black and bottled teas to

determine the reproducibility of both the extraction and analysis of the samples. The same batch of dried fresh tea leaf or black tea was extracted five separate times and each infusion was analyzed in five-fold. Five spiked solutions were prepared from one sample of bottled tea and analyzed in five-fold.

3. Results and discussion

3.1. Factors influencing the analysis

Polyphenols have pK_a values between 8 and 10. This causes them to be neutral to partially anionic in the tested pH range 6.6 to 8.0. The peak shapes of EGC, EGCg, ECg were affected significantly by an increase in pH. The polyphenols showed extensive peak tailing and distortion above pH 7.6 (results not shown). The catechins are chemically unstable in an alkaline environment and this may cause the peak distortions. Ascorbic acid showed an increased leading front with a decrease in pH. The peak shapes of gallic acid, caffeine, theanine and pNP were unaffected by pH changes in this range. The optimum pH was chosen as 7 because peak shapes did not improve with further decrease in pH. Lower pH only increased the analysis time and not the resolution of the analytes.

The SDS concentration was tested in the range 25–200 m*M*. An increase in the SDS concentration helped to resolve the different analytes. An increase in surfactant concentration showed an increase in the capacity factor (k') of all analytes except for theanine, gallic acid and ascorbic acid. They had a higher affinity for the bulk phase than for the micellar phase and their ionic properties influenced their separation. Ascorbic acid and gallic acid are organic acid anions at pH 7.0. They do not interact with the micellar phase and their negative migration mobility against the electrosmotic flow (EOF) effects their separation. Theanine, a zwitterion with an overall charge of zero, elutes near the EOF. This is probably because theanine is retained only slightly when the hydrocarbon side-chain and the α-amino group form weak interactions with the surfactant. The concentration of 100 m*M* SDS provided good resolution of almost all the compounds. Higher SDS concentrations did not increase the resolution, caused peak broadening and unacceptable high Joule heating. Phosphate and borate were tested as possible background electrolytes. It was found that the catechins showed leading fronts when borate was present in the running buffer. The use of phosphate alone as the background electrolyte improved the peak shapes. The analytes were separated on their different hydrophobic interactions with the SDS (catechins and caffeine) as well as their ionic properties at pH 7.0 (gallic acid, ascorbic acid and pNP). Phosphate was tested as a background electrolyte in the concentration range 5–45 m*M*. A concentration of 25 m*M* was found to be optimal for the separation of the analytes. Methanol was used as organic modifier. A wide range of 0–10% (v/v) methanol was tested. The k' of the catechins and caffeine decreased with an increase in percentage methanol, since the compounds became more soluble in the bulk phase. The methanol does not alter the solubility of the ionic analytes (theanine, gallic acid and ascorbic acid) in the bulk phase. A 6% (v/v) methanol concentration was considered optimal for the analysis of green and black tea samples because it provided the shortest analysis time with good resolution. Bottled tea samples required an optimal concentration of 5% (v/v) methanol to provide good resolution between ascorbic and EC. This increased the total separation time to 13 min.

The applied voltage was varied from 10 to 20 kV. The optimal voltage was selected as 14 kV because it was away from any cross-over points (near 12 and 17 kV) and the Joule heating was within the limits for the given type and length of capillary.

Table 1
Limits of detection and quantification values, linear limits and regression coefficients of all analytes

Compound	LOD (μg/ml)	LOQ (μg/ml)	Linear limit (μg/ml)	r
Theanine	4.0	10.0	600.0	0.995
Caffeine	2.0	5.0	75.0	0.998
Catechin	1.0	2.0	55.0	0.999
EGC	2.0	4.0	55.0	0.997
EGCg	6.0	11.0	110.0	0.994
Gallic acid	2.5	6.0	120.0	0.998
EC	1.0	2.0	135.0	0.996
pNP	4.0	8.0	160.0	0.998
ECg	3.0	10.0	100.0	0.994
Ascorbic acid	20.0	50.0	500.0	0.994

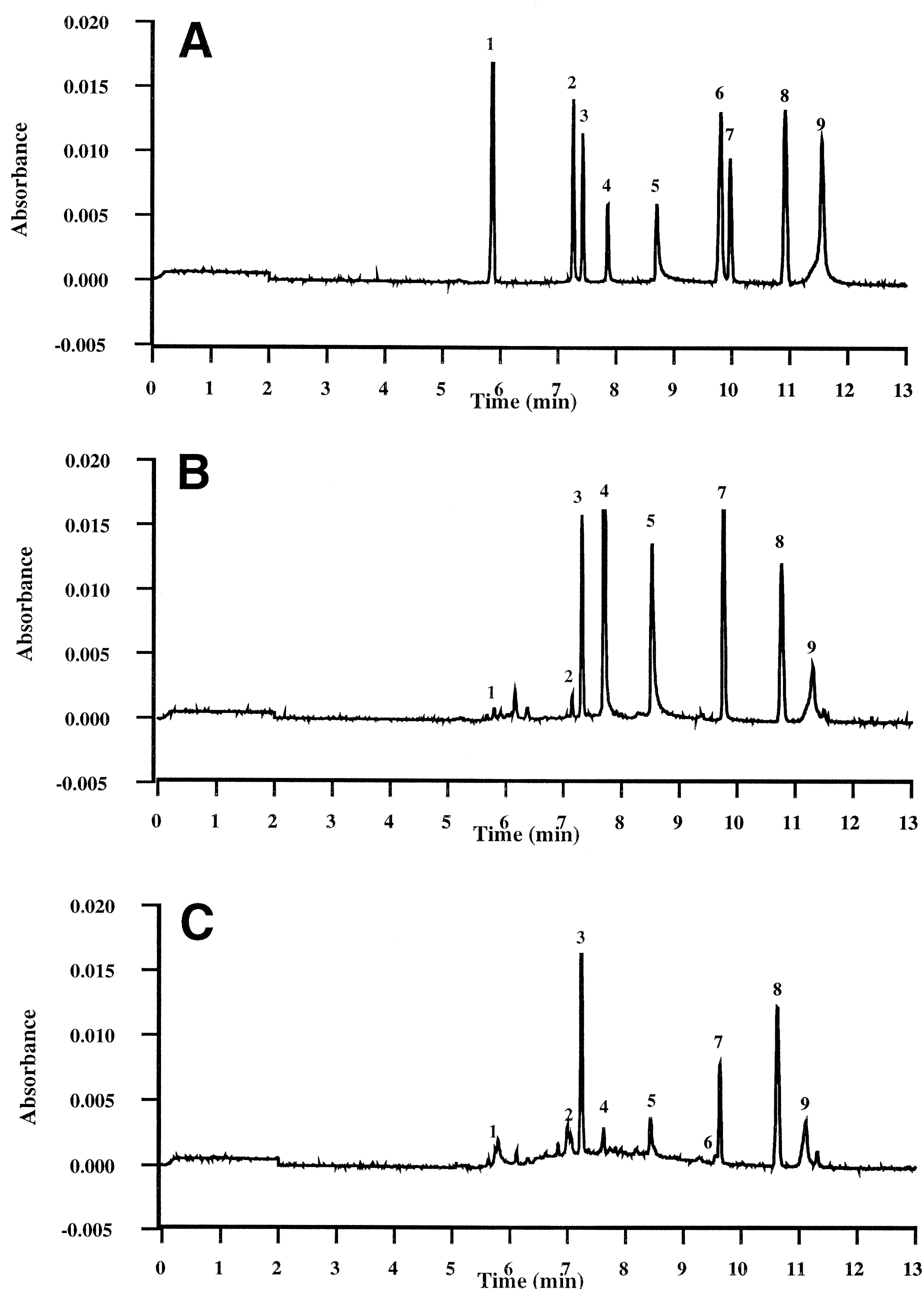

Fig. 2. (A) Separation of standard mix. The peaks are: 1=theanine, 2=catechin, 3=caffeine, 4=EGC, 5=EGCg, 6=gallic acid, 7=EC, 8=pNP, 9=ECg. (B) Separation of dried fresh leaf sample 1% (w/v) solution five times diluted. (C) Separation of black tea sample 1% (w/v) solution three times diluted. Conditions: 100 m*M* SDS, 25 m*M* phosphate, 6% (v/v) methanol pH 7.0. Separation was carried out at 14 kV. Detection at 200 nm.

3.2. Validation of analytical procedure

pNP seems to have been a good selection as internal standard and reference compound for automated peak identification. The k' of pNP was not influenced significantly under the conditions investigated here. The limits of detection (LODs) for all analytes were determined experimentally and are shown in Table 1. The limits of quantification (LOQs) are taken as the concentrations that result in 5% or less relative standard deviation (RSD) upon quantification of the peak area. The upper limits of

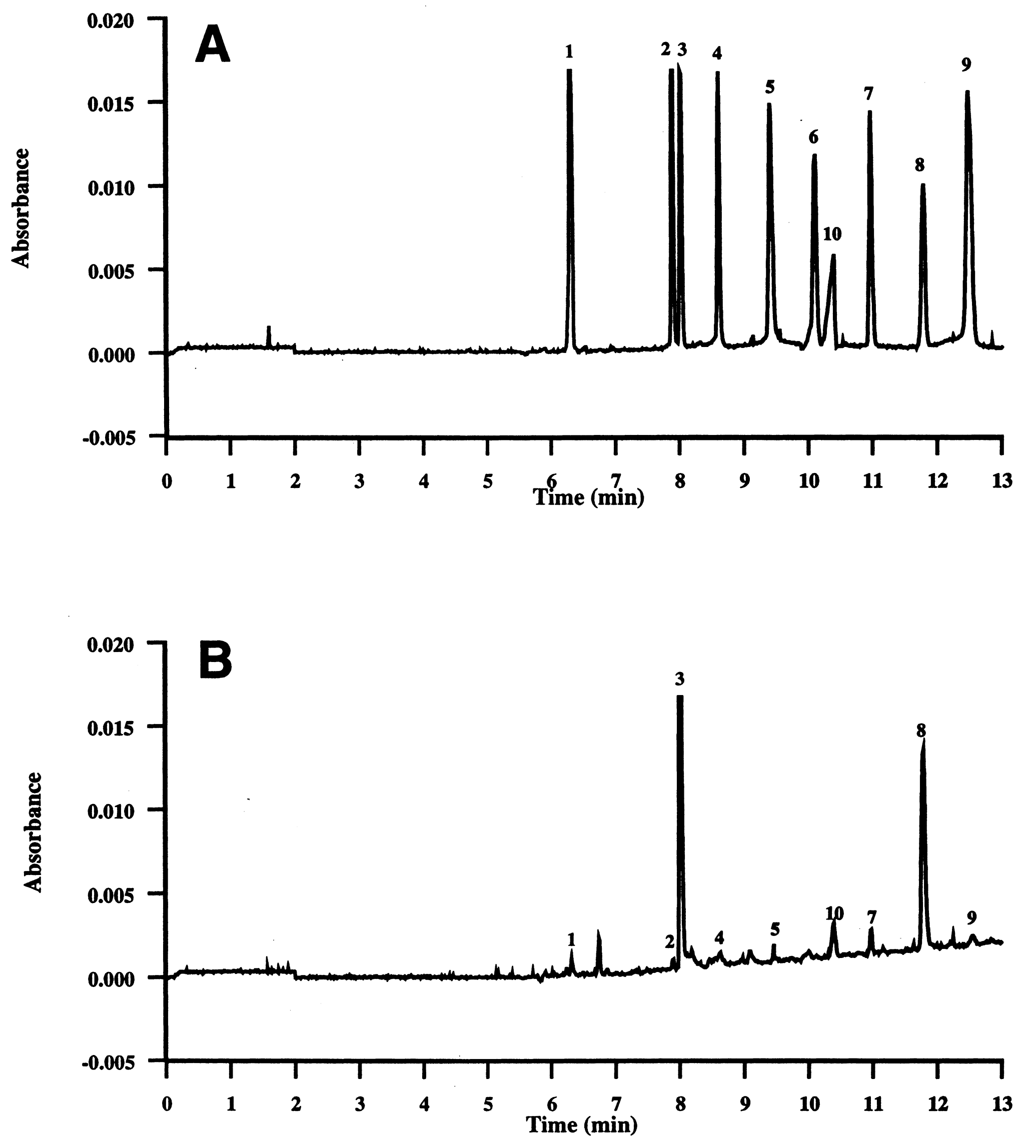

Fig. 3. (A) Separation of standard mix. All peaks are the same as in Fig. 2A and peak 10 is ascorbic acid. (B) Separation of undiluted canned tea sample. Conditions: 100 m*M* SDS, 25 m*M* phosphate, pH 7.0, 5% (v/v) methanol. Separation was carried out at 14 kV. Detection at 200 nm.

Table 2
Typical concentrations of analytes found in dried fresh tea leaf, black tea (mg/g dry mass) and bottled tea (μg/ml). A typical cup of tea (black or green) is brewed as a 1% (w/v) solution

Compound	Dried fresh tea leaf		Black tea		Bottled black tea	
	mg/g	RSD (%)	mg/g	RSD (%)	μg/ml	RSD (%)
Theanine	11.15	5.45	7.27	4.42	<LOQ	–
Caffeine	37.93	1.18	20.59	3.80	100.33	4.07
Catechin	0.87	3.71	1.21	4.92	<LOQ	–
EGC	25.68	1.75	1.14	2.46	<LOQ	–
EGCg	78.77	2.85	10.62	4.56	<LOQ	–
Gallic acid	<LOQ	–	2.29	2.33	<LOD	–
EC	6.73	1.60	2.17	3.31	2.51	4.96
ECg	15.18	2.87	7.51	1.40	<LOQ	–
Ascorbic acid	<LOD	–	<LOD	–	224.98	4.60
Total catechins	127.23	–	22.65	–	2.51	–

linearity for calibration curves were determined for each analyte. The correlation coefficients (r) of the calibration curves were better than 0.994. The precision study showed that the migration times varied by 3% or less for intra-day analyses and by less than 5% for inter-day analyses.

An electropherogram of the standards is shown in Fig. 2A, while dried fresh tea leaf and black tea profiles are shown in Fig. 2B and C, respectively. The results are shown in Table 2. Typical electropherograms of the standards (including ascorbic acid) and a sample of bottled black iced tea are shown in Fig. 3A and B, respectively. In the commercial brand of iced tea analyzed, tea catechins were detectable. All the catechins except for EC were below their LOQs.

4. Conclusion

This is a robust MEKC method enabling the analysis of the eight most important compounds in dried fresh tea leaf and black teas. A slight modification to this method was used to analyze bottled ice tea fortified with ascorbic acid. The separation order of the catechins is similar to that reported for other MEKC [13–15] and reversed-phase HPLC methods [11] where the gallated catechins elute later than their ungallated counterparts. MEKC analyses of tea samples for catechin and caffeine content are much shorter and less costly than analysis with HPLC.

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