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Isocratic elution system for the determination of catechins, caffeine and gallic acid in green tea using HPLC

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Abstract

A simple high performance liquid chromatographic analysis for tea catechins, caffeine and gallic acid with an isocratic elution system was developed. The separation system consisted of a C18 reversed-phase column, an isocratic elution system of methanol/water/orthophosphoric acid, and an UV detector. This method is ideally suited for rapid, routine analysis for the determination of catechins in green tea with good repeatability and accuracy of results. Furthermore, this method can be applied to all kinds of tea and tea products, and is especially useful for the determination of (+)-catechin, which was regarded as being in too low a concentration to detect, and (−)-gallocatechin gallate, which was regarded as a measure for heat treatment for green tea.

Keywords: Tea; Catechins; Caffeine; Gallic acid; HPLC

1. Introduction

It has been discovered that green tea contains various components with anti-oxidative and anticarcinogenic properties (Dreostic, Wargovich & Yang, 1997; Jankun, Salman, Swiercz & Skrzypczak-Jankun, 1997; Wiseman, Balentine & Frei, 1997; Yang, 1997). Of these, catechins, which make up about 20% of the dry weight of green tea, are thought to be the most important.

Green tea catechins are structurally primarily flavanols. The main catechins in green tea are (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC).

In recent years, there have been more and more applications for tea extracts, especially in the pharmaceutical and food areas. Increased demand for tea extracts has resulted in the recognition of the inherent value of routine quality control methods to determine the catechins in both the extracts and the crude plant materials. High performance liquid chromatography (HPLC) is normally used as the analytical technique to determine catechins in green tea (Bronneer & Beecher, 1998; Dalluge, Nelson, Thomas & Sander, 1998; Goto, Yoshida, Kiso & Nagashima, 1996; Price & Spitzer, 1993; Umegaki, Esashi, Tezuka, Ono, Sano & Tomita, 1996). In these systems complex mobile phases are needed. The use of HPLC/MS (Lin, Ng & Tang, 1993) and capillary electrophoresis (Horie, Mukai & Kohata, 1997) have been reported. However, as highly precise instruments were involved, their utilisation for routine analysis is limited. Recently, a study of stationary phases and elution conditions for the HPLC determination of six catechins has been reported by Dalluge et al. However, their initial efforts to develop an isocratic elution system for the separation of catechins were unsuccessful.

This paper presents an isocratic elution system for the separation of catechins in green tea. The system employs a simple mobile phase containing methanol and water. In addition, gallic acid and caffeine can be separated simultaneously by this method.

2. Materials and methods

2.1. Samples

Roasted green tea and Keemun black tea were obtained from the Tea Research Institute, Chinese Academy of Agricultural Sciences. Gunpowder tea and

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Ceylon black tea were purchased from a local teashop in Hitchin, Hertfordshire, UK. Sencha tea was a present from Japan. The tea samples were prepared according to the conventional tea brewing method, taking 3 g of sample and infusing with 150 ml of boiling distilled water for 5 min. The infusions were then filtered and kept at room temperature, and filtered again through a 0.45 μm nylon filter before HPLC analysis.

2.2. Standards

(+)-Catechin (C), (−)-EC, (−)-EGC, (−)-EGCG, (−)-ECG and (+)-Gallocatechin (GC) and caffeine were purchased from Sigma Chemical Co. Gallic acid was purchased from Merck Chemical Co. (−)-gallocatechin gallate (GCG) was separated and purified from green tea by the Tea Research Institute, Chinese Academy of Agricultural Sciences. All other reagents were standard items from reputable commercial sources. All solvents used for extraction were analytical grade.

2.3. Analytical determinations

A HP 1100 series liquid chromatograph system comprising vacuum degasser, quaternary pump, auto-sampler, thermostatted column compartment, and diode array detector was used. The column used was a C18 reversed phase Kingsorb 5 μm (150×4.6 mm). Mobile phase eventually adopted for this study was methanol/water/orthophosphoric acid (20/79.9/0.1) and the flow rate was 1.0 ml/min. Absorption wavelength was selected at 210 nm. The column was operated at 30°C. The sample injection volume was 20 μl. The UV spectra obtained for each peak, after subtraction of the corresponding UV base spectrum, were computer normalised and the plots were superimposed. Peaks were considered to be chromatographically pure when there was exact coincidence of their corresponding UV spectra. Chromatographic peaks in the samples were identified by comparing their retention time and UV spectrum with those of the reference standards. Working standard solutions (5–30 μl) were injected into the HPLC, and peak area responses were obtained. A standard graph for each component was prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the sample and corresponding standard graph.

3. Results and discussion

3.1. Selection of mobile phases

Two mobile phases, one containing methanol and the other containing acetonitrile, were tested. The methanol/water system gave a complete separation of the seven catechins, caffeine, and gallic acid. However, although reasonable separation was achieved with the acetonitrile/water system, (−)-EGC and (+)-C could not be separated.

It was reported that the presence of acid in the mobile phase is essential for the complete separation and elution of the analytes (Dalluge et al., 1998). Fig. 1 shows the effect of the presence of orthophosphoric acid in the mobile phase on the separations of catechins, caffeine, and gallic acid. It can be seen that, in the methanol/water system without orthophosphoric acid, the separation was poor, for example, gallic acid and (+)-GC could not be separated; and a baseline separation could not be achieved between (−)-EGC and (+)-C or between (−)-EGCG and (−)-EC (Fig. 1a). Similarly, in the acetonitrile/water system without orthophosphoric acid, gallic acid could not be separated from (+)-GC, and (−)-EGC could not be separated from (+)-C (Fig. 1c). If orthophosphoric acid was added to both mobile phases, the chromatographic separations could be substantially improved. Full separation could be achieved in the methanol/water system with orthophosphoric acid addition (Fig. 1b), but (−)-EGC and (+)-C still could not be separated in the acetonitrile/water system (Fig. 1d). Our results show that acid is always necessary in the mobile phase, whether a column has been equilibrated with acid-containing buffers or not. This is different from the result given by Dalluge et al. (1998). Acetic acid has been found to have the same effect on the separation, but it is not as effective as orthophosphoric acid. Therefore, a system consisting of methanol, water with orthophosphoric acid was selected. Different quantities of orthophosphoric acid in the mobile phase were tested in the present study. Experiments showed that the effective amount of orthophosphoric acid in the mobile phase is from 0.04–0.1%.

3.2. Selection of absorption wavelength

All catechins, gallic acid and caffeine were found to have maximum absorbances at 210 nm and from 275–280 nm. Fig. 2 shows the chromatograms of standard solution at 210 and 280 nm. Comparing the absorbances at the two wavelengths, the absorbances at 210 nm were 2.6, 65.8, 62.4, 15.8, 2.7, 7.9, 15.9, 7.1, and 5.8 times larger than those at 280 nm for gallic acid, (+)-GC, (−)-EGC, (+)-C, caffeine, (−)-EGCG, (−)-EC, (−)-GCG, and (−)-ECG, respectively, in the system methanol/water/orthophosphoric acid. Thus, chromatograms recorded at 210 nm showed considerable improvement in signal-to-noise ratio. This was especially useful for the determination of (+)-GC, (−)-EGC, and (−)-GCG, which were very difficult to detect, either because of their low concentration in tea samples or because of their low response.

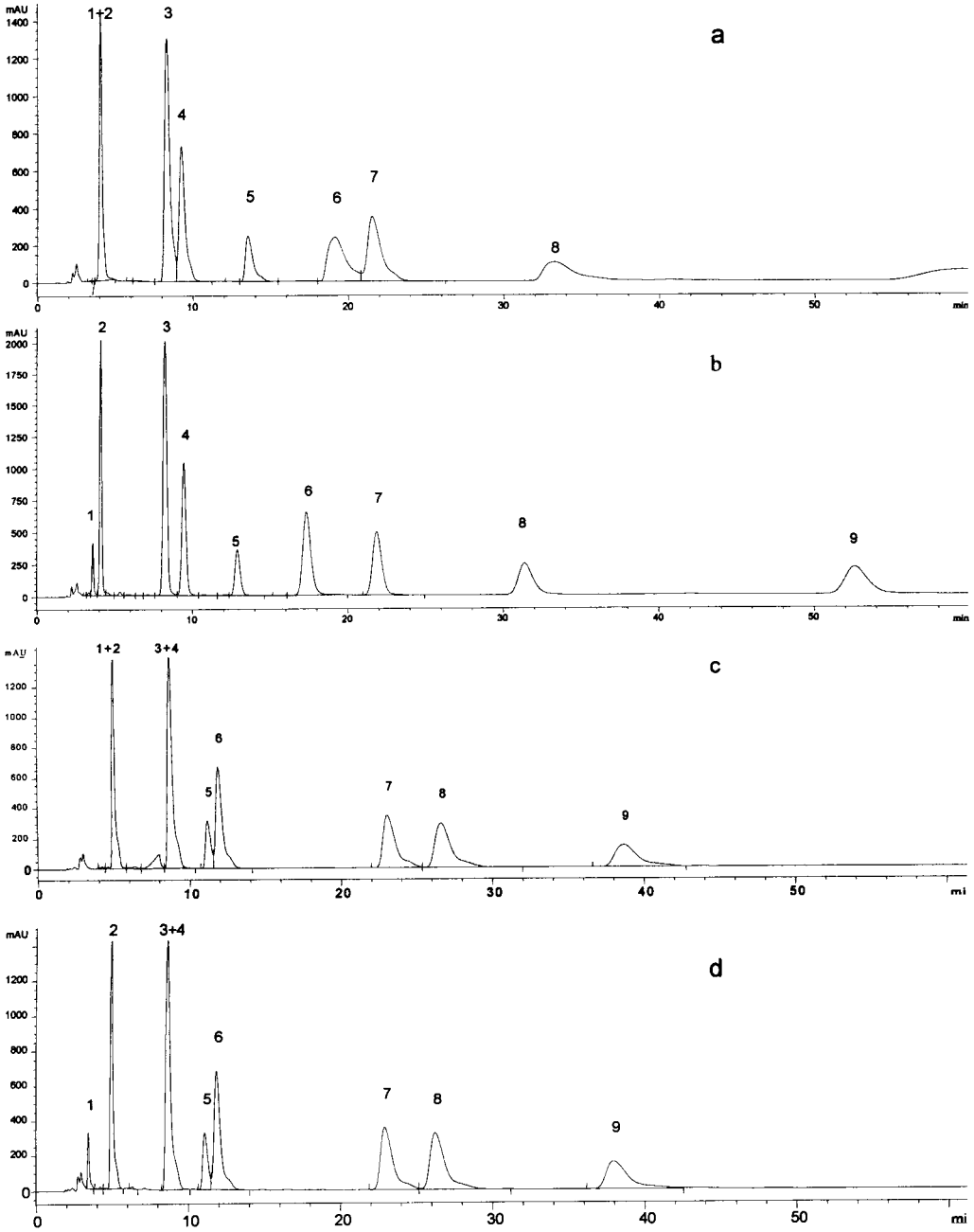

Fig. 1. The effect of orthophosphoric acid in the mobile phase on the separations of catechins, caffeine, and gallic acid. The chromatographic conditions are described under Materials and Methods. Column: Kingsorb 5 μ (150×4.6 mm); Detection: 210 nm; Flow rate: 1.0 ml/min. a: a mobile phase consisted of methanol/water (20/80) without orthophosphoric acid; b: a mobile phase consisted of methanol/water/orthophosphoric acid (20/79.9/0.1); c: a mobile phase consisted of acetonitrile/water (10/90) without orthophosphoric acid; d: a mobile phase consisted of acetonitrile/water/orthophosphoric acid (10/89.9/0.1). 1: Gallic acid; 2: (+)-GC; 3: (−)-EGC; 4: (+)-C; 5: Caffeine; 6: (−)-EGCG; 7: (−)-EC; 8: (−)-GCG; 9: (−)-ECG.

3.3. Selection of columns

Two columns, Kingsorb C18 and Nucleosil C18 were tested under the same conditions. Both columns gave similar responses. However, Kingsorb gave full separation for all the analytes, whereas Nucleosil could not achieve satisfactory resolution of (−)-EGC and (+)-C, or (−)-EGCG and (−)-EC in the methanol/water/orthophosphoric acid solvent system. It appeared that the performance of the Nucleosil column was better in the acetonitrile/water/orthophosphoric acid solvent system. As a result, the Kingsorb column was selected for this investigation.

3.4. Effect of the concentration of ethanol for sample preparation on the separation of analytes

Suematsu, Hisanobu, Saigo, Matsuda and Komatsu (1995) investigated the effect of water and aqueous

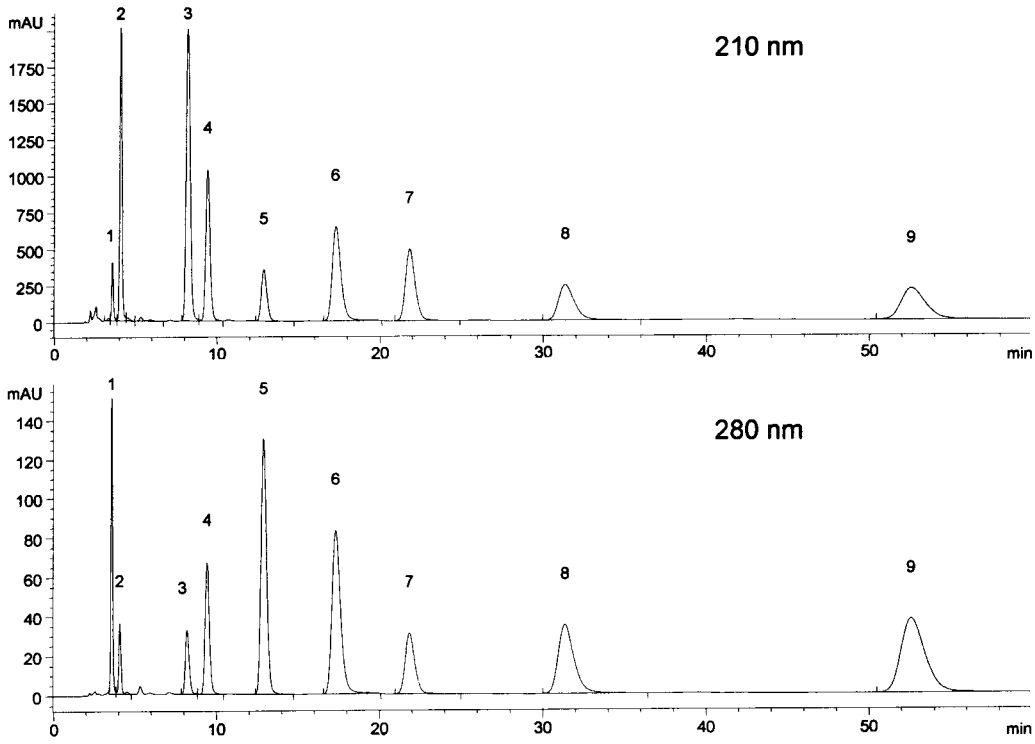

Fig. 2. The chromatograms of standard solution at 210 and 280 nm. The chromatographic conditions are described under Materials and Methods. Column: Kingsorb 5 μ (150×4.6 mm); Detection: 210 nm; Flow rate: 1.0 ml/min. 1: Gallic acid; 2: (+)-GC; 3: (−)-EGC; 4: (+)-C; 5: Caffeine; 6: (−)-EGCG; 7: (−)-EC; 8: (−)-GCG; 9: (−)-ECG.

acetonitrile of different concentrations on the extraction of catechins and caffeine in green tea, but ethanol was not investigated. As it is usual to prepare extracts from green tea using various concentrations of ethanol as the extraction solvent, we examined the effect of ethanol content, used in the sample preparation, on the separation of the analytes (Fig. 3). It can be seem from this that good separation could not be achieved if the sample was prepared with concentrations of ethanol as high as 60%. With decreasing ethanol content, the separation improved. Methanol had a similar effect on the separation. As long as the concentration of ethanol in the extraction solvent was below 15%, there was no problem with the separation of all the analytes. However, it was noticed that the result was around 20% higher for the extraction of catechins in the samples prepared with 15% ethanol than those prepared with distilled water. It was interesting that, when the sample prepared with 60% ethanol was diluted with water to make the concentration of ethanol in the sample less than 15%, good separation and quantification could be achieved. This suggested that it does not matter what concentration of ethanol is used when a tea extract is made, but it is important to make the concentration of ethanol in the sample solution less than 15% when HPLC analysis of catechins is carried out.

3.5. Validation of the method

The relationship between concentration and peak area is shown by the a, b and r values in Table 1, where a and b are the coefficients of the regression equation $y = ax + b$, x being the concentration of analytes and y the peak area, and r is the correlation coefficient of the equation. All the analytes exhibited good linearity (r) over the range tested with correlation coefficients from 0.988 to 1.000.

To test the precision of the assay method, the standard solution of catechins and one of the samples to be analysed were injected 10 times under the chromatographic conditions described above. Table 2 summarises the results obtained. It can be seen that the variation coefficients were quite low: for standard solution, the coefficients of all analytes were within 1.50%, while for an actual sample, within 2.19% except (+)-GC (8.88%) and (−)-EC (5.83%).

By running a blank injection after the analysis of tea samples, no memory effect was observed.

Stability of catechins, caffeine and gallic acid in the standard solution was also tested. The experiment showed stability when the standards of every compound were prepared just with distilled water and kept in a refrigerator. After the standard solution was kept for

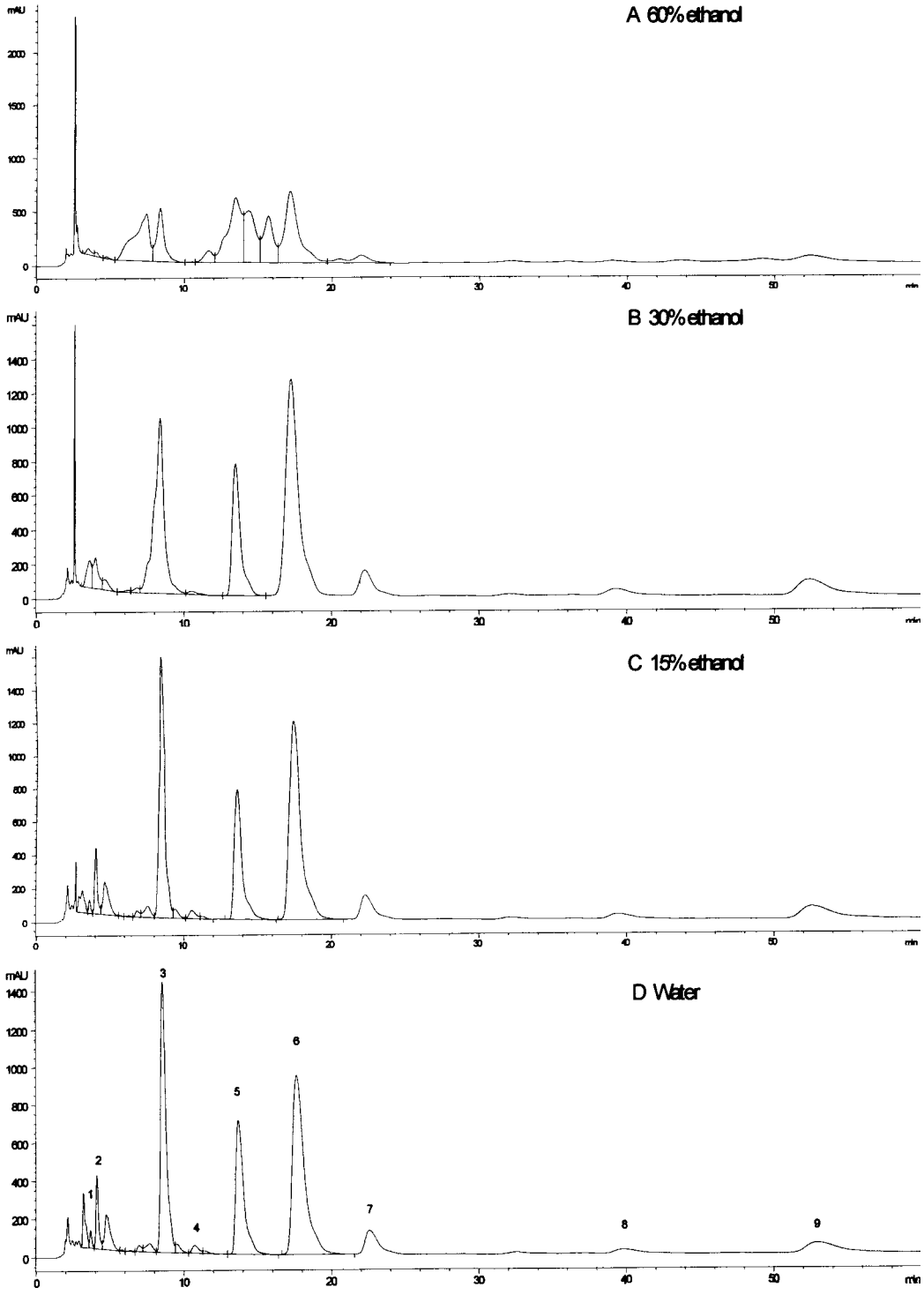

Fig. 3. Effect of solvent for sample preparation on the separation of the analytes. The chromatographic conditions are described under Materials and Methods. Column: Kingsorb 5 μ (150×4.6 mm); Detection: 210 nm; Flow rate: 1.0 ml/min. 1: Gallic acid; 2: (+)-GC; 3: (−)-EGC; 4: (+)-C; 5: Caffeine; 6: (−)-EGCG; 7: (−)-EC; 8: (−)-GCG; 9: (−)-ECG.

half a year, the decreases of each compound in concentration were 5.5, 2.8, 8.3,3.0, 1.2, 7.5, 1.9, 3.1, and 1.1% for gallic acid, (+)-GC, (−)-EGC, (+)-C, caffeine, (−)-EGCG, (−)-EC, (−)-GCG, and (−)-ECG, respectively.

3.6. Quantitative measurement of different tea samples

In China, where tea quality is judged by sensory evaluation, an infusion of tea for test is normally made from about 3 g of loose tea with 150 ml of boiling water.

Table 1
Relationships between analyte concentrations and their peak areas

Compound	*a*	*b*	*r*
Gallic acid	6513.8	205.63	0.999
(+)-GC	4893	3428.6	0.988
(−)-EGC	13743	−11155	0.990
(+)-C	5784	1233.8	0.999
Caffeine	6631.8	159.99	1.000
(−)-EGCG	2750.4	126.18	1.000
(−)-EC	6964.6	764.46	1.000
(−)-GCG	3035.9	−55.43	1.000
(−)-ECG	2798.6	−46.07	1.000

Table 2
Precision of the method after 10 injections

Compound	Standard deviation		Variation coefficient (%)	
	Standard solution	Sample	Standard solution	Sample
Gallic acid	16.1	266	0.54	8.88
(+)-GC	60.9	221	0.39	2.19
(−)-EGC	297	59.6	0.90	0.17
(+)-C	20.7	30.4	0.15	1.46
Caffeine	19.7	148	0.24	0.34
(−)-EGCG	60.7	228	0.56	0.31
(−)-EC	80.5	461	0.52	5.83
(−)-GCG	32.8	203	0.53	1.58
(−)-ECG	113	167	1.50	1.41

According to this regime, different kinds of green tea, namely, gunpowder which is popular in the UK, roasted green tea (RGT), which is popular in China, and Sencha which is popular in Japan, and two kinds of black tea, Keemun black tea and Sri Lanka black tea were prepared for analysis. The contents of catechins, caffeine and gallic acid were calculated as milligrams per 100 ml using an infusion derived from 3 g of tea in 150 ml of boiling distilled water (Table 3). As expected, green tea contained a much higher quantity of catechins than black tea. This is because, in black tea, most of the catechins have been altered to form theaflavins and thearubigins during black tea manufacture. In green tea, the most abundant catechin is usually (−)-EGCG, following (−)-EGC, (−)-EC, (−)-ECG, and (+)-GC in decreasing order. With this method, (+)-C, which was regarded as being in too low a concentration to detect (Khokhar, Venema, Hollman, Dekker & Jongen, 1997), and (−)-GCG, which was regarded as a measure for heat treatment for green tea (Wang, Lynch, Lewis, Bond & Davies, 1998), could be quantitatively detected. Furthermore, it is easier to establish the structure-retention time (capacity factor) relationship by using isocratic systems. For example, under the present system the ratio of (−)-EC to (+)-C approximately equals the ratio of (−)-EGC to (+)-GC. Similarly, the 'addition' of a gallate residue should also be expected to have a consistent effect on the retention time and thus the ratio of (−)-ECG to (−)-EC approximately equals the ratio of (−)-EGCG to (−)-EGC. Therefore, this is an additional chromatographic tool for the identification of these catechins.

Table 3
Catechins, caffeine and gallic acid in different tea samples (mg/100 ml)[a]

Components	Gunpowder	RGT	Sencha	Keemun	Sri Lanka
Gallic acid	0.78	1.65	0.74	3.33	2.79
(+)-GC	2.57	3.05	2.81	0.40	1.57
(−)-EGC	29.7	30.1	36.2	0.90	1.84
(+)-C	0.69	1.22	1.41	nd[b]	0.50
Caffeine	23.9	30.3	28.9	38.2	22.9
(−)-EGCG	32.6	31.4	28.8	0.95	1.16
(−)-EC	5.58	6.48	9.54	nd	1.45
(−)-GCG	0.51	0.91	1.02	nd	nd
(−)-ECG	4.26	5.03	4.92	1.19	2.92
Σ Catchins	76.0	78.2	84.6	3.44	9.43

[a] 3 g of tea leaves were brewed with 150 ml of boiling distilled water for 5 min. HPLC conditions: Column: Kingsorb C18 5 μ, 150×4.6 mm, Detection: 210 nm, Mobile phase: methanol:water:orthophosphoric acid = 20:79.9:0.1 at 1 ml/min.
[b] nd, not detected.

4. Conclusions

The isocratic method developed for the determination of catechins in green tea is ideally suited for rapid, routine analysis. With this method good repeatability of the results was established, and seven different catechins, caffeine, and gallic acid could be determined at the same time. Furthermore, this method is simple, sensitive and accurate and can be applied to all kinds of tea and tea products.

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References

Bronneer, W. E., & Beecher, G. R. (1998). Method for determining the content of catechins in tea infusions by high-performance liquid chromatography. *Journal of Chromatography A, 805*, 137–142.

Dalluge, J. J., Nelson, B. C., Thomas, J. B., & Sander, L. C. (1998). Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. *Journal of Chromatography A, 793*, 265–274.

Dreosti, I. E., Wargovich, M. J., & Yang, C. S. (1997). Inhibition of carcinogenesis by tea: the evidence from experimental studies. *Crit. Rev. Sci. Nutr., 37*, 761–770.

Goto, T., Yoshida, Y., Kiso, M., & Nagashima, H. (1996). Simultaneous analysis of individual catechins and caffeine in green tea. *Journal of Chromatography A, 749*, 295–299.

Horie, H., Mukai, T., & Kohata, K. (1997). Simultaneous determination of qualitatively important components in green tea infusions using capillary electrophoresis. *Journal of Chromatography A, 758*, 332–335.

Jankun, J., Selman, S. H., Swiercz, R., & Skrzypczak-Jankun, E. (1997). Why drinking green tea could prevent cancer. *Nature, 387*, 561.

Khokhar, S., Venema, D., Hollman, P. C. H., Dekker, M., & Jongen, W. (1997). A RP-HPLC method for the determination of tea catechins. *Cancer Letters, 114*, 171–172.

Lin, Y. Y., Ng, K. J., & Tang, S. (1993). Characterization of flavonoids by liquid-chromatography tandem mass-spectrometry. *Journal of Chromatography, 629*, 389–393.

Price, W. E., & Spitzer, J. C. (1993). Variations in the amounts of individual flavanols in a range of green teas. *Food Chemistry, 47*, 271–276.

Suematsu, S., Hisanobu, Y., Saigo, H., Matsuda, R., & Komatsu, Y. (1995). A new extraction procedure for determination of caffeine and catechins in green tea. *Nippon Shokuhin Kagaku Kaishi, 42*, 419–424.

Umegaki, K., Esashi, T., Tezuka, M., Ono, A., Sano, M., & Tomita, I. (1996). Determination of tea catechins in food by HPLC with an electrochemical detector. *Shokuhin-Eiseigaku-Zasshi, 37*, 77.

Wang, H. F., Lynch, P. N., Lewis, J., Bond, T. and Davies, A. P. (1998). Molecular rearrangements of tea catechins. In: *Polyphenol communications 98* (pp. 181–182), Lille, France, 1–4 September.

Wiseman, S. A., Balentine, D. A., & Frei, B. (1997). Antioxidants in tea. *Crit. Rev. Sci. Nutr., 37*, 705–718.

Yang, C. S. (1997). Inhibition of carcinogenesis by tea. *Nature, 389*, 134–135.