A METHOD FOR FAST DETERMINATION OF EPIGALLOCATECHIN GALLATE (EGCG), EPICATECHIN (EC), CATECHIN (C) AND CAFFEINE (CAF) IN GREEN TEA USING HPLC[1]

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SUMMARY

Tea has been considered a medicine and a healthy beverage since ancient times, but recently it has received a great deal of attention because of its antioxidant properties. Green tea polyphenols have demonstrated to be an effective chemopreventive agent. Recently, investigators have found that EGCG, one of the green tea catechins, could have anti-HIV effects when bound to CD4 receptor. Many factors can constitute important influences on the composition of tea, such as species, season, age of the leaf, climate, and horticultural practices (soil, water, minerals, fertilizers). This paper presents an HPLC analytical methodology development, using column RP-18 and mobile phase composed by water, acetonitrile, methanol, ethyl acetate, glacial acetic acid (89:6:1:3:1 v/v/v/v/v) for simultaneous determination and quantification of caffeine (CAF), catechin (C), epicatechin (EC) and epigallocatechin gallate (EGCG) in samples of *Camellia sinensis* (green tea) grown in Brazil and harvested in spring, in summer and in autumn, in comparison to Brazilian black tea, to samples of Japanese and Chinese green tea and to two standardized dry extracts of green tea. The method has been statistically evaluated and has proved to be adequate to qualitative and quantitative determination of the samples.
Keywords: HPLC; EGCG; catechin; *Camellia sinensis*; Brazilian green tea.

RESUMO

MÉTODO RÁPIDO PARA DETERMINAÇÃO DE GALATO DE EPIGALOCATEQUINA (EGCG), EPICATEQUINA (EC), CATEQUINA (C) E CAFEÍNA (CAF) EM CHÁ VERDE USANDO CLAE. O chá vem sendo considerado uma bebida saudável e com propriedades medicinais desde tempos bem remotos, mas recentemente tem ganhado grande interesse no meio científico devido a suas atividades como antioxidante. Os polifenóis do chá verde vêm demonstrando possuir atividades quimiopreventivas. Recentemente, pesquisadores descobriram que o EGCG, a principal catequina do chá verde, poderia ter efeito anti-HIV quando acoplado ao receptor CD4. Muitos fatores podem influenciar de forma significativa na composição do chá, como sua espécie, estação que foi colhida, idade da folha, clima e técnicas de cultura (solo, irrigação e fertilizantes). Este artigo vem apresentar uma metodologia analítica desenvolvida para análise em CLAE, usando coluna de fase reversa C-18 e fase móvel composta de água, acetonitrila, metanol, acetato de etila, ácido acético glacial (89:6:1:3:1 v/v/v/v/v) para análise simultânea de cafeína (CAF), catequina (C), epicatequina (EC) e galato de epicatequina (EGCG) em amostras de chá verde, cultivado no Brasil, e colhidas nas estações de primavera, verão e outono; comparando-os com o chá preto brasileiro, amostras de chá verde japonês e chinês e também com dois extratos padronizados importados de chá verde. O método foi avaliado estatisticamente e se mostrou adequado para analisar qualitativamente e quantitativamente as amostras.
Palavras-chave: CLAE; EGCG; catequina; *Camellia sinensis*; chá verde brasileiro.

1 - INTRODUCTION

Tea is the second most popular beverage worldwide and a major source of dietary flavonoids [18]. Tea has been considered a medicine and a healthy beverage since ancient times, but recently it has received a great deal of attention because of its antioxidant properties [2, 5, 14, 18, 25, 26]. Moreover, some epidemiological studies have associated the consumption of tea with a lower risk of several types of cancer [15, 20, 23]. Green tea polyphenols have demonstrated to be an effective chemopreventive agent [5, 9]. Other health benefits/properties, such as enhancing insulin activity [1, 5], antimicrobial [10, 20, 21], imunostimulatory [13, 20], antiinflamatory capacities [20], its protective effect against cardiovascular diseases [19, 20] and cerebral ischemic damage [22], have been suggested. Recently, investigators have found that EGCG, a green tea catechin, could have anti-HIV effects when bound to CD4 receptor [11].

Many factors can constitute important influences on the composition of tea, such as species, season, age of the leaf, climate, and horticultural practices (soil, water, minerals, fertilizers) [8, 12]. The growth of *Camellia sinensis* in Brazil is restricted to Vale do Ribeira, in São Paulo, where most of the harvest is used for black tea production [12]. Recently, Brazil has started to produce green tea as well, in order to supply the newcoming market.

Due to the rise in consumption and production, it is necessary to evaluate qualitatively and quantitatively the constituents of Brazilian green tea in comparison to foreign green tea. Several analytical HPLC methods, with different systen and detection modes, have been published for determination of catechins and caffeine in green tea, most of them using gradient elution [4, 6, 7, 16]. The aim of this paper is to develop an isocratic HPLC method to check the CAF (caffeine), C (catechin), EGCG (epigallocatechin gallate) and EC (epicatechin) (*Figure 1*) contents in Brazilian green and black tea, and compare them with the traditional Japanese and Chinese green tea and also with Green Tea Extract found in the local market.

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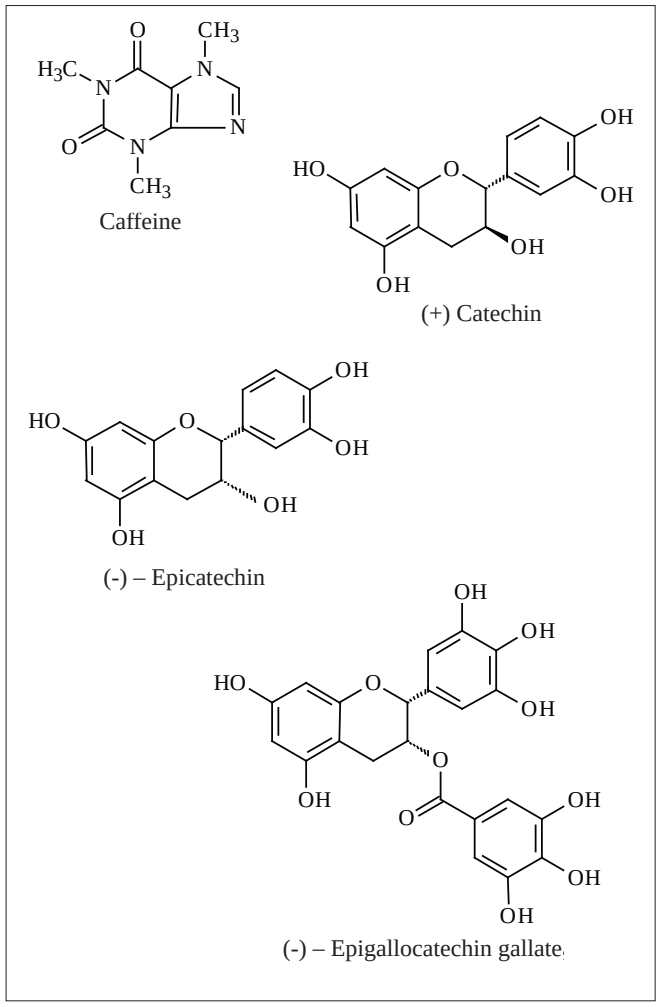

FIGURE 1 – Chemical structures of the tea components assayed

2 - EXPERIMENTAL

2.1 - Chemicals and Samples

The standard chemicals of (+)-catechin (C, 98%), (-)-epicatechin (EC, purity not specified), (-)-epigallocatechin-3-gallate (EGCG, 80%), were purchased from Sigma Chemical Co. (USA); anhydrous caffeine (CAF, 99.9%) was purchased from Nuclear (Brazil). Spectroscopy-grade acetonitrile, ethyl acetate and methanol were purchased from Merck (Darmstdt, Germany). Water was purified using a Milli-Q system (Millipore).

Samples from Brazilian green tea and Brazilian black tea were obtained from Indústria de Chá Ouro Preto (São Paulo, Brazil). Two samples of Chinese green tea were obtained from Quimer (São Paulo, Brazil). Sencha tea was a gift of a Japanese tea producer (Shizuoka, Japan).

Green tea samples harvested during the spring (first harvest after the winter trimming), summer and autumn, from the same plantation and processed in the same factory were obtained.

Green tea extracts (GTEs) were kindly donated by SP Farma (São Paulo, Brazil) and Wenda do Brasil (Brasília, Brazil) and denominated GTE-1 and GTE-2, respectively.

2.2 - Instrumentation

The HPLC system consisted of Varian pumps with a three pump gradient system – Model 9012 - combined with a fixed wavelength UV-VIS detector – Model 9050 (Varian, USA) – a Rheodyne sample injector with a 20 μL sample loop. The column was a LiChrosorb RP-18 column (12.5 cm long x 4 mm i.d. and 5 μm particle diameter, Merck, Germany) equipped with a guard column (1.0 cm long x 4.6 mm i.d., Varian). A Star v. 4.5 software (Varian) was used for both the operation of the detector and for data processing. Detection was carried out by measurement of UV absorbance at 280 nm. The mobile phase was composed of water/ acetonitrile/methanol/ethyl acetate/glacial acetic acid (89:6:1:3:1 v/v/v/v/v). Before the use, the mobile phase was filtered through a G5 sintered glass filter, then degassed for 30 min in an ultrasonic bath. The mobile phase flow rate started at 0.7 mL/min and was maintained for 15 min, and then linearly increased to 1.2 mL/min in 1 min. This flow rate was maintained for 8 min, and then decreased to 0.7 mL/min. All chromatographic analyses were performed at 19±2ºC.

2.3 - Preparation of sample and standard solutions

Stock solutions of caffeine (606 μg/mL), catechin (602 μg/mL), epicatechin (608 μg/mL), epigallocatechin gallate (602 μg/mL) were prepared by dissolving of reference standards into mobile phase. Less concentrated solutions were prepared, as needed, by dilution in the same mobile phase.

Tea samples were ground with a mill and extracted (2 g) with mobile phase (100 mL) by sonication for 5 min. Green tea extract was directly extracted (600 mg) with mobile phase (100 mL) by sonication for 5 min at room temperature. Then both were filtered through a 0.22 μm Millex filter (PVDF membrane) in order to be analysed by HPLC. The temperature for extraction of the samples was performed at 23ºC.

2.4 - Calibration curve

The method development was performed establishing the linearity for all the four substances analysed. Linearity of the methods was established by triplicate injections of solutions in the range of 60-300 $\mu g.mL^{-1}$.

3 - RESULTS AND DISCUSSION

3.1 - Development of analytical method

This study began with an attempt to reproduce some isocratic methods found in the literature, which describe the separation of green tea catechins and caffeine. In general,

it was found that the published methods were somewhat or entirely irreproducible, both with regard to complete resolution of the analytes and/or the quality of chromatography, especially when using conventional C18 column (non-deactivated 5 μm ODS stationary phase). Complete separation of the catechins and chromatographic quality are column-dependent. Better efficiency was reached using deactivated monomeric C18 than non-deactivated monomeric or non-deactivated polymeric C18 columns [6].

For these reasons, we had to develop a new suitable isocratic method, using a LiChrosorb RP-18 column (non-deactivated, monomeric C18 column, irregular silica) to separate three catechins and caffeine. DALLUGE *et al.* [6, 7] tried to develop an isocratic elution system using a MeOH-based mobile phase, but it resulted in poor resolution and low chromatographic efficiency. So we developed a system composed of water/ acetonitrile/methanol/ethyl acetate/glacial acetic acid (89:6:1:3:1 v/v/v/v/v). Methanol and ethyl acetate were used to improve the chromatographic resolution. The presence of an acid was essential to the complete resolution of the cathechins and chromatographic efficiency of these compounds, specifically the elimination of peak tailing [6, 7, 24]. The system was considered efficient only after the complete separation of the standard catechins and caffeine mixture (*Figure. 2*).

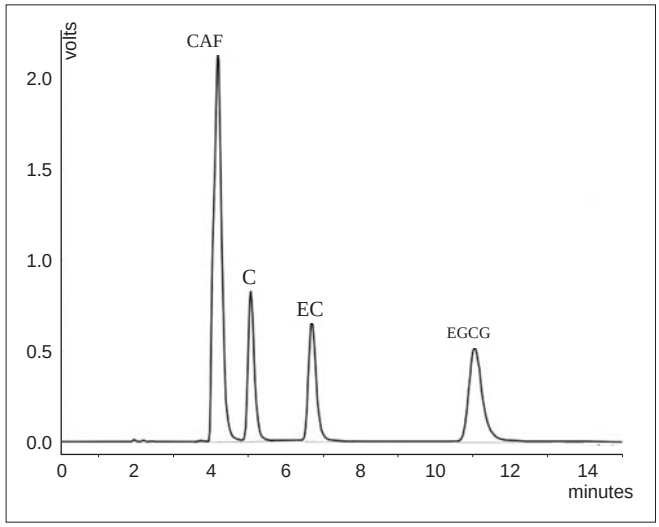

Figure 2 – HPLC profile of a standard substances mixture (180 μg/mL). C-catechin, CAF-caffeine, EC-epicatechin, EGCG – epigallocatechin gallate

Flow rate gradient system was optimized to assay the samples. When the flow rate was maintained in isocratic elution at 0.7 mL/min, all the components of the samples were eluted in 37 min; when flow rate gradient was used, all the components were eluted within 27 min (*Figure 3*).

All catechins and caffeine were found to have maximum absorbances at 210 nm and from 275-280 nm [24]. Wavelength of 280 nm was selected to reduce the interference from the noise.

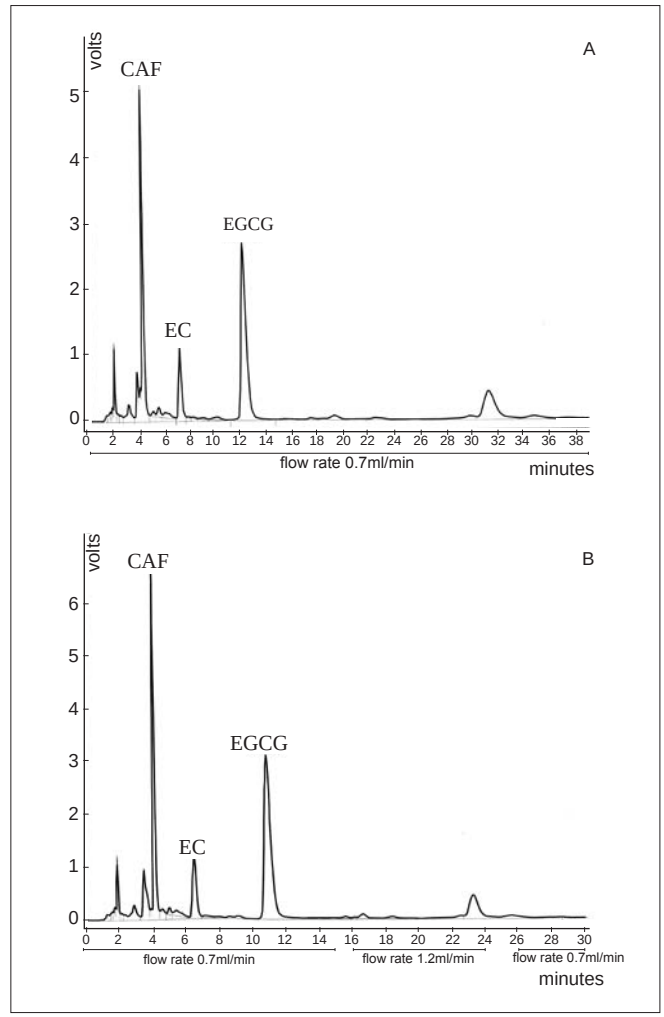

FIGURE 3 – HPLC profile of Brazilian green tea sample. (A) Isocratic elution. Flow rate: 0.7 mL/min. (B) Flow rate gradient. The flow rate started at 0.7 mL/min, was maintained for 15 min, and then linearly increased to 1.2 mL/min in 1 min. This flow rate was maintained for 8 min followed by a linear decrease of flow rate to 0.7 mL/min in 1 min. The final flow rate was held for 4 min. C – catechin, CAF – caffeine, EC – epicatechin, EGCG – epigallocatechin gallate.

3.2 - Method results

Quantitative data from HPLC analysis were compared using the relative standard deviations (R.S.D.%) and analysis of variance (ANOVA). For variables where significant F-values ($p<0.05$) were found, Fisher's Least Significant Difference (LSD) has been used to compare average [3].

Table 1 lists the mean HPLC area responses for CAF, C, EC, EGCG at different concentrations. The linearity plots are shown in *Figure 4*. As shown, the responses for the standard were strictly linear ($r^2>0.99$) in the concentration range of 60-300 μg/mL. The proposed HPLC method presented a good chromatographic performance, with tail factor below 1.5 for CAF, C, EC and EGCG. The number of plates was higher than 2,000.

TABLE 1 – Data for catechin, caffeine, epicatechin and epigallocatechin gallate from linearity studies (n=3)

Concentration (µg/mL)	Mean peak area ± S.D., (R.S.D. %)			
	C	CAF	EC	EGCG
60	333135±1697 (0.5)	1025198±20084 (1.9)	323721±2788 (0.9)	311655±6915 (2.2)
120	689350±3641 (0.5)	2138932±25627 (1.2)	627447±5730 (0.9)	713293±3279 (0.4)
180	989221±14441 (1.4)	3357899±46809 (1.4)	1147023±8855 (0.7)	1175752±3909 (0.3)
240	1400709±18992 (1.3)	4472466±29376 (0.6)	1415631±15404 (1.1)	1482194±11049 (0.7)
300	1731885±19238 (1.1)	5321323±72744 (1.4)	1703092±24364 (1.4)	2093421±54678 (2.6)

3.3 - Analysis of green tea samples

Using this analytical method, caffeine, catechin, epicathechin and epigallocatechin gallate were determined in several kinds of commercial tea samples and two green tea extracts. All samples were analysed in triplicate. The results are shown in *Table 2*. No reports about the levels of catechins in Brazilian green tea were found in the literature. The catechins and caffeine levels of the Brazilian green tea, harvested during several seasons, Brazilian black tea, Chinese green tea, Japanese green tea and two green tea extracts were compared.

A significant variation of HPLC profile between Brazilian black tea, green tea (Brazilians, Chinese and Japanese) and the GTEs (*Figure 5*) was observed.

In the analysed samples, it was observed that Brazilian green tea harvested in spring has presented the lowest EGCC content among the Brazilian samples, sequentially increasing in autumn and summer, suggesting that the EGCC content is related to the age of the leaf and the incidence of sunlight. The EC content, however, did not vary significantly throughout the seasons.

Concerning black tea, low EGCC and EC contents were verified, which can be explained by the oxidation of its fenolic compounds, including catechins, due to benzotropolon formation [17, 18], transforming them into more aromatic and brown-colored substances. This method did not prove to be adequate to dose the caffeine in the Brazilian black tea, since there was a division in the peak, suggesting that other fenolic substances, as the products of its oxidation, could present retention time very close to that of caffeine. During EGCC oxidation, there is galic acid liberation, with a small retention time [4]. An increase of the peaks close to 2 min on the B chromatogram was noted (*Figure 5*), suggesting the influence of this compound in the retention time.

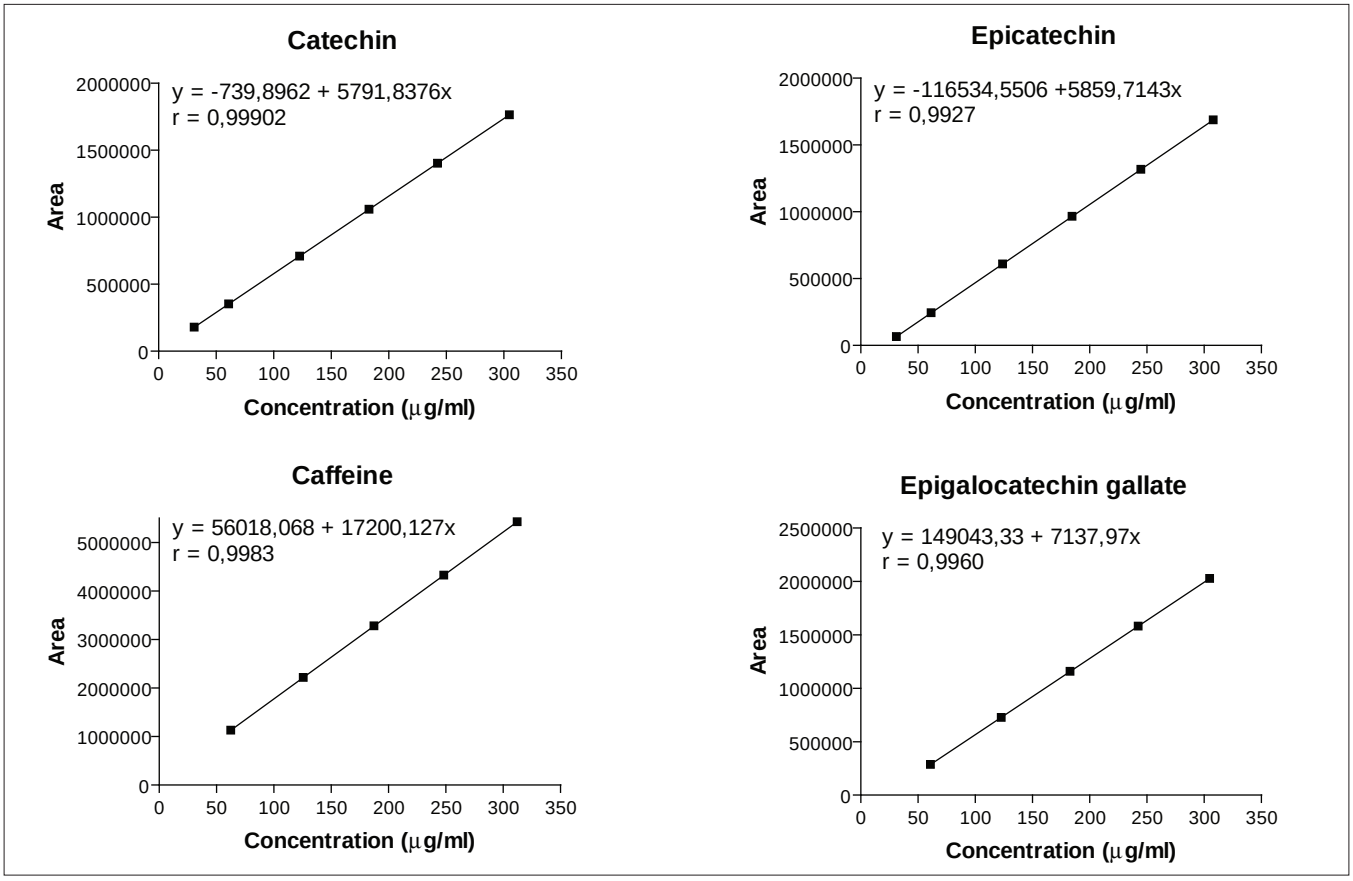

FIGURE 4 – Linearity plots for catechin, caffeine, epicatechin and epigallocatechin gallate

TABLE 2 – Caffeine, catechin, epicatechin and epigallocatechin gallate contents, standard deviations, RSD% (n=3) by HPLC-UV, in various kinds of tea and green tea extract (dry weight %)

Retention time (min)	4.0	5.1	6.5	10.6
Sample's	**CAF**	**C**	**EC**	**EGCG**
Brazilian tea				
Green tea[1] (spring)	2.35±0.07 (2.9)	0.06±0.00 (2.4)	1.28±0.06 (5.0)	5.82±0.18 (3.0)
Green tea[1] (summer)	3.02±0.08 (2.5)	0.12±0.00 (3.3)	1.28±0.05 (4.1)	8.47±0.09 (1.0)
Green tea[1] (fall)	2.80±0.06 (2.2)	0.11±0.01 (9.1)	1.18±0.07 (5.6)	8.23±0.20 (2.4)
Green tea[2] (summer)	2.50±0.16 (6.2)	n.d.	0.85±0.02 (1.9)	8.05±0.32 (3.9)
Black tea[1] (summer)	n.d.	n.d.	0.16±0.00 (3.2)	0.54±0.03 (5.2)
Japanese green tea				
Sencha (Shizuoka)	2.23±0.11 (4.8)	0.83±0.04 (4.6)	0.88±0.05 (5.2)	4.68±0.23 (4.9)
Chinese green tea				
Gunpowder	2.32±0.23 (10.1)	n.d.	0.91±0.07 (8.1)	4.67±0.39 (8.3)
Ground tea	2.09±0.07 (3.1)	0,02±0.00 (4.7)	0.55±0.01 (0.9)	4.03±0.17 (4.2)
Green tea 50% extract				
GTE-1 (China)	7.29±0.21 (2.9)	0.36±0.01 (2.3)	2.07±0.03 (1.6)	20.93±0.65 (3.1)
GTE-2 (China)	6.73±0.22 (3.3)	2.75±0.13 (4.7)	5.73±0.20 (3.5)	11.96±0.36 (2.9)

[1]Made from Camellia sinensis, var. Assam
[2]Made from Camellia sinensis
n.d. – not detected

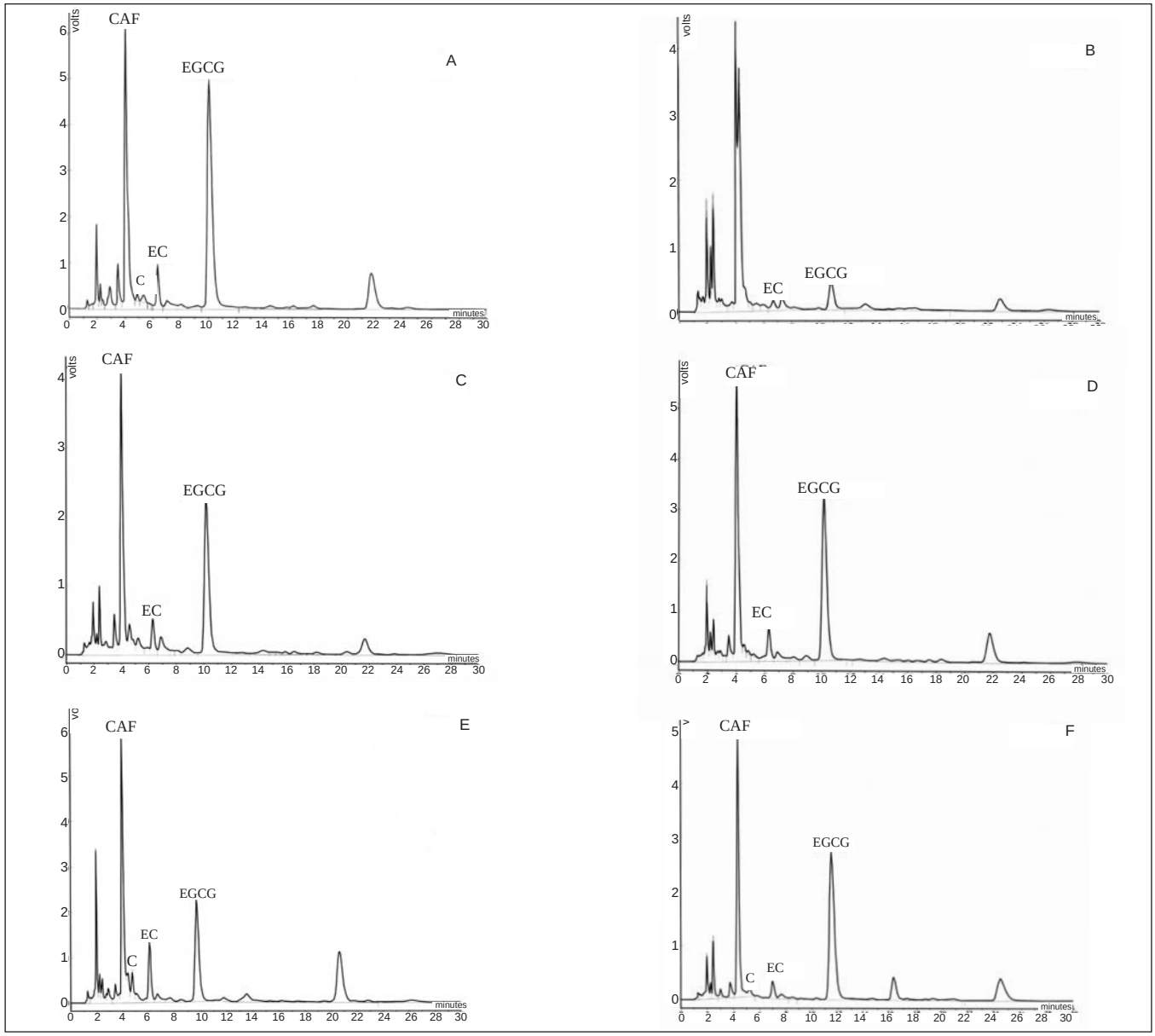

FIGURE 5 – HPLC profiles of samples. (A) Brazilian green tea – summer, (B) Brazilian black tea, (C) Japanese green tea – sencha, (D) Chinese green tea – gunpowder, (E) GTE-1, (F) GTE-2. C – catechin, CAF – caffeine, EC – epicatechin, EGCG – epigallocatechin gallate

TABLE 3 – Comparison of the results with the literature. (dry weight %)

Sample's	CAF (%)	C (%)	EC (%)	EGCG (%)
Brazilian green tea[a]	2.3-3.0	<0.1	0.8-1.2	5.8-8.4
Japanese green tea[a]	2.23	0.83	0.88	4.68
Literature[b]	1.4-2.8	<0.3	0.8-1.2	5.6-7.5
Chinese green tea[a]	2.32	n.d.	0.91	4.67
Literature[b]	2.2-3.8	0.1-0.5	0.7-2.1	4.9-10.1
Brazilian black tea[a]	n.d.	n.d.	0.16	0.54
Indian black tea[b]	4.0-4.8	<0.1	<0.07	0.4-0.9
Green tea 50% extract [a]	6.7-7.2	0.3-2.7	2.0-5.7	11.9-20.9
Literature[c]	1.8-6.0	1.3-5.2	0.3-5.6	2.6-41.2

[a]Samples assayed
[b]Reflects range of data compiled from ref [04]
[c]Reflects range of data compiled from ref [16]

The Chinese and Japanese green tea analysed samples showed lower EGCC contents and, in some cases, also EC, than the Brazilian samples harvested in spring. The range of the results found in literature (*Table 3*) was compared with the results found in this essay, which came out to be noticeably similar. Fernández *et al.* [8] have analysed several types of tea, fermented and non-fermented ones, originated from various countries, in order to trace their constituents geographically; Pelillo *et al.* [16] have analysed five types of green tea extracts and significant differences in their cathechins were found.

As the specification presented by the manufacturer, GTE1 sample showed similar contents of EGCG (18.9% labeled to 20.93% analysed) and CAF (7.6% labeled to 7.29% analysed). GTE2´s manufacturer certificate of analysis only labeled UV assay for total polyphenols (higher than 50%). The sample showed lower contents of catechins and caffeine than GTE1 sample. Standardized green tea extracts may vary the catechin content significantly, specially the EGCC, probably because its standardization is made only in relation to the total polyphenols.

Further analyses are being done with more samples of Brazilian green tea, with the aim of verify their content of catechins and caffeine to conclude this study.

4 - CONCLUSIONS

The proposed method has proved to be efficient to quantify EGCC, EC and CAF in green tea, once it has presented a good performance with the utilized column and with analysis time relatively short (27 min), when compared to other works that evaluate *Camellia sinensis* catechins. It was verified that the EGCC contents found in the Brazilian green tea samples are superior to the contents of the black tea produced in the same region and that of the imported green tea samples, which might be attributed to the different climate and soil in Brazil, as well as the variety that is grown. However, more analyses should be done to confirm it.

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6 - ACKNOWLEDGEMENTS

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