ELSEVIER

Journal of Chromatography A, 827 (1998) 113–120

JOURNAL OF CHROMATOGRAPHY A

Determination of anti-carcinogenic polyphenols present in green tea using capillary electrophoresis coupled to a flow injection system

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Received 26 May 1998; received in revised form 8 September 1998; accepted 8 September 1998

Abstract

A capillary electrophoresis (CE) method was developed for the simultaneous determination of a number of major ingredients of green tea. The components analysed were caffeine, adenine, theophylline, epigallocatechin-3 gallate, epigallocatechin, epicatechin-3 gallate, (−)-epicatechin, (+)-catechin, gallic acid, quercetin and caffeic acid. Separation was achieved using a fused capillary column with 0.15 *M* H_3BO_3 as buffer at a pH of 8.5, UV detection at 210 nm and 20 kV of voltage. Analysis was carried out after treatment (extraction, filtration and dilution) of the samples in a flow injection system which was coupled to a CE equipment via a programmable arm. The procedure allows the determination of these compounds in less than 20 min. Quantitative analysis was performed by the standard addition method. Limits of detection ranged between 0.04 μg ml^{-1} for flavonols and 1.2 μg ml^{-1} for caffeine.

Keywords: Tea; Food analysis; Flow injection–capillary electrophoresis coupling; Catechins; Polyphenols; Flavanols

1. Introduction

Epidemiological studies suggest that the consumption of green tea may help to prevent cancers in humans [1]. Jankun et al. has demonstrated that catechin, one of the major ingredients of green tea, inhibits uokinase, an enzyme crucial for cancer growth [2]. Green tea contains many polyphenols known as catechins, including epigallocatechin-3 gallate (EGCG), epigallocatechin (EGC) and epicatechin-3 gallate (ECG). The quality of green tea has been evaluated by organoleptic test of trained specialists but recently the relationship between tea quality and its chemical components, free amino acids, catechins, caffeine and ascorbic acid, is being made clear [3]. The principal components present in tea are cellulose, hemicellulose, lignin, polyphenols (flavonols, flavon-3-ols, phenolic acids), alkaloids (caffeine, theophylline, theobromine), amino acids, metals and cations. The aim of this work was to determine the main anti-carcinogenic compounds present in tea, namely EGC, EGCG and ECG which are classified as flavon-3-ols. Polyphenols are the most abundant group of compounds in tea leaves. Among these, the flavonols (catechins) constitute the major component, with up to 30% of the dry matter of fresh leaf [4]. The characteristics of many plant products, i.e., taste, palatability, nutritional value, pharmacological and toxic effects and microbial decomposition, depend substantially on their polyphenol content [5]. Catechins also contribute to the astringent and bitter taste of tea. Catechin is known to be unstable under alkaline conditions, and at

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00737-7

higher pH values of buffer solution the tailing of the electrophoretic peaks become significant. Gallic acid is the most abundant phenolic acid in tea. The amount of gallic acid increases during fermentation owing to its release from catechin gallates [4].

High-performance liquid chromatography (HPLC) is currently the most useful approach for the routine analysis and research of non-volatile tea constituents. Using HPLC several catechins and caffeine can be separated. Clean-up for HPLC analysis is performed by, e.g., using heavy magnesium oxide in a modified Kjeldahl apparatus or in a shaking bath [4]. This paper proposes their separation in a single run. As far as we are aware, there are only three papers [6–8] on the analysis of some polyphenols in tea samples by capillary electrophoresis (CE). Recently, Horie et al. [8] published an investigation on eight tea constituents, but only a single sample was analysed and no quantification was done. In this work, a new method for the simultaneous determination of eleven polyphenols in green tea infusions by flow injection (FI)–CE in a single run is proposed by coupling the FI system to a CE instrument (arrangement described previously [9,10]). The main objectives pursued in this work were: (i) to investigate a CE procedure in order to achieve an optimum separation of those components present in tea which are of interest in the prevention of cancer; (ii) to use a FI system coupled to CE equipment via a programmable arm to automate sample preparation before its analysis by CE.

2. Experimental

2.1. Chemicals

Standard and buffer solutions were prepared in 18 mΩ deionized water from a Millipore Milli-Q water purification system. An individual standard solutions of each of the following polyphenols were prepared: caffeine, adenine, theophylline, EGCG, EGC, ECG, (−)-epicatechin, (+)-catechin, gallic acid, quercetin and caffeic acid. All were supplied by Sigma. Standard working solutions were prepared from the stock solutions by dilution with Milli-Q water. A 0.1 *M* solution of sodium hydroxide (Merck) was used to condition the capillary and to adjust the pH of the buffer solution. A background electrolyte (BGE), 0.15 *M* H_3BO_3 at pH 8.5 (supplied by Sigma) was used for electrophoretic separations.

2.2. Apparatus

A Beckman P/ACE 5500 CE unit equipped with a diode array detector was used to separate and quantify the analytes. Beckman capillary tubing of 57 cm×75 μm I.D×375 μm O.D. was used. A Gilson Minipuls-3 peristaltic pump, a Rheodyne 5041 injection valve and PTFE tubing of 0.5 mm I.D. were used in the FI manifold. A continuous filtration laboratory-made system was developed and tested with different types of microfilters from Micron Separations (pore size 0.45 μm). Pre-treatment of the sample was carried out in a FI system after which sample was introduced in the CE equipment using a laboratory-made programmable arm controlled by a microcomputer via an electronic interface [9]. A household microwave oven (AEG Micromat-Duo) equipped with a magnetron of 2450 MHz with a maximum power of 800 W was used, without further modifications. This set-up accomplished a high degree of automation in all the steps implied in the process.

Extraction and filtration of the analytes of interest from the tea samples was carried out by using a FI system coupled to a CE equipment via a programmable arm (see Fig. 1). The manifold included an injection valve (IV) which was used to measure the volume of tea sample–water (250 μl) in order to dilute the sample and get results that could be compared with the ones obtained with the standard addition method. Tea was filtered through a 0.45-μm filter prior to its introduction into the manifold.

The entire fraction containing the eluted analytes was driven to a vial of the CE sampler via the activation of the programmable arm that is controlled by the CE software. The time taken by the tea sample to reach the CE equipment was measured using a dye (methylene blue). While the analysis is performed in the CE instrument, a new sample is processed in the flow system. The overall flow-rate was not found to have any significant influence. Consequently, a 4.5 ml min^{-1} flow-rate was finally chosen as a compromise between reasonable high sample throughput and the volume of sample needed.

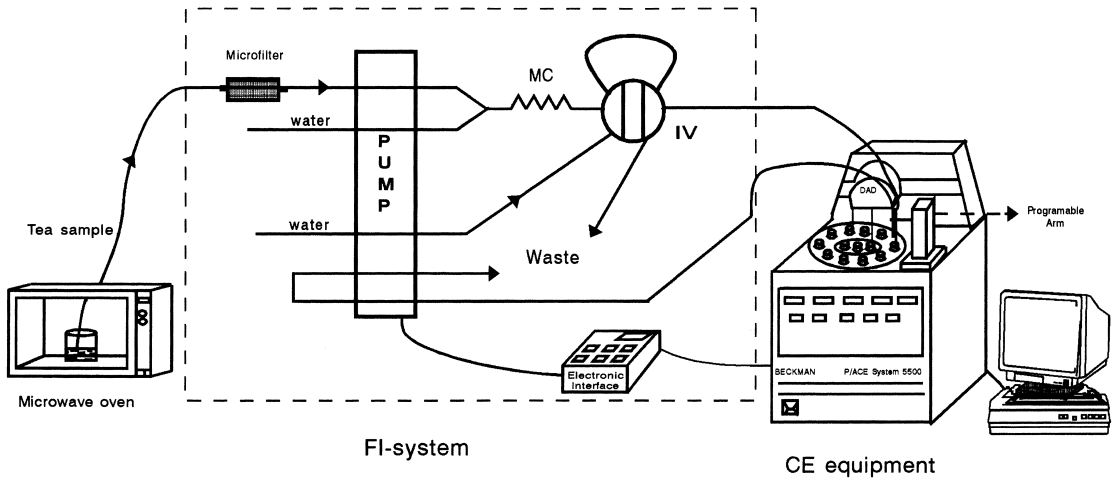

Fig. 1. Flow injection system assisted by microwave treatment used as sample preparation/introduction into the CE equipment. IV=Injection valve, MC=mixing coil.

2.3. Operating conditions

The running buffer used was a solution of 0.15 *M* H_3BO_3 at pH 8.5 (adjusted with NaOH). The applied voltage was 20 kV, the average current 40.1 μA, 20°C temperature and the selected wavelength at 210 nm. Samples were hydrodynamically injected (by high pressure) for 10 s. In order to maintain the capillary under good working conditions, its surface was regenerated once a day by consecutive washing with water (5 min), 0.1 *M* sodium hydroxide (5 min), and water (2 min) followed by the running buffer (15 min). When used under the conditions mentioned above, the capillary showed good performance for more than six months without losing its initial efficiency.

2.4. Tea samples

Two main different types of tea can be found: green tea is produced by drying and roasting the leaves, whereas black tea is produced in the same way as green tea but its leaves are further fermented [11]. In this fermentation step an enzymatic oxidation of polyphenols occurs, leading to the formation of chemicals compounds that are responsible for the characteristic aroma and colour of black tea. Eight different types of commercially available tea were analysed. Samples spiked with analyte concentrations in the typical reported ranges were used [12]. Tea samples (1.75 g) were extracted with 100 ml of boiling water.

3. Results and discussion

3.1. FI system

A sample volume of 100 ml was used to prepare a tea infusion in a microwave that was directly connected with the FI system, as described in the previous section. The microwave was turned on at power of 500 W for 1 min. The FI system allowed a rapid and easy sample preparation and no manipulation of the sample was needed.

3.2. Separation conditions of polyphenol compounds

Although the method was mainly designed for the separation and quantification of the main flavonols present in tea, some other analytes such as caffeine, theophylline, gallic acid, quercetin and adenine were separated and could be identified and quantified using the appropriate standards. The total time taken for the whole analysis was less than 20 min. For optimisation purposes the following 11 analytes were chosen: caffeine, adenine, theophylline, EGCG,

EGC, ECG, (−)-epicatechin, (+)-catechin, quercetin, gallic acid and caffeic acid. The criterion used for optimisation was to achieve the best possible separation for the compounds of interest (flavonols).

In the first place optimisation of chemical variables was carried out. With this purpose a H_3BO_3 buffer, at an alkaline pH was used. The pH was studied in the range 8–9.5. As can be seen in Fig. 2 a pH of 8.5 proved to be the optimum value. However, at this value of pH the peaks corresponding to EGC and (−)-epicatechin almost overlapped. Different solutions of H_3BO_3 (at the 0.05–0.35 *M* range) at pH 8.5 were prepared in order to achieve the best resolution of the test mixture. 0.15 *M* was the value chosen for the concentration of H_3BO_3 due to the good resolution provided for the separation of the 11 analytes (see Fig. 2F). At H_3BO_3 concentrations of 0.25 and 0.35 *M* the separation was also achieved

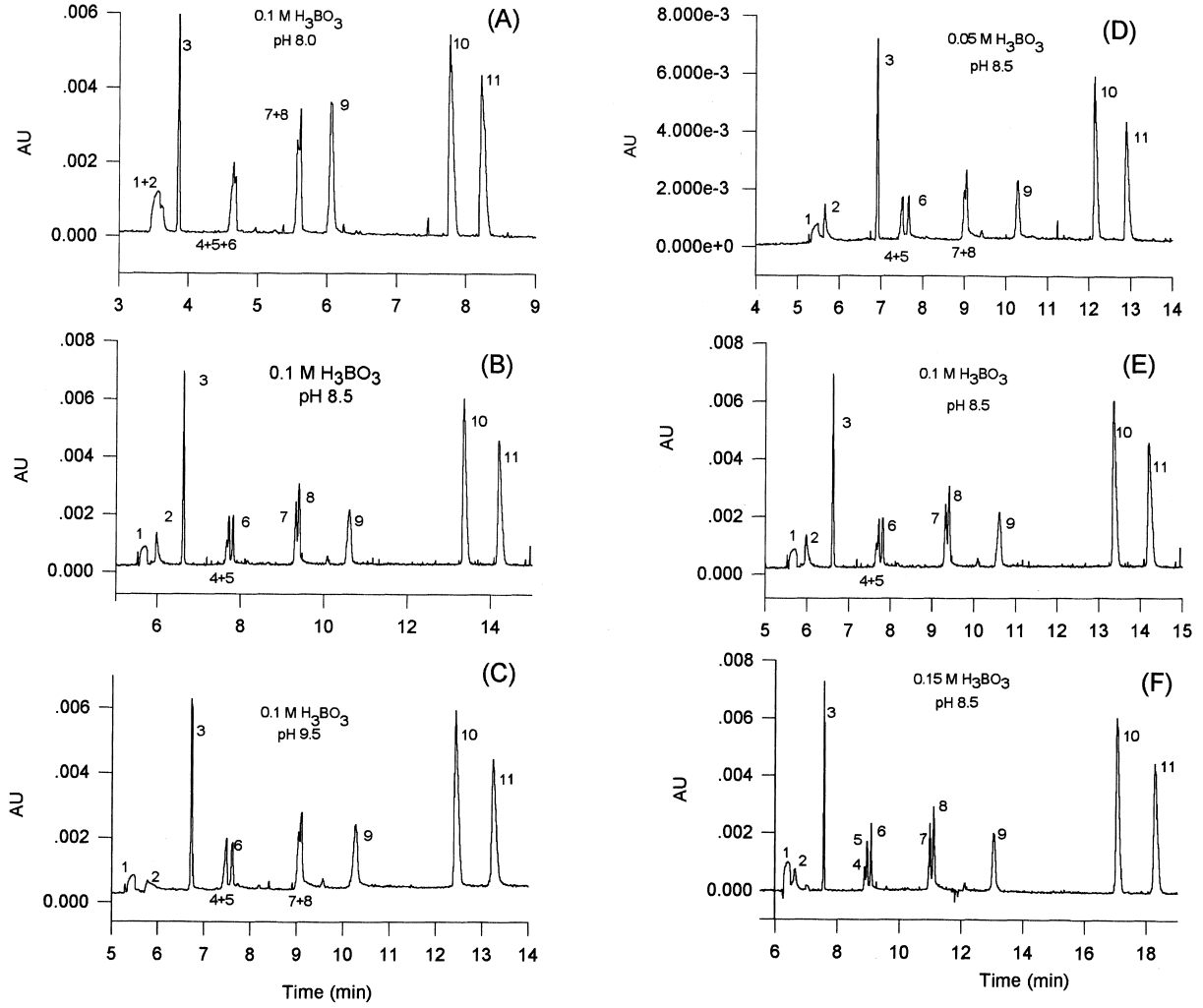

Fig. 2. (A, B, C) Influence of the pH of the buffer in the resolution of the peaks. (D, E, F) Influence of the buffer concentration in the resolution of the peaks. Electropherograms of a standard mixture of polyphenols. Peaks: 1=EOF+caffeine; 2=adenine; 3=theopylline; 4=EGC; 5=(−)-epicatechin; 6=(+)-catechin; 7=EGCG; 8=ECG; 9=quercetin; 10=gallic acid and 11=caffeic acid.

with acceptable resolution but the sensitivity was very poor and the time of analysis also increased.

Finally, the instrumental variables were also optimised. By increasing the voltage (up to 30 kV) the time of analysis was reduced but it may lead to significant looses of resolution and peak efficiencies if an excessive heating occurs within the capillary. The value finally chosen was 20 kV. This value improved detection limits of the method although longer analysis times were required. The increase in temperature from 20–30°C produced a decrease in the time of analysis due to the fact that the analytes migrate faster at higher temperatures. However, higher temperature resulted in poorer sensitivity. Separation between peaks improved when the applied temperature was raised to 20°C. A diode array detector was used to determine the maximum of absorbance of these compounds. All of them presented a maximum of absorbance at 210 nm.

3.3. Performance of the method

Calibration graphs were obtained by injecting standard solutions in the range 0.1–1 μg ml^{-1}. Each point of the calibration graph corresponds to the mean value obtained from three independent area measurements. The limit of detection (LOD) was calculated as the value obtained for the blank plus three-times its standard deviation, whereas the limit of quantification (LOQ) was calculated as the value obtained for the blank plus 10-times its standard deviation. The corresponding regression equation and other characteristic parameters for the determination of these polyphenolic compounds are shown in Table 1. The proposed method allows polyphenols to be determined at low levels (with LODs between 0.01–1 μg ml^{-1}).

Eleven replicate analyses were performed on the standard solution (10 μg ml^{-1} for each compound), in order to evaluate the precision of the method for every compound to be determined. In all cases the value of the relative standard deviation for the retention time were less than 0.7% and less than 5% for quantification. The standard deviations of residuals and curve-fitting level (in percentages) were obtained by analysis of variance (ANOVA) during the validation of the calibration model.

3.4. Analytical applications

Since the objective was to determine certain analytes present in drinking tea, extraction was achieved by using simply boiling water. Other components that could possibly be extracted when using other extractants were not considered since although perhaps this would lead to extraction of other different components, they will never be present in a tea infusion in the way it is normally consumed. The sample was then filtrated through a membrane (cellulose, 0.45 μm) and directly injected in the system.

Fig. 3 shows, as an example, the electropherogram recorded for a tea infusion (1.75 g/100 ml) in which the different peaks can be clearly identified. The migration times obtained for the standard samples and for the real samples were different due to matrix effects. The peaks were identified by using the standard addition method and the peak purity spectrum. Only the peak corresponding to caffeine overlapped with that corresponding to electroosmotic flow (EOF). However, this could be overcome by subtracting the signal obtained for the caffeine from that of the blank in all cases. Interferences due to the matrix were negligible.

Spiked tea samples were prepared in order to evaluate the accuracy of the method. Excellent recoveries were obtained in all cases (between 97–99%). The proposed method was applied to different brands of tea for the direct determination of EGC, EGCG, ECG among other components. Table 2 shows the results obtained in triplicate analyses of some of these polyphenolic compounds found in the tea samples. In order to validate the procedure the standard addition method was applied. In all cases the application of the *t*-test for the slopes of the calibration curves showed no significant statistical differences. Consequently there are no evidence of systematic error affecting the determination of these analytes in tea by the proposed method. By checking the results it can be concluded that green tea contains several polyphenols which could reduce the incidence of cancer in humans or the size of cancers already formed [1]. In all the samples, caffeine was the most abundant compound. EGC did not appear in all tea samples. It was only found in three of the

Table 1
Figures of merit of the proposed method

Analytes	$y=a+bx$	r	$S_{y/x}$	R^2	R.S.D. (%) (AU)	R.S.D. (%) (s)	LOD	LOQ
Caffeine	$a=0.08\pm0.01$ $b=0.025\pm2.0\cdot10^{-4}$	0.999	0.029	99.93	3.7	0.27	1.2	4
Adenine	$a=-0.029\pm7.28\cdot10^{-3}$ $b=0.072\pm2.26\cdot10^{-4}$	0.999	0.02	99.99	4.5	0.27	0.3	1.01
Theopylline	$a=-7.0\cdot10^{-4}\pm1.26\cdot10^{-3}$ $b=0.10\pm2.18\cdot10^{-3}$	0.997	$2.52\cdot10^{-3}$	99.55	4.6	0.12	0.03	0.12
EGC	$a=-3.5\cdot10^{-3}\pm2.6\cdot10^{-3}$ $b=0.12\pm4.45\cdot10^{-3}$	0.993	$5.16\cdot10^{-3}$	98.78	4.5	0.27	0.06	0.21
Epicatechin	$a=-7.5\cdot10^{-3}\pm2.5\cdot10^{-3}$ $b=0.17\pm4.3\cdot10^{-3}$	0.996	$4.97\cdot10^{-3}$	99.37	3.38	0.43	0.04	0.14
Catechin	$a=5.6\cdot10^{-5}\pm2.77\cdot10^{-3}$ $b=0.17\pm4.77\cdot10^{-3}$	0.996	$5.5\cdot10^{-3}$	99.27	4.18	0.42	0.04	0.15
EGCG	$a=-8.4\cdot10^{-3}\pm1.9\cdot10^{-3}$ $b=0.17\pm3.3\cdot10^{-3}$	0.998	$3.8\cdot10^{-3}$	99.66	3.7	0.45	0.03	0.1
ECG	$a=-6.9\cdot10^{-3}\pm2.2\cdot10^{-3}$ $b=0.16\pm3.9\cdot10^{-3}$	0.997	$4.51\cdot10^{-3}$	99.45	4.3	0.5	0.04	0.14
Quercetin	$a=0.037\pm0.018$ $b=0.077\pm3.66\cdot10^{-4}$	0.999	0.052	99.98	4.18	0.42	0.7	2.3
Caffeic acid	$a=-7.3\cdot10^{-3}\pm1.9\cdot10^{-3}$ $b=0.072\pm2.9\cdot10^{-3}$	0.995	$2.1\cdot10^{-3}$	99.16	3.1	0.49	0.08	0.26
Gallic acid	$a=0.01\pm2.6\cdot10^{-3}$ $b=0.09\pm4.2\ 10^{-3}$	0.993	$3.27\cdot10^{-3}$	98.67	2.6	0.52	0.08	0.3

a=Intercept; b=slope; r=correlation coefficient; $S_{y/x}$=standard deviation of residuals; R^2=curve-fitting level (in %) obtained by ANOVA for the validation of the model; R.S.D.=relative standard deviation of the retention time (s) and absorbance (AU) values. LOD=Limit of detection; LOQ=limit of quantification. Concentration, LOD and LOQ are expressed in μg ml^{-1}. (Buffer: 0.15 *M* H_3BO_3, pH 8.5, 20 kV; 20°C; 10 s hydrodynamic injection; 210 nm.)

eight samples analysed. No spurious peaks interfered with the ones of interest. The purity of each peak in the tea samples was better than 0.990, as calculated from the peak purity spectrum.

4. Conclusions

The connection of a continuous automatic sample pre-treatment module (FI) with CE has been established and constituted an easy and user-friendly approach. This system represents a new contribution in the field of automation of analytical methods. CE proved to be a suitable analytical separation technique for flavonols in tea samples. The coupling of FI with CE to automate extraction, transportation and dilution of the analytes from solid tea simplifies the manual sample pre-treatment. The method here proposed is suitable for the identification and quantification of at least 11 polyphenols in tea. Detection limits were around 0.04 μg ml^{-1} for flavonols, which is much lower than the concentrations expected to be present in tea. Therefore the use of a more sensitive method could be time consuming and not necessary for the analysis of the major flavonols in tea. Moreover, it should be noted that the specific advantages of this method are based on the fact that no pre-treatment or derivatization of the samples is required, apart from filtration and a suitable dilution,

Table 2
Analysis of polyphenols in real samples (figures correspond to concentrations in μg ml^{-1})[a]

	Pompadour	Hornimans	Burco	Akfa	CayCiceg	Filiz	Demcay	Rize
Caffeine	770.8±4.6 (773.7±3.3)	319.2±3.5 (319.5±2.9)	504.0±3.8 (510.1±1.5)	408.9±2.6 (409.4±2.7)	197.9±1.5 (192.4±2.0)	307.9±3.1 (310.4±2.2)	216.06±2.3 (218.1±2.1)	545.4±3.2 (554.0±1.9)
Adenine	45.9±1.7 (44.56±0.7)	0.64±0.06 (0.74±0.08)	–	–	19.3±0.9 (17.6±1.2)	23.43±1.6 (22.8±1.3)	20.5±0.8 (19.4±0.3)	–
EGC	85.82±2.0 (90.64±2.8)	–	80.8±0.4 (79.1±2.0)	2.2±0.1 (1.9±0.5)	–	–	–	–
Epicatechin	97.33±3.0 (93.32±2.4)	8.62±1.2 (10.1±0.9)	6.8±0.6 (5.8±0.1)	4.7±0.9 (5.4±0.4)	2.02±0.3 (2.5±0.4)	14.64±0.9 (15.4±0.6)	7.6±0.8 (7.1±0.5)	9.1±0.7 (9.9±0.8)
Catechin	32.06±0.4 (33.68±0.9)	11.2±1.6 (11.0±0.4)	8.3±0.2 (8.7±0.8)	5.8±0.2 (5.3±0.2)	0.85±0.05 (0.72±0.09)	2.19±0.8 (2.04±0.4)	1.62±0.4 (1.05±0.4)	2.2±0.3 (2.1±0.4)
EGCG	316.4±3.3 (327.0±2.5)	26.4±0.9 (26.2±1.8)	9.12±0.5 (9.4±1.2)	13.2±0.9 (12.1±1.1)	3.9±0.1 (3.5±0.8)	7.2±0.5 (7.7±0.4)	9.46±0.7 (10.0±0.4)	12.9±0.9 (13.4±0.7)
ECG	177.4±2.6 (171.9±2.0)	19.9±1.5 (21.4±0.9)	1.12±0.3 (0.98±0.2)	6.2±0.4 (6.7±0.3)	0.74±0.08 (0.8±0.01)	3.66±0.9 (3.45±0.08)	4.3±0.1 (4.9±0.3)	6.3±0.4 (6.9±0.5)
Gallic acid	88.14±2.2 (84.28±1.8)	40.4±0.3 (40.7±1.8)	32.4±1.3 (30.5±1.1)	25.4±1.0 (25.0±1.2)	7.0±0.6 (6.1±0.2)	15.0±1.1 (15.4±0.5)	18.93±1.2 (20.2±1.0)	31.7±1.6 (30.6±1.3)

[a] In parentheses concentrations found by using the standard addition method (μg ml^{-1}).
[b] – = Not detected.

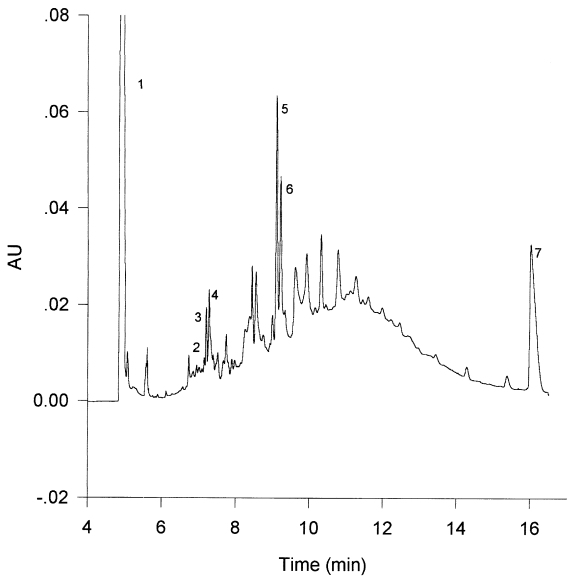

Fig. 3. Electropherogram of a tea sample (Akfa) using 100 ml boiling water as extractant. Peaks: 1=caffeine; 2=EGC; 3=(−)-epicatechin; 4=(+)-catechin; 5=EGCG; 6=ECG; 7=gallic acid.

when necessary. The resolving power of capillary zone electrophoresis (CZE) was far superior to that of HPLC and the CZE method is quicker and uses less organic solvents.

Acknowledgements

Financial support provided by Spain's DGICyT (PB95-0977) is gratefully acknowledged. Authors wish to thank Mustafa Soylak who provided us with the Turkish tea samples.

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