

Analysis of natural flavonoids by microchip-micellar electrokinetic chromatography with pulsed amperometric detection

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Catechins (catechin and other derivatives) are naturally occurring flavonoids present in a number of plants and foods. They are also part of numerous nutraceutical formulations because they are believed to have antioxidant, cancer chemo-preventative, anti-inflammatory and antimicrobial properties. The determination of catechins has traditionally been performed by HPLC. However, this methodology is both time and sample intensive and generates large amounts of organic solvent waste. In the current report, an application of MEKC using a PDMS microchip is presented for the analysis of catechins. The system uses pulsed amperometric detection for direct analysis of important naturally occurring catechins. The effect of pH, surfactant concentration, detection potential and signal stability were analyzed. Linear relationships were found between the concentration and peak current, with good stability and limits of detection of 8 μM for catechin, epigallocatechin gallate and epicatechin, and 14 μM for epicatechin gallate. Optimum conditions were applied to the detection of selected catechins in a commercially available green tea extract nutraceutical and the results were compared to HPLC analysis. The analysis using microchip micellar electrokinetic chromatography and pulsed amperometric detection was completed in 4.5 min, 10 times faster than the HPLC analysis.

Introduction

Natural products have become increasingly more sought after as ecologically safer alternatives to herbicides and pesticides as well as sources of new medicines.^{1,2} Among the many classes of products, catechins (catechin and other derivatives) have been reported to have antiatherogenic,³ antihypertensive,⁴ antioxidant,^{3,5} antimicrobial-antifungal,² antiobesity,⁶ and cancer chemopreventative⁷ activity. The structure of several common catechins is shown in Fig. 1. Catechins are naturally present in commonly consumed products such as green and black tea,⁸ mate,⁹ vegetables and fruits,¹⁰ chocolate,¹¹ and wine.¹² Due to the variety of health benefits,¹³ there is also a large number of nutritional supplements derived from plant extracts (nutraceuticals) that contain catechins.^{14–16} Since they fall between the traditional dividing lines between food and medicine, there is a very interesting debate about the commercial availability of these supplements and the corresponding regulations. In addition, quantitative data on catechin contents of food are, as yet, largely absent, incomplete, or unreliable.¹¹

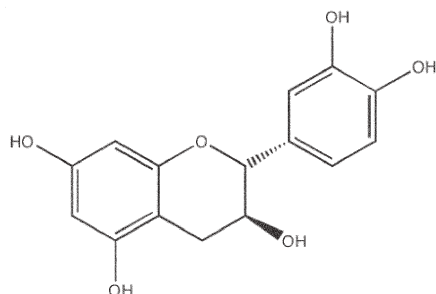
Several methodologies can be used to measure catechins, with HPLC-UV being the most used.^{8,10,17} HPLC coupled with mass spectrometry (MS) has also been used, but the expensive instrumentation limits its wide application.^{18,19} More recently, capillary electrophoresis (CE) with diode-array,²⁰ UV,⁷ and electrochemical detection²¹ has been used for the analysis of catechins. The separation of catechins by CE in the zone mode

can be challenging because of the similarities in their chemical structure and their neutral charge (at pH 7). Alkaline electrolytes have been used in the past^{22,23} but catechins are extremely unstable and can be degraded almost completely in a few minutes under those conditions.²⁴ Epimerization reactions also became significant at pH values greater than 8.²⁵ A complementary method for the separation of catechins is micellar electrokinetic chromatography (MEKC).^{26,27} MEKC can be performed by dissolving a surfactant in the CE background electrolyte (BGE) at a concentration higher than the critical micelle concentration (CMC).²⁸ In MEKC, the micelles form a pseudostationary phase with a charged surface and a non-polar core. The partitioning coefficient of the analyte is the main factor determining the selectivity because the separation is based on the time that the analytes spend inside or outside the micelle. In general, more hydrophobic analytes spend more time inside the micelle and therefore are retained more. Analytes of the same hydrophobicity can also be separated based on shape.

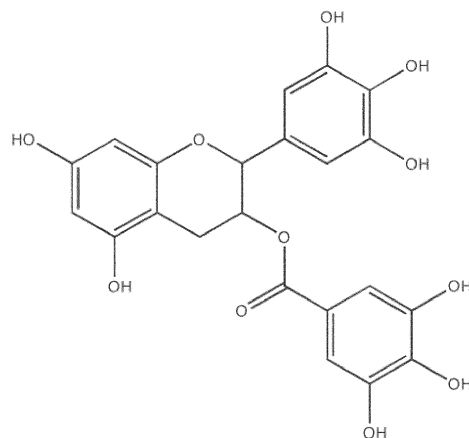
As mentioned previously, catechins can be detected electrochemically using different conditions.^{22,23,29–31} Electrochemistry coupled with microchip electrophoresis is attractive because of its low cost, ease of miniaturization and ability to perform direct detection.³² However, if DC amperometry (or simply amperometry) is used the signal may decrease rapidly due to the adsorption of the oxidation products on the electrode surface resulting in surface fouling.³³ To overcome the problem the surface can be polished periodically or coated to prevent build up.³⁴ Pulsed amperometric detection (PAD) can also be used to overcome problems associated with electrode fouling.³⁵ PAD methods integrate a

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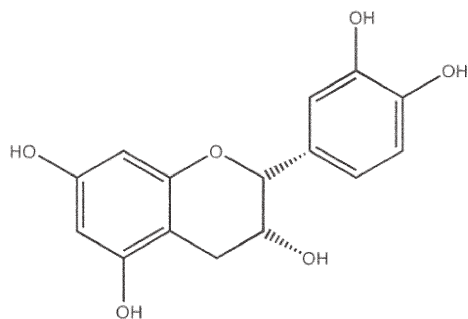
A) Catechin



B) Epigallocatechin gallate



C) Epicatechin



D) Epicatechin gallate

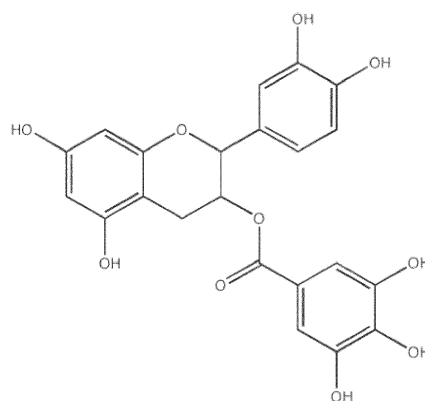


Fig. 1 Structures of the studied catechins. (A) catechin, (B) epigallocatechin gallate, (C) epicatechin and (D) epicatechin gallate.

self-cleaning cycle with the measurement to prevent build up on the electrode surface and have proven to be effective for a large number of analytes including carbohydrates, amino acids, sulfur-containing compounds, alcohols and metabolites.³⁵ Another advantage of PAD is that it can be easily incorporated into microchips, allowing fast, reproducible and low-cost direct analysis for many different analytes. Microchips also have many advantages over conventional bench-top systems including better flexibility of design, reduced reagent and sample use, lower waste generation, and portability.³⁶

In the current report, the separation of catechin, epigallocatechin gallate, epicatechin and epicatechin gallate by MEKC using a PDMS microchip is presented. PAD was used for direct detection of the four compounds, providing good sensitivity (LOD within 7–14 μM) and better signal stability than DC amperometry. The effect of pH, surfactant concentration, detection potential and signal stability were analyzed. Optimized conditions were applied to the detection of catechins in a green tea extract nutraceutical and the results compared to HPLC with UV detection.

Materials and methods

Reagents and solutions

SU-8 2035 photoresist and XP SU-8 developer were purchased from MicroChem Co. Sylgard 184 silicone elastomer and curing agent were obtained from Dow Corning. Aqueous solutions were prepared using analytical grade reagents and 18 $\text{M}\Omega\text{ cm}^{-1}$ resistance water (Milli-Q, Millipore). The running electrolytes were prepared by weighing the desired amount of sodium phosphate (either Na_2HPO_4 or NaH_2PO_4) and adjusting the pH with either 2 M NaOH (Fisher) or 2 M HCl (Fisher). Following the pH adjustment, the desired amount of surfactant was added to the running buffer. Sodium dodecyl sulfate (SDS, Aldrich) was selected for the present study. Stock solutions of catechin (C, Aldrich), epigallocatechin gallate (EGCG, Aldrich), epicatechin (EC, Aldrich) and epicatechin gallate (ECG, Aldrich) were dissolved in methanol and stored at $-4\text{ }^\circ\text{C}$ until use. Samples were prepared by the proper dilution of the stock in running electrolyte. The green tea extract (Nature's Way) was purchased locally. A 25 μm diameter, 99.99% gold wire (Goodfellow,

England) was used as the working electrode for electrochemical detection. For comparison purposes, a 35 μm carbon fiber was also used. For pH measurements, a glass electrode and a digital pH meter (Denver Instrument) were used. Methanol was of ACS certified quality and purchased from Fisher. All chemicals were used as received without any further purification. All experiments were performed at room temperature (22 ± 2 °C) and unless noted, the points and error bars on the plots represent the average and standard deviation obtained for at least 3 measurements.

Instrumentation

A 3-channel (two positives and one negative) laboratory-built high-voltage power supply, with an adjustable voltage range between 0 and ± 4000 V, was used for all the electrophoresis experiments.³⁷ Cross injection was used for all the experiments. During the injection, +410 V, -150 V and +410 V were applied to the reservoirs S (sample), SW (sample waste) and B (buffer) respectively. During the separation procedure the potential applied to reservoir B was raised to +1000 V (or the corresponding separation potential) while the potential applied to reservoir SW was changed to +410 V. The waste reservoir (W) was always grounded. In order to avoid joule heating in the S-B channel during the injection, a 1 M Ω resistor was included in series with the chip.

HPLC analyses were performed with an instrument consisting of a Hewlett Packard quaternary pump (Series 1050), a Rheodyne injection valve (model 7125), an HPLC column (Adsorbosphere XL C₁₈, 5 μm , 150 mm long, 4.6 mm id, Aldrich) and a Spectra 100 variable wavelength detector operating at 280 nm. Data were collected using a PC and ChromPerfect Software. The solvent program used for this analysis consists of a linear gradient from 100% solution A (2% acetic acid - 8% methanol - 90% water) to 80% solution A and 20% solution B (2% acetic acid - 88% methanol - 10% water) in 20 min. In order to clean the HPLC column, the gradient was ramped up to 100% solution B in 20 min and finally ramped back to 100% solution A in 5 min. A 20 μL sample loop was used and flow rate was kept constant at 1 mL min⁻¹ during the entire solvent program.

Fabrication of the PDMS microchip

For the present experiments, a previously described design³⁸ was used for the microchip (Fig. 2). Briefly, a clean 76 mm

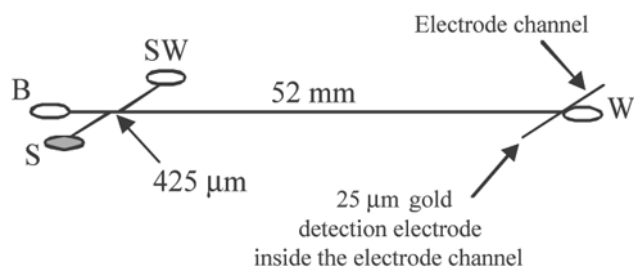


Fig. 2 Schematic drawing of the microchip-CE with PAD. Channels: 50 μm width, 50 μm depth, double-T arms: 10 mm long, double-T volume: 2 nL, separation channel: 52 mm, solution reservoirs: 6 mm diameter, detection electrode: 25 μm diameter, gold.

silicon wafer (Silicon Inc.) was coated with SU-8 2035 negative photoresist. A digitally produced mask (2400 dpi) containing the channel pattern was placed on the coated wafer, exposed to light *via* a near-UV flood source and then post-baked. The positive relief was developed by placing the wafer in propylene glycol methyl ether acetate for 15 min, rinsing with methanol and drying under a N₂ stream. The height of the positive pattern on the molding master, which is equal to the channel depth created on the PDMS layer, was 50 μm when measured with a profilometer. PDMS layers were fabricated by pouring a degassed mixture of Sylgard 184 silicone elastomer and curing agent (10 : 1) onto either a molding master or a blank wafer, followed by curing for at least 2 h at 65 °C. The cured PDMS was separated from the mold and reservoirs were made at the end of each channel using a 6 mm circular punch. A 25 μm gold wire was aligned at the end of the separation channel in a perpendicular electrode channel and served as the working electrode. After that, the two PDMS layers were placed in an air plasma cleaner (Harrick PDC-32G Plasma Cleaner/Sterilizer), oxidized for 20 s and immediately brought into conformal contact to form an irreversible seal. Finally, the extremities of the electrode channel were sealed with super-glue and electrical connection to the working electrode was made using silver paint (Structure Probe Inc, PA) and a copper wire.

Electrochemical detection

Electrochemical detection was performed using PAD with a two-electrode setup (CHI660B, CH Instruments). As mentioned previously, a gold wire (25 μm diameter) was used as the working electrode and the corresponding detection potential was optimized for each compound. A platinum wire placed in the waste reservoir was used as both an auxiliary and a reference electrode. This electrode positioning design was presented previously.³⁸⁻⁴² Quantitation of the catechins in green tea extracts was accomplished using a calibration curve for each compound from *ca.* 10–150 μM .

Results and discussion

Separation of catechins

The separation of catechins must be achieved to be able to determine their respective concentrations. The optimization of the separation of catechins by MEKC was done by varying the sodium dodecyl sulfate (SDS) concentration in 50 mM phosphate buffer (pH = 7.00). Fig. 3 shows electropherograms for four concentrations of SDS. At 0 mM SDS, one large peak with multiple shoulders was seen. When SDS was added to the background electrolyte, the migration time initially decreased due to the increase in EOF. As the SDS concentration was progressively increased, the migration times increased while preserving the migration order. At 20 mM SDS, all the peaks were resolved. However, the optimum concentration of SDS was determined to be 30 mM since the C, EGCG, EC, and ECG peaks were all baseline separated with better resolution, leaving a significant open area for additional peaks of unidentified compounds in a real green tea sample. At higher SDS concentrations (>40 mM) a little improvement in

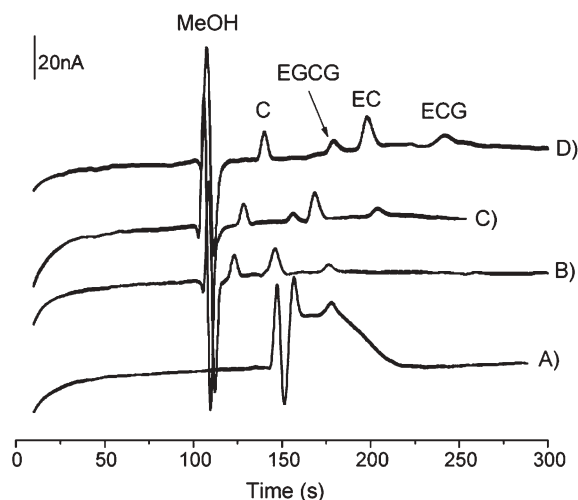


Fig. 3 Effect of the surfactant concentration on the separation of catechin (C), epigallocatechin gallate (EGCG), epicatechin (EC) and epicatechin gallate (ECG). (A) No SDS added to the BGE, (B) 10 mM SDS, (C) 20 mM SDS and (D) 30 mM SDS. Other conditions: 50 mM phosphate buffer (pH = 7.00), $E_{SEP} = 1010$ V, $T_{INJ} = 10$ s, $E_{DET} = 1.0$ V.

resolution was reported,²⁶ probably due to the high ionic strength of the BGE. It can also be observed that the presence of the micelle pseudo-stationary phase does not affect the position of the methanol peak, which allows for it to be used as an EOF marker.

Due to the high conductivity of the BGE, Joule heating studies were performed in order to determine an optimum separation potential. A linear relationship between separation potential and current was obtained between 100 and 1200 V for a 50 mM phosphate buffer with 30 mM SDS. An optimum separation potential was chosen at 1000 V in order to avoid Joule heating while preserving a reasonable analysis time of under 4 min. In addition, due to the complexity of green tea extracts, a high resolution is necessary in order to avoid co-migration of catechin peaks and possible interference.

Electrochemical detection

The magnitude of the signal for a given compound can be significantly influenced by the electrochemical technique used.^{35,40} Three electrochemical techniques were tested for the analysis of catechins including PAD, DC amperometry, and integrated pulsed amperometric detection (iPAD).³⁵ The optimum technique was determined by comparing the noise and baseline and signal stability of the different techniques. As can be observed in Fig. 4, PAD and DC amperometry demonstrated greater baseline stability than iPAD. In addition, a higher number of theoretical plates (N_{PAD} for catechin = 105536 plates m^{-1} ; N_{iPAD} for catechin = 29412 plates m^{-1}) and smaller baseline noise (PAD = 0.2 nA; iPAD = 0.9 nA) was observed for PAD than for iPAD. DC amperometry gave higher theoretical plate counts (N_{DCA} for catechin = 251180 plates m^{-1}) and lower baseline noise (0.15 nA) than PAD.

After determining the optimal baseline stability, we evaluated the signal stability in different conditions. For

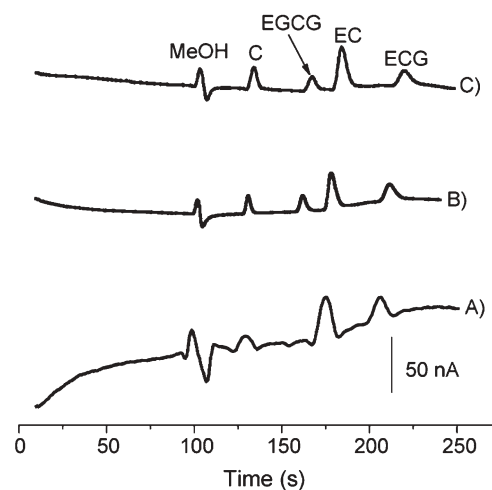


Fig. 4 Effect of different electrochemical detection methods on the signal of catechin (C), epigallocatechin gallate (EGCG), epicatechin (EC) and epicatechin gallate (ECG). (A) iPAD, (B) DC Amperometry and (C) PAD. Other conditions: 50 mM phosphate buffer with 30 mM SDS (pH = 7.00), $E_{SEP} = 995$ V, $T_{INJ} = 10$ s.

comparison purposes, a carbon fiber electrode was used since a high performance has been reported when detecting poly-phenolic compounds.^{23,34} Studies performed showed significant peak tailing and low signal stability when attempting to detect catechins (data not shown). In particular, the signal of catechin decreased to 37% of its original value over a period of 4 runs using DC amperometry, which made it unsuitable as a working electrode. This decrease in signal may be attributed to carbonaceous material fouling the electrode. PAD was also used with the carbon fiber electrode but, as expected, poor signal was observed. This can be explained by the absence of a surface layer that can be reduced and oxidized in order to regenerate the electrode surface. In contrast, when using a gold wire electrode, PAD offered good signal stability. As can be observed in Fig. 5, over a period of seven runs while using DC amperometry, the signals for catechin and epicatechin

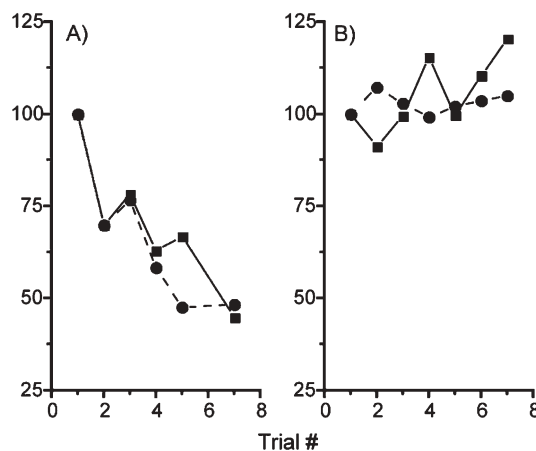


Fig. 5 Comparison of the signal stability using (A) DC amperometry and (B) PAD for catechin (■) and epicatechin (●). Other conditions: 50 mM phosphate buffer with 30 mM SDS (pH = 7.00), $E_{SEP} = 1000$ V, $T_{INJ} = 10$ s, $E_{DET} = 1.0$ V.

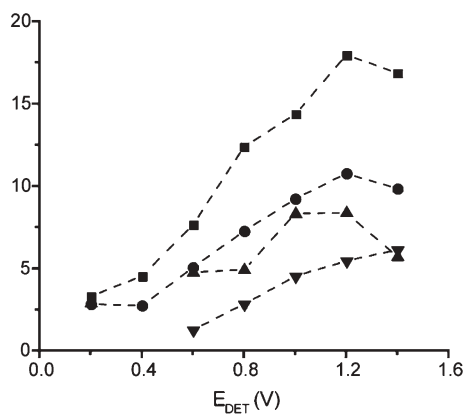


Fig. 6 Hydrodynamic voltammograms for 164 μM catechin (—■—), 159 μM epigallocatechin gallate (—▲—), 171 μM epicatechin (—●—) and 136 μM epicatechin gallate (—▼—). Other conditions: 50 mM phosphate buffer with 30 mM SDS (pH = 7.00), $E_{\text{SEP}} = 995$ V, $T_{\text{INJ}} = 10$ s, 25 μm gold electrode.

decreased to *ca.* 50% of their original values while PAD gave stable signals over the same number of runs. According to these results, gold wire was used as the electrode material and PAD was chosen as the optimum electrochemical technique for the detection of catechins.

After the optimum separation conditions and electrochemical technique were selected, the optimum detection potential was determined (Fig. 6). The detection potential was increased from 0.2 to 1.4 V in 0.2 V intervals and the peak current (I_p) was analyzed for the four catechins. The I_p increased significantly with potential until a maximum signal was achieved at 1.2 V. When potentials higher than 1.2 V were applied, a decrease in signal was observed for three of the catechins. The final decrease can be attributed to gold oxide being formed on the surface of the gold wire inhibiting the oxidation of catechins. A potential of 1.0 V was determined to be the optimum detection potential for catechins because the signal to noise ratio was also highest for all the catechins at this level. Above 1.0 V, a decrease in baseline stability and signal to noise ratio was observed (data not shown).

Analysis of green tea extract

Using the optimized conditions for separation and detection (50 mM phosphate (pH = 7.00) + 30 mM SDS, 1000 V as the separation potential, 10 s injection and 1.0 V as the detection potential), the response of the detector was analyzed as function of the concentration for the four catechins. The results are summarized in Table 1. As can be observed, the lowest detection limit was observed for catechin while the highest was obtained for epicatechin gallate. The limits of detection were in the low μM range (signal/noise = 3, Table 1). These results are

comparable with those reported by Hua *et al.*²¹ (with a similar BGE) but substantially better than the ones reported by Cao *et al.*,²² both using CE with amperometric detection. We believe the relatively high limits of detection are the result of the high concentration of SDS and the low pH, both necessary to achieve the separation. The high conductivity of the BGE increases the background noise while the pH = 7.00 may not be optimal for PAD. Additional studies where the waste reservoir solution was replaced with 0.01 M NaOH without SDS did not show a significant increase in the signal but current work is in progress to improve the limits of detection through the use of alternative surfactants and a decoupling system. It is also worth noting the good reproducibility of the migration times obtained for these analyses (see Table 1). This is probably due to the use of SDS as the surfactant to form the micelles. We have reported that SDS can increase and stabilize the EOF in PDMS microdevices, even at low concentrations (*ca.* 0.8 mM).^{39–41} Based on those results, we believe that at 30 mM SDS (*ca.* 3.75 times the CMC in water) the PDMS surface is completely saturated with SDS and therefore has a constant EOF.

In order to evaluate the capabilities of the method, a green tea extract nutraceutical was analyzed. The catechins present in 1 capsule (0.46 g) of Nature's Way Green Tea Extract were extracted with 50 mL of 80% methanol–20% water^{43–45} (with stirring) for 30 min. The insoluble matter was removed using a syringe filter (0.22 μm). Finally, 100 μL of the filtered solution was diluted to 1 mL with BGE and analyzed under the optimized conditions. As can be observed in Fig. 7A, clear peaks were obtained corresponding to methanol (solvent from the extraction), catechin, epigallocatechin gallate, epicatechin and epicatechin gallate. Two additional unidentified peaks were also observed at 132 and 171 s, most likely to correspond to other polyphenolic compounds. The concentration was calculated using the calibration curve parameters described previously. The final calculated concentrations were 3.3 ± 0.7 mg of catechin per capsule, 104.6 ± 7.1 mg of epigallocatechin gallate per capsule, 21.2 ± 2.0 mg epicatechin per capsule and 35.9 ± 9.2 mg of epicatechin gallate per capsule ($n = 4$). The amount of total catechin declared by the manufacturer in the label is 170 mg per capsule, which matches the value obtained by microchip-MEKC closely (C + EGCG + EC + ECG) 165 ± 12 mg per capsule. However, there may be other catechins that we did not identify which could contribute to the 170 mg per capsule.

In order to validate the microchip results, the same sample was analyzed using HPLC and UV detection. Peak identification was performed by comparison of the migration times with previously injected standards. As can be observed in Fig. 7B, clear peaks were also obtained, showing the presence of catechin ($t_r = 7.63$ min), epigallocatechin gallate

Table 1 Calibration curve parameters for the selected catechins using the optimum analysis conditions

	t_R (sec)	Slope/nA μM^{-1}	R^2	Linear range/ μM
Catechin	117.9 ± 1.4	0.366 ± 0.012	0.996	164.0–8.2
Epigallocatechin gallate	143.1 ± 2.3	0.157 ± 0.001	0.991	159.2–8.0
Epicatechin	157.2 ± 2.4	0.266 ± 0.013	0.992	170.5–8.5
Epicatechin gallate	178.4 ± 4.2	0.109 ± 0.003	0.998	135.6–13.6

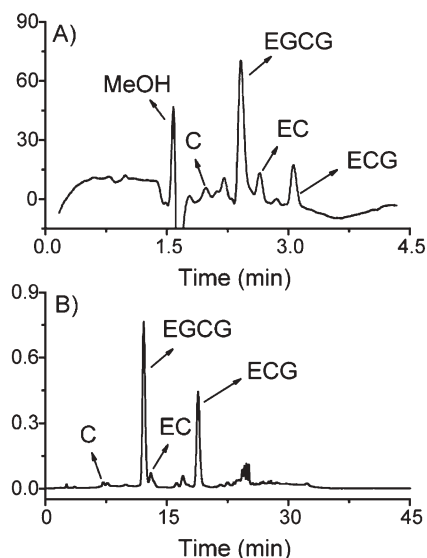


Fig. 7 (A) Electropherogram of a tea extract sample showing the presence of catechin (C), epigallocatechin gallate (EGCG), epicatechin (EC) and epicatechin gallate (ECG). Conditions: 50 mM phosphate buffer with 30 mM SDS (pH = 7.00) $E_{SEP} = 1000$ V, $T_{INJ} = 10$ s. (B) HPLC with UV detection (280 nm) of a green tea extract sample. HPLC conditions as described in the Experimental section.

($t_r = 12.09$ min), epicatechin ($t_r = 12.98$ min) and epicatechin gallate ($t_r = 18.75$ min). The comparison between microchip-MEKC-PAD and HPLC-UV showed an average correspondence of 98%, ranging from 119% for epicatechin gallate to 89% for catechin. It is also very important to note that the analysis by microchip-MEKC-PAD took just over 3 min for injection and separation to be completed and organic solvents were used only to prepare the stock solutions. By contrast, the analysis by HPLC-UV took approximately 45 min and consumed *ca.* 50 mL of solvent for each analysis. The extraction method for each step was the same in both methods.

Conclusions

The possibility of performing fast and inexpensive analysis of catechins from a nutraceutical using microchip-MEKC with pulsed amperometric detection was demonstrated for the first time. The use of SDS not only facilitated the separation by forming the micellar media but also contributed to the stabilization of EOF in the PDMS device. Using the optimized conditions a set of four catechins (catechin, epigallocatechin gallate, epicatechin, epicatechin gallate) were determined in a commercial green tea extract and the results were compared to HPLC. Faster analyses were achieved (3.5 min *versus* 45 min by HPLC) and the results obtained by both techniques were in good agreement, showing the potential of microchip-MEKC-PAD for the routine analysis of natural flavonoids.

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