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Microchip capillary electrophoresis with amperometric detection for rapid separation and detection of phenolic acids

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Abstract

A microchip capillary-electrophoresis protocol for rapid and effective measurements of food-related phenolic acids (including chlorogenic, gentisic, ferulic, and vanillic acids) is described. Relevant parameters of the chip separation and amperometric detection are examined and optimized. Under optimum conditions, the analytes could be separated and detected in a 15 mM borate buffer (pH 9.5, with 10% of methanol) within 300 s using a separation voltage of 2000 V and a detection voltage of +1.0 V. Linear calibration plots are observed for micromolar concentrations of the phenolic acid compounds. The negligible sample volumes used in the microchip procedure obviates surface fouling common to amperometric measurements of phenolic compounds. The new microchip protocol offers great promise for a wide range of food applications requiring fast measurements and negligible sample consumption. An application on a commercial red wine was performed with minimal sample preparation and promising results.

Keywords: Microchip; Phenolic acids; Wine analysis

1. Introduction

The separation and detection of phenolic acids is a challenging and important task owing to the importance of these compounds in a variety of food and beverage samples of plant origin [1]. In particular, phenolic acids are important in the wine industry due to their profound effect upon the sensory characteristics of wine (including its flavor, color or bitterness) [2,3]. The type and concentration of the phenolic acids thus reflect the overall quality of corresponding wine.

The similarity in the structural and chemical properties of phenolic acids along with the complexity of the wine samples have prompted researchers to employ high resolution separation methods for their wine analysis. In particular, high-performance liquid chromatography (HPLC), in connection to gradient elution, has been useful for providing efficient separation [4–8]. However, such separations require large volumes of organic solvents and long separation times. Capillary electrophoresis (CE) has also been employed successfully for determining phenolic acids in wine samples [9–12]. However, to our knowledge there are no reports on the use of CE microchips for the separation and detection of phenolic acids.

Microfabricated CE microchips have received growing attention in recent years [13,14]. Such analytical microsystems combine the advantages of high performance, integration, reagent economy, high throughput, miniaturization, and automation [15,16]. Electrochemical detection offers great promise for CE microseparation systems owing to its high sensitivity, low cost and power requirements, inherent miniaturization (of both the detector and control instrumentation), and high compatibility of microfabrication technology [17–20]. In particular, controlled-potential amperometric

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detectors (placed at the exit of the separation channel) are highly suited for CE microchip monitoring of analytes (such as phenolic acids) that are electroactive at modest potentials. The aim of this investigation is to develop a fast and efficient method for the rapid analysis of food-related phenolic acids in wine samples using microchip capillary electrophoresis with amperometric detection. Various parameters influencing the separation and detection were optimized including the electrolyte in the detection reservoir (DR), detection potential, separation voltage, pH and concentration of the running buffer, and injection volume. The applicability to the real samples was also demonstrated.

2. Experimental

2.1. Chemicals and reagents

Phenolic acids (ferulic, vanillic, chlorogenic, and gentisic acids), methanol (HPLC-grade), sodium tetraborate decahydrate, and nitric acid were obtained from Sigma (St. Louis, MO, USA). All chemicals were used without any further purification. Several borate buffers (at different concentrations and pH) were prepared daily in deionized water. The desired pH was obtained by titrating the buffer with 1 M NaOH or HCl as necessary. The optimal run buffer (RB) consisted of 15 mM borate (pH 9.5). Stock solutions (10 mM) of the phenolic acids were prepared by dissolving the required amount in methanol; working standard solutions were obtained by diluting corresponding stock solutions in mixture of electrophoresis buffer and methanol (final concentration of methanol in the sample is 10%). Real samples of a commercial wine (purchased at a local store) were prepared by filtering through a 0.45 μm pore size syringe filter (Cole-Parmer, Vermon Hills, IL, USA) and diluting in borate buffer (50 mM) in the ratio 7:3.

2.1.1. Apparatus

The glass microchip (88 mm × 16 mm) depicted in Fig. 1 was fabricated by Micralyne (Model MC-BF4-001, Edmonton, Canada) by means of wet chemical etching and thermal bonding techniques. The simple-cross microchip consisted of a four-way injection cross with a 74 mm long separation channel and side arms of 5 mm long (Fig. 1). The original waste reservoir was cut off, leaving the channel outlet at the end side of the chip, thus facilitating the end column amperometric detection [21]. The channels were 50 μm wide, 20 μm deep. Short pipette tips were inserted into the three (run buffer (RB), sample (S) and unused) reservoir holes on the glass chip for solution contact between the channel on the chip and the corresponding reservoir on the chip holder.

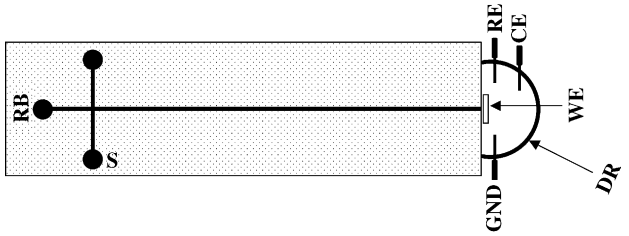

Fig. 1. Schematic diagram of the electrophoretic glass microchip system coupled with a screen-printed working-electrode detector. S, sample reservoir; RB, run buffer reservoir; WE, working electrode; RE, reference electrode; CE, counter electrode; GND, ground electrode; DR, detector reservoir.

The details of the CE/amperometric system layout and of the Plexiglas holder (accommodating the separation microchip and the end-column amperometric detector) were described elsewhere [21]. Briefly, the CE microchip was placed in a laboratory-built Plexiglas holder for housing the separation chip and detector, thereby allowing their convenient replacement. The holder contained a sample (S), running buffer (RB), and an unused reservoir. Platinum wires were inserted into each reservoir to serve as contacts for the high-voltage power supply. The detection reservoir (DR, at the channel outlet side) consists of a platinum wire and an Ag/AgCl wire (prepared by electrolytic oxidation of silver wire in 0.1 M HCl) to serve as counter (CE) and reference (RE) electrodes, respectively, for the amperometric detection. The screen-printed carbon working electrodes (WE) were printed with a semi-automatic printer (Model TF 100, MPM, Franklin, MA, USA). The Acheson ink (Electrodag 440B, Cat. No. 49AB90, Acheson Colloids, Ontario, CA, USA) was used for printing electrode strips. Details of the printing process and dimensions were described elsewhere [21]. The final screen-printed carbon working electrode had an exposed contact area of 0.3 mm × 2.5 mm. The screen-printed carbon electrode was further held in place by a plastic screw pressing the strip against the channel outlet and the distance (50 μm) of the working electrode from the chip outlet was controlled by a thin spacer. A homemade power supply, with an adjustable voltage range between 0 and 4000 V was used for injections and separations.

2.2. End-column amperometric detection

Amperometric detection was performed with an electrochemical analyzer CHI 621A (CH Instruments, Austin, Texas, USA) connected to a computer. Each electropherograms was recorded with a time resolution of 0.1 s using an applied potential of +1.0 V (versus Ag/AgCl pseudo reference wire electrode). Sample injections were performed after the stabilization of baseline. The detection reservoir (DR, Fig. 1) was filled with 1N nitric acid in order to improve the detection sensitivity [22]. All experiments were performed at room temperature.

2.3. Electrophoresis procedure

The channels were treated before use by rinsing with deionized water for 10 min, with 0.1 M NaOH for 10 min, and deionized water for additional 10 min. The electrophoresis buffer (for separating phenolic acids) was a borate buffer,

15 mM (pH 9.5). The 'run buffer' and 'unused' reservoirs were filled with electrophoresis running buffer solution, while the 'sample' reservoir with the mixture of phenolic acids. The grounded detection reservoir (GND) was filled with 1N nitric acid in order to improve the detection sensitivity. The injection was affected by applying 1500 V between the sample reservoir and the grounded detection reservoir (GND) for 5 s. This drove the sample 'plug' into the separation channel through the intersection. Separations were performed by switching the high voltage contacts and applying a potential of 2000 V to the 'running buffer' reservoir with the detection reservoir grounded and all other reservoirs floating.

2.4. Safety considerations

The high voltage power supply and associated open electrical connections should be handled with extreme care to avoid electrical shock. Phenolic compounds are toxic/irritant and should be handled with care. Skin and eye contact, and accidental inhalation or ingestion should be avoided.

3. Results and discussion

The aim of this study is to demonstrate the use and advantages of a microchip capillary electrophoresis with an amperometric detector for rapid simultaneous measurements of phenolic acids related to foods and beverages. Fig. 2 displays a typical electropherogram for a mixture solution containing four common phenolic acids under optimum conditions. The electropherogram was obtained at the screen-printed carbon amperometric detector using submillimolar concentrations of chlorogenic (a), gentisic (b), ferulic (c), and vanillic (d) acids. The four peaks are well resolved, with the entire assay

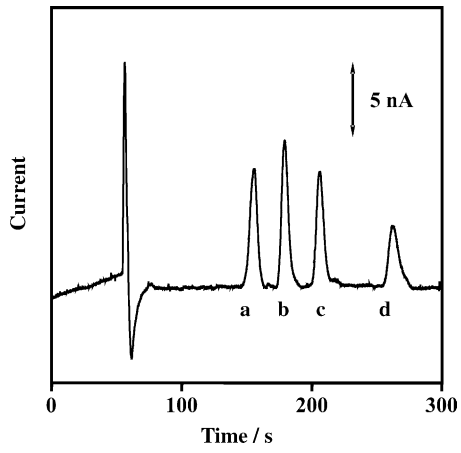

Fig. 2. Electropherograms for mixtures containing 200 μM chlorogenic acid (a); 200 μM gentisic acid (b); 200 μM ferulic acid (c); 200 μM vanillic acid (d) at the screen-printed carbon electrode. *Operation conditions*: separation voltage, 2000 V; injection voltage, 1500 V; injection time, 5 s; running buffer, 15 mM borate (pH 9.5); detection medium, 1N nitric acid; detector potential, +1.0 V (vs. Ag/AgCl wire).

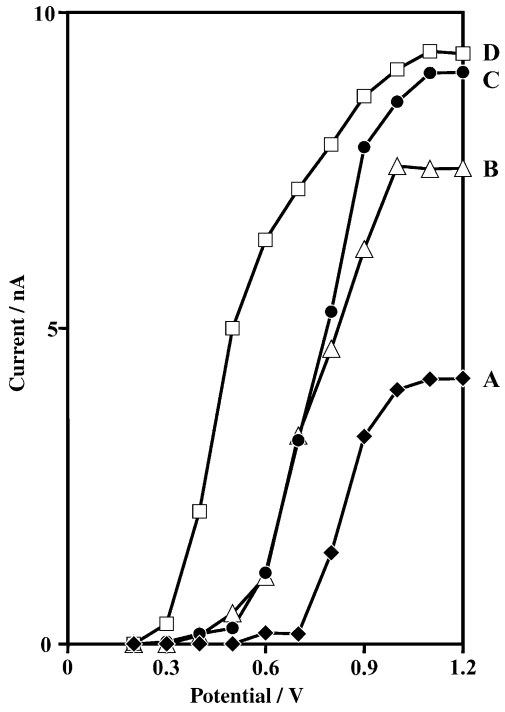

Fig. 3. Hydrodynamic voltammograms for 200 μM vanillic acid (A), 200 μM of chlorogenic acid (B), 200 μM ferulic acid (C) and 200 μM gentisic acid (D) at the screen-printed carbon electrode. Running buffer: 10 mM borate (pH 9.5). Other conditions, as in Fig. 2.

requiring less than 300 s. Notice also the favorable signal-to-noise characteristics of the phenolic acid peaks. Analogous measurements with conventional (fused silica) CE capillaries required significantly longer (>13 min) periods [22,23].

In order to establish the best detection conditions for the phenolic acids, their behavior at screen-printed carbon electrodes was investigated. The selection of the detection potential relies on the construction of hydrodynamic voltammograms (HDV). Fig. 3 depicts typical HDV for the oxidation of (A) vanillic acid, (B) ferulic acid, (C) chlorogenic acid, and (D) gentisic acid. The curves were developed pointwise by making 100 mV changes in the applied potential over the 0.3–1.20 V range, while using a separation voltage of 2000 V. A well-defined sigmoidal response is observed for all four compounds, with oxidation starting around 0.3 V (D), 0.4 V (B, C), and 0.5 V (A). The peak currents rise rapidly and level off around +1.0 V (A, B) and +1.1 V (C, D). Most favorable signal-to-noise characteristics were observed at +1.0 V, and this potential was used for all subsequent work. The HDV of Fig. 3 reflects also the different sensitivities of the phenolic acids, with the limiting current following the trend: 9.5 nA (D) > 9.2 nA (C) > 7.6 nA (B) > 4.3 nA (A).

The separation voltage affects the migration time of analytes and the overall resolution. High separation voltages decrease the analytical time, but worsen the separation of the analytes. Furthermore, it may results in a higher joule heating and, consequently, leading to formation of bubbles and compromising the performance of the channel [24]. The effect

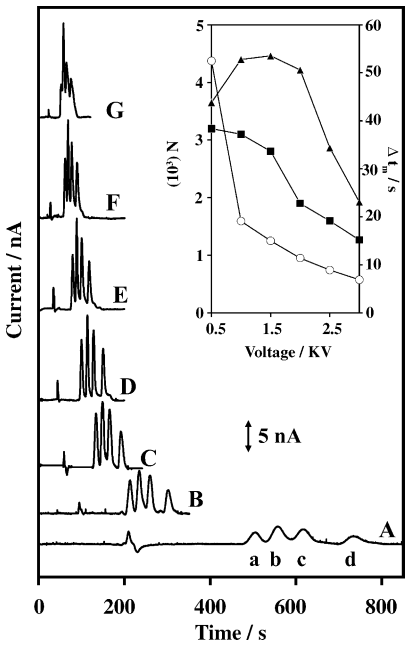

Fig. 4. Influence of the separation voltage on the response of the screen-printed carbon electrode for a mixture containing 200 μM chlorogenic acid (a); 200 μM gentisic acid (b); 200 μM ferulic acid (c); 200 μM vanillic acid (d). Also shown (inset) is the dependence of the plate number (*N*) of (■) chlorogenic acid and of (▲) gentisic acid and the difference in migration time between chlorogenic and gentisic acids upon the separation voltage (◯). Separation voltage: (A) 500 V; (B) 1000 V; (C) 1500 V; (D) 2000 V; (E) 2500 V; (F) 3000 V; (G) 3500 V; buffer concentration, 10 mM borate (pH 9.5). Other conditions, as in Fig. 2.

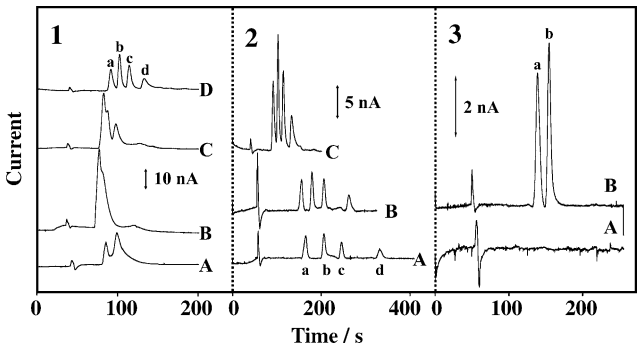

Fig. 5. (1) Influence of the pH of the buffer on the response of the screen-printed carbon electrode. pH buffer: (A) 8.0, (B) 8.5, (C) 9.0, (D) 9.5; buffer concentration: 10 mM. Other conditions as in Fig. 2. (2) Influence of the concentration of the buffer on the response of the screen-printed carbon electrode. Concentration of the buffer: (A) 20 mM (B) 15 mM (C) 10 mM; pH buffer: 9.5. Both experiments used a mixture containing 200 μM chlorogenic acid (a); 200 μM gentisic acid (b); 200 μM ferulic acid (c); 200 μM vanillic acid. (3) Electropherograms for 200 μM chlorogenic (a) and gentisic (b) acids in the presence of 1N nitric acid (A) or the 15 mM (pH 9.5) borate buffer solution (B) in the detection reservoir. Other conditions, as in Fig. 2.

of the separation potential upon the amperometric response and separation efficiency is shown in Fig. 4. As expected, increasing the separation potential from 500 to 3500 V (in 500 V increments, A–G) dramatically decreases the migration time (from 510 to 60 s for chlorogenic acid), but, consequently leads to overlapping peaks (for voltages higher than 2500 V). The plate number (for chlorogenic acid) increases from 3650 to around 4400 between 500 and 1000 V, and decreases rapidly above 2000 V (inset). A separation potential of 2000 V represented the best compromise between separation efficiency and analysis time and was thus used in all subsequent work. Higher separation voltages used in CE separations (compared to low detection voltages necessary for the detection) have been shown to influence the hydrodynamic voltammetric profiles by shifting the profile to the anodic direction [25]. Higher operating (detection) potentials would be required in order to eliminate such high separation voltage effects. The injection time is also found to have a profound effect on microchip measurements of phenolic acids. Increasing the injection time from 2 to 5 s, led to a considerable (2.5-fold) sensitivity enhancement (not shown), while retaining the peak resolution. Similar observations were made earlier by Ramsey and co-workers [26] using a floated sample loading. Subsequent work thus employed a 5 s injection.

The pH of the run buffer is an important parameter since it determines the extent of ionization and mobility of each solute [24]. The influence of the pH was studied using four buffer systems in the basic region: 10 mM borate at pH 8.0 (A), 8.5 (B), 9.0 (C) and 9.5 (D). Fig. 5(1) displays the influence of the pH on the separation of the phenolic acids considered. The most favorable separation is observed using a pH value of 9.5. This pH was used for all subsequent separations.

The influence of the concentration of the borate run buffer was examined over the 10–30 mM range. Fig. 5(2) shows the electropherogram for the four phenolic acids using buffer concentrations of 20 mM (A), 15 mM (B) and 10 mM (C). Longer assays coupled to higher resolution are observed upon increasing the buffer concentration. An even longer assay time was observed using a 30 mM level (not shown). Hence, a borate buffer 15 mM was chosen as optimum concentration of buffer, providing baseline resolution along with a 5 min analysis.

The microchip route allows the use of a different pH in the detector compartment, as recently demonstrated by Garcia and Henry [27]. Under acidic conditions (in this compartment) the phenolic acid peaks are neutral and therefore are not repelled from the electrode surface [22]. Nitric acid was used in the detection reservoir and its concentration was optimized over the 0.1 and 3.0 M range (not shown). As indicated from Fig. 5(3), the use of 1.0 M nitric acid leads to a significantly higher sensitivity. While the use of lower concentrations of nitric acid resulted in decreased sensitivity, levels higher than 1.0 M nitric acid resulted in an increase in the baseline noise coupled with an unstable signal. Therefore, the most subsequent work employed a 1.0 M nitric acid solution in the detection reservoir. In view of the negligible sample volumes and flow rates, dilution of this acid is negligible during routine operations.

While amperometric detection of phenolic compounds is commonly prone to surface fouling, due to the formation of

Table 1
Precision data: results of 10 repetitions of the four phenolic acids at a concentration of 150 μM

	Chlorogenic			Gentisic			Ferulic			Vanillic		
	Time (s)	Peak (nA)	Area (nC)	Time (s)	Peak (nA)	Area (nC)	Time (s)	Peak (nA)	Area (nC)	Time (s)	Peak (nA)	Area (nC)
Average	156.4	4.4	33.3	176.5	5.2	33.7	195.8	4.4	30.8	245.3	2.7	23.3
R.S.D. (%)	1.8	2.3	3.1	1.4	6.1	6.2	2.2	1.5	3.1	3.0	6.1	6.7

Conditions, as in Fig. 2; the respective units for peak height and peak area are nano-ampere (nA) and nano-coulombs (nC).

inhibitory polymeric films, no such passivation problem was observed using the CE/electrochemical-detector microsystem. A series of 10 repetitive injections of a mixture containing 150 μM of the four phenolic acids (using the same detector strip) was performed for estimating the relative standard deviations (R.S.D.) of the peak current and migration time for all the analytes under optimum conditions. The results are summarized in Table 1. The R.S.D. of the peak currents vary from 1.5% (for ferulic acid) to 6.1% (in the case of gentisic and vanillic acids). The exact reasons for obtaining higher R.S.D. values in the case of gentisic and vanillic acids are not clearly understood at this time and may be attributed to different interactions of the phenolic acids However, the good precision of the migration time (R.S.D., 1.4–3.0%; $n = 10$) indicate negligible surface (channel) passivation due to the injection of extremely small sample volumes. Similar improvements were reported for microchip measurements of chlorophenols [28]. Whenever needed, the design of the microsystem permits rapid (5–10 s) replacement of the detector strip.

The amperometric detection at the screen-printed electrode results in a well-defined concentration dependence. A three-dimensional electropherograms for sample mixtures containing increasing levels of chlorogenic and gentisic acid in 50 μM steps are shown in Fig. 6(a–f). Defined peaks, proportional to the concentration of both analytes, are observed. The resulting calibration plots (shown as inset on the left) are highly linear with correlation coefficients for chlorogenic and gentisic acid 0.998 and 0.996, respectively. The second inset of Fig. 6 (right) shows an electropherogram for a mixture containing 10 μM of chlorogenic and gentisic acid. On the basis of a S/N ratio of 3, such concentration represents the detection limit of both analytes.

The suitability of the CE microchip for measuring low levels of phenolic acids compounds in wine samples was examined. Fig. 7 (right) displays an electropherogram for a commercial wine sample, spiked with (a) 200 μM chlorogenic acid, (b) 200 μM gentisic acid, (c) 200 μM ferulic acid, and (d) 200 μM vanillic acid, is characterized with four well-defined and baseline resolved peaks. The total assay time

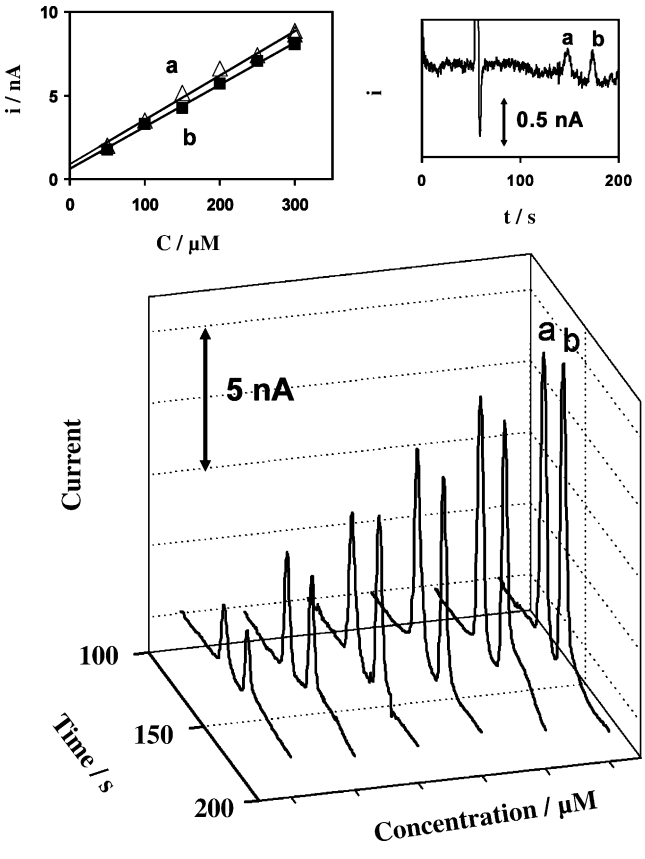

Fig. 6. Three-dimensional electropherogram of the calibration experiment at the screen-printed carbon electrode for mixture containing increasing levels, from 50 to 300 μM of chlorogenic acid (a) and gentisic acid (b) in increments of 50 μM. Also shown in the insets (left) corresponding calibration plots and (right) an electropherogram for a mixture containing 10 μM of chlorogenic and gentisic acid. Detection potential, 1.0 V (vs. Ag/AgCl wire); other conditions, as in Fig. 2.

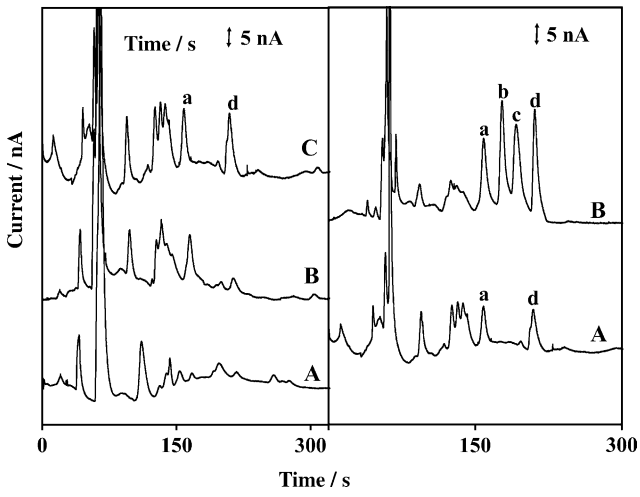

Fig. 7. Electropherogram of Californian red wine sample at various detection potentials (left). Peaks label (on the basis of migration times in comparison to standards) correspond to: (a) chlorogenic acid; (b) gentisic acid; (c) ferulic acid; (d) vanillic acid. (A) 0.8 V (B) 0.9 V; (C) 1.0 V. Analysis of the same wine sample before (A) and after (B) addition of 200 μM of the same phenolic acids (B) (right). Other conditions, as in Fig. 2.

is around 300 s. The unspiked sample exhibits well-defined chlorogenic (a) and vanillic (d) acid peaks. All four peaks are readily detected using the spiked sample. Such microchip wine analysis requires minimal sample preparation (involving filtration and dilution). Fig. 7 (left) shows electropherograms for the wine sample obtained at different detection potentials. Lower potentials provide greater selectivity while higher ones lead to increased sensitivity. For example, in the electropherogram obtained at +0.8 V (versus Ag/AgCl wire) vanillic peak is hardly detected, while it is well resolved using a potential of 1.0 V. Chlorogenic acid is still detectable at +0.8 V (versus Ag/AgCl wire) owing to its high sensitivity at the carbon screen-printed electrode, even though this potential is near to its half-wave potential value (Fig. 2B).

4. Conclusions

We have demonstrated the utility of CE microchips with screen-printed electrochemical detectors for the separation and detection of common food-related phenolic compounds. Such coupling offers great promise for monitoring of food matrices such as wines, whose quality depends on the level of phenolic acids. The fast separation and negligible waste production (compared, for example, to common liquid chromatographic protocols) is attractive for various practical applications. The microsystem offers also great promise for the quantitation of phenols in food or pharmaceutical matrices. Further work is in progress to provide a qualitative method of detection of phenolic compounds and characterization of food samples by the simultaneous use of the microchip capillary electrophoresis and pattern-recognition chemometric techniques.

Acknowledgements

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