Detection of phenolic acids in beverages by capillary electrophoresis with electrochemical detection†

The Analyst

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Capillary electrophoresis using amperometric detection is used to detect phenolic acids in beer samples. Both the detection and injection conditions were optimized. First, the electrophoretic separation requires that the phenolic acids be charged and therefore the pH be above their pK_as. However, electrochemical detection is optimal when the pH is low so that the phenolic acids are neutral and not repulsed by negative charges on the electrode surface. These divergent conditions were met by using a pH 7.2 run buffer and lowering the pH after the separation by using nitric acid in the detection reservoir. Cationic and neutral compounds in the beer samples interfered with electrochemical detection by passivating the electrode surface. These compounds were removed using a reversed-polarity injection technique to elute them from the separation capillary into the sample reservoir prior to the electrophoretic separation. These techniques were demonstrated by detecting several phenolic acids in various types of beer. Electrophoretic peaks in the samples were identified by both matching their elution time and electrochemical properties with standards. The use of voltammetric characterization provided improved peak identification for complex samples.

Phenolic acids are universally found in plants, with the specific phenolic acids present depending on the plant genera.[1] The highest concentrations of these acids are thought to be present in the surface layer of plants.[2] Foods and beverages of plant origin contain significant amounts of phenolic acids. These contribute to the flavour and stability of plant-derived beverages such as fruit juices, wines and beers.

The most commonly utilized technique for the determination of the phenolic acid content of beverages is HPLC with UV or electrochemical detection.[3–8] Preconcentration techniques are typically required to detect the phenolic acids in beverage samples using UV detection.[3–5] Significantly lower detection limits have been achieved by electrochemical detection.[6–8] The electrochemical properties of the phenolic acids can also provide a second method of identification in addition to retention time.[7,8]

Due to the structural similarity of most phenolic acids and the complex nature of the beverage matrix, gradient elution is often required to provide an adequate separation by HPLC. Capillary electrophoresis (CE) provides an alternative to HPLC for the separation of charged species. CE has been used to detect cinnamic acid and ferulic acid as metabolites of τ-orzanol in dog plasma by CE.[9] Micellar electrokinetic capillary chromatography (MECC) has also been used to determine phenolic acids in plant materials.[10]

In this report, the use of CE with electrochemical detection to detect phenolic acids in beverages is described. Amperometric detection of the phenolic acids was first optimized. Electrochemical characterization to aid in peak identification was evaluated. Neutral compounds in the beverages were found to passivate the electrode. This limitation was overcome using the polarity reversal method first described by Chein and Burgi.[11] The sample is first diluted in a low conductivity solvent and hydrodynamically injected. Under reverse-polarity, the cations and anions are eluted from the injection end of the capillary while the anions are concentrated by field amplification. The concentrated anions are then separated under normal polarity conditions.

Experimental

Apparatus

The CE system was built in-house using a high voltage dc (0–30 kV) dual polarity power supply (Spellman, Plainview, NY, USA) as described previously.[12] The anodic high voltage end was isolated in a plexiglas box fitted with an interlock for operation safety. A fused silica capillary 65 cm in length and 50 μm id was used for separation.

For amperometric detection, an end-column Nafion decoupler, prepared as described previously,[12] was used to isolate the electrochemical cell from the electrophoresis current. The outlet of the capillary, containing the Nafion decoupler, was introduced into the electrochemical cell *via* a septum. A 33 μm diameter carbon fibre working electrode was prepared as described previously.[13] The electrode was inserted into the electrochemical cell *via* a hole drilled in the wall of the cell directly opposite the capillary end-column decoupler and was held in place with a septum. The electrode was inserted into the decoupler to 0.15 mm from the capillary end using an X-Y-Z micromanipulator (Newport, Fountain Alley, CA, USA). This was carried out under a microscope. An Ag/AgCl reference electrode and a platinum wire auxiliary electrode were used. The detection cell was placed in a BAS CC-4 Faraday cage (Bioanalytical Systems, West Lafayette, IN, USA) to eliminate environmental noise. The detection potential was applied using a BAS preamplifier system connected to an IBM compatible computer. Data acquisition and control of the system was operated by software programmed locally with Turbo C2.0. Data was collected in ASCII format and further processed in Origin 3.5 (Microcal Software, Northampton, MA).

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Reagents

Gentisic acid, protocatechuic acid, vanillic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid and chlorogenic acid were all purchased from Sigma (St. Louis, MO, USA). Nafion perfluorinated ion exchange powder (5% stock) was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were of reagent grade or better and were used as received. Beer samples were obtained commercially.

All solutions were prepared using NANOpure water obtained by passing distilled water through a NANOpure water purification system (Sybron-Barnsted, Boston, MA, USA). 10 μM stock solutions of the phenolic acids were prepared in 0.1 M perchloric acid and refrigerated until use. Stock solutions were diluted as required with buffer and filtered through a 0.45 μm pore size Acrodisc syringe filter (Fisher, Fair Lawn, NJ, USA) before injection. CE run buffers were prepared by titrating the free acid to the desired pH with solid sodium hydroxide. For capillary activation, EDTA pH 13 was prepared by titrating 0.5 M disodium EDTA with sodium hydroxide to pH 13.

Capillary electrophoretic conditions

The CE run buffer was 25 mM sodium phosphate buffer, pH 7.2. For each new preparation of run buffer, the steady-state electrophoretic current at 25 kV was determined. During sample injection the electrochemical cell was filled with run buffer and during the CE separation it was filled with 1.0 M nitric acid. Hydrodynamic injection was implemented by applying a pressure of 20 psi to the sample vial for the times indicated in the text. For the method of large injection volume two vials of run buffer were used, one for the waste from the sample injection and one for fresh run buffer for the actual separation. After hydrodynamic injection of the sample, a buffer vial was placed on the injection end of the capillary and a voltage of 25 kV was applied under reversed-polarity conditions (cathode at injection end). The electrophoretic current was monitored and the voltage turned off when the current reached 99% of its steady-state value. The first buffer vial was replaced with the second run buffer vial and the detection cell filled with 1.0 M nitric acid. The electrophoretic separation was then carried out at an applied voltage of 25 kV (anode at injection end). Following each electrophoretic run, the capillary was flushed for 60 s at 20 psi with pH 13 EDTA solution and then with run buffer for 120 s.

Amperometric detection

Each new electrode was cleaned by sonication in 33% (v/v) 'Micro' cleaning solution (International Products, Trenton, NJ, USA) for 2 min. The electrode was then electrochemically activated using a ±2.0 V squarewave at 1 kHz for 30 s. The activated electrode was then inserted into the detection cell. After each electrophoretic run, the carbon fibre electrode was reactivated using a ±2 V squarewave at 10 kHz for 30 s. All potentials are reported *versus* the Ag/AgCl reference electrode.

Sample preparation

Samples were diluted 1 + 24 with NANOpure water to a final volume of 1 ml and adjusted to pH 6.5 by the addition of 0.1 M NaOH. The diluted samples were filtered through a 0.45 μm pore size Acrodisc syringe filter. For Guinness Stout a clean-up step was necessary prior to CE analysis. A Sep-pak C18 cartridge was conditioned by flushing with 20 ml of water, 5 ml of methanol and a further 20 ml of water. The diluted Guinness Stout was cycled through the Sep-pak 5 times prior to injection into the CE capillary. This step removed a significant amount of interferences that migrated as anions in the CE system and were therefore not removed during the reversed-polarity step of the injection.

Results and discussion

Voltammetry of phenolic acids

In order to establish the detection conditions for the phenolic acids, their voltammetric behavior at carbon fiber electrodes was investigated. For the phenolic acids, a significant dependence on pH was observed for the half-wave potential, the wave shape and the peak current (Fig. 1). The pH dependence of the half-wave potential is as expected for a two electron, two proton oxidation. Catechol (neutral) and dopamine (cationic) exhibited the same pH dependence of the half-wave potential. The effect of pH on the wave shape and the peak current were unique for the phenolic acids and not exhibited by catechol or dopamine. The voltammetric wave is steeper and the limiting current is larger at lower pH. Whereas, for catechol and dopamine, the limiting current decreased as the pH was lowered. This behavior of the phenolic acids is due to the anionic nature of the carbon fiber electrode resulting in electrostatic repulsion of the anionic phenolic acids at higher pH.[14] When the pH is below their pK_as, the phenolic acids are protonated and therefore neutral. Such electrostatic repulsion does not occur for either neutral or cationic species. Based on electrochemical detection considerations, low pH provides better sensitivity for electrochemical detection of anions at these carbon fiber electrodes.

The separation in CE is based on the electrophoretic migration of charged analytes. Therefore the separation of the phenolic acids must be carried out at a pH above their pK_as. A run buffer pH of 7.2 was found to provide a good separation of the phenolic acids. However, this pH is not optimal for electrochemical detection. By using 1 M nitric acid in the detection buffer reservoir optimal pH conditions for both separation and detection could be achieved.[12] A pH 7.2 run buffer was used for separation but the nitric acid lowered the pH at the working electrode to neutralize the phenolic acids for optimal electrochemical detection.

Using these conditions the voltammetry of the phenolic acids was determined using the CEEC system. Hydrodynamic voltammograms (HDVs) were acquired using amperometric detection by making several injections of the same sample solution into the CE system and varying the applied detection potential between CE runs. The hydrodynamic voltammetry of the phenolic acids under the conditions of the CEEC experiment is shown in Fig. 2. The half-wave potentials extracted from this data are tabulated in Table 1.

Large volume reversed polarity sample injection

The detection of phenolic acids in complex matrices such as beverages using CE with electrochemical detection is hindered by the large concentrations of neutral and cationic compounds present in the sample. Even though these compounds can be separated from the phenolic acids electrophoretically, they may interfere by fouling the working electrode. Fouling of the electrode can occur by absorption of the compounds themselves or their oxidation products on the electrode surface. This fouling results in a considerable decrease in electrode response. To remove these compounds from the electrode surface, *i.e.*, to regenerate the electrode surface, requires electrochemical activation procedures which often results in irreversible fracturing of the electrode. The migration order with normal mode electrophoresis is cations, then neutrals, and anions last. The

electrode fouling thus occurs during the separation before the detection of the anions making quantitation difficult. Neutral and cationic species in the sample should be removed prior to separation to avoid electrode fouling. Either liquid–liquid or solid phase extraction can be used to remove neutral and cationic species prior to the CE separation.

An alternative approach is to use the field amplification sample stacking technique reported by Chein *et al.*[11] For this technique the sample is diluted with a low conductivity solution such as water. The diluted sample is injected so as to fill or nearly fill the capillary. When a voltage is applied across the capillary, the ions move with high velocity within the sample plug due to the high electric field across the low conductivity solution. Once they reach the sample–run buffer interface, the ions move very slowly due to the weak electric field across the high conductivity run buffer. This results in stacking of the ions at the sample–run buffer interface. If a reversed polarity voltage is applied across the capillary (*i.e.*, the cathodic electrode at the injection end of the capillary), electroosmotic flow is toward the injection end. Cations and neutrals migrate towards the injection end by a combination of electrophoresis and electroosmotic flow. Anions migrate toward the anode and are stacked at the sample–run buffer interface on the detector side of the sample plug. If the reversed-polarity voltage is maintained for sufficient time, the cationic and neutral species in the sample will elute from the injection end of the capillary. If the reversed-polarity voltage is maintained for too long, the anions will also be eluted from the injection end of the capillary as a result of electroosmotic flow. The proper time for reversed-polarity voltage application can be determined by monitoring the electrophoretic current. Due to the low conductivity of the sample plug relative to the run buffer, the electrophoretic current is initially much lower than when the entire capillary is filled with run buffer. As the sample plug elutes from the injection end of the capillary and is replaced with run buffer at the detector end of the capillary, the current rises due to the increase in net conductivity. When the electrophoretic current reaches 99% of its value, essentially the entire capillary is filled with run buffer and nearly all of the cations and neutrals have

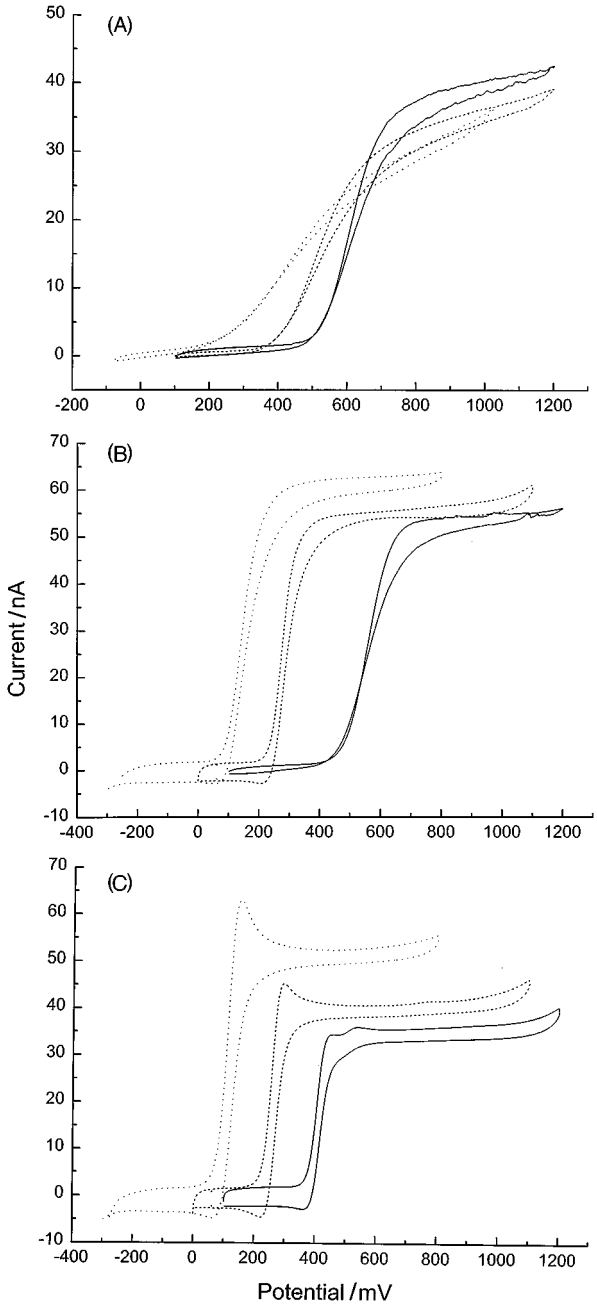

Fig. 1 Cyclic voltammetry of protocatechuic acid (A), catechol (B), and dopamine (C) at a carbon fiber electrode. The electrodes were activated using a ±2 V squarewave at 10 kHz for 10 s. The cyclic voltammetry scan rate was 10 mV s^{-1} using 10 mM solutions. Buffers: —— 100 mM sodium phosphate buffer, pH 2.5; --- 100 mM sodium acetate buffer, pH 4.75; … 100 mM sodium phosphate buffer, pH 7.2.

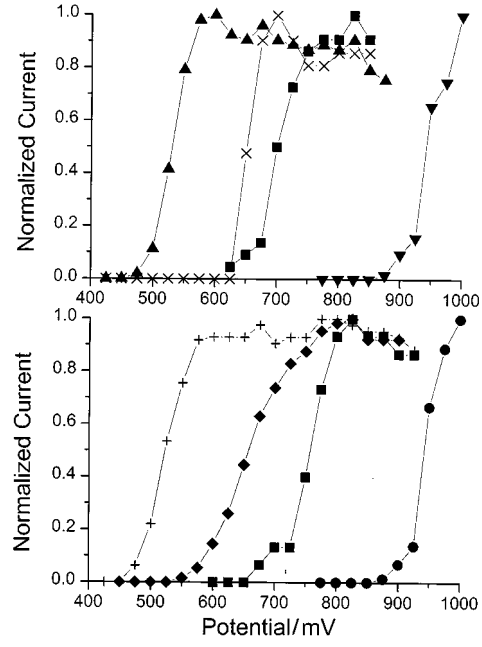

Fig. 2 Hydrodynamic voltammetry of the phenolic acids obtained using CE with amperometric detection. The CE run buffer was 25 mM sodium phosphate buffer, pH 7.2 with 1.0 M nitric acid in the detection reservoir. Symbols + gentisic acid, ● caffeic acid, × chlorogenic acid, ◆ protocatechuic acid,* sinapic acid, ■ ferulic acid, ▲ *p*-coumaric acid, ▼ vanillic acid.

Table 1 Half-wave potentials of the phenolic acids under the detection conditions

Phenolic acid	Chlorogenic acid	Sinapic acid	Ferulic acid	Caffeic acid	*p*-Coumaric acid	Vanillic acid	Protocatechuic acid	Gentisic acid
$E_{1/2}$ (mV)[a]	650	700	758	531	942	942	658	522

[a] *Versus* Ag/AgCl.

been eluted but the anions remain in the capillary. At this point the electrophoresis voltage is removed and the solution in the injection buffer reservoir is replaced with fresh run buffer. The electrophoresis voltage is then returned to its normal polarity and the separation takes place. The removal of neutral and cationic species using this reversed-polarity procedure is illustrated in Fig. 3. Dopamine and catechol are effectively removed during the injection procedure in Fig. 3(B) relative to normal electrokinetic injection as shown in Fig. 3(A). Note that the elution time is significantly affected by the injection procedure. It is therefore necessary to inject all standard solutions using the same procedure as used for the samples in order to use elution time for peak identification.

The large-volume reversed-polarity injection conditions were optimized for the phenolic acids. The length of the hydrodynamic injection of sample was optimized by comparing the peak heights for various injection times. Peak height increased with hydrodynamic injection time up to 30 s, above which peak height did not change. This time was taken to be the time necessary to fill the capillary and should be optimized for each capillary used. The sample dilution factor was also investigated. Dilutions from 1 + 9 to 1 + 99 were compared for separation efficiency and peak height. Dilutions less than 1 + 24 produced broad, poorly resolved peaks due to poor stacking efficiency because the sample ionic strength was too high. The greater the sample dilution the sharper the resulting electrophoretic peaks due to the lower conductivity of the sample. However, greater sample dilution also resulted in loss in sensitivity based on the concentration of the initial sample. The optimal condition was a dilution of 1 + 24 to provide good peak shape with the minimum loss in sensitivity. An electropherogram of a beer sample obtained using this procedure is shown in Fig. 4.

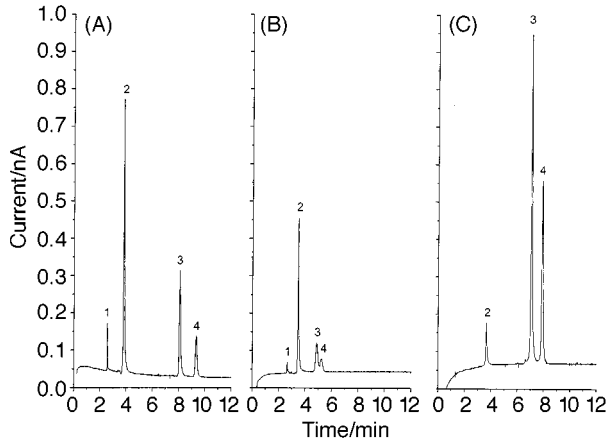

Fig. 3 Removal of cations and neutrals using the large-volume reversed-polarity injection technique. A standard solution containing 10 μM each of dopamine, catechol, caffeic acid, and protocatechuic acid was injected by: (A) electrokinetic injection for 3 s at 30 kV; (B) sample diluted 1 + 9 with water then hydrodynamically injected for 30 s at 20 psi; and (C) as in (B) but reversed-polarity applied prior to the electrophoretic separation. The run buffer was 25 mM sodium phosphate, pH 7.2 with 1.0 M nitric acid in the detection reservoir. Peak identities: 1, dopamine; 2, catechol; 3, caffeic acid; 4, protocatechuic acid.

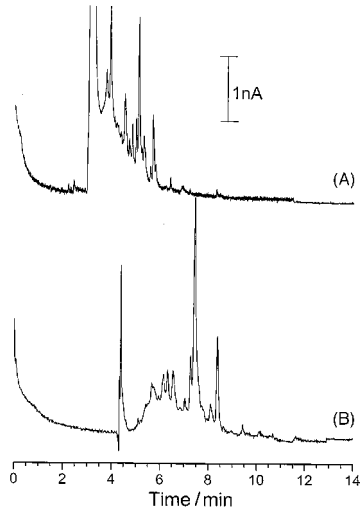

Fig. 4 CEEC electropherograms of Budweiser using normal electrokinetic injection (A) and the large-volume reversed-polarity technique (B). The sample for electropherogram was diluted 1 + 24 with water prior to injection. Separation conditions as in Fig. 3 except detection was at +850 mV *versus* Ag/AgCl.

Detection of phenolic acids in beer samples

An electropherogram of a standard phenolic acid solution is shown in Fig. 5. Electropherograms of two beer samples obtained at several applied potentials are shown in Figs. 6 and 7. Fig. 6 is for a Bud Light sample, which represents a relatively simple beverage matrix. The only sample preparation needed for this sample prior to injection into the CE system was dilution and filtration. Several peaks were tentatively identified as

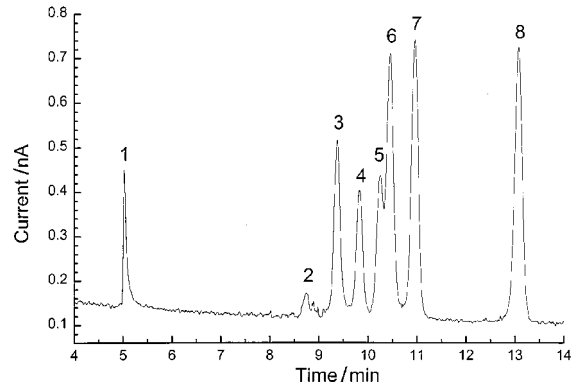

Fig. 5 CEEC electropherogram of phenolic acid standards injected using the large-volume reversed-polarity technique. Separation conditions were as in Fig. 3 except detection was at +950 mV *versus* Ag/AgCl. Peak identities: 1, chlorogenic acid; 2, sinapic acid; 3 ferulic acid; 4, caffeic acid; 5, *p*-coumaric acid; 6, vanillic acid; 7, protocatechuic acid; 8, gentisic acid.

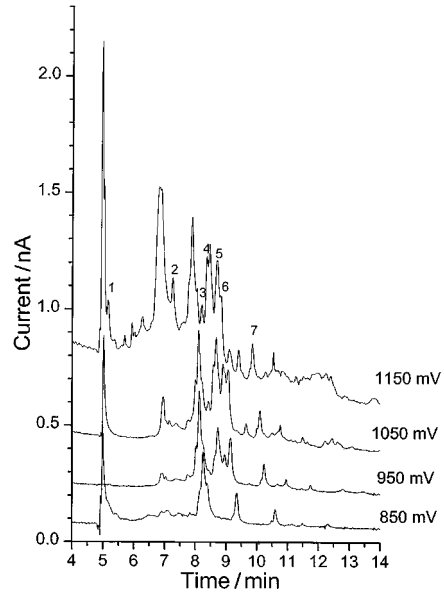

Fig. 6 CEEC electropherograms of Bud Light at various detection potentials. Separation conditions were as in Fig. 3. Peak labels correspond to tentative identities based on elution time of standards as in Fig. 5.

Table 2 Electrochemical characterization of electrophoretic peaks in beer samples

Sample peak	Characterization potentials	Current ratio		Tentative peak identity	Current ratio
		Bud Light	Guinness		
1	650/700	ND[a]	0.21	Chlorogenic acid	0.48
2	700/750	0.61	0.54	Sinapic acid	0.58
3	750/800	0.45	0.41	Ferulic acid	0.43
4	500/550	ND	ND	Caffeic acid	0.14
5	950/1000	0.70	0.67	*p*-Coumaric acid	0.67
6	950/1000	0.69	0.65	Vanillic acid	0.66
7	650/700	0.32	ND	Protocatechuic acid	0.60

[a] Peak not detected at these potentials.

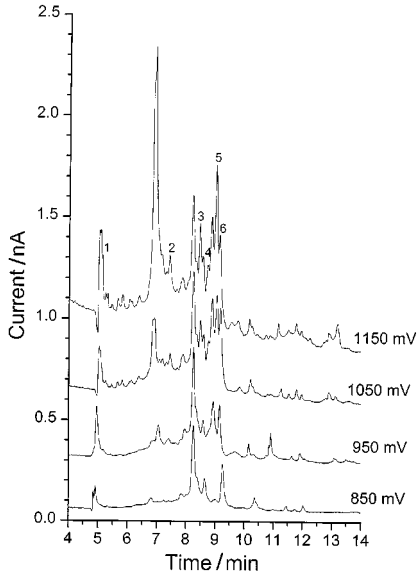

Fig. 7 CEEC electropherograms of Guinness Stout at various detection potentials. The sample was pretreated with a C18 Sep-pak as described in the text prior to injection. Separation conditions were as in Fig. 3. Peak labels correspond to tentative identities based on elution time of standards as in Fig. 5.

phenolic acids based on their elution times compared to the standards. Fig. 7 is for a Guinness Stout sample, which represents an extremely complex beverage matrix. Solid-phase extraction was necessary prior to CE analysis for this sample. Solid-phase extraction on a C18 Sep-pak removed a significant amount of hydrophobic (based on their retention on the Sep-pak) interferences, that migrated in the electrophoresis system as anions. Without this clean-up step, none of the phenolic acids were detectable in the Guinness Stout sample. However, using both solid-phase extraction and the large volume reversed-polarity injection procedure, several peaks in this sample were tentatively identified as phenolic acids based on elution time.

The selection of the detection potential is critical for amperometric detection. Lower potentials provide greater selectivity for easily oxidized compounds. This is seen in the electropherograms obtained at +850 mV relative to those obtained at +1150 mV. Far fewer peaks are seen in the lower potential electropherograms. However, the detection potential must be sufficient to oxidize the compounds of interest. In this case neither vanillic acid nor *p*-coumaric acid could be detected below a potential of +950 mV.

Peak identification by voltammetric characterization

Elution time alone provides poor reliability for peak identification, particularly with complex samples such as beverages. This is particularly true for this application because the elution time is very sensitive to the injection procedure and conditions. Electrochemical characterization provides an orthogonal technique to greatly enhance the reliability of peak identification. The peak heights obtained at various detection potentials were ratioed for the tentatively identified peaks in the two samples and compared to the peak height ratios of the standard phenolic acids. The potentials used for this electrochemical characterization by current ratio were chosen to be near the half-wave potential for the tentatively identified compound. This is the potential region where the current ratio is most sensitive to differences in voltammetry. These results are summarized in Table 2. As can be seen from the data in the table, the current ratios for the sample peaks eluting at the same time as sinapic acid, ferulic acid, *p*-coumaric acid, and vanillic acid also exhibit similar electrochemical behavior to the standard compounds. On the other hand, while peaks are observed in the samples at the proper elution time for chlorogenic acid, caffeic acid, and protocatechuic acid, the electrochemical behavior of these compounds is markedly different from the standard compounds. The identity of these sample peaks based on elution time is then incorrect or the peak corresponds to coeluting compounds.

Conclusions

Electrochemical detection in conjunction with a CE separation provides a powerful tool for detecting redox active compounds in complex samples. Electrochemistry provides more selective and sensitive detection than UV absorbance. In addition, electrochemical characterization of sample peaks can aid in identification of the sample components. The reversed-polarity injection technique allowed on-column removal of cationic and neutral interferences.

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