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Capillary electrophoresis with electrochemical detection employing an on-column Nafion joint

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ABSTRACT

The construction and evaluation of an on-column joint utilizing Nafion tubing for the isolation of the electrical circuit from the detection end of a capillary zone electrophoresis system is described. The joint enables electrochemical detection to be performed without adverse effects from the applied high voltage. The joint is both simple to construct and durable. The electrochemical detector employing a carbon fiber working electrode exhibited high coulometric efficiencies and a detection limit of $6 \cdot 10^{-9}$ *M* or 34.8 amol for hydroquinone. A high efficiency, of the order of 185 000 theoretical plates, was achieved for this compound. This system was evaluated for the detection of phenolic acids in apple juice and for the determination of naphthalene-2,3-dicarboxaldehyde derivatized amino acids in a brain homogenate. The use of voltammetry as a method of compound verification was also demonstrated.

INTRODUCTION

Since its introduction over a decade ago, capillary electrophoresis (CE) has become established as a powerful analytical tool for the separation of complex mixtures [1]. Capillaries with small diameter are advantageous over conventional slab gel electrophoresis for separations owing to the higher efficiency, lower joule heating effect and faster analysis times. One of the main areas of research is the development of sensitive detection systems. Because of the small sample volumes involved, high-sensitivity and small-volume detectors are necessary for the analysis of many real samples. Much of the work on CE and most commercial instruments use UV detection. However, as this is an optical technique and is path-length dependent, the sensitivity is limited when using small-diameter capillaries. Laser-based fluorescence detectors are more sensitive, but are expensive and limited to certain wavelengths. Electrochemical detection has an advantage over these methods in that the response is not dependent on path-length; therefore, very small capillary diameters can be used without a sacrifice in signal. It also utilizes relatively inexpensive instrumentation [2,3].

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Wallingford and Ewing [2,3] developed an off-column electrochemical detector and have reported 10^{-8} *M* detection limits for several catechol compounds. In their system, two pieces of capillary column are coupled inside a piece of porous glass capillary. This joint permits the flow of ions but not bulk electrolytic flow, enabling the detection end of the capillary to be held at ground. The fabrication of this joint assembly is difficult and intricate. Unless perfect alignment of both sections of the capillary is achieved, considerable band broadening can occur. The joint does not appear to be durable as the porous glass is extremely fragile and must be kept submerged in solution. Another limitation of this design is that the porous glass capillary is not readily available. Huang and Zare [4] designed an on-column frit which also served to isolate the final section of the capillary column from the applied electrical field. However, they reported the drawbacks of this design, including lack of capillary-to-capillary reproducibility, leakage of the frit and difficulty of fabrication (requiring the use of a carbon dioxide laser). Recently, Huang *et al.* [5] reported that it is not necessary to isolate the microelectrode from the high voltage if capillaries with very small inside diameter (5 μm) are employed. In such small capillaries, the current generated by the CE separation is low enough that it does not adversely affect the electrochemical detection. However, as a consequence of the small size, the concentration detection limits are not as low as those reported with larger inside diameter capillary columns.

This paper describes an alternative construction procedure in which the detection end of the capillary column is isolated from the high applied voltage. We believe this system to be simpler and more durable than those previously published. The capacity of the system for the analysis of real samples is explored, in addition to the use of voltammetric characterization as a method of compound identification.

EXPERIMENTAL

Construction of the joint assembly

Fused-silica capillaries (65–70 cm) with an I.D. of 50 μm and an O.D. of 360 μm were obtained from Polymicro Technologies (Phoenix, AZ, USA). A capillary cutter (Supelco, Bellfonte, PA, USA) was used to score the polyimide coating *ca.* 1.5 cm from the end of the capillary column. A 1-cm length of Nafion tubing (I.D. 0.33 mm, O.D. 0.51 mm) (Perma Pure Products, Tom's River, NJ, USA) was then carefully threaded over the score mark. Both

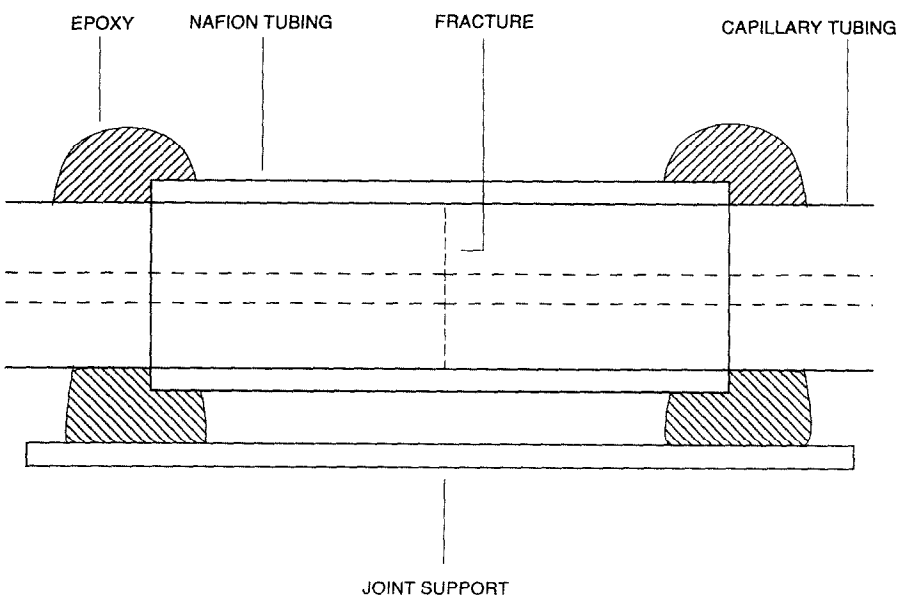

Fig. 1. Schematic diagram of Nafion joint.

ends of the Nafion tubing were sealed to the capillary tubing using 815 epoxy resin (Mid-Con Plastics, Wichita, KS, USA) with 20% (v/v) triethylenetetramine. This was cured overnight. Once cured, gentle pressure was applied to either end of the Nafion tubing, causing the capillary to fracture at the score. The Nafion tube holds the capillary joint securely in place and insures correct alignment. For additional support, the joint was epoxied to a small section of glass. A schematic diagram of the joint is illustrated in Fig. 1. We have completed the construction with a 100% success rate.

CE apparatus

Electrophoresis in the capillary was driven by a high-voltage d.c. (0–30 kV) power supply (Glassman High Voltage, Whitehouse Station, NJ, USA). The anodic high-voltage end of the capillary was isolated in a Plexiglas box fitted with an interlock for operator safety. A digital microampere current meter was positioned between the platinum wire ground cathode and ground. Experiments were performed at ambient air temperature (24°C). For UV work a CV^4 absorbance detector (ISCO, Lincoln, NE, USA) was employed. Sample introduction was performed using pressure injection, which was found to be reproducible and avoided bias associated with electrokinetic injection. The injection volume was calculated in a continuous-fill mode by recording the time required for the sample to reach the detector.

The Nafion joint was manipulated through two openings in opposite sides of a plastic beaker and subsequently sealed in place with epoxy. The joint was immersed in buffer solution and this assembly served as the cathodic buffer reservoir. The detection capillary section was then inserted into the electrochemical detection cell. An illustration of the complete system is shown in Fig. 2.

Previous investigations by Wallingford and Ewing [2] reported that back-pressure in the detection capillary section is a significant contributor to zone broadening. However, they demonstrated that if the length is shorter than 2 cm, peak distortion is negligible. Accordingly, we positioned the Nafion joint *ca.* 1.5 cm from the detection end of the column. A small section of polyimide was removed from the end of the detection capillary to provide better visualization of the insertion of the microelectrode.

Electrochemical detection

The electrochemical cell is similar in design to those described previously [2,6]. Cylindrical carbon fiber microelectrodes were constructed by aspiration of a 33-μm diameter fiber (Avco Specialty Products, Lowell, MA, USA) into a 1.0 mm I.D. capillary tube. The capillary tube was then pulled with a List-Medical Model 3A vertical pipet puller (Medical Systems, Greenvale, NY, USA). Silicone rubber adhesive (General Electric, Waterford, NY, USA) was applied to the tip of the capillary where the fiber protruded. Once cured, the sealant formed an intact seal around the fiber which was found to be resistant to all buffer solutions used. In addition, owing to the nature of the sealant, added flexibility was imparted

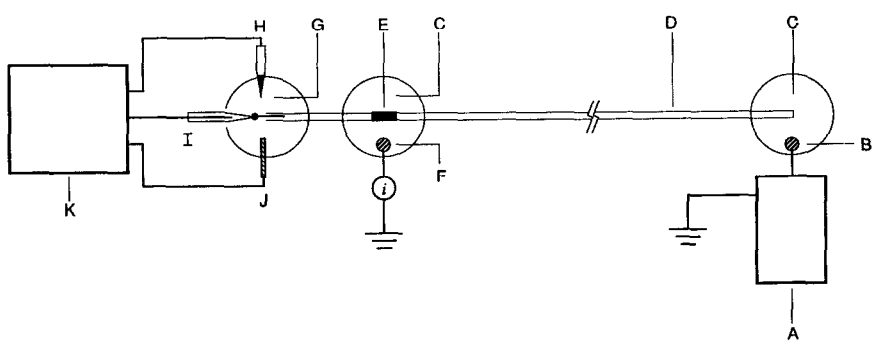

Fig. 2. Schematic diagram of CE system. A, High-voltage power supply; B, anode; C, buffer reservoirs; D, capillary column; E, Nafion joint; F, cathode; G, detection cell; H, reference electrode; I, carbon fiber microelectrode; J, auxiliary electrode; K, amperometric detector.

to the fiber, which aided in the insertion of the fiber into the capillary column. The fiber was then cut to the required length, 150–250 μm, using surgical scissors. Electrical contact was established via a copper wire cemented to the carbon fiber using silver epoxy (Ted Pella, Redding, CA, USA).

The microelectrode was then mounted on an *X–Y–Z* micromanipulator (Newport, Fountain Valley, CA, USA) and positioned in the electrochemical detection cell. With the aid of an optical microscope, the microelectrode was aligned and inserted into the capillary column. The cell was operated in a three-electrode configuration, with platinum wire auxiliary and a laboratory-built Ag/AgCl reference electrode.

Electrode connections were made to a BAS LC-4C (Bioanalytical Systems, West Lafayette, IN, USA) amperometric detector. The low currents generated at the microelectrode required the electrochemical cell to be shielded in a Faraday cage to reduce noise contributions from external sources.

Electrochemical pretreatment of the microelectrode was performed using a 50-Hz square-wave waveform of 2 V amplitude for 1 min. This was accomplished using a function generator (Exact Electronics, Hillsboro, OR, USA) connected to the external input of the BAS LC-4C. An oscilloscope was used to monitor the applied waveform. Using this arrangement, pretreatment could be performed without removing the microelectrode from the capillary column.

Chemicals

Hydroquinone, glutamic acid, aspartic acid, *p*-chlorogenic acid, caffeic acid, *p*-coumaric acid and sinapic acid were purchased from Sigma (St. Louis, MO, USA) and used as received. Naphthalene-2,3-dicarboxaldehyde (NDA) was supplied by Oread Labs. (Lawrence, KS, USA). Sodium cyanide was obtained from Fisher Scientific (Fair Lawn, NJ, USA).

All other chemicals were of analytical-reagent grade. All solutions were prepared in NANOpure water (Sybron-Barnstead, Boston, MA, USA) and filtered through a 0.45-μm pore size membrane filter before use.

Apple juice preparation

The phenolic acids present in apple juice (Tree Top, Selah, WA, USA) were separated from possible interferents by passing 4 ml of juice through a Sep-Pak C_{18} cartridge and washing the column with 10 ml of NANOpure water. A 2-ml volume of 0.01 *M* sodium borate solution (pH 9.25) was used to elute the phenolic acids. Neutral phenols remained on the column. This extract was injected directly onto the capillary.

Brain homogenate preparation

A rat was killed by cervical dislocation and the brain removed. Approximately 1.7 g of brain was homogenized in 10 ml of 50 m*M* borate buffer (pH 9.0) for 15 min. A 1-ml volume of homogenate was then removed and acidified with 80 μl of concentrated perchloric acid and centrifuged at 16 000 *g* for 10 min. The supernatant was filtered with a 2-μm filter. A 50-μl aliquot of the supernatant was derivatized in a final volume of 1 ml. The derivatization procedure was carried out as described previously [7].

RESULTS AND DISCUSSION

Several tests were performed to evaluate the Nafion joint and to characterize the effects of this modification. No substantial difference (< 1%) in the current measurement was observed between capillaries that did not contain the joint and those that had been modified when the same applied field strength and buffer were used.

No difference in electroosmotic flow was obtained when grounding was conducted either through the joint or at the detection end of the capillary column. As this experiment could not be carried out with the electrochemical detector, a UV–VIS detector was employed. Current measurements taken at both of these grounded positions were essentially the same. Reproducibility of joint-to-joint construction was examined based on the measurement of electroosmotic flow for six modified columns. The relative standard deviation was calculated to be 6.8%. No deterioration of the operation of a modified column was apparent following daily use over a 2-month period.

Although the Nafion joint completes the electrical circuit, the detection end of the column does not appear to be at "true" ground, as the noise levels were found to be proportional to the applied

voltage. Further, when buffers were used which exhibited higher electrophoretic currents (*i.e.*, buffers of a lower resistance), detector noise was observed to increase. This has also been reported by Wallingford and Ewing [2]. In order to minimize this effect, buffers of high resistance should be employed.

Linearity and detection limit for hydroquinone

To ascertain the detector response using the described sytem, hydroquinone was chosen as the test analyte. Using 0.01 *M* sodium acetate buffer (pH 6.0) and a separation voltage of 425 V/cm, linear regression analysis for concentrations ranging from $7 \cdot 10^{-8}$ to $1 \cdot 10^{-4}$ *M* provided a calibration graph with a correlation coefficient of 0.998 (n = 10). The high separation efficiency achievable with CE was apparent, with the number of theoretical plates calculated from the peak half-width for hydroquinone being of the order of 185 000. The detection limit for this compound was calculated from the electropherogram shown in Fig. 3 and was determined to be $6 \cdot 10^{-9}$ *M* based on a signal-to-noise ratio of 2. Using 5.8 nl as the injection volume, the detection limit corresponds to 34.8 amol. From a review of literature, this is the lowest concentration limit of detection reported using CE with electrochemical detection. Relative standard deviations for the reproducibility of the migration time and the detector response for hydroquinone were 0.7% and 1.8%, respectively (n = 8).

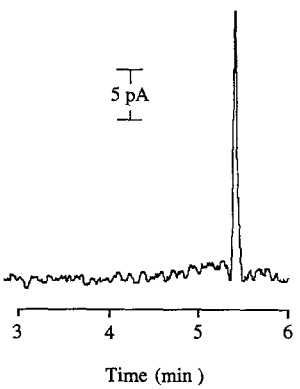

Fig. 3. Electropherogram of $7 \cdot 10^{-8}$ *M* hydroquinone. 0.01 *M* sodium acetate (pH 6.0); separation voltage, 425 V/cm; detection potential, 750 mV *vs.* Ag/AgCl.

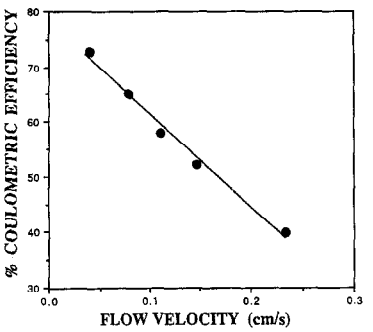

Fig. 4. Coulometric efficiency as a function of flow velocity for $1 \cdot 10^{-4}$ *M* hydroquinone. Conditions as in Fig. 3.

The coulometric efficiency of the detector was also examined. Insertion of a 33 μm O.D. carbon fiber into a 50 μm I.D. capillary column produces an annular flow width of *ca.* 8.5 μm. This, and the high sensitivity, are indicative of a thin-layer flow cell of high coulometric efficiency. To measure the coulometric efficiency as a function of flow velocity, a known volume of $1 \cdot 10^{-4}$ *M* hydroquinone was injected. Different flow velocities were achieved by adjustment of the applied electrophoretic voltage between 65 and 400 V/cm. The coulometric efficiency could be determined by knowing the number of moles, current sensitivity and chart speed, and that the oxidation of hydroquinone involves 2 F/mol. The coulometric efficiency was determined at several flow velocities. Fig. 4 illustrates the data

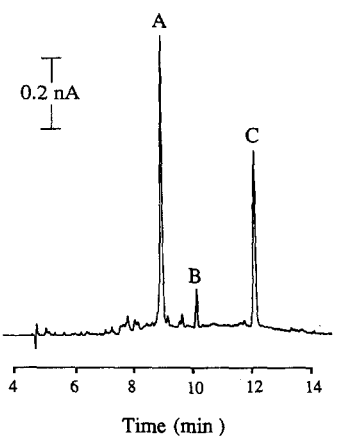

Fig. 5. Electropherograms of apple juice extract. 0.01 *M* sodium borate (pH 9.5); separation voltage, 425 V/cm; detection potential, 650 mV *vs.* Ag/AgCl.

obtained in this study and, as expected, demonstrates the high efficiencies for flow velocities typically utilized in CE separations.

Analysis of apple juice

The application of this system to real sample matrices was examined. The electropherogram obtained for the apple juice extract is shown in Fig. 5. Based on the migration times, peaks A, B and C were identified as chlorogenic acid, *p*-coumaric acid and caffeic acid, respectively. However, migration time is not always a reliable indicator of peak identity, particularly in CE where the sample matrix can have a considerable effect on the mobility of the sample constituents. For further verification of peak identity and purity assessment, voltammetric characterization was utilized. The combination of voltammetric characterization and migration time provides peak identity assignments with a high degree of certainty.

It has been shown that it is not necessary to obtain the entire voltammogram of the analyte in order to characterize sample components; the comparison of current response in the region where it changes most rapidly is sufficient [8]. To do this, the current response obtained at a potential near $E_{1/2}$ (where the current is most dependent on potential) was ratioed to the current response at a potential where the current is no longer dependent on potential (mass transport-limited value). As each phenolic acid has a different hydrodynamic response curve in terms of voltage and shape, the ratio is unique to each compound. Current ratios have been employed extensively for the voltammetric characterization of compounds in complex samples [9–11]. In this system, the current responses for both standards and sample peaks were measured at 550, 750 and 950 mV. Current ratios (ratioed to 950 mV) recorded are given in Table I. The ratios for *p*-coumaric acid and caffeic acid were virtually identical with those of the sample components eluting at the same time. However, peak A and chlorogenic acid did not exhibit similar voltammetric behavior, indicating impurity. This was further verified when sinapic acid, another phenolic constituent of apple juice, was found to co-elute with chlorogenic acid.

Analysis of brain tissue homogenate

The detection of glutamic and aspartic acid in a rat brain homogenate was investigated. These are important excitatory amino acids that can play a role as neurotransmitters in the brain [12]. Both amino acids lack electrochemically active moieties; therefore, derivatization is necessary for their detection. NDA reacts with primary amines in the presence of cyanide to produce cyano[*f*]benzoisoindole (CBI) derivatives. These have been shown to be electroactive at moderate oxidation potentials [13]. Fig. 6 illustrates electropherograms of a standard mixture of $1 \cdot 10^{-5}$ *M* of both CBI-amino acids. Fig. 7 shows the electropherogram recorded for the derivatized brain tissue homogenate in which both glutamic and aspartic acid were detected. This is the first reported use of a derivatizing agent to enhance detection in CE with amperometric detection.

It was found that the carbon fiber had to be pretreated between successive injections of brain homogenate samples in order to maintain current sensitivity. It is presumed that fouling of the electrode surface occurs owing to the formation of

TABLE I

VOLTAMMETRIC CHARACTERIZATION OF APPLE JUICE COMPONENTS

Conditions as in Fig. 5.

Sample components	Retention time (min)		Current ratio			
			550 mV/950 mV		750 mV/950 mV	
	Sample	Standard	Sample	Standard	Sample	Standard
(A) Chlorogenic acid	8.0	8.0	0.011	0.053	0.171	0.263
(B) *p*-Coumaric acid	9.1	9.0	0.025	0.026	0.254	0.246
(C) Caffeic acid	10.5	10.4	0.313	0.306	0.543	0.523

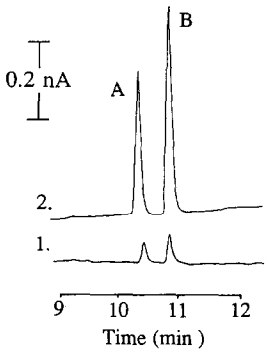

Fig. 6. Electropherograms of CBI derivatives of $1 \cdot 10^{-5}$ *M* (A) glutamic acid and (B) aspartic acid. (1) Response of untreated carbon fiber microelectrode; (2) response for pretreated carbon fiber microelectrode. 0.01 *M* sodium borate (pH 9.25); separation voltage, 425 V/cm; detection potential, 900 mV *vs.* Ag/AgCl.

insoluble reaction products and the pretreatment "cleans" these from the surface. Electrochemical pretreatment has been shown previously to have a dramatic effect on the response of carbon fiber microelectrodes [14–16]. This is demonstrated in Fig. 6. Application of a square-wave waveform to the microelectrode increased the current sensitivity nearly ten-fold over that of the untreated electrode. The detector response was also found to be very reproducible when the electrode was pretreated between injections [17].

CONCLUSIONS

An electrochemical detection system for CE has been developed that is more easily constructed than those previously reported. The design was evaluated and found to be extremely durable with no adverse effects on the CE separation. The resulting system has detection limits for hydroquinone in the low attomole range. In addition, voltammetry was used to verify peak identity and purity.

We believe that the simplicity of construction of this system should make electrochemical detection for CE more accessible to other investigators. This design also lends itself to sample collection and coupling to other end-column detectors. Future studies will be focused on the analysis of brain dialysate, which requires the high sensitivity for small sample volumes provided by the system.

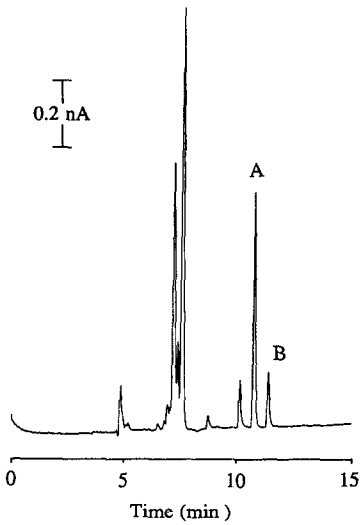

Fig. 7. Electropherograms of CBI-derivatized rat brain tissue homogenate. (A) CBI-glutamic acid; (B) CBI-aspartic acid. Conditions as in Fig. 6.

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