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Microchip capillary electrophoresis with amperometric detection for several carbohydrates

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Abstract

Microchip capillary electrophoresis (μCE) with amperometric detection at Cu electrode benefited fast separation and direct detection of carbohydrates. The working electrode of 50-μm Cu wire attached nearly against the channel outlet—4 μm, where it benefited collecting detection current and suppressing overwhelming noise. The use of alkaline medium was essential to separating and detecting carbohydrates, which dissociated into the sensitive alcolate anions. The 10-cm serpentine chip, though lengthening the migration time, it provided better efficiency. Sucrose, cellobiose, glucose, and fructose migrated from the outlet in 400 s +2000 V. The linear calibration plots ranging from 10 to 1000 μM with regression coefficients better than 0.996 were obtained. The injection-to-injection reproducibility of 1.24% ($n = 7$) for glucose in peak current and 0.6% for migration times were excellent. The detection limit was low, down to 2.3 μM for glucose ($S/N = 3$) or 27.6 attomole in mass detection.

Keywords: Carbohydrates; Microchip; Capillary electrophoresis; Amperometric detection; Copper electrode

1. Introduction

The past decade had witnessed a tremendous growth of miniaturization, which had nurtured the development of the micro total analysis system (μ-TAS). These systems incorporated part or all of the lab procedures for sample preparation, separation and detection. While downscaling labtop instruments such as GC or HPLC is difficult, fabricating the less complicated micro analyzer such as CE microchip (μCE) is relatively easy. But, μCE has weakness, too; its tiny size limits the optical path, which craves for sensitive measurements by fluorescence or laser-induced fluorescence. Both require derivitization with fluorophores and allow only a select number of wavelengths for excitation [1,2]. Mass spectrometry (MS) was another choice [1,3] but fell short of portability, economy, and sensitivity.

Electrochemical method of detection was attractive for both conventional [4–9] and microchip CE [10,11–14], since many compounds were detectable without the need for derivatization. It is less sensitive than LIF but much more versatile, economical, and easy to scale down in the micrometric range. μCE–EC has succeeded in the analysis of neurotransmitters [10], amino acid [14], peptides [15], and other compounds. However, the inherent field-induced noise was troublesome if not employing the tedious decoupling techniques. Mathies and coworkers [10] developed a plasma-sputtered Pt microelectrode for indirect detection of DNA. Wang et al. [16] used two separate designs: one with gold film sputtered around the outlet of the channel. The other design used a replaceable screen-printed carbon thick film affixed to the channel outlet [17]. In the same paper, they described an optimal electrode-to-outlet distance of 120 μm for best efficiency and sensitivity. But their later works had used 50 μm, instead [13]. Lunte and coworkers [15] reported the use of carbon paste electrode array for μCE. The electrode if positioned at 15 (±5) μm from the outlet was sufficient to suppress the noise but with band broadening. With electrode-to-outlet distance shorter than 5 μm, the noise began to soar. However, the statement was rather ambiguous since their experimental error was also ±5 μm. No conclusion about the optimized distance could be drawn from this. More importantly, all the above methods required microalignment of the electrode, which was time-consuming and skill-intensive. In the prefixed approach, however, the

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electrode was affixed permanently to the outlet, which eliminated the need for frequent in situ alignment.

Regardless of their wide distribution in nature, carbohydrates still crave for more efficient analytical methods. The difficulty arose from the lack of chromophores for photometric detection or electrophores under normal amperometric detection. Derivatization with chromophore [18–20] or use of indirect UV detection can help, but the former was time-consuming while the latter lacked the necessary sensitivity. Sensitive techniques such as fluorescence often limit their usefulness by inborn selectivity [21,22]. Analysis by enzyme-modified electrodes, known for its wide applications in bioimportant substances [23], was too specific to be useful for mixtures. Fortunately, carbohydrates, dissociated into alcolate anion in alkaline solution, was electrochemically active at the surface of noble [24,25] or transition metals [26–28]. More importantly, electrodes of Cu [29–31], Ni [32,33], Co [34,35], or Ru [36] coupled to HPLC could resist oxidation over an extended period. Cu electrode had shown selectivity toward amino acids, peptides, and proteins, besides carbohydrates [30,31]. Alternatively, pulsed amperometric detection (PAD) at gold electrode in series with conventional CE [37] was effective for carbohydrates, but presumably harmful to the electrode. Instead, detection by amperometry at constant potential posterior to capillary end was less aggressive to the electrode [38]. Wang et al. [39] had done enzymatic analysis on μCE without separation, which was effective for glucose. Schwartz et al. [40] was the first group to use Cu electrode on μCE to detect sucrose, galactose, and fructose, with estimated efficiency of 12 000 N/m. Unfortunately, they all required the time-consuming and skill-intensive microalignment of the electrode to the channel outlet—a setback to the day-to-day reproducibility. Hebert et al. [41] had further improved the method using sinusoidal voltammetry at the deposited Cu electrode to detect glucose and sucrose, with 50 000 N/m for sucrose and 70 000 N/m for glucose. However, the deposited Cu thin-film electrode, at the mercy of sinusoidal pulse, tended to foul, deteriorate and eventually breakdown. The end-channel configuration, coined by Lunte and coworkers [42], with Cu electrode placed at the outlet received considerable attention recently. It needed no micromanipulator, specific decoupling or machined cells. The built-in electrode, though exempt from such difficulty of alignment, still need to attend to the problem of noise. The distance between the electrode and the outlet mattered a great deal to noise suppression. In this study, parameters such as separation voltage, detection potential, injection potential, pH and injection time were studied to optimize the separation.

Our 10-cm μCE, 40 μm wide, and 10 μm deep, was sensitive and able to detect 2.3 μM (27.6 attomole) glucose, which was better than Schwartz et al. [40] or Hebert et al. [41]. This increase in sensitivity could be related to the integrated approach of on-chip Cu wire electrode. The approach of attaching Cu wire to the outlet after chip bonding was superior to the deposited-electrode approach in durability. The latter was more likely to break down. Although the 10-cm serpentine chip took longer analysis time, 6.7 min, than the works by Schwartz, 8.5 cm for 70 s, or Hebert, 4 cm for 150 s, it gave 120 000 N/m—a few times superior. The analysis time was seven-fold shorter than the conventional CE—6.7 min versus 45 min [38].

2. Experimental

2.1. Reagents and materials

All etchants and reagents were of analytical grade. Stock solution (1.0 mM) of all sugars, purchased from Sigma (St. Louis, MO, USA), were prepared fresh daily in pure water. Standard solutions were prepared by dilution to desired concentrations with the separation medium, immediately before use.

Pyrex glass (Corning 7740, 500 μm thickness) was obtained from I.G.S. (Sunnyvale, CA, USA). Thin film positive photoresist S1813 and developer MF-319 were purchased from Shipley (Marlborough, MA, USA). All of the solutions were prepared in a class 1000 clean room.

2.2. Apparatus

A high-voltage power supply from Bertan 230-30R (Valhalla, NY, USA) with adjustable voltage range of 0–30 kV was used for separation. Electrochemical detection was performed with an Electrochemical Analyzer 812 (CH Instruments, Austin, TX, USA), which was connected to a Pentium 166 MHz computer.

2.3. Chip fabrication, design, and operation

The experimental μCE–EC device is depicted in Figs. 1 and 2. Photolithography was done at the MEMS Facility (National Taiwan University (NTU)) followed by in-house fabrication. The microfluidic network was fabricated on an 18 mm × 58 mm × 0.5 mm thick Pyrex 7740 wafer using the standard photolithographic techniques. Pyrex glass was cleaned in acetone and then in deionized water by sonification for 10 min. Then transferred to hot Piranha acid solution (H_2SO_4/H_2O_2, 3:1) maintained at 120 °C in a class 1000 clean room. (***Caution!*** *Piranha acid solution is a powerful oxidizing agent, which reacts violently with organic compounds; it should be handled with extreme care.*) After 10 min, changed the solution to $NH_4/H_2O_2/H_2O$ (1:1:2) for another 10 min. After blown dry under nitrogen, a sacrificial etching mask layer of Cr/Au (35 nm Cr/200 nm Au) was deposited on the glass surface; here Cr served as the adhesive to Au. A S1813 positive photoresist was spun on the glass wafer at 4000 rpm for 30 s to yield a 1.5-μm-thick layer. After prebake at 90 °C for 2 min, the mask pattern was successfully transferred to the photoresist with a mask aligner (Double Side Mask Aligner MA6/MB6, Karl Suss,

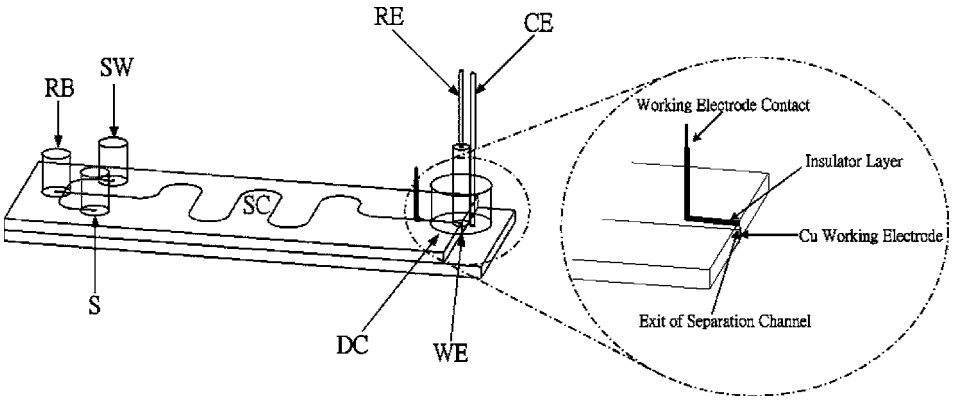

Fig. 1. Schematic diagram of μCE with electrochemical detection used in this study. SC, separation channel; RB, running buffer reservoir; S, sample reservoir; SW, sample waste reservoir; DC, detection cell; CE, counter electrode (300 μm Pt wire); RE, reference electrode (Ag/AgCl); and WE, working electrode of 50 μm Cu wire.

Germany). The exposure rate was 6 mW/cm^2 for 6 s. The wafer was developed in MF-319 for 25 s to remove the unreacted resist to expose the desired pattern, then hard baked at 120 °C for 30 min on a hot plate. Au film was then etched with KI/I_2 and Cr film with Cr etchant (KTI Chemicals, Sunnyvale, CA, USA); both of commercial grade. The HNA ($HF/HNO_3/CH_3COOH$ 1:2:1 (v/v)) etching solution was used for pattern engraving. The depth of the etched channel was periodically monitored with the Alphastep profilometer (Tencor Ind., San Jose, CA, USA) to establish basic data for future reference. The effective separation channel was 10 cm long, 40 μm wide, and 10 μm deep, with injection channel crossed at 500 μm from the running buffer reservoir. The injection volume confined in the intersection was 12 pl as estimated from the cross-sectional trapezoid, top square of 40 μm and bottom square of 20 μm. Connection from buffer or sample reservoir to the flow channel was bored by electric discharge in alkaline solution with a tungsten carbide needle (300 μm i.d.). Cleaned the glass plates accordingly as described above and blew it dry under nitrogen before bonding. A load of metal weights on the glass plate helped the bonding in a programmable furnace (Model 2-525 by J.M. Ney Co., Yucaipa, CA). The glass chip was first ramped to 650 °C at 3.4 °C/min, and then annealed to 100 °C at 5 °C/min, held for 1.5 h before cooling down to room temperature. Insufficient bonding can be spotted by the presence of rainbow. Several trials may be necessary to ensure a perfect bond. The glass tubing (0.4 cm i.d.) was inserted into the hole and glued permanently with epoxy.

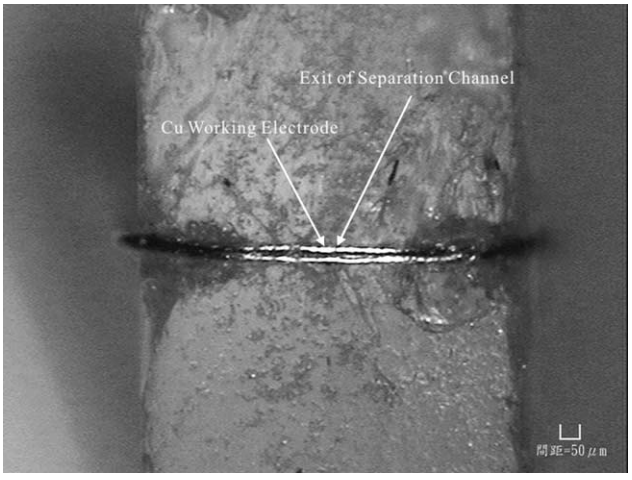

Fig. 2. Optical micrograph of the on-chip microelectrode.

2.4. On-chip detection

A schematic diagram for on-chip electrochemical detector is shown in Fig. 2. Cu wire of 2 cm 50-μm (Goodfellow Corp., Malvern, PA, USA) was mounted vertically to the prepolished channel end. In detail, Cu wire led from the bottom plate across the outlet to the top plate, with both ends glued with 15-min epoxy (Selleys Bolt, NSW, Australia). Finally, unused portion of the Cu wire was covered with epoxy for better performance.

2.5. Electrophoresis procedure

Rinse the channels before use with 0.1 M NaOH for 10 min, then deionized water 20 min. The channels were flushed with the separation medium of 80 mM NaOH by vacuum for 5 min and then sit for 15 min before use. The entire device was then flushed again with the separation medium by applying +2000 V for 20 min. Sample was drawn into the sampling channel by high voltage for 3 s, while keeping the sample waste reservoir grounded and the other reservoirs electrically floating. Apply +2000 V between the running buffer reservoir and the detection reservoir while keeping the sample reservoir floating to trigger separation.

2.6. Amperometric detection

Three-electrode configuration was employed in all experiments with a Ag/AgCl reference electrode and a platinum auxiliary electrode. Amperometric detection was performed

with an Electrochemical Analyzer 812 (CH Instruments), which was connected to a personal computer (Pentium 166 MHz, 32 MB RAM). The electropherograms were obtained at the detection potential of +0.6 V against Ag/AgCl, for injections after baseline stabilization.

3. Results and discussion

3.1. Effect of electrode position and detection potential

High separation voltage induces noise, which would in turn chew up the sensitivity gain from miniaturization and integration. The plague originates from indiscriminate positioning of the electrode; in-channel electrode arrangement invariably introduces serious noises. However, moving the electrode outside the channel would restrain noise but the sensitivity suffers. In essence, the end-channel design with the electrode positioned outside is necessary. It is a trade-off between noise reduction and signal gain. In principle, the electrode should stay as close to the outlet as possible, but to position the electrode at precisely 1 or 2 μm away from the outlet was extremely difficult. In this chip, the distance between the electrode and the outlet was 4 μm, measured by a microscope.

Carbohydrates are inert toward Pt or Au but sensitive to metals such as Cu or Ni under specified conditions; anchoring electrophore is another choice but too tedious. The use of Cu electrode in strongly alkaline solution for carbohydrate analysis [43] is sensitive and more convenient. All seven carbohydrates, characterized individually, gave similar HDV voltammograms as the detection potential stepped from +0.4 to +0.7 V, as shown in Fig. 3. Beyond this range, the current was too small for practical use or the baseline rise too severe over +0.7, not shown. The current rose in nonlinear response, except glucose, to potential increase; the optimized detection potential was +0.6 V against Ag/AgCl.

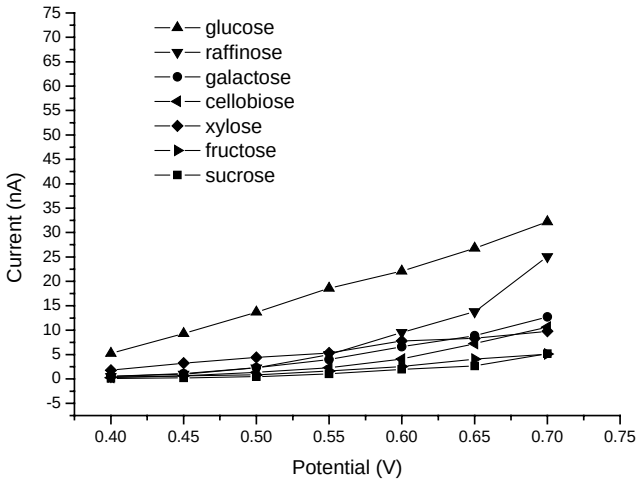

Fig. 3. Hydrodynamic voltammograms (HDVs) for 200 μM of glucose, raffinose, galactose, cellobiose, xylose, fructose, and sucrose. Conditions: separation voltage at +2000 V; electrokinetic injection for 3 s (at +2000 V); working electrode with Cu electrode; separation medium of 80 mM NaOH.

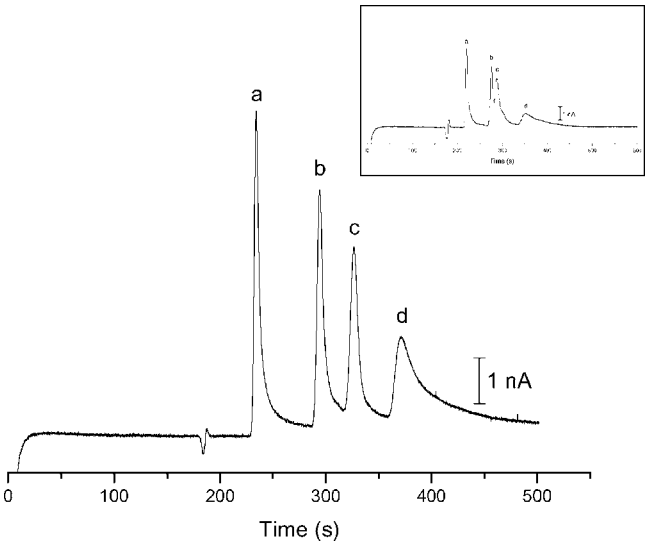

Fig. 4. Electrophoregram for 100 μM sucrose (a), cellobiose (b), glucose (c), and 200 μM fructose (d). Also shown (in the inset) are the resulting plots of 3000 injections (three months). 80 mM NaOH separation medium; sample injection time for 3 s at +2000 V; detection at +0.6 V against Ag/AgCl at Cu electrode.

3.2. Effect of separation medium

Carbohydrates are weak acids, which dissociate to negatively charged alcoholates in medium more alkaline than pH 12. This anionic transformation incurred discriminate migration and helped peak resolution [44,45]. As shown in Fig. 4, the overlap between glucose and fructose began to resolve in separation media more basic than 50 mM NaOH. Overall, the condition optimized at 80 mM NaOH.

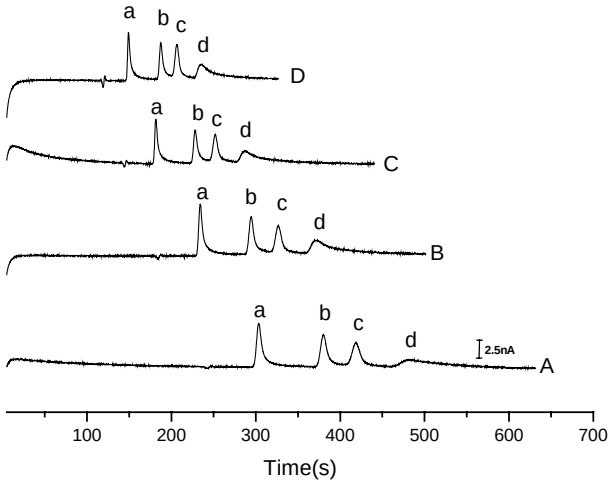

Fig. 5. Effect of separation voltage on migration time: (A) +1000 V, (B) +2000 V, (C) +2500 V, and (D) +3000 V. CE conditions: 100 μM (a) sucrose, (b) cellobiose, (c) glucose, and (d) 200 μM fructose; 80 mM NaOH separation medium; sample injection time for 3 s at +2000 V; detection at +0.6 V against Ag/AgCl at Cu electrode.

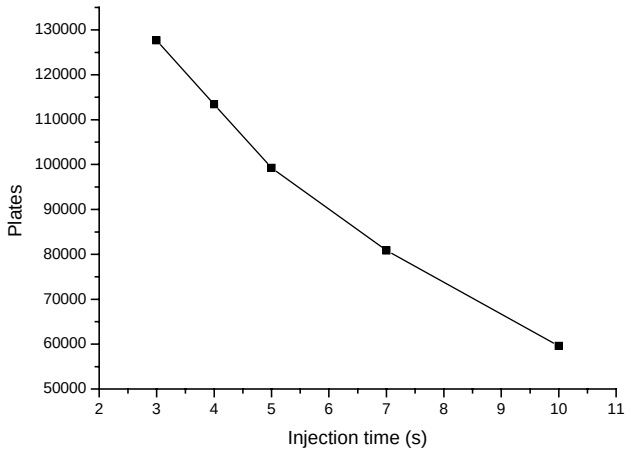

Fig. 6. Effect of injection time on the number of theoretical plate per meter for 50 μM glucose. Experimental conditions as in Fig. 4.

3.3. Effects of separation voltage and injection time

The separation voltage plays a major role in manipulating migration time and resolution, as shown in Fig. 5. Increasing separation voltage from +1500 to +3000 V increased migration and sensitivity, but at the cost of resolution and efficiency; migration times of most of the compounds declined in half while N dropped nearly 30%. A separation voltage of +2000 V optimized separation time, sensitivity, and efficiency. The injection volume, though confined by the intersection, was not constant if injection time was not controlled, as shown in Fig. 6. Discriminate migration of ions by electrophoretic field in separation was also effective when drawing the sample into the sampling channel. Peak broadened on prolonging sampling time from 3 to 10 s. Injection time shorter than 3 s was very difficult to control. A sampling time of 3 s usually ensured good resolution and sensitivity.

3.4. Reproducibility, linearity, and detection limits

Long-term use of glass at high alkalinity usually raises concerns about its legitimacy, but the chip had endured more than one month without anomalies. The analytical quality had remained almost intact after 300 injections, with migration time shift of only 0.6%. However, the sensitivity had dropped 12% over the same period. After 3000 injections over three-month period, as shown in the inset in Fig. 4, the efficiency and resolution declined with peak overlap

Table 1
The results of regression analysis on calibration curves and the detection limits[a]

Analyte	Regression equation $y = bx + a$[b]	Correlation coefficient	Linear range (μM)	Detection limit[c] (μM)
Raffinose	$y = 22.391x + 0.2085$	0.9971	10–1000	2.9
Sucrose	$y = 2.944x + 0.2204$	0.9963	50–1000	8.4
Cellobiose	$y = 9.967x - 0.4232$	0.9971	50–1000	16.6
Glucose	$y = 46.398x - 0.10$	0.9994	10–1000	2.3
Galactose	$y = 16.51x + 0.8348$	0.9989	50–1000	20.3
Fructose	$y = 16.22x - 0.5962$	0.9972	50–1000	14.2
Xylose	$y = 15.636x + 0.4193$	0.9968	50–1000	15.2

[a] Detection potential is +0.6 V against Ag/AgCl. Other conditions as in Fig. 4.
[b] y and x are the peak current (nA) and the concentration of the analyte (mM), respectively.
[c] Detection limits was based on the S/N ratio of 3.

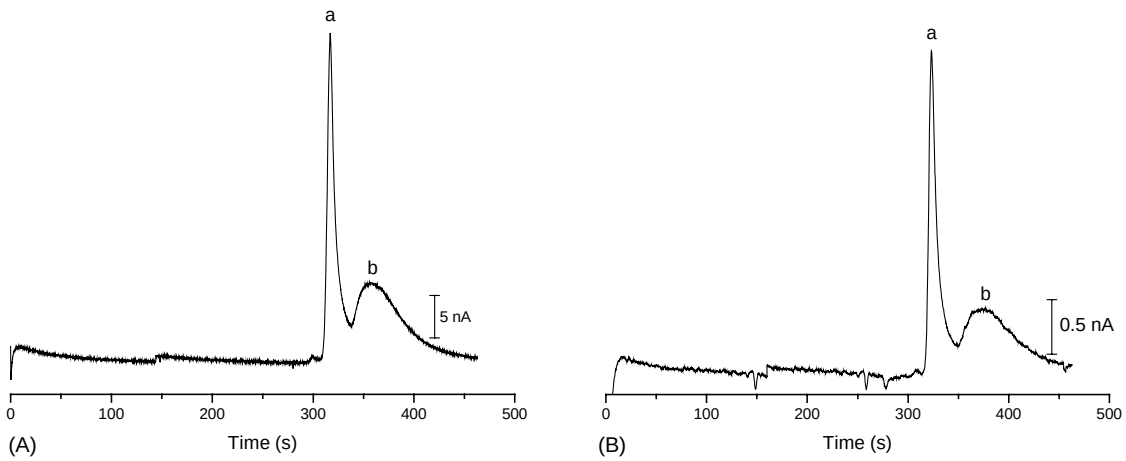

Fig. 7. Electrophoregram of carbonated soft drinks for (A) regular Coca-Cola®; (B) light Coca-Cola®. Peaks (a) glucose and (b) fructose, respectively. All samples are diluted 1:500. 80 mM NaOH separation medium; sample injection time for 3 s at +2000 V; detection at +0.6 V against Ag/AgCl at Cu electrode.

between cellobiose and glucose. NaOH solution severely attacked the channel. A year later, the peak had widened 178% with a 33% drop in separation efficiency. Table 1 showed good linearity between peak current and concentration in the range of 10–1000 μM, correlation coefficients >0.996. The detection limit was 2.3 μM for glucose, or higher for other species. Reproducibility in peak currents ($n = 7$) showed little deviation: as little as 0.04% for sucrose or 1.24% for glucose. Even the precision of migration time was controlled within 0.6% ($n = 7$).

3.5. Analytical applications

Samples from two popular soft drinks, Coca-Cola® and light Coca-Cola®, were injected for evaluation of its feasibility for routine use. Samples were diluted 500-fold with the separation medium, followed by membrane filtration before injection; as shown in Fig. 7. Identification of peaks employed spiking and peak matching. The concentration of glucose was 185 mM for Coca-Cola® and 18.5 mM for light Coca-Cola®.

4. Conclusions

This μCE was sensitive, able to detect 2.3 μM glucose or 27.6 attomole in mass detection. This boost in sensitivity owed a great deal to the integrated approach of on-chip Cu electrode and the electrode positioning. The high-field induced noise was obnoxious with the electrode sitting in the channel—at least a thousand-fold more severe. Placing the electrode near but off the outlet, 4 μm in this study, reduced the noise substantially while maintaining high sensibility.

Analysis with μCE is typically faster than the conventional ones; for example 6.7 min versus 45 min. Although our 10-cm serpentine chip took longer analysis time than the chip used by Schwartz or Hebert, the efficiency, and thus, the service a longer channel could provide was hardly matched by shorter channels. The dual roles of alkaline medium to promote the detection and separation were essential to carbohydrate analysis. The former benefited from the dissociation into detectable anions, and the latter the discriminate migration. The Cu electrode had outlasted the glass chip. After a year, the electrode's sensitivity remained largely the same but the channel, though still in service, widened 20–30%.

In summary, μCE with Cu electrode is sensitive, reliable, thrifty and advantageous of frugal consumption of reagent and sample. Its potential in other separation modes, considered no less versatile, such as micellar electrokinetic CE are under evaluation.

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