CHROMSYMP. 1776

Indirect fluorescence detection of sugars separated by capillary zone electrophoresis with visible laser excitation

TOMMY W. GARNER and EDWARD S. YEUNG*

Ames Laboratory–U.S. Department of Energy and Department of Chemistry, Iowa State University, Ames, IA 50011 (U.S.A.)

ABSTRACT

   This work extends the use of indirect fluorescence detection for capillary zone electrophoresis to the visible region. Detection is based on charge displacement and is not based upon any absorption or emission properties of the analyte, therefore the need for chemical derivatization is eliminated. Capillary zone electrophoresis–indirect fluorescence detection can separate and detect almost any compound that contains a charge. This was demonstrated by the separation and detection of a mixture of sugars which are only weakly acidic. The mass detection limit of fructose was 2 fmol using a 5-μm diameter capillary. This peak had an efficiency of more than 600 000 theoretical plates.

INTRODUCTION

   Capillary zone electrophoresis (CZE) is a new separation technique that separates ionic compounds based on their differential migration rates in an electric field[1]. CZE is known for its high separation efficiencies which can be more than 10^6 theoretical plates[2]. The separation in CZE takes place is small capillaries with internal diameters often less than 50 μm. Detection of the pl injection volumes in this technique is a challenging problem. Fluorescence and electrochemical detection have produced excellent results, but are limited to compounds which have the appropriate physical properties[3,4]. These physical properties can be obtained by chemical derivatization, but derivatization is time consuming and inefficient. There exists a need for an all-purpose detector for CZE.
   Indirect fluorescence detection (IFD) for CZE has been applied successfully to many ionic compounds including amino acids[5], proteins, nucleotides[6], tryptic digests[7] and other ionic organic and inorganic compounds[8]. Indirect detection is a universal detection scheme that can detect a wide variety of compounds without the need for chemical derivatization. CZE–IFD is also very sensitive with limits of detection (LOD) in the 50-amol range. Previously, CZE–IFD has been done in the UV region using salicylate as the indirect fluorophore. Fluorophores that fit lasers in the visible region

will aid detection by allowing the use of visible optics and more powerful visible-light sources.

In CZE a buffer is necessary to establish the potential gradient across the capillary tube. This buffer, which is usually inert, can be selected to optimize detection. In indirect fluorescence detection a fluorescing buffer is chosen. This gives a continuously high signal at the detector. When a charged analyte molecule reaches the detector region it displaces the fluorescing buffer ions to maintain constant conductance. Even though the analyte does not absorb or fluoresce it will give a signal by this displacement mechanism. This is unlike indirect detection in liquid chromatography[9], where even neutral analytes can be detected based on changes in partition at the stationary phase.

Sugars present especially challenging separation and detection problems in CZE. Sugars are only weakly acidic and thus the buffer must be made very basic before any ionization will occur. As an example the pK for glucose is 12.35[10]. The sugars can then be separated by their differences in migration velocity. Sugars also show little if any ultraviolet–visible absorption or fluorescence activity. This problem is even more challenging due to the small volumes of analytes injected in CZE.

In this work we will also introduce two new fluorophores that can be used for CZE–IFD. Fluorescein is a highly efficient fluorophore that matches very well with the argon ion 488-nm laser line. Coumarin 343 works very well with the 442-nm line of a helium–cadmium laser.

EXPERIMENTAL

The CZE system is similar to that described previously[5]. A high-voltage power supply (Spellman, Plainview, NY, U.S.A.; Model UHR50PN50 or Glassman, Whitehouse Station, NJ, U.S.A.; Model MJ30P0400-11) was used to supply the electromotive force across the capillary. The anodic high-voltage end of the capillary was isolated in a plexiglass box for operator safety while the cathodic end was held at ground potential.

Capillary columns of various lengths and diameters were used. Lengths ranged from 80 to 100 cm. The outer diameter of the columns was always 150 μm, while the inner diameters ranged from 5 to 22 μm. The polymer coating was burned off 10 cm from the cathodic end of the capillary to form the observation region.

A helium–cadmium Laser (Linconix, Sunnyvale, CA, U.S.A.; Model 4240NB) operating at 442 nm or an argon ion laser (Control Laser Corp., Orlando, CA, U.S.A.; Model 554A) operating at 488 nm was used for excitation. The laser beams (1 mW) were stabilized within 0.04% with a laser power stabilizer (Cambridge Research Institute, Cambridge, MA, U.S.A.; Model LS100). The laser was focused onto the capillary with a 1 or 0.5 cm focal length lens (0.5 cm for the 5-μm capillary). The capillary was mounted at Brewster's angle to reduce scattered radiation.

The fluorescence was collected at 90° with a 20 × microscope objective. The fluorescent image was focused onto a photomultiplier tube (Hamamatsu, Middlesex, NJ, U.S.A.; Model R928). Stray and scattered radiation were rejected by two spatial filters at either end of a blackened tube preceding the photomultiplier tube. The fluorescence was further isolated with two color filters (Corning Glass, Corning, NY, U.S.A.; Model 3-71 and Schott Glass, Duryea, PA, U.S.A.; Model GG475).

The photomultiplier tube current was monitored with a pA meter (Keithly,

Cleveland, OH, U.S.A.; Model 417). The output from the pA-meter was recorded on a chart recorder (Fisher series 5000) or on a personal computer after analog-to-digital conversion (Data Translations, Marlborough, MA, U.S.A.; Model DT 2825 or DT 2827). Digital data were smoothed with a smoothing routine based on the Savitsky–Golay smoothing algorithm[11]. A 1-s time constant was added after the pA meter.

The photomultiplier tube voltage was adjusted to maintain a 1-μA background current. Injections were made for 1 s at 30 kV and the voltage was held at 30 kV throughout the run.

Reagents

All chemicals were reagent grade unless otherwise noted. Fluorescein (Molecular Probes) and Coumarin 343 (Eastman Kodak, Rochester, NY, U.S.A.; laser grade) were prepared in deionized water (Millipore, Bedford, MA, U.S.A.; Milli-Q system) and the pH was adjusted with sodium hydroxide. Buffers were purged with nitrogen to remove carbon dioxide. The pH was checked and adjusted if necessary every 3 h. All quantitative data were obtained with freshly prepared buffer solutions. All analytes were diluted in buffer before injection.

RESULTS AND DISCUSSION

There are several factors involved in the selection of a fluorophore used for CZE–IFD. First, the molecule must have a high molar absorptivity for the excitation wavelengths available. Second, it must have a high fluorescence quantum efficiency. Thirdly, it must be compatible with the solvent system used, *i.e.* it must be soluble and inert. The molecule must also be charged, preferable a charge of one. This will ensure a value as close to unity as possible for the transfer ratio, *TR*. *TR* is defined as the number of fluorophore molecules displaced by one analyte molecule. The molecule also must be well behaved in the system. Some molecules may adsorb to the column walls causing a non-equilibrium state to exist.

The first new fluorophore studied in this work was disodium fluorescein, and is demonstrated by the separation of glutamic acid and aspartic acid. Tris was added to the buffer to increase the buffer capacity. Separation efficiencies were high for this system with plate numbers for each amino acid exceeding 100 000. This allows indirect detection to be implemented with an argon ion laser. No stabilization of the laser power was implemented. Although this system was not optimized for the best LOD high absolute sensitivities were obtained with a LOD for each amino acid of 2 fmol. Lower detection limits should be obtained by lowering the concentration of buffer and stabilization of the laser intensity[6]. As can be seen from these results, fluorescein worked well at pH 7. When the pH was increased to 9, however, the baseline became very erratic. This was believed to be caused by the interaction of fluorescein with the wall of the capillary.

The next fluorophore studied in this work was coumarin 343. Coumarin 343 was selected because of its good solubility in water, high molar absorptivity and high fluorescence yield. Coumarin 343 has a molar absorptivity of $2 \cdot 10^4$ at 442 nm which matches very well with the 442-nm line of the helium–cadmium laser. The high fluorescence yield was evaluated experimentally by injecting coumarin 343 in the direct detection mode. The buffer used was 10 mM sodium bicarbonate at pH 10.7. The
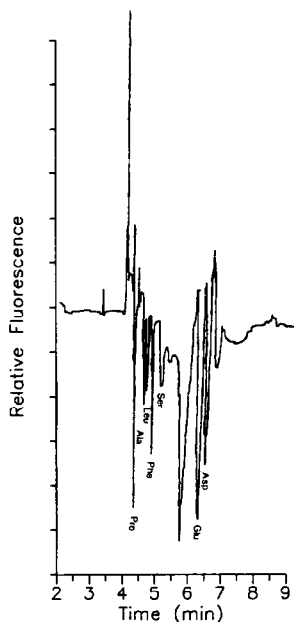
Fig. 1. Indirect detection of an amino acid mixture using coumarin 343 as the fluorophore. The buffer used was 1 mM coumarin 343 at pH 9.5. A 1-s, 30-kV injection of 75 μM each was followed by electrophoresis at 30 kV on a 80 cm × 18 μm I.D. × 150 μm O.D. column.

detection limit was $8 \cdot 10^{-10}$ M (signal-to-noise ratio, $S/N = 3$) (column 80 cm, 5 μm diameter, 1 s injection at 30 kV, 30-kV separation).

Coumarin 343 is successfully employed in the separation of a complex mixture of amono acids shown in Fig. 1. The elution order agrees with previous work[5]. The broad peak at 5.5 min is an unknown impurity. The separation efficiency is very good here also, with an average plate number of 270 000. This proves that indirect detection is based on the charge displacement and not specific interaction with the fluorescing ion. Otherwise, the peak heights would have been different from those in previous work.

Sugars have been separated by CZE as their borate complexes[12]. It is shown here that sugars can be separated and detected without any prior derivatization. CZE separates compounds based on their mobility which is related to the pK values and the buffer pH. In indirect detection the sensitivity depends on the fraction of the analyte that is ionized. This means when using indirect detection for the detection of sugars the pH of the running buffer must be approaching 12 to have any substantial fraction, α, in the ionized form. This presents another problem. When the pH of the buffer solution gets this high the concentration of hydroxide ion is no longer negligible relative to the concentration of the fluorophore. This results in a decrease of TR. These effects must be balanced to obtain the best sensitivity possible for the system.

The effect of the hydroxide ion can be approximately described by eqn. 1.

$$TR_{tot} = \frac{\alpha[sugar]}{[FL] + [OH^-]} \tag{1}$$
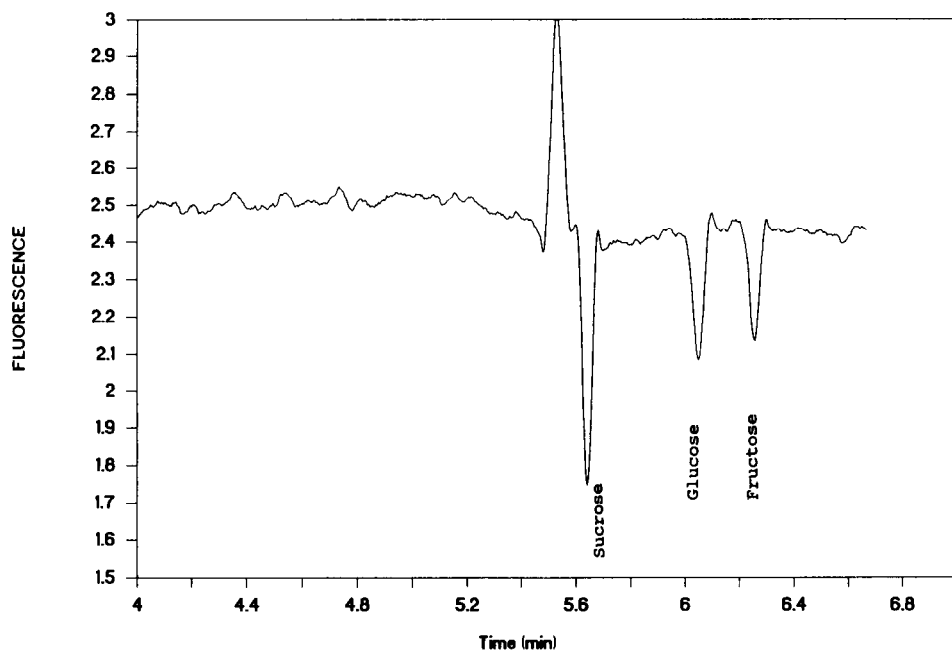
Fig. 2. Separation of a sugar mixture. Indirect detection of 640 fmol of each sugar in 1 mM coumarin at pH 11.5. The column was 90 cm × 18 μm I.D. × 150 μm O.D.

In this equation TR_{tot} is the total transfer ratio. This includes the amount of fluorophore [FL], and the amount of hydroxide ion [OH$^-$], displaced by the sugar molecules that are ionized. Ir can be seen that at constant fluorophore and sugar concentrations α in the numerator and the [OH$^-$] in the denominator are competing functions of pH. TR_{tot} goes through a maximum when it is plotted as a function of pH. The pH at this maximum is the most sensitive pH for detection.

The separation of a three-sugar mixture is shown in Fig. 2. The optimum detection pH was found to be 11.65 based on tabulated literature values of the pK for each sugar[10] and eqn. 1. A pH of 11.5 was used for detection, however, because the rate

TABLE I

SUGARS DETECTED BY INDIRECT FLUORESCENCE DETECTION

Each sugar was injected at a concentration of 1 mM in 1 mM coumarin 343 at pH 11.5. Column was 84 cm × 18 μm I.D. × 150 μm O.D.

Sugar	Migration time (min)
Raffinose	4.98
Sucrose	4.99
Maltose	5.35
Arabinose	5.47
Glucose	5.50
Lactose	5.61
Xylose	5.62
Fructose	5.66

of degradation of fluorophore was decreased without any appreciable decrease of detection efficiency. The capillary columns were found to be stable for many runs at this pH. Table I is a compilation of the sugars detected using CZE–IFD and their corresponding migration times. Each of these sugars were injected at a concentration of 1 mM. This is compared to a previous report where the N-2-pyridylglycamine derivatives of sugars are injected in the 10–100-mM range and detected by UV absorbance[12].

In this analysis of biological compounds there is often a limited supply of sample, such as in the analysis of single cells[13]. The analysis of such small volumes of analytes requires the use of smaller bore capillaries in the CZE system. The use of these smaller bore capillaries, however, requires the absolute detection limit of the detection method to decrease as the dimensions of the capillary decrease. The detection scheme should also be universal to detect the large number of biological compounds that lack analytically useful physical properties. CZE–IFD fulfills the requirements that are demanded by the use of smaller capillaries. This was demonstrated by the detection of fructose after it was injected into a 5-μm diameter capillary. The absolute limit of detection in this system was 2 fmol based on a S/N_{rms} (rms = root mean square) of 3. This is expected based on eqn. 1 and ref. 6. The high separation efficiency offered by this system (over 600 000 theoretical plates) is partially responsible for the low detection limit obtained. The dynamic range is therefore a factor of 100, from the detection limit to saturation of the background signal.

The detection limit of fructose was believed to be limited by the mechanical vibration of the system. This is because the capillary diameters are approaching the diameter of the focused laser beam[14]. Small fluctuations in the capillary relative to the beam will cause large fluctuations in the signal. This can be partially compensated for by slightly defocusing the beam by moving the capillary away from the beam focusing lens. This problem will have to be studied further as capillary dimensions decrease.

ACKNOWLEDGEMENTS

REFERENCES

1 K. D. Lukacs and J. W. Jorgenson, *Science (Washington, D.C.)*, 222 (1983) 266.
2 K. D. Lukacs and J. W. Jorgenson, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 8 (1985) 407.
3 R. A. Wallingford and A. G. Ewing, *Anal. Chem.*, 60 (1988) 258.
4 J. S. Green and J. W. Jorgenson, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 7 (1984) 529.
5 W. G. Kuhr and E. S. Yeung, *Anal. Chem.*, 60 (1988) 1832.
6 W. G. Kuhr and E. S. Yeung, *Anal. Chem.*, 60 (1988) 2642.
7 B. L. Hogan and E. S. Yeung, *J. Chromatogr. Sci.*, 28 (1990) 15.
8 L. Gross and E. S. Yeung, *J. Chromatogr.*, 480 (1989) 169.
9 J. E. Parkin, *J. Chromatogr.*, 287 (1984) 457.
10 J. A. Rendle, Jr., in H. S. Isbell (Editor), *Carbohydrates in Solution*, American Chemical Society, Washington, DC, 1973, p. 54.
11 A. Savitsky and M. J. E. Golay, *Anal. Chem.*, 36 (1964) 1627.
12 S. Honda, S. Iwase, A. Makino and S. Fugiwara, *Anal. Biochem.*, 176 (1989) 72.
13 R. A. Wallingford and A. G. Ewing, *Anal. Chem.*, 60 (1988) 1975.
14 E. J. Guthrie, J. W. Jorgenson and P. R. Dluzneski, *J. Chromatogr. Sci.*, 22 (1984) 171.