ELSEVIER

Journal of Chromatography A, 725 (1996) 361–366

JOURNAL OF
CHROMATOGRAPHY A

Automated capillary electrochromatography: reliability and reproducibility studies

Maria T. Dulay[a], Chao Yan[a], David J. Rakestraw[b], Richard N. Zare[a,*]

[a]Department of Chemistry, Stanford University, Stanford, CA 94305, USA
[b]Sandia National Laboratories, Livermore, CA 94551, USA

Received 11 July 1995; revised 5 September 1995; accepted 7 September 1995

Abstract

The routine application of capillary electrochromatography (CEC) is demonstrated by incorporating 75 μm I.D. capillaries packed with 3 μm octadecylsilica (ODS) particles into a commercial CZE instrument. A mixture of several neutral compounds is separated into its components with an average efficiency up to 181 000 plates/m in less than 8 min. Hundreds of consecutive runs are performed over a period of weeks from which it is concluded that the reproducibility of the capacity factors is better than 2% and that CEC separations can be achieved in a reliable and routine manner.

Keywords: Capillary electrochromatography; Electrochromatography

1. Introduction

The separation and analysis of environmentally and biologically important molecules, both neutral and ionic in nature, have become increasingly important. A widely used technique for this purpose is high-performance liquid chromatography (HPLC) in which separation relies on the partitioning of the analytes between the stationary and the mobile phases [1].

With the advent of capillary zone electrophoresis (CZE) [2,3], separation and analysis of complex sample mixtures can be achieved using ultra-low sample volumes with high resolution and efficiency. The peak capacity (i.e., the number of peaks separated per unit time) in CZE is high, making this analytical technique particularly attractive for separating ionic species by their electrophoretic mobilities. The separation of neutral species via electrokinetic techniques, however, has remained more problematic.

Terabe et al. [4] have demonstrated the advantages of micellar electrokinetic chromatography (MECC) in separating uncharged analytes using micelles as a pseudostationary phase. Although the use of micellar phases has been demonstrated as a useful means for improving separation selectivity in capillary electrophoresis, the choice of micellar phases as well as the resolving power is limited. Hence, MECC is not yet a routinely used technique in the laboratory.

The concept of capillary electrochromatography (CEC) was first demonstrated in 1974 by Pretorius et al. [5]. CEC has since been utilized to analyze neutral compounds that are not separable by CZE [6–12]. In CEC the separation of uncharged analytes is based on partitioning, while the separation of

*Corresponding author.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)01000-9

charged analytes is based both on partitioning and electrophoretic mobility. The mobile phase in CEC is driven via electroosmotic flow (EOF) induced by an applied electrical field [3]. EOF originates from the electrical double layer generated at the surface of the capillary wall as well as on the surface of the packing material. The flow profile of EOF in CEC is essentially flat as compared with the parabolic flow profile of pressure-driven HPLC. This flat flow profile is a contributing factor to the high efficiencies observed in CEC. The efficiency of CEC also markedly depends on the size of the particles that can be packed in the capillary [13]. The power of CEC as an analytical method is based on the advantage of combining the high efficiency and resolution of CZE with the high selectivity and universality of liquid chromatography. Unlike MECC where the number of useful types of micelles is limited, a wide variety of stationary phases are available (generally those used in HPLC), which makes this technique versatile.

Although it has been demonstrated that a typical micro-HPLC column with 320 mm I.D. can be operated by electroosmotic "pumping" [14], many practical problems and difficulties were encountered [6,8,14]. A notable problem in CEC was the difficulty in preparing and operating the packed capillary columns (e.g., frit making, column packing, bubble formation, and Joule heating). Consequently, CEC has received relatively little attention. Recently, however, Yan et al. [15] have demonstrated that CEC columns can be routinely fabricated via a novel electrokinetic packing method [16]. Very high efficiencies (120 000 to 400 000 theoretical plates/m) were achieved by using on-column, laser-induced fluorescence (LIF) detection [15].

Routine use of CEC as an analytical technique for the analysis of complex sample mixtures necessitates the reliability and reproducibility of an automated CEC system for achieving separation of a test mixture with high efficiency and high selectivity. In the present study, we describe an automated CEC separation of a mixture of neutral organic compounds in C_{18}-packed capillary columns using a Beckman P/ACE Model 2000 capillary electrophoresis instrument. Hundreds of consecutive runs over a period of several weeks were performed. Results of these studies are presented in what follows.

2. Experimental

2.1. Capillary electrochromatography apparatus

The apparatus used to perform CEC in this study is a Beckman Model 2000 P/ACE capillary electrophoresis system (Fullerton, CA, USA) equipped with a UV absorbance detector. The Beckman system was slightly modified such that the detector end of the packed capillary is constantly immersed in the buffer solution. (Under normal operating conditions, both the inlet and outlet vials are simultaneously pneumatically raised to immerse both ends of the capillary in the buffer solution.) Each packed capillary column was installed in a Beckman Model 2000 P/ACE capillary cartridge holder that was then inserted into the P/ACE instrument. A packed capillary column, positioned in the capillary cartridge holder, was pre-conditioned with the mobile phase [by pressurizing the column inlet to approximately 500 p.s.i. (3.45 MPa) with a syringe pressurized with a hand-held vise] prior to installation in the P/ACE system. With the packed capillary column installed in the P/ACE instrument, it was further conditioned by driving the mobile phase through the capillary at an applied voltage of 5 kV prior to its use. The mobile phase employed in these separations was a 5 m*M* phosphate buffer (pH 6.5) containing 80% (v/v) acetonitrile (sonicated prior to use). The mixture of analytes was electrokinetically injected into the packed capillary column at 5 kV for 5 s. Separations were performed at an applied voltage of 10 kV and at a temperature of 20°C. The analytes were detected by monitoring their absorbance at 254 nm.

2.2. Fabrication of packed capillary columns

The capillary columns employed in this study had an inner diameter (I.D.) of 75 μm and an outer diameter (O.D.) of ca. 365 μm, and they were packed (20-cm packed length) using an electrokinetic packing method described previously [16]. (A similar packing method has also been reported by Tsuda and co-workers [17–19].) First, an inlet frit was made at one end of a capillary by sintering 5-μm silica particles using either a thermal wire stripper (column 1) or a fiber optic splicer (column 2) (Fiberlign IFS-2001, Proformed Line Products, Cleveland, OH,

USA). Second, a suspension of 3-μm ODS (90% w/w) and 1-μm bare silica particles (10%, w/w) in methanol containing 4 m*M* phosphate (pH 6.5) was sonicated for approximately 10 min, then electrokinetically packed into the column for approximately 1 h. Third, after packing, a second frit (the outlet frit) was made in the column by sintering the packing material. The column was pressurized at ca. 3000 p.s.i. (ca. 20.7 MPa) during the fabrication of this second frit to minimize disturbance of the adjacent ODS particles. Each column was then inspected carefully under a microscope (at magnifications of 10× to 40×) to verify the packing density and the structural appearance of the frits.

Two different columns were used for the separation of a mixture that contains neutral molecules of thiourea, benzyl alcohol, and benzaldehyde. Both columns 1 and 2 are 20 cm×75 μm I.D. packed (27 cm total length, 20.3 cm from the inlet frit to the detection window) fused-silica capillary columns filled with 3-μm ODS particles. For column 1, both the inlet and outlet frits were made with a thermal wire stripper (sintering times of approximately 15 s). For column 2, both inlet and outlet frits were prepared using a fiber optic splicer. The splicer allows for programmable fusing parameters which were: (1) prefusion: time 10 ms, rise time 10 ms, fall time 10 ms; and (2) fusion: power 12.0 mA, rise time 10 ms, hold time 400 ms, and fall time 360 ms. Only column 2 was used for the reproducibility study.

2.3. Materials and reagents

The fused-silica capillary columns used in this study were purchased from Polymicro Technologies (Phoenix, AZ, USA). The 3-μm spherical octadecylsilica (ODS) particles were purchased from Synchrom (Lafayette, IN, USA). The 1-μm bare silica particles were purchased from Phase Separation (Norwalk, NJ, USA). The 5-μm silica particles used for frit fabrication was a gift from John O'Gara from Waters (Milford, MA, USA). Thiourea, benzaldehyde, benzyl alcohol, sodium phosphate, and acetonitrile (HPLC grade) were purchased from Aldrich (Milwaukee, WI, USA) and used without further purification. Stock solutions of the analytes were prepared in micromolar to millimolar concentrations in the mobile phase and degassed by sonication. Water was purified with an Ultra-Pure water system from Millipore (Milford, MA, USA).

3. Results and discussion

3.1. Column reliability (packed column preparation)

Two capillary columns were electrokinetically packed with 3-μm ODS particles containing 10% (w/w) 1-μm bare silica particles suspended in a mixture of methanol and buffer. In our experience, packing columns electrokinetically was more effective and more reliable (i.e., resulted in a more homogeneously packed capillary column) than a slurry (i.e., pressure-driven) packing method. Addition of 1-μm silica particles appears to aid in stabilization of the electroosmotic flow by preventing "drying out" of the capillary column during each separation run. Drying out of the capillary column is brought about by formation of bubbles within the packed capillary which results in portions of the stationary phase not being in contact with the mobile phase. The addition of these silica particles to the stationary phase may increase the pumping power of the column by increasing the EOF as well as decrease the overall hydrophobicity of the stationary phase. This decreased hydrophobicity is expected to result in a lower incidence of bubble formation within the capillary and stabilization of the EOF. Additionally, the quality of the inlet and outlet frits significantly influenced the reliability of the results (see next section). When long sintering times were used, the frits made were less "porous" resulting in a reduction of the mobile phase flow in the column as well as its efficiency (i.e., peak broadening). On the other hand, insufficient heating time led to the production of weak frits causing the stationary phase particles to migrate through the frits and pass through the detection window. More reliable frits were fabricated using a fiber optic splicer because the splicer: (1) allowed for a more precise placement of the frit in the capillary column; and (2) provided more control over the sintering (fusion) time and fusion power. The gentle prefusion (10 ms) served to dry out a small section of the column (200 μm)

followed by a more powerful fusion (400 ms) after a delay time of <1 s. When prefusion was not used, sudden heating of the mobile phase in the column caused an "explosive" effect that disturbed the packing bed in the vicinity of the frit. With the use of the fiber optic splicer, packed capillary columns with nearly identical frits were prepared. Under careful inspection using a microscope (10× to 40× magnification), the frits were found to be about 200 μm in length. (Frit lengths of approximately 2 mm were fabricated when a thermal wire stripper was employed.) Placement of an on-column frit at the outlet end of the capillary immediately before the detection window allowed us to use a single capillary for the column construction. This proved superior to connecting two capillaries by a Teflon sleeve. In our experience, a good connection was usually difficult to achieve and seldom reliable.

Particular attention was paid to thorough degassing of the mobile phase as well as the analyte samples. This simple step aided greatly in preventing bubble formation within the capillary column. Equilibrating the column with the mobile phase at a relatively lower voltage (e.g., 5 kV) prior to its use resulted in significantly lowering the incidence of bubble formation within the column.

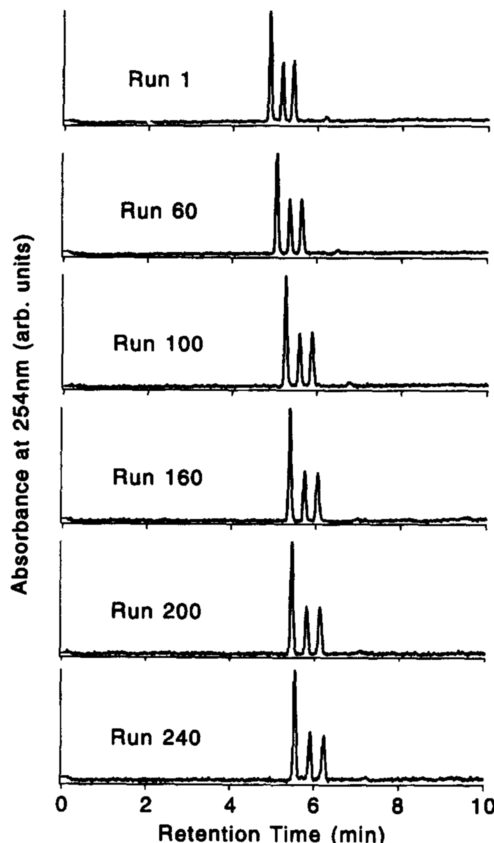

Fig. 1. Selected representative CEC electrochromatograms from hundreds of consecutive runs obtained over several weeks. Experimental conditions: column 2, 27 cm×75 μm I.D. column (20 cm section packed with 3-μm ODS particles); mobile phase, 80% (v/v) acetonitrile–20% 5 m*M* phosphate buffer (pH 6.5); sample, thiourea, benzyl alcohol, benzaldehyde (in order of elution); applied voltage, 10 kV (typical current 2 μA); injection at 5 kV for 5 s; detection wavelength, 254 nm; temperature, 20°C.

3.2. Column reproducibility (N, k', and R_s)

A systematic study of the reproducibility of a single packed capillary column for the analysis of a mixture of uncharged analytes was performed using column 2. Hundreds of consecutive runs over a period of several weeks were performed to demonstrate the reliability of a packed capillary column in terms of the efficiency, capacity factor, and resolution. Fig. 1 illustrates the reproducibility of the separation of the analyte mixture using a single capillary column over a period of several weeks. The relative standard deviations for retention times and peak heights, averaged over 150 consecutive runs using column 2, were better than 4% and 5%, respectively.

Table 1 shows the relative standard deviations of efficiency N, capacity factor k', and resolution R_s. The number of theoretical plates N for each analyte was calculated using the equation $N=5.54(t_R/\omega_{0.5})^2$,

Table 1
Relative standard deviation of efficiency, capacity factor, and resolution obtained with column 2 for 150 separations[a]

Analyte	Relative standard deviation		
	k'	N	R_s
Thiourea (T)[b]	–	4.2	–
Benzyl alcohol (BA)	1.5	3.7	1.5 (BA/T)
Benzaldehyde (B)	1.8	3.9	2.4 (B/BA)

[a] Column, 27 cm×75 μm I.D. (20-cm section packed with 3-μm ODS particles); mobile phase, acetonitrile–5 m*M* phosphate buffer (pH 6.5) (80:20, v/v).
[b] Thiourea was used as an "unretained" neutral marker.

is the full width at half height of the analyte peak, and N was normalized per meter of packed column length. Day-to-day reproducibility for k' and R_s was better than 2.5%. Resolution, as it varies with the experimental parameters k' and N, was calculated using the following equation:

$$R_s = \frac{(k'_o - k'_A)\sqrt{N}}{4(1 + k'_A)}$$

where k'_o and k'_A are the capacity factors for thiourea, which was used as the "unretained" solute marker, and the analyte (i.e., benzyl alcohol and benzaldehyde), respectively.

Shown in Fig. 2 is a plot of theoretical plate number per meter (N/m) vs. number of days over a period of two weeks. Each data point is an average of 30 runs performed during a day with each run lasting 10 min.

A conditioning period for attaining a consistent retention time for each analyte was observed with retention times becoming constant only after the first day. This observed equilibration time may correspond to a period for the alteration of the nature of the stationary phase by the application of a voltage across the capillary (i.e., the saturation or release of adsorptive species from the surface of the particles).

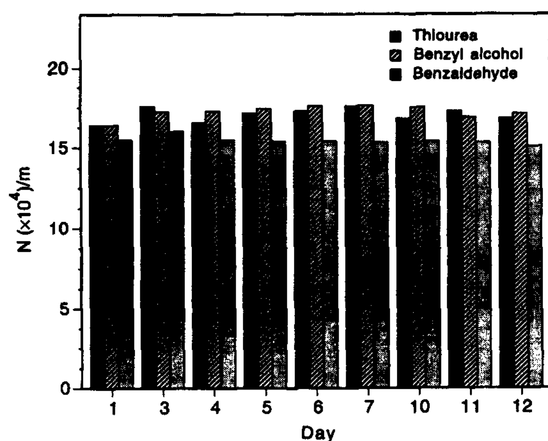

Fig. 2. Variations of day-to-day column efficiency in CEC over a period of two weeks. Each data point is an average of 30 runs. Experimental conditions are as in Fig. 1. (No runs were performed on days 2, 8, and 9.)

This behavior is consistent with observations made by Tsuda [9].

3.3. Column efficiency and resolution

The high efficiency of CEC is shown in Table 2. The number of theoretical plates per meter for thiourea, benzyl alcohol, and benzaldehyde are 178 000, 181 000, and 161 000, respectively, using column 2 where both frits were made with a fiber optic splicer. Lower efficiencies (up to 110 000 plates) were achieved for the separation of the same sample mixture using column 1 where the frits were created with a thermal wire stripper. Frit quality plays an important role in determining column efficiency. (A systematic study of the effect of frit fabrication on column efficiencies is in progress.)

The high efficiency of CEC is demonstrated by the reduced plate heights (h) of 1.90, 1.88, and 1.99 for thiourea, benzyl alcohol, and benzaldehyde, respectively, using column 2. These reduced plate heights are within the range of values for h calculated for other electrochromatographic separations of neutral compounds [13] and is smaller than reduced plate heights obtained in HPLC (typically between 3 and 5) for similar column dimensions, supporting the conclusion that the efficiency of electrically driven chromatography is higher than that of pressure-driven chromatography [6]. However, reduced plate heights of 2.0 or less attained in pressure-driven microcolumn liquid chromatography with capillary columns of small diameters under optimized experimental conditions have been reported previously [20,21].

Table 2
Column efficiency, plate height, and reduced plate height in CEC (column 2)[a]

Analyte	N/m	H (μm)	h
Thiourea	178 000	5.62	1.90
Benzyl alcohol	181 000	5.52	1.88
Benzaldehyde	161 000	6.21	1.99

[a] Column, 27 cm×75 μm I.D. (20-cm section packed with 3-μm ODS particles); mobile phase, acetonitrile–5 mM phosphate buffer (pH 6.5) (80:20, v/v).

fully automated, commercially available CZE instrument, such as the Beckman P/ACE Model 2000 CZE instrument. The use of such an automated CZE instrument offers several advantages over home-built CZE systems: (1) increased injection accuracy; (2) precise temperature control; (3) minimized Joule heating (through the use of a cooling system); and (4) decreased manual labor.

4. Conclusions

We have demonstrated that capillary electrochromatography can be used routinely in the laboratory to achieve reproducible and reliable separations of neutral species through: (1) the use of packed capillary columns with long-term stability and performance; and (2) integration of these packed columns into a fully automated commercial CZE instrument. In this regard, CEC is expected to offer the analyst the advantages of both the high efficiency of electrokinetic separations and the versatility of using different chromatographic packings in separating a wide variety of complex mixtures containing neutral as well as ionic species.

Acknowledgments

M.T.D. would like to thank NIH for a postdoctoral research fellowship. This work was supported by grants from both Beckman Instruments, Inc., Fullerton, CA, USA and Sandia National Laboratories, Livermore, CA, USA.

References

[1] R.P.W. Scott, Liquid Chromatography for the Analyst, Marcel Dekker, New York, 1994.
[2] J.W. Jorgenson and K.D. Lukacs, J. Chromatogr., 218 (1981) 209.
[3] R. Weinberger, Practical Capillary Electrophoresis, Academic Press, San Diego, CA, 1993.
[4] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 113.
[5] V. Pretorius, B.J. Hopkins and J.D. Schieke, J. Chromatogr., 99 (1974) 23.
[6] J.H. Knox and I.H. Grant, Chromatographia, 32 (1991) 317.
[7] S. Kitagawa and T. Tsuda, J. Microcol. Sep., 6 (1994) 91.
[8] N.W. Smith and M.B. Evans, Chromatographia, 38 (1994) 649.
[9] T. Tsuda, Anal. Chem., 59 (1987) 521.
[10] T. Tsuda, K. Nomura and G. Nakagawa, J. Chromatogr., 248 (1982) 241.
[11] H. Yamamoto, J. Baumann and F. Erni, J. Chromatogr., 593 (1992) 313.
[12] F. Lelievre, C. Yan, R.N. Zare and P. Gariel, J. Chromatogr. A, (1996) in press.
[13] J.H. Knox, Chromatographia, 26 (1988) 329.
[14] C. Yan, D. Schaufelberger and F. Erni, J. Chromatogr. A, 670 (1994) 15.
[15] C. Yan, R. Dadoo, H. Zhao, R.N. Zare and D.J. Rakestraw, Anal. Chem., (1995) in press .
[16] C. Yan, Electrokinetic Packing of Capillary Columns, U.S. Patent No. 08/142, 917 (1993).
[17] S. Kitagawa and T. Tsuda, Chromatography, 15 (1994) 137.
[18] M. Inagaki, S. Kitagawa and T. Tsuda, Chromatography, 14 (1993) 55R.
[19] S. Kitagawa, M. Inagaki and T. Tsuda, Chromatography, 14 (1993) 39R.
[20] K.-E. Karlsson and M. Novotny, Anal. Chem., 60 (1988) 1662.
[21] R.T. Kennedy and J.W. Jorgenson, Anal. Chem., 61 (1989) 1128.