ELSEVIER

Journal of Chromatography B, 683 (1996) 29–35

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Micellar electrokinetic chromatography in zero-electroosmotic flow environment

George M. Janini*, Gary M. Muschik, Haleem J. Issaq

SAIC Frederick, NCI–Frederick Cancer Research and Development Center, P.O. Box B, Frederick, MD 21702, USA

Abstract

Micellar electrokinetic chromatography (MEKC) is conducted in polyacrylamide-coated capillaries under almost complete suppression of electroosmotic flow. The equations of migration and resolution for neutral solutes in this mode of MEKC operation are presented. The technique is termed reversed-flow MEKC (RF-MEKC) because, in contrast to MEKC in bare-silica capillaries (N-MEKC), solute migration order is reversed and solute migration time is inversely proportional to micelle concentration. This presents an advantage for the high-efficiency separation of extremely and moderately hydrophobic solutes in a short analysis time. Examples of the separation of polycyclic aromatic hydrocarbons, aflatoxins and dansylated-amino acids are presented using sodium dodecyl sulfate (SDS) surfactant. Polycyclic aromatic hydrocarbons are separated using a relatively low micelle concentration. The detection sensitivity for these compounds is enhanced in two ways. First, the peaks are sharp because of the short analysis time and the inertness of the column surface. Second, the fluorescence background and Joule's heating are minimal because of the low concentration of SDS and other additives needed to affect the separation. While N-MEKC is mainly conducted with basic buffers, RF-MEKC can be conducted in basic as well as acidic media as illustrated in the separation of 15 dansylated-amino acids at pH 4.2.

Keywords: Reversed-flow micellar electrokinetic chromatography; Polynuclear aromatic hydrocarbons; Aflatoxins; Amino acids, Dns derivatives

1. Introduction

The recent development of zone electrophoresis in capillary format (CZE) has opened the way for the application of electrophoresis to the separation of small ions and ionizable molecules [1]. The development of micellar electrokinetic chromatography (MEKC) has allowed the extension of this technique to a wide variety of neutral solute systems [2,3]. MEKC is more like a combination of CZE and micellar liquid chromatography. Analytes are separated based on their differential partitioning between the aqueous mobile phase and the hydrophobic interior of the micelles. The multiplicity of interactions which micellar systems provide (hydrophobic, electrostatic and hydrogen-bonding) are absent in CZE and cannot be duplicated by conventional normal- or reversed-phase systems in high-performance liquid chromatography (HPLC). Theoretical plate numbers in MEKC are not as high as could be achieved by CZE because of the resistance to mass transfer that is introduced by solute partitioning between the bulk buffer and the micelles. However, column efficiency is much higher than could be achieved by HPLC. One of the main drawbacks of MEKC is its limited migration window which tends to limit the system's peak capacity. Several approaches to extend the migration window have been

*Corresponding author.

Published by Elsevier Science B.V.
PII S0378-4347(96)00130-2

attempted, including pH [4], use of short chain-length surfactants and surface-silylated fused-silica columns [5], use of polymeric surface modifiers [6], organic modifiers [7] and mixed surfactants [8]. The above-mentioned techniques have been successful, to varying degrees, in extending the migration window and improving MEKC performance. Unfortunately, this improvement comes at the expense of a substantial increase in analysis time, especially for hydrophobic solutes, which tend to stack at the end of the migration window because of their strong association with the micelle, even after the addition of large (>20%) amounts of organic solvent to the aqueous phase [7].

Under certain conditions, such as the use of acidic micellar solutions at pH below 5.0 [4,9], or the use of excessive amounts of certain organic solvents such as 2-propanol [10], the electroosmotic flow is reduced and is exceeded by the electrophoretic mobility of the micelle. This results in reversal of migration whereby the micelles migrate toward the positive electrode, and the more hydrophobic solutes migrate faster than the more hydrophilic [9]. While reversal of migration (the subject of this study) seems to be promising, these approaches are impractical, because column efficiency quickly deteriorates with excessive concentration of organic solvents [7,10], and the reproducibility of migration times in bare-silica columns is very poor in acidic solutions below pH 5.0 [4].

In a previous work from our laboratory [11], we used polyacrylamide-coated columns with zero electroosmotic flow to determine the partition coefficients of hydrophobic and slightly polar solutes in micelle–aqueous systems, and suggested that these columns may be useful for the separation of hydrophobic solutes. While our publication was in press, Chiari et al. [12] presented unique separations of neutral isotopic molecules, using this mode of MEKC. In the present work, we demonstrate the practical utility of this technique for the separation of several classes of hydrophobic compounds. The technique which, for brevity, is termed reversed-flow MEKC (RF-MEKC), will be compared and contrasted with normal MEKC in bare-silica capillaries (N-MEKC) and its advantages and limitations will be discussed. Also, the effect of variables such as surfactant concentration and organic solvents on migration time and resolution will be examined. The term migration rather than elution is used in this manuscript in reference to solute transport for consistency with MEKC literature.

2. Experimental

2.1. Chemicals

All chemicals used in this study were obtained from Sigma (St. Louis, MO, USA) except SDS, which was a Fluka product (Fluka Chemika–Bio-Chemika, Buchs, Switzerland), γ-cyclodextrin (γ-CD), which was obtained from Advanced Separation Technologies, (Whippany, NJ, USA), and the buffer components, which were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Apparatus

A Beckman CZE Model P/ACE 5510, equipped with a P/ACE diode array detector, an automatic injector, a fluid-cooled column cartridge and a System Gold data station, was used in this study. All runs were performed at 25°C. The buffers, with different concentrations of SDS acetonitrile and γ-cyclodextrin, were prepared fresh, passed through 0.2-μm Nylon filters and degassed. The capillary inlet and outlet vials were replenished after every 10 injections. Injections were made using the pressure mode for 5 s at 3.42 kPa.

The home-built capillary electrophoresis and laser-induced fluorescence system used in this study to generate Fig. 3 has been described in detail previously [13,14]. The fused-silica columns used in this study were purchased from Polymicro Technologies (Phoenix, AZ, USA) and were coated with a dense layer of 10% polyacrylamide as described in detail elsewhere [15]. The 10% polyacrylamide-coated column provided stability and migration-time reproducibility to well within 1% R.S.D. throughout the experiments. This was established by periodically monitoring the electroosmotic mobility and measuring the migration time of Sudan III (the micelle marker). The electroosmotic mobility was determined as described [15], to be around 0.1×10^{-4} cm^2 V^{-1} s^{-1}

3. Results and discussion

Electropherograms in RF-MEKC differ from those generated by N-MEKC in that the migration range is infinite. The first solute to appear is the micelle marker (a solute that is fully partitioned in the micelle) followed by analytes in order of decreasing hydrophobicity. The aqueous marker (a solute that is fully partitioned in the aqueous phase) will never appear because the aqueous phase is immobile in the absence of electroosmotic flow. In this respect RF-MEKC is very similar to micellar liquid chromatography, albeit with some theoretical and operational differences. For example, having a mobile micellar phase and a stationary aqueous phase is a unique situation that cannot be operationally duplicated by conventional chromatographic techniques.

The equation of migration in RF-MEKC is derived following basic chromatographic theory. The migration time (t_R) of a neutral solute is given by:

$$t_R = t_{mc} + t_{aq} \tag{1}$$

where t_{aq} is the time the analyte spends in the immobile aqueous phase and t_{mc} is the migration time of a micelle marker. It follows that:

$$t_R = t_{mc} + t_{mc}\frac{t_{aq}}{t_{mc}} = t_{mc} + t_{mc}\frac{n_{aq}}{n_{mc}} \tag{2}$$

where n_{aq}/n_{mc} (the ratio of the number of moles of solute in the stationary aqueous phase to that in the mobile micellar phase) is the capacity factor (k), defined the same way as in elution chromatography. Thus:

$$t_R = t_{mc}(1 + k) \tag{3}$$

For a solute that is fully partitioned in the micelle, $k = 0$ and, hence, the micelle marker in RF-MEKC serves the same purpose as the dead-time marker in elution chromatography.

This simple equation is contrasted with the equation of migration in N-MEKC [2,3]:

$$t_R = \frac{1 + \tilde{k}}{1 + \left(\frac{t_0}{t_{mc}}\right)\tilde{k}} t_0 \tag{4}$$

where $\tilde{k}$ of Eq. (4) is, by definition, the inverse of k of Eq. (3). It is to be noted that Terabe [16] also derived Eq. (3) from Eq. (4) by allowing t_0 to go to infinity.

The resolution equation in RF-MEKC is identical to that of elution chromatography:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{k + 1}\right) \tag{5}$$

where N is the number of theoretical plates and α, the separation factor for two closely-eluting solutes 1 and 2 is equal to $(\frac{k_2}{k_1} = \frac{t_{R2} - t_{mc}}{t_{R1} - t_{mc}})$. In contrast, the resolution equation in N-MEKC is given by [2,3]:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{\tilde{k}_2}{\tilde{k}_2 + 1}\right)\left(\frac{1 - (t_0/t_{mc})}{1 + (t_0/t_{mc})\tilde{k}_1}\right) \tag{6}$$

Here, $\tilde{k}$ implicitly depends on the magnitude of the electroosmotic flow through its explicit dependence on t_0 [Eq. (4)]. More significantly, the separation factor in Eq. (6) is also a function of electroosmotic flow, in contrast to RF-MEKC [Eq. (3)] where the separation factor has the same significance as in chromatography, i.e., it is a thermodynamic property of the system that is independent of operational variables of the measuring instrument.

Finally, the capacity factor in N-MEKC is related to the solute micelle–aqueous partition coefficient (P_{mw}) by the following expression [2,3]:

$$\tilde{k} = P_{mw} V_{mc} C_{mc} \tag{7}$$

where V_{mc} is the molar volume of the micelle and C_{mc} is the micelle concentration. The equivalent relationship in RF-MEKC is given as [11]:

$$k = \frac{1}{P_{mw} V_{mc} C_{mc}} \tag{8}$$

Comparison of Eq. (7) and Eq. (8) reveals that the migration time in N-MEKC increases with increasing hydrophobicity (larger P_{mw}) and increasing micelle concentration, while in RF-MEKC the opposite trend is observed for both variables. Thus, even though N-, and RF-MEKC share the same separation mechanism, there are differences that are analogous, in practice if not in principle, to the differences between normal- and reversed-phase HPLC. It should

be noted that N-MEKC generally exhibits elution orders that resemble reversed-phase HPLC while RF-MEKC exhibits elution orders that are more like normal-phase HPLC.

Several examples of separations in RF-MEKC mode using SDS, which is by far the most popular micelle-forming surfactant, are presented. When SDS is used as the micelle-forming surfactant the resultant negatively-charged micelles migrate electrophoretically towards the anodic detector end of the capillary.

Fig. 1 is an illustration of RF-MEKC. Sudan III (the micelle marker) is eluted first, followed by solutes in order of decreasing hydrophobicity. The separation was carried out at pH 4.2.

N-MEKC separations are usually carried out in neutral and acidic buffers. Separations at low pH are impractical because the electroosmotic flow is slowed down and, at some point (about pH 5), it is counterbalanced by the micelle electrophoretic mobility in the opposite direction, resulting in excessively long migration times [4]. However, this is not the case in RF-MEKC where it is possible, and in certain applications preferable, to conduct the separation at acidic pH. This is clearly illustrated in Fig. 2, which shows the separation of a set of 15 dansylated-amino acids at pH 4.2. The high column efficiency demonstrated in the figure is indicative of

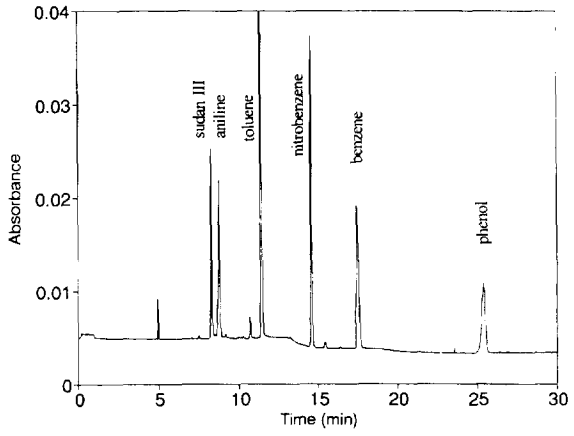

Fig. 1. Order of migration versus solute hydrophobicity in RF-MEKC mode. Column: 10% linear polyacrylamide-coated fused-silica. Column dimensions: L_{total} = 57 cm; $L_{detector}$ = 50 cm; I.D. = 75 μm; buffer: 10 mM acetate and 50 mM SDS; pH = 4.2; applied voltage: −20 kV; current: 30 μA; detection: 214 nm.

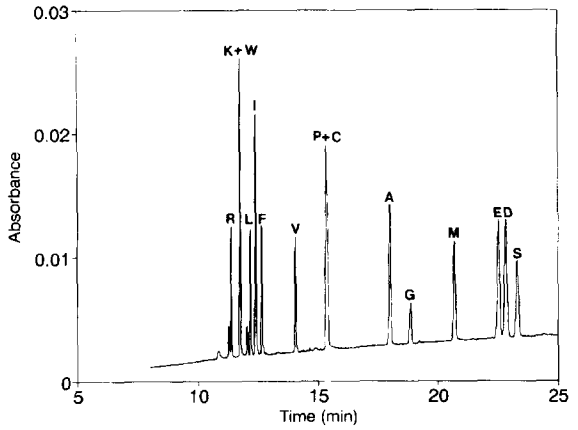

Fig. 2. RF-MEKC separation of 15 dansyl-amino acids. Column: As in Fig. 1; column dimensions: L_t = 57 cm; $L_{det.}$ = 50 cm; I.D. = 75 μm; buffer: 25 mM acetate, 25 mM SDS; pH = 4.2; voltage: −15 kV; current: 27 μA; detection: 214 nm; solute concentration: 10 μg/ml each. The peaks are identified with one-letter abbreviations.

the inertness of the neutral coating used in this study. The order of migration can be explained – largely in terms of differences in solute hydrophobicity. The positively-charged and the more hydrophobic solutes are eluted first and the negatively-charged and least hydrophobic solutes are the last to elute. The ability to conduct the separations at low pH allows for subtle manipulation of the selectivity by taking advantage of differences in the ionization constants of the carboxylic groups. In a recent review of MEKC methods for the separation of amino acids [17], only one out of seventeen methods listed is conducted at acidic pH. This was reported by Waldron et al. [18], who compared the separation of DABTH–amino acids at pH 2.5 with separations at higher pH, and concluded that the separation of amino acids at low pH in bare-silica is not ideal because of long migration times and excessive peak broadening.

MEKC is particularly suited to the analysis of polycyclic aromatic hydrocarbons (PAH), and their derivatives and metabolites from biological samples, because they are present at trace amounts in small volumes. Fig. 3 gives the separation of three polycyclic hydrocarbon compounds using a γ-CD containing buffer. Laser-induced fluorescence was used for detection in this example to illustrate a unique

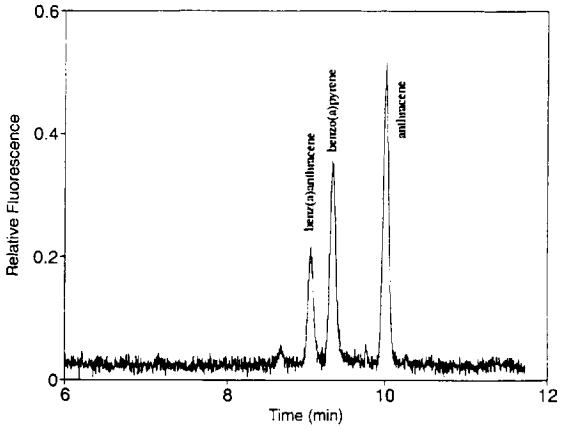

Fig. 3. Electropherogram of the separation of three polycyclic aromatic hydrocarbons using γ-CD shape selector. Column: As in Fig. 1; column dimensions: $L_t = 60$ cm; $L_{det} = 53$ cm; I.D. = 75 μm; buffer: 10 m*M* phosphate, 25 m*M* SDS, 15 m*M* γ-CD; pH = 2.8; voltage: −18 kV; current: 39 μA.; detector: LIF (ex. 248, em. 400±20 nm; solute concentration: 0.1 μg/ml. Concentration limit of detection for benzo[*a*]pyrene [B(a)P] in 5 ppb.

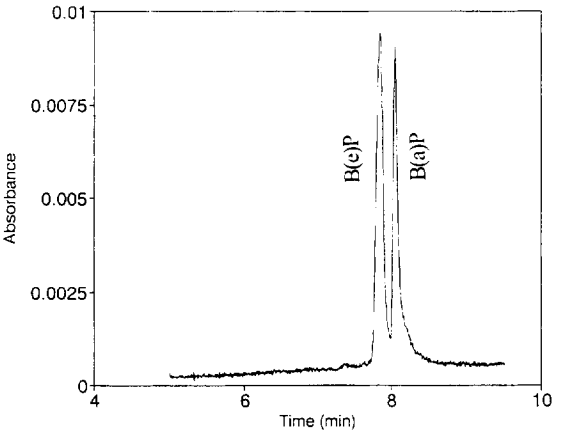

Fig. 4. RF-MEKC separation of benzo(a)pyrene and benzo(e)pyrene using a γ-CD shape selector. Column: As in Fig. 1; column dimensions: $L_t = 47$ cm; $L_{det} = 40$ cm; I.D. = 75 μm; buffer: 10 m*M* phosphate, 50 m*M* SDS, and 20 m*M* γ-CD; pH = 3.0; voltage: −15 kV; detection: 214 nm; solute concentration: 5 μg/ml.

advantage of RF-MEKC over N-MEKC, namely the capability of producing relatively sharp peaks (*N* for anthracene = 170 000 plates/m) for extremely hydrophobic solutes, with a relatively low SDS concentration. Extremely hydrophobic solutes, including those of Fig. 3, have been separated by N-MEKC only by using a high concentration of SDS and other additives such as organic solvents, urea and cyclodextrins, among others [19–22]. However, as pointed out by Yan et al. [23], the use of large amounts of SDS and other additives results in excessive fluorescence background and reduced sensitivity. The order of migration in Fig. 3 does not seem to strictly follow hydrophobicity because benzo[*a*]pyrene (5-membered ring) is larger and more hydrophobic than benz[*a*]anthracene (4-membered ring). This reversal of migration may, however, be explained on the basis that benzo[a]pyrene forms a stronger inclusion complex with the γ-CD cavity and consequently resides longer than benz[*a*]anthracene in the immobile phase. The separation of geometric isomers of polycyclic aromatic hydrocarbons is hard to achieve without the use of shape selectors as additives to the buffer. Copper and Sepaniak [21] used γ-CD and Szolar et al. [22] used a mixture of neutral and anionic β-cyclodextrins for the separation of benzo[*e*]pyrene from benzo[*a*]pyrene. In this work, this very difficult separation was achieved (Fig. 4) by using γ-CD. As expected, the order of migration in Fig. 4 is the reverse of that reported by Copper and Sepaniak [21].

It is to be emphasized that both normal and reversed modes of MEKC share the same separation mechanism, i.e., solute partitioning between the micelle and aqueous phases. If a set of solutes is separated in N-MEKC it should also be separated in reversed-flow MEKC in a shorter analysis time if both separations are conducted under the same experimental conditions of buffer composition, pH, etc. Shorter analysis time, and the fact that neutrally-coated surfaces are generally more inert and less adsorptive than bare-silica, result in sharper peaks and enhanced sensitivity for most hydrophobic solutes.

The effect of SDS concentration on migration time and separation is presented in Fig. 5. In contrast to N-MEKC, the migration time in RF-MEKC is inversely proportional to micelle concentration [Eq. (8)]. As shown in Fig. 5, the separation of the hydrophobic amino acids improves with decreasing SDS concentration. Less current and less fluorescence background are produced with lower SDS

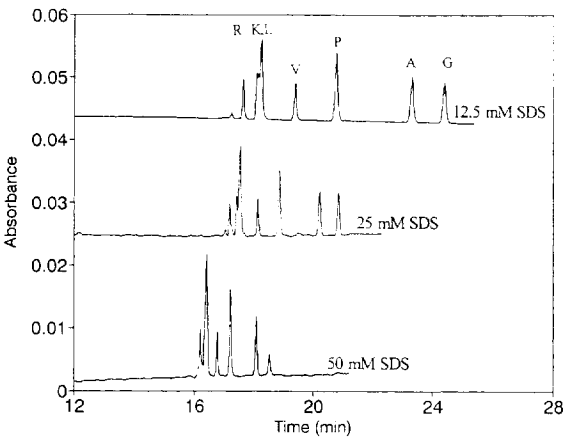

Fig. 5. Effect of SDS concentration on migration time and resolution in RF-MEKC. Column: As in Fig. 1; column dimensions: $L_t = 57$ cm; $L_{det.} = 50$ cm; I.D. = 75 μm; buffer: 50 m*M* acetate and SDS; pH = 4.2; voltage: −10 kV; detection: 214 nm; solutes: dansyl-amino acids identified with one-letter abbreviations.

concentration, which presents another advantage of RF-MEKC over N-MEKC.

The effect of the addition of organic solvents to the buffer in capillary zone electrophoresis is well studied [24]. Typically, the addition results in an increase in buffer viscosity and a decrease in electroosmotic flow. In MEKC, the situation is more complicated because of the effect of the additive on the micelle and on solute partitioning between the micelle and the aqueous phases. In general, the addition of organic solvents renders the aqueous phase more hospitable to hydrophobic solutes. This should speed up the migration of solutes in N-MEKC. However, since electroosmotic flow is slowed down to a larger extent, the net effect of these two opposing factors is a decrease in migration time with an increase in the percentage of organic modifier. The situation is different in RF-MEKC because the effect of electroosmotic flow is eliminated. Here, the addition of organic solvents favors partitioning of the hydrophobic solutes in the immobile aqueous phase, resulting in longer solute migration times and a higher degree of selectivity. This is clearly illustrated in Fig. 6, which shows the effect of the addition of ACN on the separation of aflatoxins B_1, B_2, G_1 and G_2. A significant improvement in the separation is achieved with the addition of a relatively small amount of ACN (4%). Three of these compounds (G_1, G_2, and B_2) were separated by N-MEKC in a relatively long analysis time with the use of a large concentration of SDS (100 m*M*) and 10% ACN [25]. It is interesting to note that when 8% acetronitrile (ACN) was added to the buffer, no improvement in resolution between B_2 and G_1 was observed, although a higher separation factor was achieved between B_1 and B_2 and G_1 and G_2.

The use of polyacrylamide-coated capillaries has been developed and promoted for use in the separation of biopolymers [12,26–29]. The columns used in this study have been previously tested with basic [29] as well as acidic [15,28] buffers. There seems to be a reasonable degree of concern about the stability of polyacrylamide-coated columns over extended periods of operation and a great deal of controversy about the causes of deterioration [12,27,30]. What is clear is that the coating is adversely affected by extreme experimental conditions such as high pH [30] and excessive Joule heating [27]. At high voltage the coating is stripped from the column [27] and at high pH the amide bonds are hydrolyzed [30]. Fortunately, these extreme conditions are not necessary for RF-MEKC separations. The separation of hydrophobic solutes can be conveniently conducted at low pH with buffers of low ionic strength.

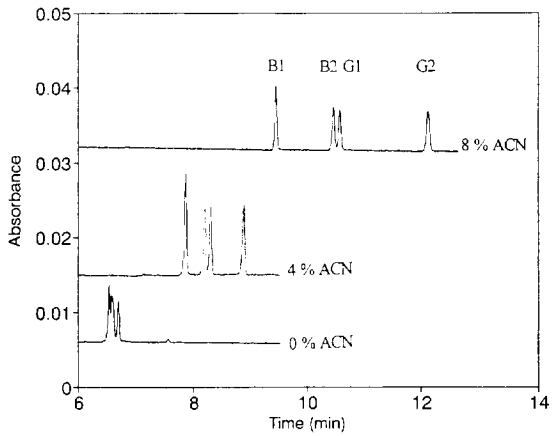

Fig. 6. Effect of acetonitrile concentration on the RF-MEKC separation of aflatoxins. Column: As in Fig. 1; column dimensions: $L_t = 47$ cm; $L_{det.} = 40$ cm; I.D. = 75 μm; buffer: 10 m*M* phosphate, 50 m*M* SDS; pH = 3.0; voltage: −15 kV; current: 25 μA; detection: 214 nm; solute concentration: 2–5 μg/ml.

Acknowledgments

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. The authors thank Dr. K.C. Chan for his help with the laser-induced fluorescence system used in this study.

References

[1] J.W. Jorgenson and K.D. Lukacs, Anal. Chem., 53 (1981) 1298.
[2] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem., 56 (1984) 111.
[3] S. Terabe, K. Otsuka and T. Ando, Anal. Chem., 57 (1985) 834.
[4] K. Otsuka and S. Terabe, J. Microcol. Sep., 1 (1989) 150.
[5] A.T. Balchunas and M.J. Sepaniak, Anal. Chem., 59 (1987) 1966.
[6] J.A. Lux, H. Yin and G. Schomburg, J. High Resolut. Chromatogr., 13 (1990) 145.
[7] A.T. Balchunas and M.J. Sepaniak, Anal. Chem., 60 (1988) 617.
[8] E.S. Ahuja, E.L. Little, K.R. Nielsen and J.P. Foley, Anal. Chem., 67 (1995) 26.
[9] H.T. Rasmussen and H.M. McNair, J. High Resolut. Chromatogr., 12 (1989) 635.
[10] R.O. Cole, M.J. Sepaniak, W.L. Hinze, J. Gorse and K. Oldiges, J. Chromatogr., 557 (1991) 113.
[11] G.M. Janini, G.M. Muschik and H.J. Issaq, J. High Resolut. Chromatogr., 18 (1995) 171.
[12] M. Chiari, M. Nesi, G. Ottolina and P.G. Righetti, J. Chromatogr. A, 680 (1994) 571.
[13] K.C. Chan, G.M. Janini, G.M. Muschik and H.J. Issaq, J. Liq. Chromatogr. A, 16 (1993) 1877.
[14] K.C. Chan, G.M. Janini, G.M. Muschik and H.J. Issaq, J. Chromatogr. A, 653 (1993) 93.
[15] G.M. Janini, K.C. Chan, G.M. Muschik and H.J. Issaq, J. Chromatogr. B, 657 (1994) 419.
[16] S. Terabe, in: N.A. Guzman (Ed.), Capillary Electrophoresis Technology, Chromatographic Series, Vol. 64, Marcel Dekker, New York, 1993, pp. 65–87.
[17] H.J. Issaq and K.C. Chan, Electrophoresis, 16 (1995) 467.
[18] K.C. Waldron, S. Wu, C.W. Earle and N.J. Dovichi, Electrophoresis, 11 (1990) 777.
[19] S. Terabe, Y. Miyashita, Y. Ishihama and O. Shibata, J. Chromatogr. A, 636 (1993) 4.
[20] S. Nie, R. Dadoo and R.N. Zare, Anal. Chem., 65 (1993) 3571.
[21] C.L. Copper and M.J. Sepaniak, Anal. Chem., 66 (1994) 147.
[22] O.H.J. Szolar, R.S. Brown and J.H.T. Luong, Anal. Chem., 67 (1995) 3004.
[23] C. Yan, R. Dadoo, H. Zhao and R.N. Zare, Anal. Chem., 67 (1995) 2026.
[24] G.M. Janini, K.C. Chan, J.A. Barnes, G.M. Muschik and H.J. Issaq, Chromatographia, 35 (1993) 497.
[25] A.T. Balchunas, D.F. Swaile, A.C. Powell and M.J. Sepaniak, Sep. Sci. Technol., 23 (1988) 891.
[26] S. Hjerten, J. Chromatogr., 347 (1985) 191.
[27] D. Schmalzing, C.A. Piggee, F. Foret, E. Carrilho and B.L. Karger, J. Chromatogr. A, 652 (1993) 149.
[28] G.M. Janini, H.J. Issaq and J. Lakszo, J. High Resolut. Chromatogr., 17 (1994) 102.
[29] G.M. Janini, R.J. Fisher, L.E. Henderson and H.J. Issaq, J. Liq. Chromatogr., 18 (1995) 3617.
[30] H. Engelhardt and M.A. Cunat-Walter, J. Chromatogr. A, 716 (1995) 27.