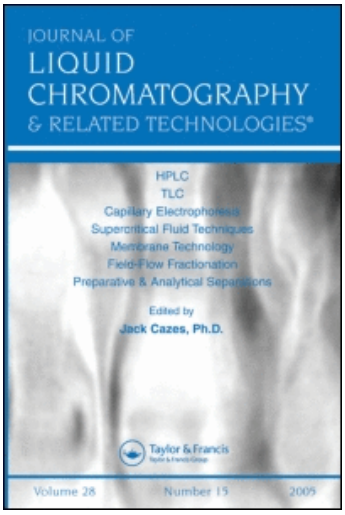


Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Identification of Nucleic Acids and Oligonucleotides by Capillary Zone Electrophoresis with the Four Marker Technique

Heli Sirén[a]; Juho H. Jumppanen[ab]; Kimmo Peltonen[c]; Marja-Liisa Riekkola[a]
[a] Department of Chemistry, Laboratory of Analytical Chemistry, University of Helsinki, Helsinki, Finland [b] Cultor Ltd. Finnsugar Development, Kantvik, Finland [c] The Institute of Occupational Health, Helsinki, Finland

To cite this Article Sirén, Heli , Jumppanen, Juho H. , Peltonen, Kimmo and Riekkola, Marja-Liisa(1995) 'Identification of Nucleic Acids and Oligonucleotides by Capillary Zone Electrophoresis with the Four Marker Technique', Journal of Liquid Chromatography & Related Technologies, 18: 18, 3577 — 3589
To link to this Article: DOI: 10.1080/10826079508014612
URL: http://dx.doi.org/10.1080/10826079508014612

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(18&19), 3577-3589 (1995)

IDENTIFICATION OF NUCLEIC ACIDS AND OLIGONUCLEOTIDES BY CAPILLARY ZONE ELECTROPHORESIS WITH THE FOUR MARKER TECHNIQUE

HELI SIRÉN[1], JUHO H. JUMPPANEN[1,2], KIMMO PELTONEN[3], AND MARJA-LIISA RIEKKOLA[1]*

[1]*Department of Chemistry*
Laboratory of Analytical Chemistry
University of Helsinki
P.O. Box 55 (A.I. Virtasen aukio 1)
FIN-00014 Helsinki, Finland
[2]*Cultor Ltd.*
Finnsugar Development
FIN-02460 Kantvik, Finland
[3]*The Institute of Occupational Health*
Topeliuksenkatu 41 aA
FIN-00180 Helsinki, Finland

ABSTRACT

The paper describes the improvement brought by the four marker technique to the repeatability of the separation of macromolecules [nicotinamide nucleotides (NADPH and NADH), one adenine compound (ATP), two oligonucleotides (135-oligomer and 84-primer containing 135 and 20 bases, respectively) and N2-5'-deoxyquanosine-benzo[a]pyrene-diolepoxide (dG5'BaDE)] by capillary zone electrophoresis with UV detection at the wavelength of 220 nm. Optimized conditions [3-(cyclohexylamino)-1-propanesulphonic acid as the organic electrolyte solution with pH of 11.2, and concentration of 50 mM] were used for the separation. The counter-ion in the system was sodium. The macromolecules could be separated within 20 minutes. The four marker

compounds were (-)mandelic acid, *meso*-2,3-diphenylsuccinic acid, 1,2-phenylenediacetic acid and *o*-phthalic acid, which were used for the calculations of the mobilities for the analytes. The identification of the macromolecules was confirmed with the coefficients of identification. The reliability of the identification was increased up to 75-fold relative to the use of the absolute migration times.

INTRODUCTION

Living cells produce an impressive variety of macromolecules, chiefly proteins, nucleic acids and polysaccharides, which serve as structural components, biocatalysts, hormones and repositories of energy. Nucleic acids are the fundamental genetic material of all living organisms. Normally they are long-chained polymers consisting of several different components, including phosphoric acid, sugar, and purine and pyrimidine bases. Nucleic acids, including single-stranded DNA, are widely varied in structures.

In general, the procedures for separating nucleic acids and synthetic oligonucleotides are complex processes that differ according to the type of material to be separated. The use of CE for the separation of nucleic acids has been reviewed in a few papers [1,2]. Many recent investigations have been devoted to the development of CE methods for the isolation, purification and sequencing of nucleic acids and their constituents [2]. Dolnik et al. [3] have separated oligonucleotides in CZE and also discovered that spermine added to the electrolyte solution enhances the separation. Yamamota et al. [4] have suggested that mononucleotides of calf thymus DNA could be separated under similar conditions to those used in isotachophoretic separations of proteins by adding ampholine to the background buffer solution. Later on, Heiger et al. [5] suggested that use of gels would provide better separations of the proteins than in CZE. One example of the excellent efficiency of CZE is the separation of 19- to 60-mers of DNA within 30 min [6]. Senda et al. [7] have described MEKC-separations with fluorescence detection for nicotinamide nucleotides and adenine compounds.

Poor repeatability of absolute migration times of analytes has been a major drawback of capillary electrophoretic techniques, in both qualitative and quantitative analyses. Numerous attempts have been made to improve quantitation by applying internal standards [8-16]. Also, the use of retention indices [17] and attention to the concept of pseudo-effective mobility [18] have been reported to improve the level of quantitation in MEKC. Although the fluctuation of electroosmosis (v_{eo}) is well known to be the major source of variation in analyte absolute migration times, little effort has been made to study the time dependence more closely. Lee et al. [19] and Jumppanen et al. [20] have recently proposed that the problem could be solved by taking the effect of v_{eo} into account by determining its time dependency rather than by trying to suppress it. Our approach relies on marker compounds of known electrophoretic mobilities. Another important factor is the effective electric field strength, which is the net force affecting an ion and depends on the composition of the electrolyte solution as well as on the applied voltage [21,22].

The basic requirement for the use of marker techniques in CZE and MEKC is that the marker compounds do not undergo changes in mobility if small changes occur in the composition of the electrolyte solution [20,23]. In practice, this means that the marker compounds must have pK_a values far from the pH of the buffer, they must not undergo thermal degradation or conformational changes, and, in the case of MEKC, they must not partition strongly into micelles. When these requirements are met, the marker techniques should work well. If they are not, the repeatability will suffer.

Difficulties have been encountered in CZE separations of macromolecules, especially proteins and peptides. Many proteins possess positive, multiply charged domains that may cause proteins to adsorb onto silica capillary walls [24]. Furthermore, proteins and other macromolecules may undergo chemically induced or temperature induced conformational changes that alter their electrophoretic mobilities [25]. Such behaviour also limits the ability of CE to separate compounds in a repeatable way.

Many methods, such as the Randerath ^{13}P-postlabelling assay, are used for the screening of DNA adducts in biological systems [26, 27]. Although the method is very sensitive, however, the assay is not structure-specific, which means that the procedures are unreliable.

The marker techniques developed in our laboratory have worked well for small, rigid molecules. However, the applicability of the technique to enhance the repeatability of the separation of larger molecules by capillary electrophoresis has not yet been tested. Accordingly, the purpose of this work was to compare the reliability of the identification of selected macromolecules by CZE when using the four marker technique and when using absolute migration times.

EXPERIMENTAL

Chemicals

Nicotinamide-adenine dinucleotide phosphate (NADPH, MW 663 g/mol) was from Boeringer Mannheim (Germany). 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS), adenosine triphosphate (ATP, MW 507 g/mol) and nicotinamide-adenine dinucleotide (NADH, MW 743 g/mol) reduced from β-DPNH and containing β-NADH were purchased from Sigma (Dorset, UK). HPLC-grade methanol, KOH (Titrisol), 1,2-phenylenediacetic acid and *o*-phthalic acid were from Merck (Darmstadt, Germany), *meso*-2,3-diphenylsuccinic acid from Trade TCL (Japan), and (-)mandelic acid from EGA-CHEMIE (Steinheim, Germany). The racemic mixture of dG5-BaDE (N2-5'-deoxyquanosine-benzo[a]pyrenediolepoxide, MW about 571 g/mol) adduct, the 135-oligomer (MW about 43880 g/mol) and the 84-primer (MW about 6349 g/mol) of oligonucleotides were synthesised, extracted and purified at the laboratory of the Institute of Occupational Health (Helsinki, Finland). The 84-primer products were synthesised by using a conventional nucleic acid synthesiser and the oligonucleotides were cloned by using PCR. N2-5'-

deoxyquanosine-benzo[a]pyrenediolepoxide adduct was synthesised as described in the literature [27]. The structures of the analytes are presented in Figure 1.

Apparatus

CE was performed in a 87.0-cm uncoated fused silica capillaries (80.0 cm to the detector) with 50 μm i.d. and 360 μm o.d. (Composite Metal Services Ltd., The Chase, Hallow, Worcs., UK). The capillary electrophoresis apparatus was a Beckman 2050 P/ACE capillary electrophoresis system with a UV/visible detector (wavelength 220 nm) and a liquid cooling system for the capillary (Beckman Instruments, Fullerton, CA, USA). The data were printed and analysed with an HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA). All experiments were carried out at +23 °C. The samples were injected hydrostatically by pressure (0.500 psi) for 10 s. The running voltage was 22 kV.

Treatment of the capillary

Before starting the analysis a new capillary was successively purged with 0.1 M NaOH for 2 min, followed by water for 2 min and then with the running buffer for 5 min. Before each injection the capillary was flushed for 5.0 min with the running buffer.

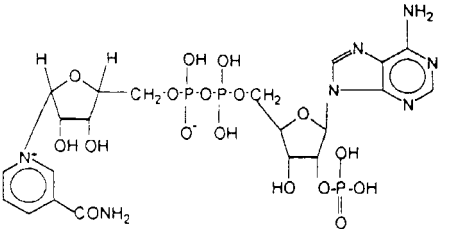
Preparation of electrolytes

The pHs of the solutions were adjusted with a Jenway 3030 pH meter connected to a Jenway electrode (Jenway Ltd., Felsted, England) containing 3

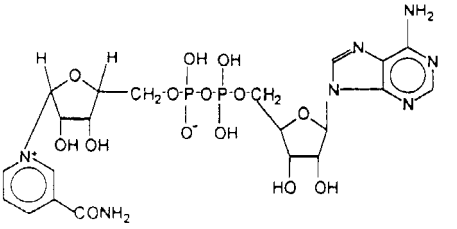
Preparation of electrolytes

The pHs of the solutions were adjusted with a Jenway 3030 pH meter connected to a Jenway electrode (Jenway Ltd., Felsted, England) containing 3 M KCl in saturated AgCl solution. The electrode system was calibrated with Convol BDH buffer solutions (BDH Chemicals Ltd., Poole, UK) and FI-XANAL solutions (Riedel-deHaën AG, Seelze, Germany) of pHs 10.00 and

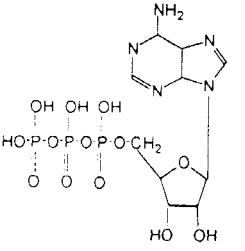
NADPH



NADH



ATP



BASE SEQ TO 20 MER
(84-PRIMER)

5'- gca gca ttt acc tct atc gt

BASE SEQ TO 135 OLIGOMER

ag gcctgctgaa aatgactgag tataaacttg tggtagttgg agctggtggc
gtaggcaaga gtgccttgac gatacagcta attcagaatc actttgtgga tgaatatgat
cctacgatag aggtaaatgc tgc

dG5-BaDE

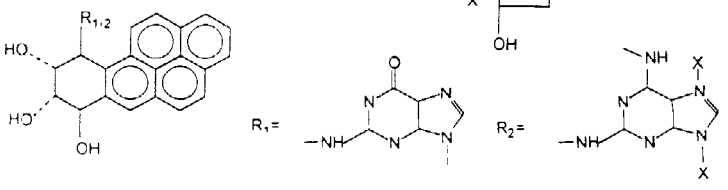


Figure 1. Structures of the macromolecules studied.

11.00. The electrolyte solutions were filtered through 0.45 μm membrane filters (Millipore, Molsheim, France) and degassed before use in an ultrasonic bath.

Determination of viscosity

The absolute viscosity of the electrolyte solutions in CZE separations was determined with an S.I.L.-type viscometer and a pycnometer. The average kinematic viscosity of the 50 mM CAPS solution (pH 11.2) was 0.9273 $\pm$ 0.0001 cSt (n=6, 23 °C) and the average density was 1.00155 $\pm$ 0.00002 g/cm^3 (n=6, 23 °C). The average absolute viscosity was 0.9288 $\pm$ 0.0001 cP. All calculations were carried out with "in-house" designed programs operating in MATLAB (Mathworks Inc.) [20-23].

Preparation of the electrolyte solutions

0.050 M CAPS solution was prepared by weighing 11.2656 g of CAPS powder into 1000 ml total volume of deionized distilled water. The pH was adjusted with 35 ml of 1.000 M NaOH solution to 11.2. After filling the volumetric flask to the one-mark level, the solution was homogenized and kept for 10 min in an ultrasonic bath before filtering it with a Millipore system with 0.45 μm filters. The ionic strength of the solution was 0.085 M.

Preparation of the samples

1000 ppm stock solutions of each nucleic acid and oligonucleotides were prepared to distilled, deionized and filtered water. The 1000 ppm stock solutions of the marker compounds were prepared to HPLC-grade methanol. The final sample was prepared from the stock solutions by pipetting 40 μl of each analyte solution into the sample vial and adding 40 μl of each marker compound and purified, filtered water up to a total volume of 350 μl.

Electrophoretic separations

The final sample solution was analysed nine times by introducing fresh electrolyte solutions after every three injections.

Marker techniques

During one analysis the matrix operation can be expressed as

$$X = A^{-1} B , \quad (1)$$

where the matrices are

$$A = \begin{matrix} 1/3t_1^3 & 1/2t_1^2 & t_1 & \mu_1 t_1 \\ 1/3t_2^3 & 1/2t_2^2 & t_2 & \mu_2 t_2 \\ 1/3t_3^3 & 1/2t_3^2 & t_3 & \mu_3 t_3 \\ 1/3t_4^3 & 1/2t_4^2 & t_4 & \mu_4 t_4 \end{matrix} \quad B = \begin{matrix} L_{det} \\ L_{det} \\ L_{det} \\ L_{det} \end{matrix} \quad X = \begin{matrix} c \\ b \\ a \\ E_{eff} \end{matrix} , \quad (2)$$

where t_1-t_4 are the the migration times and μ_1-μ_4 are the electrophoretic mobilities of the four markers, respectively. L_{det} can be calculated from Eq. 3. Constants a, b and c are values for electroosmotic flow velocity (v_{eo}), linear acceleration of v_{eo} and non-linear acceleration of v_{eo}, respectively. E_{eff} is the effective electric field strength.

However, when four markers were used, v_{eo} were approximated to accelerate non-linearly. The mobilities were calculated with Eqs. 2-4. The basic equation is

$$L_{det} = ct_x^3/3 + bt_x^2/2 + at_x + E_{eff}\, \mu_{ep(x)}\, t_x , \quad (3)$$

where $\mu_{ep(x)}$ is the electrophoretic mobility of the unknown (x) analyte.

After solving for X in Eq. 1, the electrophoretic mobilities of the macromolecules can be calculated from

$$\mu_{ep(x)} = (L_{det}/t_x - ct_x^2/3 - bt_x/2 - a)/E_{eff} \quad (4)$$

Reliability of the identification

The reliability of the identification can be expressed by the coefficient Q_{id}.

$$Q_{id} = (x_2-x_1)/(\sigma_1 + \sigma_2), \quad (5)$$

where x_1 and x_2 are the responses of interest (e.g. migration times), and σ_1 and σ_2 are their standard deviations. If the Q_{id} value exceeds 2, the differentiation between two analytes can be considered reliable [21].

RESULTS AND DISCUSSION

We studied the use of marker compounds to improve the reliability of CZE analysis of NADH, NADPH, ATP and three oligonucleotides. The calculations were based on the data obtained from the separations of the mixture containing the analytes and the four aromatic mono- and dicarboxylic acids, (-)mandelic acid, *meso*-2,3-diphenylsuccinic acid, 1,2-phenylenediacetic acid and *o*-phthalic acid under basic conditions (50 mM CAPS, pH 11.2, 23 °C, Na as the counter-ion, Fig.2). With the four marker technique (4m), it was possible to approximate the electro-osmotic flow velocity (v_{eo}) to be non-linearly accelerating, while the effective electric field strength (E_{eff}) was approximated to stay constant during each run. The marker compounds were selected so that their mobilities were more or less evenly distributed, ($-3.1*10^{-8}$ $m^2V^{-1}s^{-1}$ to $-5.2*10^{-8}$ $m^2V^{-1}s^{-1}$, Table 1) to calibrate for electrophoretic mobilities of the analytes ranging from $2.0*10^{-8}$ $m^2V^{-1}s^{-1}$ to $5.5*10^{-8}$ $m^2V^{-1}s^{-1}$ (Table 2).

In addition, in applying the marker techniques it has proven to be important not to use marker compounds that have electrophoretic mobilities very close to each other in any particular system.

The analytes studied in this work were very different from the marker compounds of relatively small molecular size. The analytes, in turn, were large compounds (MW 500-43880 g/mol), of less rigid structure, and their pKa-values were far closer to the pH of the electrolyte solution than those of the marker compounds. Figure 2 shows that the nucleic acid, adenoside triphosphate and the oligonucleotides migrated in the order of increasing molecular mass in CZE technique.

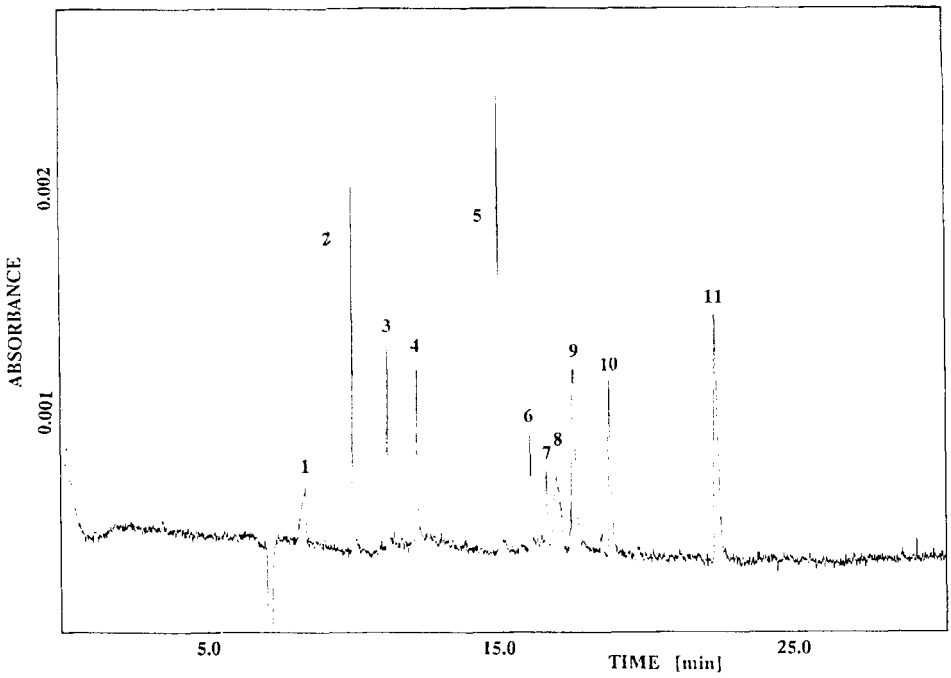


Figure 2. Electropherogram of 35 μg/ml of 1= system peak, 2=135-oligomer, 3= NADPH, 4=(-)mandelic acid, 5=*meso*-2,3-diphenylsuccinic acid, 6=NADH, 7=84-primer, 8=ATP, 9= dG5'BaDE, 10=1,2-phenylenediacetic acid, 11=*o*-phthalic acid. Migration conditions as described in the Experimental section.

TABLE 1.
Marker compounds and their mobility values in 50 mM CAPS (pH 11.2).

Marker compound	Mobilities μ_{ep}, 10^{-8} $m^2V^{-1}s^{-1}$
(-)mandelic acid	-3.1249
meso-2,3-diphenylsuccinic acid	-3.9799
1,2-phenylenediacetic acid	-4.7544
o-phthalic acid	-5.1964

TABLE 2.
Average values and standard deviations for absolute migration times and for electrophoretic mobilities determined by four marker technique.
E_{eff} = 2.3 x 10^4 Vm^{-1}

Analytes	Migration time [min] $t_{a,ave}$	SD	RSD%	Mobilities [10^{-8} $m^2V^{-1}s^{-1}$] μ_{ep}(ave)	SD	RSD%
135-oligomer	10.12	0.19	1.9	-2.2111	0.0037	0.166
NADPH	11.35	0.24	2.1	-2.7401	0.0018	0.067
NADH	16.47	0.53	3.2	-4.2397	0.0010	0.024
84-primer	17.07	0.59	3.5	-4.4236	0.0125	0.284
ATP	17.38	0.56	3.2	-4.5206	0.0020	0.044
dG5'BaDE	17.95	0.55	3.1	-4.6966	0.0248	0.527

To the macromolecules in this study, the four marker technique enhanced the reliability of the analysis dramatically. The average absolute migration times and electrophoretic mobilities and their relative standard deviations for all the analytes are presented in Table 2. It can be seen, that the repeatabilities of the mobilities of the analytes were very reliable and at promille level. However, the relative standard deviations of the oligonucleotides are higher than those of nucleic acids and ATP, most presumably because their pK_a-values are too close to the pH of the electrolyte solution. It has been shown elsewhere, [20] that the repeatability of analysis can be increased by a factor up to 350 for aromatic carboxylic acids at pH 10.6. The same research revealed that such excellent repeatabilities are more difficult to achieve for compounds that undergo changes when small changes are induced in the carrier electrolyte. For such compounds there are some practical limits on the use of marker compounds.

TABLE 3.
Values of coefficients for identification of the macromolecules.

Pair of analytes	Coefficient of identification Q_{id}	
	Migration times	Mobilities
135-oligomer - NADPH	2.87	96.1
NADPH - NADH	6.69	525
NADH - 84-primer	0.533	21.8
84-primer - ATP	0.271	3.21
ATP - dG5'BaDE	0.511	7.34

Even though, in this study it can clearly be seen, that the electrophoretic mobilities determined by the four marker technique are clearly more reliable than the absolute migration times themselves, the reliability is more accurately studied by using the coefficient for identification Q_{id} (Eq. 5). The Q_{id} value can be determined from the analyte absolute migration times compared with the Q_{id} value from the electrophoretic mobilities, for each pair of successively eluting analytes. The results are presented in Table 3. For each analyte pair Q_{id} value is much greater when calculated from the electrophoretic mobilities of the analytes by 4m than when calculated from the absolute migration times. All the values obtained on the basis of the mobilities of the marker compounds show that the analyses are very reliable. However, when the values are calculated from the absolute migration times, only few of the results are above 2.

As a conclusion, our results demonstrate the effectiveness of the 4m technique to improve the reliability of CZE analysis, even for analytes of large molecular weights lacking rigid structures. Good repeatabilities (0.04-0.52%) were obtained for the macromolecules studied. The coefficient for identification of the analyte pairs, Q_{id}, was clearly better, when identification was based on electrophoretic mobilities rather than absolute migration times.

REFERENCES

1. Z. Deyl, R. Struzinsky, J. Chromatogr., 569: 63-122 (1991)
2. W. Kuhr, Anal. Chem., 62: 403R-414R (1990)
3. V. Dolnik, J.-P. Liu, J.F. Banks, M.V. Novotny, P. Bocek, J. Chromatogr., 480: 321-330 (1989)
4. H. Yamamoto, T. Manabe, T. Okuyama, J. Chromatogr., 480: 277-283 (1989)
5. D.N. Heiger, A.S. Cohen, B.L. Karger, J. Chromatogr., 516: 33-48 (1990)
6. A.M. Chin, J.C. Colburn, The 2nd Int. Symp. on HPCE, 1990, San Fransisco, CA, Abstract, TL 106.
7. M. Senda, T. Sasaki, T. Hiyama in Proceedings of the 14th Symposium on Capillary Chromatography, Eds. P. Sandra, M.L. Lee, F. David and G. Devos, Baltimore, Maryland, USA, pp. 545-551.
8. S. Fujiwara, S. Honda, Anal. Chem., 58: 1811-1814 (1986)
9. S. Honda, S. Iwase, S. Fujiwara, J. Chromatogr., 404: 313-320 (1987)
10. X. Huang, J.A. Luckey, M.J. Gordon, R.N. Zare, Anal. Chem., 61: 766-(1989)
11. E.V. Dose, G.A. Guiochon, Anal. Chem., 63: 1154-1158 (1991)
12. T. Lee, E.S. Yeung, Anal. Chem., 63: 2842-2848 (1991)
13. J.L. Beckers, F.M. Everaerts, M.J. Ackermans, J. Chromatogr., 537: 407-428 (1991)
14. R. Vespalec, P. Gebauer, P. Bocék, Electrophoresis 13: 677-682 (1992)
15. S. Fujiwara, S. Honda, Anal. Chem. 59: 2773-2776 (1987)
16. K. Otsuka, S. Terabe, T. Ando, J. Chromatogr., 396: 350-354 (1987)
17. P.G.H.M. Muijselaar, H.A. Claessens, C.A. Cramers, Anal. Chem., 66: 635-644 (1994)
18. M.T. Ackermans, F.M. Everaerts, J.L. Beckers, J. Chromatogr. 585: 123-131 (1991)
19. T.T. Lee, R. Dadoo, R.N. Zare, Anal. Chem., 66: 2694-2700 (1994)
20. J.H. Jumppanen, M.-L. Riekkola, Anal. Chem., 67: 1060-1066 (1995)
21. J.H. Jumppanen, H. Sirén, M.-L. Riekkola, O. Söderman, J. Microcol. Sep., 5: 451- 457 (1993)
22. J.H. Jumppanen, M.-L. Riekkola, Electrophoresis (1995), in press.
23. H. Sirén, J.H. Jumppanen, K. Manninen, M.-L. Riekkola, Electrophoresis, 15: 779-784 (1994)
24. J.W. Jorgenson, K.D. Lukacs, Science, 222: 266-273 (1983)
25. R.S. Rush, A.S. Cohen, B.L. Karger, Anal. Chem., 63: 1346-1350 (1991)
26. M.V. Reddy, K. Randerath, Carsinogenesis, 7: 1543-1551 (1986)
27. K.A. Canella, K. Peltonen, A. Dipple, Carsinogenesis, 12: 1109-1114 (1991)

Received: July 10, 1995
Accepted: August 6, 1995