Validation of enantiomeric separations by micellar electrokinetic capillary chromatography using synthetic chiral surfactants

Michael E. Swartz[a,*], Jeffrey R. Mazzeo[a], Edward R. Grover[a], Phyllis R. Brown[b]

[a]Waters Corporation, Department TG, 34 Maple Street, Milford, MA 01757, USA
[b]Department of Chemistry, University of Rhode Island, Kingston, RI 02881, USA

Abstract

The first report on the feasibility of method validation for the separation of enantiomeric mixtures by chiral micelle electrokinetic capillary chromatography is described. Method validation data elements were investigated according to U.S. Pharmacopeia protocol and are reported for the separation of ephedrine enantiomers using the synthetic chiral surfactants (S)- and (R)-N-dodecoxycarbonylvaline as buffer additives. Excellent linearity from 1–150% of the target concentration was obtained. A 1.0% limit of quantitation for an enantiomeric impurity with acceptable precision is shown. Method robustness and ruggedness is also presented. Migration order reversal was used for the determination of a trace enantiomeric impurity, and to identify the enantiomeric compound in a multiple active ingredient formulation. The capability to reverse migration order is shown to be a valuable tool to satisfy the U.S. Pharmacopeia specificity requirement as well as improving detection and quantitation limits.

Keywords: Micellar electrokinetic chromatography; Enantiomer separation; Buffer composition; Ephedrine; Surfactants; Pseudoephedrine

1. Introduction

Validated methods are required by the U.S. Food And Drug Administration (FDA) as part of any new drug application (NDA). In its 1987 guideline on submitting analytical data for methods validation, the FDA has specified the U.S. Pharmacopeia (USP) Validation of Compendial Methods as the legally recognized specifications to be used to determine compliance [1]. The USP defines the validation of an analytical method as the process of establishing performance characteristics and their corresponding suitability for an analytical application [2]. These performance characteristics, or data elements, expressed in terms of analytical parameters, are precision, accuracy, selectivity (specificity), linearity and range, limit of detection (LOD), limit of quantitation (LOQ), ruggedness and robustness. Method validation provides an assurance of reliability and is completed to insure that an analytical methodology is accurate, reproducible, and rugged over the specific range that an analyte will be

* Corresponding author.

analyzed. In a practical sense, method validation is accomplished by measuring and documenting the data elements once a method is developed and optimized. The data elements required for assay validation are further divided into three categories according to the requirements of individual test methods. Category One is for the analysis of major components, and Category Three is for the determination of performance characteristics such as dissolution. Category Two is a quantitative assay for the determination of the purity of bulk drug substances or degradation compounds in finished pharmaceutical products. The analysis of trace level enantiomeric impurities falls into this category.

Capillary electrophoresis (CE) is a technique that has been shown to be ideally suited for the separation of a wide variety of neutral and charged drug molecules [3–5]. When compared to other techniques such as liquid or gas chromatography, CE has the advantages of efficiency, selectivity, ease of method development, speed, and overall applicability and flexibility. One of the niches where capillary electrophoresis has become routinely investigated in the separation of enantiomeric mixtures [6,7]. High efficiencies in CE lead to a much broader applicability in comparison to other analytical techniques, encouraging its widespread use. However, while the application of CE has grown significantly over recent years there is a general lack of acceptance of CE as a routine analytical tool, particularly in the regulatory environment.

To gain broader acceptance of CE as a routine analytical tool, especially in a regulated environment, methods in support of NDAs and method transfer must be validated. Perceived limitations in sensitivity and reproducibility have limited efforts in the area of validation. However, as CE technology matures and becomes better understood, these perceptions have lead to increased CE method validation endeavors with applications in both achiral [8–13] and chiral [10,14–17] compound analyses. Results comparable to [11,13] or better than [10] those obtained by high-performance liquid chromatography have been reported.

All of the validation efforts for the determination of enantiomeric purity to date, however, have utilized natural products such as cyclodextrins at low pH [low electroosmotic flow (EOF)] conditions. It has recently been shown that the use of synthetic chiral surfactants as buffer additives affords significant advantages when utilized in the micellar electrokinetic chromatographic (MEKC) mode of CE [18–21]. These advantages include the analysis of achiral and chiral compounds in a single run, and the ability to reverse enantiomer migration order for improved quantitation and identification of enantiomers in a complex sample. In validation, migration order reversal can improve specificity, detection, and quantitation limits.

The objective of this study was to investigate the feasibility of validating an MEKC method using synthetic chiral surfactants for the determination of trace level enantiomeric impurities. For this purpose the chiral compound ephedrine and its diastereomer pseudoephedrine were chosen as potential worst-case scenarios because of their limited absorbance in the ultraviolet (UV) range used for detection. The separation of the enantiomers of ephedrine and/or the enantiomers of pseudoephedrine have been previously reported using both cyclodextrins [22–24] and synthetic chiral surfactants [18]. In most cases, however, the samples were racemic mixtures. In pharmaceutical formulations, the (−)-enantiomer of ephedrine and the (+)-enantiomer of pseudoephedrine (the pharmacological active ingredients) are present in high enantiopurity. It is therefore necessary to be able to measure low levels of the enantiomeric impurity in the presence of high levels of enantiomeric excess. Once the method is developed and optimized for resolution, the data elements necessary for USP method validation can be evaluated. In this paper, we describe the first report of a validated CE method using synthetic chiral surfactants with ephedrine and pseudoephedrine as test compounds. It is also shown that enantiomer migration order reversal can be used to significantly improve specificity, and limits of quantitation and detection.

2. Experimental

2.1. Apparatus

All separations were performed on a Waters Quanta™ 4000E capillary electrophoresis system (Waters Corp., Milford, MA, USA). The electrophoretic system was controlled by the Millennium Chromatography Manager (Waters) which was also used for data collection (five points per second) and processing. Standard untreated AccuSep™ capillaries, 60 cm (52.5 cm effective length to detection) × 50 μm I.D. were used throughout. The capillaries were prepared prior to use by rinsing (purging by vacuum) with 0.5 M sodium hydroxide (10 min), followed by water (10 min), and run buffer (10 min). Hydrostatic (10 cm height) injections from five to 15 s in duration with an applied voltage of +15000 V were employed. A 3-min purge of fresh buffer was routinely employed between individual runs. All analyses were performed at 30°C with UV detection at 214 nm.

2.2. Chemicals and supplies

Electrophoresis buffers and all standards used were purchased commercially from either Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) in the highest purity available and were used without further purification. The ephedrine sulfate injectable solution and cough cold preparation samples were obtained locally. Sample preparation consisted only of dilution.

2.3. Buffer solutions

(S)- and (R)-N-dodecoxycarbonylvaline (DDCV) were synthesized in our laboratories as described previously [18]. Buffers were prepared in Milli-Q water (Millipore, Bedford, MA, USA) with surfactant and disodium phosphate and/or disodium tetraborate. The pH was adjusted with either 10% sodium hydroxide or 0.47 M phosphoric acid. Buffers were filtered (0.45 μm Millex™ filters, Millipore) and degassed (with vacuum and sonication) on a daily basis.

3. Results and discussion

3.1. Method development, optimization, robustness, and specificity

It has been shown previously that pH and surfactant concentration play a significant role in the separation of enantiomers by chiral MEKC [18–21]. Therefore, to develop and optimize the separation for the trace level analysis of ephedrine and pseudoephedrine enantiomers the influence of both surfactant concentration and pH on resolution was evaluated. Since the robustness of a method is the measure of its capacity to remain uneffected by variations in method (buffer) parameters, the evaluation of pH and surfactant concentration also serves the purpose of measuring robustness with respect to these two variables. The variations of resolution as surfactant concentration is increased is shown in Table 1. As surfactant concentration increases (10–75 mM), k' (partitioning into the surfactant) increases, resulting in higher resolution. Although resolution occurs at surfactant concentrations as low as 10 mM it is not optimized, and it was necessary to maximize resolution for trace level enantiopurity determinations. Therefore, subsequent work used a surfactant concentration of 50 mM to maximize resolution. Surfactant concentrations higher than 50 mM results in higher resolution, but at the expense of significantly longer run times.

The effect of pH on resolution was also studied and the data is presented in Table 2. In each instance, k', α (enantioselectivity), and resolution decreases with increasing pH. Since both ephedrine and pseudoephedrine become less positively charged as the pH increases, charge attraction to the negatively charged micelles decreases, thereby lowering k' (partitioning) and resolution. For all subsequent work, a pH of 8.0 was chosen to maximize resolution. While pH values lower than 8.0 result in additional resolution, the run times are longer. Resolution (specificity) using optimized conditions for separation of a racemic mixture of ephedrine enantiomers is illustrated in Fig. 1.

Table 1
Effect of surfactant concentration on resolution, k', and α

Compound	Surfactant concentration (mM)	R_s	k'_1	k'_2	α
Ephedrine	10	2.30	1.63	1.81	1.11
Ephedrine	25	3.13	3.20	3.54	1.11
Ephedrine	50	3.63	4.03	4.44	1.10
Ephedrine	75	5.36	5.05	5.45	1.08
Pseudoephedrine	10	3.10	1.25	1.55	1.24
Pseudoephedrine	25	3.10	2.53	3.11	1.23
Pseudoephedrine	50	4.96	3.32	4.04	1.22
Pseudoephedrine	75	6.67	4.29	5.10	1.19

Conditions: Free zone conditions: 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$, pH 9.2. Chiral MECC conditions: 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$, pH 9.2, varying concentrations of (R)-DDCV. Sample: 100 μg/ml of each compound as a racemic mixture in water, 15 s hydrostatic injection. Values are the result of duplicate measurements. All calculations performed according to Ref. [18]. Abbreviations are: R_s = resolution; k' = partitioning, and α = alpha (selectivity). All other conditions as described in the Experimental section.

3.2. Method validation

3.2.1. Linearity

The USP defines linearity as the elicitation of test results that are directly proportional to the concentration of analyte within a given range (USP). Linearity was determined for (−)-ephedrine over the range 1–150% of the nominal target concentration of 1.0 mg/ml. Duplicate injections at nine intervals were made. Acceptable linearity with a correlation coefficient of 0.997 was obtained.

3.2.2. Limit of quantitation and detection (LOQ, LOD)

The LOQ is defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy (USP), while LOD is the lowest concentration of sample that can be clearly detected above the baseline noise. An R.S.D. of 6.1% (area) was obtained for six replicate injections of a solution of (+)-ephedrine, equivalent to 1.0% (10 μg/ml, signal-to-noise ratio of 4.1) of the target concentration of (−)-ephedrine, and was defined as

Table 2
Effect of pH on resolution, k', and α

Compound	Buffer pH	R_s	k'_1	k'_2	α
Ephedrine	7.5	2.40	14.43	16.24	1.13
Ephedrine	8.0	2.26	15.03	16.80	1.12
Ephedrine	8.5	2.39	12.94	14.37	1.11
Ephedrine	9.0	2.32	7.55	8.27	1.10
Pseudoephedrine	7.5	5.32	13.47	16.69	1.24
Pseudoephedrine	8.0	4.44	9.41	11.64	1.24
Pseudoephedrine	8.5	3.75	9.23	11.34	1.23
Pseudoephedrine	9.0	3.73	6.39	7.52	1.18

Conditions: Free zone conditions: 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$, at various pH values. Chiral MECC conditions: 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$ (various pH values), 50 mM (R)-DDCV. Sample: 100 μg/ml of each compound as a racemic mixture in water, 15 s hydrostatic injection. All calculations performed according to Ref. [18]. Values are the result of duplicate measurements. All other conditions as described in the Experimental section.
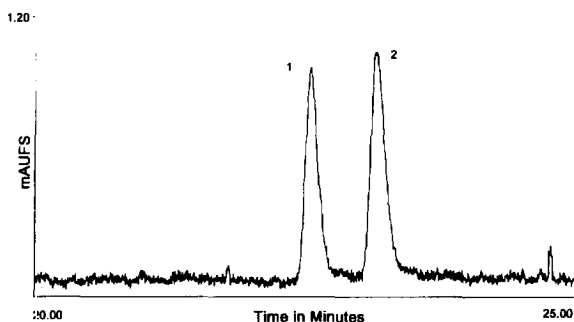
Fig 1. Chiral MEKC separation of ephedrine enantiomers. A buffer of 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$ pH 8.0, 50 mM (R)-DDCV and a 15-s hydrostatic injection were used. The sample is 100 μg/ml racemic ephedrine in water. Peak identification: 1 = (+)-ephedrine, 2 = (−)-ephedrine. All other conditions as described in the Experimental section.

the LOQ. Reproducibility of less than 10% at the LOQ has been shown to be acceptable [5,8]. The LOD can then be calculated to be 0.5% at a signal-to-noise ratio of two. An example electropherogram of a sample of (−)-ephedrine spiked with 1% (+)-ephedrine that was used to generate this data is presented in Fig. 2.

3.2.3. Precision of peak area and migration time

Precision of peak area and migration time for (−)-ephedrine at the target concentration, and for (+)-ephedrine at the LOQ (1.0% or 10 μg/ml) were determined for six replicate injections. An R.S.D. of 0.63% for peak migration time and
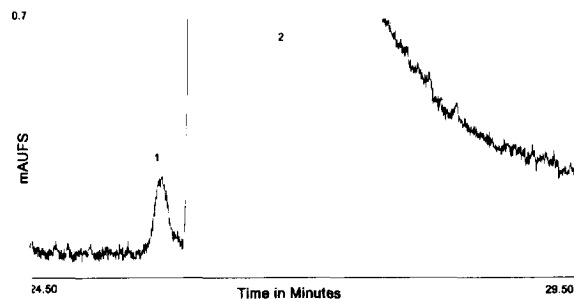

Fig. 2. Determination of LOQ for ephedrine enantiomers. A buffer of 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$ pH 8.0, 50 mM (R)-DDCV and a 15-s hydrostatic injection were used. The sample is 1.00 μg/ml (−)-ephedrine (peak 2) spiked with 1% (10 μg/ml) (+)-ephedrine (peak 1) in water. All other conditions as described in the Experimental section.

1.10 for peak area were obtained at the target concentration respectively. For the LOQ concentration, an R.S.D. of 0.40% for peak migration time and 6.09% for peak area was realized. Acceptable results were obtained without the use of time corrected area counts, relative migration time calculations, or internal standards.

3.2.4. Specificity and accuracy

A sample of (−)-ephedrine was spiked with (+)-ephedrine to produce a 60:40 ratio of the two enantiomers (−): (+). Six replicate injections were made using the conditions in Fig. 1. Time corrected peak area measurements confirmed the spiking levels; 59.8% (−) and 40.2% (+) with 1.2 and 0.8% R.S.D. respectively.

3.2.5. Ruggedness

Ruggedness is a measure of the reproducibility of results obtained by analysis of the same samples under a variety of normal test conditions (USP). For this study, buffer preparation to buffer preparation, capillary to capillary and instrument to instrument migration time reproducibility was measured. Buffer to buffer ruggedness plays an important role in migration order reversal as discussed later. Capillary to capillary and instrument to instrument ruggedness are important from the standpoint of the method transfer. The ruggedness results are reported in Table 3. These data were obtained without the aid of ratios or other corrections. As seen in Table 3, buffer to buffer and instrument to instrument migration time reproducibility falls

Table 3
Reproducibility of method ruggedness

Experiment	%R.S.D. t_{M1}	%R.S.D. t_{M2}
Buffer to Buffer	0.31	0.36
Instrument to Instrument	0.66	0.61
Capillary to Capillary	1.81	3.95

Conditions: 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$ pH 8.0, 50 mM (R)-DDCV. Sample: 100 μg/ml racemic pseudoephedrine in water, 15 s hydrostatic injection. Values are the result of five replicate measurements. Abbreviations are: R.S.D. = relative standard deviation; t_M = migration time. All other conditions as described in the Experimental section.

within the range of normal precision as presented earlier. Capillary to capillary reproducibility is somewhat higher, however, and may be a result of small lot to lot variations in internal diameter or silica surface.

3.3. Enantiomer migration order reversal: improved quantitation of enantiopurity

It has been shown that by using the two enantiomers of DDCV individually, enantiomer migration time reversal can be achieved [18,19]. For quantitation of enantiopurity, it is desirable that the trace enantiomeric impurity be eluted before a tailed parent enantiomer peak [25]. Conversely, in the case of fronted peaks, it would be desirable that the trace enantiomer be eluted last. A comparison electropherogram of a (−)-ephedrine solution spiked at the 1% level with (+)-ephedrine run with both (R)- and (S)-DDCV (under otherwise identical conditions) is shown in Fig. 3A and B. For the determination of enantiopurity of ephedrine formulations, (R)-DDCV is the desired buffer additive (Fig. 3A), since the migration order of the ephedrine enantiomers is (+) followed by (−). Using (S)-DDCV, migration order of the enantiomers is reversed, limiting selectivity, LOQ and LOD. The determination of enantiopurity of an ephedrine sulfate injectable using both (R)- and (S)-
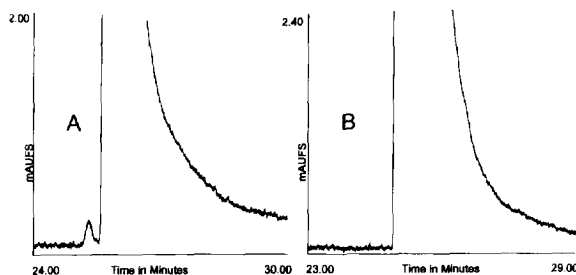
DDCV is shown in Fig. 4. No (+)-ephedrine (0.5% or 5 μg/ml LOD) could be detected in the formulation.

3.4. Enantiomer migration order reversal: identification of enantiomeric compounds in a complex mixture

Another way to use migration order reversal is in the identification of enantiomeric compounds in a complex mixture. In a sample with multiple peaks, enantiomeric compounds can be identified by migration time shifts when individual runs using (R)- and then (S)-DDCV are compared. Migration order reversal was used in this way to identify the pharmacologically active pure (+)-pseudoephedrine peak in an over-the-counter (OTC) generic "night-time" cough/cold preparation. The OTC formulation consisted of four active ingredients, and multiple inactive ingredients and excipients. Following a fifty fold dilution, the sample was run first in (R)-DDCV, and then in (S)-DDCV under conditions optimized for the separation of ephedrine and pseudoephedrine enantiomers. The overlay electropherogram of the runs in each surfactant is shown in Fig. 5. Of the five peaks obtained, only one shifts migration time between the two surfactants; this peak therefore corresponds to the (+)-pseudoephedrine active ingredient. The
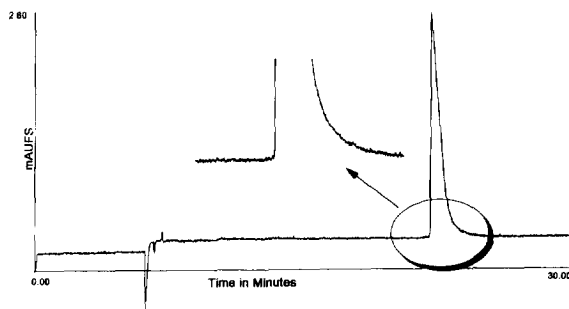

Fig. 3. Effect of enantiomeric migration order on LOQ. A buffer of 25 mM Na₂HPO₄–NaB₄O₇ pH 8.0, 50 mM (R)-DDCV (A) or (S)-DDCV (B) with a 15-s hydrostatic injection were used. The sample is 1.00 μg/ml (−)-ephedrine spiked with 1% (10 μg/ml) (+)-ephedrine in water. Peak assignments in (A) are identical to those in Fig. 2. In (B), only the (−) enantiomer can be distinguished. All other conditions as described in the Experimental section.

Fig. 4. Enantiopurity of ephedrine sulfate injectable solution. A buffer of 25 mM Na₂HPO₄–NaB₄O₇ pH 8.0, 50 mM (R)-DDCV and a 15-s hydrostatic injection were used. The sample is a 1/50 dilution of a 50 mg/ml ephedrine sulfate injectable solution [(−)-ephedrine]. All other conditions as described above in the Experimental section.
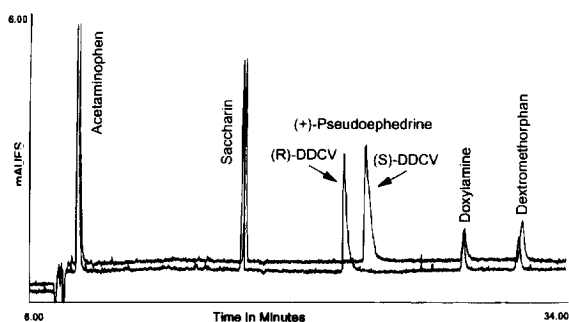
Fig. 5. Enantiomeric compound identification by migration order reversal. A buffer of 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$ pH 8.0, 50 mM (R)-DDCV or (S)-DDCV with a 15-s hydrostatic injection were used. The sample is a 1/50 dilution of a OTC cough/cold formulation. Approximate final concentrations in mg/ml are: acetaminophen, 20; saccharine (unknown-inactive ingredient); pseudoephedrine, 1.2; doxylamine, 0.25; and dextromethorphan, 0.6.

identity was also confirmed by the injection of an external standard. The identity of each of the three remaining active ingredients (acetaminophen, doxylamine and dextromethorphan) as well as that of the fifth ingredient, saccharin, were also identified by separate external standard injections. To quantify the migration time difference used for enantiomeric peak identification, the sample was injected five times in each surfactant and the results in Table 4 were obtained. The shift in migration time for pseudoephedrine relative to the other components is evident in the increased R.S.D. obtained. The higher R.S.D. would not be obtained for

Table 4
Peak identification by enantiomeric migration order reversal

Compound	Mean t_M	RSD (%)
Acetaminophen	7.460	0.167
Saccharin	16.722	0.306
Pseudoephedrine	22.811	2.180
Doxylamine	28.837	0.875
Dextromethorpham	31.966	0.781

Conditions: 25 mM Na$_2$HPO$_4$–NaB$_4$O$_7$ pH 8.0, 50 mM (R)- or (S)-DDCV. Sample: 1/50 dilution of generic cough-cold formulation in water, 15 s hydrostatic injection. Values are the result of five replicate measurements for each surfactant. All other conditions as described in the Experimental section.

pesudoephedrine if migration order reversal were not obtained.

4. Conclusion

The feasibility of validating a chiral MEKC method for the determination of the enantiomers of ephedrine and pseudoephedrine is shown. Acceptable levels of performance in terms of precision, accuracy, linearity, specificity and LOQ and LOD were obtained. The role of migration order reversal in method validation for the determination of trace enantiomeric impurities and to identify enantiomeric compounds in a multiple active ingredient formulation was also established.

References

[1] Food and Drug Administration, Guidelines For Submitting Samples and Analytical Data for Methods Validation, February 1987, United States Government Printing Office: 1990-281-794:20818.
[2] The United States Pharmacopeia, Vol. 23, 1995, pp. 1982–1984.
[3] P. Camilleri (Editor), Capillarity Electrophoresis Theory and Practice, CRC Press, Bosca Raton, FL, 1993.
[4] J.P. Landers (Editor), Handbook of Capillary Electrophoresis, CRC Press, Boca Raton, FL, 1993.
[5] M.E. Swartz, J. Liq. Chromatogr., 14 (1991) 923.
[6] T.J. Ward, Anal. Chem., 66 (1994) 633A.
[7] M.V. Novotny, Anal. Chem., 66 (1994) 646A.
[8] K.D. Altria and Y.L. Chanter, J. Chromatogr. A, 652 (1993) 459.
[9] B.R. Thomas and S. Ghodbane, J. Liq. Chromatogr., 16 (1993) 1983.
[10] C.L. Ng, C.P. Ong, H.K. Lee and S.F.Y. Li, J. Chromatogr. A, 680 (1994) 579.
[11] K.D. Altria, N.G. Clayton, M. Hart, R.C. Harden, J. Hevizi, J.V. Makwana and M.J. Portsmouth, Chromatographia, 39 (1994) 180.
[12] R. Nellore and H.R. Bhagat, J. Pharm. Biomed. Anal., 12 (1994) 1363.
[13] B.R. Thomas, X.G. Fang, X. Chen, R.J. Tyrrell and S. Ghodbane, J. Chromatogr. B, 657 (1994) 383.
[14] K.D. Altria, A.R. Walsh and N.W. Smith, J. Chromatogr., 645 (1993) 193.
[15] K.D. Altria, M. Hart, R.C. Harden, J. Hevizi, P.A. Hailey, J.V. Makwana and M.J. Portsmouth, J. Chromatogr., 641 (1993) 147.

[16] K.D. Altria, D.M. Goodall and M.M. Rogan, Electrophoresis, 15 (1994) 824.

[17] E.C. Rickard and R.J. Bopp, J. Chromatogr. A, 680 (1994) 609.

[18] J.R. Mazzeo, E.R. Grover, M.E. Swartz and J.S. Petersen, J. Chromatogr. A, 680 (1994) 125.

[19] M.E. Swartz, J.R. Mazzeo, E.R. Grover and P.R. Brown, Anal. Biochem., (1995) submitted for publication.

[20] M.E. Swartz, J.R. Mazzeo, E.R. Grover and P.R. Brown, J. Chromatogr. A, (1995) submitted for publication.

[21] J.R. Mazzeo, M.E. Swartz and R.R. Grover, Anal. Chem., (1995) submitted for publication.

[22] S. Fanali, J. Chromatogr., 474 (1989) 441.

[23] C. Dette, E. Siegfried and S. Terabe, Electrophoresis, 15 (1994) 799.

[24] C.L. Flurer, L.A. Lin, R.D. Satzger and K.A. Wolnik, J. Chromatogr. B 669 (1995) 133.

[25] J.A. Perryt, J.D. Rateike and T.J. Szczerba, J. Chromatogr., 389 (1987) 57.