Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1047 (2004) 299–303
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

# Electrochromatographic studies of etched capillaries modified with a cyano pentoxy biphenyl liquid crystal

G. Brent Dawson[a], Maria T. Matyska[b], Joseph J. Pesek[b,*], Richard R. Seipert[b]

[a] *Department of Chemistry, University of North Carolina, Greensboro, Greensboro, NC 27403, USA*
[b] *Department of Chemistry, San Jose State University, One Washington Square, San Jose, CA 95192, USA*

Received 10 March 2004; received in revised form 15 June 2004; accepted 16 June 2004

**Abstract**

A liquid crystal stationary phase for open tubular capillary electrochromatograpy (OTCEC) is fabricated by etching a fused silica tube and then bonding 4,4′-cyanopentoxy biphenyl by a silanization/hydrosilation process. The versatility of this electrophoretic capillary is demonstrated by separations of proteins, peptides, basic pharmaceuticals and the metabolites of tryptophan. Chromatographic interactions are verified by resolution of two neutral peptides. Variable temperature studies are used to understand the liquid crystal properties of the bonded moiety. EOF measurements as a function of pH and temperature further characterize this unique separation media.


*Keywords:* Open tubular capillary electrochromatograpy; Electrochromatograpy; Liquid crystals; Stationary phases, electrochromatograpy; Capillary columns; Proteins; Peptides; Tryptophan

## 1. Introduction

Over the past 15 years researchers have shown that capillary electrochromatography (CEC) can be utilized for separations of many classes of molecules [1,2]. This technique, which combines the use of an electrically-driven flow of a mobile phase through a stationary phase, gives high efficiency and resolution of analytes similar to capillary electrophoresis (CE) while also providing selectivity that is often comparable to high performance liquid chromatography (HPLC). The small amounts (picomoles) of sample needed for the analysis make the technique well-suited for use in biological arenas where sample volumes are limited. Furthermore strategies employing fluorescent [3], mass spectrometric [4], and electrochemical [5] detection schemes have reduced concentration limits of detection as well.

Several methods of incorporating stationary phases into CEC are available, each with their advantages and disadvantages. Early strategies focused on the packing of HPLC-type stationary phases into fused silica capillaries containing frits [6]. While high selectivity was recorded with these techniques, many problems with bubble formation and frit stability have limited the use of such techniques. Recent developments have concentrated on the formation of monolithic columns of sol–gels [7] or rigid polymers [8] inside the fused silica capillary. With these strategies, ligands, which will act as the stationary phase, can be incorporated into the separation media to give the desired selectivity.

A final strategy that has been used attaches the stationary phase to the wall of the capillary [9,10]. This procedure, termed open tubular capillary electrochromatography (OTCEC), produces a monolayer thick coating into which analytes can partition as they migrate through the capillary. Many classes of compounds including pharmaceuticals, peptides and proteins can be resolved, and the nature of the coating process produces a surface that resists adsorption onto the capillary wall [11–17]. A convenient way of attaching the stationary phase to the capillary wall is via silanization/hydrosilation [18]. Silanization covalently attaches a silicon hydride layer to the reactive silanol groups of the capillary. This hydride layer can then be reacted with a variety

* Corresponding author. Tel.: +1 4089244939; fax: +1 4089244945.
*E-mail address:* pesek@sjsu.edu (J.J. Pesek).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.06.091

of reagents to form the stationary phase. Typically, catalysts such as free radical initiators or Speier's catalysts are used to increase the rate of the reaction. To increase the surface area of the capillary, it is first reacted with ammonium bifluoride at high temperatures, a process that can etch and redeposit silica to form protrusions from the capillary wall [10].

Capillaries prepared in this way have many benefits. First they resist the adsorption of basic molecules and give highly symmetric peaks. Second, they are stable and give reproducible retention times after several injections. Third, they give selectivity and resolution that can be tuned with mobile phase additives and changes in the pH of the background electrolyte. This tunable resolution is due to changes in both the electrophoretic mobilities of the analytes as well as changes in the amount of partitioning into the coating of the capillary.

One class of molecules that has received little attention as possible stationary phases in CEC is thermotropic liquid crystals [19]. Early studies with gas chromatography demonstrated the utility of liquid crystals as stationary phases [20,21]. Recent NMR studies in our group of the $T_2$ relaxation times of C18 stationary phases versus liquid-crystal-based stationary phase have shown that liquid crystals such as cholesteryl moieties show a non-linear decrease in molecular motion with temperature [22]. Additionally, non-linear $\log k$ versus $1/T$ (van't Hoff) plots were recorded for polar analytes in chromatographic studies [23]. While these results do not represent true phase changes due to the tethered nature of the stationary phase ligand, they indicate that the degree of association of cholesteryl and biphenyl-type phases changes with temperature and that these changes can greatly affect chromatographic behavior.

The work described in this paper extends the examination of the 4,4′-cyanopentoxy biphenyl phases used in open tubular capillary electrochromatography. New applications of the phase to challenging analyte mixtures as well as temperature dependent electroosmotic flow measurements are reported.

## 2. Experimental

### 2.1. Reagents

Tris base, bovine and equine cytochrome *c*, angiotensin I, II, and III, bradykinin, trypsinogen (bovine pancreas), α- and β-chymotrypsins (bovine pancreas), chymotrypsinogen A Type II, trypsin (porcine pancreas) and the tricyclic antidepressants were all purchased from Sigma (St. Louis, MO, USA). Stock solutions of analytes were prepared in water at a concentration of 100–1000 ppm. All samples were filtered with 0.45 μm membrane syringe filters prior to use.

### 2.2. Instrumentation

Separations were performed on either an HP 3D CE system (Agilent Technologies, Waldbronn, Germany) or an ABI 270 A Capillary Electrophoresis system (Applied Biosystems, Foster City, CA, USA). Data from both systems were acquired with Agilent Chemstation software with the ABI system using an HP 35900 Interface. Absorbance at 210 nm (unless noted otherwise) was used for detection to give the best sensitivity. Modified capillaries were typically rinsed with run buffer for 8 min prior to the electrophoretic separation. Bare fused silica capillaries were preconditioned with 0.1 M sodium hydroxide, Millipore water, and run buffer prior to each electrophoretic separation. Temperature dependent resolution studies were performed by heating or cooling the capillary by a forced-air Peltier apparatus.

### 2.3. Buffers

Buffers were prepared from empirically determined recipes that gave the appropriate conductivity and absorbance values [24]. Running buffers were diluted from concentrated stock solutions and were filtered through a 0.22 μm nylon membrane and degassed for 20 min using a helium sparge.

### 2.4. Capillary modification

Biphenyl capillaries were prepared as previously reported [25]. Briefly the fused silica surface was etched with a 5% ammonium bifluoride/methanol solution for a total of 4 h at elevated temperature in a gas chromatographic oven. A silica hydride layer was then covalently attached by reacting the etched surface with ∼18% (v/v) triethoxysilane solution in dioxane with a hydrochloric acid catalyst (115 μmol). The 4,4′-cyanopentoxy biphenyl layer was attached to the hydride through a free radical initiated process. The biphenyl (1.15 g) was added to 2.0 mL of toluene and 70 μL of di-*t*-butyl peroxide and heated to 70 °C for 1 h. The solution was then passed through the capillaries, and the capillaries were heated at 100 °C for 24 h. Solution was passed through the capillaries each day for 5 days with the capillaries being stored in the GC oven at 100 °C. At the end of the process the capillaries were rinsed with toluene and methanol.

### 2.5. Electroosmotic flow studies

The electroosmotic flow in the biphenyl capillary was determined as previously described [26]. Briefly a sample of dimethyl sulfoxide was injected for 5 s at 50 mbar and voltage was applied for either 10 or 15 min. Then another sample of DMSO was injected for 5 s at 50 mbar and then 50 mbar of pressure was applied to the buffer vial to force the samples past the detection window. Absorbance was monitored at 220 nm, and the time between the peaks was used to determine the distance traveled by the first injection plug during the applied voltage as well as the electroosmotic mobility.

## 3. Results and discussion

Fig. 1 demonstrates the utility of this biphenyl modified capillary in the separation of a mixture of short peptide

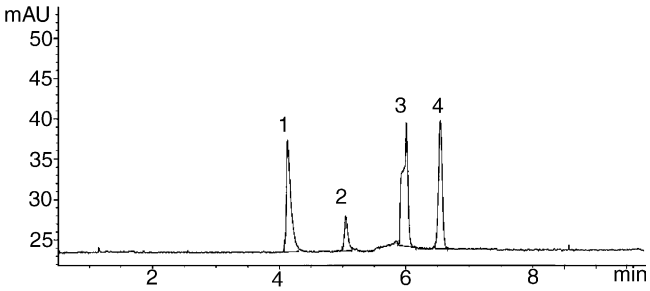


Fig. 1. Electrochromatographic separation of peptides in the etched cyanopentoxy biphenyl capillary. Experimental conditions: pH 7.00; applied voltage = 15 kV; $L$ = 50 cm; $l$ = 25 cm; injection 3.0 s at 50 mbar. Solute 1: 50 ppm bradykinin; solute 2: 100 ppm angiotensin III; solute 3: 50 ppm angiotensin I; and solute 4: 25 ppm angiotensin II.

hormones. The capillary is able to resolve the 4 peptides based on their charge in solution in the pH 7.00 run buffer. The bradykinin is predicted to have a charge of +2 and migrates the fastest followed by angiotensin III (+1) and angiotensin I then angiotensin II. The detection of the neutral species, angiotensin I and II, demonstrates that a small amount of residual electroosmotic flow remains in the capillary and that resolution of neutral species is possible due to their interaction with the coating on the capillary wall. Interestingly, angiotensin II, with a phenylalanine at its C-terminus migrates slower in the capillary than angiotensin I, presumably due to interactions with the biphenyl coating. Angiotensin I displays a shoulder on the leading edge of the peak in both the mixture and the individual sample. This feature could be an unresolved impurity, the result of sample decomposition, or a sample solvent peak.

Fig. 2 displays the ability of the capillary to resolve a mixture of digestive enzymes. The analysis of this mixture of digestive enzymes was complicated by the cleavage of the enzymes by each other. However the mixture of five enzymes was resolved into 5 peaks (three overlapping) with the peaks for trypsin, α- and β-chymotrypsin not baseline resolved under these experimental conditions. These proteins are positively charged at pH 3.00, yet no peak tailing was observed indicating that the silanol groups have been effectively covered by the silanization/hydrosilation bonding reactions.

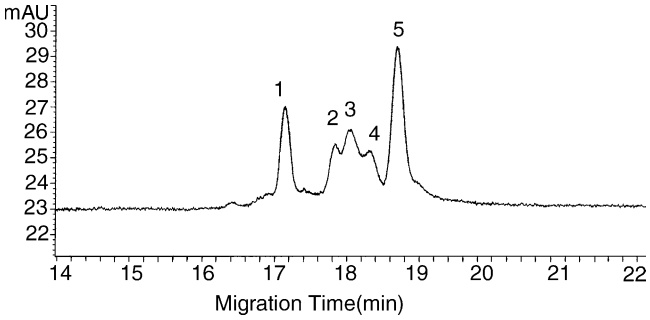


Fig. 2. Electrochromatographic separation of digestive enzymes in the etched cyanopentoxy biphenyl capillary. Experimental conditions same as Fig. 1 except pH 3.00 with 2.0 s injection at 15 kV and an applied voltage = 6 kV. Solutes (all 100 ppm): 1: trypsinogen; 2: α-chymotrypsin; 3: β-chymotrypsin; 4: trypsin; and 5: chymotrypsinogen.

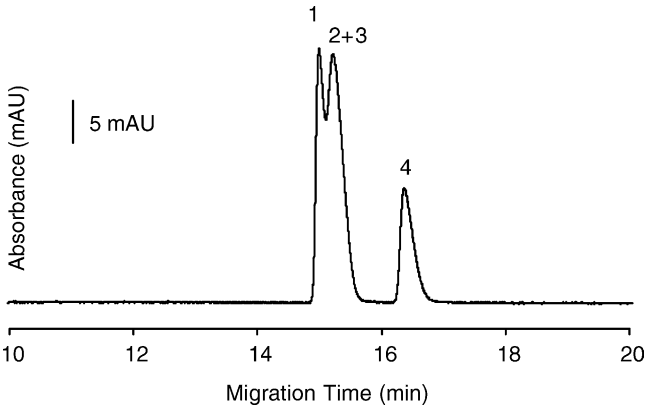


Fig. 3. Partial electrochromatographic separation of basic pharmaceuticals in the etched cyanopentoxy biphenyl capillary. Experimental conditions: applied voltage = 30 kV; injection 3.0 s vacuum; $L$ = 50 cm; $l$ = 25 cm; pH 2.14. Solutes 1: desipramine; 2: nortryptiline; solute 3: imipramine; and 4: clomipramine.

Fig. 3 shows the electrochromatogram of several basic pharmaceuticals: clomipramine, imipramine, desipramine, and nortryptiline. While only clomipramine was completely resolved from the other components under these experimental conditions, no tailing of the peaks was observed. The longer migration time of clomipramine is most likely due to its larger hydrodynamic radius giving it a lower electrophoretic flow than the other three solutes. When these drugs are separated by high performance liquid chromatography, extensive method development is required, and the optimum mobile phase consists of several organic modifiers. If one excludes column preparation, then a CEC method might only require optimization of background electrolyte pH and applied voltage. The use of an etched capillary chemically modified with another liquid crystal, cholesterol, has been demonstrated for the separation of another class of basic analytes, benzodiazepines [11].

Fig. 4 shows the separation of serotonin and tryptamine—two metabolites of the amino acid tryptophan at pH 2.14.

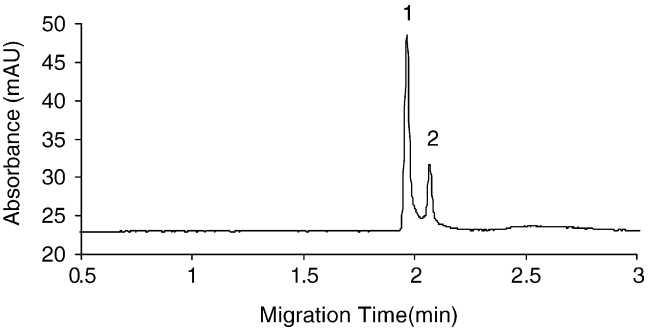


Fig. 4. Electrochromatographic separation of serotonin and tryptamine in the etched cyanopentoxy biphenyl capillary. Experimental conditions: pH 2.14; applied voltage = 25 kV; $L$ = 50 cm; $l$ = 25 cm; detection at 221 nm. Solute 1: tryptamine and solute 2: serotonin.

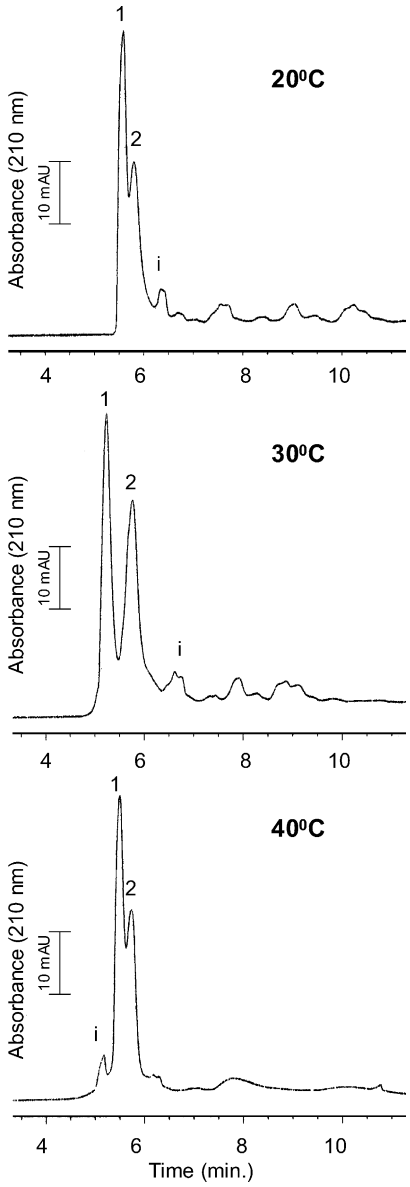


Fig. 5. Electrchromatographic separation of bovine and equine cytochrome *c* on the etched cyanopentoxy biphenyl capillary as a function of temperature. Experimental conditions: $L$ = 33.4 cm; $l$ = 24.9 cm; applied voltage = 10 kV; pH 7.0; injection 5.0 s at 50 mbar. Solute 1: bovine cytochrome c; solute 2: equine cytochrome c; and i: impurity(ies).

These molecules only differ by the presence of a hydroxyl group in the 5 position on serotonin which is absent in tryptamine. At this low pH both molecules would be singly charged and the difference in their migration times would be due to variations in their hydrodynamic radii or the extent of their interactions with the biphenyl moiety bonded to the inner capillary surface.

Fig. 5 gives the most interesting result with this capillary. This electrochromatogram shows the temperature-dependent resolution of cytochrome *c* molecules from two different species. These molecules differ by only one amino acid yet they can be resolved by OTCEC with the biphenyl capillary. Furthermore the resolution changes with the temperature of the separation. A maximum in resolution is obtained at 30 °C. A number of smaller components are also seen in these electrochromatograms. These also shift with temperature and one peak (i) can be seen to move from a longer migration time than the two major components at lower temperature to a shorter migration time at the highest temperature (40 °C). This non-linear behavior in resolution with liquid crystal stationary phases has also been observed by our group in HPLC experiments. In one study, the capacity factor ($k$) of two forms of gentamycin varied non-linearly with temperature [23].

The change in resolution of the protein peaks is obviously due to a change in the difference of the effective mobilities of the two proteins. The effective mobilities of the protein would consist of the sum of the electrophoretic mobility and the electroosmotic mobility times the fraction of time the protein spends in the mobile phase. The last factor is dependent upon the chromatographic contribution to the overall migration. The electroosmotic velocity should be the same for all species and is easily measured.

To further substantiate the chromatographic effect responsible for this change in resolution with variations in temperature, a study of the electroosmotic flow in the capillary was performed at various pH, temperatures, and voltages. This study was performed by using dimethyl sulfoxide (DMSO) as a neutral marker and applying the voltage for either 10 or 15 min. Experiments performed at 10 and 20 kV gave similar results for the intrinsic mobility of DMSO. As expected the EOF was towards the anode at low pH. This phenomenon has been recorded before for bare and chemically modified etched capillaries [27]. For bare, fused silica capillaries, any anodic EOF observed is attributed to protonated silanol groups and the presence of metal ion impurities in the capillary wall. For etched and modified capillaries, the hydride surface greatly reduces the concentration of silanol groups, and the etching process has incorporated $NH_4^+$ ions into the capillary wall. It is the presence of these cations that give rise to the reversal of the electroosmotic flow. Fig. 6 shows the measured electroosmotic flow at pH 8.14 for the cyanopentoxy biphenyl capillary. This data indicates that the electroosmotic flow in

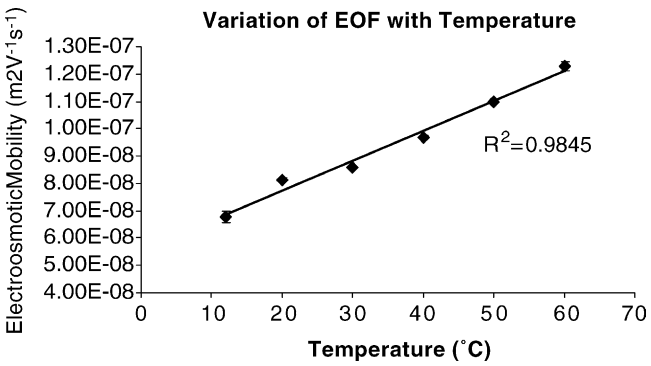


Fig. 6. Electroosmotic flow vs. temperature in the etched cyanopentoxy biphenyl capillary at pH 8.14. Experimental conditions: $L$ = 33.4 cm; $l$ = 24.9 cm; two injections of DMSO for 2 s at 50 mbar separated by 60 s application of 10 kV for 30 s, then application of 50 mbar for 10 min to elute peaks. Detection at 220 nm.

the biphenyl capillary increases in a fairly linear manner with increases in temperature. Therefore the changes in EOF with temperature do not explain the changes in resolution of the proteins and hence it can be attributed to a chromatographic or electrophoretic mechanism. Similar linear dependencies of EOF as a function of temperature were obtained at other pH values.

## 4. Conclusions

This study further demonstrates the favorable properties for basic analytes and the biocompatible nature for proteins and peptides of etched chemically modified capillaries in open tubular electrophoretic analyses. These desirable features are the result of both the etching step that produces a new surface matrix on the inner wall of the capillary and the silanization/hydrosilation modification process that leads to a hydride layer on the surface with virtually no silanols and an attached organic moiety above it. The presence of chromatographic interactions is confirmed in peptide separations and variable temperature protein migration data. The unique characteristics and usefulness of a liquid crystal bonded moiety on the surface is demonstrated in the variable temperature experiments. Further work is in progress on more complete temperature characterization of liquid crystal bonded capillaries as well as the possibility of isomer (geometric and chiral) separations.

## Acknowledgements

The financial support of the National Institutes of Health (NIH MBRS Grant 0819210-21 and Grant GM 0063519-01) and the National Science Foundation (CHE 0094983) is gratefully acknowledged. J.J.P. would like to acknowledge the support of the Camille and Henry Dreyfus Foundation through a Scholar Award.

## References

[1] S. Eeltink, G.P. Rozing, W.T. Kok, Electrophoresis 24 (2003) 3935.
[2] V.T. Remcho, S.L. Clark, A. Doneau, G.S. Chirica, Electrokinetic Phenomena (2004) 345.
[3] C. Yan, R. Dadoo, H. Zhao, R.N. Zare, Anal. Chem. 67 (1995) 2026.
[4] J.-T. Wu, P. Huang, M.X. Li, M.G. Qian, D.M. Lubman, Anal. Chem. 69 (1997) 320.
[5] A. Hilmi, J.H. Luong, Electrophoresis 21 (2000) 1395.
[6] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[7] H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka, N. Tanaka, Anal. Chem. 68 (1996) 3498.
[8] E.C. Peters, M. Petro, F. Svec, J.M.J. Frechet, Anal. Chem. 69 (1997) 3646.
[9] Y. Guo, L.A. Colon, Anal. Chem. 67 (1995) 2511.
[10] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 763 (1996) 255.
[11] A.P. Catabay, H. Sawada, K. Jinno, J.J. Pesek, M.T. Matyska, J. Capillary Electrophor. 5 (1998) 89.
[12] J.J. Pesek, M.T. Matyska, G.B. Dawson, J.I.-C. Chen, R.I. Boysen, M.T.W. Hearn, Anal. Chem. 76 (2004) 23.
[13] J.J. Pesek, M.T. Matyska, L. Mauskar, J. Chromatogr. A 763 (1997) 307.
[14] J.J. Pesek, M.T. Matyska, J.E. Sandoval, E.J. Williamsen, J. Liq. Chromatogr. Relat. Technol. 19 (1996) 2843.
[15] J.J. Pesek, M.T. Matyska, J. Chromatogr. A 736 (1996) 313.
[16] J.J. Pesek, M.T. Matyska, J. Capil. Electrophor. 4 (1997) 307.
[17] J.J. Pesek, M.T. Matyska, S. Cho, J. Chromatogr. A 845 (1999) 237.
[18] J.E. Sandoval, J.J. Pesek, US Patent 5 326 738 (1994).
[19] S. Chandrasekhar, Liquid Crystals, Cambridge University Press, New York, 1977.
[20] W.L. Zielinski Jr., D.H. Freeman, D.E. Martire, L.C. Chow, Anal. Chem. 42 (1970) 176.
[21] E.M. Barrall, R.S. Porter, J.F. Johnson, J. Chromatogr. 21 (1966) 392.
[22] J.J. Pesek, M.T. Matyska, E.J. Williamsen, R. Tam, Chromatographia 41 (1995) 301.
[23] J.J. Pesek, M.T. Matyska, G.B. Dawson, A. Wilsdorf, P. Marc, M. Padki, J. Chromatogr. A 986 (2003) 253.
[24] J.C. Reijenga, T.P.E.M. Verheggen, J.H.P.A. Martens, F.M. Everaerts, J. Chromatogr. A 744 (1996) 147.
[25] M.T. Matyska, J.J. Pesek, A. Katrekar, Anal. Chem. 71 (1999) 5508.
[26] J.E. Sandoval, S.-M. Chen, Anal. Chem. 68 (1996) 2771.
[27] M.T. Matyska, J.J. Pesek, L. Yang, J. Chromatogr. A 887 (2000) 497.