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Capillary electrophoresis of some tetracycline antibiotics

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Abstract

Data on the separation of tetracycline antibiotics by capillary electrophoresis are rather limited and have not been reported for micellar electrokinetic capillary chromatographic separation (MECC). In the present study, the separation of tetracycline, oxytetracycline and chlortetracycline by capillary zone electrophoresis and MECC was investigated. Adding non-ionic surfactants such as Triton X-100 to a 0.2 *M* phosphate migration buffer of pH 2.2 greatly improved separation. The use of mixed micelles enlarged the variety of the micellar phases, *e.g.* a combination of Tween 20 and Tween 80 provided a similar separation pattern. The addition of β-cyclodextrin to a Triton X-100 and Brij-35 surfactant combination did not result in an improved separation. A Triton X-100 and Brij-35 combination could separate tetracycline and its degradation products 4-epitetracycline (ETC), anhydrotetracycline and 4-epianhydrotetracycline. This enabled us to identify ETC in a commercial tetracycline sample.

1. Introduction

Tetracycline antibiotics are an important class of therapeutic compounds as they represent a key component in the strategy used to control bacterial infections in both humans and animals. Although many high-performance liquid chromatographic (HPLC) methods for the analysis of antibiotics have been reported and reviewed [1,2], the use of capillary electrophoresis (CE) has gained considerable importance in recent years. This topic was recently reviewed by Bobbitt and Ng [1] and Grossman and Colburn [3]. This includes reports by Nishi and co-workers [4–6] using micellar electrokinetic capillary chromatography (MECC) to separate both penicillin and cephalosporin antibiotics, Tsikas *et al.* [7] using capillary isotachophoretic chromatography to separate penicillin and cephalosporin antibiotics and their precursors, and Ackermans and co-workers using capillary zone electrophoresis (CZE) to separate aminoglycoside antibiotics [8] and eleven sulphonamides [9]. Most of the reported studies usually involve only one family of the antibiotics. Yeo *et al.* [10] reported the CZE separation of six selected antibiotics of different types, including chlortetracycline.

For further enhancement of selectivity, cyclodextrins [11,12] and organic solvents [12–14] can be added to the electrophoretic media. The inclusion complexating properties of cyclodextrins have been discussed in detail, espe-

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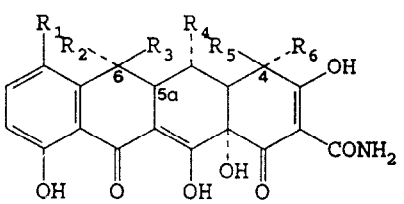
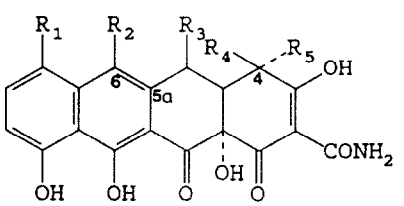
cially their applications to perform chiral separations [15]. Interactions between additives and solutes may cause the latter to migrate at different velocities owing to differences in the magnitude of solute–additive associations. Nevertheless, none of these additives have been applied to the CE separation of tetracyclines. To our knowledge, only one CZE method has been published for the separation of tetracycline and its degradation products [16]. By this method, complete baseline separation of tetracycline and its degradation products was achieved using EDTA as an additive in a phosphate buffer solution (pH 3.9). In view of this, attempts were made to establish the optimum conditions for CZE separation of three widely used tetracyclines: tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC).

One aspect of current interest is the addition of micelles to the electrophoretic buffer to perform a chromatography-like separation [17], which was first described by Terabe *et al.* [18] in 1984 as micellar electrokinetic chromatography. Nevertheless, this MECC was not mentioned before for tetracycline antibiotics. The present study was undertaken to determine the CZE and MECC behaviour of TC, OTC and CTC. Indeed, the separation of TC, OTC and CTC by MECC provided a better resolving power than CZE due to the interaction or partition of the analytes with the micellar phase [19].

In Fig. 1 the structural formulae of the investigated tetracycline antibiotics and tetracycline degradation products are given. At pH values lower than 2, especially upon heating, a dehydration reaction at C5a–C6 leads to the formation of anhydrotetracycline (ATC). In aqueous solutions at pH 2–6 a reversible epimerization reaction at C4 takes place to form 4-epitetracycline (ETC) and 4-epianhydrotetracycline (EATC), respectively [20]. The separation of TC and its degradation products was similarly studied by MECC in the present investigation. ETC could be detected in a commercial tetracycline sample.





Compound	R_1	R_2	R_3	R_4	R_5	R_6	Compound	R_1	R_2	R_3	R_4	R_5
TC	H	CH_3	OH	H	H	$N(CH_3)_2$	ATC	H	CH_3	H	H	$N(CH_3)_2$
OTC	H	CH_3	OH	OH	H	$N(CH_3)_2$	EATC	H	CH_3	H	$N(CH_3)_2$	H
CTC	Cl	CH_3	OH	H	H	$N(CH_3)_2$						
ETC	H	CH_3	OH	H	$N(CH_3)_2$	H						

Fig. 1. Structural formulae of some representative tetracycline antibiotics: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and tetracycline degradation products 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC).

Its presence could be confirmed applying a validated HPLC method for purity control of tetracycline samples [21].

2. Experimental

2.1. Instrumentation

CE analysis

CE was performed on a HPE 100 high-performance CE instrument from Bio-Rad (Nazareth, Belgium), equipped with a variable-wavelength UV detector. Commercial cartridges (Bio-Rad) containing coated fused-silica columns of 20 cm × 25 μm I.D. were used. Sample solutions were injected into the capillary on the anodic side by electromigration for 10 s at 12 kV. A constant-voltage mode was applied for the separation of the analytical solutions. For detection, the absorbance was measured at 265 nm, and the signal from the detector was processed with a Shimadzu (Kyoto, Japan) Chromatopac C-R3A integrator system.

HPLC analysis

HPLC was performed on a Varian (Walnut Creek, CA, USA) 9010 solvent-delivery system with a 20-μl loop, coupled with a Hewlett-Packard (Waldbronn, Germany) Series 1050 multiple-wavelength detector. A Shimadzu CTO-6A column oven was used to maintain column temperature at 60°C. The chromatograms were recorded and integrated on a Hewlett-Packard (Grenoble, France) Vectra QS/16S integrator.

2.2. Chemicals

CE analysis

CTC hydrochloride, ETC hydrochloride, EATC hydrochloride and ATC hydrochloride were purchased from Janssen Chimica (Beerse, Belgium). TC hydrochloride and OTC were kindly provided by Pfizer (Brussels, Belgium). EDTA and sodium dodecyl sulphate (SDS) were obtained from Merck (Darmstadt, Germany). Cetyltrimethylammonium bromide was purchased from UCB (Drogenbos, Belgium). Sodium taurocholate, sodium deoxycholate, β-cyclodextrin (β-CD) and Triton X-100 were obtained from Janssen Chimica. Polyoxyethylene (20) sorbitan monooleate (Polysorbate 80, Tween 80) was from Laboratoria Flandria (Zwijnaarde, Belgium), polyoxyethylene sorbitan monolaurate (Tween 20) was from Sigma (St. Louis, MO, USA) and polyoxyethylene dodecyl ether (Brij-35) was from Merck–Schuchardt (Munich, Germany).

All other reagents and solvents, of analytical-reagent grade, were purchased from Janssen Chimica or from Merck. Distilled deionized water was used to prepare solutions for CE purposes.

HPLC analysis

The reference TC·HCl sample, which was certified to contain 98.1% of the hydrochloride salt, was available from Janssen Chimica. Dipotassium hydrogenphosphate and 2-methyl-2-propanol were from Merck, tetrabutylammonium hydrogensulphate was from Janssen Chimica. HPLC-grade water was from Labscan (Dublin, Ireland). Sample solutions were stored protected from light not more than 12 h at about 5°C.

2.3. Procedures

CZE analysis

An aqueous solution of TC, OTC and CTC each at a concentration of 20 μg ml^{-1} was prepared daily and subjected to CZE and MECC.

Phosphate buffers in the pH range 1.6–2.2 and concentrations between 0.01 and 0.2 *M* were composed of sodium dihydrogenphosphate solutions with the pH adjusted using a 85% phosphoric acid solution. Other phosphate buffers with pH values of 6.0 (0.05 *M*) and 9.0 (0.2 *M*) consisted of disodium hydrogenphosphate solutions adjusted to pH using a 4 *M* sodium hydroxide solution.

The effect of buffer additives on the separation of TC, OTC and CTC in the CZE mode

was studied with addition of EDTA, organic modifiers and β-CD. EDTA was added in a concentration of 0.005 *M* to a 0.05 *M* phosphate buffer (pH 6.0). The addition of acetonitrile and methanol to a 0.1 *M* phosphate buffer system (pH 2.2) at concentrations of 30 and 10% (v/v), respectively, was studied. The effect of the addition of β-CD in a concentration range between 0.5 and 20 m*M* to the same buffer system was also tested.

MECC analysis

Anionic micelle systems consisted of a 12 m*M* sodium deoxycholate solution in a phosphate running buffer 0.2 *M* (pH 9.0), 17 m*M* sodium taurocholate solution in a phosphate running buffer 0.05 *M* (pH 6.0) and 40 m*M* SDS in the same buffer system.

The cationic micelle system tested was a 10 m*M* cetyltrimethylammonium bromide solution in a 0.1 *M* phosphate running buffer (pH 2.2).

In this investigation, all non-ionic surfactants were added to a 0.2 *M* phosphate running buffer of pH 2.2 and their concentration values are expressed as % (m/m). The effect of serial dilutions of the investigated micelle systems in the same migration buffer (1:2) was studied.

For the separation of TC, OTC and CTC a micelle system containing 0.48% Triton X-100 was applied. Also a mixed micelle system containing a mixture of Tween 20 (0.52%) and Tween 80 (0.56%) was applicable. The addition of β-CD (10 m*M*) to another mixed micelle system which combined Triton X-100 (0.48%) and Brij-35 (0.16%) was also tested.

For the separation of TC and its degradation products a combination of Brij-35 (0.035%) and Triton X-100 (0.10%) was applied.

HPLC analysis

HPLC was carried out as described by Hendrix *et al.* [21]. The column used was a PRP-1 column (25 cm × 4.6 mm I.D., 10 μm spherical 75 Å poly(styrene–divinylbenzene) particles, Hamilton, Reno, USA). The mobile phase contained 8.5% (m/v) of 2-methyl-2-propanol, 10% (v/v) of a 3.5% (m/v) dipotassium hydrogenphosphate solution adjusted to pH 9.0, 20% (v/v) of a 1.0% (m/v) tetrabutylammonium hydrogensulphate solution adjusted to pH 9.0, 1% (v/v) of a 4.0% (m/v) EDTA solution adjusted to pH 9.0 and the volume was made up to 100% (v/v) with water. Dilute phosphoric acid (10%, m/m) or dilute sodium hydroxide solution (8.5%, m/m) were used to adjust the solutions to the required pH. The mobile phase was degassed by ultrasonication. The flow-rate was set at 1.0 ml min^{-1}.

A solution containing 1.0 mg ml^{-1} of the TC sample (Pfizer) in 0.01 *M* hydrochloric acid was prepared.

3. Results and discussion

3.1. CZE separation of TC, OTC and CTC: choice of parameters

Several electrophoretic parameters were considered and their influence on the overall performance of the separation studied. The results can be summarized as follows.

Influence of migration buffer pH

The pK_a values relevant to acid–base equilibrium in aqueous solution of the tetracycline antibiotics are agreed to be approximately 3.3, 7.5 and 9.3 [20]. At buffer pH values below the value of the isoelectric point at pH 5.5, the positively charged tetracyclines migrate towards the cathode. In order to fully utilize the electrophoretic mobility differences as a result of this ionization, an acid buffer of pH 2.2, using phosphate as the buffering ion, was chosen.

Influence of migration buffer concentration

It has been shown that the ionic strength of the buffer has significant effects on solute mobilities and separation efficiency [22]. In general, the migration times of the tetracyclines increased with increasing buffer concentration. A phosphate buffer concentration of 0.2 *M* was finally chosen as the most suitable.

Influence of running voltage

During the separation a constant voltage mode was applied and migration occurred under rather constant currents of about 12 μA. Running voltages in the range 4–12 kV were tested. As expected, decreasing migration times were obtained with increasing applied voltages but varying the voltage did not influence the selectivity. A running voltage of 12 kV was chosen for further experiments, because this was the maximum value allowed by the instrument and this significantly shortened the migration times.

3.2. Effect of buffer additives on the separation of TC, OTC and CTC

Since CZE experiments did not provide baseline separation, the effect of buffer additives was investigated. Zhang *et al.* [16] added 0.005 *M* EDTA to a 0.02 *M* CZE migrating phosphate buffer (pH 3.9) to improve the resolution between TC and its degradation products ETC, ATC and EATC. To avoid solubility problems of EDTA at pH 2.2, a similar addition of EDTA to a phosphate buffer pH 6.0 was tested. Organic modifiers as acetonitrile and methanol were added to the running buffer as well, as was β-CD based on its extensive application in various CZE systems [11,12]. None of the additions provided improved resolution.

3.3. MECC separation

Separation of TC, OTC and CTC

MECC has the advantage that changing the micellar phase is very easy, requiring only that the capillary be rinsed and filled with the micellar solution. In practice, the applicability of a surfactant to MECC will mainly depend on its solubility and its critical micellar concentration (CMC) [22]. Anionic and cationic micelle systems are the most commonly employed micellar phases, though non-ionic surfactants are employed as well. SDS, sodium deoxycholate and sodium taurocholate added to a phosphate running buffer, did not improve the separation, neither did cetyltrimethylammonium bromide. However, when introducing non-ionic surfactants distinct effects upon the separation could be observed. Fig. 2 illustrates the striking effect of Triton X-100 on the separation of a solution of TC, OTC and CTC. Initially, resolution increased strongly with decreasing surfactant concentration but then levelled off at a maximum value. During migration, the micelles can interact with the tetracyclines in a chromatographic manner, possible through hydrophobic interactions with the alkyl chain. The more hydrophobic compounds interact more strongly with the micelle and are "retained" longer.

Also surfactant combinations were tested for

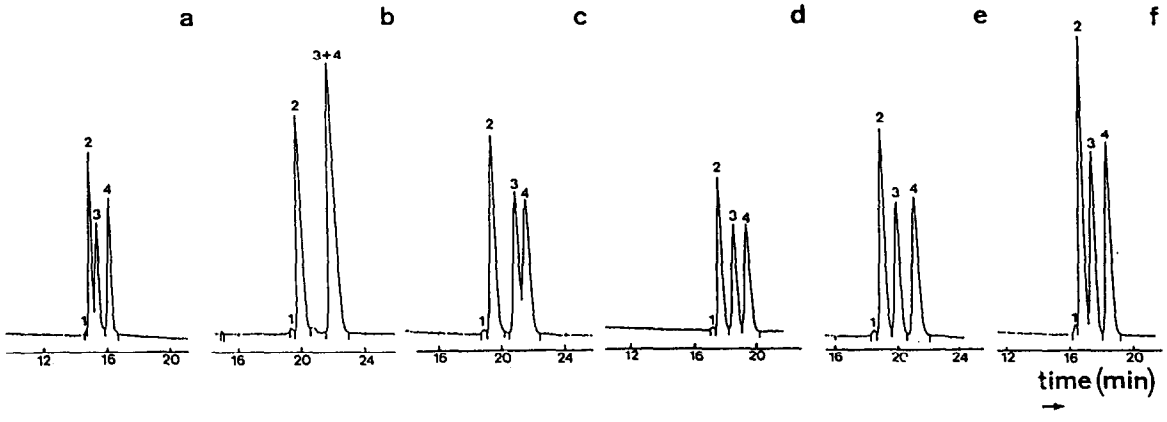


Fig. 2. Effect of Triton X-100 concentration on the MECC separation of a solution of 20 μg ml^{-1} TC, OTC and CTC each. Experimental conditions: 20 cm × 25 μm I.D. coated column; 0.2 *M* phosphate migration buffer of pH 2.2; running voltage: 12 kV; 12 kV 10 s injection conditions; detection: 265 nm. Peaks: 1 = ETC; 2 = TC; 3 = CTC; 4 = OTC. Concentration Triton X-100 (%, m/m) included in the migration buffer: (a) 0%, (b) 0.48%, (c) 0.24%, (d) 0.12%, (e) 0.06%, (f) 0.03%.

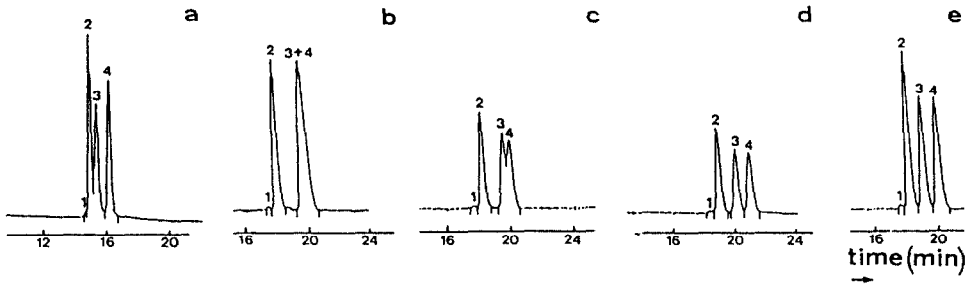


Fig. 3. Effect of a Tween 20 and Tween 80 combination on the separation of a solution of 20 μg ml^{-1} TC, OTC and CTC each. Experimental conditions and peaks as in Fig. 2. Concentration Tween 20 and Tween 80 (%, m/m) included in the migration buffer: (a) 0%, (b) 0.52% Tween 20 and 0.56% Tween 80, (c) 0.26% Tween 20 and 0.28% Tween 80, (d) 0.13% Tween 20 and 0.14% Tween 80, (e) 0.065% Tween 20 and 0.07% Tween 80.

further enhancement of selectivity. The effect of a Tween 20 and Tween 80 combination is shown in Fig. 3, but analogue separations as in Fig. 2 can be noted.

Addition of β-CD to a surfactant combination did not effect selectivity and similar separation patterns were observed.

Separation of TC and its degradation products

As the electropherograms showed a peak co-eluting with the TC peak, the question raised whether this compound was either a degradation product created during the electrophoretic run or was already present in the commercial TC sample.

Therefore, a separation of a standard mixture of TC and its degradation products ETC, ATC and EATC was needed to check the migration behaviour of the degradation products. This separation was performed using a similar combination of non-ionic micelles as for the separation of TC, OTC and CTC. The MECC system combined Triton X-100 and Brij-35 added to a 0.2 *M* phosphate buffer of pH 2.2 (Fig. 4). As can be seen, peak shapes of ATC and EATC are not optimal, but allow anyhow confirmation of their presence or absence in the sample. Further

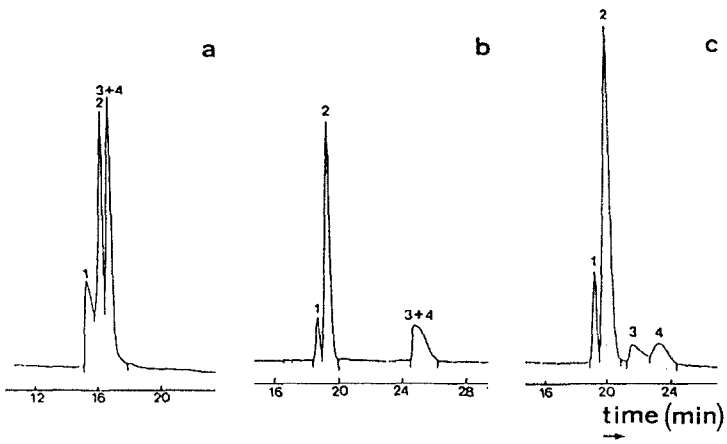


Fig. 4. Effect of a Triton X-100 and Brij-35 combination on the separation of a standard mixture of TC and its degradation products ETC, EATC and ATC. Concentration TC 30 μg ml^{-1}; ETC, ATC and EATC: (a) 10 μg ml^{-1}, (b and c) 2 μg ml^{-1}. Experimental conditions as in Fig. 2. Peaks: 1 = ETC; 2 = TC; 3 = EATC; 4 = ATC. Concentration Triton X-100 and Brij-35 (%, m/m) included in the migration buffer: (a) 0%, (b) 0.10% Triton X-100 and 0.035% Brij-35, (c) 0.05% Triton X-100 and 0.017% Brij-35.

experiments are required to optimize the MECC separation pattern of the decomposition products. The mentioned co-eluting peak could be identified as ETC after standard addition.

HPLC confirmation of ETC presence

Under conditions of acid pH (pH 2–6) and heating (Joule effect in the capillary [23]), TC can degrade to ETC [20].

Possible degradation of TC to ETC during the CE analysis providing erroneous results was examined with the same TC sample using another separation technique. Therefore a method reported for the analysis of TC by liquid chromatography on poly(styrene–divinylbenzene) was applied [21]. A chromatogram obtained from the TC sample is given in Fig. 5. The relative amounts of ETC found by the HPLC method corresponded well with those found by MECC, so the MECC separation method proved to be a non-destructive method.

4. Conclusions

The present findings demonstrate the power of MECC for the separation of TC, OTC and CTC as their cations using an acid running buffer solution. Adding non-ionic surfactants such as Triton X-100, Brij-35, Tween 20 and Tween 80 to the running buffer solution provided improved separation. A MECC buffer system combining

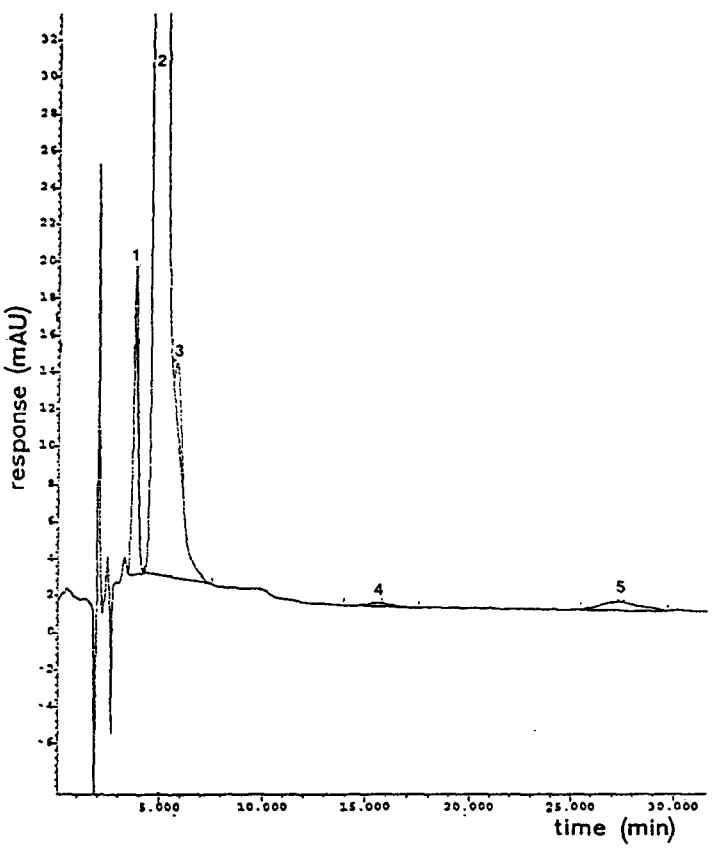


Fig. 5. HPLC chromatogram of a TC sample. Conditions: column: PRP-1 column; mobile phase: 2-methyl-2-propanol (8.5%, m/v), 3.5% (m/v) dipotassium hydrogenphosphate pH 9.0 (10 ml), 1.0% (m/v) tetrabutylammonium hydrogensulphate pH 9.0 (20 ml), 4.0% (m/v) EDTA pH 9.0 (1 ml), water (up to 100 ml); temperature: 60°C; injection loop: 20 μl; flow-rate: 1 ml min^{-1}; detection: 254 nm. Peaks: 1 = ETC; 2 = TC; 3 = 2-acetyl-2-decarboxamidotetracycline (ADTC); 4 = EATC; 5 = ATC.

Triton X-100 and Brij-35 was suitable for the separation of TC, ETC, ATC and EATC.

The separation of these tetracycline antibiotics by MECC could be achieved in less than half an hour requiring only small volumes of analyte solutions and limited quantities of electrolytes and additives.

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References

[1] D.R. Bobbitt and K.W. Ng, *J. Chromatogr.*, 624 (1992) 153.
[2] S.A. Barker and C.C. Walker, *J. Chromatogr.*, 624 (1992) 195.
[3] P.D. Grossman and J.C. Colburn, *Capillary Electrophoresis —Theory and Practice*, Academic Press, San Diego, CA, 1992.
[4] H. Nishi, N. Tsumagari, T. Kakimoto and S. Terabe, *J. Chromatogr.*, 477 (1989) 259.
[5] H. Nishi, N. Tsumagari and S. Terabe, *Anal. Chem.*, 61 (1989) 2434.
[6] H. Nishi, T. Fukuyama and M. Matsuo, *J. Chromatogr.*, 515 (1990) 245.
[7] D. Tsikas, A. Hofrichter and G. Brunner, *Chromatographia*, 30 (1990) 657.
[8] M.T. Ackermans, F.M. Everaerts and J.L. Beckers, *J. Chromatogr.*, 606 (1992) 229.
[9] M.T. Ackermans, J.L. Beckers, F.M. Everaerts, H. Hoogland and M.J.H. Tomassen, *J. Chromatogr.*, 596 (1992) 101.
[10] S.K. Yeo, H.K. Lee and S.F.Y. Li, *J. Chromatogr.*, 585 (1991) 133.
[11] T.E. Peterson, *J. Chromatogr.*, 630 (1993) 353.
[12] D.N. Heiger, *High Performance Capillary Electrophoresis: An Introduction*, Hewlett-Packard, Waldbronn, 1992.
[13] S. Honda, A. Taga, K. Kakehi, S. Koda and Y. Okamoto, *J. Chromatogr.*, 590 (1992) 364.
[14] G.M. Janini, K.C. Chan, J.A. Barnes, G.M. Muschik and H.J. Issaq, presented at the *5th International Symposium on High Performance Capillary Electrophoresis, Orlando, FL, January 25–28, 1993.*
[15] P. Jandik and G. Bonn, *Capillary Electrophoresis of Small Molecules and Ions*, VCH, New York, 1993.
[16] C.-X. Zhang, Z.-P. Sun, D.-K. Ling and Y.J. Zhang, *J. Chromatogr.*, 627 (1992) 281.
[17] J. Vindevogel and P. Sandra, *Introduction to Micellar Electrokinetic Chromatography*, Hüthig, Heidelberg, 1992.
[18] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 113.
[19] Y.J. Yao, H.K. Lee and S.F.Y. Li, *J. Chromatogr.*, 637 (1993) 195.
[20] L.A. Mitscher, *The Chemistry of the Tetracycline Antibiotics*, Marcel Dekker, New York, 1978.
[21] C. Hendrix, E. Roets, J. Crommen, J. De Beer, E. Porqueras, W. Van den Bossche and J. Hoogmartens, *J. Liq. Chromatogr.*, 16 (1993) 3321.
[22] S.F.Y. Li, *Capillary Electrophoresis —Principles, Practice and Applications (Journal of Chromatography Library*, Vol. 52), Elsevier, Amsterdam, 1992.
[23] H.-T. Chang and E.S. Yung, *J. Chromatogr.*, 632 (1993) 149.