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Use of metal complexation in non-aqueous capillary electrophoresis systems for the separation and improved detection of tetracyclines

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Abstract

Metal complexation in non-aqueous capillary electrophoresis systems was evaluated for the separation and improved detection of tetracycline antibiotics using laser-induced fluorescence detection. It was found that three factors were important for the choice of complexing agent: (i) it should be soluble in the organic solvent used for the separation, (ii) it should have a sufficient fast complexing rate so as not to invalidate the electrophoretic separation and, (iii) it should give a large increase in the fluorescence intensity. Mg^{2+} ions were found to be the most suitable ions for the separation of the tetracyclines as the acetate salt of magnesium is very soluble in organic solvents and only a relatively low current was generated during electrophoresis making it possible to use high concentrations of the complexing metal ion. Metal complexation strongly intensified the fluorescence of tetracyclines and all organic solvents investigated further intensified the fluorescence, e.g. dimethylformamide improved the fluorescence of the oxytetracycline metal complex by a factor of 34 compared to water. However, magnesium acetate was not sufficiently soluble in dimethylformamide and therefore *N*-methylformamide, improving the fluorescence intensity by only a factor of 9, was used. It was demonstrated that the method can be used for the detection of tetracyclines at the ppb level in milk and plasma. © 1997 Elsevier Science B.V.

Keywords: Metal complexation; Tetracyclines; Metal complexes

1. Introduction

The use of non-aqueous solvents has recently been shown to offer advantages over aqueous systems regarding selectivity adjustment of small ionic species in capillary electrophoresis (CE) [1–7]. Some of the more important properties of the solvents that may influence the selectivity of the separation are the dielectric constant, the viscosity, as well as the auto protolysis. The dielectric constant strongly influences the electrical properties of the solvent and thereby the electrophoretic mobilities obtained. A high viscosity of the solvent may decrease the electroosmotic flow. However, a high dielectric constant of a solvent is partly able to compensate for a high viscosity by giving the solvent improved electrical properties [8]. The auto protolysis constant pK_{auto} of water is 14. Many organic solvents exhibit larger pK_{auto} values examplified by 17.2 for methanol and 33.3 for dimethylsulphoxide. A higher value provides a wider operating span compared to aqueous systems and thus an improved possiblity for obtaining increased selectivity towards the solutes separated in the

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system. Furthermore, the levelling effect of water [9] is absent or at least very weak in organic solvents and minor differences between the solutes are therefore expressed to a larger extent. Moreover, solvation of the solutes differ considerably from aqueous systems and this may have a profound effect on the separation selectivity [10].

The means for adjusting selectivity in non-aqueous CE have primarily been the choice of solvent or solvent mixture combined with addition of electrolytes with acidic or basic properties [1–12]. Furthermore, formation of ion-pairs in organic solvents has been used as a principle for achieving separation of chiral substances [13] and for minor adjustments of selectivity in the separation of long-chain surfactants [7] and polyethers [14]. Moreover, a non-ionic surfactants have been added to non-aqueous systems in order to slow down the electro-osmotic flow [13]. Metal complexes are known to be formed in non-aqueous solvents [15,16], but metal complexation has not previously been used in non-aqueous CE systems for obtaining separation of tetracycline and related compounds. Tetracycline antibiotics and related compounds are known to combine with many metal ions in aqueous systems [17]. The chelates formed with Mg^{2+}, Ca^{2+} or Eu^{2+} are fluorescent under neutral or alkaline conditions [17–19]. Fluorescent chelates of the tetracyclines may also be formed in acidic solutions if Zr^{4+} or Al^{3+} are used [20,21]. HPLC [20–24] as well as aqueous CE [25–29] and non-aqueous CE [30] have succesfully been applied to separate tetracyclines. A few HPLC methods with fluorescence detection using Ca^{2+}, Al^{3+} or Zr^{4+} as the complexing ions have been reported [20,21,24]. In the present paper a selective and sensitive technique for the separation of tetracycline antibiotics is presented. The tetracyclines are separated as stable and strongly fluorescent metal chelates in the electrophoresis medium.

2. Experimental

2.1. Apparatus

A Beckman P/ACE 5010 (Beckman, Fullerton, CA, USA) equipped with a UV detector (operated at 280 nm) or a laser-induced fluorescence (LIF) unit connected with a He–Cd laser (Omnicrome, Chino, CA, USA) emitting light at a wavelength of 325 nm was used. After excitation at 325 nm the fluorescence was measured at an emission wavelength of 514 nm. The samples were loaded by applying a pressure (0.5 psig; ca. 3.5 kPa) for 3 s unless otherwise stated and the separations were carried out at 20°C by applying a voltage gradient of 15 kV. The air circulation over the samples in the autosampler carrousel was reduced in order to minimize evaporation of the sample as well as of the electrophoresis medium during the experiments. Furthermore, the part of the leverarms entering the vials was replaced with one made of teflon in order to make it more resistant to the organic solvents used. Data acquisition was accomplished with a NEM personal computer 486 with System Gold software.

A Kontron SMF 25 spectrofluorometer (Tegimenta, Switzerland) was used for obtaining emission and excitation spectra of 1 m*M* of oxytetracycline.

2.2. Reagents

Tetracycline hydrochloride (TC), chlortetracycline hydrochloride (CTC) and oxytetracycline hydrochloride (OTC) were kindly donated by Nycomed–DAK (Roskilde, Denmark) and Dumex (Copenhagen, Denmark). Magnesium acetate tetrahydrate, zinc acetate dihydrate, strontium acetate hemihydrate and trichloroacetic acid were purchased from E. Merck (Darmstadt, Germany). Calcium acetate and ammonium acetate were from Riedel de Haën (Seelze, Hannover, Germany). Demeclocycline hydrochloride (DeC) and β-naphthol were purchased from Sigma (St. Louis, MO, USA). 4-Epianhydrotetracycline hydrochloride (EATC) and 4-epitetracycline hydrochloride were CRS-standards obtained from the Council of Europe (Strasbourg, France). Anhydrotetracycline hydrochloride (ATC) was purchased from Agros Chemicals (Beerse, Belgium).

All organic solvents used were of analytical grade and used as received from the supplier.

2.3. Procedures

The capillaries used were obtained from Polymicro Technologies (Phoenix, AZ, USA). Prior to

use new capillaries were rinsed with 1 *M* sodium hydroxide for 1 h, 0.1 *M* sodium hydroxide for 20 min, distilled water for 20 min and subsequently with methanol for 20 min followed by 15 min flushing with the electrophoresis medium. The capillary were flushed with the electrophoresis medium for 0.5 min prior to each run. The electrophoresis medium was replaced after 6 runs. Prior to long term storage the capillary was flushed with 0.1 *M* HCl followed by 0.1 *M* NaOH, water, MeOH and finally air.

β-Naphthol was used as a fluorescent flow marker when using LIF-detection.

2.4. Test solutions

The test solutions containing the tetracyclines were prepared daily in the organic solvent corresponding to the organic solvent used for the separation.

2.5. Milk and plasma samples

One volume of plasma or milk was precipitated with 0.2 volumes of 10% trichloroacetic acid in water and centrifuged for 4 min at 1000 *g*. A 3 ml SPEC 15 mg MP1 solid-phase extraction microcolumn (Ansys, Irvin, CA, USA) was conditioned with 1.0 ml of methanol followed by 1.0 ml of distilled water. A 5.0 ml volume of supernatant was applied to the slightly wet microcolumn at a flow-rate of ca. 2 ml per min. The microcolumn was then washed with 500 μl of water and dried under vacuum (approx. 40 kPa) for 5 min or more if necessary. The analytes were then eluted by applying 200 μl of 50 m*M* magnesium acetate in NMF. The sample was injected at 3.5 kPa for 25 s and separated using the following experimental conditions: fused-silica capillary: 75 μm I.D.×27 cm (20 cm to the detector); electrophoresis medium: 500 m*M* magnesium acetate tetrahydrate in NMF; voltage: 15 kV; temperature: 20°C; detection: laser induced fluorescence with excitation at 325 nm and emission measured at 514 nm.

Calibrations curves were prepared by spiking milk with known concentrations of OTC. The standard solutions in milk were treated the same as the samples analysed.

Calibrations curves for the recovery studies were prepared by analysing standard solutions of OTC in 50 m*M* magnesium acetate tetrahydrate in NMF and using the conditions as described above.

3. Results and discussion

3.1. Selection of solvent and electrolytes

Organic solvents are known to have a great impact on the separation selectivity in capillary electrophoresis [5]. Moreover, the solvent can strongly effect the fluorescence characteristics of a solute [31,32]. The influence of the nature of the solvent on the fluorescence intensities of the metal complex of oxytetracycline using various electrolytes is shown in Table 1. DMF, DMSO and NMF provided the highest fluorescence intensity compared to water. Thus these solvents were chosen for further studies.

Table 1
The relative fluorescence of the metal complex of 1 m*M* of OTC in various solvents compared to water. The complexing ions were added as the acetates

Medium	Relative fluorescence	Emission λ_{max}	Excitation λ_{max}
50 m*M* Mg^{2+} in water	1.0	500	420
50 m*M* Mg^{2+} in DMSO	20.8	498	460
50 m*M* Mg^{2+} in formamide	4.7	500	428
50 m*M* Mg^{2+} in DMF	34.4	493	448
50 m*M* Mg^{2+} in NMF	9.0	493	425
50 m*M* Zn^{2+} in methanol:acetonitrile (1:1)	5.4	495	434
50 m*M* Mg^{2+} in methanol:acetonitrile (1:1)	2.3	500	428
50 m*M* Mg^{2-} in methanol:DMF (1:1)	5.3	500	432

The nature of the complexing metal ion has also an influence on the fluorescence intensity e.g. it can be seen in Table 1 that Zn^{2+} enhances the fluorescence compared to magnesium. The effect of Mg^{2+}, Ca^{2+} and Sr^{2+} on the relative fluorescence of OTC were compared in electrophorsis systems with various concentrations of the corresponding acetates added to NMF. Sr^{2+} ions were found to quence the fluorescence even of the flow marker β-naphthol whereas the use of Mg^{2+} or Ca^{2+} ions gave strong fluorescence of the OTC metal complexes as well as of the β-naphthol.

The electrolyte to be chosen should be soluble in the organic solvent used for the separation. Acetates of metal ions from the second main group in the Periodic Table are generally fairly soluble even in organic solvents. Furthermore, they provide neutral to weak alkaline conditions favouring the complexation between tetracycline and the metal ion. However, it was found that the solubility of the acetates in the organic solvents used was a limiting factor. The solubility of the acetates used decreased in the order $Mg^{2+}>Ca^{2+}>Sr^{2+}$ and the ability of the solvents to dissolve the metal acetates decreased in the order NMF>formamide>DMSO>DMF. A full comparison of the metal ions was only possible in NMF using the electrolytes in concentrations of 25 m*M*. TC, OTC and CTC co-migrated when using strontium acetate in a concentration of 25 m*M* calcium or magnesium acetate in concentrations of 25 m*M*, 50 m*M* or 100 m*M* in NMF. However, when using increasing concentrations of magnesium acetate above 100 m*M* in NMF (Fig. 1A) the selectivity in the separation of TC, OTC and CTC increased. Magnesium acetate tetrahydrate is very soluble in NMF and it is possible to dissolve approximately 800 m*M* in NMF. However, due to a high current, 500 m*M* was found to be the practical limit. Electropherograms of the separation of TC and related substances using metal complexation as well as the previously described acidic non-aqueous CE-system [30] are shown in Fig. 2A,B. In the acidic CE-system (Fig. 2A) the migration order of the pairs ATC and TC as well as EATC and ETC correspond to the expected mass-to-charge ratio based on the the molecular weight of the solutes. Compared to Fig. 2A, the migration order of ATC and TC has been reversed in the metal complexation non-aqueous CE-system (Fig. 2B). Moreover, the migration order of TC and EATC has been reversed. Thus the metal complexation may induce significant selectivity changes. The mass-to-charge ratios of the analytes may be influenced by differences the degree of complexation, basicity of the electrophoresis medium or differences in the solvation of the analytes in the organic solvent used.

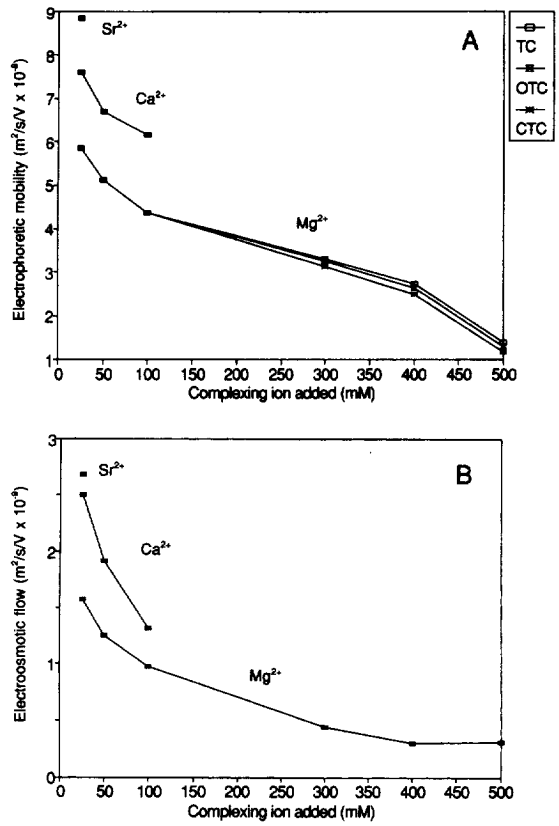

Fig. 1. (A) Effect of strontium acetate hemihydrate (Sr^{2+}), calcium acetate (Ca^{2+}) as well as magnesium acetate tetrahydrate (Mg^{2+}) on the electrophoretic mobilities of TC, OTC and CTC in NMF. (B) The effect of the complexing agents Sr^{2+}, Ca^{2+} or Mg^{2+} on the electroosmotic flow measured using β-naphthol. Experimental conditions: Fused-silica capillary 75 μm I.D.×27 cm (20 cm to the detector); voltage: 15 kV; temperature: 20°C; detection: laser-induced fluorescence with exitation at 325 nm and emission at 514 nm; injection time: 3 s.

The tetracyclines were dissolved in NMF with concentrations from 0 to 500 m*M* of magnesium acetate added in order to study the effect of the sample matrix as well as the injection time (1–30 s)

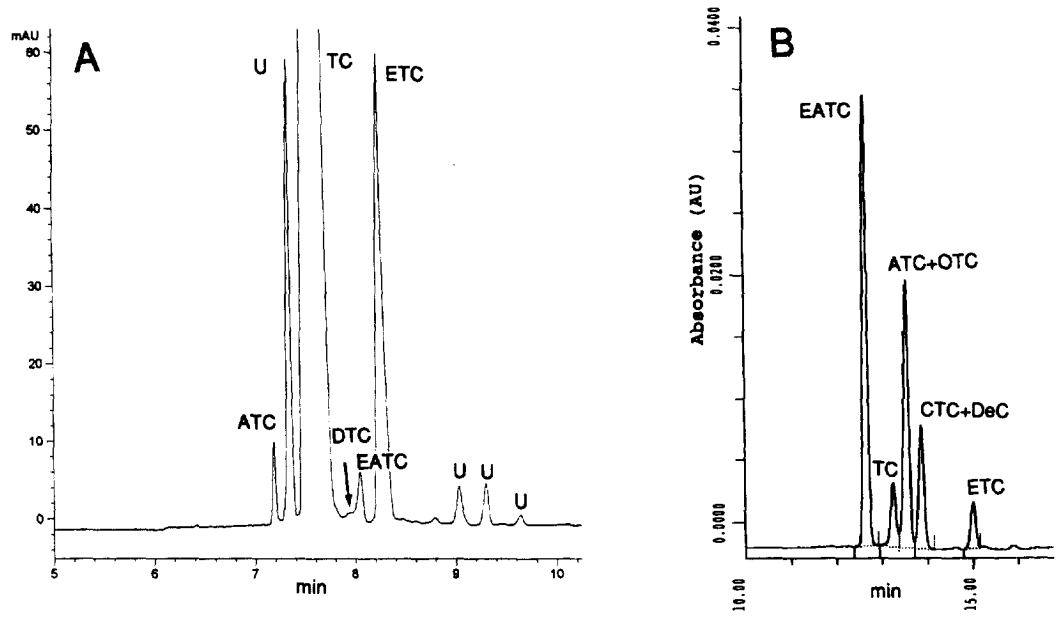

Fig. 2. Separation of tetracycline and related substances. (A) Electropherogram of tetracycline hydrochloride raw material 5.0 mg ml^{-1} dissolved in methanol–acetonitrile–DMF (45:49:6, v/v/v) obtained using an acidic non-aqueous CE system [30]. Experimental conditions: apparatus HP^{3D} CE-system; fused-silica capillary 50 μm×64 cm (55.5 cm to the detector); electrophoresis medium: 25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid in methanol–acetonitrile–DMF (45:49:6 v/v/v); voltage: 30 kV; temperature: 25°C; detection at 254 nm; injection: 1 s at 5 kPa; produced current: ca. 65 μA. (B) Electropherogram of tetracycline and selected related substances in concentrations in the range 0.2–0.8 mg ml^{-1} in NMF. Experimental conditions: fused-silica capillary 75 μm I.D.×27 cm (20 cm to the detector); electrophoresis medium: 500 m*M* magnesium acetate tetrahydrate in NMF; voltage: 15 kV; temperature: 20°C; detection: UV at 280 nm; injection: 3 s at 3.5 kPa; produced current: ca. 100 μA. TC=tetracycline, CTC=chlortetracycline, OTC= oxytetracycline, DeC=demeclocycline, ETC=4-epitetracycline, ATC=anhydrotetracycline, EATC=4-epianhydrotetracycline, DTC= desmethyltetracycline, U=unknown.

on the separation performance in an electrophoresis medium consisting of 500 m*M* magnesium acetate in NMF. Sample stacking was found to be most efficient in this system when injecting a sample containing 50 m*M* magnesium acetate. Separation with a good efficiency was achieved even when injecting the tetracylines in neat NMF. This indicates rapid formation of positively charged complexes in the electrophoresis medium. No attempt was made to investigate if the formation of the complexes were reversible. The number of theoretical plates obtained with the system described in Fig. 2B range from 65 000 to 80 000.

3.2. Electroosmotic flow

The electroosmotic flow decreased with increasing concentrations of magnesium acetate tetrahydrate or calcium acetate in NMF (Fig. 1B) opposed to the fact that the electrophoresis medium becomes more basic with an increasing concentration of acetate. However, the EOF is expected to decrease with increasing ionic strength in the electrophoresis medium at constant pH due to changes in the electric double layer resulting in a decrease of the Zeta potential. In aqueous systems magnesium ions as well as calcium ions are known to form complexes with silicic acid [33] and to adsorb to the silica surface [34]. Thus the magnesium ions may partly mask the silanol groups responsible for the electroosmotic flow. Furthermore, it has earlier been demonstrated that the presense of water in NMF decreases the electroosmotic mobility [8,35]. The water of hydration added with 500 m*M* magnesium acetate tetrahydrate corresponds to a water content in NMF of 3.6%. According to earlier described results this

may reduce the electroosmotic mobility by approximately 20% compared to neat NMF [8,35]. The observed decrease of the electrophoretic mobility is 81% comparing 25 m*M* magnesium acetate in NMF to 500 m*M* magnesium acetate in NMF. Thus the decrease can only partly be explained by the water added due to the magnesium acetate tetrahydrate. Hence, masking of the silanol groups as well as the increasing ionic strength leading to a reduction of the zeta potential may be the more important factors in the decrease of the EOF.

3.3. Detection of tetracyclines in biological samples

The applicability of the method is shown by two examples, namely the detection of tetracyclines in milk and in plasma. The method is not validated thoroughly as development of rugged methods for determination of tetracyclines in biological fluids are beyond the scope of this paper. Thus only a few but essential validation data for determination of oxytetracyline in milk were determined. The linear regression coefficient (r^2) was found to be 0.999 in the range from 50 ng ml^{-1} to 1000 ng ml^{-1} of OTC in milk. However, the calibration curve was not linear above ca. 1000 ng ml^{-1}. Thus the samples with a high concentation of OTC were diluted to fit the range of the calibration curve. The recoveries obtained at the concentration level of 100 ng ml^{-1} of OTC were 97.2% (R.S.D.=4.2%, n=6) and 63.3% (R.S.D.=3.6%, n=6) for 2.5-ml and 5.0-ml milk samples applied to the extraction microcolumn, respectively. The decrease in the recovery when increasing the volume of the sample applied was found to be due to overload of the microcolumn at the sample application step. The usual capacity of the microcolumns used are 1–2 ml of a serum or an urine sample. The repeatability of the method was evaluated and the relative standard deviations (n=6) were found to be 3.6%, 5.4% and 10.2% at 100 ng ml^{-1}, 1000 ng ml^{-1} and 10 000 ng ml^{-1} of OTC

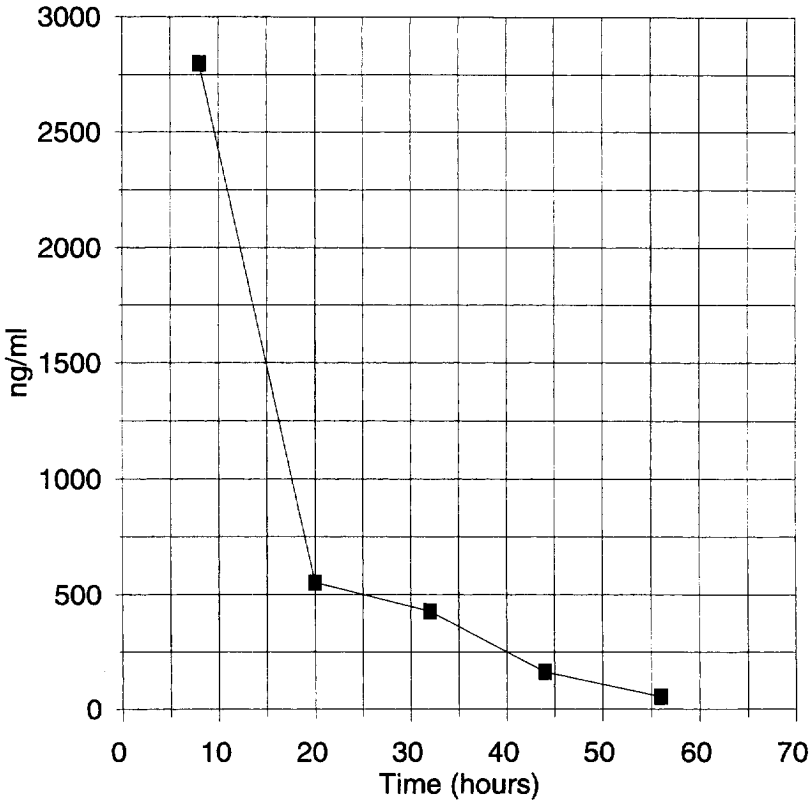

Fig. 3. Concentrations of OTC in cow milk after treatment with a single dose of 4000 mg OTC i.v.

in milk, respectively. The semi-validated method was used to determine the excretion of tetracycline in cows milk (5-ml samples) after treatment with a single dose of 4 g of oxytetracycline (Fig. 3).

An increase of the injection time from two to 25–30 s resulted in less efficiency and decreasing migration times (Fig. 4) but the reduction of the resolution is less than that expected from the fact that ca. 15% of the capillary is filled with the sample. Sahota and Khaledi have previously demontrated that the band broadning arising from injecting of a large sample volume into 250 m*M* of sodium dihydrogen phosphate in formamide was small compared to the result obtained using an aqueous medium with comparable conductivity [1]. An injection time of 25 s combined with a concentration procedure (factor of 25) enabled us to detect 25 ng ml^{-1} of TC, OTC or CTC in a spiked milk or plasma sample. The electropherograms obtained are shown in Fig. 5A,B. The relatively poor resolution of the three tetracyclines is due to the large volume injected. The detection limits reported for the most sensitive HPLC method decribed for the determination of tetracyclines (TC, OTC and CTC) in milk are in the range from 1 ng ml^{-1} to 4 ng ml^{-1} [20]. This separation was performed within 12 min and the sample preparation provided a concentration factor of 5 and used an injection volume of 100 μl of sample onto the chromatographic column. Detection limits for TC's using capillary electrophoresis with UV-detection of 1.3–5.3 ng ml^{-1} has recently been reported [26], but a very tedious sample preparation procedure of concentrating 10 ml of milk to a volume 20 μl was needed and an injection time of 5 s corresponding to 5 nl injected, was applied.

4. Conclusions

The results in this study suggest that metal com-

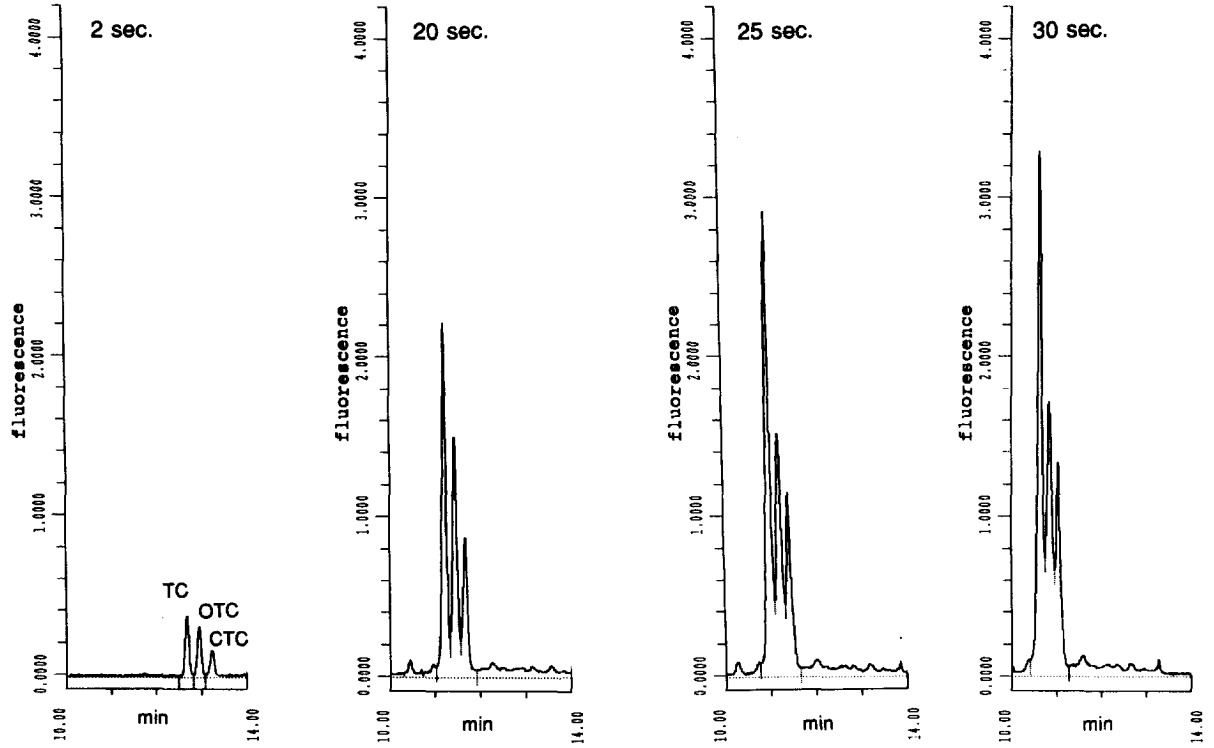

Fig. 4. Effect of increasing the injection time on the separation of 10 μg ml^{-1} of TC, OTC and CTC in NMF. Experimental conditions: fused-silica capillary 75 μm I.D.×27 cm (20 cm to the detector); electrophoresis medium: 500 m*M* magnesium acetate tetrahydrate in NMF; voltage: 15 kV; temperature: 20°C; detection: laser-induced fluorescence with exitation at 325 nm and emission at 514 nm; produced current: ca. 100 μA; injection time: as indicated on the figure.

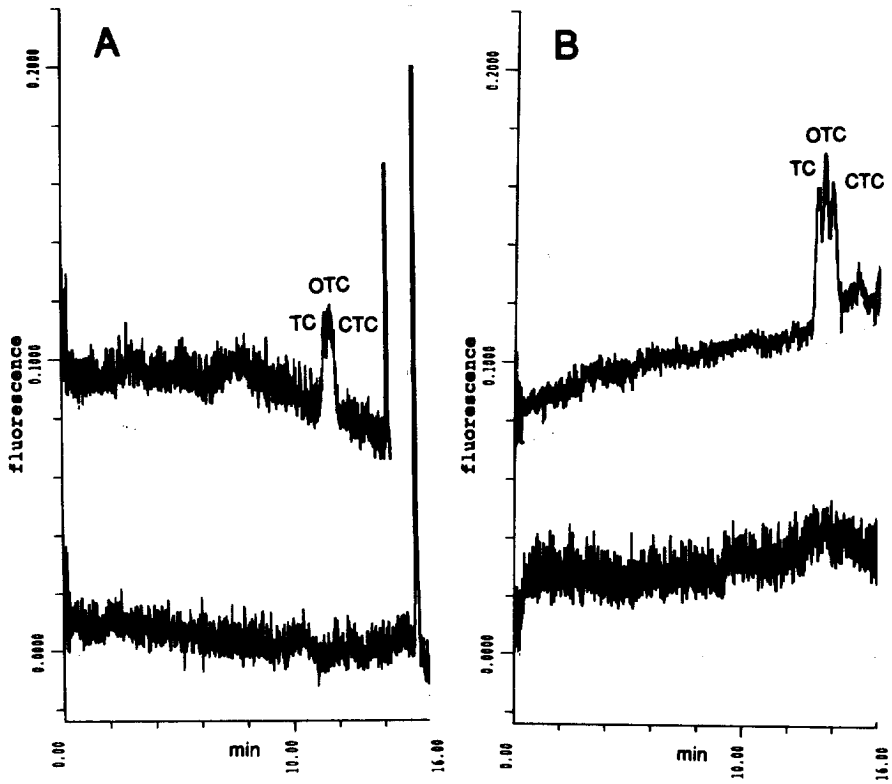

Fig. 5. Electropherograms of plasma and milk samples. (A) Blank milk sample (lower) and milk spiked with 25 ng ml^{-1} TC, OTC and CTC (upper). (B) Blank plasma sample (lower) and plasma spiked with 25 ng ml^{-1} TC, OTC and CTC (upper). Experimental conditions as in Fig. 4. Injection time: 25 s.

plexation of organic molecules can be used to obtain separation selectivity in non-aqueous CE systems. Furthermore, it is demonstrated that the use of organic solvents in place of water may further enhance the fluorescence and thus improve the detections limits obtained in CE.

Acknowledgments

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