Journal of Chromatography, 608 (1992) 85–92
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 258

Indirect time-resolved luminescence detection in capillary zone electrophoresis

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ABSTRACT

Time-resolved luminescence of the terbium(III)–acetylacetone chelate has been used as an indirect detection method for capillary zone electrophoresis. Three different modes were investigated: dynamic quenching of the background signal by selected anions; ligand displacement of acetylacetone by other complexing agents; and electrophoretic displacement of acetylacetonate by anions in general. The latter two modes required the use of a post-capillary reactor; in this study, a coaxial flow-type reactor was used for this purpose. The results of dynamic quenching were good for the determination of nitrite, yielding detection limits of $3 \cdot 10^{-9}$ *M* (0.2 ppb). In addition, the feasibility of ligand and electrophoretic displacement was demonstrated. However, improvement of the post-capillary reactor and substitution of the xenon lamp by a focused laser beam is required to exploit fully the potential of the ligand and electrophoretic displacement options.

INTRODUCTION

Capillary zone electrophoresis (CZE) gives a fast and efficient separation of ionic compounds. Indirect detection methods [1] can be applied for those compounds without a suitable chromophore. Among the different approaches described so far are indirect UV absorbance [2–5], indirect laser-induced fluorescence [6–8] and indirect amperometric methods [9]. According to Yeung and Kuhr [1], the attainable detection limit, C_{lim} (in concentration units), is given by the following equation

$$C_{lim} = \frac{C_b}{DR \cdot TR} \quad (1)$$

where C_b represents the concentration of the buffer ion which generates the background signal, *DR* is the dynamic reserve and *TR* the transfer ratio. From eqn. 1 it can be seen that C_b should be as low as possible while still generating a sufficient background signal. The transfer ratio will depend on the mobilities of the analyte and the background ion and might deviate significantly from unity [5]. The dynamic reserve should be as high as possible. Some representative data are summarized in Table I.

The UV detector gives a good performance with respect to *DR*; its limited sensitivity, however, demands high concentrations of the background ion. Both conventional and laser-induced fluorescence methods yield relatively low *DR* values due to instability of the source and scattered light. Only after extensive modifications [7] can the situation be improved.

As an alternative, indirect time-resolved luminescence methods using pulsed sources and gated photomultipliers can be considered. The absence of scattered excitation light will be beneficial, especially at cylindrical flow cells such as CZE capillaries.

Relatively inexpensive luminescence detectors, designed for high-performance liquid chromatography (HPLC), allow time-resolved measurements in the microsecond domain. Thus fluorophores or phosphorophores having relatively long lifetimes

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TABLE I

DYNAMIC RESERVE AND DETECTION LIMITS FOR INDIRECT DETECTION MODES IN CZE

Limits of detection (LOD) at a signal-to-noise ratio of 2.

Detection mode[a]	Background ion	C_b (m*M*)	*DR*	LOD (mol/l)	Ref.
UV	Veronal	6	1100	$1.5 \cdot 10^{-5}$	5
Flu	Quinine	0.1	170	$1.0 \cdot 10^{-6}$	[b]
Flu	Salicylate	0.25	180	$3.0 \cdot 10^{-6}$	[b]
LIFlu	Quinine	0.4	380	$2.0 \cdot 10^{-6}$	8
LIFlu	Salicylate	0.25	270	$2.0 \cdot 10^{-6}$	6
LIFlu	Salicylate	0.05	>1000	$1.0 \cdot 10^{-7}$	7

[a] Flu = fluorescence; LI = laser-induced.
[b] Using the detector as described in this paper.

are required. Europium chelates fulfill this requirement and are widely used as labels in time-resolved fluorescence immunoassays [10]. Terbiumchelates have been used in spectrofluorimetric determinations of trace amounts of protein [11] and of salicylate in biological samples [12]. Baumann *et al.* [13] proposed dynamic quenching of europium(III) and terbium(III) luminescence as a detection method for ion chromatography. A decrease in background signal is observed for those analytes able to reduce the luminescence quantum yield, *i.e.* the detection limit is not restricted by the concentration of the background ion and does not follow eqn. 1. The method can be improved by excitation of the lanthanide acetylacetone chelates (instead of direct excitation of the lanthanide ions) [14]. Only a few anions [nitrite, chromate, hexacyanoferrate(II) and hexacyanoferrate(III)] respond, thus the dynamic quenching mode offers both sensitivity and selectivity in ion chromatography.

In this study, the time-resolved luminescence of the terbium(III)–acetylacetone (Tb–acac) chelate was used as a background signal for indirect detection in CZE. Three different modes have been investigated: dynamic quenching of the signal (selective mode); ligand displacement (less selective mode); and electrophoretic displacement (universal mode). Only the latter mode is comparable with the other systems in Table I and follows eqn. 1.

Both ligand and electrophoretic displacement required the use of a post-capillary reactor [15].

EXPERIMENTAL

Apparatus

Experiments were carried out using a laboratory-made CZE system consisting of an F.u.G. (Rosenheim, Germany) Model HCN 35-35000 power supply operated at +25 or −25 kV, a Plexiglas cabinet equipped with safety interlocks and a Perkin-Elmer (Beaconsfield, UK) Model LS-40 luminescence detector. Unless stated otherwise, the detector was operated at an excitation wavelength of 295 nm. The emission monochromator was replaced by the total emission mirror accessory combined with the 430 nm cutoff filter. Time-resolved luminescence was obtained using a delay time of 0.1 ms and a gating time of 0.5 ms. The original flow cuvette of the detector was removed and replaced by the CZE capillary, without additional optimization or focusing of the optical system. The detection window on the CZE capillary was about 4 mm long. CZE capillaries were either fused silica (Polymicro Technologies, Phoenix, AZ, USA), 120 cm × 50 μm I.D./180 μm O.D., or a synthetic hollow fibre [16], 88 cm × 50 μm I.D./350 μm O.D. Samples were injected either hydrodynamically by raising the injection end and the sample at a specific height and time, or electrokinetically by applying a voltage of −10 kV for a specific time.

Some preliminary experiments were carried out using an Applied Biosystems (San Jose, USA) Model 270A capillary electrophoresis system [17] coupled with the detector described above.

Post-capillary reactor

Ligand and electrophoretic displacement required the use of a post-capillary reactor. The reactor was of the coaxial flow type and was constructed according to Rose and Jorgenson [15]. The CZE capillary, 50 μm I.D./180 μm O.D., was inserted into a 200 μm I.D./350 μm O.D. reaction capillary. In this work, the reaction is actually a displacement only, thus hardly any reaction time is required. To allow mixing, the CZE capillary was inserted to 1 mm before the beginning of the detection window in the reaction capillary. The reagent was delivered via a stainless-steel tee using an Applied Biosystems Model 140B syringe pump, equipped with an Acurate (LC-Packings, Amsterdam, Netherlands) Model 100 flow splitter and a restriction capillary of 5 m × 50 μm I.D.

The post-capillary reactor was not optimized in this work. Optimization can be carried out by etching the outer diameter of the CZE capillary and by improved matching with the inner diameter of the reaction capillary [15], or by the use of a focused laser beam [18].

Chemicals

Terbium(III) chloride hexahydrate and acac were obtained from Aldrich (Steinheim, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany). Distilled water was purified in a Milli-Q apparatus (Millipore, Bedford, MA, USA).

Methods

Buffers were prepared in Milli-Q water and adjusted to a specific pH with 0.1 *M* sodium hydroxide solution using a Philips (Cambridge, UK) Model PW 9409 pH meter. Stock solutions of the analytes under investigation were prepared in Milli-Q water at $1 \cdot 10^{-3}$ *M*. Samples were freshly diluted or their pH was adjusted in accordance with the buffer system under investigation, or both. Buffers and samples were filtered through 0.45 μm Spartan 30/B filters (Schleicher & Schuell, Dassel, Germany) before use.

RESULTS AND DISCUSSION

Dynamic quenching mode

Initial experiments were performed using direct excitation of Tb(III), *i.e.* without acac ligands, at 225 nm in an electrolyte system consisting of 5 m*M* $TbCl_3$ in water.

Not surprisingly, the performance was poor. The maximum *DR* value obtained was 70 due to the noisy baseline and the low background signal obtained. The output of the xenon lamp and the molar extinction coefficient of Tb(III) are both low at this wavelength. Indirect excitation of Tb(III) via its acac chelate was expected to yield better results. The excitation and emission maxima were determined and found to be 295 and 545 nm, respectively. The optimum detector settings as described under Experimental, combined with a buffer system consisting of 0.35 m*M* $TbCl_3$, 0.8 m*M* acac, 6 m*M* NaCl at pH 8.3 yielded a *DR* value of 360. Compared with the (conventional) fluorescence data in Table I, the *DR* value has increased significantly as a result of the time-resolved measurement. An even bigger increase might have been expected. However, it should be noted that the optics of the detector used were not designed nor optimized for the CZE capillary.

In addition, the chemistry involved might significantly contribute to the baseline noise (chemical noise). Tb ions tend to adsorb onto the capillary wall, complex with acetylacetonate and migrate, free or complexed, in the electric field.

A mixture of nitrite, chromate and hexacyanoferrate(III), $3 \cdot 10^{-5}$ *M* each, was separated by CZE at −25 kV and detected using the dynamic quenching mode. A typical electropherogram is shown in Fig. 1. The negative peaks obtained cannot be attributed to electrophoretic displacement of acac because of the excess of chloride ions in the buffer and the complexation of acac with the Tb(III) ions. The results for nitrite were promising but the other two ions, especially chromate, showed a lot of tailing. Tailing has also been observed in HPLC systems for the analysis and detection of these compounds by dynamic quenching of Tb–acac [14,19]. Contrary to the suggestion in these papers, interaction with metal parts cannot be responsible for tailing observed in this fused-silica capillary. The behaviour of these two ions in this CZE system resembles an adsorption mechanism: the respective electrophoretic mobilities are much lower than the theoretical values and a typical concentration dependency occurs on dilution of the mixture in Fig. 1.

In contrast to nitrite, chromate and hexacyanoferrate(III) tend to disappear completely at $1 \cdot 10^{-6}$

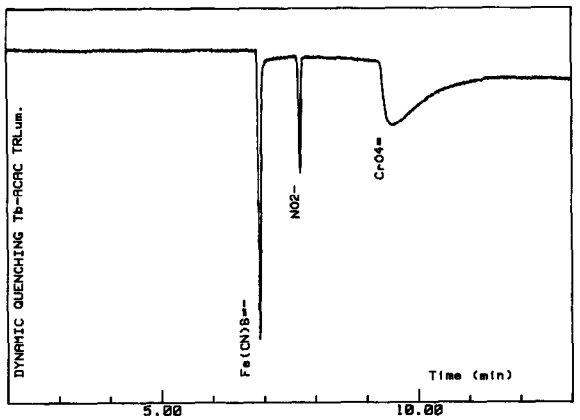

Fig. 1. Electropherogram of $3 \cdot 10^{-5}$ *M* nitrite, chromate and hexacyanoferrate(III) using dynamic quenching of Tb–acac luminescence. Conditions: Applied Biosystems Model 270A apparatus equipped with a fused-silica capillary, 120 cm × 50 μm I.D., coupled with the luminescence detector; CZE at −25 kV in 0.8 m*M* acac, 0.35 m*M* $TbCl_3$, 6 m*M* NaCl, pH 8.3 (in water); other conditions as given under Experimental.

M. These observations might be explained by one or more of the following mechanisms: adsorption onto wall-adsorbed Tb(III) ions; precipitation after complexation with Tb–acac; unintended electrochemical reactions in the CZE system. In this study, aimed at determining the general principles and feasibility of indirect detection in CZE using Tb–acac, chromate and hexacyanoferrate(III) were simply excluded from the test mixture and experiments in the dynamic quenching mode were continued with nitrite alone.

The performance of this system was investigated using a CZE capillary of 113 cm length (71 cm to detector) and a buffer system consisting of 6 m*M* NaCl, 1 m*M* acac, 0.2 m*M* $TbCl_3$ at pH 7.4. Nitrite samples ($3 \cdot 10^{-5}$ *M* in water) were injected hydrodynamically (14 cm, 20 s). The nitrite peaks obtained showed a plate number of 130 000. The repeatabilities of the migration time and the peak height were ±1% relative standard deviation (R.S.D.) (n = 6) and ±3.8% R.S.D. (n = 6), respectively. The lower limit of detection (signal-to-noise ratio-2) was $2 \cdot 10^{-7}$ *M*, which is fairly good for a capillary separation technique (*cf.* Table I) and comparable with the results obtained with high-performance liquid chromatographic (HPLC) instruments [13, 14]. It should be noted that the theoretical detection limit for 1% dynamic quenching of the Tb(III)–acac signal by nitrite is $1 \cdot 10^{-7}$ *M* [14], so the results presented here are in good agreement. The lower limit of detection can be simply improved in CZE by using focusing techniques.

We have successfully applied field amplified sample injection [20]. A tapwater sample spiked with $1 \cdot 10^{-7}$ *M* nitrite was injected electrokinetically at −10 kV for 15 s. The electropherogram obtained (Fig. 2) shows a good selectivity towards other ionic species normally present in this matrix. The lower limit of detection can be calculated using the Stern–Volmer equation [13] and was found to be $3 \cdot 10^{-9}$ *M* or 0.2 ppb, which is, as far as is known, superior to any other separation technique for the selective determination of nitrite in aqueous samples. In addition, the method presented here will cost much less than a comparable ion chromatographic system. An interesting application would be the determination of nitrite (often used as a food preservative and a known precursor of carcinogenic N-nitrosamines) in aqueous extracts of processed meats [21].

Ligand-exchange mode

The second indirect detection option using Tb (III)–acac time-resolved luminescence is based on ligand-exchange principles. Any analyte which is able to form a complex or a mixed complex, *i.e.* any analyte having a complexation constant with Tb(III) which is higher than (or comparable with) Tb(III)–acac and which is unable (or less able) to provide indirect excitation of Tb(III) ions at 295 nm, will cause a negative peak on the background signal. A polycarboxylic acid, known as a complexing agent, showed good sensitivity in both batch and flow injection experiments [22].

In this CZE experiment, it was preferred to separate the complexing agents EDTA and nitrilotriacetic acid (NTA) as such, *i.e.* in their uncomplexed form. Consequently, $TbCl_3$ had to be excluded from the CZE separation system and added after the capillary using the coaxial flow reactor (see under Experimental). The performance of the post-capillary reactor was tested using hydrodynamic injections of nitrite ($3 \cdot 10^{-5}$ *M*). At a reagent flow-rate of 0.0 μl/min the plate number was 1600, which is in good agreement with published data for a 200 μm I.D. reaction capillary at low flow-rates [15]. The plate number increased towards 7000 at the reagent flow-rate of 0.55 μl/min used in this study.

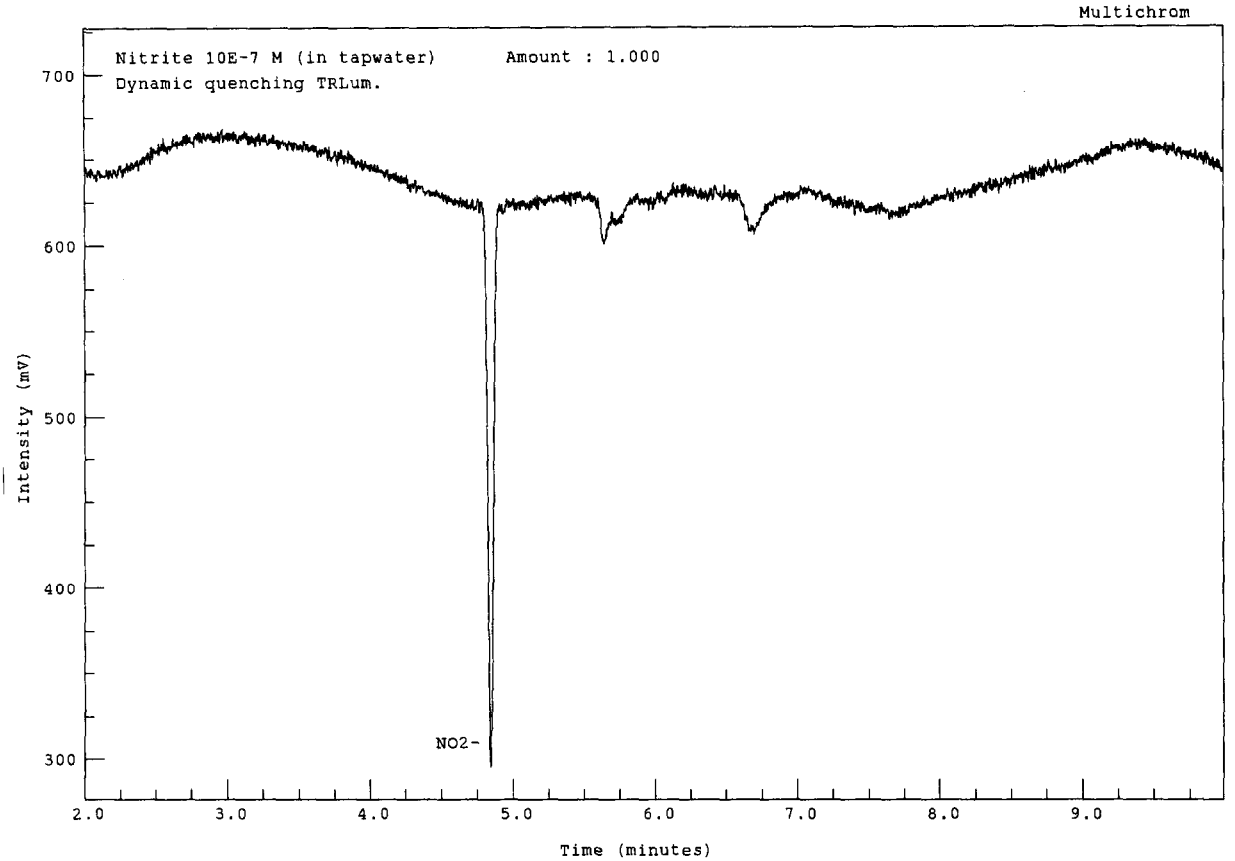

Fig. 2. Electropherogram of tapwater spiked with $1 \cdot 10^{-7}$ *M* nitrite using dynamic quenching of Tb–acac luminescence. Conditions: CZE at −25 kV in 1 m*M* acac, 0.5 m*M* $TbCl_3$, 10 m*M* NaCl, pH 7.1 (in water–methanol, 75:25); capillary, synthetic hollow fibre, 88 cm × 50 μm I.D. Apparatus and other conditions as given under Experimental.

The efficiency of the coaxial flow reactor can be significantly improved (plate numbers up to 50 000–600 000) by etching the outer diameter of the CZE capillary and decreasing the inner diameter of the reaction capillary, or using a focused laser beam as an excitation source, or both [15,16]. However, this plate number was considered to be sufficient for the purpose of demonstrating the ligand-exchange option using Tb–acac luminescence.

Complexing agents ($5 \cdot 10^{-4}$ *M*) were injected hydrodynamically into a CZE electrolyte system consisting of 10 m*M* NaCl and 1.4 m*M* acac at pH 7.1. $TbCl_3$ (0.5 m*M*, pH 7) was added coaxially at a flow-rate of 0.55 μl/min. Matching the pH of the CZE electrolyte and the reagent was a prerequisite for adequate mixing and a stable background signal. The results obtained are shown by the electropherograms in Fig. 3. In contrast with Figs. 1 and 2, a strong electro-osmotic flow in the direction of the cathode is induced due to the absence of Tb^{3+} ions in the CZE capillary. The complexing agents EDTA (Fig. 3A), NTA (Fig. 3B) and the commercially available technical mixture (Fig. 3C) show relatively large responses (peak area/time), demonstrating the potential of this ligand-exchange detection mode. The negative peaks are not caused by electrophoretic displacement of acac because of the excess of chloride ions present in the electrolyte system. The plate numbers and peak shapes are poor compared with Fig. 2. These discrepancies can be explained by the poor performance of the coaxial flow reactor in general and electromigration dispersion caused by differences in effective mobilities between analytes and buffer ions [23].

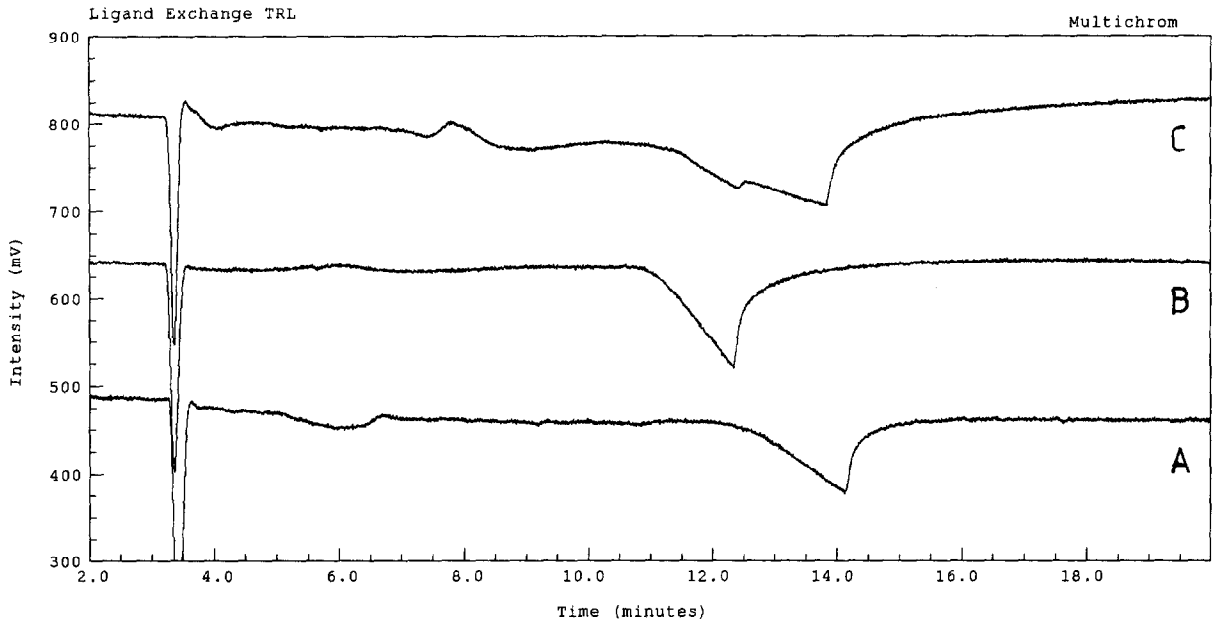

Fig. 3. Electropherogram of (A) $5 \cdot 10^{-4}$ *M* EDTA, (B) NTA and (C) a technical mixture using ligand-exchange Tb–acac luminescence. Conditions: CZE at +25 kV in 1.4 m*M* acac, 10 m*M* NaCl, pH 7.1 (in water); post-capillary addition of 0.5 m*M* $TbCl_3$ at 0.55 μl/min; total length of capillaries, 108 cm, CZE fused-silica capillary 63 cm × 50 μm I.D., reaction capillary 200 μm I.D. Apparatus and other conditions as given under Experimental.

Electrophoretic displacement mode

The third option for indirect detection using the Tb–acac luminescence is electrophoretic or charge displacement. The principles of this mode are the same as for the indirect UV or fluorescence methods (see Table I). In this instance only acac should be present as background electrolyte in the CZE system, so $TbCl_3$ should be added after the capillary (*cf.* ligand-exchange mode).

The pK_a value of acac was determined by recording a titration curve and was found to be 8.76. This value is attractive for CZE separation using electroosmotic flow and, in addition, the buffering capability will provide a stable system.

C_9 and C_{12} linear alkylsulphates ($1 \cdot 10^{-3}$ *M* in water) were injected hydrodynamically in a CZE electrolyte consisting of 10 m*M* acac buffer at pH 8.5. $TbCl_3$ (0.25 m*M*) was added after the capillary at a flow-rate of 3.0 μl/min.

CZE was performed at +25 kV and indirect detection was easily obtained (Fig. 4). The plate number increased to 12 000 as a result of the increased reagent flow-rate. It can be seen that these linear alkylsulphates are partly separated in this system. This unoptimized post-capillary reaction system cannot compete with the indirect UV detection system [5] in which baseline resolution and 400 000 theoretical plates were obtained. Nevertheless, the present indirect detection option might become a serious alternative when the post-capillary reactor [15,18] has been improved or the indirect UV system [5] cannot be used because of interfering compounds with a chromophore, or both.

CONCLUSIONS

Indirect time-resolved luminescence detection using the Tb(III)–acac chelate has been applied to CZE using a conventional HPLC luminescence detector. Three different indirect detection modes have been demonstrated: dynamic quenching of the background signal; ligand displacement by complexing agents; and electrophoretic or charge displacement. The dynamic quenching mode offers unequalled sensitivity (less than pbb levels can be detected) and selectivity for nitrite in aqueous

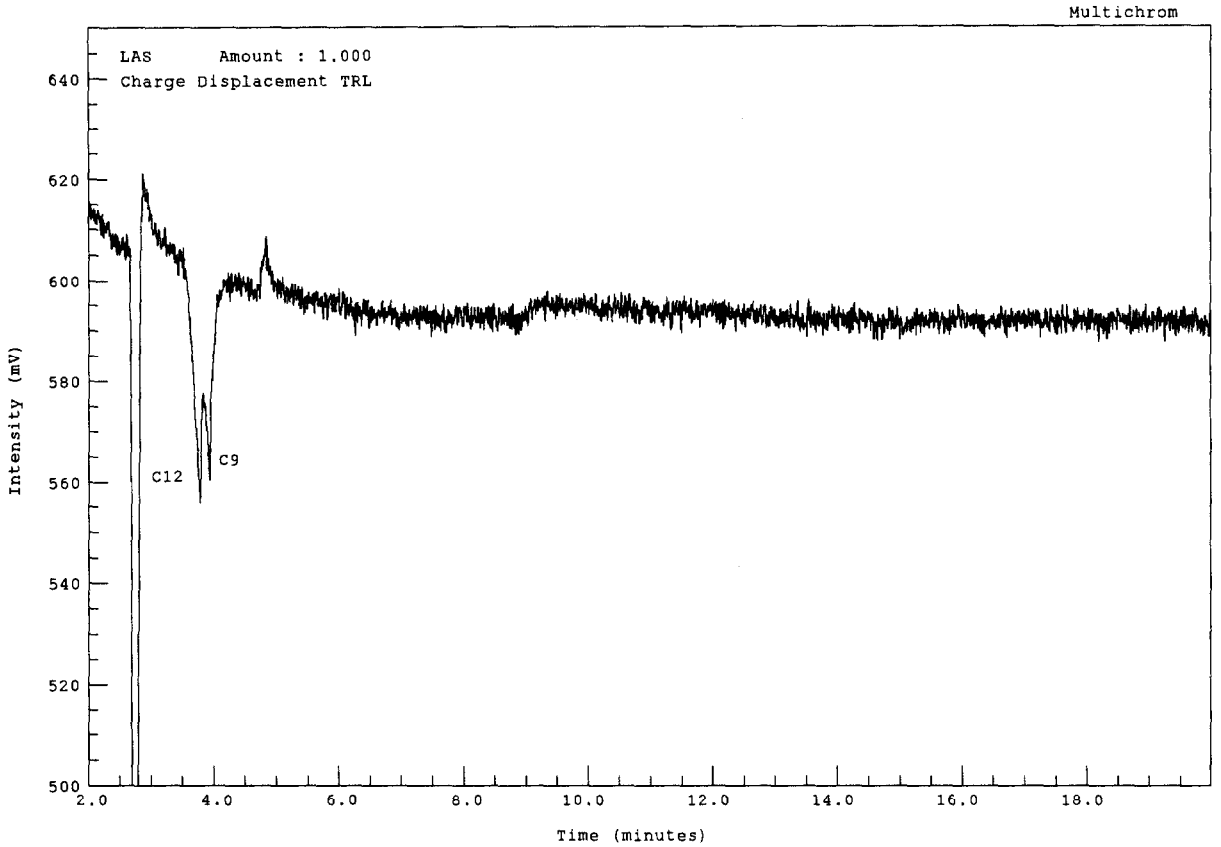

Fig. 4. Electropherogram of a mixture of $1 \cdot 10^{-3}$ *M* nonyl- and dodecylsulphate using electrophoretic displacement of acac. Conditions: CZE at +25 kV in 10 m*M* acac, pH 8.5 (in water); post-capillary addition of 0.25 m*M* $TbCl_3$ at 3.0 μl/min. Other conditions as in Fig. 3.

samples. The feasibilities of the ligand and electrophoretic displacement options have been preliminarily shown, but were adversely affected by the low plate numbers caused by the unoptimized coaxial flow reactor. Further improvement of the post-capillary reactor following the guidelines of Jorgenson and co-workers [15,18] will be required to exploit fully the potential of the ligand and electrophoretic displacement modes.

ACKNOWLEDGEMENTS

M. Schreurs, C. Gooijer and N. H. Velthorst (Free University, Amsterdam, Netherlands) are acknowledged for stimulating discussions.

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