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Analysis of dimeric cyanine–nucleic acid dyes by capillary zone electrophoresis in *N*,*N*-dimethylacetamide as non-aqueous organic solvent

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Abstract

A method based on capillary zone electrophoresis is presented for the determination of the purity of commercial dimeric cyanine dyes (TOTO, YOYO, BOBO, all -1 and -3 species, LOLO-1, POPO-1) that are common as fluorescent probes for nucleic acid staining. These dyes are tetracharged cations, and have a strong tendency to interact with negatively charged centres, where they are rapidly adsorbed, especially from aqueous solutions. Thus anionic sites at the capillary wall must be avoided, and aqueous buffers are not suitable. The method introduced here avoids both complications, using non-aqueous *N*,*N*-dimethylacetamide as solvent, and suppressing the dissociation of silanol groups at the capillary surface due to selection of acidic separation conditions (20 mmol/l perchloric acid as background electrolyte). The present method enables the determination of the purity of all 10 dyes in less than 15 min. The selectivity of the method allows separation of at least five main and differentiating a number of unresolved minor contaminants as demonstrated in detail for TOTO-3 as an example. Quantitation (with 100% normalisation of the peak areas) of nine lots of this dye results in a purity between 33 and 87%.

Keywords: Non-aqueous capillary electrophoresis; Dyes; Cyanine–nucleic acid; Cyanine-nucleic acid dyes; Nucleic acid dyes; *N*,*N*-Dimethylacetamide

1. Introduction

Dimeric cyanine dyes are compounds, which form complexes with nucleic acids with strong fluorescing activity, and are thus used as fluorescent probes for staining of DNAs and RNAs. They have four permanently charged nitrogen atoms aligned in a stretched molecule. In Fig. 1 the structural formula for TOTO-3 is depicted, taken as a typical example pars pro toto.

Due to this structural property it can strongly interact electrostatically with negative charges, one of the reasons why it has high complex forming ability with polynucleic acids. TOTO, e.g., binds to single- or double-stranded DNA (ssDNA or dsDNA) [1–4]. Virtually non-fluorescing in solution, com-

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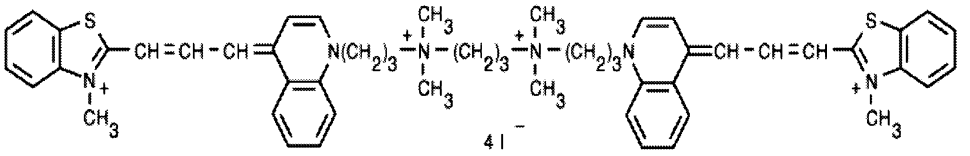

Fig. 1. Structural formula of TOTO-3.

plexing with DNAs leads to an enormous fluorescence enhancement [5]. Therefore these dimeric cyanine dyes gained an extraordinary importance for the detection, determination and quantitation of DNAs, with confocal laser scanning microscopy [6–8], or laser-induced fluorescence detection in electrophoresis [5,9–14].

Obviously the strong tendency to interact electrostatically is not limited to DNAs, but leads, e.g., to a pronounced adsorptivity to negatively charged sites of surfaces. When such active centres are present in separation systems like in chromatographic columns or in capillaries for electrophoresis, adsorption effects will lead to severe peak distortion or even to a loss of the sample due to irreversible adsorption. This is the main limitation that must be overcome when working out an appropriate analytical method for the determination of such dyes.

Another aspect is the limited stability of the dye in aqueous solutions, at least in the long-term range. Possible hydrolysis, but more pronounced the enhanced adsorptivity from aqueous solution cause this. For these reasons the dyes are stored and handled in non-aqueous solutions upon application to DNA samples, mostly in dimethylsulfoxide (DMSO). Although being unstable in free aqueous solution, the dye gains stability against hydrolysis once complexed with DNA. However, the risk of instability or adsorptive loss during analysis leading to biased quantitative results limits the application of aqueous phases in the separation systems.

In order to obtain correct results for the qualitative and quantitative determination of polynucleic acids after labelling, these agents must have a defined purity. Therefore an accurate, reproducible (and, favourably, a fast) method is needed to characterise the quality of commercial dye samples. Capillary zone electrophoresis (CZE) seems well suited for this goal, because the dyes are ionic. However, due to the restrictions discussed above the selection of the separation conditions seems to be not obvious. In the present method both main limitations were overcome by working in non-aqueous background electrolytes (BGEs) and avoiding negatively charged surfaces.

2. Experimental

2.1. Instrumentation

CZE analysis were carried out with a ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany) which was equipped with a diode array detector. Uncoated capillaries (Composite Metal Services, Hallow, UK) of 0.305 m (0.220 m to the detector)×50 μm I.D.×375 μm O.D., were used. Voltage was +10 kV, leading to a current of about 8.2 μA in the background electrolyte used. Injection of the sample was for 300 mbar s. Detection was at 254 and 590 nm (bandwidth 20 nm) in the case of TOTO-3, or at 254 nm and the particular λ_{max} for the other dyes.

2.2. Chemicals and BGE

The analytes were the commercially available dimeric cynanine–nucleic acid dyes (Molecular Probes, Eugene, OR, USA) BOBO-1, BOBO-3, JOJO-1, JOJO-3, POPO-1, POPO-3, TOTO,-1, TOTO-3, YOYO-1, and YOYO-3, all tetraiodides. They were dissolved in organic solvent at a concentration of 250 μmol/l (POPO-3 in DMF, all other dyes in DMSO), and were injected without further dilution.

Perchloric acid (70–72%, analytical grade; E. Merck, Darmstadt, Germany) and *N*,*N*-dimethylacetamide (DMA, 99.9%; Aldrich, Steinheim, Germany) were used. The organic BGE was a 20 mmol/l solution of $HClO_4$ in DMA. The water content of the

BGE was about 0.05% (v/v), as calculated from the composition of perchloric acid.

3. Results and discussion

3.1. Selection of the separation conditions

For reasons of stability as pointed out in the Introduction, and to enhance their solubility, non-aqueous solvents are preferable for the BGE in CZE of the analytes. As we have obtained good performance in previous work [15,16], DMA was selected here. Although this solvent is UV absorbing below 240 nm, detection of the dye components in the visible range, and even at 254 nm, is not hindered. This was one prerequisite for the selection of the BGE. The other was the avoidance of negatively charged sites in the capillary. Such adsorptive sites could be suppressed, e.g., by coating the capillary wall with an appropriate layer. However, this method might have restrictions concerning the stability of the coating in organic solvents. Moreover, the negative charges might not be quantitatively shielded by the layer, and thus adsorption is not fully suppressed especially in case of strong interaction. Thus we decided to avoid negatively charged surface simply by the selection of a sufficiently low pH*. The accurate adjustment of the pH* (that is problematic in non-aqueous solvents [17,18]) is out of the scope of the present problem; here it was sufficient to establish conditions under which the capillary surface has no electrostatic attraction to the cationic analytes. Under acidic conditions the surface charge stemming from silanolate ions can be eliminated, and even the reversal of the charge is possible. It is well known that perchloric acid is a strong electrolyte also in aprotic solvents like acetonitrile [19]. Therefore it can be assumed that in DMA (which is seemingly more basic than acetonitrile [20,21]) perchloric acid fully dissociates as well. A solution of this acid in the organic solvent will result in a positively charged capillary surface. This was indeed indicated by the weak electroosmotic flow (EOF) directed towards the anode with a BGE consisting of 20 mmol/l perchloric acid. It is clear that this BGE has no buffering ability, but this is not required due to the chemical properties of the analytes (the dyes are permanent ions). However, even weaker basic analytes will be protonised and move as cations in the CZE system selected.

3.2. CZE of dye samples

In Fig. 2 the electropherograms of samples of nine dyes obtained with the BGE as described above are shown (TOTO-3 is not included, because the analysis of this dye is treated in more detail below). It can be seen that all samples (with POPO-1 as exception) show one main peak at migration time around 8 min, with a variable number of minor peaks stemming from impurities or by-products. POPO-1, in contrast, gives no main peak, but two peaks with about the same area, and a number of smaller peaks as well. As it is not of interest for the present work to discuss the analysis of all dyes with the same detail; we

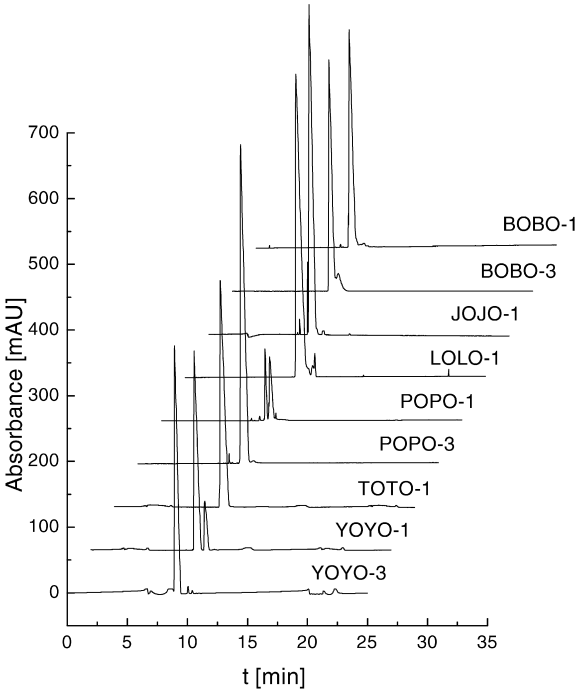

Fig. 2. Electropherograms of nine different dyes in DMA as solvent of the BGE. Conditions: uncoated fused-silica capillary, length 0.305 m (effective length 0.220 m)×50 μm I.D.; BGE, 20 mmol/l perchloric acid; sample concentration, 250 μmol/l TOTO-3 in DMSO. Injection at 300 mbar s. Voltage, +10 kV (~ 8.1 μA). Detection at λ_{max} of each dye.

demonstrate the applicability of the method on the example of TOTO-3 in the following.

3.3. Analysis of TOTO-3 samples

Electropherograms obtained from nine different lots of commercial TOTO-3 are shown in Fig. 3, together with the blank run. In addition to the main peak, a number of other peaks can be observed. It is obvious that accurate quantitation of the samples needs the response factors of the individual components. However, as even degradation products of the parent compound should contain an aromatic system (cf. Fig. 1), we assume a similar response factor for all compounds at least at 254 nm. The results of quantitation are given in Table 1, where the sum of the contaminants is related to TOTO-3. Under the given assumption the purity of the dye varies between 33 and 87%. The majority of the samples contain the main compound in the range of 70%.

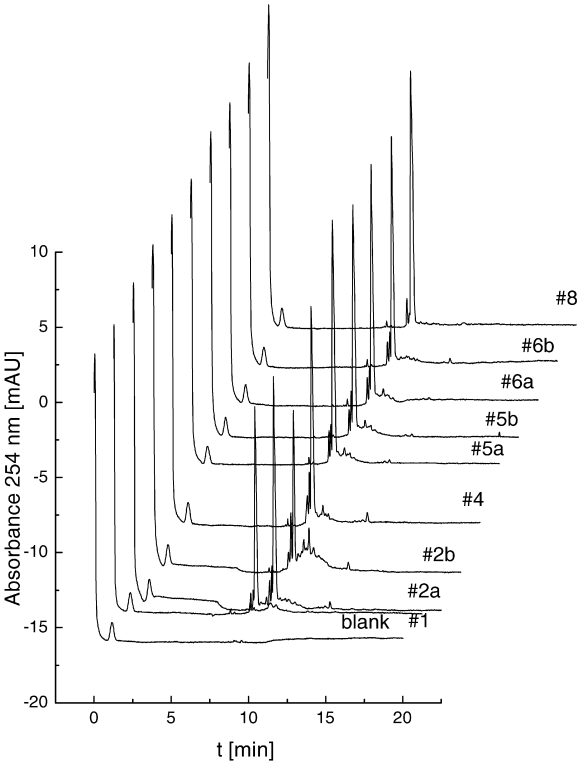

Fig. 3. Electropherograms of TOTO-3 from nine different lots. Conditions as in Fig. 2. UV detection at 254 nm.

Table 1
Purity of TOTO-3 in nine different lots

Lot	Main peak (span), %
1	69.6 (2.0)
2a	69.2 (0.1)
2b	33.3 (0.2)
4	67.5 (1.6)
5a	71.4 (0.4)
5b	72.1 (1.8)
6a	72.8 (3.3)
6b	76.7 (0.3)
8	87.4 (1.3)

Quantitation is based on 100% normalisation of the peak areas. The content is calculated from the mean peak area of the main peak from injections in duplicate; the span is given in the parentheses.

Information about the chemical nature of the contaminants was obtained from their optical properties derived from the CZE peaks. This is shown with one example; here analysis was made in capillaries with larger inner diameter (100 μm), because the sensitivity at higher wavelengths was not sufficient with the 50 μm I.D. tube. Better sensitivity due to selection of λ_{max} of the TOTO-3 spectrum could not be reached, because the maximum wavelength the detector records (600 nm) is lower.

The electropherograms recorded at 254 and 590 nm, given in Fig. 4, show that seemingly all sample contaminants have a significant absorbance at 590 nm. This finding allows the conclusion that (like the main component) the impurities have a large delocalised π electron system. This would not be the case when the compounds were the result of cleavage into only small molecules, say consisting from only one aromatic ring. We will prove this presumption by photolytical decomposition of TOTO-3.

3.4. Photodegradation of TOTO-3

The dyes are known to be light-sensitive, and must therefore not be stored in bright light. For photo-induced decomposition one solution (lot 8) was exposed to sunlight; aliquots were taken after certain periods and analysed. The resulting series of electropherograms is shown in Fig. 5.

It can be seen that the peak of the parent compound rapidly decreases, and a number of other

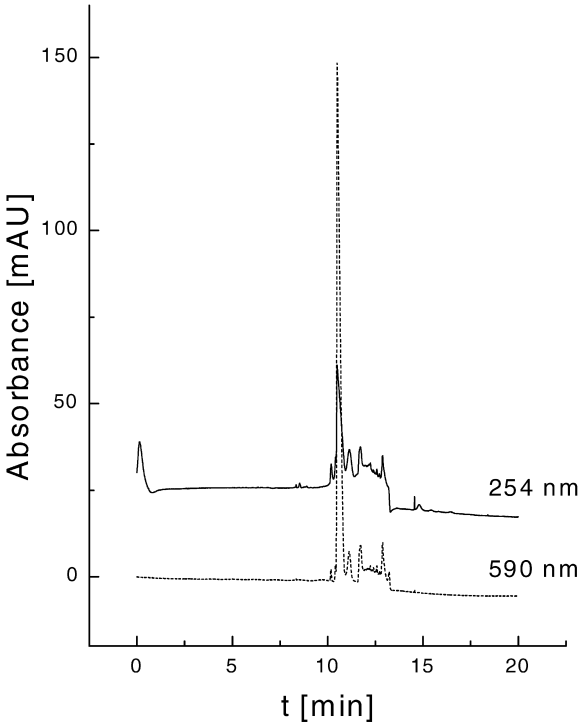

Fig. 4. Records of the electropherogram of a sample of TOTO-3 in a 100 μm I.D. capillary. Detection at 254 and 590 nm. Sample concentration, 250 μmol/l in DMSO. Injection at 200 mbar s. Capillary lengths as in Fig. 2. Voltage, +10 kV (~34.1 μA).

peaks arise in the electropherograms recorded at 254 nm, a wavelength that is indicative for aromatic systems. After 1 h the appearance of additional five peaks of the same size as the parent peak can be observed. Indication about the chemical composition of the reaction products delivers the electropherogram recorded at 590 nm in Fig. 6. The rapid decrease of the parent compound can be recognised, however, within 1 h no peak is visible anymore at this wavelength, in contrast to 254 nm. This result strongly indicates the fragmentation of the molecules with the large delocalised π-electron systems into smaller units.

The change in the concentration of the parent compound and the degradation products can be seen from Fig. 7. Here the contaminants are quantified as a function of the time of exposure to daylight. The area of peak 1 (present in all samples) remains nearly constant, which means that it does not undergo

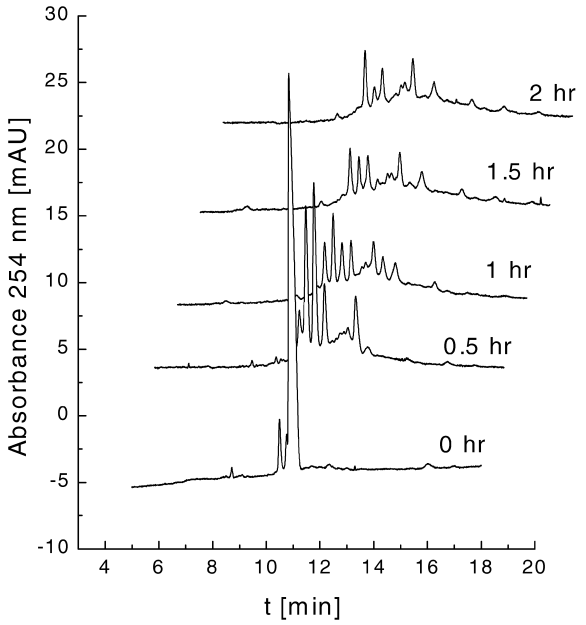

Fig. 5. Photodegradation of TOTO-3. The sample was placed in direct sunlight for different times (as indicated). UV detection at 254 nm.

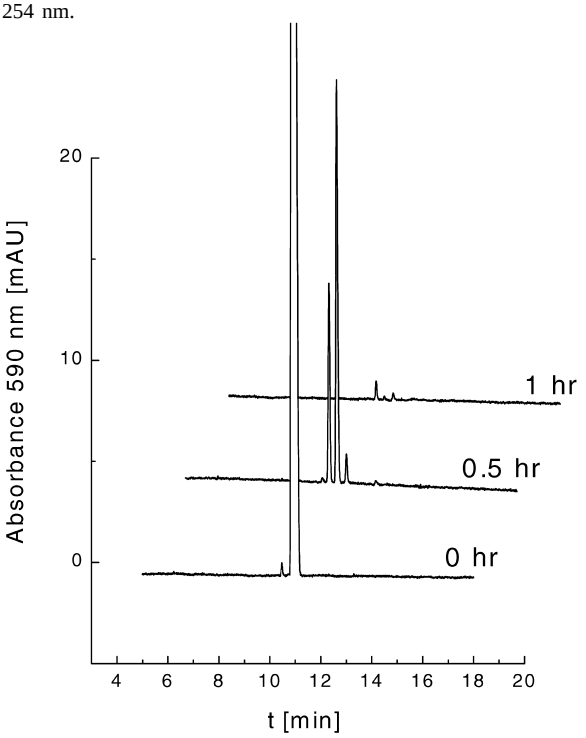

Fig. 6. Electropherograms of TOTO-3 upon photodegradation in direct sunlight. Detection at 590 nm (compare with the corresponding traces recorded at 254 nm shown in Fig. 5).

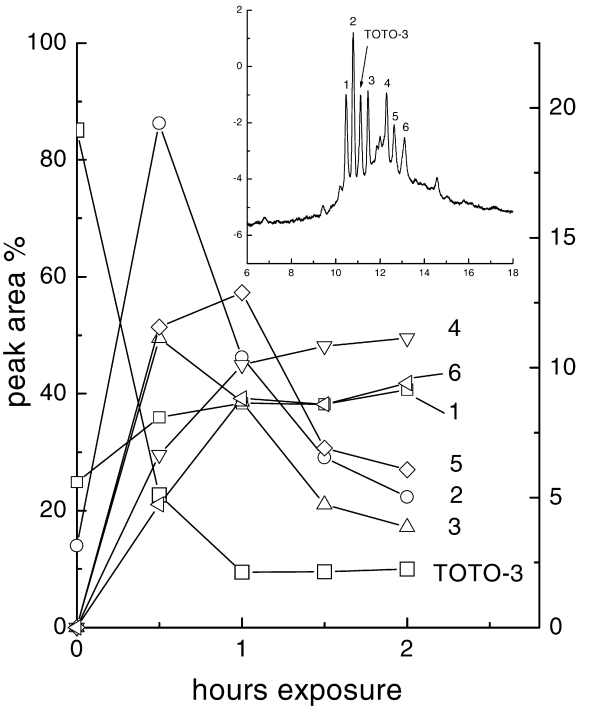

Fig. 7. Time course of photodegradation of TOTO-3. Percentage of peak areas of the main peak (left axis) and of peak numbers 1–6 (right axis) in the inserted electropherogram. The electropherogram was recorded after 1 h of exposure of lot 8 to direct sunlight.

photolysis. The peak areas of the main degradation products (peak numbers 2 and 3) are constantly increasing. Accordingly, the area of the TOTO-3 peak decreases from initial 85% to less than 10% after 2 h.

4. Conclusions

The present method is suited to determine the purity in commercial dimeric cyanine–nucleic acid dye samples. Up to 12 contaminants can be differentiated by CZE in 50 μm I.D. capillaries with diode array detection at 254 nm. Spectral information at 590 nm reveals that the majority of contaminants have large delocalised π electron systems, similar to the main compound. Although not all peaks are resolved in the electropherograms, quantitation is possible. For purity control by the present method, which is based on 100% normalisation of the peak areas, equal response factors are assumed for all compounds. Photodegradation leads to compounds that have no significant absorbance at 590 nm, but are detectable at 254 nm, indicating the presence of smaller aromatic systems, caused by the cleavage of the large parent molecule into smaller units.

In order to confirm this assumption, and in order to get a better insight into the chemical nature of the contaminants, coupling of CZE, e.g., with mass spectrometry would be desirable. As the BGE used by the present method (20 mmol/l perchloric acid in DMA) is not suitable for this combination, the development of better suitable background electrolytes is a topic of current work.

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