Quenched Phosphorescence Detection in Cyclodextrin-Based Electrokinetic Chromatography

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Quenched phosphorescence detection is a sensitive detection method recently introduced in capillary zone electrophoresis. It is based on the dynamic quenching interaction of the analytes (quenchers) with a phosphorophore, 1-bromo-4-naphthalenesulfonate (BrNS), present in the separation buffer. In this study, it is shown that this detection method can also be used in cyclodextrin-based electrokinetic chromatography (CD-EKC) despite the presence of cyclodextrins, which are known to reduce the luminescence quenching rate constants. Experiments indicate that BrNS mainly resides in the aqueous phase, while the analytes are distributed between both phases. In principle, the observed quenching might arise from the interaction of BrNS with uncomplexed as well as complexed analytes. However, from the dependence of the fractional quenching on the capacity factor (the normalized fractional quenching was found to be equal to the fraction of analyte in the aqueous phase), it was concluded that only aqueous-phase quenching contributes significantly to the observed quenching. Nevertheless, separation and detection can be regarded as fully compatible, because the capacity factors encountered in CD-EKC are generally low (in this study they ranged from about 0.1 to 2.5). Indeed, with nitroaromatic compounds as the target analytes, limits of detection in the $10^{-8}$ M range were achieved.

Since its introduction in 1984 by Terabe et al., electrokinetic chromatography (EKC) has become an important capillary electrophoretic technique for the separation of neutral analytes. Initially, the technique made use of a micellar “pseudophase” in the separation buffer (MEKC), the separation being based on the partitioning of the analytes between the micellar and the aqueous phase. Subsequently, it was found that derivatized, anionic cyclodextrins (CDs) can also be used to separate neutral analytes and this technique—cyclodextrin-based electrokinetic chromatography (CD-EKC)—has been widely used ever since. In addition, much work was done on the use of charged (and uncharged) CDs for the separation of enantiomeric compounds.

As in other modes of CE, there is an obvious need for sensitive detection in CD-EKC. In previous papers, we introduced quenched phosphorescence detection in capillary zone electrophoresis (CZE) and obtained limits of detection (LODs) down to $10^{-8}$ M. In the present work, the coupling of CD-EKC and quenched phosphorescence detection was studied. The method is based on the dynamic quenching interaction between the analytes (quenchers) and the phosphorophore, 1-bromo-4-naphthalenesulfonate (BrNS). For this purpose, a manually switched buffer deoxygenation/sample injection device was constructed. The small buffer volume of this system was expected to provide rapid deoxygenation and, thus, short start-up times. Moreover, it allowed the use of expensive buffer constituents such as CDs.

The suitability of quenched phosphorescence detection for CD-EKC is far from evident: early studies on CD-induced phosphorescence reported partial protection against quenching by oxygen for compounds included in the cavity of the CD. More detailed reports on the protection of CDs against luminescence quenching by aqueous-phase quenchers have appeared in the literature also. Although the situation in our system is considered to be reversed, with the quenchers partly in the CD phase and BrNS in the aqueous phase, the protective effect can be similar.

Nevertheless, sensitive detection was achieved using nitroaromatic compounds as the target analytes. These compounds were chosen because of their relevance in environmental analysis: nitropolyaromatic compounds are well known for their carcinogenic and mutagenic properties. In addition, their concentration levels are usually much lower than those of the parent PAHs. Sensitive detection methods are therefore required. GC and LC—combined with several detection methods—have been used most often, but HPLC separations with bile salts in the separation buffer have also been reported. Nitromonoaromatic compounds are also known for their toxicity; they are targeted in environmental analysis and for explosives detection using GC and LC,21 and MEEK.24,25

**EXPERIMENTAL SECTION**

**Chemicals.** Carboxymethyl-β-cyclodextrin (CM-β-CD; degree of substitution 3.0–3.5) and 9-nitroanthracene (9-Na; ~95%) were obtained from Fluka (Buchs, Switzerland). Nitrobenzene (NB; 99%), 2-nitrotoluene (2-NT; 99%), 3-nitrotoluene (3-NT; 99%), 4-nitrotoluene (4-NT; 99%), 1,3-dinitrobenzene (1,3-DNB; 97%), 2,4-dinitrotoluene (2,4-DNT; 97%), 2-nitrofluorene (2-NF; 98%), 1-nitronaphthalene (1-NN; 99%), 1-nitropyrene (1-NP; 99%), anthracene (99%), and γ-cyclodextrin were purchased from Riedel-de Haën (Seelze, Germany). For the preparation of standard solutions and buffers, demineralized and distilled water was used. 1-Bromo-4-naphthalene (1-NN; 99%), 1-nitropyrene (1-NP; 99%), anthracene (99%), and γ-cyclodextrin were purchased from Fluka (Buchs, Switzerland). Nitrobenzene (NB; 99%) and 9-nitroanthracene (9-Na; ~95%) were obtained from Riedel-de Haën (Seelze, Germany). For the preparation of standard solutions and buffers, demineralized and distilled water was used. 1-Bromo-4-naphthalenesulfonic acid was synthesized in house.

**Analytical Procedures.** Fresh stock solutions of the target analytes (10⁻³ M in methanol) and buffers containing CM-β-CD were prepared weekly. Standard solutions in water/methanol (20:80 v/v) were prepared each day. For some experiments, water/methanol (50:50 v/v) was used. Buffers containing CM-β-CD and stock solutions were stored at 4°C. The buffers were filtered over 0.2-μm syringe filters (Schleicher & Schuell, Dassel, Germany). Capillaries were rinsed for 15 min (1000 mbar) with 0.1 M NaOH at the start of each day.

**Capillary Electrophoresis.** A Prince CE system (Prince Technologies, Emmen, The Netherlands) was used with 50- or 75-μm-inner diameter, uncoated capillaries (BGB Analytik, Anwil, Switzerland) with a total length of 120 cm and an effective length of 60 cm. Because of the adapted CE setup (vide infra), a negative voltage was applied to perform separations.

**Detection.** For detection, an LS-40 luminescence detector (Perkin-Elmer, Norwalk, CT) was used in the time-resolved mode with a delay time of 0.05 ms and a gating time of 5.00 ms. Excitation of BrNS, present in the separation buffer, was performed with a total emission mirror (TEM) and 390-nm cutoff filter provided with the instrument. Electropherograms of CM-β-CD were obtained with a UV absorption detector (SpectraPhysics, Mountain View, CA) at 200 nm.

**RESULTS AND DISCUSSION**

**Buffer Deoxygenation/Sample Injection Device.** A device was designed to allow for injection of samples, while the deoxygenated buffer conditions required for obtaining phosphorescence were maintained (Figure 1). Deoxygenation of the separation buffer was achieved by purging with nitrogen gas. The nitrogen gas flow was continuously measured during experiments using a flow meter (Porter, Hatfield, PA). The CE program typically used is presented in Table 1. During step 1 of the program, a voltage is applied for 2 min, after which the positions of the vials are exchanged during step 2 to allow injection (step 3). During step

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**Table 1. Typical CE Program Used for CD-EKC with Quenched Phosphorescence Detection**

<table>
<thead>
<tr>
<th>step</th>
<th>vial position</th>
<th>pressure (mbar)</th>
<th>voltage (kV)</th>
<th>duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>0</td>
<td>-20</td>
<td>2.0</td>
</tr>
<tr>
<td>2</td>
<td>B → S</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>S → B</td>
<td>-50</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>S → B</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>0</td>
<td>-20</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*a B, buffer vial; S, sample vial.*
Table 2. Nitrogen Flows Applied for Deoxygenation under Different CE Conditions

<table>
<thead>
<tr>
<th>capillary i.d. (μm)</th>
<th>[CM-(d-CD)] (M)</th>
<th>current (μA)</th>
<th>nitrogen flow (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>5.0</td>
<td>−17</td>
<td>24</td>
</tr>
<tr>
<td>50</td>
<td>5.0</td>
<td>−7</td>
<td>13</td>
</tr>
<tr>
<td>50</td>
<td>10.0</td>
<td>−12</td>
<td>18</td>
</tr>
<tr>
<td>50</td>
<td>12.5</td>
<td>−14</td>
<td>18</td>
</tr>
<tr>
<td>50</td>
<td>12.5</td>
<td>−23 (−30 kV)</td>
<td>24</td>
</tr>
</tbody>
</table>

a Buffer: 25 mM borate, 1 mM BrNS, pH 8.5. b Separation voltage, −20 kV.

4, the vial positions are changed again, and next, the separation is started (step 5).

Since the LODs in phosphorescence detection are strongly dependent on the oxygen concentration in the separation buffer, one should quantify this parameter under various experimental conditions. When the buffer deoxygenation/sample injection device is used, the total oxygen production (M·s⁻¹) in a fixed volume of deoxygenated buffer is equal to the influx of oxygen from outside and the oxygen produced at the anode

\[
\frac{d[O_2]}{dt} = \phi_{ox} + I/4FV
\]

where \(\phi_{ox}\) is the influx (M·s⁻¹) of oxygen from outside, \(I\) the current (C·s⁻¹) generated during separation, \(F\) the Faraday constant (C·mol⁻¹), and \(V\) the volume (L) of the buffer. The number of oxygen molecules generated per second equals \(I/4\), since 4 electrons are required to produce 1 oxygen molecule. Thus, division by \(F\) and \(V\) gives the number of moles per liter per second. Equation 1 is slightly different from that used before, because of differences in the setups used. In the former setup, deoxygenated buffer flowed through the interface (anode), where oxygen is generated during electrophoresis, resulting in a steady-state oxygen concentration. To obtain a low oxygen concentration in the present setup, the nitrogen gas flow used for deoxygenation must be high enough to counterbalance both contributions from eq 1. It was observed that the influx of oxygen from outside can be quite high during exchange of the vial positions, when the buffer is exposed more directly to the outside air. As a result, the phosphorescence signal directly after the electroosmotic flow (EOF) signal can become smaller; however, the effect is eliminated almost completely when an adequate nitrogen flow is used. The flow required is also dependent on the buffer system, separation voltage, and inner diameter of the capillary used, since they determine the current that is generated. Nitrogen gas flows found to be adequate for different separation conditions are presented in Table 2.

After filling the capillary, a voltage can be applied and a stable baseline is obtained within only 20 min due to the small buffer volume. This is a significant improvement compared to the system we used before to couple CZE and quenched phosphorescence (start-up time, 2 h).

Determination of Capacity Factors. In this section, equations are given for the calculation of the capacity factors, \(k'\), and the fractions of analytes in the aqueous phase, \(R\) for neutral and anionic compounds. For the neutral compounds, \(k'\) values have to be known to establish the dependence of the detection sensitivity on the degree of inclusion in the CD phase. The equations for anionic compounds are needed to determine in which phase(s) the phosphorophore, BrNS, will be present. The capacity factor in a CD-EKC system is given by

\[
k' = n_{CD}/n_{aq} = K(V_{CD}/V_{aq})
\]

where \(n_{CD}\) and \(n_{aq}\) are the number of molecules in each phase and \(V_{CD}\) and \(V_{aq}\) are the corresponding volumes; \(K\) is the distribution coefficient for the partitioning process. The capacity factor is related to the fraction of the analytes in the aqueous phase, \(R\), by the well-known equation \(R = 1/(1 + k')\). For neutral compounds, mobility is induced only during the time they are in the (negatively charged) CD phase. The induced mobility, \(\mu_{ind}\), is therefore given by

\[
\mu_{ind} = (1 - R)\mu_{CD}
\]

where \(\mu_{CD}\) is the mobility of the CD. Upon rearrangement this gives

\[
R = (\mu_{CD} - \mu_{ind})/\mu_{CD}
\]

For anionic compounds, the mobility in the presence of CD, \(\mu_{CD}\), is given by

\[
\mu_{CD} = R\mu_{fs} + (1 - R)\mu_{CD}
\]

where \(\mu_{fs}\) is the mobility of the anion in the absence of CD, i.e., in free solution. Rearranging gives

\[
R = (\mu_{CD} - \mu_{fs})/\mu_{CD}
\]

In these equations, it is assumed that inclusion of a guest molecule does not significantly change the mobility of the CD. The mobilities are all intrinsic values, i.e., values corrected for mobility induced by electroosmosis. With phosphorescence detection in CE, \(\mu_{EO}\) can easily be determined because \(\mu_{EO}\) is marked by a large dip in the baseline, due to the fact that the samples are not deoxygenated. Unfortunately, it is well known that the determination of \(\mu_{EO}\) is the main problem in calculating capacity factors in CD-EKC. This is because the derivatized CDs are in general complex mixtures with different degrees of substitution and different positional isomers, although one recent report describes the use of single-isomer anionic CD derivatives. Indirect absorption detection has been used in CE to characterize charged CDs, but an average value of \(\mu_{CD}\) was not provided in that study. Approximate values for the mobilities of charged CDs

have been inferred from the induced mobility of anthracene, based on the assumption that inclusion of this hydrophobic compound in the CD cavity is almost complete.\(^3\)

We observed that the anthracene peak shifted toward the front of the electropherogram when the methanol content of the injected anthracene solution was increased. Consequently, \(\mu_{CD}\) may be underestimated when this procedure is used. Instead, we recorded electropherograms of CM-\(\beta\)-CD to obtain \(\mu_{CD}\) using direct UV absorption detection at 200 nm. An electropherogram of 10 mM CM-\(\beta\)-CD, obtained with a 25 mM borate, pH 8.5 buffer, is presented in Figure 2. To calculate the average value of \(\mu_{CD}\), the center of mass of the cluster of peaks was determined from the electropherograms. To this end, the areas of the peaks were corrected for the migration time (division by \(t\)) and for their estimated degree of substitution; the latter correction is needed because the absorption at 200 nm is dominated by the carboxymethyl groups. It was assumed that the degree of substitution is roughly proportional to the observed mobilities.\(^27\) The average value of \(\mu_{CD}\) was thus estimated to be \(3.1 \times 10^{-4}\) cm\(^2\) (V·s)\(^{-1}\) (RSD: 1.3\% \(n = 4\)). It should be noted that the buffer used to determine \(\mu_{CD}\) is not exactly the same as the buffer used in the actual separations, to which BrNS (1 mM) and CM-\(\beta\)-CD (concentrations from 5 to 12.5 mM) were added. Consequently, the mobility determined in this way might be higher than in the buffer used to separate the test compounds, due to the lower ionic strength.\(^29\) BrNS was not added because it interferes with absorption detection of the weakly absorbing CM-\(\beta\)-CD. Moreover, electropherograms of 50 mM CM-\(\beta\)-CD, recorded with 5 mM CM-\(\beta\)-CD in the separation buffer, showed a broad featureless peak, inhibiting reliable determination of \(\mu_{CD}\). However, the calculated mobility obtained in this way was similar to the above value, which indicates that the addition of CM-\(\beta\)-CD does not exert a large influence on \(\mu_{CD}\).

**Separation of Test Analytes.** For a first assessment of the feasibility of our technique, four nitropolyaromatic compounds were used as test analytes: 1-NN, 2-NF, 9-NA, and 1-NP. Electropherograms obtained with a 25 mM borate, 1 mM BrNS, 5 mM CM-\(\beta\)-CD buffer at pH 8.5, using a 75-\(\mu\)m-i.d. capillary, are shown in Figure 3 (traces a and b). 1-NN (\(k'\), 0.57), 9-NA (\(k'\), 0.98), and 2-NF (\(k'\), 1.30) were separated, but 1-NP (\(k'\), 0.96) almost completely overlapped with 9NA. The hydrophobic polyaromatic moieties of 1NN and 2-NF fit well into the cavity with the nitro group remaining outside, which is energetically the most favorable situation. Nevertheless, the capacity factor of 1-NN is rather low because this compound is not very hydrophobic. For 9-NA and 1-NP, the hydrophobic part of the molecule will be partially excluded from the cavity, which explains the relatively small interaction of these strongly hydrophobic compounds with the CD. In accordance with this explanation, addition to the separation buffer of a small amount of underivatized \(\gamma\)-CD—which has a larger cavity—resulted in a strong shift of the 1-NP and 9-NA peaks toward the front of the electropherogram. Thus, all four compounds were separated when 0.5 mM \(\gamma\)-CD was added to the buffer. However, the peak for 1-NP became \(\sim\)2-fold broader and showed significant tailing (Figure 3, trace c), which may be due to slow mass transfer from the hydrophobic CD cavity to the aqueous phase.\(^3\)

The LODs (3\(\sigma\); root-mean-square noise) were determined using a standard with low concentration (2 \(\times\) 10\(^{-7}\) M), because the response in quenched phosphorescence detection is linear over a relatively short concentration range. This can be read from the rearranged form of the well-known Stern–Volmer equation \(I_0/I = 1 + k_0[Q]c^0\), in which the fractional quenching, \((I_0 - I)/I_0\), is

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**Figure 2.** Electropherogram of 10 mM CM-\(\beta\)-CD. Buffer, 25 mM borate, pH 8.5; separation voltage, \(-20\) kV; detection, UV absorption at 200 nm; EOF, electroosmotic flow signal.

**Figure 3.** Electropherograms of selected nitropolyaromatic compounds (\(\sim\)1 \(\times\) 10\(^{-6}\) M) detected with quenched phosphorescence. 1, 1-Nitronaphthalene; 2, 9-nitroanthracene; 3, 2-nitrofluorene; 4, 1-nitropyrene. EOF, electroosmotic flow signal; separation voltage, \(-20\) kV (\(-17\ \mu\)A). Buffer: (a) and (b) 25 mM borate, 1 mM BrNS, 5 mM CM-\(\beta\)-CD, pH 8.5; (c) 25 mM borate, 1 mM BrNS, 5 mM CM-\(\beta\)-CD, 0.5 mM \(\gamma\)-CD, pH 8.5.

\(^{29}\) Kuhn, R.; Hofstetter-Kuhn, S. Capillary Electrophoresis: Principles and Practice; Springer: Berlin, 1993; Chapter 3.
where \( I \) and \( I_0 \) are the phosphorescence intensities in the presence and absence of quencher, respectively, and \( k_0 \) is the bimolecular quenching rate constant, \([Q]\) the quencher (analyte) concentration, and \( r^0 \) the phosphorescence lifetime in the absence of quencher. A plot of the fractional quenching versus the product \( k_0[Q]r^0 \) is linear only for low values of this product.\(^9\)

The LODs were found to be \( \sim 2 \times 10^{-8} \) M for all test analytes, although a slight dependence on the capacity factor was observed. These values are close to the lowest LODs obtained in CZE with quenched phosphorescence detection (1 \times 10^{-8} \) M).\(^9\) When 0.5 mM \( \gamma\)-CD was present in the separation buffer, the LOD of 1-NP was increased \( \sim 3\)-fold, i.e., to \( 6 \times 10^{-8} \) M. Electropherograms of nitropolyaromatic compounds were also obtained using 50-åm i.d. capillaries. In this case, the relative noise of the baseline is increased, which will lead to higher LODs. Nevertheless, concentration LODs were still as low as \( \sim 5 \times 10^{-8} \) M, while absolute LODs were \( \sim 0.1 \) pg for all compounds. An advantage of using 50-åm capillaries is the better long-term baseline stability, which is due to the smaller nitrogen flow required for deoxygenation (cf. Table 2).

As a second example, six nitromonoaromatic compounds (1,3-DNB, 2,4-DNT, NB, 4-NT, 2-NT, 3-NT) were subjected to CD-EKC (\(-20 \) kV). All these compounds easily fit into the cavity of the CM-\( \beta\)-CD with their nitro groups outside. Nevertheless, smaller \( k' \) values can be expected since they are less hydrophobic than the nitropolyaromatic compounds. Indeed, with a 25 mM borate, 1 mM BrNS, 5 mM CM-\( \beta\)-CD, pH 8.5 buffer, \( k' \) values ranged from 0.07 to 0.48. The peak of 1,3-DNB partially overlapped with the EOF signal, and 4-NT and 2-NT also showed overlap (Figure 4, trace a).

The separation was improved by increasing the concentration of CM-\( \beta\)-CD (Figure 4, traces b and c), which increased \( k' \) for all compounds without changing the migration order and resulted in better resolution. When 12.5 mM CM-\( \beta\)-CD was used in the separation buffer, 4-NT and 2-NT were resolved almost completely, but the analysis time was significantly increased. As shown in trace d of Figure 4, the analysis time can be reduced while the increased resolution as well as the migration order is maintained, by using a higher voltage (\(-30 \) kV). However, an adequate separation was achieved also by using 10 mM CM-\( \beta\)-CD (Figure 4, trace b). Figure 4 also serves to illustrate the room for optimization of separations in CD-EKC with quenched phosphorescence detection. Higher concentrations of CM-\( \beta\)-CD lead to higher currents and, consequently, to increased oxygen production at the anode during electrophoresis. This in turn leads to a decrease in the phosphorescence background and quenching efficiency,\(^9,10\) both resulting in increased LODs. However, as can be observed from Figure 4, the effect of increased CM-\( \beta\)-CD appears to be small over the concentration range considered here. In other words, separations can be optimized without seriously compromising the detection. With a 25 mM borate, 1 mM BrNS, 10 mM CM-\( \beta\)-CD, pH 8.5 buffer, LODs of \((4 - 5) \times 10^{-8} \) M were obtained for all nitromonoaromatics, except 2-NT (8 \times 10^{-8} M). That is, quenched phosphorescence is \( \sim 10\)-fold more sensitive for these compounds than UV absorption detection in CE.\(^3\)

**Quenching Mechanism and Sensitivity.** The favorable LODs obtained in this study indicate that quenching is efficient, even when CDs are present. In this section, an explanation for this will be provided. In principle, four mechanisms are possible for the quenching of the BrNS phosphorescence by a quencher, Q, in the presence of CM-\( \beta\)-CD:

\[
\begin{align*}
\text{BrNS}^\ast(aq) + Q(aq) &\rightarrow \text{BrNS(aq)} + Q^\ast(aq) \quad \text{(A)} \\
\text{BrNS}^\ast(aq) + Q:CD &\rightarrow \text{BrNS(aq)} + Q^\ast:CD \quad \text{(B)} \\
\text{BrNS}^\ast:CD + Q(aq) &\rightarrow \text{BrNS:CD} + Q^\ast(aq) \quad \text{(C)} \\
\text{BrNS}^\ast:CD + Q:CD &\rightarrow \text{BrNS:CD} + Q^\ast:CD \quad \text{(D)} 
\end{align*}
\]

Mechanism A describes free solution quenching. This has been
shown to occur at diffusion-limited rates for various compounds, resulting in sensitive detection in CZE.\textsuperscript{5-10} Mechanism B may well contribute much less to the overall quenching process than does mechanism A because of shielding by the CD molecules. Several detailed studies on luminescence quenching of luminophores included in CDs by aqueous-phase quenchers have been published,\textsuperscript{13-15} most of these discuss fluorescence quenching,\textsuperscript{13,14} but phosphorescence quenching has also been studied.\textsuperscript{15} It was proposed that the quenching mechanism—whether being long range or short range in nature—and the degree of screening by the host molecule determine the reduction of the quenching efficiency.\textsuperscript{13} Triplet quenching by electron transfer of α-terthiophene included in β-CD was observed to be reduced 3–4-fold.\textsuperscript{15}

To investigate whether mechanism B plays a significant role, the dependence of $k_q$ on $k'$ was measured for the test compounds and anthracene. The former group induces phosphorescence quenching of BrNS by electron transfer while anthracene causes quenching by triplet–triplet energy transfer. All compounds can be assumed to yield diffusion-controlled quenching, although some scatter in the data may be induced by this assumption for the nitroaromatics, because of the influence of substituents on the quenching rate constants.\textsuperscript{30,31} To obtain the observed (average) quenching rate constants, $k_{q,av}$, in a system containing CDs, it is assumed that $k_{q,av}$ is equal to the weighted average of the quenching rate constants in the aqueous and CD phases. The weighing factors are given by the fraction of quenchers in both phases ($R$ and $R - 1$ for the aqueous and CD phase, respectively). From this, and from comparison with eq 5, it is clear that

$$k_{q,av} = Rk_{q,fs} + (1 - R)k_{q,CD}$$  \hspace{1cm} (8)

where $k_{q,fs}$ and $k_{q,CD}$ are the quenching rate constants for quenchers in the aqueous ("free solution") and CD phase, respectively. In the special case that $k_{q,CD}$ is negligibly small compared to $k_{q,fs}$, eq 8 becomes

$$k_{q,av} = RK_{q,fs}$$  \hspace{1cm} (9)

Since at low quencher concentration the bimolecular quenching rate constant, $k_q$, is proportional to the fractional amount of quenching (see eq 7), \((I_0 - I)/I_0\) can be taken as a measure of $k_q$. Figure 5 shows the experimentally found dependence of \((I_0 - I)/I_0\) on the fraction of quencher in the aqueous phase, $R$. The values of \((I_0 - I)/I_0\) were normalized to give unity for $R = 1$. In Figure 5a, the results for the nitroaromatic compounds and anthracene are presented. Clearly, the fit through the data points ($y = 0.9943x + 0.0057; r^2 = 0.85$) closely resembles the theoretical line $y = R$ as predicted by eq 9, with $y$ being the normalized fractional quenching. Therefore, it can be concluded that quenching by analytes in the CD phase does not significantly contribute to the overall quenching. This conclusion is confirmed by the results obtained for the nitroaromatics (Figure 5b): in this case, the fit through the data points is given by the line $y = 0.9705x + 0.0295$ ($r^2 = 0.88$). The outlying result for 2-NT was not included in the fit. For this compound, the sensitivity is ~2-fold less than for the other nitroaromatics, which may be due to steric hindrance (ortho effect).\textsuperscript{30} On the other hand, such a steric effect was not observed for the quenching of BrNS by 2-, 3-, and 4-nitrophenol: similar responses were obtained for these compounds.

Mechanisms C and D can probably be excluded, since BrNS is expected to have poor interaction with CM-β-CD because of electrostatic repulsion (both molecules are negatively charged). This assumption is supported by the following observations: first, the observed phosphorescence lifetime, $\mu_s$, was ~300 $\mu$s, which is very similar to that in buffer systems without CDs under similar electrophoretic conditions.\textsuperscript{5} In other words, no protective effect of the CDs on the phosphorophore was observed, which points to poor complexation of BrNS with CM-β-CD. Second, the mobilities of BrNS in the presence and absence of 5 mM CM-β-CD (25 mM borate buffer, pH 8.5) were found to be equal within experimental error; values of $2.7 \times 10^{-3}$ and $2.8 \times 10^{-4} \text{ cm}^2 \text{ (V-s)}^{-1}$ were obtained for $\mu_{CD}$ and $\mu_{fs}$, respectively, again suggesting that the degree of inclusion of BrNS in the CD phase is close to zero. Therefore, mechanisms C and D can probably be excluded. It should be noted that mechanism D is also ruled out by the observation that mechanism B does not play an important role, since in both cases the quenchers are included in the cavity of the CD; obviously, mechanism D is even more unlikely because

![Figure 5](https://example.com/figure5.png)

Figure 5. Response (normalized fractional quenching) as a function of the fraction of quencher in the aqueous phase. (a) Nitroaromatic compounds and anthracene: buffer, 25 mM borate, 1 mM BrNS, 5 mM CM-β-CD, pH 8.5; capillary i.d., 75 μm. (b) Nitroaromatic compounds: buffer, 25 mM borate, 1 mM BrNS, 10 mM CM-β-CD, pH 8.5; capillary i.d., 50 μm.


in this case the phosphorophore is included in the CD phase also. Thus, the overall conclusion is that quenching predominantly occurs in the aqueous phase.

The above conclusion is in marked contrast to that of a recent study on the quenching behavior of nitromonoaromatic compounds in the absence and presence of β-CD. Here, it was concluded that the quenching of a tris(2,2′-bipyridine)ruthenium(II) complex was not significantly reduced by the β-CD because the nitro groups are not included in the CD. However, in our opinion, this conclusion is not justified, since the authors also reported that the interaction of these quenchers with β-CD was rather weak (K∞ typically ~100). This implies that the largest fraction of the quenchers will be present in the aqueous phase. Therefore, it is not surprising that the observed quenching rate constants are not much lower than those obtained in the absence of CDs. In other words, the data do not conflict with our conclusion that aqueous-phase quenching is the most important mechanism in the phosphorophore/quencher/CM-β-CD system. Moreover, it should be noted that the inhibition of efficient quenching can be even stronger for CM-β-CD than for neutral β-CD, because of electrostatic repulsion between the negatively charged BrNS and CM-β-CD.

CONCLUSIONS

The use of quenched phosphorescence detection in CE, which until now was restricted to charged analytes (CZE), has been extended to the detection of neutral analytes by using CD-EKC as the separation technique. Although efficient quenching appears to be restricted to the aqueous phase, quenched phosphorescence detection can provide sensitive detection in CD-EKC with LODS of ~2–5 × 10⁻⁸ M, due to the low-capacity factors encountered. For a more detailed study of the quenching interaction in the presence of CDs, it may be advantageous to use single-isomer charged CD derivatives, which recently became commercially available.

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