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Flavin mononucleotide for indirect laser-induced fluorescence detection of anions separated by capillary electrophoresis

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Abstract

Flavin mononucleotide (FMN), also known as riboflavin-5′-phosphate, has been characterized as an indirect laser-induced fluorescence (LIF) detection reagent for inorganic anions, organic acids, anionic surfactants and polyphosphates after separation by capillary electrophoresis (CE). FMN provides a good wavelength match for laser excitation at 488 nm, is readily soluble in aqueous or aqueous/organic solutions, and unlike fluorescein provides strong fluorescence at both acidic and basic pH values. Analyte peaks due to a loss in FMN fluorescence are generated at weakly alkaline pH values as expected, but peak direction is switched at more alkaline pH values such as 8.6 or 9.0. A separation of 21 inorganic anions and organic acids is possible in about 20 min using the indirect LIF mode using 10 μ*M* FMN with 2 m*M* diethylenetriamine as the electroosmotic flow suppressor. Detection limits for these analytes are in the 10–20-μg/l range without any required preconcentration. The use of methanol improves resolution and facilitates the simultaneous separation of aliphatic/aromatic surfactant standard mixtures or commercial shampoos in less than 20 min.

Keywords: Laser-induced fluorescence detection; Flavin mononucleotide; Inorganic anions; Organic acids; Anionic surfactants; Polyphosphates

1. Introduction

Capillary electrophoresis (CE) of weakly or non-UV absorbing ionic compounds with indirect detection continues to be an important method. Although most of the research has been in the characterization of reagents to permit indirect photometric detection (IPD), CE instruments equipped with laser-induced fluorescence (LIF) detection has spurred the characterization of reagents for indirect LIF and this topic has been reviewed [1]. Some compounds which can be excited using a UV laser, such as salicylate for amino acids [2] or inorganic anions [3] and quinine [4] for cations have been reported. However, reagents such as fluorescein [5] compatible with visible laser excitation at 488 nm are more useful because of the commercial availability of CE instruments equipped with such a laser. Inorganic cations as EDTA complexes [6], as well as inorganic anions [7], organic acids [7,8] and anionic surfactants [8], have also been determined using indirect LIF with fluorescein. One disadvantage of fluorescein is that it fluoresces strongly at alkaline pH changing dramatically from no fluorescence at pH 3 to weak fluores-

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cence at pH 6, and to maximum fluorescence at pH 8 or above [6]. Cyanide plus related compounds [9] and phenols [10] only anionic at high pH near 10–11 can be determined by CE with indirect LIF using fluorescein. However the indirect determination of sugars by CE [11] at high pH was done best using coumarin and not fluorescein. Because the fluorescence of fluorescein is stable over only the alkaline pH range, CE with indirect LIF detection at lower pH (desirable for a more selective determination of some samples) would be problematic.

In this study, we have characterized flavin mononucleotide (FMN) or riboflavin-5′-monophosphate as a versatile reagent for indirect LIF of various classes of anions after CE separation. Although riboflavin has been used for CE of sugars with indirect UV detection at high pH [12], to the best of our knowledge FMN has not been used for CE with either indirect UV or fluorescence detection. FMN provides a good match with commercial laser-based CE instruments with excitation at 488 nm and emission at 520 nm, is stable in fluorescence response from pH 3.5–8, as shown in Fig. 1 [13], and is readily soluble in aqueous or aqueous–methanol electrolyte solutions. Fluorescence response generated by the separated anions as a function of such parameters as concentration of FMN, pH and % (v/v) methanol were studied by CE with LIF detection. A separation of 21 inorganic anions and organic acids in about 20 min was possible for the first time with indirect LIF and detection limits for these analytes were in the 10–20-μg/l range without any required preconcentration.

2. Experimental

2.1. Instrumentation

Separations were performed with a Beckman (Fullerton, CA, USA) P/ACE System 5510 CE instrument equipped with software System Gold for instrument control and data handling. Fluorescence excitation was provided at 488 nm with 4 mW of power using the argon ion laser and emission was isolated at 520 nm using a band pass filter. The fused-silica capillary also from Beckman had dimensions of 57 cm×75 μm I.D. with an effective length to the detector of 50 cm. The temperature of the capillary was solution controlled at 23°C. The detector polarity was switched, so most of the electropherograms showed positive peaks and data collection was performed at 5 Hz.

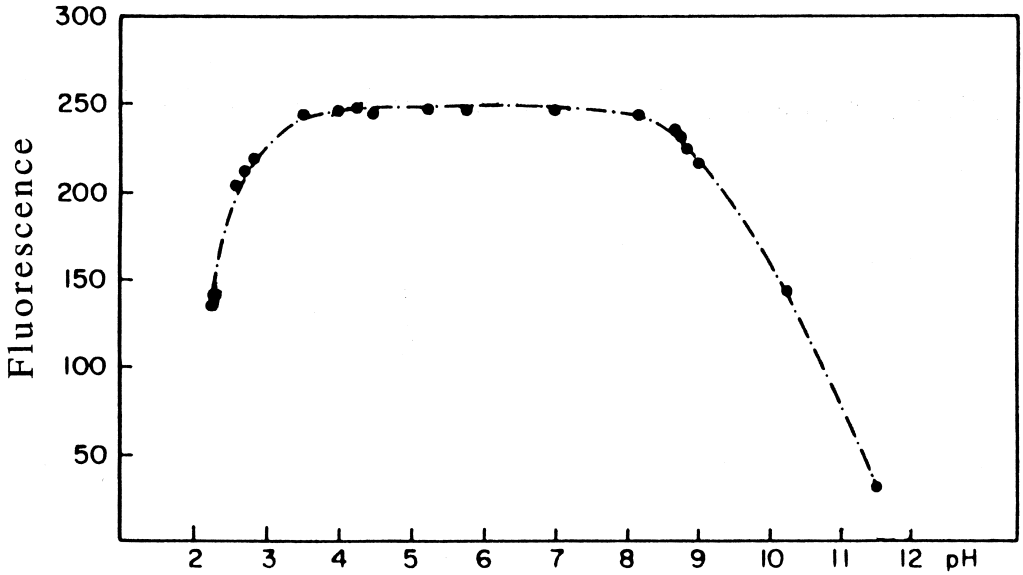

Fig. 1. Effect of pH on the fluorescence intensity of FMN. Reprinted from Ref. [13] with permission.

2.2. Reagents and samples

Flavin mononucleotide (FMN) was obtained as the monosodium salt from Sigma (St. Louis, USA). Technical grade diethylenetriamine (DETA) and boric acid (99+%) were purchased from Aldrich (Milwaukee, WI, USA). The sodium salts of *n*-alkyl sulfates, aromatic sulfonates and polyphosphates were available from either Sigma or Aldrich. Sodium salts of the common inorganic anions and organic acids were obtained from various manufacturers. Perth Plus shampoo was purchased from a local grocery store.

2.3. Preparation of electrolytes and standard solutions

A 1 m*M* stock solution of FMN electrolyte was prepared in triply distilled water and used after the appropriate dilution to make the run buffer. All run buffers contained 100 m*M* boric acid and were adjusted to the desired pH with 1 m*M* NaOH. For the separation of *n*-alkyl sulfates and aromatic sulfonates, an appropriate percentage (v/v) of methanol was added after the pH adjustment. All the final operating electrolytes were filtered using 0.2-μm syringe filters from Gelman Science (Ann Arbor, MI, USA) by creating a vacuum inside the syringe. The stock standard solutions of inorganic anions, organic acids and polyphosphates were prepared in triply distilled water at concentrations of 1000 mg/l. The surfactant sample solutions were prepared in 50% (v/v) of methanol–water at concentrations of about 500 mg/l. Appropriate dilutions of all stock standard sample solutions were made with triply distilled water.

2.4. CE procedures

A new capillary was subjected to a standard wash cycle for 2 h using 1 *M* NaOH. As a daily routine procedure, the capillary was flushed with 1 *M* NaOH for 10 min followed by another 5 min with triply distilled water. Finally the capillary was equilibrated with the operating buffer for 5 min before the first sample injection. In between injections, the capillary was flushed with the run buffer for 3 min.

3. Results and discussion

In Fig. 2, using a negative polarity voltage, the migration order and separation times of the 21 inorganic anions plus organic acids are similar to those reported previously with naphthalenedisulfonate in the IPD mode [14]. However, the sensitivity and direction of the fluorometric signal for the peaks in this sample separation are affected by pH in the range of 7.8–9.0. At pH of 7.8 and 8.2, a decreased fluorescence occurs upon peak elution as expected and the baseline noise is quite low. At pH values of 8.6 and 9.0, an increased fluorescence upon peak elution with greater baseline noise is observed. The reason for this reversal in peak direction over a fairly narrow pH range cannot be explained with certainty. Because the pK_a values for the phosphate group of FMN are about 1.3 and 6.5, any significant change in mobility of FMN is not likely. Mobility differences between the fluorophore and analyte ions can result in peak broadening but not peak direction change [15,16]. Previously it has been shown that an enhancement of fluorescence due to electrostatic effects can occur if the analyte ions are opposite in charge to the fluorophore charge [8]. In addition, if a high concentration (m*M* level) of a fluorophore is used, this corresponds to the self-quenched region and any dilution of the fluorophore due to replacement by the analyte will result in an increased fluorescence [17]. The direction of some peaks representing phenols was switched as a function of the applied electric field when a 0.1 m*M* fluorescein fluorophore was used, but no such effect was observed when the concentration was raised to 1 m*M* [10]. However, these three previous scenarios are not present in this situation. One possible explanation may be the replacement of OH^- as well as FMN by the sample anions could cause a temporary localized decrease in pH. At pH 8.6 or 9.0, this will lead to a fluorescence increase, while at pH 7.8 or 8.2 the fluorescence remains quite constant (Fig. 1). This same fluorescence profile from pH 7.3 to 9.0 as shown in Fig. 1 was also experimentally confirmed by us. Therefore, positive peaks (decreased fluorescence) are observed due to the decrease in FMN in Fig. 2a and b, while negative peaks (increased fluorescence) are observed due to the more important decrease in OH^- in Fig. 2c and d. Because of the

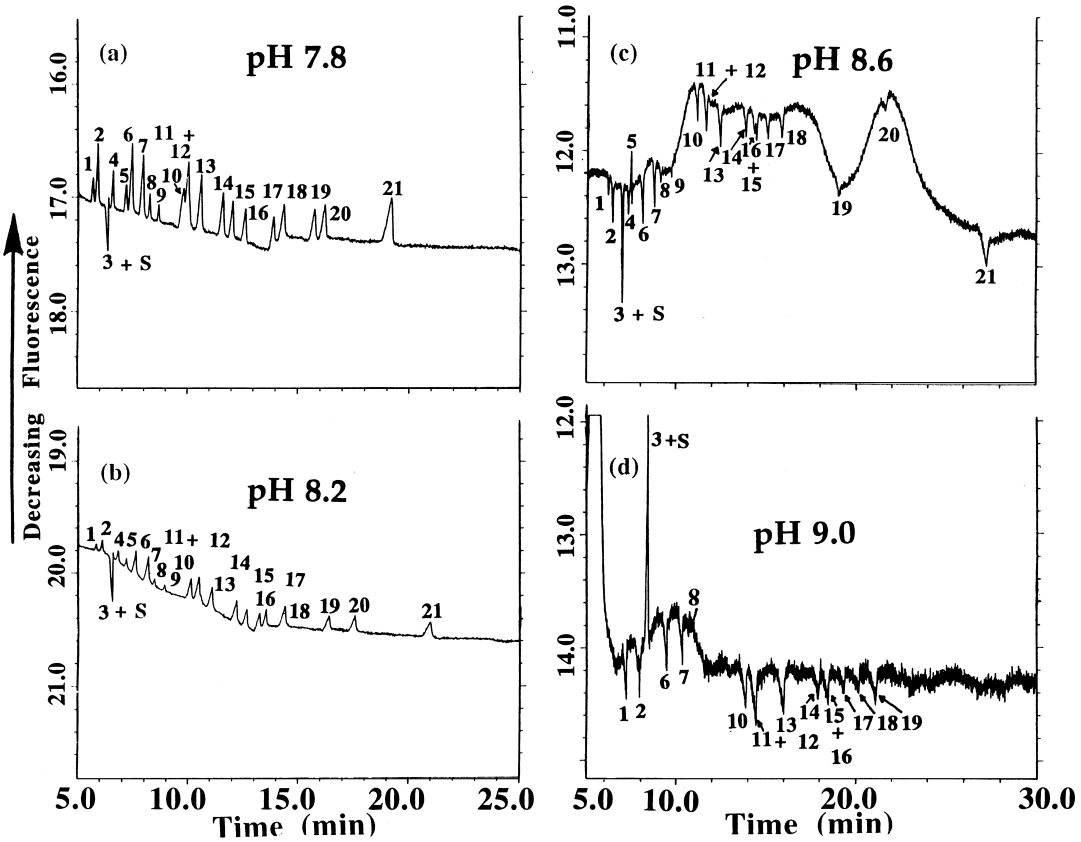

Fig. 2. Effect of pH on separation, sensitivity, and direction of fluorometric signal of a mixture of 21 inorganic anions and organic acids. Electrolyte composed of 20 μ*M* of FMN in 100 m*M* H_3BO_3, 2 m*M* DETA adjusted to various pH values with 1 m*M* NaOH. All anion peak concentrations are 2 mg/l for inorganic anions and 5 mg/l for organic acids. (1) Bromide, (2) chloride, (3) nitrite, (4) nitrate, (5) chromate, (6) sulfate, (7) oxalate, (8) molybdate, (9) tungstate, (10) malonate, (11), fluoride, (12) fumarate, (13) formate, (14) succinate, (15) malate, (16) citrate, (17) tartarate, (18), phosphate, (19) hypophosphate, (20) phthalate, (21) carbonate; pressure injection for 4 s at pH 7.8–8.6 and 8 s at pH 9.0; voltage of −15 kV applied for separation; current varied from 7.0 to 41 μA.

Table 1
Detection characteristics of flavin mononucleotide (FMN)

Cm of FMN ($\times 10^6$ mol/l)	Background fluorescence[a] (RFU)	Background noise[b] ($\times 10^2$ RFU)	Dynamic reserve (DR)[c]	Baseline drift ($\times 10^2$ RFU/min)
5	36.0	1.6	2250	1.2
10	75.2	3.2	2300	2.5
15	110	4.7	2340	5.2
20	152	6.4	2380	6.3
30	229	9.5	2420	6.8
50	382	20	1910	14
70	523	21	2490	20
100	744	22	3380	23

[a]Measured during the rinse of fused-silica capillary for 2–3 min until a stable output is displayed. RFU, relative fluorescence units.
[b]Measured as peak-to-peak noise.
[c]Calculated as background fluorescence/background noise.

borate buffer, the increase in baseline noise at pH 8 and 9 was quite unexpected. Previously the baseline noise increase for fluorescein at pH 9 compared to 7 has been attributed to some interaction of the fluorophore with the silica capillary [11]. The optimum pH for the best S/N ratio was found to be 8.0 or lower to pH 5.5 where primarily replacement of FMN by the sample anions causes the signal change.

The magnitude of the analyte detection limit is proportional to the concentration of the fluorophore but inversely related to the transfer ratio (the number of molecules of detectable ions displaced by each

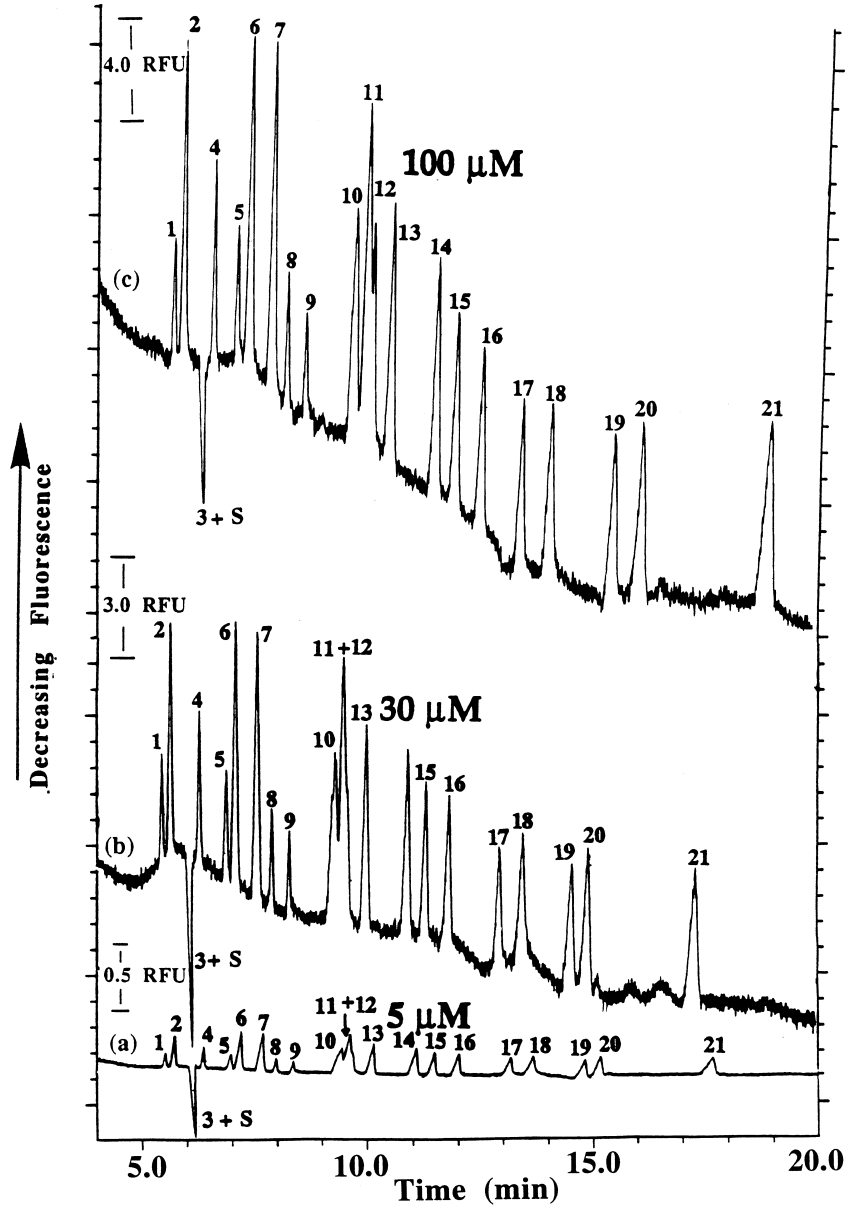

Fig. 3. Effect of FMN concentration on separation, sensitivity, and baseline stability of a mixture of 21 inorganic anions and organic acids. Electrolyte composed of 5, 30 and 100 µM FMN in a 100 mM H_3BO_3, 2 mM DETA, pH 7.8, buffer; pressure injection for 4 s; voltage of −15 kV applied for separation. Current varied from 6.5 to 9.0 µA. Peak identification same as in Fig. 1; RFU, relative fluorescence units.

analyte molecule) and the dynamic reserve or DR (the ratio of background absorbance to noise) [18]. The indirect LIF detection characteristics of FMN from 5 to 100 μ*M* are shown in Table 1. Both background fluorescence and noise increase proportionally with FMN concentration to 30 μ*M* so the DR is quite constant. A dramatic increase in noise and baseline drift occurs at 50 μ*M* indicating a minimum in the DR. However, at 70 and 100 μ*M*, the background noise is constant but the dynamic reserve is much improved because of the increased fluorescence. Because the maximum DR ratio occurs at 100 μ*M*, this would appear to be the optimum assuming the baseline drift is tolerable. However, the effect of FMN concentration at 5, 30 and 100 μ*M* on the indirect LIF peaks as shown in the electropherograms of Fig. 3 should be examined. No change in peak direction as a function of concentration is noted indicating fluorescence self-quenching is not a problem to at least 0.1 m*M* FMN. At 5 μ*M* FMN, baseline noise is low (0.016 RFU) but peak response for sulfate is weak (0.275 RFU) giving a *S*/*N* ratio of 17.2. At 100 μ*M* FMN, peak response for sulfate is strong at 2.45 RFU and the baseline noise is increased at 0.198 RFU, but the *S*/*N* ratio is 12.3. At 30 μ*M* FMN, the peak response for sulfate has diminished to 1.96 RFU with a baseline noise of 0.109 RFU but the baseline drift is better as compared to 100 μ*M* FMN. The optimum concentration of FMN was considered to be 30 μ*M* because of the *S*/*N* ratio (18.0 for sulfate) and the reasonable baseline drift.

Fig. 4 shows the separation of polyphosphates in about 15 min, using Mg^{2+} as a complexing agent and application of a positive polarity voltage. We have shown previously [19] by IPD, and also in this work using FMN, that in the absence of Mg^{2+} no separation is observed. The migration order is simply based on the ability of Mg^{2+} to reduce the negative charge of the polyphosphate causing it to be less attracted to the positive injector side.

The effect of methanol in the run buffer on the peak distribution of both aliphatic and aromatic surfactants is shown in Fig. 5. Due to the change in the dielectric constant and viscosity of the run buffer as methanol is added, the electroosmotic flow (EOF) as expected decreases, as evidenced by the increase in separation time [20]. The change in the system peak (*S*) migration time with solvent composition is evidence of a change in the mobility of FMN causing a mobility mismatch with the aromatic surfactants (peaks 10, 11, 12) and peak broadening. At low methanol concentrations below 30% (v/v), the long chain aliphatic sulfonates C_{18}^{-} and C_{16}–SO_4^{-} (peaks 1 and 2) are either absent or poorly resolved. In our earlier CE study using naphthalenedisulfonate as the

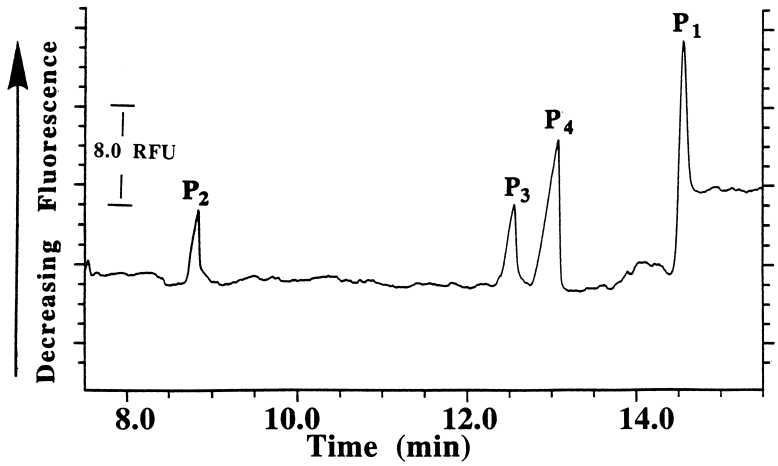

Fig. 4. Separation of polyphosphates with indirect LIF detection. Electrolyte composed of 30 μ*M* FMN in 100 m*M* H_3BO_3, 2 m*M* Mg^{2+}, 0.2 m*M* DETA, pH 7.0; vacuum injection was 1.5 s (4 nl on 75 μ*M* I.D. capillary); 425 V/cm applied across capillary. Peak identification: P_1, orthophosphate (25 mg/l); P_2, pyrophosphate (15 mg/l); P_3, tripolyphosphate (15 mg/l); P_4, tetrapolyphosphate (15 mg/l); RFU, relative fluorescence units.

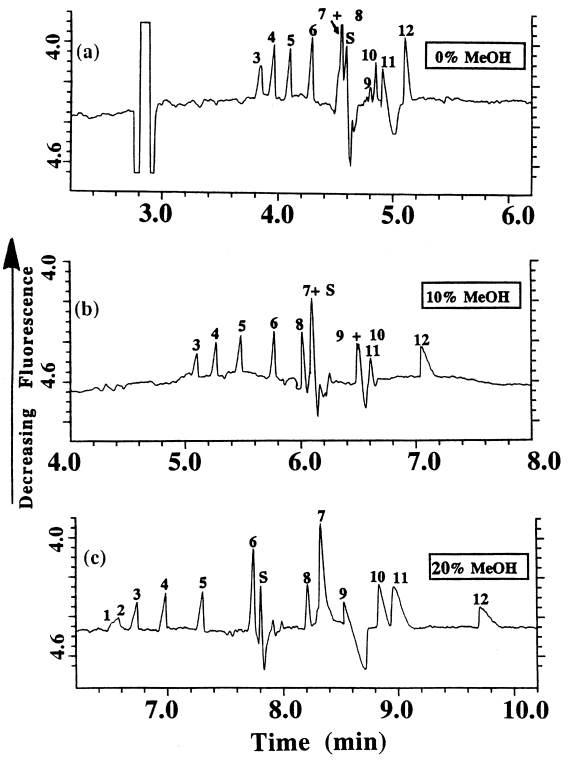

Fig. 5. Effect of methanol (MeOH) on the simultaneous separation of aliphatic and aromatic surfactants. Electrolyte composed of 30 μ*M* FMN in a 100 m*M* H_3BO_3, pH 8.0, with various % (v/v) of MeOH; pressure injection for 5 s; separation voltage of +25 kV; current varied from 8 to 12 μ*A*; all peaks represent 25 mg/l for *n*-alkyl sulfates and 50 mg/l for aromatic sulfonates: (1) $C_{18}SO_4^-$, (2) $C_{16}SO_4^-$, (3) $C_{14}SO_3^-$, (4) $C_{12}SO_3^-$, (5) $C_{10}SO_4^-$, (6) $C_8SO_4^-$, (7) $C_6SO_4^-$, (8) xylene sulfonate, (9) *p*-phenol sulfonate, (10) *p*-toluene sulfonate, (11) *o*-chlorobenzene sulfonate, (12) benzene sulfonate.

IPD reagent, detection and resolution of $C_{18}SO_3^-$ and $C_{16}SO_3^-$ was evident using 50% methanol in the run buffer [14]. Using 50% methanol (data not shown), baseline resolution and good detectability of peaks 1, 2 and 3 were found between 15 and 20 min. However, broadening of the other peaks due to the increased separation time (38 min) is a problem. Because resolution of the aromatic sample component peaks 8–12 is best without overlap of the system peak and the aliphatic surfactants (C_6–C_{14}–SO_4^-) are generally well resolved, the optimum separation of this mixture is considered to be that using 20% methanol as shown in Fig. 5c.

Fig. 6 shows the separation of ethoxylated lauryl sulfate using 0 and 50% (v/v) methanol. The importance of methanol in improving sample solubility as well as slowing the EOF is clearly seen as at least 25 of the 30 oligomers can be distinguished. Fig. 7 shows the separation of sulfonated surfactants in a commercial shampoo sample. A series of ethoxylated lauryl sulfonates can be distinguished as well as C_{12}–SO_4^- and xylene sulfonate. The dye responsible for the green color of the Perth shampoo can also be isolated, although the migration time is long and the peak shows a positive fluorescence (data not shown).

It has been reported for the determination of isoprenyl phosphates that indirect LIF using salicylate gives a detection limit of 0.5 μ*M*, which is a factor of 14 better than indirect IPD using phthalate [21]. Detection limits for the different classes of anions using CE with FMN and indirect LIF are shown in Table 2. For the small ions, low detection

Table 2
Detection limit summary of various classes of anions

Anion class	Detection limits (μg/l)[a]	
	Pressure injection	Electrokinetic injection
Inorganic anions[b]	20–30	10–15
Organic acids[b]	40–50	20–25
Polyphosphates (P_1–P_4)[c]	250–500	100–250
Alkyl sulfates (C_6–C_{12})[d]	500–1000	200–500

[a] S/N=3.0 based on peak height.
[b] Using 30 μ*M* FMN, 100 m*M* H_3BO_3, 2m*M* DETA (pH 7.80); pressure injection 15 s, voltage injection 5 s (−10 kV); −15 kV applied for separation.
[c] Using 30 μ*M* FMN, 100 m*M* H_3BO_3, 2 m*M* Mg^{2+} (pH 7.20); pressure injection 6 s, voltage injection 5 s (+10 kV); +20 kV applied for separation.
[d] Using 30 μ*M* FMN, 100 m*M* H_3BO_3, (pH 8.00); pressure injection 6 s, electrokinetic injection 5 s (+10 kV); +25 kV applied for separation.

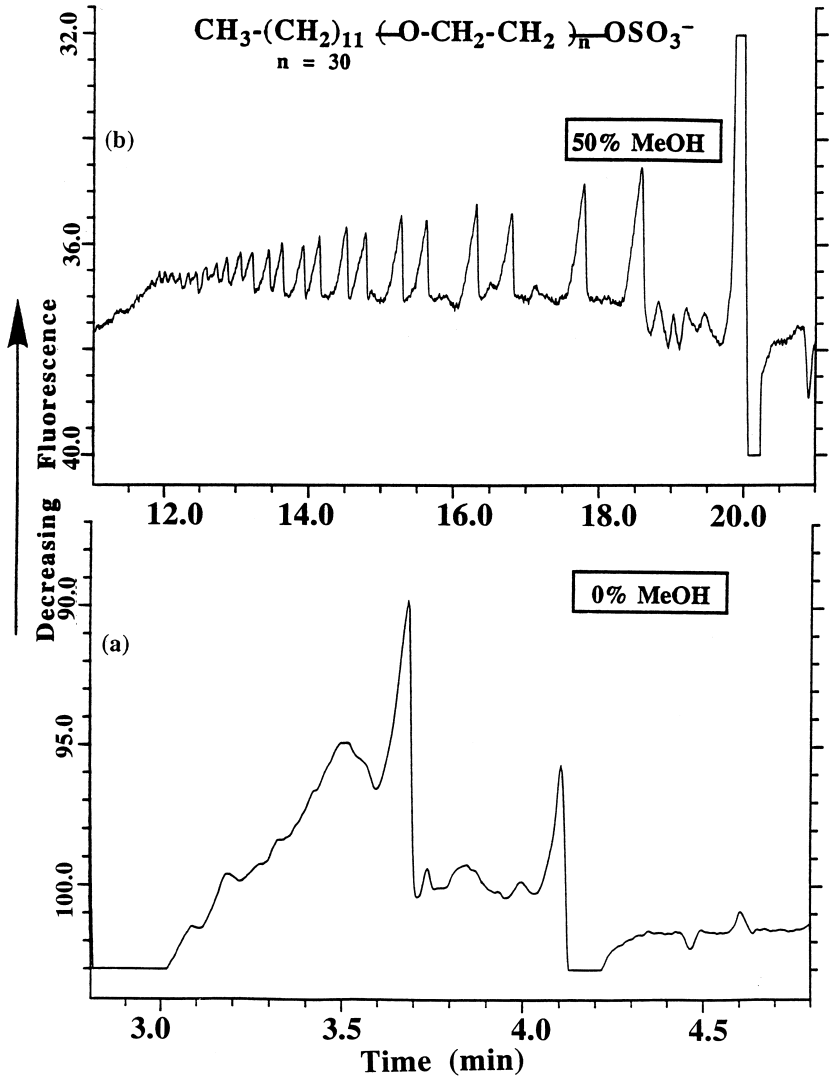

Fig. 6. Comparison of electropherograms for the separation of ethoxylated lauryl sulfonate (n=30) in the absence (bottom) and presence (top) of MeOH; pressure injection for 3 s; analyte, 100 mg/l of lauryl ether sulfate (n=30). Other conditions the same as Fig. 5 except the FMN electrolyte contains a fixed volume, i.e., 50% (v/v) MeOH.

limits in the 10–25-μg/l or 0.5 μ*M* range are comparable to those previously found by CE with IPD using naphthalenedisulfonate [14]. In this work, baseline drift using negative polarity is a major reason why detection limits for small ions are not improved using indirect LIF as compared to IPD. With positive polarity, the polyphosphates and surfactants can be detected at lower levels than IPD because baseline drift is no longer a problem. These indirect detection limits of 500–1000 μg/l for surfactants are similar to those found previously using fluorescein [8]. However, selectivity in anion or alkyl sulfate detection in the presence of organic acids could be gained by CE with LIF using FMN at

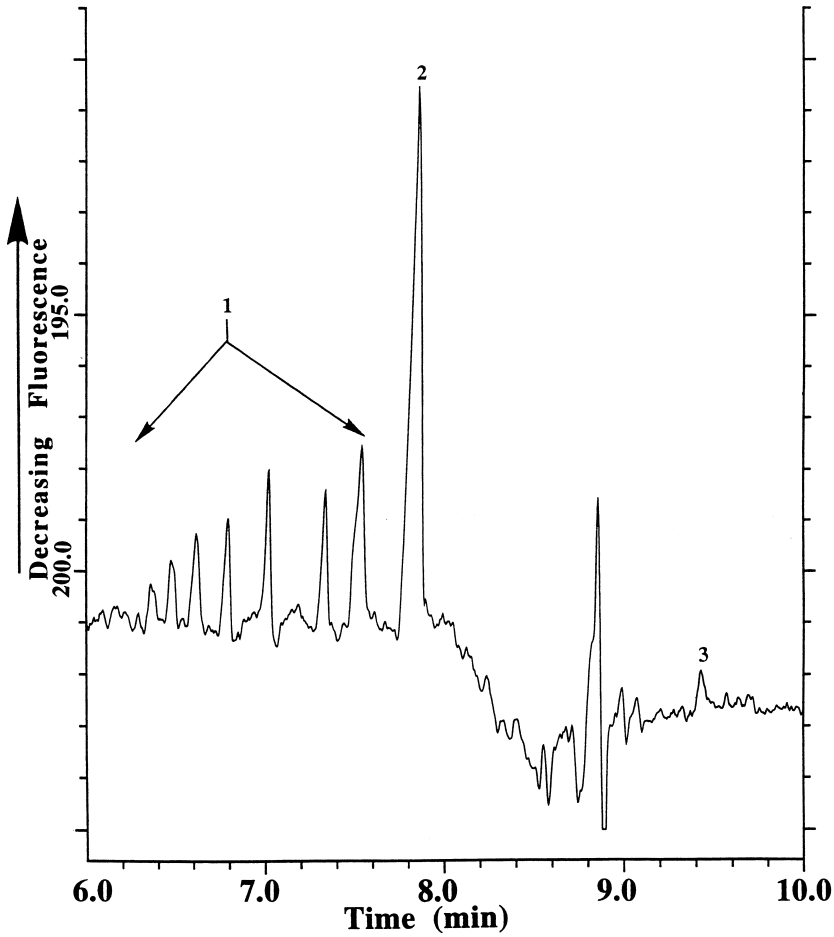

Fig. 7. Analysis of 0.1 g/100 ml of Perth Plus shampoo containing (1) laureth sulfate with *n* degree of ethoxylation, (2) $C_{12}SO_4^-$, (3) xylene sulfonate; pressure injection for 3 s. Other conditions same as Fig. 5, except the FMN electrolyte contains a fixed volume, i.e., 25% (v/v) MeOH.

an acidic pH. Many of the organic acids would be neutral at a pH of about 4 and migrate with the neutral marker.

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