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On-Line Focusing of Flavin Derivatives Using Dynamic pH Junction-Sweeping Capillary Electrophoresis with Laser-Induced Fluorescence Detection

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Simple yet effective methods to enhance concentration sensitivity is needed for capillary electrophoresis (CE) to become a practical method to analyze trace levels of analytes in real samples. In this report, the development of a novel on-line preconcentration technique combining dynamic pH junction and sweeping modes of focusing is applied to the sensitive and selective analysis of three flavin derivatives: riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Picomolar (pM) detectability of flavins by CE with laser-induced fluorescence (LIF) detection is demonstrated through effective focusing of large sample volumes (up to 22% capillary length) using a dual pH junction-sweeping focusing mode. This results in greater than a 1200-fold improvement in sensitivity relative to conventional injection methods, giving a limit of detection (S/N = 3) of ~4.0 pM for FAD and FMN. Flavin focusing is examined in terms of analyte mobility dependence on buffer pH, borate complexation and SDS interaction. Dynamic pH junction-sweeping extends on-line focusing to both neutral (hydrophobic) and weakly acidic (hydrophilic) species and is considered useful in cases when either conventional sweeping or dynamic pH junction techniques used alone are less effective for certain classes of analytes. Enhanced focusing performance by this hyphenated method was demonstrated by greater than a 4-fold reduction in flavin bandwidth, as compared to either sweeping or dynamic pH junction, reflected by analyte detector bandwidths <0.20 cm. Novel on-line focusing strategies are required to improve sensitivity in CE, which may be applied toward more effective biochemical analysis methods for diverse types of analytes.

There has long been an interest in developing effective on-line preconcentration techniques ever since Jorgenson and Lukacs first demonstrated CE in the early 1980s.[1] Although the narrow capillary used in CE is vital for obtaining rapid high-resolution separations, it has the disadvantage of generating poor concentration sensitivity when using UV detection as a result of small injection volumes and narrow optical path length. This is often insufficient to detect submicomolar levels of analyte without using more sensitive detection methods, such as laser-induced fluorescence (LIF)[2] or subjecting the sample to on-line chromatographic preconcentration[3−5] (i.e., solid-phase extraction) by attaching a short segment of sorbent to the capillary inlet. On-line solid-phase extraction methods allow for high sample enrichment, but compatibility issues regarding sample elution may reduce the CE separation performance. On-line preconcentration methods based on electrophoresis (without sorbents) represent one of the most facile ways for sample enrichment in CE (direct injection of large sample volumes), since the preconcentration step is performed within the same capillary used for analysis. Normally, injection of large volumes of sample (>1% capillary length) results in band broadening of analyte peaks due to various dispersion processes.[6,7] Since the electrophoretic mobility of the analyte is dependent on the local properties of the electrolyte, appropriate selection of different electrolyte matrixes may be used to induce focusing of sample zones prior to detection. Under favorable conditions, analytes having different mobilities (velocities) in the sample and background electrolyte (BGE) zones may focus into narrow bands electrokinetically. The same properties used to optimize separation selectivity, namely buffer pH, ionic strength, or micelle (additive) concentration, may be considered when designing effective on-line focusing strategies in CE.

Several on-line preconcentration techniques have been reported in CE that exploit changes in analyte mobility in two or more different electrolyte matrixes. By far, the most common approach to on-line analyte focusing in CE has been sample stacking, which utilizes a sample dissolved in a low conductivity

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† Kyoto University.

(1) Jorgenson, J. W.; Lukacs, K. D. *Anal. Chem.* **1981**, *53*, 1298.

(2) Zhang, J.; Fang, Y.; Hou, J. Y.; Ren, H. J.; Jiang, R.; Roos, P.; Dovichi, N. J. *Anal. Chem.* **1995**, *67*, 4589−4593.

(3) Tomlinson, A. J.; Benson, L. M.; Guzman, N. A.; Naylor, S. *J. Chromatogr. A.* **1996**, *744*, 3−15.

(4) Strausbauch, M. A.; Madden, B. J.; Wettstein, P. J.; Landers, J. P. *Electrophoresis* **1995**, *16*, 541−548.

(5) Figeys, D.; Ducret, A.; Aebersold, R. J. *J. Chromatogr. A* **1997**, *763*, 295−306.

(6) Terabe, S.; Otsuka, K.; Ando, T. *Anal. Chem.* **1989**, *61*, 251−260.

(7) Peng, X.; Chen, D. D. Y. *J. Chromatogr.* **1997**, *767*, 205−216.

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electrolyte relative to BGE, thereby generating a local enhanced analyte velocity in the sample zone.[8] Various formats of sample stacking, including large-volume sample stacking,[9−13] head-column field-enhanced sample stacking,[14,15] and pH-mediated stacking,[16−18] have been developed to focus charged analytes, resulting in up to a 100-fold sensitivity enhancement. Sample stacking may also be extended to neutral analytes when using charged micelles in the BGE.[19,20] However, in analysis of real biological or environmental samples, sample stacking has found limited application without prior desalting. Shihabi reported a modified stacking technique using acetonitrile that operates under high salt conditions in the sample.[21,22] Transient isotachophoresis (t-ITP) is an alternative on-line preconcentration format that uses a discontinuous electrolyte system consisting of leading and terminating co-ions that are selected to have mobilities greater than and less than the analyte, respectively.[23−25] Sample self-stacking has been reported to occur naturally when an analyte is in a sample matrix containing high concentrations of a suitable terminating or leading co-ion.[26−28] Early reports have also indicated that pH differences between the sample and BGE may be a promising approach for on-line focusing of some zwitterionic peptides and protein.[29,30] Recently, a dynamic pH junction of several weakly acidic analytes, including catecholamines, nucleosides, and nucleotides, has been shown to extend concentration sensitivity approaching nanomolar levels with UV detection.[31,32] In general, weakly acidic species are dissolved in an acidic or neutral sample matrix (analyte has low mobility), and separation is performed using a basic BGE in which the analyte has a high negative mobility, thereby forming a discrete pH step (i.e., junction) between the sample and BGE zones. Unlike conventional isoelectric focusing methods[33,34] that use ampholytes to generate continuous pH gradients for protein preconcentration, analyte focusing and zone separation occur simultaneously in a dynamic pH junction format using common buffers. Recently, Yeung et al.[35] reported a novel one-step analyte-focusing method using a dynamic pH gradient generated by electrolysis of water (using a dilute buffer in BGE) after inserting a short piece of platinum wire inside the capillary. However, methods based on dynamic pH changes in BGE are not applicable to focusing neutral analytes, since their mobility is unaffected by buffer pH. Sweeping[36,37] is a versatile on-line focusing method in CE that uses micelles or other complexation additives to modify analyte mobility through analyte−additive binding. In sweeping, the sample matrix is normally devoid of additive but has a conductivity similar to BGE in which there are high concentrations of micelle. Sweeping is effective for strongly interacting analytes, with the magnitude of focusing being inversely related to magnitude of analyte retention factor.[37] In addition to micellar sweeping, recent reports have also applied other types of additives to focus analytes, including charged cyclodextrin derivatives,[38,39] borate,[32,40] and poly(ethylene oxide) polymers.[41] Thus, a number of complementary approaches can be used to focus specific analytes of interest electrokinetically, thereby counteracting normal band-broadening processes. Since previous on-line preconcentration methods in CE induce focusing through changes in an analyte's velocity (mobility) in discontinuous electrolyte systems, velocity-difference-induced focusing (V-DIF) has recently been coined to describe their common underlying mechanism.[32] Better understanding of general strategies to modify analyte velocity is needed to guide researchers on the most effective ways to improve concentration sensitivity in CE without deterioration of separation performance, as was proposed by Hjertén et al.[42] for peptides and protein.

Flavins represent an important class of biomolecule that are present in most cells and tissue. The three main derivatives of flavins include the water-soluble vitamin precursor riboflavin (RF) and its two biologically active metabolites, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD serve as essential redox-active coenzymes associated with flavoenzymes that catalyze a variety of biochemical reactions, involving carbohydrate, protein, or fat metabolism.[43] Flavins are also one of the few biomolecules that exhibit native fluorescence, which makes their detection by fluorescence very convenient, since there is no need for chemical derivatization. Recently, flavin analysis by CE-LIF detection has been reported in plasma and tissue samples.[44,45] Since the concentration of flavins in plasma is often very low (nanomolar level), CE methods have normally required an off-line sample preconcentration step[45] that is time-consuming and may also decrease assay reproducibility. In this

(8) Burgi, D. S.; Chien, R. L. *Anal. Biochem.* **1992**, *202*, 306−309.
(9) Burgi, D. S. *Anal. Chem.* **1993**, *65*, 3726−3729.
(10) McGrath, G.; Smyth, W. F. *J. Chromatogr. B* **1996**, *681*, 125−131.
(11) Chien, R.; Burgi, D. S. *Anal. Chem.* **1992**, *64*, 1046−1050.
(12) Palmarsdottir, S.; Mathiasson, L.; Jonsson, J. A.; Edholm, L. E. *J. Chromatogr. B* **1997**, *688*, 127−134.
(13) Baryla, N. E.; Lucy, C. A. *Electrophoresis* **2001**, *22*, 52−58.
(14) Zhang, C. X.; Thormann, W. *Anal. Chem.* **1996**, *68*, 2523−2532.
(15) Wey, A. B.; Zhang, C. X.; Thormann, W. *J. Chromatogr. A* **1999**, *853*, 95−106.
(16) Hadwiger, M. E.; Torchia, S. R.; Park, S.; Biggin, M. E.; Lunte, C. E. *J. Chromatogr. B* **1996**, *681*, 241−249.
(17) Weiss, D. J.; Saunders, K.; Lunte, C. E. *Electrophoresis* **2001**, *22*, 59−65.
(18) Xiong, Y.; Park, S.; Swerdlow, H. *Anal. Chem.* **1998**, *70*, 3605−3611.
(19) Liu, Z. S., P.; Sirimanne, S. R.; McClure, P. C.; Grainger, J.; Patterson, D. G., Jr. *J. Chromatogr. A* **1994**, *673*, 125−132.
(20) Quirino, J. P.; Terabe, S. *Anal. Chem.* **1998**, *70*, 1893−1901.
(21) Shihabi, Z. K. *J. Chromatogr. A* **1993**, *652*, 471−475.
(22) Shihabi, Z. K. *J. Chromatogr. A* **1996**, *744*, 231−240.
(23) Kriváková, L.; Boček, P. *J. Chromatogr. B* **1997**, *689*, 13−34.
(24) Thompson, T. J.; Foret, F.; Vouros, P.; Karger, B. L. *Anal. Chem.* **1993**, *65*, 900−906.
(25) Foret, F.; Szoko, E.; Karger, B. L. *J. Chromatogr.* **1992**, *608*, 3−12.
(26) Kriváková, L.; Vrana, A.; Gebauer, P.; Boček, P. *J. Chromatogr. A* **1997**, *772*, 283−295.
(27) Beckers, J. L. *J. Chromatogr.* **1993**, *641*, 363−373.
(28) Gebauer, P.; Thormann, W.; Boček, P. *Electrophoresis* **1995**, *16*, 2039−2050.
(29) Aebersold, R.; Morrison, H. D. *J. Chromatogr.* **1990**, *516*, 79−88.
(30) Schwer, C.; Lottspeich, F. *J. Chromatogr.* **1992**, *623*, 345−355.
(31) Britz-McKibbin, P.; Chen, D. D. Y. *Anal. Chem.* **2000**, *72*, 1242−1252.
(32) Britz-McKibbin, P.; Chen, D. D. Y. *Anal. Chem.* **2000**, *72*, 1729−1735.
(33) Hjertén, S.; Zhu, M. J. *J. Chromatogr.* **1985**, *346*, 265−270.
(34) Righetti, P. G.; Bossi, A. *Anal. Chim. Acta* **1998**, *372*, 1−19.
(35) Wei, W.; Xue, G.; Yeung, E. S. *Anal. Chem.* **2002**, *74*, 934−940.
(36) Quirino, J. P.; Terabe, S. *Science* **1998**, *282*, 465−468.
(37) Quirino, J. P.; Terabe, S. *Anal. Chem.* **1999**, *71*, 1638−1644.
(38) Quirino, J. P.; Terabe, S.; Otsuka, K.; Vincent, J. B.; Vigh, G. *J. Chromatogr. A* **1999**, *838*, 3−10.
(39) Munro, N. J.; Palmer, J.; Stalcup, A. M.; Landers, J. P. *J. Chromatogr. B* **1999**, *731*, 369−381.
(40) Quirino, J. P.; Terabe, S. *Chromatographia* **2001**, *53*, 285−289.
(41) Hsieh, M. M.; Tseng, W. L.; Chang, H. T. *Electrophoresis* **2000**, *21*, 2904−2910.
(42) Hjertén, S.; Liao, J. L.; Zhang, R. *J. Chromatogr. A* **1994**, *676*, 409−420.
(43) De Leenheer et al.; *Modern Chromatographic Analysis of Vitamins*, 3rd ed.; Chromatographic Series; Marcel Dekker: New York, 2000; Series Vol. 84.
(44) Pérez-Ruiz, T.; Martínez-Lozano, C.; Sanz, A.; Bravo, E. *Electrophoresis* **2001**, *22*, 1170−1174.
(45) Hustad, S.; Ueland, P. M.; Schneede, J. *Clin. Chem.* **1999**, *45*, 862−868.

report, the development of a novel on-line preconcentration technique that combines dynamic pH junction and sweeping is used as a simple method to focus trace amounts of flavins by CE using LIF detection. Dynamic pH junction-sweeping is defined when the sample is devoid of SDS (sweeping condition) and has a different buffer pH (dynamic pH junction condition) relative to BGE. To the best of our knowledge, this is the first reported example of a dynamic pH junction-sweeping focusing method used in CE. Analyte focusing is determined to be mediated by buffer pH, borate complexation, and SDS partitioning, which allows for greater than a 1200-fold improvement in sensitivity compared to conventional injections. Under optimum conditions, the limit of detection (LOD) with S/N = 3 is ~4.0 pM for the coenzymes FAD and FMN. Enhanced focusing performance was demonstrated by greater than a 4-fold reduction in flavin detector bandwidth in comparison to either sweeping or dynamic pH junction techniques alone. On-line focusing by dynamic pH junction-sweeping represents an effective way to enhance concentration sensitivity in CE as well as modifying selectivity that may be suitable to complex sample mixtures, including hydrophobic and weakly acidic species.

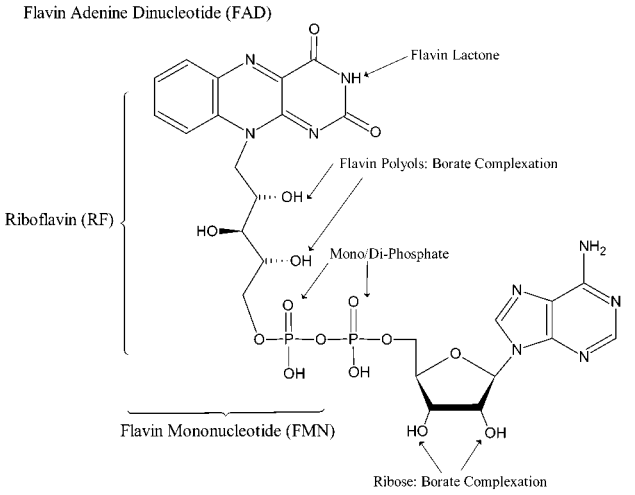

Figure 1. Chemical structures of RF, FMN, and FAD depicting weakly acidic functional groups and vicinal diol moieties capable of borate complexation. SDS micelle may also be added to buffer to modify flavin mobility for enhanced focusing by dynamic pH junction-sweeping in CE.

EXPERIMENTAL CONDITIONS

Apparatus and Procedure. Separations were performed on a Beckman P/ACE 2000 automated capillary electrophoresis system (Beckman Instruments, Tokyo, Japan). Uncoated capillaries (Polymicro Technologies, Phoenix, AZ) with 75-μm i.d., 375-μm o.d., and 57-cm length were used. New capillaries were first rinsed with 1.0 M NaOH (5 min, 20 psi, or 140 kPa), followed by rinsing with the separation BGE (10 min, 140 kPa). The capillary was then allowed to equilibrate overnight in the separation BGE prior to use. Each separation was preceded by a 1.5-min rinse with 0.1 M NaOH, followed by a 3-min rinse with BGE. The sample was introduced using a low pressure (0.5 psi or 3.5 kPa) injection ranging from 1 to 300 s. The average flow rate of the low-pressure injection in a 57-cm capillary was determined to be 0.157 and 0.137 cm/s for 140 mM borate, pH 10.0, and 140 mM borate, 100 mM SDS, pH 8.5, buffers, respectively, which was used to estimate injection bandwidth (w_{inj}). Detector bandwidths (w_{det}) of analytes were calculated from flavin migration time, baseline peak width, and length of capillary to detector in order to normalize measured bandwidths for differential analyte migration velocities.[31] The conductivity of solutions was calculated by measuring the current generated when a capillary of known dimensions (50-cm length, 75-μm i.d.) was filled with solution at a specified voltage. To test flavin focusing under suppressed EOF conditions, polyacrylamide-coated capillaries that were prepared in-house as described previously were used.[46] All separations were carried out at 25 °C using a voltage of 15 kV. Laser-induced fluorescence excitation was carried out with a 488-nm line of a 4 mW Beckman P/ACE System argon ion laser, which was coupled to the CE instrument via a fiber optic cable. Emission was monitored at 520 nm with a Beckman fluorescence detector. Data were collected and processed using P/ACE Station software (Beckman).

Chemicals and Reagents. Water used for buffer and sample preparations was obtained by using a Milli-Q water purification system (Millipore, Bedford, MA). The aqueous BGE consisted of 140 mM borate (Borax, Wako, Osaka, Japan). The pH of the separation buffer was adjusted by using 1.0 M NaOH and boric acid (Nacalai Tesque, Kyoto, Japan) within a range of pH 8.5 to 10.0. The BGE used for experiments was 140 mM borate at pH 8.5 or 10.0, unless otherwise indicated. Sudan III and caffeine (Wako) were used as the SDS and EOF markers, respectively, to determine flavin electrophoretic mobilities and the SDS retention factor with UV absorbance detection (Beckman). Sodium dihydrogen phosphate, sodium dodecyl sulfate (SDS), and riboflavin (vitamin B2) were all purchased from Wako. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were purchased from Sigma (St. Louis, MO) and TCI Chemicals (Tokyo, Japan), respectively. It is important to note that because flavins are light- and alkali-sensitive, stock solutions (8×10^{-5} M for RF, 1×10^{-3} M for FMN and FAD) were prepared in amber glass bottles and diluted with deionized water while stored refrigerated at 4 °C. These stock solutions were then further diluted and mixed with the appropriate sample matrix in opaque black ependorf tubes (Treff AG, Degerscheim, Switzerland) prior to injection into the capillary. Peaks were identified by spiking the sample solution with standard solutions of each flavin.

RESULTS AND DISCUSSION

Flavin Chemical Structure and Focusing Rationale. Buffer pH and additive (e.g., micelle) represent two of the most effective ways to modify analyte selectivity in CE that may also be applied together toward new strategies for on-line sample preconcentration. As depicted in Figure 1, the unique chemical structure of flavins and their variable electrophoretic mobility in different buffer pHs (see Table 1) and micelle concentration permit their on-line focusing by CE using dynamic pH junction-sweeping. Figure 2 depicts three different CE setups used for performing flavin (RF shown) on-line preconcentration in this report. Since Figure 2a utilizes a continuous electrolyte system, large volumes of injected RF results in broad peaks, since there is no mobility change in the sample and BGE zones to counteract sample overloading. In contrast, on-line preconcentration of RF by dynamic pH junction,

(46) Kim, J. B.; Otsuka, K.; Terabe, S. *J. Chromatogr. A* **2001**, *912*, 343−352.

Table 1. Electrophoretic Mobility (μ_{Ep}) of Flavins as a Function of Buffer pH

buffer conditions	$-\mu_{ep,RF} \times 10^{-4}$ cm^2/Vs	$-\mu_{ep,FMN} \times 10^{-4}$ cm^2/Vs	$-\mu_{ep,FAD} \times 10^{-4}$ cm^2/Vs
(a) 75 mM carbonate, pH 10.0	0.545	2.02	2.28
(b) 140 mM borate, pH 10.0	1.86	2.88	3.01
(c) 140 mM borate, pH 8.5	1.34	2.62	2.82
(d) 75 mM phosphate, pH 6.0	0	1.72	2.00

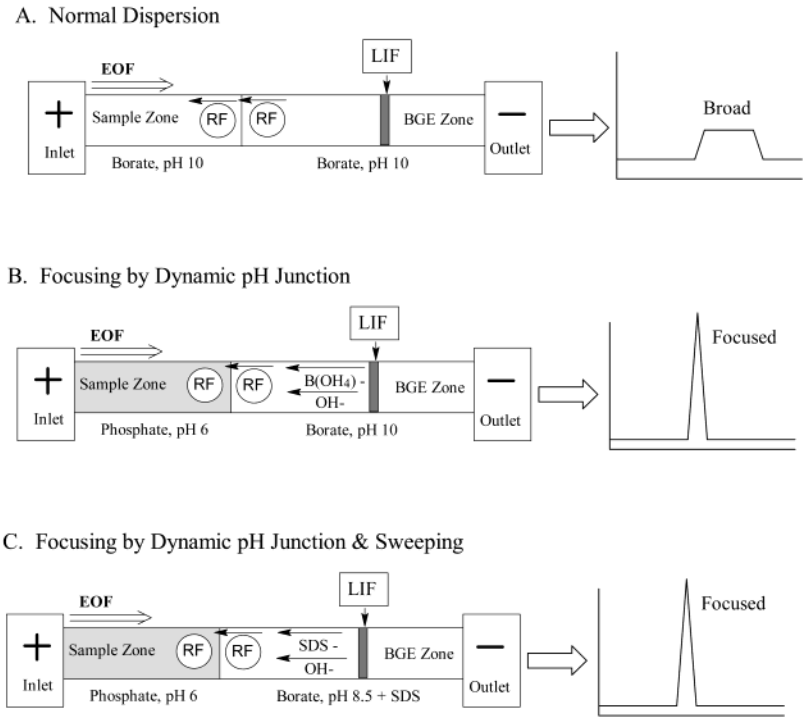

Figure 2. Schematic representation comparing the setup conditions used for three different approaches for on-line focusing of large injection volumes of RF: normal band dispersion with (a) continuous electrolyte system and band narrowing using (b) dynamic pH junction and (c) dynamic pH junction-sweeping with discontinuous electrolyte systems. Improved band focusing of RF may be generated by analyte mobility changes as a result of pH, borate complexation, and SDS partitioning differences in the sample and BGE zones when using dynamic pH junction-sweeping.

as shown in Figure 2b, utilizes a discontinuous electrolyte system consisting of a weakly acidic phosphate pH 6 buffer in the sample relative to borate pH 10 in BGE. RF, which contains a weakly acidic lactam group on the fluorescent isoalloxazine tricyclic ring system ($pK_a = 10.2$)[47] and ribose polyol group, is neutral in phosphate pH 6, but is negatively charged in alkaline borate buffer, pH 10 ($\mu_{ep} = -1.86 \times 10^{-4}$ cm^2/Vs) as a result of borate−vicinal diol complexation and partial lactam deprotonation. Borate has the unique property of serving as both the alkaline buffer as well as selective complexation agent for vicinal diol compounds. Borate−flavin complexation was confirmed by measuring the mobility of RF in 75 mM carbonate buffer, pH 10 ($\mu_{ep} = -5.45 \times 10^{-5}$ cm^2/Vs), which was significantly lower than in borate at the same pH. FMN and FAD possess a higher negative mobility than RF because of the additional phosphate and adenine monophosphate groups, respectively. Other buffer types may be used in the sample and BGE to select the most appropriate pH range to modify analyte mobility. SDS may be also added to BGE to further increase the negative mobility of flavins (such as RF) on the basis of analyte partitioning into anionic micelle via hydrophobic interactions. Micelle sweeping may be combined with dynamic pH junction to simultaneously focus neutral (hydrophobic) and ionic analytes, as well as to improve the focusing performance for certain analytes as compared to either dynamic pH junction or sweeping formats alone. For example, as a way to enhance SDS partitioning and analyte sweeping, flavins may be dissolved in acidic phosphate buffer, pH 6, in order to reduce their negative charge (RF is neutral) while retaining the selectivity of borate separation under alkaline conditions. This is in contrast to conventional sweeping formats that use the same buffer type and pH in the sample and BGE zones. In fact, the magnitude of RF−SDS partitioning is over 8-fold greater in phosphate pH 6 ($k = 5.0$), as compared to borate pH 8.5. Figure 2c depicts the RF focusing setup using a combined dynamic pH junction-sweeping technique when the sample electrolyte used is phosphate, whereas the BGE consists of borate pH 8.5 with SDS. Enhanced flavin focusing may be realized by using dynamic pH junction-sweeping format, since band narrowing is induced by several distinct processes (which may be additive), including buffer pH, borate complexation, and micelle partitioning. This combined focusing approach is aimed at overcoming the often poor band-narrowing efficiency of conventional sweeping (using anionic micelles) and

(47) *Handbook of Biochemistry: Selected Data for Molecular Biology*; CRC Press: Cleveland, 1970.

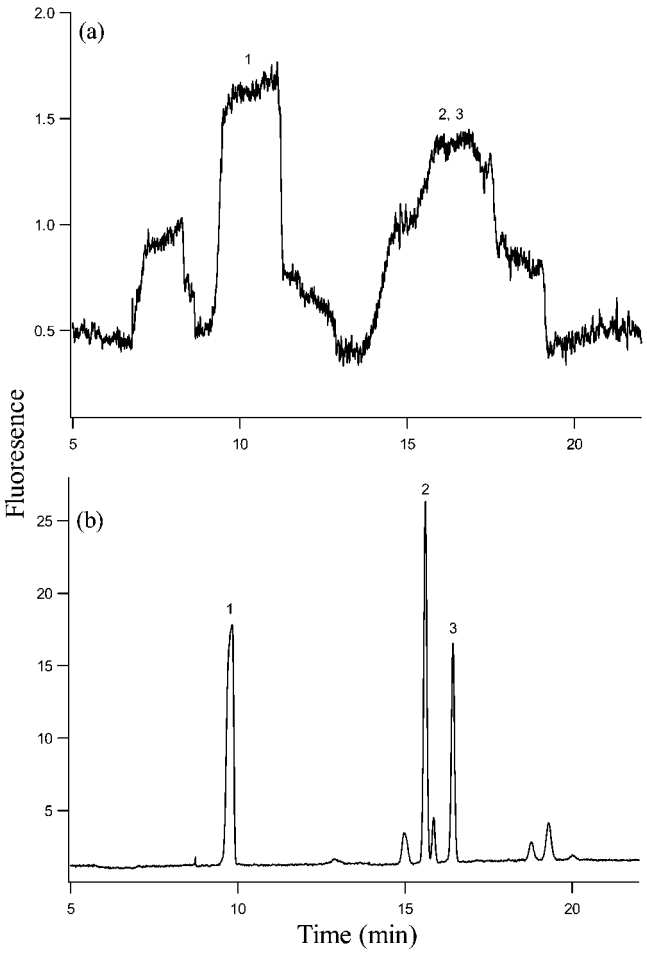

Figure 3. Two electropherograms comparing flavin focusing using large injection plugs (9.4 cm) with (a) continuous electrolyte system (no pH differences) and (b) dynamic pH junction in CE. The BGE used in both electropherograms was 140 mM borate at pH 10. Sample solutions contained 0.2 μM flavins dissolved in either (a) 140 mM borate, pH 10, or (b) 75 mM phosphate, pH 6.0. Conditions: aqueous 140 mM borate buffer; voltage, 15 kV; capillary length, 57 cm; injection, 60s. Analyte peak numbering corresponds to 1, RF; 2, FMN; and 3, FAD.

dynamic pH junction for hydrophilic and neutral analytes, respectively.

Flavin Focusing Using Dynamic pH Junction. Preliminary CE experiments revealed concentration sensitivity for flavins was poor even when using LIF detection. When using conventional small injections (1 s) with 140 mM borate, pH 10, the LOD was determined to be ~0.01 μM for FAD. This relatively low fluorescence sensitivity is due to the poor excitation of flavins (absorption maximum at 450 nm) using the 488-nm line of an argon ion laser. In addition, flavins exhibit lower fluorescence quantum efficiencies under alkaline conditions[44] (optimum pH < 8), but this disadvantage is offset by an improvement in the selectivity of the separation when using borate at pH > 9. Larger volumes of sample injected into the capillary may be used to increase concentration sensitivity; however, this normally leads to detrimental broadening when there does not exist a specific electrokinetic focusing mechanism to counteract dispersion. This is highlighted in Figure 3a, which shows a broad w_{det} of 19.7 cm for FAD and FMN (overlapped) when using w_{inj} of 9.4 cm. It should be noted that Figure 3a uses a continuous electrolyte system in which both sample and BGE have similar matrix properties. Conventional sample stacking of flavins was also attempted to increase concentration sensitivity (w_{inj} = 9.4 cm) when the sample is dissolved in a 10-fold diluted buffer. Although significant focusing was noticed for RF, sample overloading generated poor resolution and separation performance (overlapping FMN/FAD peaks) as a result of the high electric field strength caused by the low-conductivity sample plug. Thus, a more effective and simple method for focusing all three flavin derivatives is required to improve concentration sensitivity in CE.

Previous reports of dynamic pH junction have demonstrated it to be a useful on-line preconcentration method for focusing large volumes of weakly acidic species, including analytes having vicinal diol functionalities.[31,32,48] On-line focusing of flavins by dynamic pH junction was investigated using a discontinuous electrolyte system consisting of 75 mM phosphate, pH 6, and 140 mM borate (pH was varied from 8.5 to 10.5) in the sample and BGE zones, respectively. In all cases, significant flavin focusing by dynamic pH junction was observed, as compared to using a continuous buffer as shown in Figure 3a. Interestingly, a pH difference of only 2.5 units between sample and BGE (using 140 mM borate, pH 8.5) was sufficient to focus flavins, most notably, FMN and FAD (w_{det} = 0.40 cm). However, FMN and FAD migrated as sharp but overlapping (unresolved) peaks. As the pH of the BGE was increased from 8.5 to 10.5, improved focusing of RF and resolution of FMN/FAD was observed. Optimal flavin focusing, in terms of both resolution and sensitivity enhancement, was obtained using 140 mM borate pH 10. Figure 3b depicts an electropherogram under optimum conditions using a dynamic pH junction to focus a w_{inj} of 9.4 cm containing 0.2 μM flavins. Higher currents and lower flavin fluorescence signals were observed when pH of BGE was >10.

Table 1 shows the measured electrophoretic mobilities of flavins as a function of borate pH, in comparison to phosphate pH 6 and carbonate pH 10 buffers. Flavins were measured to have the lowest electrophoretic mobilities in phosphate, pH 6, with RF being neutral in this buffer, whereas FAD and FMN are still negatively charged because of the strong acidity of the phosphate group. The mobility of flavins increase gradually as the pH of borate was increased from 8.5 to 10.0 because of deprotonation of lactam group and enhanced borate complexation with vicinal polyol groups.[49] Focusing of large volumes of flavins by dynamic pH junction is induced by analyte mobility changes in the sample and BGE zones. Previous reports have used the detector-to-injection-bandwidth ratio (DIBR) as a quantitative measure for assessing analyte focusing that corrects for different migration velocities.[31,32] Analyte focusing is defined when DIBR values are <1. For example, FAD peak in Figure 3b has a w_{det} of only 0.91 cm, which results in a DIBR of 0.097. This indicates that the original sample plug for FAD is narrowed by over 10-fold during focusing when using a dynamic pH junction (see Table 2).

Flavin Focusing Using Dynamic pH Junction-Sweeping. As demonstrated in Figure 3b, focusing by dynamic pH junction was less successful for low-mobility analytes, such as RF (w_{det} = 2.4 cm), as compared to flavin coenzymes, as well as being generally ineffective for neutral species. This trend is in contrast

(48) Britz-McKibbin, P.; Wong, J.; Chen, D. D. Y. *J. Chromatogr. A* **1999**, *853*, 535−540.
(49) Hoffstetter-Kühn, S.; Paulus, A.; Gassman, E.; Widmer, H. M. *Anal. Chem.* **1991**, *63*, 1541−1547.

Table 2. Bandwith Values to Quantitate FAD Focusing Using Different On-Line Focusing Methods

on-line focusing method	injection bandwidth w_{inj} (cm)	detector bandwidth w_{det} (cm)	w_{det}/w_{inj} (DIBR)	sample buffer conditions[a]
(a) conventional	0.16	0.70	4.4	140 mM borate, pH 10.0[b]
(b) conventional	9.4	19.7	2.1	140 mM borate, pH 10.0
(c) sample stacking	9.4	12.1	1.3	14 mM borate, pH 10.0
(d) sweeping	8.2	4.25	0.52	140 mM borate, pH 8.5
(e) dynamic pH junction	9.4	0.91	0.097	75 mM phosphate, pH 6.0
(f) dynamic pH junction-sweeping	8.2	0.20	0.024	75 mM phosphate, pH 6.0

[a] BGE used in lines (a)−(c) and (e) is 140 mM borate, pH 10, and (d) and (f) is 140 mM borate, 100 mM SDS, pH 8.5, using a low-pressure injection of 60 s. [b] Normal sample injection using a low pressure of 1 s.

to the focusing performance of sweeping using anionic micelles, such as SDS. It is for this reason that dynamic pH junction-sweeping was considered to represent a unique way to improve focusing efficiency or peak sharpness of all three flavins for increased sensitivity. Conventional sweeping using SDS was first examined to compare its focusing performance relative to dynamic pH junction. To improve overall sensitivity of flavin analysis by CE, the use of a less alkaline buffer system is favored, since flavin fluorescence quantum yield is enhanced when pH is <10.[44] The lower borate pH is also important to facilitate SDS partitioning, since flavins have a lower negative charge at this pH. Figure 4a depicts optimal sweeping conditions when using 100 mM SDS as the additive in borate pH 8.5 (sample is devoid of SDS). Concentrations of SDS >100 mM generated excessive current as well as longer analyte migration times. As shown in Figure 4a, a large injection of flavins resulted in partial focusing of only RF (w_{det} = 2.1 cm) by SDS sweeping, whereas both FMN and FAD are broad overlapping peaks. Because the retention factor of flavins is relatively low for SDS (k = 0.6 for RF at pH 8.5) under alkaline buffer conditions, conventional sweeping using anionic micelles is normally not effective for focusing weakly interacting hydrophilic analytes. Sweeping is normally performed under strong acidic conditions to suppress EOF and enhance micelle partitioning, but this unnecessarily restricts buffer pH that is often essential in CE selectivity. The use of different types of micelles, such as cationic CTAB[46] or nonionic micelles,[50] has been reported to improve sweeping of anionic analytes under alkaline conditions; however, in the case of cationic micelles, the capillary must be coated to prevent EOF reversal.

As a simple way to improve the sensitivity of flavin analysis by CE compared to either dynamic pH junction or sweeping, a combined dynamic pH junction-sweeping approach was examined. Dynamic pH junction-sweeping is defined when the sample has a different buffer pH (dynamic pH junction condition) and is devoid of SDS (sweeping condition) relative to BGE. As higher concentrations of SDS (20−120 mM) were added to 140 mM borate, pH 8.5 BGE, gradual improvement in the resolution and focusing of FMN and FAD was observed. Moreover, the migration time and peak focusing of RF was observed to dramatically increase when SDS concentration was >60 mM. Optimum focusing and separation of flavins was realized when using 100 mM SDS in BGE, as depicted in Figure 4b. Despite similar migration times, FMN and FAD peaks are baseline-resolved (R_s = 0.92) because of their extremely sharp peak widths. It is apparent that flavin-focusing by dynamic pH junction-sweeping greatly outperforms conventional sweeping by efficiently focusing hydrophilic flavin coenzymes, as well as by improving RF peak bandwidth within shorter analysis times. Furthermore, up to a 12-fold improvement in flavin sensitivity is achieved when using the combined dynamic pH junction-sweeping format in Figure 4b, as compared to dynamic

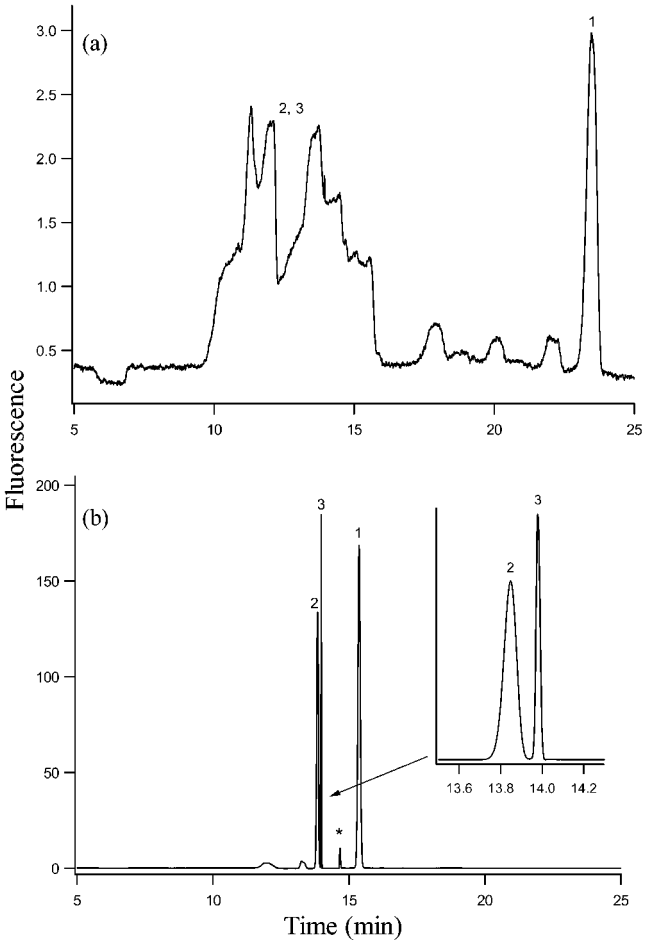

Figure 4. Two electropherograms comparing flavin focusing using large injection plugs (8.2 cm) with (a) conventional sweeping and (b) dynamic pH junction-sweeping. The BGE used in both electropherograms was 140 mM borate, 100 mM SDS, pH 8.5. Sample solutions contained 0.2 μM flavins dissolved in either (a) 140 mM borate, pH 8.5, or (b) 75 mM phosphate, pH 6.0. Analyte peak numbering and conditions are the same as in Figure 3, except that the system peak is noted by an asterisk, ∗. Note the change in selectivity of RF relative to dynamic pH junction in Figure 3b.

(50) Monton, M. R. N.; Quirino, J. P.; Otsuka, K.; Terabe, S. *J. Chromatogr. A* **2001**, *939*, 99−108.

pH junction alone in Figure 3b, as a result of the generation of sharper peak bandwidths, as well as higher flavin fluorescence at lower borate pH 8.5 conditions. Two system peaks were observed when using dynamic pH junction-sweeping with SDS micelles that were present in both flavin sample and blank sample electropherograms. One minor system peak comigrated with the FAD peak, in addition to a second system peak (noted by an asterisk, ∗) that migrated before RF peak, as depicted in Figure 4b. System peaks have been previously observed in other on-line focusing techniques in CE and may represent discrete electrolyte boundaries that develop when using discontinuous electrolyte systems.[51]

The relative order of flavin migration in Figure 4b implies that RF has the highest affinity for SDS, followed by weak interaction to FAD and negligible binding to FMN. This trend may be reflected by the relative hydrophobicity of flavins. The detector bandwidths of flavins in Figure 4b are all extremely narrow with RF, FMN and FAD having w_{det} of 0.85, 0.70, and 0.20 cm, respectively. In the case of FAD, this results in a DIBR of 0.024 or greater than a 40-fold reduction in original w_{inj}. Table 2 summarizes w_{inj}, w_{det} and DIBR values measured for FAD in this report when using different on-line preconcentration techniques to focus large volumes of sample used. It should be noted that the relatively smaller w_{inj} reported when using 140 mM borate, 100 mM SDS, pH 8.5, buffer is caused by the increased viscosity of the solution. It is apparent that the largest degree of FAD band focusing is obtained using the dynamic pH junction-sweeping format. Interestingly, the FAD w_{det} of 0.20 cm when using a 60-s injection of sample by dynamic pH junction-sweeping is nearly four times more narrow than the smallest injection bandwidth possible when using a 1-s injection, as shown in Table 2. The ability to obtain extremely sharp peaks serves to greatly improve not only the concentration sensitivity but also the resolution of a separation.

Further experiments were carried out to determine the effect of changes in sample conductivity on flavin focusing. It was noticed that optimum flavin focusing occurred in both dynamic pH junction and dynamic pH junction-sweeping formats when the conductivity of the sample and BGE were of similar magnitude, as was observed in a previous report.[32] For example, the sample (75 mM phosphate, pH 6.0) had a slightly lower conductivity of 8.0 mS/cm, as compared to BGE having 10.4 mS/cm and 9.7 mS/cm for 140 mM borate, pH 10, and 140 mM borate, 100 mM SDS, pH 8.5, respectively. The influence of phosphate ionic strength (10−150 mM) in the sample on flavin focusing by dynamic pH junction was also examined to better understand the impact of conductivity. Gradual broadening of flavin peaks was observed when the ionic strength of phosphate, pH 6, used in the sample was >100 mM. Lower phosphate concentration (<75 mM) resulted in poor flavin focusing and resolution as a result of the detrimental effects of sample stacking. Thus, this focusing mechanism operates under nonfield enhancement conditions and may be applicable to real samples in high salt matrixes. Flavin focusing by dynamic pH junction-focusing was also performed using a polyacrylamide-coated capillary in order to test the influence of EOF on analyte focusing. Under suppressed EOF conditions (negative polarity), the order of flavin migration was reversed, with RF, FMN, and FAD having w_{det}'s of 0.53, 0.44, and 0.28 cm, respectively. In the case of RF and FMN, peak sharpness was improved substantially under suppressed EOF conditions. This may be a result of extra band-broadening processes caused by an EOF mismatch when using mixed buffers in uncoated (strong EOF) capillaries. In general, dynamic pH junction-sweeping represents an effective on-line preconcentration technique that can be applied under both strong and suppressed EOF conditions to generate extremely narrow analyte bandwidths.

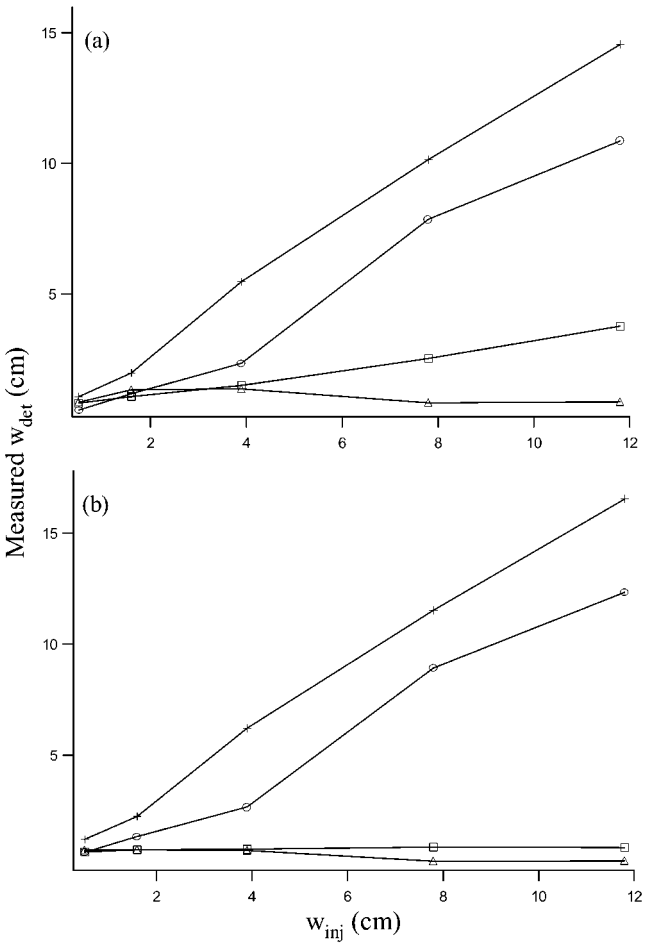

Figure 5. Two graphs comparing the measured detector bandwidth (w_{det}) of (a) RF and (b) FAD as a function of injection bandwidth (w_{inj}) using four different on-line preconcentration formats studied in this report. Shapes on plot lines represent (+) continuous electrolyte system, (○) sample stacking, (□) dynamic pH junction, and (△) dynamic pH junction-sweeping. Note that about a 4-fold enhancement in flavin focusing was observed when using dynamic pH junction-sweeping, as compared to dynamic pH junction with large sample injection volumes.

Effect of Injection Length on Flavin Focusing and Method Validation. Figure 5 compares the effect of injection length on the measured flavin w_{det} using different on-line focusing techniques studied in this report, which include continuous electrolyte system (normal broadening), sample stacking, dynamic pH junction, and dynamic pH junction-sweeping. Figure 5a depicts the measured w_{det} of RF as a function of w_{inj} ranging from 0.5 to ∼12 cm (75 s or 22% capillary length), which was the maximum injection length that permitted focusing and base separation of flavin peaks. Figure 5b compares the same effect measured for FAD in this report. It is apparent in Figure 5 that as w_{inj} was increased, both RF and FAD exhibited deleterious band broadening as a result of sample

(51) Quirino, J. P.; Terabe, S. *J. Microcolumn Sep.* **1999**, *11*, 513−520.

overloading when using continuous electrolyte system. In the case of conventional stacking, flavin focusing was only effective when using relatively smaller w_{inj}, much less than 4 cm. In contrast, dynamic pH junction greatly enhanced flavin focusing, as compared to sample stacking, even when using a large w_{inj}. Although dynamic pH junction was effective in focusing FAD (w_{det} = 0.83 cm or DIBR = 0.070) in Figure 5b, it was clearly less successful for RF (w_{det} = 3.8 cm or DIBR = 0.26) when using the largest injection plug, as shown in Figure 5a. However, dynamic pH junction-sweeping greatly enhanced focusing efficiency of both flavins, most notably for RF. Figure 5a demonstrates that RF bandwidth using dynamic pH junction-sweeping is further narrowed by ~4.4-fold to w_{det} = 0.86 cm, in comparison to dynamic pH junction. Although not as readily apparent as the RF graph, Figure 5b also shows that FAD bandwidth is sharpened by ~3.6-fold relative to dynamic pH junction, resulting in a w_{det} of 0.23 cm. Consequently, dynamic pH junction-sweeping resulted in better focusing performance of all three flavins, as compared to dynamic pH junction or sweeping alone when using large sample injections.

Excellent reproducibility (n = 10) of migration times and peak area for flavins when using dynamic pH junction-sweeping with long sample injections were reflected by RSDs of 0.7 and 3.8%, respectively. The limit of detection (LOD, defined as S/N = 3) for flavin coenzymes, FAD and FMN, while retaining baseline resolution, was determined to be ~4.0 pM when using a 8.2-cm injection plug with dynamic pH junction-sweeping. This represents about a 60-fold improvement in sensitivity for FAD by CE-LIF, as compared to a previous method,[44] and permits direct analyses of flavins from biological samples without off-line solid-phase extraction, which will be presented in a following report.[52] In contrast to normal injections, in this study, greater than a 1200-fold improvement in concentration sensitivity for flavin coenzymes was achieved when using dynamic pH junction-sweeping because of the combined benefits of large sample injection volumes, narrow analyte w_{det}, and a higher fluorescence yield at lower alkaline conditions. Further work is necessary to better understand the focusing mechanism of dynamic pH junction-sweeping as well as assessing its potential to focus other types of analytes with UV detection. Ways to directly visualize pH changes occurring in the capillary, such as whole-capillary optical imaging,[53] including computer modeling of analyte and buffer co-ion mobility behavior, may prove valuable for the rational design of more effective techniques to focus analytes in CE for enhanced sensitivity.

(52) Britz-McKibbin, P.; Markuszewski, M.; Nishioka, T.; Terabe, S. *Anal. Chem*., submitted.

(53) Mao, Q.; Pawliszyn, J.; Thormann, W. *Anal. Chem*. **2000**, *72*, 5493−5502.

CONCLUSION

A dynamic pH junction-sweeping focusing technique was developed as a new strategy for on-line preconcentration of three flavin derivatives, RF, FMN, and FAD, by CE-LIF. This technique further extends the usefulness of on-line focusing methods in CE to simultaneously preconcentrate and separate both weakly acidic (hydrophilic) and neutral (hydrophobic) analytes using inexpensive buffers and conventional instrumentation. Focusing is electrokinetically driven and is dependent on analyte mobility (velocity) changes in which the sample has a different buffer pH (dynamic pH junction) and is devoid of surfactant (sweeping) relative to BGE. Buffer pH, borate complexation, and SDS partitioning were determined as the most important parameters influencing flavin focusing when the conductivity (ionic strength) of buffer junction is optimized. Greater than a 4-fold enhancement in band narrowing of flavin zones was demonstrated by dynamic pH junction-sweeping relative to either conventional sweeping or dynamic pH junction formats alone. Picomolar detectability of flavins by CE-LIF with a LOD of ~4.0 pM for flavin coenzymes, FAD and FMN, was achieved with good reproducibility. Further studies of CE using dynamic pH junction-sweeping on other classes of analytes is needed to assess its potential as an efficient on-line focusing technique that may be important in cases when either dynamic pH junction or sweeping techniques are less effective for certain sample types.

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