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Electrophoretic analysis of amines using reversed-phase, reversed-polarity, head-column field-amplified sample stacking and laser-induced fluorescence detection

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Abstract

This paper describes the use of reversed-phase, reversed-polarity head-column field-amplified sample stacking (HCFASS) for on-line sample concentration in conventional capillary electrophoresis. The effective stacking efficiency was determined as a function of sodium hydroxide concentration in the sample matrix. Results concur with theoretical predictions where stacking efficiency depends on the conductivity (electric field strength) and electrophoretic mobility in the sample matrix solution. Fluorescein isothiocyanate-derivatized aniline and 2,4-dimethylaniline were dissolved in sodium hydroxide (800 μM), separated in a phosphate running buffer (0.05 *M*, pH 9.0) and detected utilising laser-induced fluorescence. The use of reversed-phase, reversed-polarity HCFASS with laser-induced fluorescence detection yielded sensitivity improvements with respect to normal injection schemes in excess of three orders of magnitude, and a limit of detection as low as 10^{-13} M.

Keywords: Field-enhanced sample stacking; Amines

1. Introduction

Over the past two decades, capillary electrophoresis (CE) has emerged as a powerful alternative to high-performance liquid chromatography (HPLC) in separation science. CE methods afford high-speed and high-efficiency separations, utilise relatively inexpensive and long lasting capillary columns, and consume small volumes of sample and reagent. Since samples can be introduced into the capillary via electrokinetic mechanisms extremely small volumes (pL–nL) can be injected easily. This results in improved component resolution and also a high mass sensitivity. Unfortunately, the small injection volumes and short optical pathlengths (25–75 μm) encountered in most systems dictate that concentration sensitivity (using UV absorption) is relatively poor when compared to HPLC methods. In practice, the optical pathlength can be increased by incorporating bubble [1–4], Z-shaped [5], or multi-reflection [6] flow cells. These modifications generally yield a moderate (10-fold) pathlength extension but are accompanied by a reduction in component resolution.

More recently, laser-induced fluorescence (LIF) methods have become increasingly popular due to the availability of a diversity of highly stable, low cost laser sources. Conventionally, LIF methods afford concentration detection limits three orders of magnitude better than absorption techniques [7]. Alternative approaches to overcome the problems

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associated with nanoliter injection volumes have utilised the phenomenon of on-column sample preconcentration. Of these, three techniques have been most frequently applied to the analysis of charged analytes; isotachophoresis (ITP) [8,9], intra-capillary solid-phase extraction (SPE) [10,11] and field-amplified sample stacking (FASS) [12–45]. ITP is the electrophoretic counterpart of displacement chromatography and involves the use of two distinctive electrolyte buffers, a leading and a terminating electrolyte. Sample is sandwiched between the two and on application of an electric field the sample components distribute themselves based on their individual electrophoretic mobilities. If a component zone is dilute, ITP compresses this zone and can lead to significant sample enrichment. However, ITP–CE methods suffer from a number of drawbacks including the fact that only anions or cations can be separated in a single run. Consequently, ITP–CE is only used for specialised analyses. SPE is a physical concentration technique that is commonly employed for pre-treatment of crude samples in order to isolate a given analyte from matrix components. SPE involves specific binding of the analyte to an immobilised phase prior to electrophoretic analysis. Although, highly efficient at pre-treating large sample volumes (up to 250-fold enrichment has been reported) SPE methods are both complex and costly. A more practical and effective way of sample preconcentration is via on-line FASS. FASS was first demonstrated by Burgi and Chien [18,22,46,47]. FASS results from the movement of sample ions across a boundary of low and high conductivity zones. As an example, the principle of sample stacking of cations is summarised in Fig. 1. Sample ions are initially contained within a low conductivity solution whilst the background buffer region is of high conductivity (Fig. 1a). Upon application of a voltage, the low conductivity region experiences a higher electric field in relation to the background buffer region. Consequently, sample ions move more quickly in the low conductivity region than in the high conductivity region. The abrupt change in sample ion velocity across the concentration boundary results in a reduction of sample zone length and therefore an increase in the sample concentration (Fig. 1b). Sample stacking with anions usually follows the same principle with negative electrode on the inlet side. Separation is then performed by reversing the polarity of the applied voltage or by reversing electroosmotic flow (EOF) using additives in the running buffer. Sample stacking with neutral compounds can also be performed through FASS and is termed ‘sweeping’. This phenomenon was initially observed by Gilges [48] and further developed by Quirino et al. [49,50]. Sweeping is defined as the

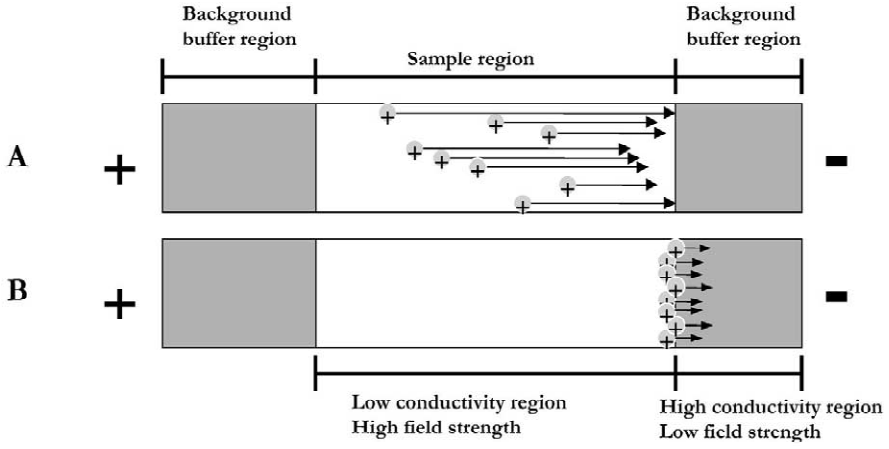

Fig. 1. Principle of sample stacking of cations in capillary electrophoresis: (a) A sample plug of cations is injected in a low conductivity buffer (e.g. de-ionised water). When a voltage is applied, the electric field in the sample solution is higher than in the rest of the capillary, cations migrate rapidly through the sample zone until they reach the low electric field in the separation buffer; (b) the cations then slow down and become stacked at the boundary between the sample region and buffer region.

picking and accumulating of analyte molecules by pseudostationary phases (PSPs) that enter and fill the sample zone upon application of a voltage. This result in stacked PSP carrying neutral analytes at the interface between sample and running buffer zones.

There are two primary modes of FASS. The first involves the introduction of sample into the capillary via hydrodynamic mechanisms [12,16,19,20,23–27,30–34,36–40,42,51]. Analyte ions are then stacked by application of a voltage. After stacking has occurred, the sample matrix must be removed prior to separation. The process is complex and involves temporary application of reverse voltage with respect to that used for separation (polarity switching). Sensitivity in this technique is based on the total sample volume that can be injected into the capillary. Since, there is a maximum length of the capillary which can be filled without any loss of analytes there exists a maximum sample volume that can be effectively stacked [18]. The second and more effective mode of sample stacking is HCFASS [13,29,51]. In head-column (HC) FASS, analytes are injected into the capillary without the introduction of a significant amount of solvent. The stacking process is based on the presence of a short zone of low conductivity matrix at the capillary inlet, where the electric field strength is up to several hundred times higher than that employed in normal CE. In this region, charged analytes are permitted to be injected at high velocity. During the electrokinetic injection process, analytes are concentrated at the interface between the low conductivity zone and the running buffer. Large amount of cations can be stacked with no polarity change prior to separation under positive polarity CE operation. When anions are analysed, negative polarity is needed for the electrokinetic injection. By combining this approach with a reversed-phase CE separation, no polarity switching is required and the mechanism is shown in Fig. 2. At the starting situation (Fig. 2a), the capillary is filled with background running buffer followed by injection of a short water plug. Anions prepared low conductivity matrix are then injected electrokinetically for a long time at negative polarity (Fig. 2b). Electrophoretic separation is carried out subsequently under negative polarity (Fig. 2c).

In this paper, we demonstrate a novel online sample concentration technique, using reversed-phase, reversed-polarity HCFASS with laser-induced fluorescence (LIF) detection for the electrophoretic analysis of FITC derivatized aniline and 2,4-dimethylaniline. The use of sodium hydroxide as the sample matrix for reverse phase, reversed polarity HCFASS is also investigated. Optimised separation and stacking conditions are obtained with good reproducibility of signal and migration time.

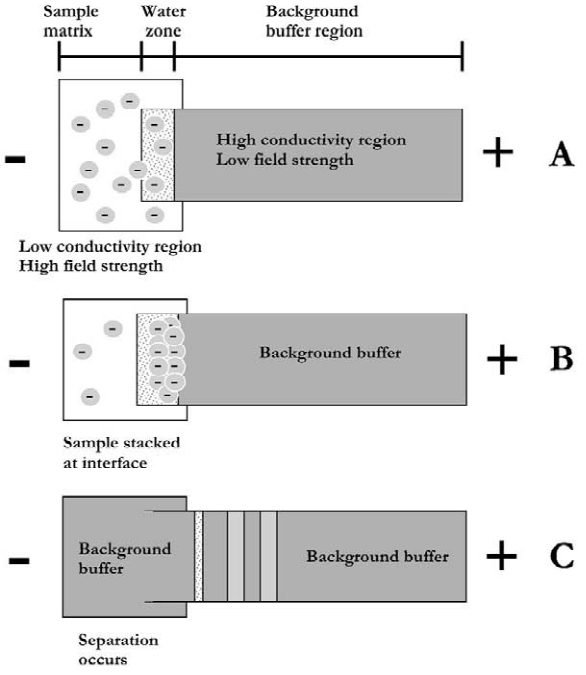

Fig. 2. Principle of reversed-phase, reversed-polarity HCFASS. (a) Starting situation; capillary is first filled with background buffer followed by injection of a short water plug. (b) Electrokinetic injection for at negative polarity of anions prepared in a low conductivity matrix. (c) Separation carried out under negative polarity.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and the organic solvents ethanol, acetonitrile were HPLC grade. All solvents were tested for extraneous fluorescence prior to use. Aniline, 2,4-dimethylaniline and fluorescein isothiocyanate (FITC) (Sigma–Aldrich, Dorset, UK) were used as received. All

aqueous solutions were made up using high-resistivity (18 MΩ) deionised water (Elga, Buckinghamshire, UK) and orthophosphoric acid, sodium hydroxide, acetonitrile and ethanol (Merck, Dorset, UK) were used as received.

2.2. Labelling amine

The procedure for the labelling of amine with FITC followed a protocol described elsewhere [52]. For standard derivatisation, an excess of FITC and 10 m*M* of amine are mixed in 0.05 *M* phosphate buffer (pH 9.0) for 4 to 24 h at 4 °C. The resulting mixture was diluted in the desired solvent for CE analysis.

2.3. Preparation of sample solution and running buffer

For studies assessing sodium hydroxide concentration in the sample matrix, solutions of FITC-labelled aniline (1.5 n*M*) and of 2,4-dimethylaniline (1.5 n*M*) were prepared by mixing appropriate aliquots of analyte solutions and 0.4 *M* NaOH followed by dilution with deionised water. The phosphate running buffer was prepared by mixing appropriate aliquots of 0.5 *M* H_3PO_4 and 1 *M* NaOH to the desired concentration and pH.

2.4. Capillary electrophoresis

All experiments were performed on an P/ACE 2050 capillary electrophoresis system (Beckman Coulter, Buckinghamshire, UK) equipped with an argon ion laser operating at 488 nm. A notch filter (488 nm) was employed at the detection window to eliminate transmission of excitation radiation. All equipment control and data recording was performed using P/ACE Station software (Beckman Coulter). Separations were performed in amine-coated fused-silica capillaries of 27 cm (effective length 20 cm)× 75 μm I.D. (Supelco, Dorset, UK). A constant voltage of 16 kV (600 V/cm, current, 93–104 μA) was used for all separations with the positive electrode located on the sampling side. Sample injection was effected by applying a positive voltage of 8 kV for 40 s and pre-injecting a water plug (by immersion in a water vial for 5 s). The temperature of the capillary was maintained at 25 °C during the course of all experiments. Prior to use, new capillary columns were preconditioned by rinsing with 0.01 *M* HCl for 30 min, followed by water for 10 min and running buffer for 5 min using positive pressure. In addition, capillaries were rinsed with HCl for 5 min followed by running buffer for 2 min between separations. To prevent capillary blockage, buffers and sample solutions were filtered through 0.2 μm filters (Millisart, Fisher Scientific, Loughborough, UK) prior to entry. Running buffer and analyte concentrations, sample matrix composition and concentrations were as stated in each experiment.

3. Results and discussion

The stacking efficiency (E_s) in FASS can be expressed as the ratio of the solute concentration in the sample zone (C_1) and its concentration in the stacking area (C_2, buffer zone) [21]. If electromigrational transport phenomena are applied, this ratio can be expressed in terms of the electric field strength and effective electrophoretic mobility in both the sample and buffer zones. This relationship is described in Eq. (1),

$$E_s \equiv \frac{C_2}{C_1} = \frac{E_1}{E_2} \cdot \frac{\mu_1}{\mu_2} \quad (1)$$

where E_1 and μ_1 are the electric field strength and the effective electrophoretic mobility of the solute in the sample zone, and E_2 and μ_2 are the corresponding parameters in the adjacent buffer zone. According to Eq. (1), it can be seen that a large difference in the ionic strengths of the buffer and sample zones will lead to a large increase in E_1/E_2 and consequently an improved stacking efficiency. As noted by Zhang and Thormann the mobility ratio (μ_1/μ_2) is equally important and can be increased by additives such as organic solvents, acids and ions in the sample matrix [21].

Fig. 3 illustrates the dependence of peak height and peak area for an injected plug of aniline and 2,4-dimethyaniline as a function of sodium hydroxide concentration in the sample matrix. As the concentration of sodium hydroxide in the sample matrix increases, both the peak height and area increase to a maximum value at 800 μ*M* sodium

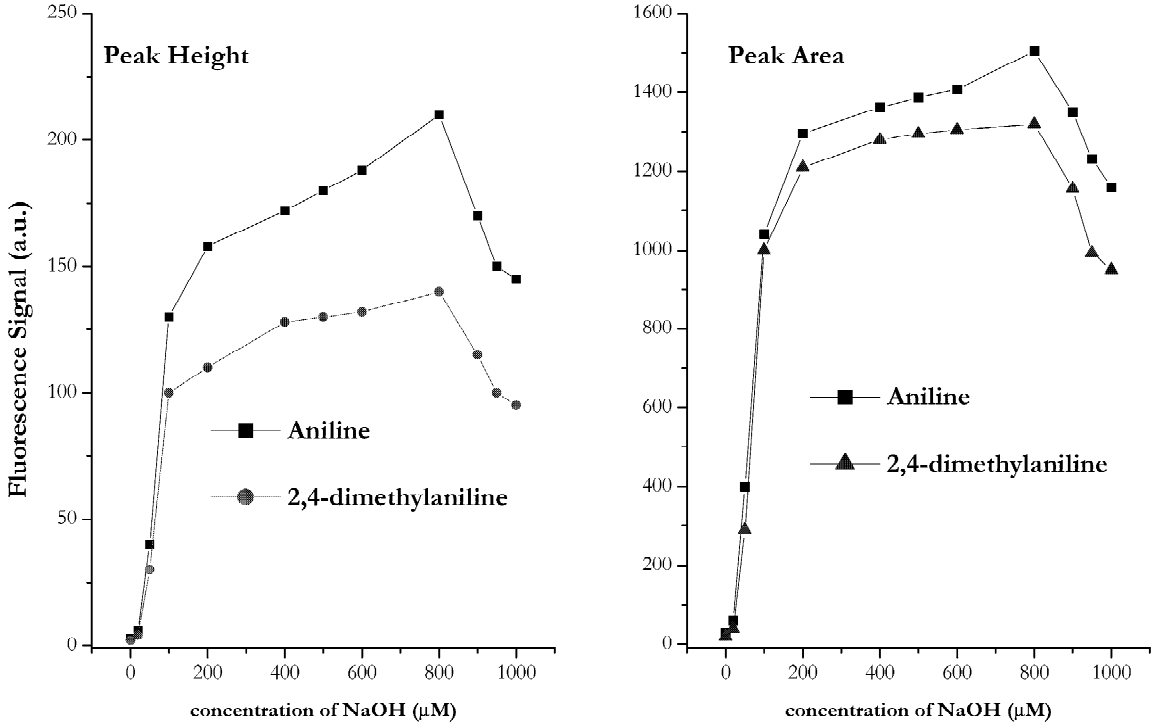

Fig. 3. Effect of sodium hydroxide concentration in the sample matrix on the stacking efficiency of aniline–FITC and 2,4-dimethylaniline–FITC: (a) peak height, and (b) peak area. Separation conditions: amine-coated capillary 27 cm (20 cm effective length)×70 μm I.D.; 1.5 n*M* aniline and 1.5 n*M* 2,4-dimethylaniline; 0.05 *M* phosphate, pH 9.0 running buffer; electrokinetic injection, −8 kV, 40 s; separation voltage −16 kV; temperature, 25 °C.

hydroxide. They then decrease with any further increase in base concentration. The initial increase in injection efficiency results from an increase in electrophoretic mobility (and hence higher injection velocity) with increasing base concentration. This leads to a higher number of analyte molecules entering and stacking at the tip of the capillary inlet. However, as base concentration is increased there is a corresponding decrease in electric field strength within the sample matrix. This consequently acts to reduce the stacking efficiency. The combination of these two effects yields an optimum base concentration that in turn provides for a maximum stacking efficiency. For the current experiments, this was found to be 800 μ*M*.

The running buffer concentration also affects the stacking efficiency. It is observed that peak height increases linearly with increasing phosphate buffer concentration within the range studied. This is consistent with basic theory, since the higher the concentration of phosphate, the higher the conductivity in the background running buffer and the larger the difference in conductivity between the background running buffer and sample matrix. However, it should be noted that higher buffer concentrations result in longer migration times and thus reduced resolution due to diffusion. Phosphate buffer concentrations higher than 0.2 *M* were analysed but due to excessive intra-capillary Joule heating separations were of a poor quality. Based on these observations, a 0.05 *M* phosphate buffer concentration was chosen for further experiments to reconcile resolution, retention time and stacking efficiency.

Under ‘non-stacking’ conditions the model compounds were easily separated and detected. Fig. 4(a) illustrates a representative electrophoretic separation of aniline (1.5 n*M*) and 2,4-dimethylaniline (1 n*M*) in a 0.05 *M* phosphate running buffer (pH 9.0). Sample was introduced via electrokinetic injection (8 kV for 40 s) and separated using a voltage of 16 kV.

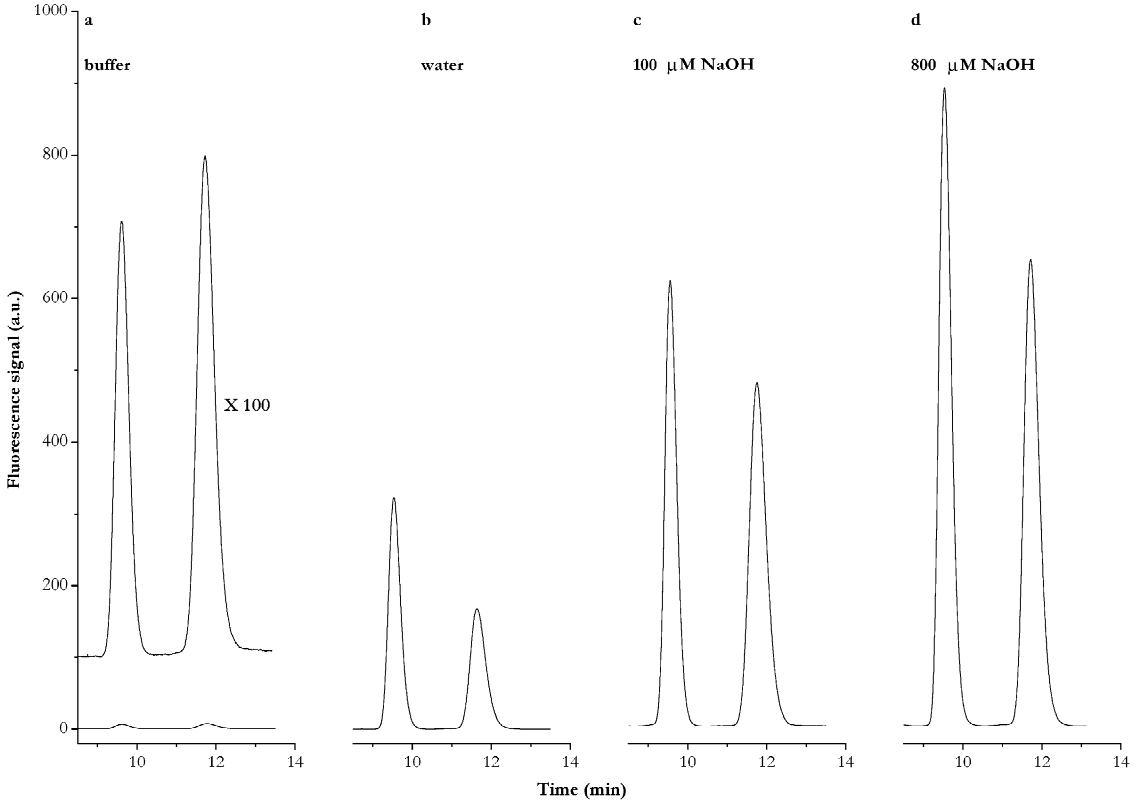

Fig. 4. Electrophoretic separations of aniline and 2,4-dimethylaniline under (a) non-stacking conditions (running buffer), (b) stacking conditions (water), (c) stacking conditions (100 μ*M* NaOH) and (d) stacking conditions (800 μ*M* NaOH). Separation conditions: amine-coated capillary 27 cm (20 cm effective length)×75 μm I.D.; 1.5 n*M* aniline and 1.5 n*M* 2,4-dimethylaniline in 800 μ*M* sodium hydroxide; 0.05 *M* phosphate, pH 9.0 running buffer; electrokinetic injection, −8 kV, 40 s; separation voltage, −16 kV; temperature, 25 °C.

Two clearly separated peaks are obtained with $S/N_{\text{aniline}} = 100$ and $S/N_{\text{2,4-dimethylaniline}} = 120$, respectively. When the samples were dissolved in a low conductivity matrix containing water, efficient stacking of the analytes occurred as demonstrated by Fig. 4(b). Prior to sample introduction, the capillary inlet was immersed in a water vial for 5 s to avoid contamination and provide a short plug of water to improve sensitivity (by providing a trap in which the solutes are collected before being separated in the running buffer) [21]. Both aniline and 2,4-dimethyaniline are detected with good sensitivity ($S/N_{\text{aniline}} = 1506$ and $S/N_{\text{2,4-dimethylaniline}}$ 1308). Compared to non-stacking conditions a signal enhancement of two orders of magnitude can be observed, with no apparent loss in resolution. When the samples were dissolved in a low conductivity, high electrophoretic mobility matrix containing 100 and 800 μ*M* NaOH ($S/N_{\text{aniline}} = 3015$ and 4628, $S/N_{\text{2,4-dimethylaniline}} = 2952$ and 4319), further 2- and 3-fold improvements in signal intensity are observed as shown in Fig. 4c and d, respectively. A total improvement in detection limit of up to three orders of magnitude can be observed when optimum stacking occurred. In principle, application of a higher voltage and a longer injection time period should result in more solute injected [35]. However, in practice, the applied voltage is limited by Joule heating and for our system should be less than 600 V/cm to avoid excessive heating in the low-conductivity zones [53]. Furthermore, exceeding a threshold of the product of injection voltage and time was found to yield only boarder peaks (sample overloading).

Table 1
Reproducibility of migration time and peak height[a]

	Peak height (RFU)		Migration time (min)	
	Aniline	2,4-Dimethylaniline	Aniline	2,4-Dimethylaniline
Mean	$1.37 \cdot 10^6$	$1.87 \cdot 10^6$	9.54	11.74
SD	$2.83 \cdot 10^4$	$1.72 \cdot 10^4$	0.058	0.12
RSD (%)	2.065	0.921	0.608	0.986

[a] Separation conditions: amine-coated capillary 27 cm (20 cm effective length)×75 μm I.D.; 1.5 n*M* aniline and 1.5 n*M* 2,4-dimethylaniline in 800 μ*M* sodium hydroxide; 0.05 *M* phosphate, pH 9.0 running buffer; electrokinetic injection, −8 kV, 40 s; separation voltage, −13.5 kV; temperature, 25 °C.

It has been reported that the addition of an organic solvent to the sample solution can decrease the conductivity and viscosity of the sample matrix and thus result in signal enhancements due to increased electric field strengths and electrophoretic mobility in the sample matrix zone (Eq. (1)). In the current studies ethanol, 1-propanol and acetonitrile were tested as additives. Acetonitrile (20%) in conjunction with 800 μ*M* sodium hydroxide in the sample matrix further increased S/N ratios 3-fold. Higher concentrations of acetonitrile were not considered due to excessive increases in buffer viscosity.

Calibration was subsequently performed under the optimised stacking conditions; electrokinetically injecting aniline and 2,4-dimethylaniline in 800 μ*M* sodium hydroxide into 0.05 *M* phosphate buffer. Results of this study generate concentration detection limits of 500 and 400 f*M* ($S/N=3$), respectively. Reproducibility was determined by performing five consecutive runs on a sample containing aniline and 2,4-dimethylaniline in 800 μ*M* sodium hydroxide sampled at 1.5 and 1.5 n*M*, respectively. Injection and separation were carried out at 8 and 16 kV respectively, with a 0.05 *M* phosphate running buffer (pH 8.0). The mean standard deviation and RSDs (%) of migration time and peak heights for aniline and 2,4-dimethylaniline under stacking are shown in Table 1. The RSDs of migration time and peak height for aniline and 2,4-dimethylaniline were 0.608 and 0.986% and 2.065 and 0.921%, respectively.

4. Concluding remarks

The studies presented herein demonstrate that by combining reversed-phase, reversed-polarity HCFASS with the LIF yields improved detection sensitivities (>1000-fold) without any loss in resolution or precision. Without modification of a commercial CE instrument, reversed-phase, reversed-polarity HCFASS was achieved by preparing samples in a low conductivity matrix and injecting electrokinetically into a high conductivity separation buffer (without polarity switching). Stacking efficiencies can be increased not only by harnessing differences in conductivity in the sample and buffer regions, but also by altering the electrophoretic mobility within the sample matrix. In the current study, a sample solution containing 800 μ*M* sodium hydroxide and 20% acetonitrile leads to a maximum stacking efficiency with the use of 0.05 *M* phosphate (pH 9.0) as running buffer. We are currently applying the described technique to the electrophoretic analysis of complex samples such as drugs, carbohydrates, amino acids and proteins.

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