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Approaches used in the reduction of buffer electrolysis effects for routine capillary electrophoresis procedures in pharmaceutical analysis.

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Abstract

Electrolysis of the electrolyte in the buffer reservoirs can occur when performing multiple assays in capillary electrophoresis (CE). This effect, which has been termed buffer depletion, results in a pH gradient being formed across the capillary. This gradient can effect both migration time, peak efficiency and selectivity and therefore can cause problems of reproducibility which leads to poor quantitative assays in pharmaceutical analysis. In this study the factors affecting the extent of buffer depletion were investigated. This involved performing a series of multiple assay runs where a number of factors were varied such as the use of dilute or concentrated buffer solutions and single or separate vials for pre-rinse and separation. In addition, the effect of the volume of buffer in the separation vials on the degree of depletion was also examined using standard (4 ml) and small volume (0.3 ml) CE vials. From these studies, it was observed that long multiple assay runs should not be carried out with small volumes of dilute buffer when pre-rinse and separation steps are from the same vial. The rate of electrolysis also increased when using wide-bore capillaries and at elevated temperatures. The use of short-end injections, zwitterionic buffers and reversing the polarity were found to appreciably reduce the effects of buffer depletion. From this work a number of recommendations are provided to maximise the number of acceptable separations that can be obtained in routine CE injection sequences.

Keywords: Buffer electrolysis; Buffer depletion; Pharmaceutical analysis; Buffer composition

1. Introduction

Extensive application of a voltage across an electrolyte solution can cause electrolysis of the buffer to occur [1,2]. This phenomenon has been termed 'buffer depletion' which results in hydrodynamic flow as a result of ion migration and water electrolysis [3] with reduced assay performance. The use of high voltages, high ionic strengths and long run times all increase this oxidation–reduction induced pH drift at the electrodes. In aqueous solutions, electrolysis of water produces soluble protons at the anode and hydroxide ions at the cathode (Fig. 1). As analysis time increases, the solution in the anode reservoir will become increasingly acidic while the solution in the cathode reservoir becomes increasingly alkaline. pH influences both the charge of the ionizable species and the magnitude of electroosmotic flow (EOF) and therefore controls the effective electrophoretic migration of the solutes. Any alteration of pH will have considerable impact on reproducibility and precision in CE.

The variation in the pH of the background electrolyte was reported by Zhu et al. [2] in a series of

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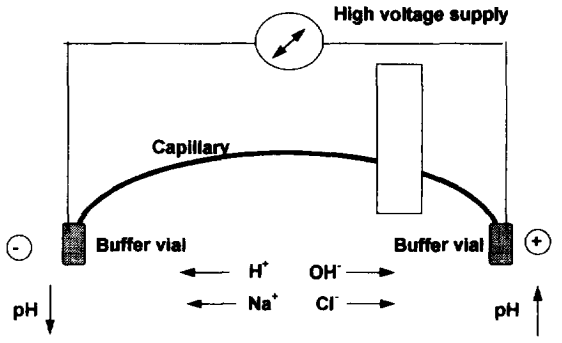

Fig. 1. Principles of buffer depletion.

electrolytic experiments on the effect of pH drift in CE. Svensson [4] proposed using this effect to create a dynamic pH gradient for iso-electric focusing. Corstjens et al. [5] presented a theoretical treatment of pH variations due to electrolytic processes. Yin et al. [6] encountered siphoning effects and buffer depletion in their preparative CE work with tryptic-digests when using wide-bore capillaries. Significant depletion occurred using dilute buffers (5 m*M*) in capillary electrochromatography (CEC) [7] and when using buffers out of their pH range [1].

Changes of buffer pH during analytical CE sequences can be a significant problem as the charge on the solute and the rate of the EOF will be dramatically altered. This change will affect the migration time and since the peak area is proportional to migration time will result in variable peak areas throughout prolonged injection sequences, therefore affecting the assay performance and reducing its quantitative capabilities.

The extent of electrolysis is in-part dependent on the buffering capacity of the buffer system and also on the volumes in the buffer reservoirs. Unfortunately, this is not helped in some reported methods where the selected buffer is used at an inappropriate pH value [1,8].

The number of runs which can be carried out with the same buffer vials is a function of the buffer capacity, buffer concentration, and ionic strength. It is generally advantageous to perform separations using electrolytes with the highest possible concentration. This can have benefits in terms of sensitivity and resolution, apart from prolonged routine performance. Sensitivity and resolution increases are obtained due to increased 'sample stacking' [9] resulting from differences in the conductivities between the sample solution and electrolyte. However, the maximum permissible electrolyte concentration is limited by the Joule heating which can produce unacceptably high levels of current and therefore non-uniform temperature gradients and local changes in viscosity which results in zone broadening. Thus temperature increase is dependent on the power generated and is determined by the capillary dimensions, conductivity of the buffer and applied voltage. Dilute electrolyte concentrations could avoid the heating problems but conversely can give rise to significant buffer depletion leading to poor electrophoretic reproducibility. An alternative is the use of low mobility zwitterionic buffer solutions which contain large minimally charged ions, such as tris-(hydroxymethyl)methylamine (Tris), and 2-(N-morpholino)ethanesulphonic acid (MES). Zwitterionic buffers contain both positively and negatively charged groups, therefore at the buffer p*I* value the buffering ions will not migrate towards the electrodes and carries little or no current. These buffers can allow a ten-fold increase in buffer concentration without generation of excessive Joule heating [7].

From the viewpoint of reductions in buffer depletion and its associated effects from an instrumental perspective, a number of changes can be introduced in the operational programming from filling the buffer vials [10], rinsing, injecting and running the capillaries in the electrophoretic system. These aspects can be optimised to reduce the detrimental effects of buffer depletion on the efficiency of the electrophoretic process [11].

Therefore buffer depletion effects can be highly significant in obtaining routine robust assays in areas such as bulk drug and pharmaceutical preparation analysis by CE. To date however there has been no comprehensive analytical study of the possible approaches to reduce these depletion effects. In this study the factors affecting buffer depletion have been assessed experimentally by using a mixture of acidic solutes. These factors included: multiple assays using dilute and concentrated buffer solutions, zwitterionic buffers, and the use of different vials for the pre-rinse and separation step, the effect of vial volume, and the influence of using short-end injections on the quality of the separation. From this work a number

of recommendations are made on how to reduce buffer depletion in routine CE assays.

2. Experimental

2.1. Instrumentation

A Beckman P/ACE 5100 (Beckman Instruments, Fullerton, CA, USA) CE instrument was used in all experiments except the short-end work which used a Hewlett–Packard 3-D (Waldbronn, Germany) CE instrument. Fused-silica capillaries were obtained from Composite Metal Services (Hallow, UK) 75 or 100 μm I.D. and total length of 27 cm (20 cm to window). Each new capillary was pre-conditioned prior to its first use by rinsing for 20 min with 0.1 *M* NaOH [12]. A Hewlett–Packard (Bracknell, UK) LAS 1000 data collection system was used for integration and data handling purposes.

2.2. Chemicals

Acetylsalicylic acid, benzoic acid and aminophenylacetic acid were obtained within GlaxoWellcome Research and Development. Water was obtained from a Millipore Milli-Q deioniser system (Watford, Herts., UK), and the organic solvents used were of HPLC grade. Sodium dihydrogenorthophosphate was obtained from BDH (Poole, Dorset, UK) and MES and Tris buffers were obtained from Sigma (Poole, Dorset, UK).

2.3. Analysis conditions

Disodium hydrogenorthophosphate (pH 7.0) was used as the buffer in different concentrations and with different applied voltages; 50 m*M* at 7.5 kV and 5 m*M* at both 7 and 23 kV to maintain a constant current of 40 μA. The capillaries were subjected to two pre-rinse steps, the first using 0.1 *M* NaOH and the second with the electrolyte. The sample was injected using pressure for 1 s with a total analysis time of 10 min. Other experiments were carried out at a range of voltages and currents and are shown in Section 3. The temperature was set at 25°C (or varied in certain experiments) and detection wavelength was at 214 nm. The acidic compounds, acetylsalicylic acid, phenylacetic acid and benzoic acid, were prepared at an initial concentration of 1 mg ml^{-1} solution in methanol and then diluting with water to give 100 μg ml^{-1} of each component.

3. Results and discussion

3.1. Influence of electrolyte concentration

The initial experiments involved examination of the effect of concentrated and dilute buffers on the extent of buffer depletion. This involved continuous analyses using the same set of two buffer vials for each separation. Fig. 2a shows a typical separation obtained using 50 m*M* disodium hydrogenorthophosphate at 7 kV and Fig. 2b shows the separation obtained using the 5 m*M* disodium hydrogenorthophosphate solution at 23 kV. The separation profile is different largely due to the lower EOF at the higher buffer concentration. The number of successful separations obtained for each buffer type are shown in Fig. 3a and b, which shows a plot of injection number against migration time. In both cases there was a gradual increase in migration time and in Fig. 3b a slight dip at injection number 15–20. With the higher concentration buffer 70 injections were possible from the set of two buffer vials using the concentrated buffer solution before the separation unacceptably diminished (Table 2) and the migration times became unpredictable. However, only 20 injections were possible using the dilute electrolyte where the same problems of migration time variation was observed. The overall effects of changing migration time is the variation in precision of the method (Table 1) and therefore at the higher electrolyte concentration more consistent migration times and therefore improved precision is obtained.

Another series of experiments were carried out to investigate the rate of depletion of the 5 m*M* buffer at a lower voltage i.e. 7 kV, which produced a current of 9 μA. It was expected that the rate of depletion would decrease roughly by a factor of three when compared to the same buffer system run at 23 kV. However the number of acceptable sample runs was only 33, compared to 70 with the more concentrated buffer (Table 2) and therefore the reduced buffer

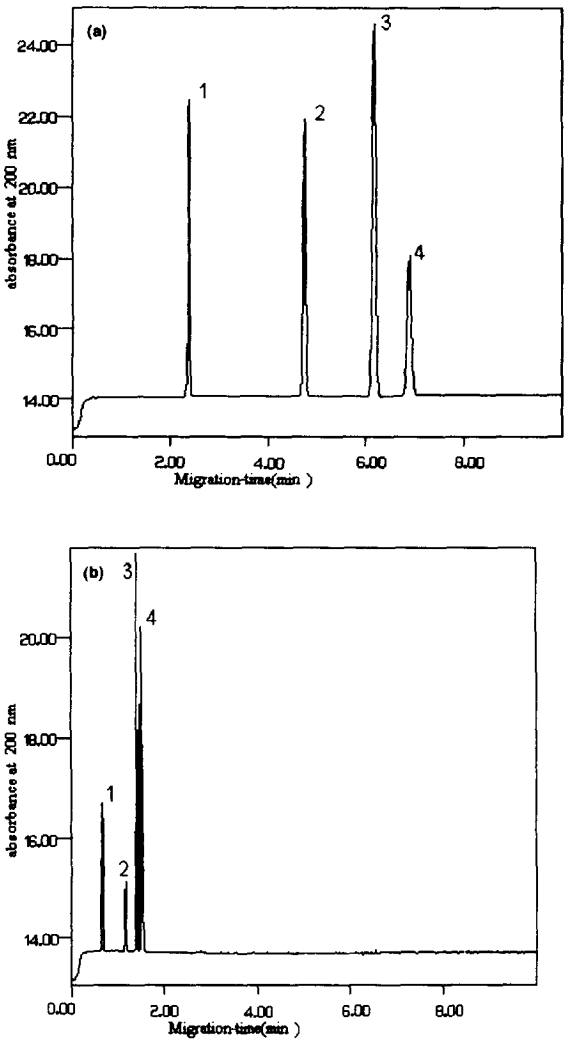

Fig. 2. Separations of acidic drug compound mixture. (a) Separation of the first injection of the acidic test mixture containing (1) acetylsalicylic acid, (2) phenylacetic acid and (3) benzoic acid. Separation conditions: 27 cm×75 μm capillary length, 50 m*M* sodium dihydrogenphosphate buffer (pH 7.0), detection at 214 nm, voltage 7 kV, temperature 25°C and injection 3 s. (b) Separation of the first injection of the acidic test mixture. Separation conditions: 27 cm×75 μm capillary length, 5 m*M* sodium dihydrogenphosphate buffer (pH 7.0), detection at 214 nm, voltage 23 kV, temperature 25°C and injection 3 s.

capacity is likely to have been a larger factor than first suggested.

The kind of problems associated with the incorrect choice of buffer was demonstrated using a phosphate buffer pH 4.0. Although separation initially occurred at this pH, resolution decreased rapidly after the fifth injection due to the low buffering capacity of the phosphate buffer at this pH. The pK_a values of the compounds involved are at or around 4 (for example benzoic acid 4.6 and phenyl acetic acid 3.07) so any change in the pH will affect the degree of ionisation. As analysis time increases, the solution in the anode reservoir becomes increasingly acidic while the solution in the cathode reservoir becomes increasingly alkaline. This will ultimately lead to a deterioration in separation efficiency after a short period of time and therefore demonstrates the importance of choosing and preparing a strong buffering system at the relevant pH [13].

3.2. Zwitterionic buffers

Zwitterionic buffers are an alternative to the inorganic buffers such as phosphate or borate as they provide low mobilities and contain large minimally charged ions. The advantages of such buffers is that high concentrations are possible as they produce low currents compared to the inorganic buffers. Therefore a series of repeated injections was carried out using a 100 m*M* Tris buffer (pH 7.0) which generated only 7 μA under these same conditions in which 50 m*M* disodium hydrogenorthophosphate produced 40 μA. In operation an excess of 100 injections were possible (Table 2) for a single set of vials using the Tris buffer system. The ion migration will be significantly reduced, however, water electrolysis will still take place.

If biological buffer systems are not appropriate to the requirements of the assay then buffer mixtures could offer a possible alternative. The effective buffer range for a weak acid or base approximately covers $pH=pK_a\pm1$ and two or more buffers would allow buffering over a wider pH range. Here sodium tetraborate ($Na_2B_4O_7\cdot10H_2O$) and phosphate buffer ($NaH_2PO_4\cdot2H_2O$) were combined to give a 50 m*M*, pH 7.0 buffer mixture. Over sixty injections were possible with the acidic compounds using the same set of buffer vials (Table 2). However the current generated was slightly higher (about 52 μA) than the respective phosphate buffer at the same concentration. This possibly explains the slight decrease in the number of acceptable injections through prob-

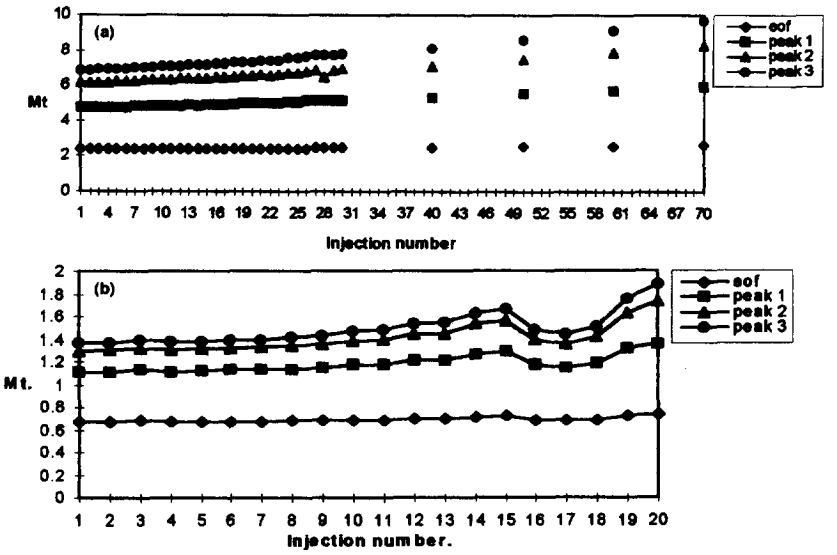

Fig. 3. (a) Plot of injection number against migration time (t_M) for the 50 m*M* phosphate buffer solution using the acidic test mixture, with separate pre-rinse and separation vial. Separation conditions: 27 cm×75 μm capillary length, 50 m*M* sodium dihydrogenphosphate buffer (pH 7.0), detection at 214 nm, voltage 7 kV, temperature 25°C and injection 3 s. (b) Plot of injection number against migration time for the 5 m*M* sodium dihydrogenphosphate buffer solution using the acidic test mixture, with separate pre-rinse and separation vials. Separation conditions as (a) except 23 kV.

lems of depletion, when compared to the 50 m*M* phosphate buffer.

3.3. Buffer replacement

Fresh vials of electrolyte [10] or the replenishment system available on certain instruments can be used throughout a sequence to prolong injection performance. A replenishment system was set up and ran continuously until the number of injections was restricted by the buffer volumes in the reserve bottles (100+ injections) (Table 2). With a large buffer replenishment system minimal vial locations in the autosampler are needed for buffer, leaving more locations for samples. Replenishment minimises buffer depletion, however not all systems possess this facility and larger volumes of electrolyte and increased analysis time is required for each injection as time is required to empty/refill the vials.

Table 1
R.S.D. (%) of migration time repeatability for the first 20 injections using both dilute and concentrated phosphate buffer solutions

Injection no.	R.S.D. (%)	
	5 m*M* phosphate buffer	50 m*M* phosphate buffer
1–10	1.52	1.03
11–21	3.53	1.5
1–20	6.03	2.21

3.4. Influence of siphoning effect

The extreme position to induce siphoning is when the same vial is used for the rinsing step and the run buffer in the method. The acidic compounds were assayed with 5 m*M* phosphate buffer and the overall number of injections possible was ten before the migration times began to increase irregularly and after a further eight injections the reliability of separation was unacceptable as shown in Fig. 4. On physical inspection of the separation vials the ground electrolyte reservoir had a significantly larger volume than the outlet reservoir having started at the same levels. This difference produced a siphoning effect from the detector end reservoir which superimposed laminar flow into the system which opposed the

Table 2
Current generated and number of injections possible under the various conditions

Buffer	I.D. (μm)	Rinse and separation vial	Buffer volume (ml)	Voltage (kV)	Current (μA)	No. of injections
50 m*M* phosphate (pH 7)	75	Different	4	7	41	70
5 m*M* phosphate (pH 7)	75	Different	4	23	39	20
5 m*M* phosphate (pH 7)	75	Different	4	7	4	33
5 m*M* phosphate (pH 4)	75	Different	4	23	39	5
100 m*M* Tris (pH 7)	75	Different	4	23	7	100+
50 m*M* phosphate/borate (pH 7)	75	Different	4	7	52	65
50 m*M* phosphate (pH 7)[a]	75	Different	4	7	41	100+
5 m*M* phosphate (pH 7)	75	Same	4	23	39	8
5 m*M* phosphate (pH 7)	75	Same	0.3	23	39	15
50 m*M* phosphate (pH 7)	100	Different	4	7	75	26
5 m*M* phosphate (pH 7)	100	Different	4	23	62	8
5 m*M* phosphate (pH 7)	100	Different	4	7	72	15
50 m*M* phosphate (pH 7)[b]	75	Different	4	−7	41	100
5 m*M* phosphate (pH 7)[c]	75	Different	4	23	38	29
5 m*M* phosphate (pH 7)[d]	75	Different	4	23	38	40
5 m*M* phosphate (pH 7)[e]	75	Different	4	23	48	15

[a] Replenishment.
[b] Short-end injections (analysis time 3 min).
[c] Reversal of polarity (HP instrument).
[d] Reversal of polarity (Beckman instrument).
[e] Temperature set at 30°C.

EOF. This is not only detrimental to migration time but also to separation efficiency.

3.5. Buffer reservoir volumes

The effect of the overall volume of buffer in the reservoir vials was also examined using the standard (Beckman) 4 ml CE vials and smaller insert vials, 0.3 ml. With the smaller vials only 15 injections (Table 2) were possible which suggests the micro-vials are unsuitable for multiple runs and in addition evaporation loss can be significant over a relatively short period of time. The movement of ions using large vials is significantly reduced as the electrolysis products will be more diluted in the larger buffer reservoir volumes.

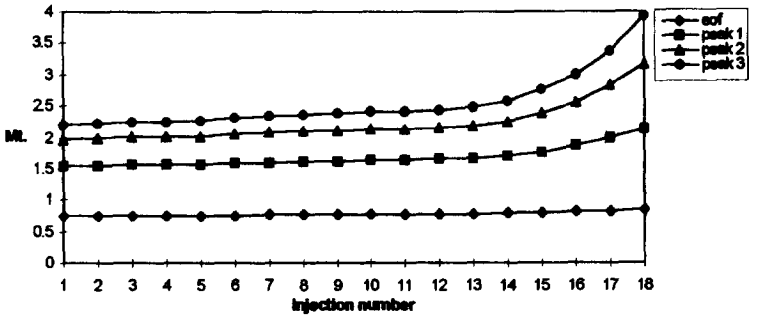

Fig. 4. Plot of injection number against migration time for the diluted buffer solution using the acidic test mixture, with the same pre-rinse and separation vial. Separation conditions: 27 cm×75 μm capillary length, 5 m*M* sodium dihydrogenphosphate buffer (pH 7.0), detection at 214 nm, voltage 23 kV, temperature 25°C and injection 3 s.

3.6. Capillary bore

Increased buffer depletion effects were observed with wider 100 μm I.D. capillary than conventional 50 and 75 μm capillaries as there is an increase in current associated with the use of larger bore capillaries. Twenty-six injections were possible with the concentrated buffer solution (50 m*M*) and only eight injections were possible using the dilute buffer (5 m*M*) (Table 2). Experiments were also carried out to examine the rate of depletion of the dilute buffer at a lower voltage (7 kV) using this wider bore capillary and only fifteen injections were possible. The rate of electrolysis is accelerated due to the increased current and movement of ions due to the increased diameter of the capillary. In addition the volumes of liquid moved by the electro-osmotic flow also increases significantly with increased capillary bore which results in magnification of any siphoning effects.

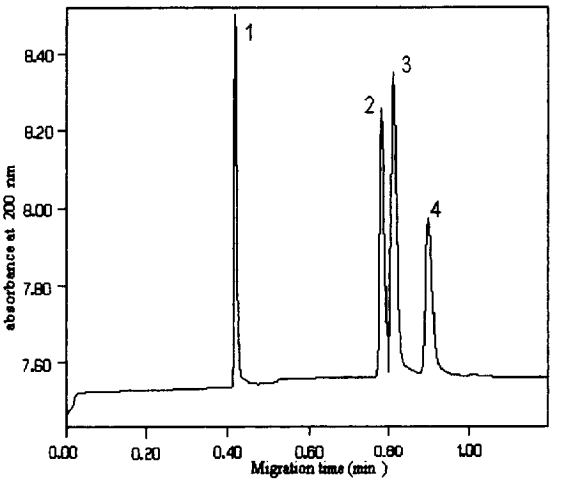

Fig. 5. 'Short-end' injection of the acidic test mixture. Separation conditions: 34 cm×75 μm capillary length with an l_d of 25.5 and a short-end (l_s) of 8.5 cm, 50 m*M* sodium dihydrogenphosphate buffer (pH 7.0), detection at 214 nm, voltage −7 kV, temperature 25°C and injection 3 s at −50 mbar.

3.7. Reduction of depletion effects through use of short-end injections

Depletion is dependent on the current produced and also on the total analysis time. One way to achieve performance gains is with shorter analysis times and an option is to use short-end injections [14]. Fig. 2a shows a typical separation using a conventional injection and Fig. 5 shows the same separation, but injected at the 'short-end' of the capillary. The reduction in analysis times will reduce the length of time that voltage is applied across the capillary and will therefore minimise depletion affects.

Table 3 provides a summary of approaches to maximise the number of acceptable separations that can be obtained in routine CE operation.

3.8. Reversal of voltage polarity or separation vials

As ion movement is the determining factor in electrolysis the possibility of reversing the flow of the ions after each analysis was investigated. This involved programming the instrument (Hewlett–Packard) to reverse the polarity after each injection and to apply a negative voltage of −20 kV for 2 min. In this way the flow of ions will be from the outlet to the inlet. It was observed that the rate of electrolysis slowed down considerably and 29 injections against 20 previously was obtained (Table 2). However on some instruments it is not possible to reverse the polarity without first turning the instrument off. Using the Beckman instrument the inlet and outlet vials were manually swapped after the last acceptable separation and the injection sequence recommenced. By using this procedure twice as many injections were possible compared to the conventional procedure, however some time was required for equilibration of the system.

3.9. Temperature effects

The effects of temperature were also investigated using either 25°C and 30°C. The current increased from 39 μA at 25°C to 48 μA at 30°C which increased ion migration and therefore a smaller number of injections were possible at the higher temperatures (Table 2). The increase in temperature also reduced viscosity which increased the rate of EOF and therefore accelerated siphoning effects.

Table 3
Suggestions on how to maximise the number of acceptable separations obtainable in CE sequences

Solution	Comment
Higher buffer concentration	Increased current but able to maintain pH more efficiently
Select the optimum buffer capacity	Optimise buffer pH at electrolyte pK_a
Zwitterionic buffers produce low current	Higher buffer concentrations possible
Buffer mixtures	Improved pH range
Replenishment	Only available on certain instruments
Use separate vials for pre-rinse and separation steps	Reduces depletion and siphoning effects
Pre-rinses should be flushed into a waste vial	Prevents contamination of reservoir
Use large vials with buffer levels equal	Increased volumes dilute depletion effects — siphoning occurs if levels are uneven
Narrow bore capillary	Reduces current but also sensitivity
Short-end injections minimise depletion	Reduces analysis time
Inter-analysis voltage reversal	Reverses depletion effects
Increased temperature	Increases the rate of depletion

4. Conclusions

The magnitude of electrolysis is dependent on the buffering capacity of the system. The rate of electrolysis is more pronounced in the dilute buffer solution due to the decrease in buffer capacity which is therefore less able to counteract the pH changes. Buffer electrolysis can be reduced using a high capacity buffer or zwitterionic buffers and also by using fresh electrolyte in the reservoirs throughout a prolonged injection sequence to maintain acceptable analytical performance.

The rate of electrolysis is also dependent on the total time the voltage is applied across the capillary. When the buffer levels are not equal, pressure induced hydrodynamic flow results and may have an effect on the CE efficiency. There is an advantage in using short capillaries to allow the application of lower voltages and significantly reduce depletion effects.

From this work it is evident that the number of runs carried out from the same set of buffer vials should be determined for each buffer system prior to the start of multiple assay runs and is directly related to the buffer capacity and the operating parameters employed. In procedures such as quality control runs, drug development programmes and bioanalytical studies where multiple runs are carried out it is suggested that buffer depletion effects should be determined as part of the validation study and included in the standard operating procedure.

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