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# Impact of organic solvents on the resolution of synthetic peptides by capillary electrophoresis

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**Abstract**

The effect of variations in the concentrations of different organic solvents, including acetonitrile, methanol, ethanol, propanol and isopropanol, with aqueous buffer electrolytes of defined composition and pH on the electroosmotic flow velocity, $\upsilon_{EOF}$, of uncoated fused silica capillaries and on the electrophoretic mobility, $\mu_e$, of synthetic peptides in high-performance capillary electrophoresis (HPCE) has been systematically investigated. In these experiments, the volume fractions of the organic solvent in the aqueous buffer electrolyte were changed from $\psi = 0.0$ to 0.80. The addition of these organic solvents to the aqueous buffer electrolyte reduced the electroosmotic flow (EOF) of the system, but to significantly different extents. For the protic solvents as the alkyl chain of the alcohol increased, at the same volume fraction the greater was the influence on the electroosmotic flow. However, for the aprotic solvent, acetonitrile, the EOF did not change substantially as the volume fraction was varied. The electrophoretic mobility of synthetic peptides under the different buffer electrolyte conditions showed similar trends, confirming that the content and type of the organic modifier can be rationally employed to subtly manipulate the separation selectivity of synthetic peptides. These results, therefore, provide fundamental insight into the experimental options that can be used to maximise resolution of synthetic peptides in HPCE with aqueous buffer-organic solvent mixtures as well as a basis to select optimal binary or ternary buffer electrolyte compositions for the analysis of peptides when hyphenated techniques, such as HPCE–electrospray ionisation mass spectrometry (ESI-MS), are contemplated for the analysis of peptide samples of low abundance as can often be experienced in proteomic investigations.
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*Keywords:* Electroosmotic flow; Electrophoretic mobility; Selectivity; Buffer composition; Peptides

## 1. Introduction

High-performance capillary electrophoresis (HPCE) has become over the past decade a very popular separation technique in the life sciences for the resolution of charged and apolar molecules. Because of the inherent capability to achieve high separation efficiencies, short analysis times and ease of interfacing with mass spectrometry [1,2], HPCE and the related technique, high-performance capillary electrochromatography (HPCEC), offer a number of potential advantages for the analysis of peptides [3,4], proteins [5,6] and other biological mixtures [7]. Conventionally, most HPCE separations with charged molecules have been performed with fully aqueous buffer electrolytes, containing in some cases a charged or polar additive, such as an alkylamine or alkylsulphate. The separation of apolar or neutral compounds, including cholesterol [8], higher fatty acids and steroid hormones [9] and other compounds that are not readily soluble in aqueous media, initially proved to be somewhat recalcitrant in HPCE with fully aqueous buffer electrolytes, necessitating the adaptations of the HPCE format such as micellar electrokinetic separations [10]. Moreover, with fully aqueous buffer electrolytes of defined ionic strength and composition there are only a limited number of experimental variables, e.g. adjustments to the temperature, ionic strength or pH of the buffer electrolyte, that can be employed to manipulate the viscosity or dielectric properties of the buffer electrolyte in the HPCE system and to optimise the separation selectivity with charged molecules such as peptides.

The addition of an organic solvent as a modifier to an aqueous buffer solution offers a number of advantages for analytical HPCE tasks. Besides increasing the solubility of

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some analytes, enhancing the stability of other analytes that may be labile in aqueous solution and reducing the interaction of hydrophobic compounds with the capillary wall, the main goal driving the application of organic solvents as an additive to the buffer electrolyte in HPCE is to modulate separation selectivity [11,12] through direct effects on the physicochemical properties of the buffer electrolyte, the capillary wall zeta potential and the analytes themselves. The wide selection of organic solvents, with their very different physical and chemical properties, significantly broadens the scope to select more appropriate solution chemistries for the buffer electrolyte to improve the resolution of analytes that exhibit very similar electrophoretic mobilities in pure aqueous media. In addition, the lower currents that occur with aqueous-organic solvent mixtures make possible the use of higher electric field strengths (producing higher separation efficiencies with shorter analysis times), and also allow scale up of the sample loading by permitting capillaries of wider internal diameters to be used, thus improving the detection sensitivity. When HPCE is coupled with electrospray ionisation mass spectrometry (ESI-MS), aquo-organic solvent buffer electrolyte combinations often provide a more stable electrospray with overall higher detection sensitivity for the derived ions, since the lower surface tension and higher volatility favour formation of more stable nanodroplets of longer mean half lives in the electrospray [13].

To be acceptable as components in HPCE buffer electrolytes the organic solvent must meet certain requirements. The ideal organic solvent for use in HPCE should be miscible with water, not cause precipitation of the analytes or the electrolytes, should not be flammable, toxic or reactive in small quantities and, yet, should have a relatively high dielectric constant, low viscosity and low vapour pressure. In addition, when UV spectroscopy is used as the primary detection tool the solvents should have good transparency at ca. 210–215 nm. Organic solvents that partially meet these criteria for use as aqueous-organic solvent buffer electrolytes in HPCE include acetonitrile, the alcoholic solvents (such as methanol, ethanol, 1-propanol, 2-propanol or 1-butanol), *N*,*N*-dimethylformamide, dimethylsulphoxide and formamide [11].

Buffered water-organic solvent combinations have been used for the separation of oligosaccharides, [14] aromatic acids [15] and drugs [16] in HPCE. However, the basis for choosing a particular organic solvent in the separation of peptides by HPCE has attracted less attention [17]. Proper choice of the organic solvent not only requires knowledge of its physical and chemical properties, but also knowledge on how it impacts on the electromigration of analytes including the extent of interaction with the analytes through solvational, dissociative or multimolecular complex formation phenomena. Unprotected peptides are amphoteric compounds that contain different types of ionogenic groups, e.g. partially ionised carboxyl groups (depending on the pH value of the buffer electrolyte) at the C-terminal position of the amino acid sequence or as part of the side chains of aspartic and glutamic acids, and partially ionised amino groups at the N-terminal position and as part of the side chains of histidine, lysine, arginine, etc. Consequently, unprotected peptides can undergo a variety of interactions with anionic and cationic species and solvent molecules present in the buffer electrolyte, at the capillary wall double layer or with silanol groups on the surface of the capillary wall. Under some solvent conditions, larger peptides may also undergo changes to their secondary and tertiary structures that define their shape and size. Despite these ramifications, the effects of different organic solvents on the ionisation status of peptides, or the impact on peptide size, shape, electromobility and associated solution features in HPCE separations have not yet been well delineated. Idei et al. [18] utilized acetonitrile, methanol, ethanol and 2-propanol as organic modifiers for the analysis of several synthetic analogues of somatostatin. These investigators showed that the migration time and selectivity of these peptides was influenced by modifying the composition of the electrophoretic buffer with these organic solvents, with the effects of the alcohols on the HPCE migration time of the peptides opposite to that observed when acetonitrile was used. Castagnola et al. [19] have studied the dissociation constants and Stokes radius of several simple di- and tripeptides in water–2,2,2-trifluoroethanol (TFE) mixtures. Their results indicated that TFE is an effective solvent to manipulate separation selectivity. Subsequently, the use of water–TFE mixtures has been extended [20] to the HPCE analysis of larger peptides, documenting the ability of water–TFE combinations to affect the conformational stability of larger peptides analysed in a HPCE format as assessed from the influence on the dissociation constants of the amino acid residues and the Stokes radius. The change in the electroosmotic flow (EOF) for a series of mixed aqueous-organic solvents consisting of up to 80% (v/v) methanol, ethanol, 2-propanol, acetonitrile, dimethyl sulfoxide and acetone has also been investigated by Schwer and Kenndler [21] with fused-silica capillaries. It was found by these investigators that the addition of organic solvents generally reduced the EOF in all cases, even when the silanol groups of the capillary wall were fully ionised at high pH.

In this work, we have systematically studied the impact of various aqueous-organic solvent combinations on the HPCE separation of two sets of synthetic peptides of closely related sequence and containing at least two basic amino acid residues and net charge >1.0. Acetonitrile and a group of alcoholic solvents, e.g. methanol, ethanol, 1-propanol and 2-propanol, were employed as the organic modifier, and the aqueous buffer selected was ammonia acetate, pH 4.0, at a final concentration of 10 mM. The main purposes of this research was to examine the impact of these mixed solvent buffer electrolyte systems on the electromigration characteristics of sets of closely related synthetic peptides and to generate a practical framework for the HPCE separation of peptides with aqueous-organic solvent buffer electrolytes that are specifically targeted for application with interfaced HPCE-ESI-MS systems for the separation and structural

characterisation of low abundance peptides or their analogues.

## 2. Materials and methods

### 2.1. Chemicals and reagents for capillary electrophoresis

Ammonia acetate was purchased from BDH (Kilsyth, Australia); NaOH and acetic acid were obtained from AJAX (Sydney, Australia); acetonitrile, methanol, ethanol, 1-propanol and 2-propanol (HPLC grade) were obtained from Biolab Scientific (Sydney, Australia). Unless otherwise stated, all the above reagents were analytical grade. All the peptides used in this study were synthesised and purified using procedures reported previously [22].

### 2.2. Capillary electrophoresis instrumentation

Studies were performed with a $HP^{3D}CE$ capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany) at ambient temperatures in an air-conditioned laboratory with room temperature maintained at around $22 \pm 1\,^{\circ}C$. The temperature of the cassette containing the capillary was controlled by a circulating air fan and registered by a monitor (U-lab, Melbourne, Australia) at between 21 and 23 °C during the period over which the measurements were made. The detection of peptides was performed using a diode-array detector at 214 and 254 nm.

### 2.3. Capillary electrophoresis

A stock solution of 100 mM ammonia acetate buffer, pH 4.0 was prepared by titrating 100 mM ammonia acetate with acetic acid, and filtered through a 0.22 mm pore sized filter. All the aqueous-organic buffers were prepared by mixing appropriate proportions of stock solution, Milli-Q water and the organic solvent, and degassed by ultrasonicating for 10 min before use. In all cases, the final concentration of the background electrolyte was kept constant, e.g. at 10 mM. Bare fused silica capillaries, with an internal diameter of 100 μm and outer diameter of 360 μm, were obtained from Chromatographie Service (Langerwehe, Germany). The total length of the capillary was 34.0 cm, and the effective length, from the outlet to the detection window, was 8.5 cm. For all the analysis the following methods were routinely employed with these capillaries: (i) −5 mbar, 2 s pressure injection of the sample from the outlet end of the capillary; (ii) constant voltage (−10 kV) separation for 5–15 min; (iii) a 1 min wash with water, 3 min wash with 0.1 M NaOH, a 2 min wash with water, a 10 min wash with separation buffer, with a 50 mbar internal pressure was applied to the inlet to perform the above wash steps. All electrophoretic separations were carried out at least in duplicate. Uracil (1 mg/ml) was used as the EOF marker in all the experiments.

### 2.4. Computational procedures

The physical characteristics of peptides ***1–17***, their molecular masses, $M_r$, net charge values, $q$, and their charge-to-size-ratio parameters, $\xi_{fric}(\xi_{fric} = q/M_r^{2/3})$, at pH 4.0, were calculated utilising the p$K_a$ values for the amino acid side chains from Dawson et al. [23], and for the C- and N-terminus from Rickard et al. [24] implemented in the Charge software developed in this laboratory, coupled to the Excel version 5.0 program (Microsoft), whilst the statistical analysis involved the Sigmaplot 4.01 program (Jandel Scientific) linear and non-linear regression analysis.

## 3. Results and discussion

### 3.1. Effect of the organic solvent content on the electroosmotic flow velocity

The application of an electric field along a fused silica capillary filled with an aqueous buffer electrolyte generates an electroosmotic flow. The magnitude of the EOF velocity, $\upsilon_{EOF}$, is given by the Smoluchowski equations:

$$\upsilon_{EOF} = \mu_{EOF} E \quad (1)$$

$$\mu_{EOF} = \frac{\varepsilon_0 \varepsilon_r \zeta}{\eta} \quad (2)$$

where $\mu_{EOF}$ is the electrophoretic mobility of the EOF, $E$ the electric field strength, $\varepsilon_0$ the permittivity of a vacuum, $\varepsilon_r$ the relative permittivity (dielectric constant) of the media, $\zeta$ the zeta potential at the liquid–solid interface, and $h$ is the viscosity of the media.

When an organic solvent is added to an aqueous buffer, it not only changes the physicochemical properties of the background electrolyte solution, for example, the pH, dielectric constant and viscosity, but also affects the zeta potential of the system in a complex manner. Changes in temperature, either through the generation of Joule heat temperature gradients within the capillary or due to the application of an external temperature ramp will also impact on $\upsilon_{EOF}$ and hence on $\upsilon_{e,i}$, the migration velocity of the analyte. With a HPCE system of defined buffer electrolyte composition and field strength, the product $\varepsilon_0 \varepsilon_r \zeta$ only varies slightly [25] with temperature, $T$. As a consequence, with a HPCE system of defined field strength, pH and buffer electrolyte composition the shape of the migration velocity profile of a charged peptide as a function of $T$ will be determined almost entirely by the temperature dependence of the viscosity and the solvated organisation of the buffer electrolyte when an organic solvent is present. Since the variation in the viscosity with temperature takes the form, $\eta \propto Ae^{B/T}$, where $A$ and $B$ are systems constants, subtle changes in the values of $\mu_{EOF}$ and $\mu_{e,i}$ are anticipated as a direct consequence of variations in the choice and composition of the organic solvent, and whether temperature variations arise as part of the

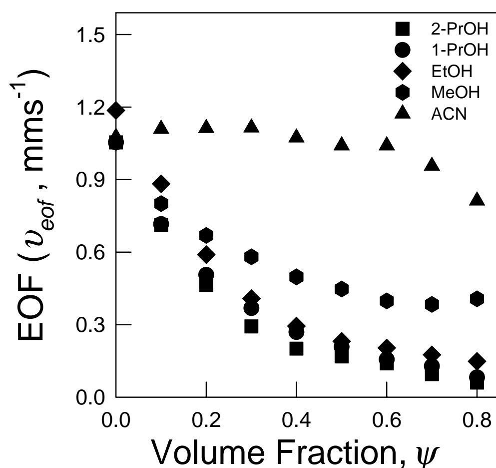


Fig. 1. Dependence of the EOF on the volume fraction ($\psi$) of different organic solvents. The experimental conditions were: buffer electrolyte: 10 mM ammonium acetate, pH 4.0, containing different contents of organic solvents; capillary: total length, $L = 34.0$ cm with a separation length of $l = 8.0$ cm, 100 μm i.d.; sample injection: −5 mbar, 2 s; UV detection at 214 nm.

separation protocol. For these reasons, particular attention was given to control the temperature of the capillaries used in the present investigations, thus minimising the influence of this variable.

Fig. 1 shows the influence of different organic solvents, e.g. acetonitrile and four alcoholic solvents on the EOF utilising 10 mM ammonium acetate, pH 4.0, as the fully aqueous buffer electrolyte. Under these conditions the silanol groups of the capillary wall are partially ionised. Acetonitrile is an aprotic solvent that tends to form solvent clusters rather than an extensive hydrogen bonded network in aqueous environments and has a different effect on the EOF compared to the alcoholic solvents. With the addition of this solvent, the EOF showed only small changes up to a volume percentage of about 40% (v/v), and then decreased slowly. When the content of acetonitrile reached 80% (v/v), the EOF had decreased by ca. 20% comparing to the value with a purely aqueous buffer electrolyte. The alcoholic solvents, on the other hand, all caused a significantly reduction in the EOF, especially up to volume percentages of 40% (v/v). The solvent 2-propanol had the largest impact on the EOF, reducing its value by a factor of nearly 20, whilst methanol had the least effect, resulting in a three- to four-fold decrease in the magnitude of the $\upsilon_{EOF}$. According to these results as well as the earlier findings of Schwer and Kenndler [21], variations in the EOF following the addition of an organic solvent cannot be attributed solely to changes in viscosity, $\eta$, dielectric constant, $\varepsilon_r$, of the resulting buffer electrolyte or their ratio, $\varepsilon_r/\eta$. The zeta potential, $\zeta$, of the electric double layer will also decrease upon addition of almost all organic solvents [21]. This outcome is due to the multiple effects of the organic solvent, namely:

(1) The addition of organic solvents to the aqueous ammonium acetate buffer alters the pH of the buffer electrolyte since the p$K_a$ values of the weak acid used to prepare these buffer electrolyte changes with the solvent composition [26]. For example, the p$K_a$ of acetic acid in water, methanol, ethanol and acetonitrile is 4.73, 9.7, 10.3 and 22.3, respectively [27], respectively, whilst the p$K_a$ of other anionic functionalities such as amino acid side chains increases with the methanol [26] or acetonitrile [28] content in mixed solvents as well. For the aqueous ammonia acetate buffers used in this study, we measured the change in the pH value of the buffer electrolyte as the content of methanol and acetonitrile was increased from 0 to 90% (v/v), and documented a 2–3 pH unit increase over this range (data not shown). This pH shift will generally promote greater ionisation of silanol groups at the inner wall of capillary and therefore raise the zeta potential.
(2) The addition of organic solvents to the buffer electrolyte can also change the zeta potential of the electric double layer via an increase of the p$K_a$ value of the silanol groups. An increase in p$K_a$ of the silanol groups will lower the $\zeta$ value at the surface of the capillary.
(3) Williams et al. [29] have shown that the molecular nature of the solvent plays a dominant role in determining the structure of the electric double layer close to the solid surface. In mixed organic solvent–water systems, the majority of counterions in the Stern layer are separated by two partially ordered layers of water molecules. When organic solvent molecules replace water molecules adsorbed to the silanol groups, the surface charge declines as most organic solvents have lower dielectric constants than water. Their presence in the Stern layer will thus tend to suppress ionisation of the silanol groups. On the other hand, these organic solvent molecules upon adherence to the solid surface will influence the adsorption of co- and counter-ions, including the ionised analytes, depending on their solvation properties.

Compared to the results reported by Schwer and Kenndler [21], our findings with acetonitrile and methanol show different effects on the EOF. With an increase in the content of acetonitrile in the buffer electrolyte, the magnitude of the EOF changed by only ca. 20% over the volume fraction range of 0.8, rather than the decrease of about two-fold as previously described [21]. Moreover, we observed a decrease of about 60% in the magnitude of the EOF upon addition of methanol, with the EOF rising slightly when the volume fraction of methanol increases from 70 to 80% (v/v) contrary to the reported 10-fold decline [21]. Since these studies were performed under different experimental conditions with regard to the choice of manufacturer of the fused silica capillary, the pH of the aqueous buffer and the final buffer composition, this difference is not unexpected. In both cases, the change in the EOF can be attributed to the different ionisation states of the silanol groups at the inner wall of capillary, i.e. the $\zeta$-potential and the ion flux that is es-

tablished, which in turn generates the EOF. These findings reveal that the extent of ionisation of the silanol groups and the characteristics of the $\zeta$-potential can be used to significantly differentiate the effects of organic solvents, especially commonly used solvents such as acetonitrile and methanol, on the EOF.

The measurement of the EOF and the electrophoretic mobilities of synthetic peptides were carried out as alternating experiments at different organic solvent conditions. All peptides investigated in this study bear positive charges at pH 4.0, and they may interact electrostatically with the negatively charged surface of the capillary wall. When higher amounts of peptide samples were injected, the possible arises that the properties of the capillary wall could change due to these adsorptive effects, as evident from changes in the zeta potential and EOF [30]. Measures were thus taken to eliminate these wall effects by rinsing the capillary with 0.1 M NaOH between experiments and at the end of each day's experiments. On the other hand, the long time contact of the two capillary ends in buffer electrolytes of different organic solvents, especially acetonitrile, can according to the literature [31] cause swelling of polyimide coating of fused-silica capillaries and this may lead to partial clogging or breakage of the capillary ends. This swelling and changes in the inlet and outlet conditions of the capillary may influence the reproducibility and performance of a separation. Generally, in the current HPCE studies, these constraints were not evident with repetitive experiments over several hundred runs exhibiting excellent reproducibility.

### 3.2. Electrophoretic mobility of peptides *1–17*

All peptides used in this study were synthesised on either the Wang-resin with the first amino acid attached or the Glu-(rink amide MBHA) resin by Fmoc-based solid-phase synthesis protocols, according to their specific amino acid sequence. The peptides have free N- and C-termini and are positively charged at pH 4.0. For the first group, peptides ***1–6*** and peptide ***8*** are fragments of parent peptide ***7*** and involve truncations from either the N- or C-terminus. Similarly, peptides ***9–15*** and peptide ***17*** are fragments of peptide ***16*** truncated from either the N- or C-terminus. The amino acid sequences, molecular masses, calculated effective charges, $q$, and charge-size ratio parameters, $\xi_{\text{fric}}$, at pH 4.0 of these structurally related synthetic peptides are presented in Table 1. As apparent from these data, the net charge carried by peptides ***1–8*** under these buffer electrolyte conditions of 10 mM ammonium acetate, pH 4.0, range between +1.01 and +3.99 with frictional ratios, $\xi_{\text{fric}}$, between 0.0092 and 0.0356. Based on these considerations, because of the similarity in their $q$ and $\xi_{\text{fric}}$, values the separation of peptides ***2–4*** by HPCE would be expected to be challenging with low ionic strength, fully aqueous buffer electrolytes at pH 4.0. However, when organic solvent modifiers are included at intermediate to relatively high concentrations, resolution of these peptides can be realised. Such solvent-dependent selectivity variations represent a powerful aspect of such HPCE systems and, as discussed below, reflect changes in protonic equilibrium of the buffer electrolyte when an organic solvent is included. As described elsewhere, solvent-dependent effects also impact on peak efficiencies [Yang and Hearn, 2003, unpublished results]. Similarly, the net charge carried by peptides ***9–17*** under these buffer electrolyte conditions of 10 mM ammonium acetate, pH 4.0, range between +1.48 and +5.53 with frictional ratios, $\xi_{\text{fric}}$, between 0.0163 and 0.0418. In these cases sufficiently large differences exist between the respective $q$ and $\xi_{\text{fric}}$, values of the various peptides, which are therefore more straightforward to resolve under low ionic strength, fully aqueous buffer electrolyte HPCE conditions.

For peptides separated in HPCE, the apparent electrophoretic mobility, $\mu_{\text{app}}$ is the summation of the contribu-

Table 1
Amino acid sequence, molecular mass $M_r$, net-charge values $q$ at pH 4.0, and charge-mass ratio parameter $\xi_{\text{fric}} = q/M_r^{2/3}$ of peptides ***1–17***

| Peptide code | Amino acid sequence | $M_r$ | $q$ (pH 4.0) | $\xi_{\text{fric}}$ (pH 4.0) |
|---|---|---|---|---|
| 1 | DHDINR | 768.78 | 1.01 | 0.0121 |
| 2 | WDHDINR | 955.00 | 1.01 | 0.0104 |
| 3 | SWDHDINR | 1042.08 | 1.01 | 0.0099 |
| 4 | NSWDHDINR | 1156.18 | 1.01 | 0.0092 |
| 5 | HNSWDHDINR | 1293.32 | 2.00 | 0.0169 |
| 6 | HHNSWDHDINR | 1430.46 | 3.00 | 0.0236 |
| 7 | HHHNSWDHDINR | 1567.60 | 3.99 | 0.0295 |
| 8 | HHHNSW | 816.83 | 3.11 | 0.0356 |
| 9 | QHNHFHR | 975.04 | 4.11 | 0.0418 |
| 10 | DQHNHFHR | 1090.13 | 3.55 | 0.0335 |
| 11 | QDQHNHFHR | 1218.26 | 3.55 | 0.0311 |
| 12 | HQDQHNHFHR | 1355.40 | 4.54 | 0.0371 |
| 13 | IHQDQHNHFHR | 1468.56 | 4.54 | 0.0352 |
| 14 | NIHQDQHNHFHR | 1582.66 | 4.54 | 0.0335 |
| 15 | TNIHQDQHNHFHR | 1683.77 | 4.54 | 0.0321 |
| 16 | HTNIHQDQHNHFHR | 1820.91 | 5.53 | 0.0371 |
| 17 | HTNIHQD | 863.89 | 1.48 | 0.0163 |

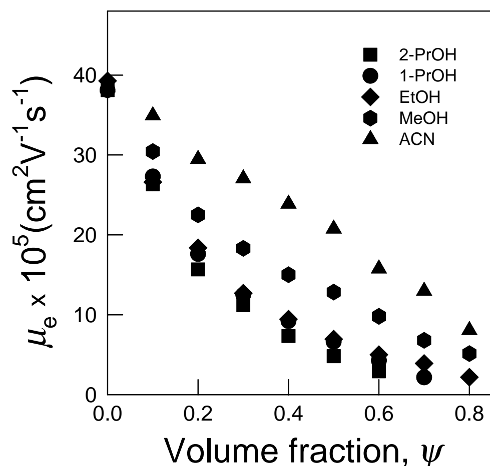


Fig. 2. Variation in the effective mobility, $\mu_e$, of peptide ***9*** with solvent composition. The experimental conditions are the same as given in the legend to Fig. 1.

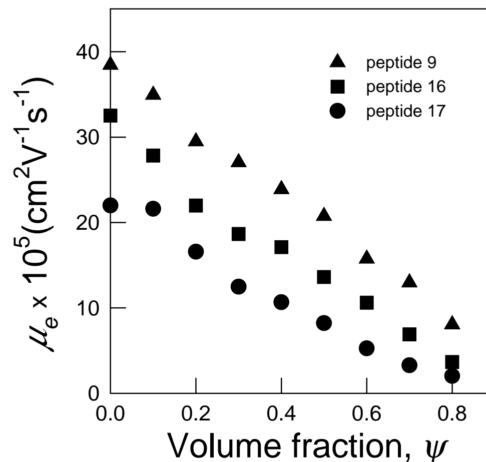


Fig. 3. Influence of the volume fraction of acetonitrile on the effective mobility, $\mu_e$, of the peptides ***9***, ***16*** and ***17***. The experimental conditions are the same as given in the legend to Fig. 1.

tions from the electroosmotic flow mobility, $\mu_{EOF}$, and the effective electrophoretic mobility, $\mu_e$, therefore:

$$\mu_e = \mu_{app} - \mu_{EOF} \tag{3}$$

Changes from an aqueous buffer to an aqueous-organic solvent buffer electrolyte system will directly impact on the electrophoretic mobility of a charged peptide through the dependency:

$$\mu_e = \frac{e}{6\pi\eta} \sum_{k=j}^{k=-i} \frac{k}{r_{s,k}} \chi_k \tag{4}$$

where $\chi_k$ is the molar fraction of any ionised species, $j$; $e$ the electron charge ($1.60 \times 10^{-19}$ C), $\eta$ the solution viscosity (water: $8.95 \times 10^{-4}$ Ns/m$^2$ at 298 °K), $r_{s,k}$ is the Stoke's radius (i.e. the radius of the sphere equivalent to the hydrated peptide) of any species $j$; and $k$ the dimensionless value of the species integer charge.

Fig. 2 shows the variation of effective electrophoretic mobility, $\mu_e$, of peptide ***9*** with changes in the content of different organic solvents. The overall trend for all solvents is similar with a progressive decrease in the electrophoretic mobility with an increase in the volume fraction of the organic solvents in the buffer electrolyte. For acetonitrile, the $\mu_e$ of peptide ***9*** decreased nearly linearly, whilst for the alcoholic solvents the magnitude of the $\mu_e$ of peptide ***9*** show a steeper decrease up to 20% (v/v) organic solvent, reducing the electrophoretic mobility of this peptide by a factor of ca. 2. Further addition of organic solvent to the buffer electrolyte caused a progressive but relatively smaller decrease in the electrophoretic mobility. Among the five organic solvents examined, acetonitrile had the least effect, reducing the electrophoretic mobility by a factor of about 4.8 at a volume percentage of 80% (v/v), while a factor of 13 was found with 60% (v/v) 2-propanol in the buffer electrolyte. The influence of organic solvents on the electromobility of the other peptides, as illustrated in Figs. 3–5, is similar to that found for peptide ***9***.

The addition of an organic solvent to an aqueous buffer changes the electrophoretic mobility of a peptide, according to Eq. (4), in three ways; e.g. the effect on the viscosity of the buffer electrolyte, the effect on the peptide's size as reflected in the change in the Stokes radius and the effect on the overall charge of the peptide. As noted above, variations in viscosity and the impact of small temperature gradients that could be generated within the capillary on viscosity can be readily rationalised. The viscosity of an acetonitrile–water mixture, at high acetonitrile content, is lower than that of pure water, yet the electrophoretic mobilities, $\mu_e$, of all peptides investigated still declined dramatically at higher acetonitrile contents compared to the $\mu_e$ values in aqueous buffer (c.f. the data for peptides ***9***, ***16*** and ***17*** shown in Figs. 3–5 for acetonitrile, methanol and ethanol). The (near linear) decreases

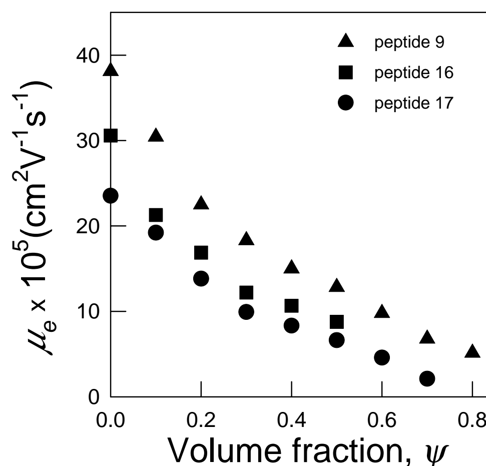


Fig. 4. Influence of the volume fraction of methanol on the effective mobility, $\mu_e$, of peptides ***9***, ***16*** and ***17***. The experimental conditions are the same as given in the legend to Fig. 1.

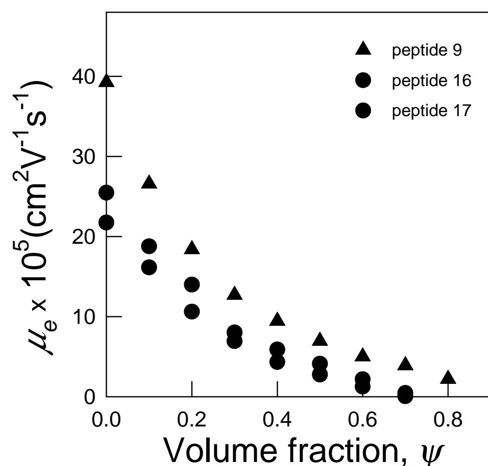


Fig. 5. The influence of the volume fraction of ethanol on the effective mobility, $\mu_e$, of peptides ***9***, ***16*** and ***17***. The experimental conditions are the same as given in the legend to Fig. 1.

in electrophoretic mobilities of these peptides with different types of solvents cannot be simply attributed to a decreased viscosity per se. Similarly, changes in peptide partial molal volume due to solvational effects, although strongly dependent on the amino acid sequence [19], will also not be anticipated to be significant [19,20] in these HPCE systems. However, for larger peptides that have the propensity to adopt stable secondary structures in solution, the addition of an organic solvent, such as acetonitrile [32], can modify their shape aspect, and this may lead to the peptide adopting thermodynamically more stable folded structures under some solvent conditions.

Elsewhere, we have shown [33] by CD spectrophotometry that the conformational preferences of peptide ***1–17*** do not significantly change over the range of solvent combinations used in the present HPCE investigations. In contrast to the small impact on the electromigration behaviour of peptide ***1–17*** due to changes in the Stoke's radius and/or conformation, the significant decreases in the electrophoretic mobilities of these peptides when organic solvents are added to aqueous buffer electrolytes up to a volume percentages of 80% (v/v) can be ascribed to the reduction of the overall charge of the peptides. In order to calculate the charge of a peptide, accurate knowledge on the ionisation constants ($pK_a$) of all ionisable groups within the peptide and the partial molar fraction, $\chi$, of each ionised species of the peptide in a particular solvent environment must be obtained. Since the dependencies of these ionisation constants and $\chi$ values on buffer electrolyte compositions with peptides of different sequence will vary and also differ from those for the corresponding free amino acid, as a general principle it is difficult to ab initio predict the correct ionisation constant and $\chi$ value for each ionisable group of a peptide in aquo-organic solvent environments. The addition of organic solvents to an aqueous buffer reduces its dielectric constant, generally leading to an increase in the dissociation constants of carboxyl group(s) of C-terminal amino acid and the side chains of aspartic and glutamic acid residues, and a decrease in the dissociation constants of amino group(s) of the N-terminal amino acid and the side chain of lysine, histidine or arginine [19,26,34] As a consequence, the calculation of the accurate charge of a peptide becomes more problematic for aqueous-organic solvent combinations when used as buffer electrolytes in HPCE. In some instances microtitration methods can be applied [35]. Another way this issue can be addressed is to measure the relative electromobility of a peptide as the pH of a buffer electrolyte of fixed solvent composition is systematically changed, and then repeating the same experiments for buffer electrolytes of different organic solvent content. Such approaches have been reported in several investigations with small peptides or polypeptides with very stable secondary structures, i.e. peptides of 3–5 amino acid residues or polypeptides with >65% α-helical content in aqueous buffers containing trifluoroethanol or acetonitrile [19,20]. This approach, however, is time consuming and for this reason has tended to be applied to an individual peptide rather than peptide sets. More empirical approaches have relied upon suppression of ionisation of a particular functional group through the choice of pH conditions.

There are several advantages with the approach followed in the present investigation, particularly as it was our intention is to interface the HPCE system with mass spectrometric detection [unpublished results]. The charge status of the peptide is under better control and surface interactions with the inner wall of the capillary can be readily monitored. Thus, at pH 4.0, the amino groups of the free N-terminal amino acid and the side chain of arginine or lysine will effectively be totally protonated because of the high $pK_a$ values of these basic amino acids. As the pH increases due to the addition of the organic solvent, these centres will progressively deprotonate, leading to a decrease in net charge on these amino groups. In contrast, the addition of an organic solvent on the ionisation of the carboxyl groups within a peptide has two competing effects. Firstly, ionisation of the C-terminal or side chain carboxyl groups will tend to be enhanced due to the higher effective pH of the mixed solvent compared to that of pure aqueous buffer and, secondly, the decrease in the dielectric constant of the aquo-organic solvent buffer electrolyte system will tend to suppress their ionisation. These counterbalancing effects will reduce the contribution of carboxyl group ionisation to the change in the charge of peptide ***1–17*** and its electrophoretic mobility as the organic solvent content is increased.

One common aspect of the peptides investigated in this study is that they contain at least one histidine residue. The $pK_a$ of the histidine side chain is quite variable and typically lies near a value of six, but it may vary from five to at least eight depending on its environment [24]. Richard et al. adopted an adjusted $pK_a$ value of 6.2 for the calculation of the charge of histidine-containing peptides and obtained good correlations between the observed and calculated electromobilities [24]. Since the addition of organic solvents in-

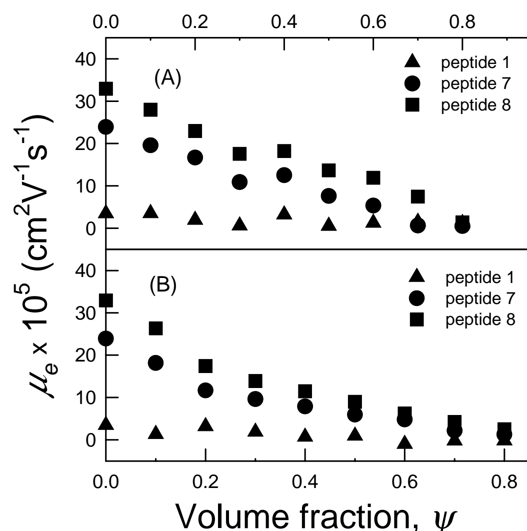


Fig. 6. Influence of solvent composition (A) acetonitrile and (B) methanol on the electrophoretic mobility, $\mu_e$, of peptides ***1***, ***7*** and ***8***. The experimental conditions are the same as given in the legend to Fig. 1.

creases the pH of an aqueous buffer electrolyte and also reduces the p$K_a$ of the histidine side chain, both effects may promote deprotonation of the histidine side chain, and thus lead to a lower peptide charge and reduced electrophoretic mobility. Consistent with this conclusion, peptides containing more than one histidine residue (Table 1) demonstrated more pronounced decreases in their electrophoretic mobility in aqueous-organic solvent buffer systems as illustrated in Fig. 6A and B for peptides ***1***, ***7*** and ***8***.

### *3.3. Separation selectivity of peptides*

From the above analysis, it can be seen that the effect of organic solvents on the electrophoretic mobility of peptides is different, depending on the peptide sequence and choice of solvent. Fig. 7 shows an example of this selectivity effect,

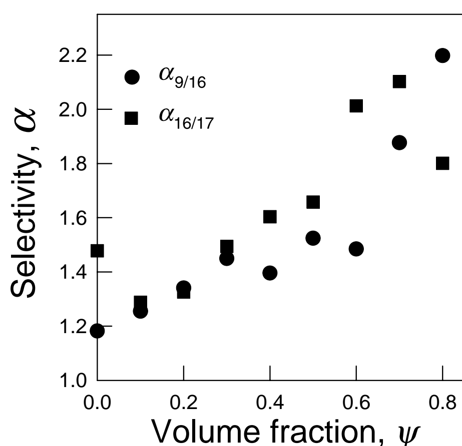


Fig. 7. The influence of the volume fraction of acetonitrile on the separation selectivity, *a*, of peptides ***9***, ***16*** and ***17***. The experimental conditions are the same as given in the legend to Fig. 1.

as defined as the ratio of relative electromobilities, *a*, of the peptides ***9***, ***16*** and ***17*** in aqueous-acetonitrile mixed buffer electrolyte systems. As evident from Fig. 7 the separation selectivity, generally, increased with the volume fraction of acetonitrile, although this trend was not always observed for the peptides with the other solvents. Again, this feature can be utilized to enhance the separation performance through the selection of the appropriate organic solvent for a particular group of peptides.

## 4. Conclusions

This study shows that the electroosmotic flow in aqueous-organic media can be effectively modulated by changes in the nature and content of the organic solvent added to the buffer electrolyte. When fast and efficient separations are required, acetonitrile can be utilized to achieve this goal, whilst alcoholic solvents can be employed to achieve reduced electroosmotic flow in those cases where higher resolution factors are needed for the HPCE separation. In addition, these studies show that the p$K_a$ values of the ionisable groups presented in a peptide can be varied according to the choice of the organic solvent in a way that depends considerably on the specific peptide sequence. Therefore, the magnitude of the changes in peptide charge and electrophoretic mobility can be manipulated over a wide range through the use of different organic solvent combinations to achieve enhanced separation selectivity.


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## References


[1] B.L. Zhang, F. Foret, B.L. Karger, Anal. Chem. 72 (2000) 1015.
[2] G.A. Valaskovic, N.L. Kelleher, F.W. McLafferty, Science 273 (1996) 1199.
[3] V. Kasicka, Electrophoresis 20 (1999) 3084.
[4] M.T.W. Hearn, Biologicals 29 (2001) 159.
[5] P.G. Righetti, Biopharm. Drug Dispos. 22 (2001) 337.
[6] F.E. Regnier, S. Lin, Chem. Anal. N. Y. 146 (1998) 683.
[7] S. Hu, N.J. Dovichi, Anal. Chem. 74 (2002) 2833.
[8] J.E. Noroski, D.J. Mayo, M. Moran, J. Pharm. Biomed. Anal. 13 (1995) 45.
[9] G.A. Valbuena, L.V. Rao, J.R. Petersen, A.O. Okorodudu, M.G. Bissell, A.A. Mohammad, J. Chromatogr. A 781 (1997) 467.
[10] K. Otsuka, S. Terabe, Mol. Biotechnol. 9 (1998) 253.
[11] M.-L. Riekkola, Electrophoresis 23 (2002) 3865.
[12] K. Sarmini, E. Kenndler, J. Chromatogr. A 792 (1997) 3.
[13] S. Cherkaoui, J.-L. Veuthey, Electrophoresis 23 (2002) 442.
[14] Z. Shen, C.D. Warren, D.S. Newburg, J. Chromatogr. A 921 (2001) 315.

[15] K. Sarmini, E. Kenndler, J. Cap. Electrophor. 5 (1998) 103.
[16] W. Ding, J.S. Fritz, Anal. Chem. 70 (1998) 1859.
[17] C. Miller, J. Rivier, J. Pept. Res. 51 (1998) 444.
[18] M. Idei, I. Mezo, Z. Vadasz, A. Horvath, I. Teplan, G. Keri, J. Liq. Chromatogr. 15 (1992) 3181.
[19] M. Castagnola, L. Cassiano, I. Messana, M. Paci, D.V. Rossetti, B. Giardina, J. Chromatogr. A 735 (1996) 271.
[20] M.T.W. Hearn, H.H. Keah, R.I. Boysen, I. Messana, F. Misiti, D.V. Rossetti, B. Giardina, M. Castagnola, Anal. Chem. 72 (2000) 1964.
[21] C. Schwer, E. Kenndler, Anal. Chem. 63 (1991) 1801.
[22] Y. Yang, R.I. Boysen, J.I.C. Chen, H.H. Keah, M.T.W. Hearn, J. Chromatogr. A 1009 (2003) 3.
[23] R.M.C. Dawson, D.C. Elliot, W.H. Elliot, K.M. Jones, Data for Biomedical Research, third ed., Clarendon Press, Oxford, 1986.
[24] E.C. Rickard, M.M. Strohl, R.G. Nielsen, Anal. Biochem. 197 (1991) 197.
[25] I. Watanabe, N. Ui, M. Nakamura, J. Phys. Colloid Chem. 54 (1950) 1366.
[26] I. Canals, J.A. Portal, E. Bosch, M. Roses, Anal. Chem. 72 (2000) 1802.
[27] K. Sarmini, E. Kenndler, J. Biochem. Biophys. Methods 38 (1999) 123.
[28] S. Espinosa, E. Bosch, M. Roses, Anal. Chem. 72 (2000) 5193.
[29] G.D. Williams, A.K. Soper, N.T. Skipper, M.V. Smalley, J. Phys. Chem., B 102 (1998) 8945.
[30] J.K. Towns, F.E. Regnier, Anal. Chem. 64 (1992) 2473.
[31] F. Baeuml, T. Welsch, J. Chromatogr. A 961 (2002) 35.
[32] L.G.J. Hammarstrom, T.J. Gauthier, R.P. Hammer, M.L. McLaughlin, J. Pept. Res. 58 (2001) 108.
[33] J.I-Chen Chen, R.I. Boysen, M.T.W. Hearn, 2003, unpublished results.
[34] V. Sanz-Nebot, I. Toro, F. Benavente, J. Barbosa, J. Chromatogr. A 942 (2002) 145–156.
[35] M. Castagnola, L. Cassiano, I. Messana, G. Nocca, R. Rabino, D.V. Rossetti, B. Giardina, J. Chromatogr. B 656 (1994) 87–97.