EFFECTS OF ORGANIC SOLVENT AND CATIONIC ADDITIVE ON CAPILLARY ELECTROPHORESIS OF PEPTIDES

L. Noumie Surugau[1*], Ed T. Bergström[2], David M. Goodall[3]

[1]*School of Science and Technology, Universiti Malaysia Sabah, Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia.*
[2]*Department of Chemistry, University of York, Heslington, York, YO10 5DD, UK.*
[3]*Paraytec Ltd., 1a St. George's Place, Tadcaster Road, York, YO24 1GN, UK.*

**Corresponding author: lnoumie@ums.edu.my*

Abstract

Capillary electrophoresis (CE) of nine peptides namely, bradykinin, bradykinin fragment 1-5, substance P, Arg[8]-vasopressin, luteinizing hormone-releasing hormone (LHRH), bombesin, leucine-enkephalin, methionine-enkephalin and oxytocin were carried out using 0.5 % and 1.0 % formic acid (FA) as the separation buffers, added with acetonitrile (ACN) and triethylamine (TEA) as an additive at low pH. The electrophoretic behaviour of these peptides was examined at different concentration of TEA (0, 10, 20, 30, 40 and 50 mM), and ACN (30, 40, 50, 60, 70 %) at their respective measured final pH. The results showed that all nine peptides were fully resolved with addition of 10 – 20 mM TEA. Peak efficiency was improved significantly by increasing TEA concentration up to 40 mM where 800 000 m^{-1} was obtained. Without TEA, the closely related enkephalins were co-migrating. Interestingly, by addition of as little as 5 mM TEA has sufficient to separate them almost at baseline. Increasing ACN to 40 % has shortened the analysis time by ca. 1 min. However, further increase of ACN can cause peak broadening and current instability.

Keywords: Peptides; Triethylamine, Formate, Acetonitrile, Selectivity; Resolution

Introduction

Electrostatic interactions are believed to play a key role in the adsorption of peptides and proteins on the capillary wall. The analyte may be attracted to the capillary surface by Coulombic forces and once there hydrogen bonding and hydrophobic interactions can join in immobilizing the protein at the interface between the capillary wall and the electrolyte solution. Therefore, masking the negatively charged silanol groups on the capillary wall by an amino compound incorporated in the electrolyte solution has been considered by several researchers as a method of choice to avoid protein adsorption.

There are a number of reports available in the literature about this topic; one particular review is by Corradini (1997). The numerous types of amino compounds that have been employed as additives can be summarized in two main groups: one comprising diaminalkanes and monovalent amines, the other including non-polymeric and polymeric polyamines. This class comprises a vast number of compounds, starting from mono-amines, such as triethylamine and propylamine (Bullock & Yuan, 1991). In reverse-phase chromatography of ionogenic substance, amino compounds are generally recommended as additives of the mobile phases to suppress the untoward effect of the residual silanol groups at the stationary phase (Nahum & Horvath, 1981). These additives are believed to act primarily by hydrogen bonding to non-derivatized silanol groups, thereby reducing adsorption and ion-exchange effects (Biij *et al*., 1987).

Previously, many CE applications have been restricted to the use of aqueous background buffer electrolytes (BGE), although the application of fully organic or aqueous-organic solvent buffer electrolytes is attracting increased attention for the separation and quantitative analysis of anionic and cationic drug substances (Riekkola, 2002; Miller & Rivier, 1998) and other classes of charged molecules. The addition of an organic solvent to modify the composition of an aqueous buffer electrolyte often increases the solubility of organic analytes in the BGE and reduces either self-self association or interactions of hydrophobic compounds with the negatively charged capillary wall (Sarmini & Kenndler, 1997; Miller & Rivier, 1998). Full separation of positional isomers of substituted benzoic acids was achieved in aqueous-organic solvents by mixing up to 60% (v/v) methanol (MeOH) or acetonitrile (ACN) with the BGE (Fujiwara, 1986). Also, increase in peak efficiency and reduction of analysis time were observed by Idei *et al*. (1992) when mixing organic solvent such as ACN, MeOH, ethanol (EtOH) and iso-propanol (*i*-PrOH) up to 30 % (v/v). Moreover, when interfaced with electrospray ionization-mass spectrometry (ESI-MS), aquo-organic solvent BGE in CE frequently generate an

overall improvement in mass detection sensibility since the lower surface tension and higher volatility favour electrospray formation (Cherkaoui & Venthey, 2002).

Because charged analytes migrate in CE as a consequence of the additive effects of their intrinsic electromobility and the electroosmotic flow (EOF), variations in the composition of the BGE as well as the field strength are expected to have profound impact on resolution (Sarmini & Kenndler, 1997). Also, the electrical conductivities of aquo-organic BGEs are different from their corresponding fully aqueous buffers, and this feature allows application of higher electric field strengths, producing higher separation efficiencies with shorter analysis time.

This report presents a systematic study on the effect of triethylamine (TEA) and ACN on the separation efficiencies of a peptides mixture using formate buffer at low pH. It is demonstrated here that separation of the test peptides can be easily manipulated by adding ACN and TEA at optimum amounts.

Experimental

Chemicals and Reagents

ACN and formic acid (FA) 98 % were obtained from Fisher Chemicals (Loughborough, UK). Peptides standard mixture (P2693), TEA, and 1,4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (Poole, UK). All reagents were of analytical grade.

For the experiments to check the effect of TEA, two sets of BGE were tested; each contains 0.5% (v/v) and 1.0% (v/v) FA, respectively. The BGE was added with differing concentration of TEA (0, 10, 20, 30, 40 and 50 mM), and then added with 30% ACN. To check the effect of ACN, the BGE contains 0.5% FA, 10 mM TEA, and differing amount of ACN (40, 50, 60 and 70 % (v/v)). All BGEs were prepared in purified water (Elgastat, High Wycombe, UK). The final pH of each buffer was measured using a Corning ion analyzer 150 (Halstead, UK). All buffers were sonicated for 20 min, filtered through a 0.2 μm microfilter (Sartorius, Göttingen, Germany) and kept in airtight bottle at room temperature if not used immediately.

The stock solution of peptide standards mixture was prepared by adding 500 μl purified water into the vial to give a concentration of 50 ng μl^{-1} each peptide. Further dilution was done to give the required concentrations. Prior to CE separation, 10 mM of DMAP was mixed into the sample as a reference.

Apparatus and procedures

CE separations were performed in 50 cm, 50 μm i.d. and 365 μm o.d. uncoated fused silica capillary (Composite Metal Services Ltd., Hallow, UK) on a Beckman Coulter P/ACE MDQ CE (High Wycombe, UK) equipped with an UV diode array detector. The scan range of UV absorbance from 190 to 300 nm took place at 10 cm from outlet end through a window created by removal of 1 cm of polyimide coating. The polyimide coating was also removed 2-3 mm from both ends to minimize adsorption of the positively charge peptides on the coating (Ensing *et al*., 1999). Prior to analysis, the capillary was washed with deionized water and 1 M HCl, and then conditioned with 0.1 M NaOH and the BGE. All analyses were carried out in triplicate of more. Other experimental details are described in Section 3.

Results and discussion

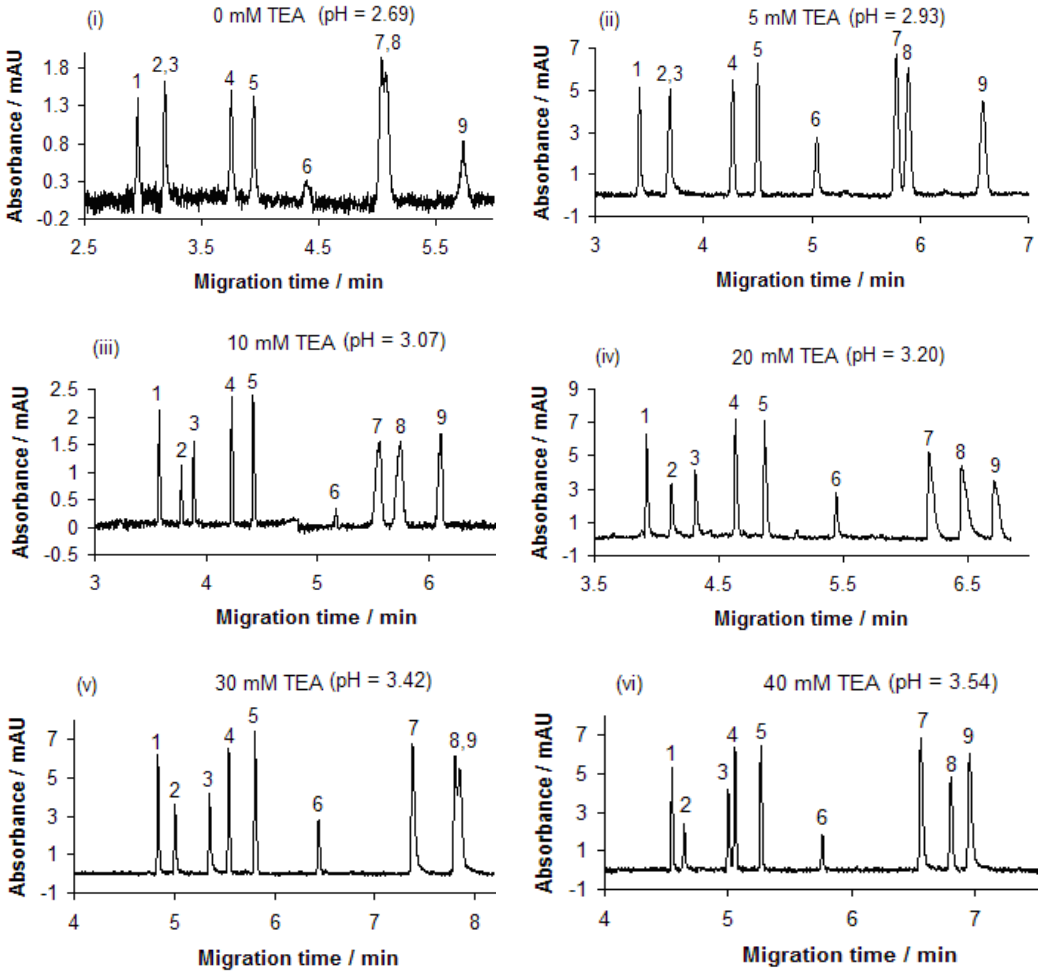
In some earlier works, MeOH was seen to cause peak broadening in peptide separation (Tachibana *et al*., 2003). Also, Peyrin and Guillaume (1999) found that ACN, instead of in MeOH, improved CE efficiency in the separation of ten benzodiazepines. However, in our experiments investigating MS-compatible buffers, it was found that peak efficiencies were not significantly different in ACN and MeOH. Probably the high peak efficiency in this study was due to the presence of TEA ions that blocks the negatively charged silanol groups on the capillary wall. In the studies by Ensing *et al.* (1999), it was found that peak shape of neuropeptide Y was improved by addition of 50 mM TEA into formic acid. Yoshida and Okada (1999) have also reported that addition of TEA gave symmetrical peaks for the strongly retained peptides in normal phase liquid chromatography. Thus, to check the effects of TEA and ACN on the peptides separation, experiments were carried with increasing concentration of TEA and ACN in BGE.

Effects of TEA

As mentioned earlier, TEA was employed to improve peak efficiency by reducing wall interactions between the positively charge peptides (at low pH) and the ionized silanol on the capillary wall. Table 1 shows the range of peak efficiency, N_{Gauss}, and Fig 1 shows the peptide separations for the nine peptides at different TEA concentrations.

Table 1: Peak efficiency, N_{Gauss}, of peak peptides at different TEA concentration.

TEA conc. / mM	N_{Gauss} / 10^5
0	0. 5 – 0.9
5	0.6 – 1.0
10	0.8 – 3.7
20	0.5 – 1.8
30	1.5 – 3.7
40	2.0 – 4.0
50	0.5 – 0.9



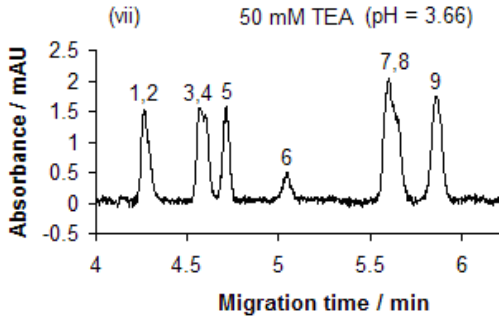


Figure 1: Electropherograms of the peptides mixture in 0.5 % FA + 30 % ACN + different concentrations (0 - 50 mM) of TEA, at respective measured pH. Sample conc.: 0.65 – 2.5 ng μl^{-1}, voltage: 30 kv (i) – (iv), 25 kv (v) – (vii); data rate: 16 Hz; and UV detection at 190 nm. Peaks identification: 1 – bradykinin; 2 – substance P; 3 – bradykinin F1–5; 4 – Arg^8-vasopressin; 5 – LHRH; 6 – bombesin; 7 – Leu-enkephalin; 8 – Met-enkephalin; 9 – oxytocin.

Although there was no straightforward trend, but it is shown in Table 1 that peak efficiencies were essentially improved in higher concentration of TEA. Peak efficiencies were particularly high at 30 and 40 mM TEA. For instance, at 40 mM TEA, N_{Gauss} obtained was 400 000 or 800 000 m^{-1}. Lee and Desiderio (1997) also reported that optimum separation of five proteins in ammonium formate at pH 2.5 was obtained at 30 mM TEA. At 50 mM, peak efficiencies of all peptide were hindered by the poor resolution (see Fig. 1(vii)). Inspecting the peaks shape in Fig. 1, all peptide peaks were symmetrical up to 10 mM TEA. At 20 mM TEA, the last three peaks were very broad and not symmetrical. Interestingly, peak efficiencies were generally improved again at 30 and 40 mM TEA.

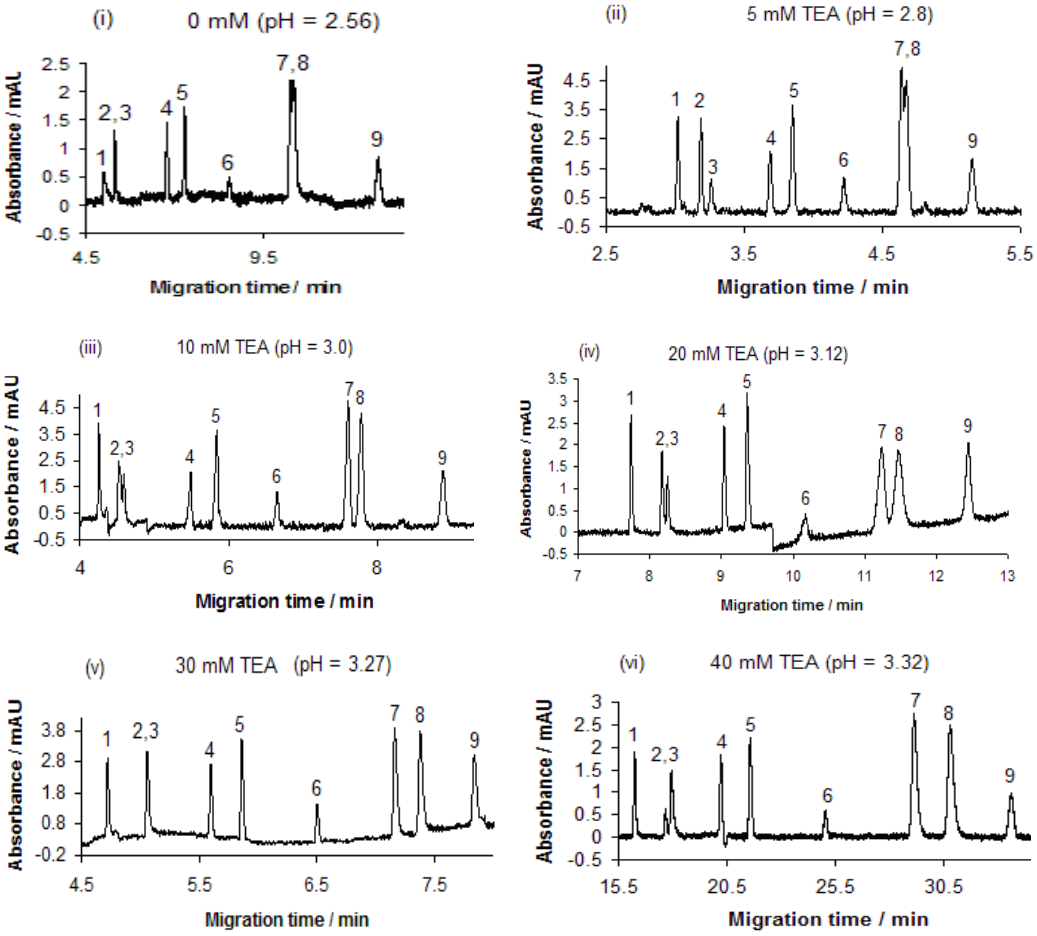
On separation resolution, Fig. 1(i) shows that without TEA, a full separation of the nine peptides mixture was not achieved where peptides 2,3 and 7,8, co-migrated. When 5 mM TEA was added to the buffer, the latter pair (7,8) was almost baseline-separated, and peptides 4,5 were resolved further apart. However, peptides 2,3 were still completely unresolved. By increasing the concentration of TEA to 10 mM, all nine peptides were fully separated. This improvement in separation was expected when using higher ionic strength. When the concentration of TEA was doubled (20 mM), better resolution was seen for all peptide. Peptides 2,3 were separated further apart when TEA was increased to 30 mM. However, resolution of peptides 8,9 was poorer. Also, the resolutions of peptides 1,2 and 3,4 decreased. At 40 mM TEA, interestingly, peptides 8,9 were fully resolved again, though resolution of peptides 1,2 and 3,4 were decreasing. At 50 mM TEA, the separation turned to its worst with only six visible peaks, where peptides 1,2, 3,4 and 7,8 were all co-migrated. Fig. 1 (i-iv) illustrates that separation was generally better as TEA concentration increases up to 40 mM, except at 30 mM TEA where met-enkephalin (peak 8) and oxytocin (peak 9) co-migrated. Resolutions of the three pairs of peptide (1&2, 3&4 and 7&8) were completely loss in higher TEA concentration (Fig. 1(vii)). It is also worth to mention that analysis time increases by increasing the concentration of the additive as the result of decreasing in the EOF.

Initially, it was expected that leu-enkephalin and met-enkephalin (peaks 7,8) would be the hardest to resolve due to their closeness in mobility, i.e. only different in one amino acid. However, from the results above, it was shown that by adding as little as 5 mM TEA was sufficient to resolve them almost at baseline. Substance P and bradykinin F1-5 (peptides 2,3), although were not expected to co-migrate, only fully separated after the addition of 10 mM or more TEA.

Looking at the separation trend, resolution of the two closely related enkephalins (peaks 7,8) was improved by increasing the concentration of TEA in buffer but the resolution was completely loss when using 50 mM TEA. Similarly, separation of substance P and bradykinin F1-5 (peaks 2,3) was improved by increasing the concentration of TEA. Resolution of peptides 4,5 was relatively constant up to 40 mM TEA. It was surprising to see met-enkephalin and oxytocin co-migrating when using 30 mM TEA. The latter is twice heavier than the former, and both have neither basic nor acidic amino acid. And the fact that this was only observed at 30 mM TEA is very suspicious. One possible explanation is an occurrence of changes in the peptides migration order,

where peaks 8,9 were actually not as they are, namely leu-enkephalin and oxytocin, respectively, but probably the both closely related enkephalins.

TEA is generally employed in peptide separation to reduce peak broadening due to wall interaction. The pK_a of TEA is 10.78 indicating that this monovalent amine will primarily exist in cation form at the pH range employed in this study. As seen here, increasing the TEA concentration in the BGE also increases its final pH. Hence, there were two factors dictating the peptides separation; pH changes and concentration of TEA ions. The pK_a of FA is 3.75, indicating that, ideally, if FA dissociation is controlled at minimum (pH < 3.75), TEA ions should be mainly interacting with the negatively charged silanol ($-SiO^-$) on the capillary wall. Thus, another series of experiment was carried out at lower pH (using 1.0 % FA with pH range of 2.56 - 3.50). The electropherograms are shown in Figure 2.



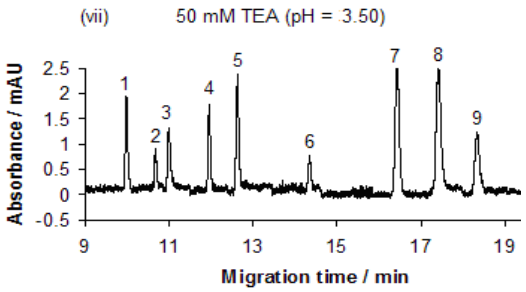


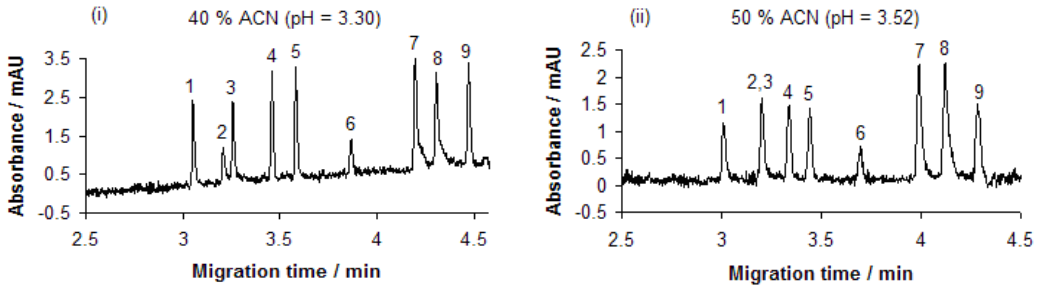
Figure 2: Electropherograms of the peptides mixture in 1.0 % FA + 30 % ACN + different concentrations (0 - 50 mM) of TEA at respective pH. Sample conc.: 5.0 ng μl^{-1}, voltage: 25 kv (i) – (iii) & (v), 20 kv (iv), 15 kv (vii), 10 kv (vi). Other experimental details are similar to Fig 1.

As in the earlier experiments, without TEA a complete separation of the peptide mixture was not achieved, where peptides 2,3 and 7,8 co-migrated. Also, peptides 1,2 were migrated closely. When 5 mM TEA added to the BGE, peptides 2,3 resolved at baseline, peptides 1,2 resolved further but peptides 7,8 were still co-migrated. At 10 mM TEA, interesting observations were seen where peptides 7,8 were baseline-resolved, but unfortunately peptides 2,3 were co-migrated again. By doubling the concentration of TEA, peptides 2,3 were beginning to separate. While all the other peptides were separated further apart, peptides 2,3 were, however, co-migrated again when TEA was increased to 30 mM TEA. In 40 mM TEA, peptides 2,3 resolved again though not at baseline. Unlike in the experiments using 0.5% FA, all nine peptides were fully separated at 50 mM TEA. Again, the occurrence of changes in migration order may explain some of inconsistence observations.

Comparing the electropherograms in Figs. 1 and 2, it can be seen that pH does plays role in the peptide separation characteristics. For instance, in 5 mM TEA, peptides 2,3 were not separated but peptides 7,8 were separated almost at baseline in 0.5 % FA (pH 2.93) (see Fig. 1(ii)). However, it was the other way around in 1.0 % FA (pH 2.80) (see Fig. 2(ii)). Also, as shown in Figs. 1(vii) (pH 3.66) and 2 (vii) (pH 3.50), although the amount of TEA in BGE was constant (50 mM) but the peptide separations were completely different. On the other hand, if pH is the dictating factor, separation characteristics at similar pH but different TEA concentration should give similar observations. For instance, the electropherograms in Figs 1(vi) and Fig 2(vii) were obtained at more or less similar pH (i.e. 3.54 (40 mM TEA) and 3.50 (50 mM TEA), respectively). Although full separations were obtained in both experiments, but closer inspection of the electropherograms revealed that resolution of peptides 1,2, 2,3, and 3,4 were all different.

Effects of ACN

Organic solvents are favourably applied to enhance the separation selectivity of CE by influencing the effective mobility of the separands and the mobility of the EOF. Also, as seen in our previous work, peptide separation was faster due to its lower viscosity compared to in aqueous alone. It can be seen in section 3.1 that, at 30 % ACN, peak efficiency was generally improved by increasing the amount of TEA in the BGE. To check whether further increase of ACN would benefit the peptide separation further, several experiments using 40 %, 50 %, 60 % and 70 % were carried out. Results are shown in Fig 3. BGE containing 0.5 % FA and 10 mM was chosen for this experiment because peak efficiencies and resolution were the best in the earlier experiments.



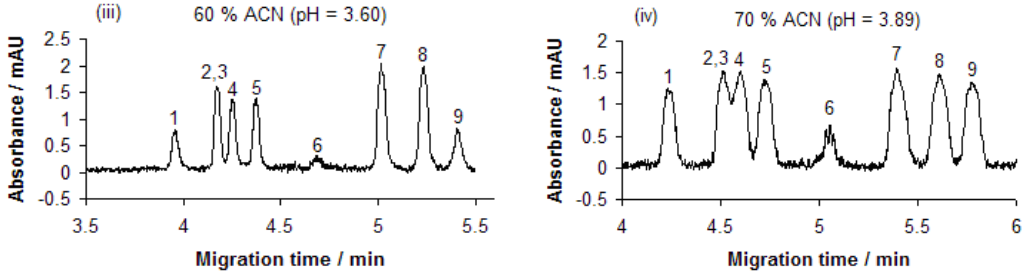


Figure 3: Electropherograms of peptide mixture in 0.5 % FA + 10 mM TEA + different amounts of ACN (40 - 70 %). Voltage: 30 kv (i) – (ii), 25 kv (iii) 20 kv (iv). Other experimental details are similar to Fig. 1.

By increasing ACN from 30 % to 40 % (compare Figs. 1 (iii) & 3 (i)), separation was faster by *ca*. 1 min but resolution of peptides 2,3 decreased. Further increase of ACN to 50 % has caused peptides 2,3 co-migrated, and furthermore the analysis time was not significantly sped up from the previous experiment. Resolution was not getting any better in higher ACN (60 %); peptides 2,3 were still not separated, resolution of peptides 3,4 and peak broadening was becoming more profound (70 %). Further increase of ACN (80 %) would not benefit the separation (data not shown) due to worsen peak broadening and current instability.

The profound effects of incorporating organic solvent in aqueous BGE are alterations in dielectric constant, viscosity and acid-base equilibrium. These factors strongly affect net charges and Stokes' radii of ionic species, thus, modify relative migration of analytes (Yang *et al*., 1999). The ACN-aqueous buffer used here would have lower dielectric constant and viscosity compared to aqueous. The pK_a of the individual acids and bases in the test peptides vary at different composition of the organic modifier in BGE, thus, provide additional selectivity. This was noted in Fig 3 where selectivity is affected as the amount of ACN varies. It is reported (Yang *et al*., 1999) that peak efficiency should be higher in non-aqueous buffer due to lower current, which creates greater difference in ionic strength between sample and the buffer. However, in this study, although the currents were generally lower in 40 – 70 % ACN, but they were not stable. This is probably due to high volatility, which cause bubble formation during the CE separation.

As with TEA, increase of ACN in the BGE will also change its final pH. Looking at the overall separation characteristics in this study, it seems that combination of pH changes, amount of TEA and ACN do influence the selectivity. Comparing Figs. 1(vi), 2 (vii) and 3(ii), although at almost the same pH, the separation characteristics were different. Probably this was partly due to differences in their ionic strengths.

Conclusion

It was shown here that increasing TEA concentration in the formate buffer affecting both peptide peak efficiency and selectivity. By adding as little as 5 mM TEA, the closely related enkephalins were resolved almost at baseline. Addition of 10 mM TEA was sufficient to resolve all nine peptide at baseline in less than 7 min with peak efficiency up to 740 000 m^{-1}. Peak efficiency was even higher, which is 800 000 m^{-1}, with addition of 40 mM TEA, however, resolution of bradykinin F1-5 and Arg^8-vasopressin were reduced by 87 %. Increasing ACN from 30 % to 40 % has fastened the analysis time by *ca.* 1 min. However, more than 40 % ACN can cause peak broadening and instability in the separation current. Changes in pH due to addition of TEA and ACN were also potentially contributed in the separation selectivity.

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