# SEPARATION OF PEPTIDES USING CAPILLARY ELECTROPHORESIS: COMPARISON BETWEEN SIMULATION AND EXPERIMENTAL DETERMINATION

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**ABSTRACT**. *Theoretical and experimental electropherograms of nine peptides in a standard mixture have been matched. The capillary electrophoresis separation of the peptides mixture was carried out in 80 mM phosphate buffer, adjusted to pH 2.3 with 1.0 M lithium hydroxide, in the range of 25 – 30 kV. The capillary was performed in 50 cm × 50 µm i.d. × 365 µm o.d. uncoated fused silica capillary. All experiments were carried out 25°C. The theoretical peptides charge were calculated using the Hendersen-Hasselbach equation at the experimental pH using the individual amino acid $pK_a$ values as report by Rickard et al. (1991). The theoretical electrophoretic mobility of each peptide was then calculated using the multi-variable model described by Janini et al. (2001). The theoretical mobilities were subsequently converted to migration times using the experimental conditions used in this study. The peaks were modelled with Gaussian functions assuming peak area for each peak is proportional to the number of peptide bonds in the peptide and peak widths based on half the maximum theoretical efficiency as described in Kenndler and Schwer (1992). The simulated electropherogram showed a close similarity to the experimental electropherogram. The correlation between the theoretical and experimentally determined mobility of all the nine peptides was good, i.e. $r^2 = 0.9781$. In this study, closest-neighbour algorithm correction on the test peptides theoretical mobilities did not show any improvement of the correlation.*

***KEYWORDS.*** *Capillary electrophoresis, peptides, theoretical electrophoretic mobility.*

## INTRODUCTION

Capillary electrophoresis (CE) offers simplicity and predictable migration patterns as charged molecules are resolved due to their differences in electrophoretic mobility that are proportional to their charge to size ratio. The simple relationship between mobility and molecular properties of charged solutes provides an opportunity for prediction of migration patterns that could facilitate method development and optimization of separation. Complimentarily, peptides are relatively simple structures that are efficiently separated by CE, and their electrophoretic migration characteristics as a function of pH are amenable to prediction by theoretical models (Smith *et al*., 1990; Grossman *et al*., 1988; Grossman *et al*., 1989; Grossman and Soane, 1990; Compton, 1991; Cifuentes and Poppe, 1994; Castagnola *et al*., 1998; Janini *et al*., 1999; Janini *et al*., 2001, Simo *et al*., 2002; Simo and Cifuentes, 2003, Winzor, 2003). The subject has been extensively reviewed (Kasicka, 1999; Adamson and Reynolds, 1997; Messana *et al*., 1997; Cifuentes and Poppe, 1997).

The utilization of CE in peptide mapping in place of high performance chromatography (HPLC) has increased tremendously over the recent years (Van de Goor *et al*., 1997). In peptide

mapping, a protein is fragmented into a pool of smaller peptides by proteolytic enzymes or specific reactions. The peptides mixture is then separated by HPLC or CE resulting in a peptide map, displayed in an electropherogram in the case of CE separation, that serves as a fingerprint for protein identification. Peptide mapping is useful in protein translational studies, and could provide information on protein post-translational modifications and chemical degradations.

In this work, a multi-variable model developed by Janini and co-workers (Janini *et al*., 2001) was employed to calculate theoretical electrophoretic mobility of peptides mixture. The mixture consists of nine standard peptides, with number of amino acids ranges from five to 14, has been used as the model peptides.

## MATERIAL AND METHODS

Peptide standards (P2693), dimethylaminopyridine (DMAP), concentrated orthophosphoric acid (85 % w/v), lithium hydroxide and benzyl alcohol were purchased from Sigma-Aldrich (Poole, UK).) All reagents were of analytical grade.

### Preparations of buffer and test peptides solutions

The 80 mM phosphate buffer was prepared from concentrated phosphoric acid. The final pH was then adjusted to 2.3 with 1.0 M LiOH. The pH meter was a Corning ion analyzer 150 (Halstead, UK), which was calibrated in aqueous buffers (pHs 4 and 9.2) prior to use. The buffer solution was sonicated for 20 min or more, filtered through a 0.2 μm microfilter (Sartorius, Göttingen, Germany) and kept in airtight bottle at room temperature if not used immediately.

A stock solution of nine peptide standards (containing 25.0 μg each peptide) was prepared by adding 500 μL HPLC-grade water into the vial to give 50.0 μg $mL^{-1}$ each peptide. An appropriate volume of the stock solution was pipetted out and diluted in 10 % 80 mM phosphate buffer to give the desired concentration. DMAP was used as an electrophoretic mobility reference where it was added into the standard solution (final concentration was 10.0 mM) before injection. Benzyl alcohol with a concentration of 0.1 % was used as an electroosmotic flow (EOF) marker.

### Apparatus and procedures

The CE experiments were performed in 50 cm x 50 μm i.d. x 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 ultra-violet (UV) diode array detector and a 32 Karat version 5 data station. UV absorbance was obtained over the range 190 to 300 nm at the detection point 10 cm from the capillary outlet end through a window created by removal of about 2 - 3 mm of polyimide coating. The polyimide coating was also removed 1 cm from both ends to minimize adsorption of the positively-charged peptides on the coating (Ensing *et al*., 1999). The voltages applied over the capillary during the CE analyses were in the range 25 – 30 kV, and the capillary thermostatting temperature was set at 25°C. Between runs, the capillary was rinsed with the separation buffer for at least 2 min. The capillary was reconditioned with 0.1

M LiOH, normally after five runs, or more frequently in the few cases where reproducibility of the migration time was poor.

## Simulation of theoretical electropherogram

### *Theoretical electrophoretic mobility*

The theoretical (or predicted) electrophoretic mobilities of peptides were calculated using the multi-variable model as described by Janini *et al*. (2001), without and with the closest-neighbour algorithm correction. According to this model, the electrophoretic mobility, $\mu_{\text{theor}}$, of a peptide is represented by a product of three functions:

$$\mu_{\text{theor}} = n(N)q(Q)w(W) \tag{1}$$

where $n(N)$ is a peptide length function, $q(Q)$ is a peptide charge function and $w(W)$ is a peptide average residue width function. The length function was represented by

$$n(N) = N^{-0.5} \tag{2}$$

where $N$ is the number of amino acid residues in the peptide.

The width function, $w(W)$, obtained from polynomial data fitting

$$w(W) = -6.76\,x\,10^{-6}W^3 + 1.90x10^{-3}W^2 - 21.6x10^{-2}W + 21.86 \tag{3}$$

where $W$ is the average relative molecular mass of the side chain residues. For example, with A, AA etc, with side chain $CH_3$, $W$ = 15; for G, GG etc, with side chain H, $W$ = 1; for AG, the average side chain $M_r = (15 + 1)/2 = 8$.

The peptide charge function $q(Q)$ was obtained from 15 selected peptides (Table 4 in Janini *et al*., 2001) covering the charge range of the whole 102 basis set. The function was expressed as

$$q(Q) = 2.9\,x\,10^{-3}Q^3 - 9.59x10^{-2}Q^2 + 1.45Q + 0.373 \tag{4}$$

where $Q$ is the charge calculated from the p$K_a$ values and the experimental pH (symbol $q$ in the previous papers by Janini *et al*., 2001).

Full descriptions of how each function in equations 1 – 4 was parameterised are given in papers from the Janini group (Janini *et al*., 1999; Metral *et al*., 1999; Janini *et al*., 2001). As mentioned earlier in the introduction, the predictive ability of the multi-variable model is better when incorporated with a computer algorithm that matches an unknown peptide to its closest neighbour in the basis set. Therefore, equation 1 was replaced with 5 as follows:

$$\mu_{\text{theor}}(\text{pep}) = \mu_{\text{ep}}(\text{cn}) \frac{[w(W)n(N)q(Q)]\text{pep}}{[w(W)n(N)q(Q)]\text{cn}} \tag{5}$$

where $\mu_{\text{ep}}(\text{cn})$ is the experimental mobility of the closest neighbour.

***Calculation of peptide charge***

As in the previous studies, the theoretical charge of the basis set peptides used to develop the multi-variable model were calculated using the p$K_a$ values reported by Rickard *et al*. (1991). At low pH (i.e. pH < 3.0), it was assumed that the basic amino acids, i.e. histidine (H), arginine (R) and lysine (K), and the N-terminal are fully-protonated to give positive charge of +1 each. The Hendersen-Hasselbalch equation is used for the calculations of net charge on each peptide. For the calculation of charge contribution from the terminal carboxyl and the acidic amino acids, glutamic acid (E) and aspartic acid (D), p$K_a$ values used were 3.20, 3.50 and 4.50, respectively.

***Peptide basis set***

Experimental data for the peptide basis set presented by Janini and co-workers (Table 1 in Janini *et al*., 2001) were used in this study to calculate theoretical mobilities. The polynomial of equation 5 was recalculated using the new predicted charges for the peptides in Janini's Table 4; this leads to a new set of theoretical mobilities for both the data base and the 'unknown' peptides.

***Modelling of electropherogram***

In order to simulate an electropherogram, the theoretical mobilities of the peptide standards were first calculated using the multi-variable model (using equation 5) and subsequently converted to migration times using the experimental conditions described in this study. The measured EOFs have been used to convert from theoretical electrophoretic mobilities, $S_{ep}$, to theoretical observed mobilities, $S_{obs}$, using the equation $S_{obs} = S_{ep} + S_{eo}$, where $S_{eo}$ is the measured electrophoretic mobility of the EOF marker. The peaks were then modelled with Gaussian functions assuming that peak area for each peak is proportional to the number of peptide bonds, $n - 1$, where $n$ is the number of amino acids in the peptide and peak widths based of half the maximum theoretical efficiency of $N = 20qV$ (Kenndler and Schwer, 1992), where $q$ is charge and $V$ is the applied voltage.

## RESULTS AND DISCUSSION

The buffer used in Janini *et al*. (1999; 2001) was made up using 50 mM phosphoric acid that was adjusted to pH 2.5 with triethylamine. The phosphate buffer used in this work was 80 mM phosphoric acid, adjusted to pH 2.3 with 1.0 M LiOH, which ionic strength was 50.6 mM. In the our preliminary studies (data not shown), it was found that 80 mM phosphate buffer, pH 2.3, with $Li^+$ as co-ion systematically gave the best peak efficiency and mobility in peptides. This was in comparison with 80 mM and 100 mM phosphate, both pH 2.3, with $Na^+$ as co-ion. The benefits here are ascribed to the lower mobility of $Li^+$ than $Na^+$, giving lower current, lower power dissipation, lower Joule heating and also better mobility matching to the peptide. Therefore, 80 mM phosphate was used, pH 2.3, with $Li^+$ as co-ion in this study.

### *Calculated theoretical peptide charge*

The test peptides used for this study were a mixture of hydrophilic and hydrophobic peptides with between five and 14 amino acids, and charge between ~+1 and +3. Their amino acid sequences, relative molecular masses masses, $M_r$, and charges, $Q$, are shown in Table 1.

**Table 1. Calculated charges, *Q*, (using Hendersen-Hasselbalch equation) of the test peptides at pH 2.3 using the $pK_a$ values from Rickard *et al.* (1991).**

| No | Peptide | Amino acid sequence ($M_r$ / Da.) | *Q* |
|---|---|---|---|
| 1 | Bradykinin | RPPGFSPFR (1060.6) | 2.888 |
| 2 | Substance P | RPKPQQFFGLM-$NH_2$ (1347.6) | 3.000 |
| 3 | Bradykinin F1-5 | RPPGF (572.7) | 1.888 |
| 4 | $Arg^8$-vasopressin | CYFQNCPRG-$NH_2$ (1084.2) | 2.000 |
| 5 | Luteinizing Hormone Releasing Hormone (LHRH) | *p-EHWSYGLRPG-$NH_2$ (1182.3) | 2.000 |
| 6 | Bombesin | *p-EQRLGNQWAVGHLM-$NH_2$ (1619.8) | 2.000 |
| 7 | Leucine enkephalin | YGGFL (555.6) | 0.888 |
| 8 | Methionine enkephalin | YGGFM (573.7) | 0.888 |
| 9 | Oxytocin | CYIQNCPLG-$NH_2$ (1007.2) | 1.000 |

Note: **p* means *pyro* i.e. the carboxyl group of the glutamic acid (E) is in a ring or cyclic form, and not as a free carboxyl group.

Interestingly, peptides 1, 2, 3, 7 and 8 are members of the basis set of peptides used by Janini *et al*. (2001) (see Table 1 in their publication) for the development of the multi-variable

model. Based on the information obtained from Sigma-Aldrich (the test peptides' supplier), the C-terminal of peptide 2 (substance P) used in this study was amidated (shown as $-NH_2$ in Table 1), i.e. the terminal carboxyl group was "capped" or "blocked". However, the previous researchers (Janini *et al*., 2001) did not mention whether the substance P they used in their work was amidated at C-terminal. It should be noted that the C-termini of peptides 2, 4, 5, 6 and 9 are amidated with no acidic side-chain groups, and therefore the charges arise from the protonated basic groups (+1 each). At pH 2.3, the only group that was partially ionized was the C-terminal carboxyl of peptides 1, 3, 7 and 8. The p$K_a$ values of the carboxyl were 3.20 for all peptides according to Rickard *et al*. (1991).

***Comparison of theoretical and experimental electropherograms***

The values of the observed (experimental) and predicted (theoretical) electrophoretic mobilities of the test peptides are shown in Table 2, and the simulation of the theoretical electropherogram, along with its counterpart, are shown in Figure 1. Peak identifications were obtained from the information provided by the supplier of the peptide standards, Sigma-Aldrich. The observed mobilities have been corrected using the measured EOF of $2.75 \times 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$ to give electrophoretic mobilities. Inspection of Figure 1 reveals a close resemblance between the simulated and experimental electropherograms, with similar migration order in all the peptides. Each of the simulated peaks has a counterpart in the experimental electropherogram. As shown in Table 2, there is a fairly good agreement between the observed and theoretical mobilities for the faster migrating peptides, but wider divergence for the slow ones, with greatest mismatch for oxytocin (peptide 9). Also, the gap between peptides 3 (bradykinin F1-5) and 4 ($Arg^8$-vasopressin) in the simulation (Figure 1) was a bit different from the experimental. The possible explanation for this is most likely due to inaccurate prediction of the peptides theoretical mobilities. As described in the experimental section, the theoretical mobilities were calculated using the multi-variable model developed by Janini *et al.* (2001) which combined three functions i.e. peptide length, charge and width. Therefore, inaccurate prediction of any of these functions may cause mismatching between the two electropherograms. Of these three functions, an accurate prediction of peptide net charge is particularly difficult, if not impossible, to obtain. This is because peptide charge is, among other factors, dependent upon the pH of the separation buffer due to the ionization of the acidic and basic side chains as well as the carboxyl and amino terminal groups of peptides.

As described earlier, many researchers calculate net charge of peptides or proteins using the Hendersen-Hasselbalch equation based on the ionization constants ($pK_a$s) of the ionizable groups. Ionization constants compiled by Rickard *et al.* (1991) are widely used. As in the previous works (Janini *et al*., 1991; 2001), the $pK_a$s values used to predict net charge in the current study is based on Rickard *et al.* (1991). In the Rickard's compilation, the $pK_a$ value for the terminal carboxyl is 3.2 for any given peptide regardless of its molecular size and shape. A more recent finding (Cifuentes & Poppe, 1997), however, suggests that beside net charge and mass, peptide mobility in CE is influenced by many factors such as peptide structure. For instance, the effects of cysteine bridge between the two cysteine residues in $Arg^8$-vasopressin (peptide 4) and oxytocin (peptide 9) were not taken into account in the Janini model. Also, according to Cifuentes and Poppe (1997), there are a number of uncertainties in the intrinsic properties of peptides.

**Table 2. Observed and theoretical electrophoretic mobilities ($\times 10^{-9}$ $m^2$ $V^{-1}$ $s^{-1}$) of the test peptides in phosphate, pH 2.3.**

| Peptide | Observed | Theoretical | |
|---|---|---|---|
| | | No closest neighbour algorithm | With closest neighbour algorithm |
| 1 | 19.3 | 19.0 | 20.0 |
| 2 | 18.0 | 18.6 | 18.8 |
| 3 | 15.8 | 17.3 | 17.0 |
| 4 | 13.7 | 14.1 | 14.6 |
| 5 | 12.1 | 13.4 | 12.4 |
| 6 | 9.3 | 11.4 | 10.8 |
| 7 | 7.4 | 9.9 | 10.3 |
| 8 | 7.1 | 9.7 | 10.1 |
| 9 | 4.9 | 8.1 | 8.3 |

The formation of the first peptide bond brings about a large variation on the terminal p$K_a$ values. Another complication stems from the mutual electrostatic interaction of charged groups. That is, for any peptide, if the charged groups are not far apart, the p$K_a$ value of given group will be affected by its neighbouring ionized groups. This means that using one $pK_a$ value for terminal –COOH of 3.2 for all peptides (as in Rickard *et al*.) may not give an accurate prediction of peptide net charge. This is conveniently supported by the recent findings by Benavente *et al*. (2006) where they experimentally measured $pK_a$ values of terminal -COOH of bradykinin (peptide 1), Met- and and Leu-enkephalins (peptides 7 and 8) using CE mobilities as function of pH. They found that $pK_a$s for terminal –COOH of bradykinin, Met- and Leu-enkephalins were 2.68, 3.17 and 3.31, respectively. The $pK_a$ values for Leu- and Met-enkephalins are fairly closer to the values predicted by Rickard *et al*. (1991). However, for bradykinin, the measured p$K_a$ is substantially lower than the calculated. It is, therefore, suggested here that inaccurate $pK_a$s values and/or possible effects of intrinsic properties and electrostatic interactions of the peptides may have contributed to the slight differences between the simulated and experimental electropherograms.

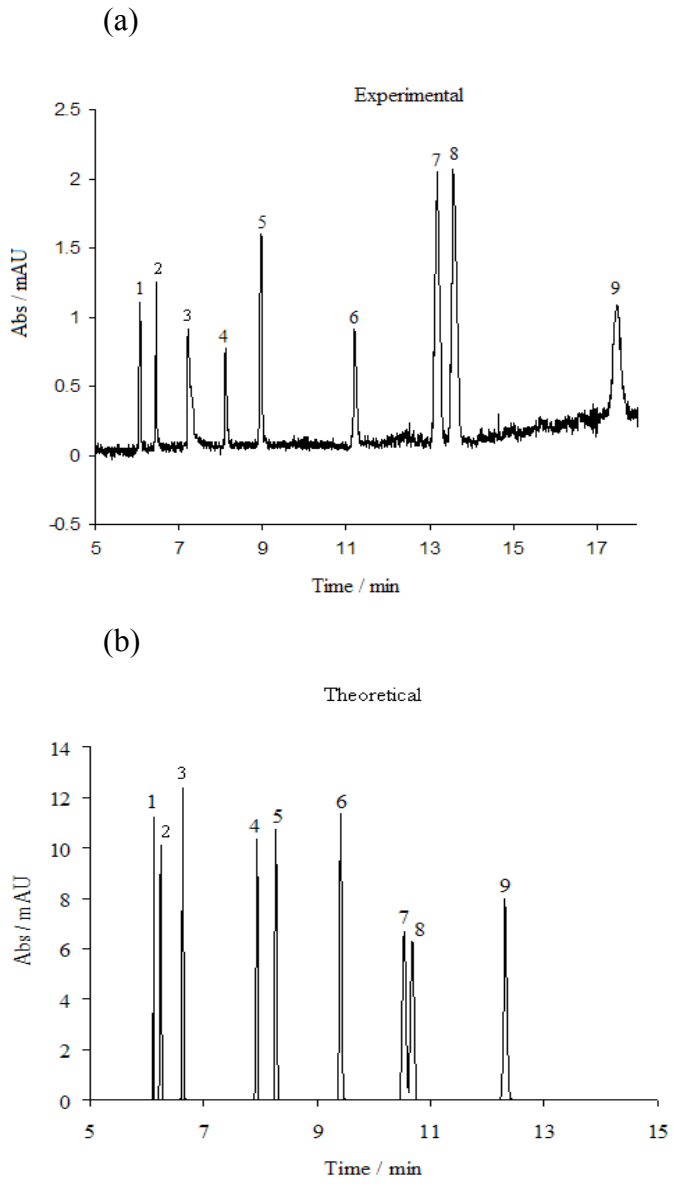
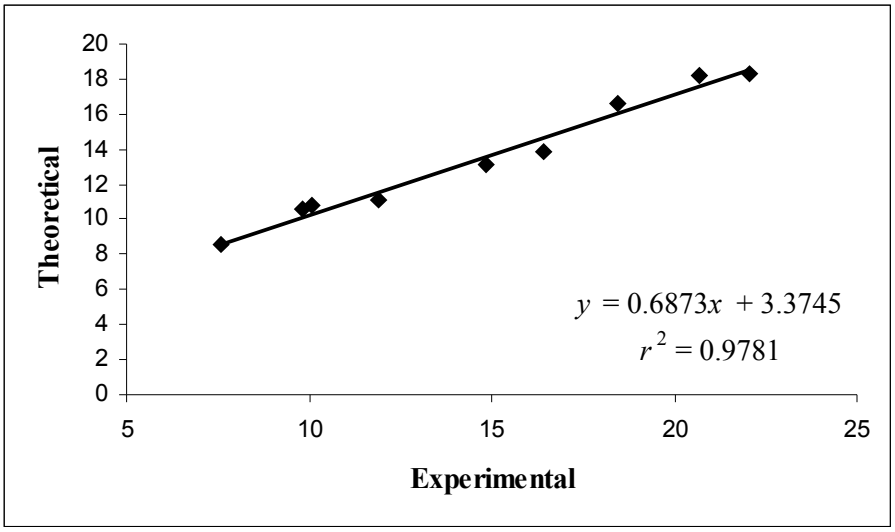



**Figure 1. Experimental (a) and theoretical (b) electropherograms of test peptides in phosphate BGE (80 mM $H_3PO_4$ adjusted to pH 2.3 with 1.0 M LiOH). Capillary, 50 cm x 50 µm i.d., 40 cm to detector; applied voltage / current, 25 kV / 56.3 µA; temp., 25°C. Sample conc., 5.0 µg $mL^{-1}$ each peptide in 10 % BGE; inj., 0.5 psi, 5 s; UV detection at 190 nm; data rate, 16 Hz. Peak 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.**

Another interesting observation here is that, on these particular set of peptides, under the experimental conditions described here, use of the closest-neighbour correction did not provide any significant benefits. The matching correlations of the theoretical and experimental mobilities of the test peptides are shown graphically in Figure 2.

(a)




(b)

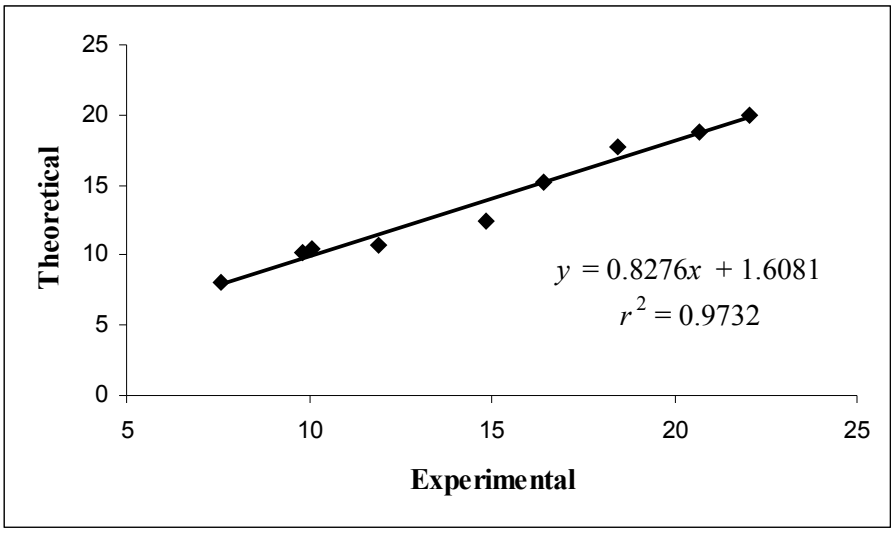



**Figure 2. Correlation of peptide theoretical and experimental electrophoretic mobilities ($\times 10^{-9}$ $m^2$ $s^{-1}$ $V^{-1}$) in phosphate buffer, pH 2.3, (a) without and (b) with closest-neighbour algorithm correction**.

Here the experimental values are given including EOF: subtraction of EOF mobility would merely shift experimental values along the *x* axis. The matching correlations of the theoretical and experimental mobilities of the test was $r^2$ = 0.9781 without using the closest-neighbour algorithm, and $r^2$ = 0.9731, which were not significantly different. Therefore, simulated electropherogram with correction using the closest-neighbour algorithm was not shown here.

## CONCLUSIONS

The theoretical electropherogram of the nine test peptides was simulated. The theoretical electrophoretic mobility was calculated using the multi-variable model developed by Janini and co-workers (Janini *et al*., 2001) at pH 2.3 in phosphate buffer. The simulated theoretical electropherogram shows a fairly close matching to the experimental electropherogram, with slight differences in the peaks resolution. It is suggested that using more accurate $pK_a$s values may improve matching between simulation and experimental electropherograms. It has been shown in this work on the peptide standards that the closest-neighbour algorithm did not improve the correlation. Despite the slight differences in the predicted mobilities, this simple technique is applicable to predict migration order of peptides. The use of mobility prediction model, such as the Janini's multi-variable model used in this work, is indeed a useful tool to optimize CE separation of peptides. In fact, it has been employed in our extensive studies for the simulation of peptide maps of real samples such as protein digests. This is especially useful for proteomics studies.


## ACKNOWLEDGEMENTS

The author would like to thank Dr Ed Bergström (Analytical Science Group, Department of Chemistry, the University of York, UK) for his assistance in the electropherogram simulations.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