Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1038 (2004) 77–84
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

# Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography

David V. McCalley*

*Faculty of Applied Sciences, Centre for Research in Biomedicine, Frenchay Campus, University of the West of England, Coldharbour Lane, Bristol BS16 1QY, UK*

Received 21 November 2003; received in revised form 11 March 2004; accepted 15 March 2004

**Abstract**

The effect of changing the buffer at constant low pH in the mobile phase is investigated with respect to the separation of a mixture of basic peptides. Considerably worse peak shapes, leading to poorer resolution of complex peptide mixtures, were obtained when using formic acid favoured in LC–MS applications compared with non volatile phosphate buffers or with trifluoroacetic acid (TFA). Poorer peak shapes were largely attributable to reduced column capacity for the peptides when using mobile phases of low ionic strength, due to the increased mutual repulsion of ions held on the hydrophobic column surface which is facilitated in these buffers. However, ion-pairing between the peptides and additives such as TFA or even phosphate may also lessen mutual repulsion effects, leading to greater column capacity. Overloading effects could be observed when sample masses around only 0.1 μg were injected on to standard size analytical columns in formic acid containing mobile phases; sample masses around only 1.5 μg may cause loss of half the system peak capacity in such mobile phases. Results were broadly comparable (after scaling sample size according to column diameter) on columns of both conventional (4.6 mm i.d.) and capillary (0.075 mm i.d.) dimensions. Ammonium formate may be a useful alternative buffer for some applications due to its higher ionic strength.
© 2004 Elsevier B.V. All rights reserved.

*Keywords:* Peak shape; Mobile phase composition; Buffer composition; Peptides

## 1. Introduction

Proteomics involves the global analysis of protein expression and function. Proteomics should allow the discovery of new biomarkers and therapeutic targets for the diagnosis and treatment of diseases of humans, and generally increase understanding of the mechanism of biological processes [1]. The analysis of protein mixtures poses a complex problem, and may involve sequential multidimensional techniques including 2D gel electrophoresis, HPLC and MS or $MS^n$. RP-LC at low pH [2] in conjunction with electrospray ionisation (ESI) for analysis of peptide digests often form an important stage in the analytical scheme. Improvements in separation selectivity and column efficiency are major objectives for separation of these complex mixtures. For example, Huber and co-workers have investigated possible chromatographic advantages of the use of organic polymer monolithic stationary phases in the RP-HPLC stage [3]. The choice of mobile phase buffer or additive is also important. While some HPLC–MS interfaces are more tolerant of non-volatile buffers like phosphate, such compounds give ion source contamination and loss of performance. Trifluoroacetic acid (TFA) is a volatile additive that gives good chromatographic results, but may cause signal suppression in ESI–MS systems due to ion pair and surface tension effects in some cases necessitating post-column addition of counteracting reagents [4]. However, there is currently much debate about the real effects of TFA on the performance of mass spectrometers. A complicating factor is that these effects may be instrument-dependent. Formic acid is considered to give reasonable chromatographic and MS performance, with low concentrations giving rather small MS signal suppression [5,6]. Issaq et al. [7] reported minor improvements in peak shape when using TFA compared with formic acid. Huber and Premstaller [8] reported peak widths for proteins at half height 69–104% larger using 0.5% formic acid compared with 0.1% TFA, and further increases in peak width of 25–51% when using 0.1% formic acid instead

* Fax: +44-117-3442904.
*E-mail address:* david.mccalley@uwe.ac.uk (D.V. McCalley).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.03.038

of 0.5% formic acid. Nevertheless, formic acid was recommended due to less signal suppression in electrospray MS. However, in a later paper, the same group concluded that 0.2–5% formic or acetic acid gave poor resolution of proteins in comparison with 0.1% TFA [9]. Signal suppression could be reduced by use of 0.05% TFA albeit with a small decrease in chromatographic performance. The use of TFA was therefore recommended. However, no particular reasons were given for the improved chromatographic performance shown by TFA other than the general statement that silanol activity is reduced in the presence of this additive. It is well known that TFA is an ion pair agent, and this factor has contributed to its successful use in the analysis of peptides.

Stationary phase overloading can be an important contributor to poor peak shape in HPLC. Overloading is much more problematic with ionogenic samples than with neutral molecules. Snyder and co-workers [10–12] showed that the column saturation capacity (the maximum sample mass that the column can hold) of a RP column at low pH was about 60 times less for the basic peptide Angiotensin II compared with the neutral molecule benzyl alcohol. The authors attributed this finding *either* to the increased likelihood of overloading the small number of silanols existing at low pH *or* a mutual repulsion effect between sorbed ions of the same charge. Our recent finding that purely polymeric columns (no silanol groups) experience similar overloading behaviour to silica-based columns lends much weight to the latter hypothesis, at least on modern RP columns which are likely to have very few, or even no ionised silanols at low pH [13].

In recent publications we have noted unusual selectivity and overloading effects in formic acid compared with phosphate buffers and TFA for a variety of (small molecule) basic drugs [14,15]. For example, we noted that reduced column efficiency could be obtained when sample masses greater than only 50 ng were introduced on to RP columns of conventional dimensions (25 cm × 0.46 cm i.d.) when using 0.02 M formic acid buffers. This sample mass is at least 10 times less than that generally needed to show overloading effects with protonated bases when using phosphate buffers of the same molar concentration. While peptide analysis may often involve the chromatography of solutions of low concentrations, the poor peak shapes of more abundant peptides may cause overlap with other peptides in the mixture.

The aim of the present study was to investigate the effect of different mobile phase buffers on the chromatography of some basic peptides. For the study, we chose a commercially available test mixture (Alberta basic peptide mix) which is well characterised and therefore we believed more suitable for such an investigation.

## 2. Experimental

An 1100 binary high pressure mixing gradient HPLC system (Agilent, Waldbronn, Germany) with Chemstation, UV detector (1 μl flow cell), and Rheodyne 7725 valve (5 μl injections) were used in all experiments with the standard bore column. Connections were made with minimum lengths of 0.01 cm i.d. tubing to minimise extra-column volume. Temperature was maintained at 30 °C by immersing the column and injector in a thermostatted water bath. A 3 m × 0.5 mm i.d. length of stainless steel tubing connected between the pump and injector and also immersed in the bath was used to preheat the mobile phase; flow was 1.0 $cm^3$ $min^{-1}$. Gradient retention times were not corrected for the small delay volume this procedure produces. The column was Discovery C18, 5 μm particle size, pore diameter 19 nm, 25 cm × 0.46 cm i.d. (Supelco, Bellafonte, USA). Peak widths at half height were determined using the Chemstation. The asymmetry factor ($A_s$) was calculated at 10% of the peak height from the ratio of the widths of the rear and front sides of the peak. Column void volume was measured by injection of uracil. Capillary LC was performed on an LC Packings/Dionex Nano-HPLC (Amsterdam, Netherlands) equipped with a SWITCHOS concentrator system and FAMOS autosampler. The column was PepMap 15 cm × 0.075 mm i.d. packed with 3 μm silica ODS, 10 nm pore diameter; the flow rate was 0.20 μl $min^{-1}$.

Phosphate buffers were prepared by weighing out the appropriate quantity of $KH_2PO_4$ and adjusting the pH with concentrated phosphoric acid, prior to the addition of organic solvent. The Alberta peptide mixture (RPS-10020) and individual peptide standards were obtained from the Alberta Peptide institute (Edmonton, Canada); the approximate composition of the mixture is indicated in Table 1 [16]. Buffer additives were incorporated in both "A" and "B" solvents in the gradient to maintain a constant concentration throughout the gradient. Ionic strength calculations were performed using the Phoebus program (Analis, Orleans, France) using correction of activity coefficients according to the Debye/Hückel equation.

Table 1
Approximate composition of Alberta peptide mixture

| | Concentration (mg $l^{-1}$) | Mass[a] (μg) |
|---|---|---|
| P1 ac-GGGLGGAGGLK-amide | 500 | 2.5 |
| P2 ac-KYGLGGAGGLK-amide | 270 | 1.4 |
| P3 ac-GGALKALKGLK-amide | 360 | 1.8 |
| P4 ac-KYALKALKGLK-amide | 260 | 1.3 |

ac indicates that the N-terminal group in each peptide is acetylated. A, alanine; G, glycine; K, lysine; Y, tyrosine; L, leucine. Quantitative composition is not given by the supplier since the mixture is supplied as a qualitative test; the composition is likely to vary somewhat from standard to standard.

[a] For 5 μl injection volume.

## 3. Results and discussion

The Alberta mixture (see Table 1) consists of four synthetic basic peptides formulated as a *qualitative* test of the silanophilic acitivity of RP columns. As such, the *quantity* of

each peptide is not specified by the supplier (and thus could vary somewhat from batch to batch), although we determined the approximate composition of the particular mixture used in our studies by injection of standards of the individual peptides. The quantity of the peptides in a 5 μl injection (approximately 1–2 μg) would not at all be considered likely to produce overload on standard size columns if these were neutral compounds [10] and the mixture is intended for use without further dilution. P1-P4 contain 1–4 residues respectively of the basic amino acid lysine. Thus, over the pH range used for the majority of RP separations (pH 2–7) the peptides P1-P4 will have a charge of +1 to +4 respectively [17]. All the carboxy-terminals of the peptides in this mixture have been amidated, thus eliminating a possible contributory negative charge. However, at the low pH values generally used in this study (pH 2.7 or less) the ionisation of weakly acidic free carboxyl groups would be expected to be suppressed. Thus, similar ionisation properties would be expected whether the peptides had free or amidated terminal carboxyl groups (a possible exception is the case of the use of ammonium formate buffer pH 3.3, see below). The more highly charged peptides should show increasing detrimental interactions, especially on older RP columns, due to ionic interactions with ionised acidic silanol groups [17,18]. However, more modern phases, made from pure silica substrates with very low concentrations of metals have low silanol activity at low pH (pH 3 or less) [13]. On such phases, we demonstrated that the tailing and poor peak shape found when using low ionic strength mobile phases such as formic acid may be attributed instead to overloading [15], at least with the pharmaceuticals and relatively low MW bases we used previously. The Alberta mixture was designed such that the hydrophobicity of the peptides spans the range normally encountered in protein hydrolysates. Peptide hydrophobicity is stated to increase from P1 to P4 [17]. However, in the present study using Discovery C18, a modern pure silica RP column, P4 often eluted before P3 (see below). It is possible with older phases that the retention of P4 was increased due to increased ionic retention caused by ionised silanol groups which are more prevalent on such phases, even at relatively low pH. This ionic retention is likely to be much smaller, or even negligible on Discovery C18, at least at a pH value of 2.7 or lower pH.

The retention factor $k$ in an isocratic separation can be related to the volume fraction of organic solvent in the mobile phase $\phi$ using the empirical equation:

$$\log k = \log k_w - S\phi \quad (1)$$

where $k_w$ is the retention of the solute in pure water and $S$ is a factor depending on the solute and the modifier [2]. For peptides, $S$ values tend to be much larger than for smaller molecules. As a result, small changes in $\phi$ give rise to large changes in retention. For this reason, gradient elution is generally used for the separation of peptides. A problem is that conventional methods of measuring column efficiency such as the equation for the number of theoretical plates $N$:

$$N = 16\left[\frac{t_r}{w_b}\right]^2 \quad (2)$$

are not valid in gradient elution. It is possible instead to calculate the *peak capacity* $P$ from [20]:

$$P = 1 + \frac{t_g}{w_b} \quad (3)$$

where $t_g$ is the gradient time. For Gaussian peaks this relationship becomes:

$$P = 1 + \frac{t_g}{1.699 w_{0.5}} \quad (4)$$

where $w_{0.5}$ is the peak width at half height. For tailing or overloaded peaks Eq. (4) may give an optimistic value of the peak capacity, just as measurement of the peak width at half height exaggerates the true number of theoretical plates in isocratic separations. However, we have used Eq. (4) due to the difficulty of reproducible measurement of the peak width at baseline, as is also the case in isocratic separations. If no detrimental interactions which affect an individual substance occur, then all peaks in a gradient should have approximately the same width. This holds if all peaks have the same $k^*$, which provided all other conditions are the same, requires each solute to have a similar value of S. $k^*$ is the instantaneous value of the retention factor when the solute has migrated half-way through the column or can be thought of as the average retention factor [2]; see Eq. (5) below. Also, for peaks to have the same width the first peaks must be sufficiently retained such that no movement of any solute occurs down the column at the start of the gradient. This means that in an isocratic analysis using the initial gradient mobile phase, all peaks should have infinite retention.

Fig. 1 shows the analysis of the peptide mixture at normal working strength ("undiluted") and at 10× dilution

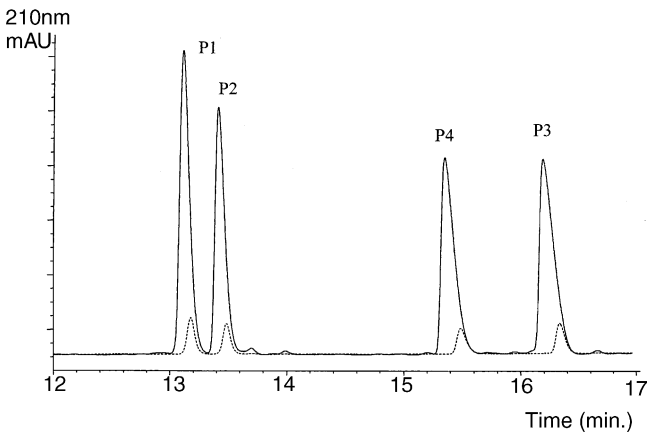


Fig. 1. Analysis of Alberta peptide mix at normal working strength (continuous line) and diluted 10 times in the mobile phase (dotted line). Solvent A: 0.02 M formic acid in water (pH 2.7). Solvent B: 0.02 M formic acid in acetonitrile. Gradient 5% B to 42.5% B in 30 min. (1.25% acetonitrile per minute). Detection: 210 nm, flow rate: 1 ml $min^{-1}$.

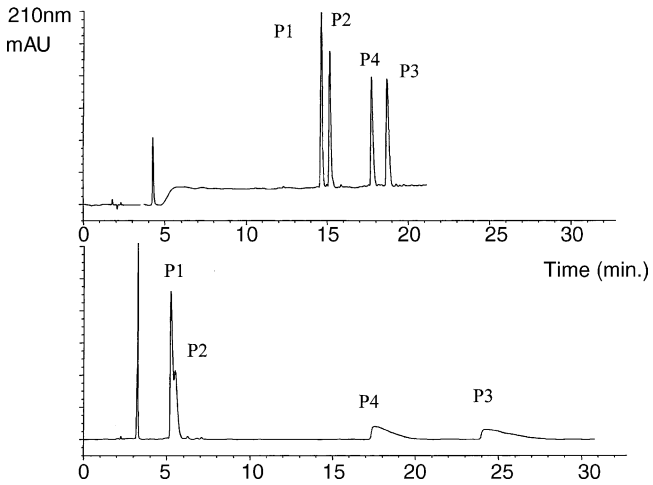


Fig. 2. Comparison of analysis of undiluted peptide mixture using gradient elution (upper plot-same mobile phase gradient as Fig. 1) and (lower plot) isocratic analysis using 14.5% acetonitrile containing overall 0.02 M formic acid. Other conditions as Fig. 1.

using an acetonitrile gradient with formic acid as the buffer, using the Discovery C18 column. This phase has been shown previously [19] to have low silanol activity. Furthermore, it also has a rather larger pore size (approximately 20 nm) than many standard RP columns. No evidence of size-exclusion effects was shown by any of the peptides in the present study. All four peptides (but especially P4 and P3) show clear evidence of overloading in the undiluted mixture. Peaks are broadened in the undiluted mixture with shapes approaching right-angle triangles, characteristic of overloading, rather than the exponential tailing which is characteristic of interaction with ionised silanols, as shown by Snyder and co-workers [10–12]. Peak maxima are shifted to lower retention in the undiluted mixture, another characteristic of overloading. Fig. 2 compares analysis of the undiluted test mixture using gradient and isocratic conditions using the same time scale. Overloading appears to be worse using isocratic conditions. The average retention factor $k^*$ in gradient elution is the analogue of the retention factor $k$ used in isocratic analysis, given by [2]:

$$k^* = \frac{87\, t_g F}{\Delta\%B V_m S} \quad (5)$$

where $F$ is the flow rate, $\Delta\%B$ is the gradient range expressed as the change in volume fraction of $B$, $V_m$ is the column volume and $S$ is as described above; the calculation assumes that the isocratic retention factor for the solute using the solvent composition at the beginning of the gradient run (as is the case in all our experiments) is very large. Fig. 3 shows a plot of $\log k$ against volume fraction of acetonitrile for isocratic analysis of the four peptides over the range of acetonitrile content of 14.5–16.8% $B$ where both acetonitrile and the aqueous buffer component contained 0.02 M formic acid. Values of $S$ determined according to Eq. (1), and $k^*$ according to Eq. (5) are shown in Table 2. Due to the different

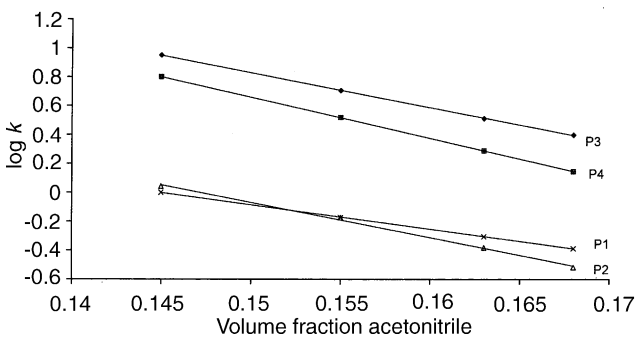


Fig. 3. Variation of $\log k$ as a function of volume fraction of acetonitrile in the mobile phase using isocratic analysis over the range 14.5–16.8% acetonitrile (v/v). Formic acid concentration maintained at 0.02 M overall throughout. Other conditions as Fig. 1.

values of $S$, a change in elution pattern occurs for peptides P1 and P2 over the range studied; at higher acetonitrile concentration, P1 elutes after P2, but at lower concentration, P1 elutes before P2, as is also the case in the gradient run (see Fig. 1). As expected, the values of $S$ are much higher for these peptides than is usual for smaller molecules, where the value of $S$ is typically ~4 [2]. In Fig. 2, the worst peak shapes in the isocratic run are clearly for P3 and P4. The values of $k$ for P3 and P4 (8.9 and 5.1, respectively) are much higher than those for P1 and P2 in this separation. Since $k$ is a measure of the amount of solute in the stationary phase divided by the amount in the mobile phase at any instant, overloading is expected to be greater for P3 and P4 in the isocratic separation. An additional factor could be that the higher charge on P3 and P4 would be expected to increase mutual repulsion and thus overloading of these two peptides. Furthermore, peaks especially for P3 and P4 are clearly worse in the isocratic separation compared with the gradient separation. It is possible that the higher values of $k$ in the isocratic run compared with $k^*$ in the gradient run could have some influence on overload. Such a result would also imply that overloading might increase when using shallower gradients, which would lead to larger values of $k^*$ (see Eq. (5)). However, at the beginning of the gradient, all the sample is adsorbed on the stationary phase, a factor which tends to complicate the hypothesis that overload could depend on $k^*$. A further important factor to consider is that tailing is generally reduced in gradient elution due to compression of the band, because the rear side of the peak is eluted using a stronger mobile phase than the front side of the peak [21]. Clearly,

Table 2
$S$ (calculated from Fig. 3) and $k^*$ values (from Eq. (5)) for individual peptides

| Peptide | $S$ | $k^*$ |
|---|---|---|
| P1 | 17 | 1.5 |
| P2 | 24 | 1.1 |
| P3 | 24 | 1.1 |
| P4 | 28 | 0.92 |

the comparison of overloading in isocratic versus gradient elution is a rather complex issue that warrants further study.

Table 3 shows the retention for the Alberta peptides prepared normally ("undiluted") and diluted by 10 times, using identical gradients of 5% acetonitrile increasing at 1.25% $min^{-1}$ gradient time 30 min.) but with different buffers/additives. The gradient retention times of the peptides are rather similar in each of the six different buffers (note that these buffers cover a pH range 2.3–3.3, although lysine is expected to be fully protonated over this pH range). However, retention is somewhat greater using TFA which can be attributed to its well-known ion pair effects (or alternatively, the adsorption of TFA on the RP surface and consequent ion interaction effects). Table 3 also shows peak shape data. Highly symmetric peaks for the diluted peptide mix were obtained using 0.02 M phosphate buffer with asymmetry factors of only 1.12–1.20 and narrow peak widths. Further dilution of the sample mixture beyond 10 times did not produce improved peak shapes with 0.02 M phosphate buffer. The peak capacity for all peptides P1–P4 in the diluted sample using 0.02 M phosphate buffer was very similar (approximately 250), as would be expected for solutes with similar *S* values (see above). We used the highest value of the peak capacity in the diluted mix (for P2, peak capacity = 252 for the 30 min. separation) to calculate a fractional peak capacity for all other measurements. This value is 0.96–1.0 for all the peptides in the diluted mix with phosphate buffer. Even with the undiluted sample, the fractional peak capacity drops only to a minimum value of 0.92 for peptides P3 and P4 in the phosphate buffer, demonstrating that overloading is hardly problematic for any of the peptides using this buffer. In contrast, the fractional efficiencies when using 0.02 M formate buffer of identical pH 2.7 show very different results. Although P1 and P2 in the diluted mixture give similar peak capacities compared with phosphate buffer, P3 and P4 give significant drops in the fractional peak capacity (0.89 and 0.82, respectively) when using formate. In addition, peak asymmetry factors for P4 and P3 approach a value of 2, compared with results close to 1.0 for the same compounds in phosphate buffer. For the undiluted sample, much larger decreases in performance are noted, especially for P3 and P4 which give fractional peak capacities of barely half those in phosphate accompanied by seriously asymmetric peaks (asymmetry factors approaching 4.0). These performance decreases may be greater than suggested by these figures, due to the measurement of peak width at half height (see above). Although "asymmetry factor" in gradient elution has probably even less theoretical significance than it has in isocratic chromatography [22], it still gives a simple practical indication of relative peak shapes when comparing peaks of the same gradient retention using the same gradient time, as in the case of our measurements. Clearly, overloading is a much more serious problem using formate rather than phosphate buffer.

The concentrations of peptides P1, P2, P3 and P4 in the mixture are of the same order (Table 1) and their $k^*$ values are also similar. There is no reason therefore to expect very large differences in overloading behaviour for the individual peptides in gradient elution with a given mobile phase, based on their retention characteristics. However, Table 3 clearly shows that the loss of efficiency when using the undiluted solution for P3 and P4 with formic acid (and also with the other mobile phases) is much greater than for P1 and P2. It seems a possibility (as mentioned for isocratic elution above) that lower column capacity for P3 and P4 is due to the increased charge on these peptides. P3 and P4 have three and four basic lysine residues respectively compared with P1 and P2 which have only one and two basic residues. Mutual repulsion might be expected to increase between more highly charged species leading to lower column capacities. However, it is possible that this relationship between multiple charge and overloading may be coincidental, and needs further investigation with other different related groups of peptides. Note that the concentration of P3 in the mixture is higher than P4 which may explain the slightly higher tendency to overload of P3 compared with P4, despite its smaller charge. In general, these results are further evidence for our proposal that mutual repulsion of similarly charged species, rather than silanol overload is the major factor which needs to be considered for explaining loss of performance with sample load on highly inert silica based RP columns operated at low pH [13,15]. It is not at all widely appreciated that only 0.1 μg or less (less than ∼100 pmol) of a basic peptide (e.g. P4 in the dilute peptide mix) may show significant overloading effects and subsequent performance loss with formic acid, even with a large diameter (0.46 cm i.d.) analytical column. Even lower sample masses would be expected to show the same degree of overloading on somewhat smaller diameter (e.g. 0.3 cm i.d.) analytical columns.

The decrease in column performance which occurs especially at higher sample loads when using formic acid compared with phosphate can be attributed at least partially to differences in buffer ionic strength, although other factors such as ion-pairing may be involved [15,23]. The 0.02 M formic acid in water has ionic strength approximately 1.9 mM which is less than one tenth that of 0.02 M aqueous phosphate buffer (approximately 22 mM when made up as described above). The low ionic strength of the formic acid is relatively ineffective in reducing mutual repulsion effects between ionised peptides held on the surface of the stationary phase. Low concentrations of buffer additives (even for formic acid) are recommended to give best mass spectrometer sensitivity [5,6]. Reducing the formic acid concentration to 0.004 M (Table 3) gave worse performance still with fractional peak capacity less than half for S3 in the undiluted mix. The undiluted sample again gave right-angled triangle type peaks, indicative of overloading. However, the higher pH of this mobile phase (pH 3.1) leads to greater likelihood of silanol ionisation and detrimental ion-exchange effects. Indeed P4 (the most sensitive peptide to ionic interactions) gave evidence of exponential tailing especially in the diluted sample (results not shown); consequently it was not

Table 3
Comparison of peak shapes using different mobile phases

| Peptide | Strength | $t(r)$ (min) | $A_s$ | $W(0.5)$ (min) | Pk capacity | Fractional Pk capacity[a] |
|---|---|---|---|---|---|---|
| 0.02 M formic acid pH 2.7, ionic strength = 1.9 mM | | | | | | |
| P1 | Normal | 13.11 | 2.36 | 0.0910 | 195 | 0.77 |
| | Dil. 10× | 13.18 | 1.32 | 0.0710 | 250 | 0.99 |
| P2 | Normal | 13.41 | 2.56 | 0.0898 | 198 | 0.78 |
| | Dil. 10× | 13.48 | 1.33 | 0.0679 | 261 | 1.04 |
| P4 | Normal | 15.35 | 3.46 | 0.120 | 148 | 0.59 |
| | Dil. 10× | 15.48 | 1.93 | 0.0861 | 206 | 0.82 |
| P3 | Normal | 16.18 | 3.89 | 0.136 | 131 | 0.52 |
| | Dil. 10× | 16.33 | 1.74 | 0.0795 | 223 | 0.89 |
| 0.004 M formic acid pH 3.1, ionic strength = 0.78 mM | | | | | | |
| P1 | Normal | 13.44 | 3.32 | 0.109 | 163 | 0.65 |
| | Dil. 10× | 13.55 | 1.56 | 0.0734 | 242 | 0.96 |
| P2 | Normal | 13.78 | 4.37 | 0.114 | 156 | 0.62 |
| | Dil. 10× | 13.92 | 2.04 | 0.0837 | 212 | 0.84 |
| P4 | Normal | 15.49 | 4.50 | 0.125 | 142 | 0.56 |
| | Dil. 10× | 15.85 | – | – | – | |
| P3 | Normal | 16.50 | 4.59 | 0.159 | 112 | 0.44 |
| | Dil. 10× | 16.73 | 3.70 | 0.117 | 152 | 0.60 |
| Ammonium formate (0.02 M formic acid + 7 mM ammonia) pH 3.3, ionic strength = 7.4 mM | | | | | | |
| P1 | Normal | 13.80 | 1.54 | 0.0801 | 221 | 0.88 |
| | Dil. 10× | 13.80 | 1.23 | 0.0746 | 238 | 0.94 |
| P2 | Normal | 14.80 | 1.58 | 0.0783 | 227 | 0.90 |
| | Dil. 10× | 14.80 | 1.21 | 0.0704 | 252 | 1.00 |
| P4 | Normal | 17.80 | 1.69 | 0.0825 | 215 | 0.85 |
| | Dil. 10× | 17.90 | 1.49 | 0.0758 | 234 | 0.93 |
| P3 | Normal | 18.30 | 2.00 | 0.0922 | 193 | 0.76 |
| | Dil. 10× | 18.50 | 1.25 | 0.0746 | 238 | 0.94 |
| 0.02 M phosphate pH 2.7, ionic strength = 22 mM | | | | | | |
| P1 | Normal | 13.62 | 1.32 | 0.0746 | 238 | 0.94 |
| | Dil. 10× | 13.66 | 1.12 | 0.0728 | 244 | 0.97 |
| P2 | Normal | 14.69 | 1.34 | 0.0728 | 244 | 0.97 |
| | Dil. 10× | 14.75 | 1.15 | 0.0704 | 252 | 1.00 |
| P4 | Normal | 18.30 | 1.35 | 0.0764 | 232 | 0.92 |
| | Dil. 10× | 18.30 | 1.20 | 0.0734 | 242 | 0.96 |
| P3 | Normal | 18.41 | 1.44 | 0.0764 | 232 | 0.92 |
| | Dil. 10× | 18.45 | 1.12 | 0.0716 | 248 | 0.98 |
| 0.02 M formic acid + 0.02 M KCI pH 2.7, ionic strength = 22 mM | | | | | | |
| P1 | Normal | 13.95 | 1.29 | 0.0758 | 234 | 0.93 |
| | Dil. 10× | 13.95 | 1.18 | 0.0728 | 244 | 0.97 |
| P2 | Normal | 15.23 | 1.31 | 0.0740 | 240 | 0.95 |
| | Dil. 10× | 15.24 | 1.19 | 0.0740 | 240 | 0.95 |
| P4 | Normal | 19.02 | 1.38 | 0.0781 | 227 | 0.90 |
| | Dil. 10× | 19.04 | 1.10 | 0.0758 | 234 | 0.93 |
| P3 | Normal | 19.10 | 1.40 | 0.0901 | 197 | 0.78 |
| | Dil. 10× | 19.12 | 1.07 | 0.0734 | 242 | 0.96 |
| 0.0079 M (0.9 g/l) TFA pH: 2.3, ionic strength = 7.8 mM | | | | | | |
| P1 | Normal | 15.13 | 1.33 | 0.0770 | 230 | 0.91 |
| | Dil. 10× | 15.15 | 1.15 | 0.0755 | 235 | 0.93 |
| P2 | Normal | 16.85 | 1.37 | 0.0758 | 234 | 0.93 |
| | Dil. 10× | 16.88 | 1.14 | 0.0734 | 242 | 0.96 |
| P4 | Normal | 21.27 | 1.35 | 0.0761 | 233 | 0.92 |
| | Dil. 10× | 21.31 | 1.13 | 0.0746 | 238 | 0.94 |
| P3 | Normal | 20.95 | 1.64 | 0.0822 | 216 | 0.86 |
| | Dil. 10× | 21.00 | 1.22 | 0.0732 | 242 | 0.96 |

[a] Peak capacity expressed as a fraction of peak capacity for P2 in dilute mix with phosphate buffer, calculated for $t_g = 30$ min. Other conditions and gradient as Fig. 1.

possible to record usual peak shape data for P4 in this sample (Table 3). For P4 in the undiluted sample, it is possible that silanol interactions play a part in determining peak shape at the higher pH in this buffer. The saturation and masking of the silanol sites by part of the sample may explain in this unique case, the better overall peak shape with higher sample load.

To further investigate whether mobile phase ionic strength effects and overloading were usually responsible for poor peak shape, we repeated the formic acid gradient but with addition of 0.02 M KCl to both solvent channels. The total ionic strength of this mobile phase was now similar to that in the phosphate mobile phase. Peak shapes were indeed (Table 3) now comparable to those with phosphate buffers, as indicated by the similar fractional peak capacity and low asymmetry factors for the peaks. Significant overloading was only shown for P3 in the undiluted solution. It is possible however that ion-pair effects, even with anions such as chloride may occur, resulting in a diminution of charge repulsion effects and thus increased column capacity [23].

Addition of inorganic salts to improve peak shape is hardly of practical use in HPLC–MS applications where volatile additives are highly desirable. However, ionic strength can be increased while maintaining buffer volatility by addition of ammonia to formic acid. Addition of ammonia (to yield a 7 mM solution) to 0.02 M formic acid gives a solution of pH 3.3 and ionic strength 7.4 mM. This mobile phase gave good peak shapes for the diluted sample and much improved peak shape for the undiluted mixture (fractional peak capacity of P3 and P4 0.76 and 0.85 compared with 0.52 and 0.59 for 0.02 M formic acid) indicating that overload problems were considerably less in this mobile phase. The critical peak P4 showed relatively little evidence of exponential tailing (silanol interaction), giving an asymmetry factor of 1.49, despite a slightly higher pH than for 0.004 M formic acid. This result may be due to the beneficial deactivating effect of the ammonium ion. Peptides with a free carboxyl group terminating the chain could show significant ionisation of this group at a pH of 3.3. This ionisation might lead to slightly different behaviour to that shown for the model peptides used here, in which the carboxyl group is amidated (see above). The effects of such ionisation warrant further study. However, it is possible that the reduced net positive charge of basic peptides with ionised carboxyls at higher pH could lead to reduced mutual repulsion and thus somewhat smaller overloading effects.

A gradient using TFA as mobile phase additive (Table 3) again produced peak capacity results similar to phosphate, and formic acid spiked with 0.02 M KCl. Fig. 4 shows a comparison of the test mix analysed at full strength and 10 times diluted. Little change in retention or peak shape occurs when changing the injected mass over this range compared with the results for formic acid in Fig. 1. However, Table 3 indicates that peak shape is slightly worse than with 0.02 M phosphate. Note we used 0.09% TFA by weight i.e. the same weight percent as for 0.02 M formic acid. However,

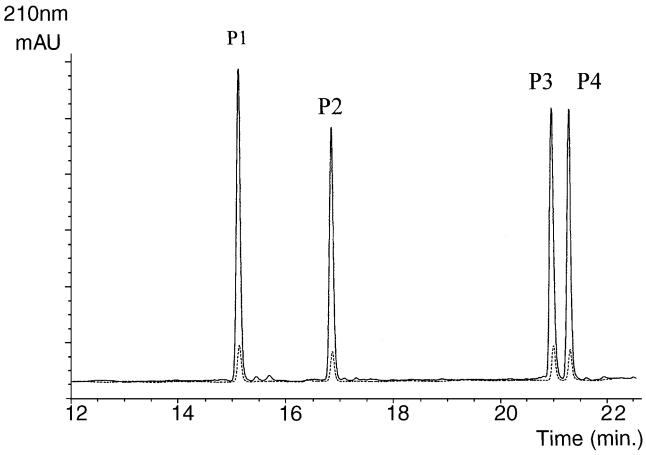


Fig. 4. Analysis of Alberta peptide mix at normal working strength (continuous line) and diluted 10 times in the mobile phase (dotted line). Solvent A: 0.09 g $l^{-1}$ TFA in water (pH 2.3). Solvent B: 0.09 g $l^{-1}$ TFA in acetonitrile. Other conditions as Fig. 1.

this weight percent is equivalent to a lower molar concentration, only 0.0079 M (pH 2.3). We used this smaller concentration for fear that a 0.02 M solution would give a pH that might damage the column; in any case, 0.1% TFA or less is generally used in mass spectrometry to limit suppression effects. The ionic strength of a 0.0079 M TFA solution is approximately 7.8 mM. This is more than four times the ionic strength of 0.02 M formic acid, but still less than that of the phosphate mobile phase. It is possible however that the known "ion-pair" effects of TFA may contribute to this reduced overloading in comparison with formate buffers, perhaps by partial neutralisation of the net charge on protonated bases, thus reducing repulsion effects.

Due to very low flow rates, capillary LC is used frequently with mass spectrometry. Capillary LC also increases sensitivity when UV detection is used, if the same sample mass is injected. If a (hypothetically) identical UV detector is used with the same path length and noise characteristics, sensitivity should increase by a factor:

$$\frac{(r_1)^2}{(r_2)^2} \quad (6)$$

where $r_1$ is the internal radius of the large column and $r_2$ the radius of the smaller column. A 0.075 mm capillary should in theory lead to an increase in sensitivity of almost 3800, compared with the 4.6 mm column used above. Intuitively, it seems overloading should be negligible using sample masses some thousands of times smaller than with standard size columns. However, the volume and mass capacity of the capillary will also be reduced by the same factor. Volume overload can be overcome by use of a concentrator column and switching valve, which allowed us to inject the same volume as used for the large column (5 μl) on to the concentrator, and backflushing on to the analytical column. Although comparisons are not very meaningful due to the differences in length and efficiency of the two columns, and

Table 4
Comparison of Peak shapes using a capillary column with 0.02 M formic acid

| Peptide | Strength | $t(r)$ (min) | W(0.5) (min) | As |
|---|---|---|---|---|
| P3 | Dil. 100× | 15.7 | 0.189 | 2.61 |
| | Dil. 1000× | 15.3 | 0.127 | 2.12 |
| | Dil. 10,000× | 16.0 | 0.0814 | 1.59 |
| P4 | Dil. 100× | 22.8 | 0.220 | 3.20 |
| | Dil. 1000× | 22.4 | 0.160 | 1.56 |
| | Dil. 10,000× | 23.1 | 0.140 | 1.55 |

Gradient conditions as Fig. 1 but scaled according to Eq. (5).

the different instruments used, as expected, we found the sensitivity gain (to achieve similar signal/noise) at least 10 times less than expected in theory, which we attribute to the increase in noise in the capillary detection system. Table 4 shows peak widths for P3 and P4 from analysis of the peptide mixture at 100, 1000 and 10,000 times dilution using 0.02 M formic acid. The gradient was scaled according to Eq. (5) to yield approximately the same $k^*$ values as on the standard size column, although possible differences in $S$ values on the two columns were ignored. Clear evidence is shown of overload of the 100 and 1000 times diluted mixture. Note that injection of 5 μl of 10,000 diluted mixture on to a 0.075 mm column corresponds to in theory 3.8 times the mass load equivalent of 5 μl of the 10× diluted sample injected on to an exactly equivalent 4.6 mm column. It is possible therefore that further reduction in peak width might be obtained using even more dilute solutions; however, the traces were too noisy to allow reliable measurement of peak widths at greater than 10,000 times dilution. A 5 μl injection of 10,000 times dilution of the peptide mixture represents approximately 0.1 ng or about a tenth of a picomole of P3 and P4 injected on to the column. We emphasise the difficulty of direct comparison with the large column results; in addition packing densities may be different in the different diameter columns. However, it is clear that equivalent overload of basic peptides in low ionic strength mobile phases can also occur in capillary LC when using typical concentrations as in proteomics.

## 4. Conclusion

Overloading of basic peptides can occur in gradient RP–HPLC even when sample masses around 0.1 μg (~100 pmol) are injected on to standard size analytical columns, when using low ionic strength buffers such as formic acid in the mobile phase. The problem of overload appears worse in isocratic separations of peptides, although rationalisation of this observation requires further study. Overload is of equal concern also in capillary columns with proportionally lower amounts of injected sample. Low ionic strength buffers are not effective in preventing the mutual repulsion of charged peptides which occurs on the stationary phase surface, limiting sample capacity. Mutual repulsion effects appear to increase for peptides with multiple positive charges at low pH. For reasons at least partially of higher ionic strength, phosphate buffers and TFA give better peak shapes for peptides. Other properties of buffers/additives such as ion-pair effects (very likely with TFA, but also a possibility with phosphate or chloride) may influence column capacity due to reduction of charge repulsion [15,23]. Clearly, despite improved chromatography, phosphate and other inorganic salts are non volatile, whereas TFA, though volatile can reduce MS sensitivity. Use of ammonium formate buffers at somewhat higher pH (e.g. pH 3.3) may be a useful alternative. Here the ionic strength of the mobile phase may be sufficiently high to reduce overload, but the pH is still sufficiently low, in combination with the possible deactivating effect of the ammonium ion, to prevent undesirable silanol interactions, at least on modern, highly inert stationary phases.

## References

[1] R. Aebersold, M. Mann, Nature 422 (2003) 198.
[2] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, Wiley, New York, 1997.
[3] W. Walcher, H. Oberacher, S. Troiani, G. Hoelzl, P. Oefner, C.G. Huber, J. Chromatogr. B 782 (2002) 111.
[4] A. Appfel, S. Fischer, G. Goldberg, P.C. Goodley, F.E. Kuhlmann, J. Chromatogr. A 712 (1995) 177.
[5] D. Temesi, B. Law, LC-GC (Int.) 12 (1999) 175.
[6] D. Temesi, B. Law, J. Chromatogr. B 748 (2000) 21.
[7] H.J. Issaq, S.D. Fox, M. Mahadevan, T.P. Conrads, T.D. Veenstra, J. Liq. Chromatogr. Related Technol. 26 (2003) 2255.
[8] C.G. Huber, A. Premstaller, J. Chromatogr. A 849 (1999) 161.
[9] D. Corradini, C.G. Huber, A.M. Timperio, L. Zolla, J. Chromatogr. A 886 (2000) 111.
[10] J.E. Eble, R.L. Grob, P.E. Antle, L.R. Snyder, J. Chromatogr. 384 (1987) 45.
[11] L.R. Snyder, G.B. Cox, P.E. Antle, Chromatographia 24 (1987) 82.
[12] L.R. Snyder, G.B. Cox, J. Chromatogr. 483 (1989) 95.
[13] S.M.C. Buckenmaier, D.V. McCalley, M.R. Euerby, Anal. Chem. 74 (2002) 4672.
[14] D.V. McCalley, J. Chromatogr. A 987 (2003) 17.
[15] D.V. McCalley, Anal. Chem. 75 (2003) 3404.
[16] Application Note 168, Supelco, Bellafonte, USA.
[17] C.R. Mant, R.S. Hodges, Chromatographia 24 (1987) 805.
[18] D.V. McCalley, J. Sep. Sci. 26 (2003) 187.
[19] D.V. McCalley, J. Chromatogr. A 844 (1999) 23.
[20] U.D. Neue, J.R. Mazzeo, J. Sep. Sci. 24 (2001) 921.
[21] U.D. Neue, HPLC Columns, Wiley-VCH, New York, 1997.
[22] L.S. Ettre, LC-GC (Europe) 16 (2003) 192.
[23] F. Gritti, G. Guiochon, J. Chromatogr. A 1033 (2004) 57.