

Introduction

Mobile phase additives have a strong influence on sensitivity and spectral quality when HPLC is coupled to mass spectrometry. For example, under acidic conditions, trifluoroacetic acid is known to lead to ionization suppression for certain groups of analytes like peptides and is therefore frequently avoided despite its well-known benefits to chromatographic peak shape. Another issue is the formation of adducts with alkali metal ions, in particular with traces of sodium, that complicate data analysis and interfere with automated LC-MS/MS analysis work flows.

When reversed-phase HPLC is performed under neutral conditions, ammonium acetate is the additive of choice because of its volatility. However, the limited solubility of ammonium acetate in commonly employed organic solvents makes it impossible to prepare solutions of more than ~0.05% in pure acetonitrile, so usually a small amount of water is added for dissolution. In addition, acetic acid can be added to obtain a buffered system.

We compared six different acetonitrile / ammonium acetate blends (see Table 1), differing in their water and acetic acid content and evaluated their performance for HPLC-ESI-MS analysis of peptides under neutral conditions.

Experimental

Instrumentation

HPLC-ESI-MS was performed either on an Agilent 1100 HPLC system interfaced to an Agilent 1100 MSD Trap SL ion trap instrument equipped with a standard ESI source or on an Agilent 1100 HPLC system interfaced to a PE Sciex API 365 triple quadrupole instrument equipped with a standard ESI source.

Sodium levels in CH₃CN / NH₄Ac blends were determined by ICP-OES. The instrument was a Thermo TJA IRIS/AP HR DUO.

HPLC conditions

stationary phase: Thermo BetaBasic-18, 150 x 4.6 mm, 3 μm, 25 °C

mobile phase: isocratic at 5% B for 5 min., then to 50% B in 20 min., where A = H₂O + 0.1% NH₄Ac and B = CH₃CN / NH₄Ac blend (see Table below), flow rate 0.8 ml min⁻¹. All solvents (Riedel de-Haën) and additives (Fluka) used were of LC-MS grade.

Table 1. Composition of the acetonitrile / ammonium acetate blends.

Blend No.	% v/v CH ₃ CN	% v/v H ₂ O	% v/v NH ₄ Ac 10%	% v/v CH ₃ COOH
1	95	4	1	0
2	96	3	1	0
3	96	2.5	1	0.5
4	96	2	1	1
5	97	1	1	1
6	98	0	1	1

ESI-MS conditions

positive ionization, scan mode m/z 100-1500

Model peptide mixture:

30 μg ml⁻¹ each of bradykinin, des-Arg¹-bradykinin, Lys-Ala³-bradykinin, bradykinin 1-6 (all from Bachem), injection volume 10 μl

Formation of sodiated peptide ions in LC-ESI-MS

The solubility of ammonium acetate in the organic part of a solvent gradient system (i.e. acetonitrile) is extremely poor and the resulting mixture (blend) is unstable, losing ammonia over time. In order to keep the additive concentration constant over the whole gradient range, it is necessary to prepare a stable acetonitrile-ammonium acetate blend by adding an excess amount of the corresponding acid, i.e. acetic acid. A well accepted side effect of this procedure is that the gradient system is buffered in a medium acid pH range between 4.5 and 6.5, which allows both positive and negative ionization of the analytes.

Analysis of a model peptide mixture on an ion trap system revealed significant dependence of sodium adduct formation on the blend composition. Reduction of the water content in mobile phase B and addition of acetic acid to buffer the system led to an increase in sodium adducts by a factor of two to four (see Figures 1 and 2). ICP-OES analyses of the blends resulted in comparable Na levels below the detection limit of 100 ppb, in accordance with the high purity of the LC-MS grade solvents employed for the study. The adduct formation was found to be solely dependent on the presence of acetic acid which on the other hand strongly improved separation of the peptides (see right column) and was essentially unaffected by variation in instrumental parameters.

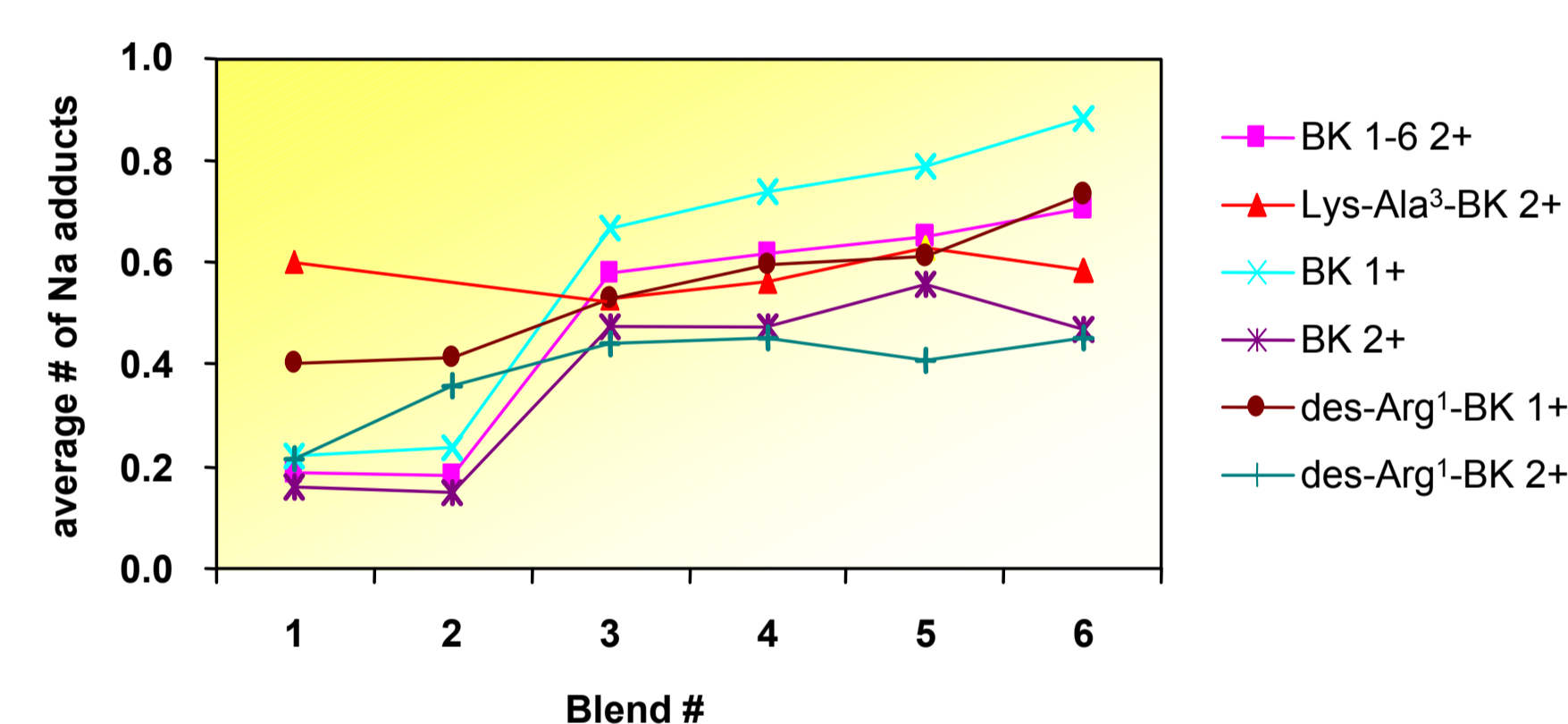


Figure 1. Sodium adduct formation for the four model peptides in dependence of the CH₃CN / NH₄Ac blend used as mobile phase B.

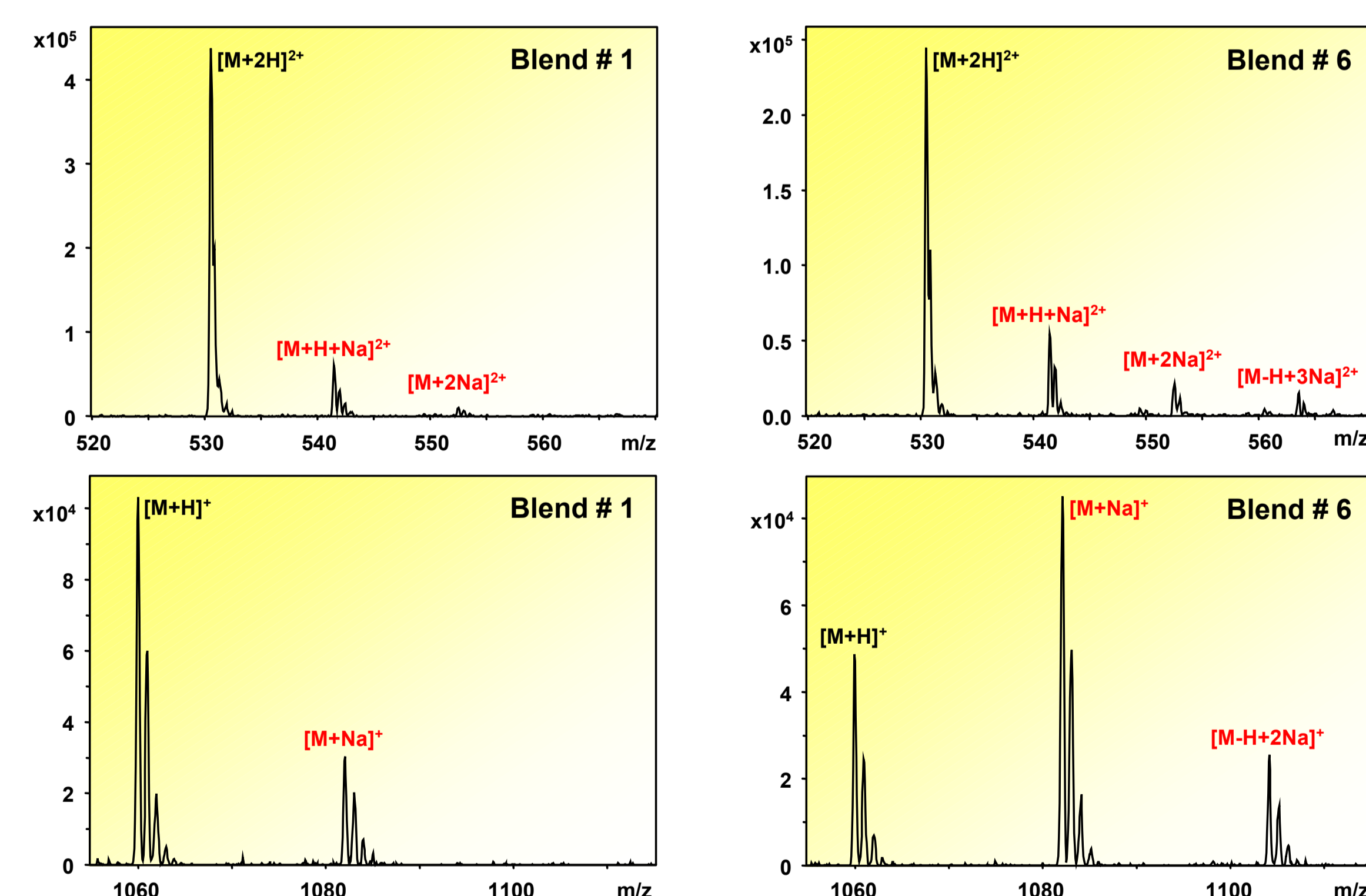


Figure 2. Sample spectra for bradykinin (RPPGFSPFR) taken from LC-ESI-MS analyses of the four peptide-mixture demonstrating the degree of sodium adduct formation using Blends # 1 and # 6 as mobile phase B. Shown are regions of doubly (top) and singly charged (bottom) peptide ions.

This effect was strongly dependent on the type of instrument used, as analysis of the same samples with identical solvents on a triple quadrupole instrument yielded negligible amounts of Na adducts for all solvent blends (data not shown). We therefore conclude that instrument-specific design elements in the desolvation region are responsible for the differences observed, and such surprising effects need to be considered for LC-MS analysis even when using high purity solvents.

Influence of blend composition on chromatographic separation

Among the four model peptides, only the hexapeptide RPPGFS (Bradykinin Fragment 1-6) is chromatographically separated from the rest when a mixture of 95% acetonitrile and 5% water + 0.1% ammonium acetate (Blend # 1) is used as solvent B (Figure 3, left). Addition of acetic acid, as in blend # 3 to # 6, has a dramatic effect on separation of the other three peptides that otherwise partially coelute (Figure 3, right). In addition, an impurity present in one of the peptides (marked with *), was also separated from the bulk.

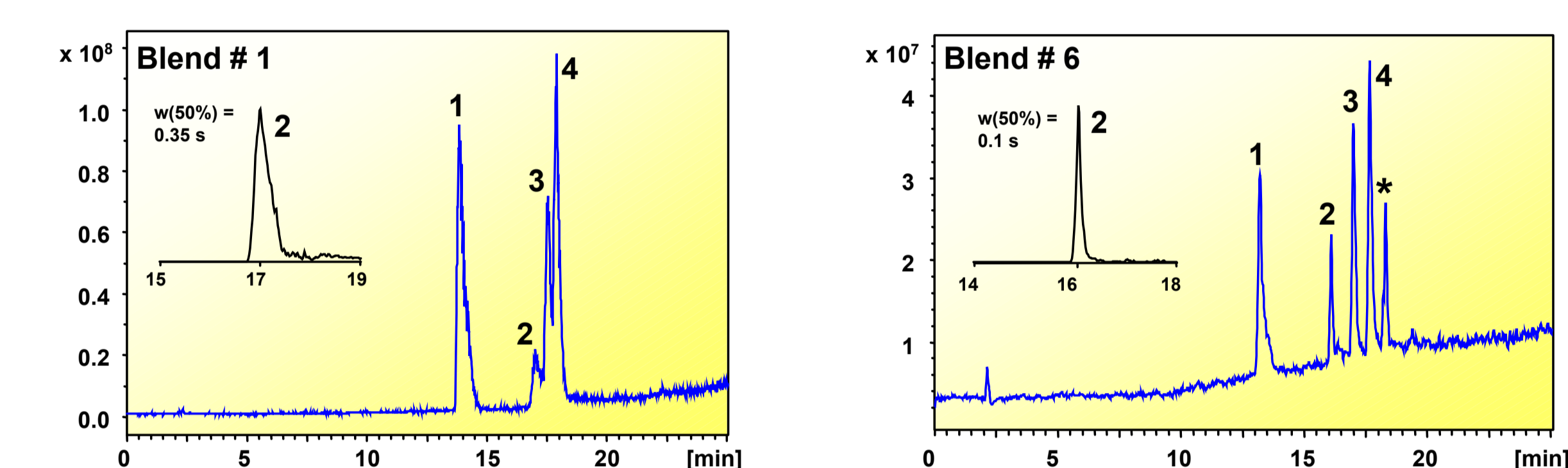


Figure 3. Total ion current chromatograms for the four peptide-mixture using Blends # 1 and # 6 as mobile phase B, respectively. Numbered peaks correspond to the following peptides: 1 = bradykinin, 2 = Lys-Ala³-bradykinin, 3 = bradykinin, 4 = des-Arg¹-bradykinin, * = impurity. Insets show extracted ion chromatograms for Lys-Ala³-bradykinin ([M+2H]²⁺, m/z = 581.6).

Figure 4 demonstrates this effect for all six blends that were studied. In the presence of acetic acid the pH curve during gradient elution is significantly altered, avoiding a shift towards the alkaline range (Figure 5). This not only affects the separation of analytes with very similar chromatographic properties, but also led to a reduction in peak widths, which was most significant for the strongly basic peptide, Lys-Ala³-bradykinin (KRPAGFSPFR, pI 12.01), as shown in Figure 3.

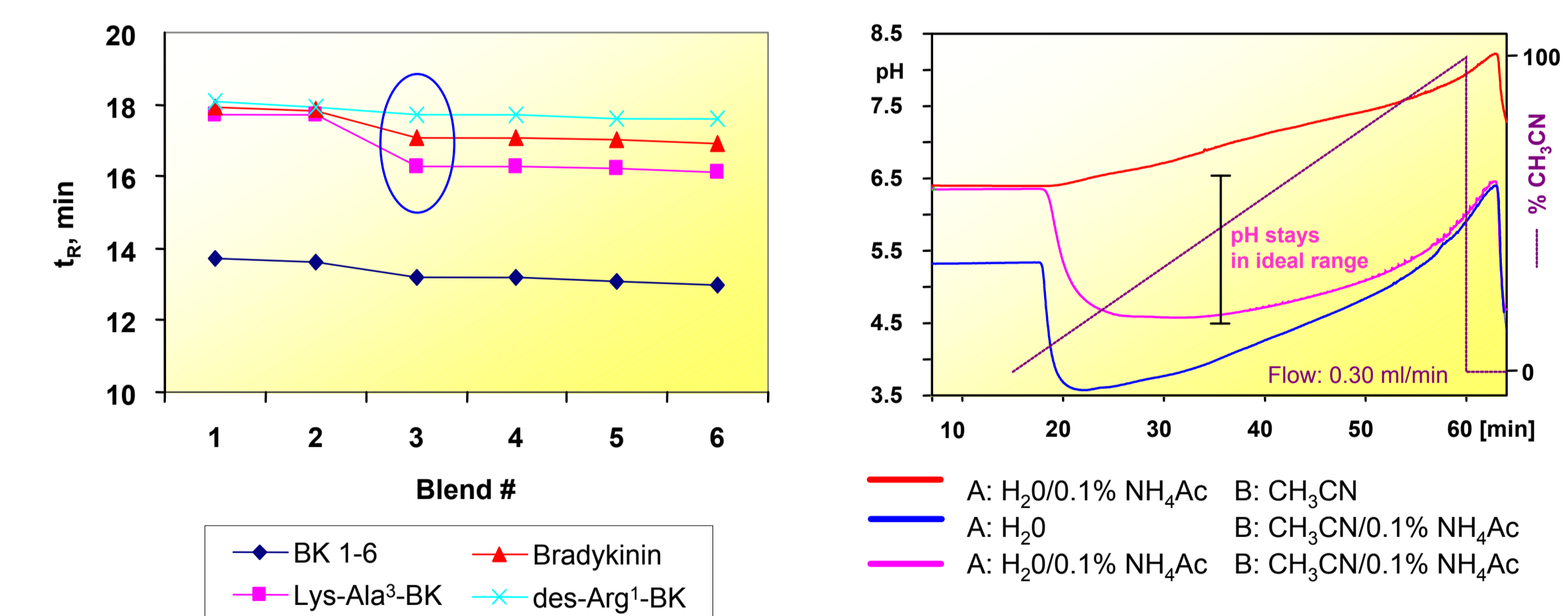


Figure 4. Overview of retention times for the four peptide-mixture using blends # 1 to # 6 as mobile phase B. Figure 5. Change of apparent pH during gradient with different mixtures of ammonium acetate and acetonitrile.

Conclusions

- The way of preparing acetonitrile / ammonium acetate blends for gradient HPLC-ESI-MS strongly influenced the degree of sodium adduct formation for a test mixture of four peptides when analyzed on an ion trap mass spectrometer.
- The absence of sodium adducts when the samples were analyzed on a triple quadrupole instrument under otherwise identical conditions demonstrates the instrument dependence of adduct formation.
- Improved chromatographic separation of the test mixture and reduced peak widths were observed upon addition of acetic acid to aid in dissolution of ammonium acetate and buffer the mobile phase.

Acknowledgements

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