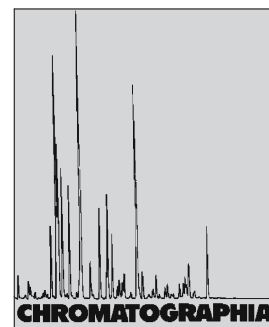


Influence of Solvent Additive Composition on Chromatographic Separation and Sodium Adduct Formation of Peptides in HPLC–ESI MS



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A. Leitner^{1,✉}, J. Emmert², K. Boerner³, W. Lindner¹

¹ Department of Analytical Chemistry and Food Chemistry, University of Vienna, Währinger Straße 38, 1090 Vienna, Austria;
E-Mail: alexander.leitner@univie.ac.at

² Sigma-Aldrich Production, Industriestraße 25, 9471 Buchs SG, Switzerland

³ Sigma-Aldrich Laborchemikalien, Wunstorfer Straße 40, 30926 Seelze, Germany

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Abstract

Mobile phase additives have a strong influence on sensitivity and spectral quality when HPLC is coupled to mass spectrometry. They may cause ion suppression, as frequently observed with trifluoroacetic acid, or may lead to increased adduct formation, complicating data analysis and interfering with automated LC–MS–MS analysis. When reversed-phase HPLC is performed under neutral conditions, ammonium acetate is the additive of choice because of its volatility. Because of the limited solubility of ammonium acetate in commonly employed organic solvents like acetonitrile usually a small amount of water is added for dissolution, and in addition, acetic acid can be added to obtain a buffered system. We compared six different acetonitrile/ammonium acetate blends, differing in their water and acetic acid content and evaluated their performance for HPLC–MS analysis of model peptides (bradykinins) under neutral conditions. The results demonstrate that mobile phase preparation strongly influences chromatographic separation and adduct formation in electrospray ionization MS, as shown for a series of bradykinin peptides. By varying the solvent composition only slightly to influence the pH curves during gradient elution, three nearly coeluting peptides could be baseline separated. At the same time, the formation of sodiated instead of protonated peptides was found to vary by up to a factor of four, depending on the preparation of the solvent blends.

Keywords

Liquid chromatography–mass spectrometry
Electrospray ionization
Sodium adduct formation
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Introduction

Since the first description by Yamashita and Fenn in [1–3] LC–MS has emerged as

a widely used technique for identification and quantification of molecules separated by liquid chromatography. Two main issues have been addressed and improved

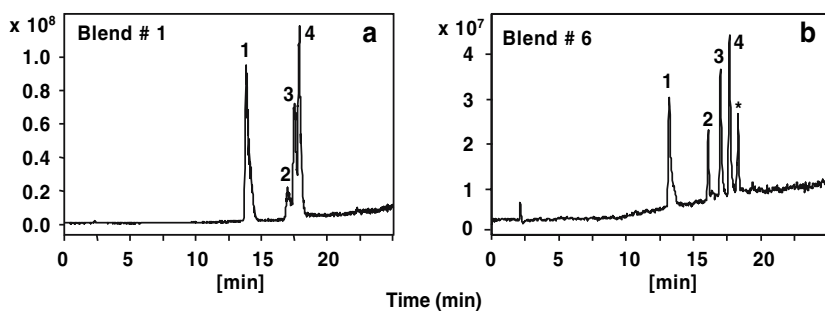
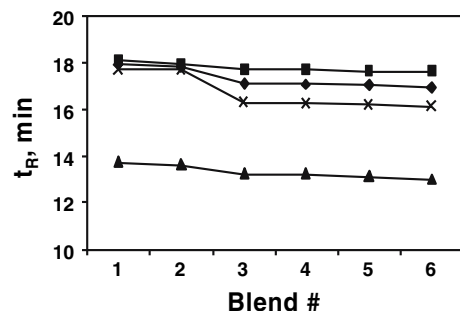
in many aspects since then: the instrumental side, driven by increased understanding of the desolvation and ionization process within the electrospray interface. And the practical side, making the use of these instruments more easy, mainly by means of automation and software capabilities.

Less emphasis was put on the chemical side concerning solvent quality and use of additives. Some papers describe the influence of certain molecules, present in trace amounts as impurities, or added to act as additives. The effect of alkali ions was studied by Wang and Cole [4] and can be critical [5] or used in a positive direction [6], especially with glycosides. Differences in the cationizing agent (proton vs. alkali metal cation) may also influence the fragmentation behavior of various analyte classes, as has been shown for peptides [7] and phospholipids [8], among others. As another example, trifluoroacetic acid, which is often used in chromatography of peptides to improve peak shape due to its ion pairing abilities, leads to ion suppression in LC–MS and has to be avoided or counteracted [9].

Many separations, performed under acidic conditions, use formic acid as ionization supporting agent [10]. But some molecules or separations require neutral conditions, in such cases ammonium formate or acetate are the additives of choice [11]. First because of their volatility, second due to their buffer capabilities. But unfortunately their solubility

Table 1. Composition of the acetonitrile/ammonium acetate blends used in the study

Blend No.	CH ₃ CN, % v/v	H ₂ O, % v/v	NH ₄ Ac 10%, % v/v (in water)	CH ₃ COOH, % v/v	Na content ppb (ICP-MS)
1	95	4	1	0	200
2	96	3	1	0	180
3	96	2.5	1	0.5	150
4	96	2	1	1	180
5	97	1	1	1	165
6	98	0	1	1	140

**Fig. 1.** Total ion current chromatograms for the four peptide-mixture using **a** Blend # 1 and **b** Blend # 6 as mobile phase B. Numbered peaks correspond to the following peptides: **1** = bradykinin 1–6, **2** = Lys–Ala³–bradykinin, **3** = bradykinin, **4** = des–Arg¹–bradykinin, * impurity**Fig. 2.** Overview of retention times for the four peptide-mixture using blends # 1 to # 6 as mobile phase B. **▲** bradykinin 1–6, **×** Lys–Ala³–bradykinin, **◆** bradykinin, **■** des–Arg¹–bradykinin. Refer to Table 1 for the composition of the blends

in organic solvents like acetonitrile is very limited. This causes a decrease in concentration and buffering capability during a gradient or the need to add water to the organic solvent. Besides, these mixtures are unstable and set free ammonia after a certain time. Addition of the corresponding acid (i.e. acetic acid) improves the dissolution of the salt and the stability of the resulting salt–acetonitrile mixture (“blend”); furthermore it keeps the pH-range during a gradient run between 4.5 and 6.5. This allows separations under mild acidic conditions and mass spectrometric detection in both positive and negative ion mode.

In this study we used 0.1% (v/v) solutions of ammonium acetate in acetonitrile, prepared with different amounts of

water and acetic acid for separation and mass spectrometric detection of a peptide mixture of bradykinin and its analogs. We studied the influence of the organic solvent composition on separation and ionization behavior, in respect to chromatographic resolution, sensitivity of mass spectrometric detection (only qualitative) and formation of sodium clusters.

Experimental

Materials

All solvents and additives were of LC–MS grade and were obtained from Riedel-de Haën (Seelze, Germany) and Fluka (Buchs, Switzerland), respectively.

The following model peptides were purchased from Bachem (Weil am Rhein, Germany) and were used without further purification: Bradykinin fragment 1–6 (RPPGFS, pI 9.75), des–Arg¹–bradykinin (PPGFSPFR, pI 10.18), bradykinin (RPPGFSPFR, pI 12.00) and Lys–Ala³–bradykinin (KRPAGFSPFR, pI 12.01). The pI values were calculated by the ProtParam tool available at <http://www.expasy.org/tools/protparam.html>.

Liquid Chromatography–Electrospray Ionization Mass Spectrometry (LC–ESI–MS)

Liquid chromatography–mass spectrometry experiments were carried out on an Agilent 1100 HPLC system together with an Agilent 1100 MSD Trap SL quadrupole ion trap mass spectrometer equipped with a standard electrospray source (Agilent Technologies, Waldbronn, Germany). Control experiments were also carried out using the same HPLC system coupled to a PSciex API 365 triple quadrupole mass spectrometer with a standard electrospray source (Applied Biosystems/MDS Sciex, Concord, Canada).

Mixtures of the model peptides were prepared at 30 µg mL^{−1} each in water/acetonitrile, 95:5 (v/v). Injection volumes were 10 µL in all cases. The peptides were separated on a Thermo BetaBasic-18 column (150 × 4.6 mm, 3 µm; Thermo Electron, Runcorn, UK) using gradient elution at a flow rate of 0.8 mL min^{−1}. Elution conditions were as follows: mobile phase A = water + 0.1% ammonium acetate, B = acetonitrile + 0.1% ammonium acetate blend prepared according to Table 1; isocratic elution at 5%B for 0–5 min, then linear increase from 5 to 50%B from 5 to 25 min. All analyses were carried out at 25 °C and at least in duplicate. After changing solvents, the system was equilibrated for a minimum of one hour, and blank runs were performed to avoid any potential cross-contamination. Selected experiments were reproduced after several weeks, conforming the initially obtained results.

Electrospray ionization–mass spectrometry detection was performed in positive ionization mode with a scan range of *m/z* 100–1,500. Ionization and desolvation parameters were optimized for peptide analysis and the given HPLC flow rate. For the triple quadrupole instrument, the HPLC eluent was split in

a ratio of approximately 1:100 using a mixing tee prior to the introduction into the mass spectrometer.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Sodium levels in the solvents were determined by ICP-MS using a VG Plasma Quad (Thermo Electron, Dreieich, Germany).

pH Measurements

Solvent pH values were determined on a WTW pH 540 GLP pH meter (WTW, Weilheim, Germany) calibrated using Hamilton Duracal buffers (Hamilton, Bonaduz, Switzerland).

Results and Discussion

Influence of Solvent Blend Composition on Chromatographic Performance

Using typical gradient elution conditions and blend #1 (95% acetonitrile, 5% water + 0.1% ammonium acetate) as solvent B, only the hexapeptide RPPGFS (Bradykinin Fragment 1–6) is chromatographically separated from the rest of the model peptide mixture, as shown in Fig. 1a. Only partial resolution is observed for the three remaining peptides. Varying the composition of solvent B according to Table 1 led to a dramatic change in resolution upon the addition of acetic acid to buffer the mobile phase. Figure 2 compares the retention times of the four peptides using the six acetonitrile/ammonium acetate blends used in this study. The chromatogram in Fig. 1b, obtained with blend #6 (98% acetonitrile, 1% water, 1% acetic acid + 0.1% ammonium acetate) not only demonstrates a complete baseline separation of all four peptides under otherwise identical chromatographic conditions compared to Fig. 1a, but also allows the separation of a coeluting impurity present in one of the peptides. The noticeable effect on retention is most likely due to the differences in apparent solvent pH during the course of the separation.

The strongly basic peptides (all with pI values of 9.75 or higher [12]) are a

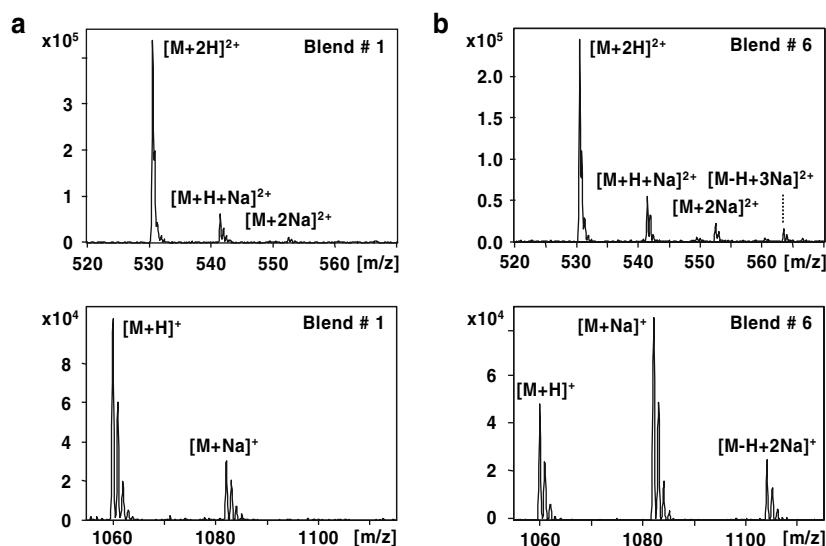


Fig. 3. Ion trap mass spectra for bradykinin (RPPGFSPFR) taken from LC-ESI-MS analyses of the four peptide-mixture demonstrating the degree of sodium adduct formation using a Blend # 1 and b Blend # 6 as mobile phase B. Shown are regions of doubly (*top*) and singly charged (*bottom*) peptide ions

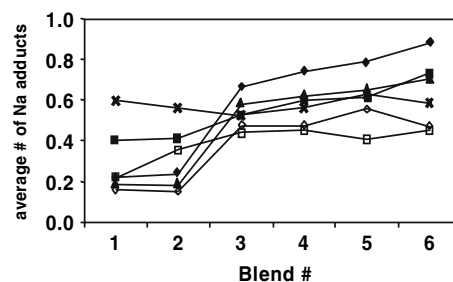


Fig. 4. Sodium adduct formation for the four model peptides in dependence of the CH₃CN/NH₄Ac blend used as mobile phase B. ▲ bradykinin 1–6 (2+), × Lys-Ala³-bradykinin (2+), ◆ bradykinin (1+), ◇ bradykinin (2+), ■ des-Arg¹-bradykinin (1+), □ des-Arg¹-bradykinin (2+). Composition of the blends is given in Table 1

typical example for analytes with non-ideal chromatographic behavior, causing noticeable peak asymmetry (tailing). Along with the effect on separation, the use of acetic acid-containing blends also resulted in improved peak shapes. As an example, the peak width at half maximum for Lys-Ala³-bradykinin (KRPAGFSPFR, pI 12.01) was reduced from approximately 0.35 s using blend #1 to 0.10 s with blend #6, resulting in an almost symmetrical peak in the latter case. Reductions in peak widths were also observed for the other analytes.

Sodium Adduct Formation in ESI-MS

Along with the changes in chromatographic behavior (see above), mass spec-

trometric signal quality was strongly influenced by the composition of the organic solvent B. In contrast to the beneficial effects of acetic acid addition on chromatography, an increase in sodium adduct formation was observed in blends #3 to #6. Figure 3 gives an example for sodium adducts of singly and doubly charged bradykinin (RPPGFSPFR), using blends #1 and #6, respectively. While for the acetic acid-free run, adducts represented less than 30% of the base peak for both charge states with blend #1, the [M + Na]⁺ signal was more abundant than the protonated [M + H]⁺ ion for blend #6. A significant increase in sodium adduct formation was also observed for doubly charged ions of this analyte. Figure 4 compares the average number of sodium adducts observed for different charge states of the

Table 2. pH values of the solvents used in the study

Solvent	pH
A	6.93
B, blend 1	7.17
B, blend 2	7.25
B, blend 3	5.22
B, blend 4	4.93
B, blend 5	4.94
B, blend 6	4.94

Given are values for pure mobile phase A and 1:1 mixtures of mobile phases A and B (acetonitrile blends). All values are uncorrected, measurements were performed at 22 °C

four peptides used in this study. With the exception of Lys-Ala³-bradykinin, which was almost unaffected, sodium adducts increased (by up to a factor of four) when acetic acid was added to solvent B. There is no apparent correlation between the pI of the peptides and the Na adduction, as the pI values of Lys-Ala³-bradykinin and bradykinin are almost identical (12.01 vs. 12.00), while the latter was much more influenced by the changes in solvent composition. Also the least basic peptide, bradykinin 1–6 (pI 9.75), showed strong increases in adduct formation in the presence of acetic acid. This coincided with a decrease in sensitivity, as the summed signal intensities of protonated and sodiated signals of all charge states for a given peptide were reduced as much as 50%.

To rule out an unintended contamination of the solvents, sodium levels were determined in all solutions, revealing comparably low levels in the range of 100–200 ppb (see Table 1), thus excluding any effect of adding trace levels of alkali metal ions by acetic acid, either by the acid itself or by leaching the glass bottles. As a consequence, differences in solution pH seem to be responsible for the different behavior of the blends within the electrospray interface. Table 2 lists the pH values of the acetonitrile blends as a 50/50 (v/v) mixture with mobile phase A. From these data, it is clearly visible that the addition of acetic acid (in blends #3 to #6) shifts the pH to a more acidic region at the endpoint of the solvent gradient used in this study. For blends #4 to #6, the apparent pH is more than 2 pH units lower compared to the acetic acid-free blends. Accordingly, sodium adduct formation correlates with the lower solution pH more than with any other variable of the experimental setup, at least for the ion trap system.

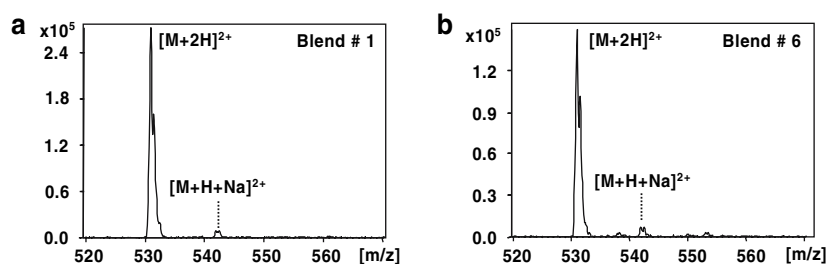


Fig. 5. Triple quadrupole mass spectra for bradykinin (RPPGFSPFR) taken from LC-ESI-MS analyses of the four peptide-mixture demonstrating the degree of sodium adduct formation using **a** Blend # 1 and **b** Blend # 6 as mobile phase B. Only doubly charged ions were observed with significant abundance

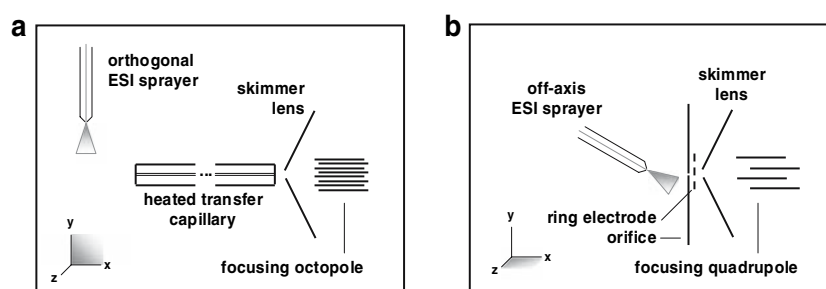


Fig. 6. Ion source and interface designs of the instruments used in this study. **a** Ion trap instrument (*side view*), **b** triple quadrupole instrument (*top view*). Drawings are not to scale. For details, see text

Attempts were made to reduce the degree of adduct formation by optimizing the operating parameters of the ion trap mass spectrometer, however, without any success. In order to investigate the prevalence of this phenomenon, the experiments were repeated on a different mass spectrometer, using identical chromatographic conditions, including the same solvents. On this triple quadrupole instrument, formation of sodiated ions was almost absent (Fig. 5).

In absence of any variation from the chromatography side, these results highlight the significance of differences in ion source and (atmospheric pressure-to-vacuum) interface region design [13]. The ion trap system uses a sprayer design orthogonal to the inlet into the interface region (Fig. 6a), while the triple quadrupole instrument is equipped with a classic “off-axis” sprayer as described by Henion and co-workers [14] (Fig. 6b). In addition, the mass spectrometers differ in the design of the desolvation stage; in the ion trap MS, a heated transfer capillary (with a countercurrent nitrogen flow) followed by a skimmer lens serves to remove remaining solvent and break up clusters while in the triple quadrupole MS desolvation is achieved by passage through the orifice plate, a skimmer and

countercurrent nitrogen gas flow. Differences in the actual flow rate into the two sources may also contribute to the observed effects to some degree because of variations in initial droplet size.

While the data obtained in this study are not sufficient to obtain a detailed picture of the ion formation process taking place under the experimental conditions, it is evident that such surprising differences need to be considered for LC-MS analysis even when using high purity solvents. Competition between protons and sodium ions as charge carriers in ESI-MS is a highly complex process that has been previously studied for selected model systems in the presence of much higher sodium levels [15–16]. The fact that very low Na levels already influence the distribution between protonated and sodiated forms of a peptide warrants further experimental and theoretical studies.

Conclusion

The use of ammonium acetate as additive for LC-ESI-MS is not as simple as for, e.g., formic acid, due to its limited solubility in the organic part of a gradient system. This causes considerable changes

in pH during a gradient run, thus influencing the separation and ionization capabilities. This can be counteracted by either totally or partly buffering the system with the corresponding acid, here acetic acid. But LC-ESI-MS experiments revealed that effects are multi-parametric: not only separation was affected, but also the quality of mass spectrometric data.

It was found that the enhancement of ammonium acetate solubility in acetonitrile by adding acetic acid led to improved separation of closely related model peptides (bradykinins), which was attributed to differences in solution pH during gradient elution compared to an acetic acid-free mobile phase. On the other hand increased sodium adduct formation was observed upon addition of acetic acid despite comparably low Na levels in all solvents used in the study. Thus, subtle influences of the solvent composition appear to affect the electrospray ionization and desolvation process, i.e., by suppression and competition between H^+ and Na^+ ions. This was confirmed by the fact that sodium adducts were abundant on one instrument—even under optimized conditions—while they were al-

most absent on another system with a different ion source and desolvation stage design.

It can therefore be concluded that it may be beneficial to use a buffered ammonium acetate gradient system instead of commonly used addition of formic acid, but the effect on ionization cannot be predicted in general, as it seems to be dependent on the MS instrument used, more exactly on the design of the electrospray interface. Furthermore, the use of an APCI interface may diminish or even avoid the unwanted effect of Na-cluster formation.

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