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Selection of buffers and of an ion-pairing agent for thermospray liquid chromatographic–mass spectrometric analysis of ionic compounds

A. L. L. DUCHATEAU*, B. H. M. MUNSTERS, G. T. C. KWAKKENBOS and R. G. J. VAN LEUKEN
DSM Research, P.O. Box 18, 6160 MD Geleen (The Netherlands)

ABSTRACT

The applicability of ammonium formate, ammonium acetate and ammonium bicarbonate as volatile electrolytes for thermospray liquid chromatographic–mass spectrometric analysis of three decomposition products of α-aspartame is demonstrated. With these buffers, the pH range of the mobile phases for silica-based reversed-phase columns can be covered. With respect to the buffer capacity and ultraviolet transparency, a buffer salt concentration of 50 m*M* was used in the mobile phase. The retention times of the compounds studied in the volatile buffers were compared with those obtained in sodium acetate and sodium phosphate buffers and were found to match quite well. For the reversed-phase ion-pair separation of α-aspartame and β-aspartame in combination with thermospray mass spectrometry, a method is described using trifluoroacetate as pairing ion. Liquid chromatographic–mass spectrometric analysis of L-aspartyl-L-phenylalanine shows that the highest signal-to-noise ratio is obtained in ammonium formate. For the compounds studied, no correlation could be found between the signal-to-noise ratio and source temperature, or between signal-to-noise ratio and vaporizer temperature.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a powerful technique for the separation of both neutral and ionic compounds. Mobile phase additives, such as buffers and ion-pairing agents, are frequently used in the daily practice of this technique.

For thermospray liquid chromatography–mass spectrometry (LC–TSP-MS), however, the non-volatile inorganic buffer salts and ion-pairing agents are detrimental to the MS. By means of post-column suppressor techniques [1,2], it has been shown that certain non-volatile substances may be removed before entering the MS system. Another approach is the replacement of the non-volatile additives in the mobile phase by volatile ones.

Some papers related to the selection of such solvent systems have been presented. Voyksner and Haney [3] evaluated the use of formate, acetate, carbonate and bicarbonate as their ammonium salts for LC–TSP-MS. A comparison between the use

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of acetate and formate buffer was also made by Barceló [4]. For the LC–TSP-MS analysis of sulphonated surfactants, ammonium acetate was used both as a volatile buffer and as an ion-pairing agent [5].

Patthy and Gyenge [6] reported the use of trifluoroacetate (TFA) and heptafluorobutyrate as pairing ions for the RP ion-pair separation of monoamine transmitters. In their study, amperometric detection was used. Their conclusion was that short-chain perfluorinated carboxylic acids are a very attractive alternative to alkylsulphonates for the separation of the monoamines studied.

In the above-mentioned studies [3–5], the choice of volatile buffers was mainly related to the MS performance. However, important chromatographic aspects such as buffer capacity and selectivity of volatile buffers in relation to non-volatile buffers were not described.

Another important aspect is the selection of volatile buffers which can be used in combination with both MS and UV detection. In this way chromatographic optimization of the sample compound can be performed by HPLC–UV, meanwhile relieving the MS system. After optimization, HPLC–MS can be applied for identification.

The aim of our work was: (a) to select a set of volatile buffers to cover the entire pH range of silica-based RP material; (b) to investigate the effect on the capacity factor of changeover from a non-volatile buffer to a volatile one; and (c) to select a volatile ion-pairing agent suitable for the LC–TSP-MS analysis of carboxylic compounds.

For this, we chose volatile buffer solutions consisting of formate, acetate and bicarbonate. Ammonia was used as the cationic part of the buffers. The following chromatographic aspects were investigated: concentration of the volatile buffer in relation to UV response and buffer capacity, and comparison of the retention behaviour of ionic compounds in volatile buffers and in non-volatile ones.

With respect to the MS, the effects of the buffer choice on signal-to-noise ratio and spectral characteristics were evaluated. The test compounds used in this study were three decomposition products of the α-dipeptide sweetener aspartame: L-aspartyl-L-phenylalanine (AP), L-phenylalanine (Phe) and 5-benzyl-3,6-dioxo-2-piperazine acetic acid (DKP). For ion-pair LC–TSP-MS, we describe the use of TFA for the separation of α-aspartame (α-APM) and β-aspartame (β-APM).

EXPERIMENTAL

Instrumentation

The chromatographic system consisted of a Gilson (Villiers-le-Bel, France) Model 302 pump, a Rheodyne (Cotati, CA, U.S.A.) Model 7010 injection valve with a 20-μl loop and a Waters Assoc. (Milford, MA, U.S.A.) Model 481 UV detector for detection at 220 nm. The columns used were Nucleosil 120-C_{18} (250 × 4.0 mm I.D., 5 μm, and 50 × 4.0 mm I.D., 5 μm, for the TFA experiment) from Macherey, Nagel (Düren, Germany). The flow-rate was 1 ml/min and the separations were carried out at ambient temperature.

Post-column addition of buffer solution was effected with a Gilson Model 302 pump. The flow-rate was 0.5 ml/min. The solution was added to the column effluent by a Lee (Frankfurt, Germany) visco-jet micromixer. The composition of the solution used for post-column addition was always identical to the composition of the mobile phase.

The MS system consisted of a Finnigan MAT TSQ-70 triple quadrupole mass spectrometer equipped with a thermospray interface (Finnigan MAT, San José, CA, U.S.A.). Trifluororacetic acid was used for calibration of the mass spectrometer up to 1000 daltons [7]. The ion source temperature and the vaporizer temperature varied, depending on the experiment, and are indicated in the figures. The repeller voltage was kept at 75 V. The electron multiplier was operated at 800 V. Scanning was performed from 110 to 410 daltons with a scan time of 1 s.

Chemicals

α-APM and β-APM were obtained from Tosoh (Nanyo, Japan). AP and DKP were supplied by Bachem (Bubendorf, Switzerland). Phe was obtained from Sigma (St. Louis, MO, U.S.A.). Trifluororacetic acid was obtained from Janssen (Beerse, Belgium). HPLC-grade acetonitrile was supplied by Merck (Darmstadt, Germany). Water was purified with a Milli-Q system. All other chemicals were of analytical reagent grade.

Buffers

Sodium phosphate buffers (pH 3.0 and 7.0) and the sodium acetate buffer (pH 5.0) were prepared by dissolving the corresponding acids in water and titrating to the required pH with sodium hydroxide solution.

The ammonium bicarbonate buffer was prepared by dissolving the ammonium bicarbonate salt in water and titrating to pH 7.0 with acetic acid. Ammonium formate buffer (pH 3.0) and ammonium acetate buffer (pH 5.0) were prepared by dissolving the corresponding acids in water and titrating to the required pH with ammonia.

The ammonium formate–trifluoroacetate buffer was prepared by dissolving formic acid and trifluoroacetic acid in water and titrating to pH 3.0 with ammonia. The concentration of trifluoroacetic acid was 8 m*M*.

For all buffers, the final concentration of the acid was 0.05 *M*. The ammonium bicarbonate buffer consisted of 0.04 *M* carbonic acid and 0.01 *M* acetic acid. For chromatography, acetonitrile was added to the buffer solutions. The composition of the mobile phases is indicated in the figures.

RESULTS AND DISCUSSION

Buffer capacity of the volatile buffers

According to the Henderson–Hasselbalch equation, a weak acid and its conjugated base have their maximum buffer capacity at pH = $pK_a \pm 1$. For this reason formate (pK_a 3.75), acetate (pK_a 4.75) and bicarbonate (pK_a 6.37) were chosen as volatile buffers for RP-LC–MS. With these three buffers, the pH range of the mobile phases for silica-based RP columns can be covered.

The molar extinction coefficients of formate, acetate and bicarbonate are higher than those of the non-volatile phosphate buffers.

To obtain a comparable UV transparency for the volatile buffers chosen, a decrease in concentration is necessary. Too low a concentration may, however, lead to a situation where the eluent pH varies because of poor buffering. As a consequence, the retention times of the ionizable solutes may vary from one chromatographic run to another. This effect will also depend on the amount of the solute injected.

As we were interested in the low microgram range, the buffer capability at a chosen buffer concentration was experimentally checked. It was found experimentally that an injection of 1 μg of α-APM into a mobile phase consisting of at least 50 m*M* volatile buffer did not give peak distortion or irreproducible retention times. Therefore a concentration of 50 m*M* formate, acetate or bicarbonate was chosen in the mobile phase. For this concentration, the capacity (β) of the buffers was calculated according to the following formula [8]

$$\beta = 2.30 \frac{C_A \times C_B}{C_A + C_B} \quad (1)$$

where C_A and C_B are the molar concentrations of the acid chosen and its conjugated base, respectively. By means of the Henderson–Hasselbalch equation, C_A and C_B were calculated for several pH values and substituted into eqn. 1.

The capacity of the ammonium formate, ammonium acetate and ammonium bicarbonate buffers as a function of the pH is shown graphically in Fig. 1. From these curves it can be concluded that, for making a mobile phase with pH < 4.2, ammonium formate is the buffer of choice. For the pH range 4.2–5, both ammonium acetate and ammonium bicarbonate are suitable. For a mobile phase with pH > 5, ammonium bicarbonate is the most appropriate buffer.

Chromatographic selectivity

For the test compounds AP, DKP and Phe, RP separations in combination with UV detection were carried out with the following non-volatile mobile phases: sodium phosphate (pH 3.0)–10% (v/v) acetonitrile, sodium acetate (pH 5.0)–5% (v/v) acetonitrile and sodium phosphate (pH 7.0)–2.5% (v/v) acetonitrile.

To examine the influence on the capacity factor (k') of the test compounds, the non-volatile sodium phosphate buffers (pH 3.0 and 7.0) and sodium acetate buffer were replaced by ammonium formate, ammonium bicarbonate and ammonium acetate. The pH of the volatile mobile phases and the acetonitrile content were kept the same.

The calculated k' values of AP, DKP and Phe in both the non-volatile and volatile solvent systems are given in Table I. From these data it can be seen that the

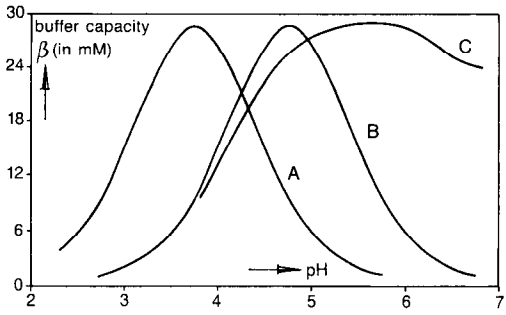

Fig. 1. Buffer capacity (β) as a function of pH. (A) 0.05 *M* ammonium formate solution; (B) 0.05 *M* ammonium acetate solution; (C) 0.05 *M* ammonium bicarbonate solution (pH adjustment with acetic acid).

TABLE I

k' VALUES OF AP, Phe AND DKP AND S/N RATIO OF AP IN VOLATILE AND NON-VOLATILE MOBILE PHASES

Mobile phase[a]	k'			S/N[b]
	Phe	AP	DKP	
I	0.6	1.8	3.2	105
II	0.6	2.0	3.3	11
III	1.1	0.8	2.2	45
IV	1.1	0.8	2.3	45
V	1.9	1.1	2.8	159
VI	1.8	0.9	2.3	120

[a] Mobile phases: I = 0.05 *M* sodium phosphate (pH 3.0)–10% (v/v) acetonitrile; II = 0.05 *M* ammonium formate (pH 3.0)–10% (v/v) acetonitrile; III = 0.05 *M* sodium acetate (pH 5.0)–5% (v/v) acetonitrile; IV = 0.05 *M* ammonium acetate (pH 5.0)–5% (v/v) acetonitrile; V = 0.05 *M* sodium phosphate (pH 7.0)–2.5% (v/v) acetonitrile; VI = 0.05 *M* ammonium bicarbonate (pH 7.0)–2.5% (v/v) acetonitrile.

[b] S/N ratio of AP at 220 nm (UV detection). The solution injected contains 1.3 μg of AP, 2.3 μg of Phe and 1.9 μg of DKP.

retention times of the compounds match quite well in the non-volatile and the corresponding volatile solvent systems.

For AP, the signal-to-noise (S/N) ratio was calculated in the different mobile phases (Table I). From this table it can be concluded that replacement of the sodium phosphate buffer (pH 7.0) by the ammonium bicarbonate buffer leads to a decrease of the S/N ratio of 25%. On the other hand, changing from sodium acetate to ammonium acetate does not influence the S/N ratio of AP.

The largest decrease of the S/N ratio occurs when replacing the sodium phosphate buffer (pH 3.0) by the ammonium formate buffer. A loss of 90% is observed here.

MS characteristics of the volatile buffers

For Phe and DKP, the quasimolecular ion peaks are the most abundant peaks in the spectra of all three buffer solutions. The most abundant peak in the AP spectrum is the quasimolecular ion for ammonium formate and protonated Phe (m/z = 166) for both ammonium acetate and ammonium bicarbonate.

Fragmentation and clustering in the three buffers are alike. However, there are significant differences in relative intensities and in the noise level. The mass spectrum of Phe consists of the peak of the quasimolecular ion $[M + H]^+$ (m/z = 166) and the cluster ions $[M + H + CH_3CN]^+$ and $[2M + H]^+$ (m/z = 207 and m/z = 331, respectively). The intensity of the double molecular cluster ion m/z = 331 is lower at higher source and vaporizer temperatures.

When source and vaporizer temperatures are increased, more AP molecules thermally decompose into DKP (m/z = 263) and Phe (m/z = 166). Consequently, the $[M + H]^+$ peak (m/z = 281) becomes less abundant.

The mass spectra of DKP give peaks for the ions $[M + H]^+$ and $[M + NH_4]^+$ (m/z = 263 and m/z = 280, respectively). Fragmentations were not detected. In the

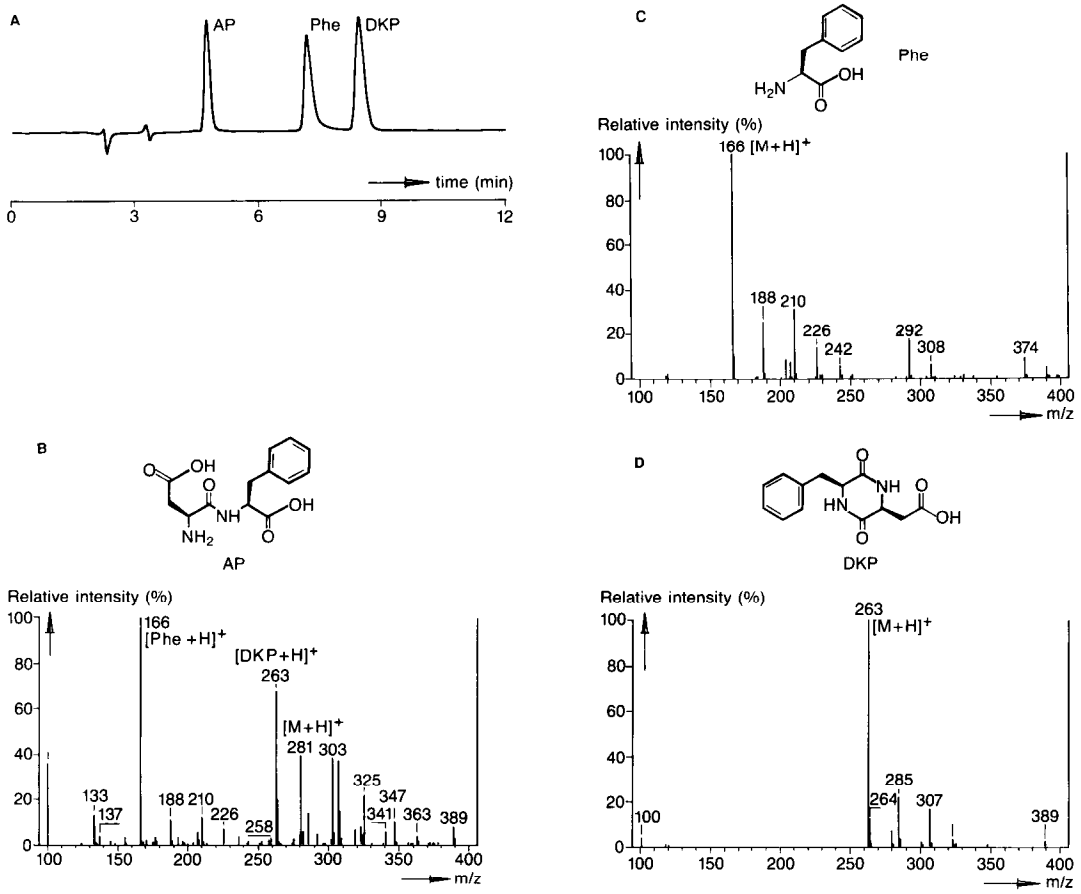

Fig. 2. (A) HPLC profile of a mixture of AP (1.3 μg), Phe (2.3 μg) and DKP (1.9 μg), and (B–D) the corresponding mass spectra. Mobile phase: 0.05 *M* ammonium bicarbonate (pH 7.0)–2.5% (v/v) acetonitrile.

mass spectra of all three compounds, several sodium and potassium adducts are present, *i.e.*, $[M + 22]^+$, $[M + 38]^+$ and $[M + 60]^+$. Since no sodium or potassium salts of the compounds are used, these ions probably come from the sodium buffer experiments on the HPLC column.

In all spectra, a peak of unknown identity at $[M + 127]^+$ is present.

As an example, the mass spectra of AP, DKP and Phe in ammonium bicarbonate buffer, together with the chromatographic separation, are given in Fig. 2.

For each buffer, maximum sensitivity for AP is obtained at different source and vaporizer temperatures.

The maximum S/N ratio of the quasimolecular ion of AP is found at vaporizer and source temperatures of 110 and 230°C for ammonium formate, 90 and 170°C for ammonium acetate and 110 and 200°C for ammonium bicarbonate, respectively. Ammonium formate gives the highest S/N ratio of the $[M + H]^+$ of AP compared with the other two buffers at their optimum conditions: S/N = 1790 (ammonium formate),

TABLE II

RELATIVE INTENSITIES AND S/N RATIOS OF AP (m/z = 281) IN THREE MOBILE PHASES

For mobile phases and concentration of AP, see Table I.

Mobile phase	RI[a] (%)	S/N[a] (%)	S/N
II	100	100	1430
IV	7.3	22	315
VI	1.8	73	1040

[a] Relative to ammonium formate (100%).

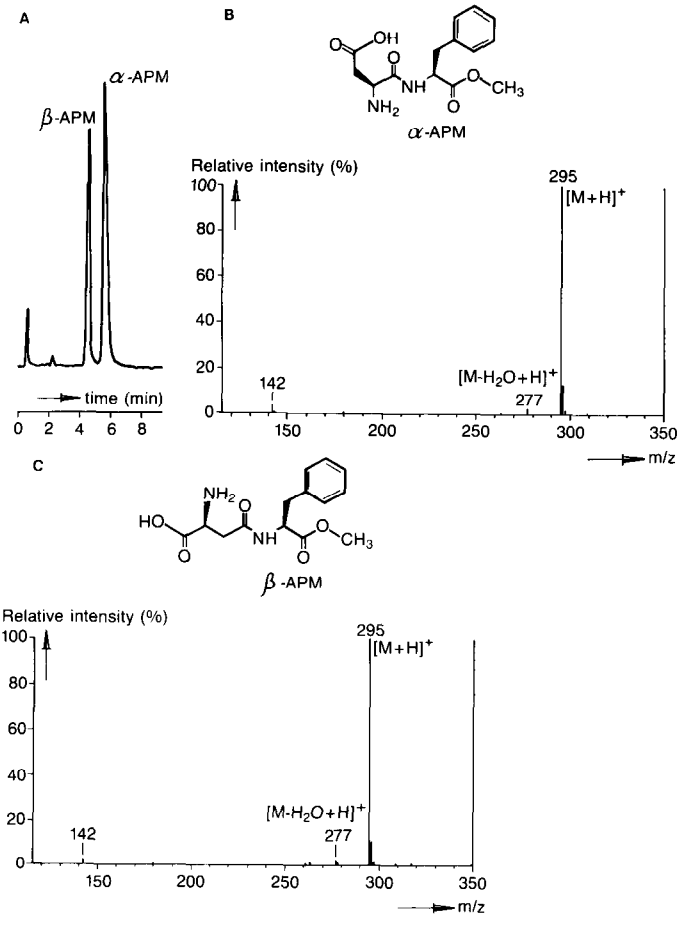

Fig. 3. (A) HPLC profile of the separation of α-APM (1.6 μg) and β-APM (2.2 μg), and (B and C) the corresponding mass spectra. Mobile phase: 50 mM ammonium formate buffer–8 mM TFA–10% (v/v) acetonitrile.

S/N = 1345 (ammonium acetate) and S/N = 1430 (ammonium bicarbonate) for 1.3 μg of AP.

Table II gives the relative intensity (RI), the S/N ratio and the relative S/N ratio of the quasimolecular ion of AP in the three mobile phases at a vaporizer temperature of 110°C and a source temperature of 200°C. From this table it appears that, depending on the mobile phase used, different values of RI are found. The variation in chemical noise between the various mobile phases is responsible for the difference in S/N ratio.

For the compounds studied, no correlation could be found between the S/N ratio and source temperature, or between S/N ratio and vaporizer temperature.

Choice of an ion-pairing agent for LC–TSP-MS

Several HPLC–UV methods have been described for the separation of α-APM and β-APM. Good resolution and peak shape have been obtained by using alkylsulphonates as ion-pairing agents in the mobile phase [9,10]. For LC–TSP-MS analysis of ionic compounds no volatile ion-pairing agent that can act as a substitute for alkylsulphonates has been reported to our knowledge. We studied the influence of TFA on the separation of α-APM and β-APM. A typical chromatogram is given in Fig. 3. Use of a 50 m*M* ammonium formate buffer (pH 3.0) and a 50 m*M* ammonium acetate buffer (pH 5.0), both without the addition of TFA, resulted in a poorer resolution than that obtained in Fig. 3.

The mass spectra of α-APM and β-APM are also shown in Fig. 3. Owing to the low concentration of TFA in the eluent, the background signal of TFA clusters is very low in the mass spectrum, and is in fact eliminated by background subtraction. There are no corresponding mass fragments from TFA and APM, so the detection level of APM is not affected by the addition of TFA.

CONCLUSIONS

With respect to the buffer capacity and chromatographic selectivity, the ammonium salts of formate, acetate and bicarbonate are suitable buffers for the LC–TSP-MS analysis of ionic degradation products of α-APM. For AP as test compound, the largest S/N ratio is obtained with ammonium formate as LC–TSP-MS buffer. Trifluororacetic acid is a good substitute for alkylsulphonates in the LC–TSP-MS analysis of α-APM and β-APM.

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