Mobile Phase Additives for LC-MS. Part 3: The Neutral Salts ..... This is the third article in a five part series on mobile phase additives for LC-MS to appear in each issue of Analytix in 2006

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Although organic acids are the most common mobile phase additive for HPLC separations that employ MS detection, it may be necessary under certain circumstances to use more neutral conditions, either because the analytes are sensitive to acids or do not exhibit optimal resolution at low pH. When acids are not suitable, volatile salts, like ammonium formate or ammonium acetate, may be the additives of choice (**Table 1**). However, compared to organic acids their use is much more complex. One issue is the limited solubility of the salts in organic solvents; another issue is the changing pH value during a gradient. On the other hand, the mildly acidic pH provided by the salts permits both positive and negative ion mode detection.

This short article will discuss the characteristics, benefits and practical use of the ammonium salts of acetic and formic acid as LC-MS mobile phase additives. All analytical conditions and test compounds were the same as described in part 1 of this series [1], except the concentration of raffinose, which was 100 ng/mL in this study. Additionally, a four peptide mixture of bradykinin analogues was used in one experiment. The salts were dissolved in the aqueous part of the mobile phase at a concentration of 0.1% w/v. The organic part of the mobile phase was used either without any additive or as ready-to-use LC-MS CHROMASOLV® blends also containing 0.1% w/v additive (**Table 2**).

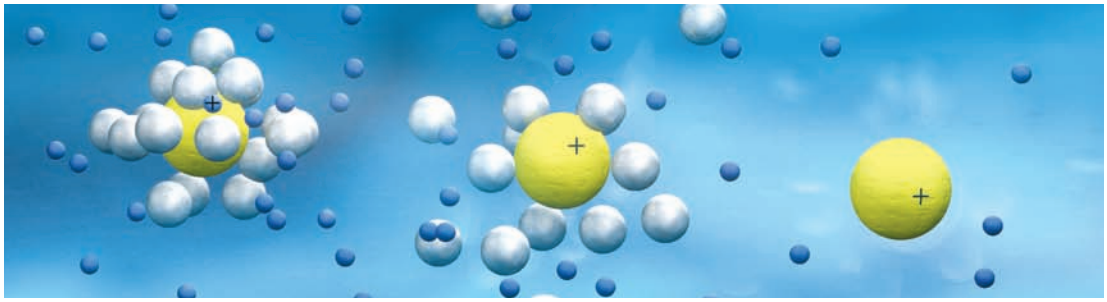


Table 1 ..... List of Sigma-Aldrich LC-MS additives

Cat. No.	Brand	Description*	Package Size	Packaging
40967	Fluka	Trifluoroacetic acid, puriss p.a., eluent additive for LC-MS	50 mL	HDPE bottle
40967	Fluka	Trifluoroacetic acid, puriss p.a., eluent additive for LC-MS	10 x 1 mL	Glass ampuls
56302	Fluka	Formic acid, puriss p.a., eluent additive for LC-MS	50 mL	HDPE bottle
49199	Fluka	Acetic acid, puriss p.a., eluent additive for LC-MS	50 mL	HDPE bottle
49916	Fluka	Propionic acid, puriss p.a., eluent additive for LC-MS	50 mL	HDPE bottle
55674	Fluka	Ammonium formate, puriss p.a., eluent additive for LC-MS	50 g	HDPE bottle
49638	Fluka	Ammonium acetate, puriss p.a., eluent additive for LC-MS	50 g	HDPE bottle
61333	Fluka	Sodium citrate tribasic dihydrate, puriss p.a., eluent additive for LC-MS	50 g	HDPE bottle
40867	Fluka	Ammonium bicarbonate, puriss p.a., eluent additive for LC-MS	50 g	HDPE bottle
44273	Fluka	Ammonium hydroxide solution 25%, puriss p.a., eluent additive for LC-MS	100 mL	HDPE bottle
65897	Fluka	Triethylamine, puriss p.a., eluent additive for LC-MS	50 mL	HDPE bottle

* "puriss" quality grade is defined as >98.5% assay, <0.1% ash, and specification n + 0.001, d + 0.001 with no extraneous color and a homogeneous appearance. "p.a." or pro analysi denotes a product with guaranteed trace impurity levels and/or suitability for the indicated analytical application.

Table 2 ..... Selection of LC-MS CHROMASOLV® blends

Cat. No.	Brand	Description	Package Size	Packaging
34674	Riedel-de Haën	Water with 0.1% ammonium acetate LC-MS CHROMASOLV®	2.5 L	amber bottle
34670	Riedel-de Haën	Methanol with 0.1% ammonium acetate LC-MS CHROMASOLV®	2.5 L	amber bottle
34669	Riedel-de Haën	Acetonitrile with 0.1% ammonium acetate LC-MS CHROMASOLV®	2.5 L	amber bottle
34668	Riedel-de Haën	Acetonitrile with 0.1% formic acid LC-MS CHROMASOLV®	2.5 L	amber bottle

The main issue when using ammonium acetate or ammonium formate as additives is their solubility, which is very good in water, sufficient in methanol to obtain a concentration of 0.1% w/v, but not in acetonitrile. This is a problem since acetonitrile is the organic solvent of choice for most separations. The effect is shown in **Figure 1**. When using pure acetonitrile as the organic part in gradient elution against 0.1% ammonium acetate in water, the apparent pH will rise and influence the separation, worsening it in most cases (curve A). The same is true when running a gradient with methanol containing 0.1% ammonium acetate in both solvents (curve B). To address the solubility issue, Sigma-Aldrich has developed a special blend (Cat. No. 34669, patent pending), which contains 0.1% w/v of ammonium acetate in acetonitrile stabilized with acid. This acid-stabilization has three desirable effects: the salt is kept in solution, the blend is stable against decomposition and the system is buffered. It also keeps the pH in the mildly acid range when using both the aqueous and organic components as buffered blends (curve C) and when using the acetonitrile blend not as intended, but with pure water as the aqueous solvent (curve D).

Figure 2 ..... EIC (positive ion mode) of test compounds with ammonium acetate as additive in both aqueous and organic components (conditions C in Figure 1)

Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine, and dark green is propazine.

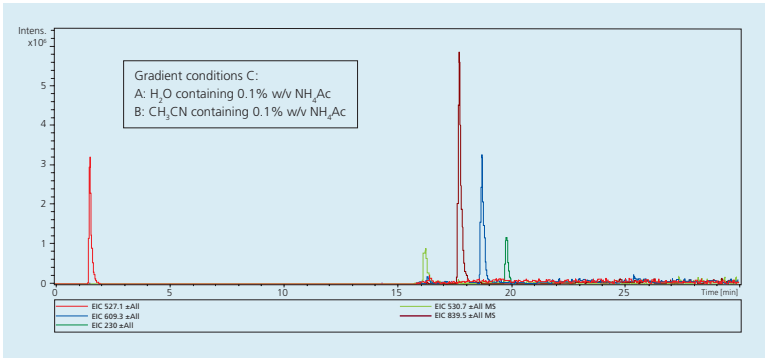
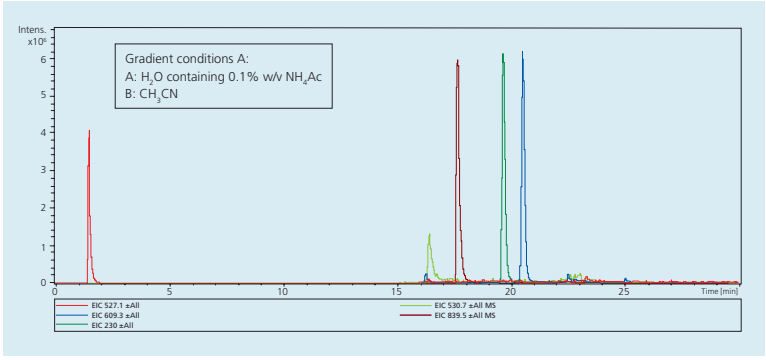


Figure 3 ..... EIC (positive ion mode) of test compounds with ammonium acetate in the aqueous component only (conditions A in Figure 1)

Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine, and dark green is propazine.



Besides affecting the apparent pH, using buffered mobile phase components has a significant impact on the separation and ionization of the test compounds in this study. Under conditions C (**Figure 2**) and conditions A (**Figure 3**), reserpine (blue peak) is shifted in retention time, and both reserpine and propazine (dark green) exhibit different degrees of ionization. The effect is even more pronounced on the four bradykinin analogues (**Figure 4**). Resolution was greatest using the buffered conditions C (upper trace). However, unfortunately it also had a higher tendency to form sodium adducts when using ion trap instruments compared to triple quads [2].

Similar observations are made for ammonium formate. **Table 3** lists the changes in pH when using 0.1%

Figure 1 ..... Apparent pH curves using buffered and unbuffered blends

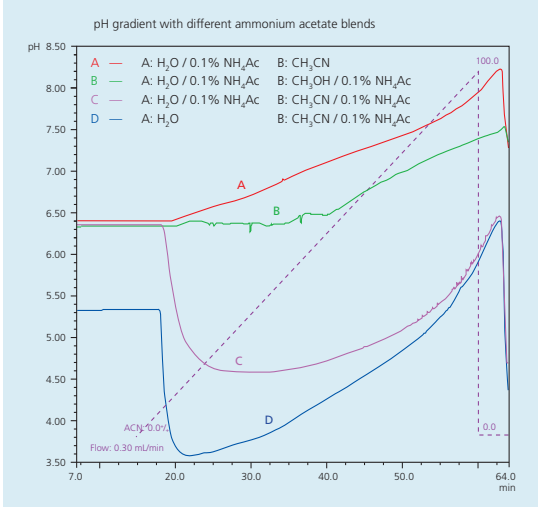


Figure 4 ..... EIC (positive ion mode) of bradykinin analogues with both components containing buffered ammonium acetate (upper trace) and with 0.1% ammonium acetate / acetonitrile, not buffered (lower) (conditions C and A in Figure 1)

1 = bradykinin 1-6, 2 = Lys-Ala^3-bradykinin, 3 = bradykinin, 4 = des-Arg^1-bradykinin, 5 = impurity

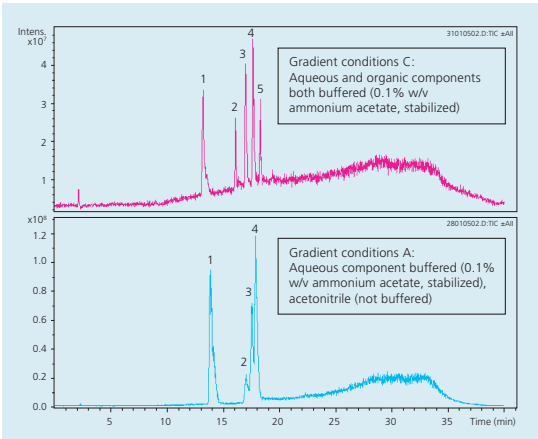


Figure 5 ..... EIC (positive ion mode) of test compounds with ammonium formate in the aqueous component only

Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine and dark green is propazine.

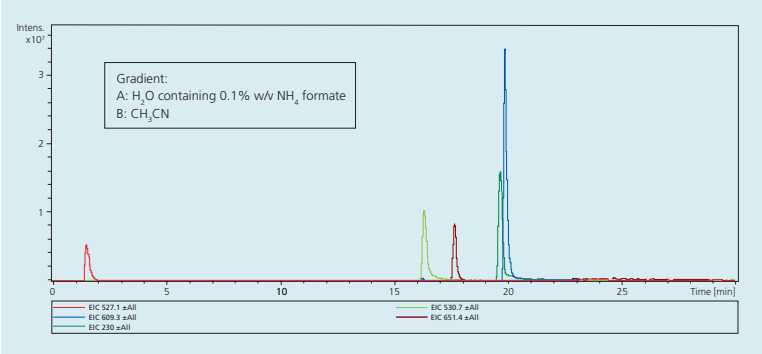


Figure 6 ..... EIC (negative ion mode) of test compounds with ammonium formate in the aqueous component only Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine; propazine is not detected in neg. ion mode.

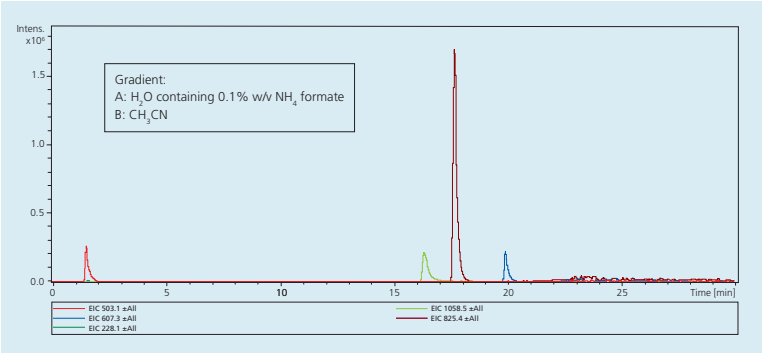


Figure 7 ..... EIC (positive ion mode) of test compounds with ammonium formate in the aqueous component and formic acid in the organic component

Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine and dark green is propazine.

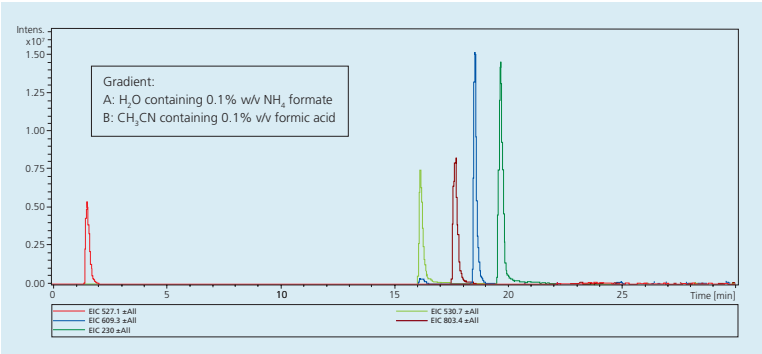
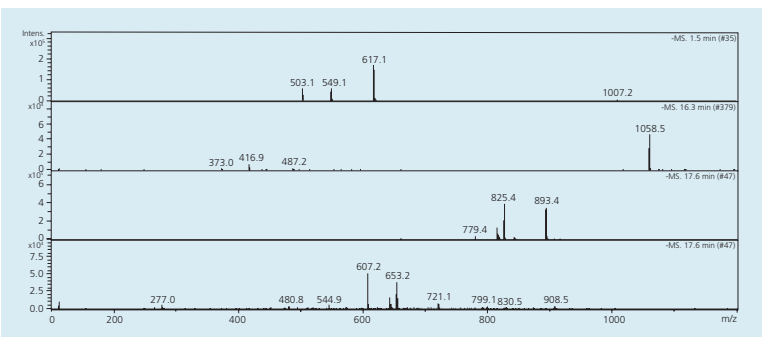


Figure 8 ..... Mass spectra of test compounds in negative ion mode shown in Figure 6



ammonium formate in gradients with either pure acetonitrile or with acetonitrile spiked with 0.1% formic acid (FA). This latter combination functions as kind of "on the fly buffering," which significantly affects the separation and ionization, although the apparent pH differences are not that dramatic.

Table 3 ..... pH change during gradient of acetonitrile against ammonium formate (aqueous component: 0.1% w/v ammonium formate in water)

% aqu.	% CH_3CN	pH CH_3CN	pH CH_3CN / 0.1% FA
100	0	6.3	6.3
50	50	6.9	6.7
10	90	7.5	7.1

Figure 5 shows the test mix separation using a gradient between 0.1% ammonium formate and pure acetonitrile. Under these conditions detection in negative ion mode is also possible, which often results in a more specific and less noisy signal (**Figure 6**). In **Figure 7** perfect resolution is achieved when using water with 0.1% w/v ammonium formate and acetonitrile with 0.1% v/v formic acid.

An interesting observation worthy of discussion are the mass spectra of the test components obtained in negative ion mode (**Figure 8**). The normal molecular ion is $[M-H]^-$, 503.2 for raffinose, 779.4 for digoxin, 607.3 for reserpine and 1058.6 for bradykinin. In this case only the singly charged molecular ion is observed for the peptide bradykinin, contrary to positive ion mode, where the doubly charged ion is dominant. For the other test compounds addition of one formate anion, $[M+45]^-$, is also observed.

In conclusion, the neutral volatile salts, ammonium acetate and ammonium formate, offer a much broader influence on analyte separation and ionization than do the acids. Their use, of course, is dictated by the particular LC-MS separation objectives or problems being addressed. Any limitations to their solubility may actually turn into the possibility of doing the separation or detection in a really unusual way.

References:

[1] "Mobile Phase Additives for LC-MS. Part 1: Acids – The Most Common Choice," Analytix 2006/2, 8-9.
(See also: "Mobile Phase Additives for LC-MS. Part 2: How to Overcome Suppression Effects of TFA," Analytix 2006/3, 16-17. Both downloadable from:
http://www.sigma-aldrich.com/analytix

[2] "Influence of solvent additive composition on chromatographic separation and sodium adduct formation of peptides in HPLC-ESI-MS", Poster at HPLC 2006 San Francisco, June 2006; will appear in J. Chromatogr. A, symposium issue.