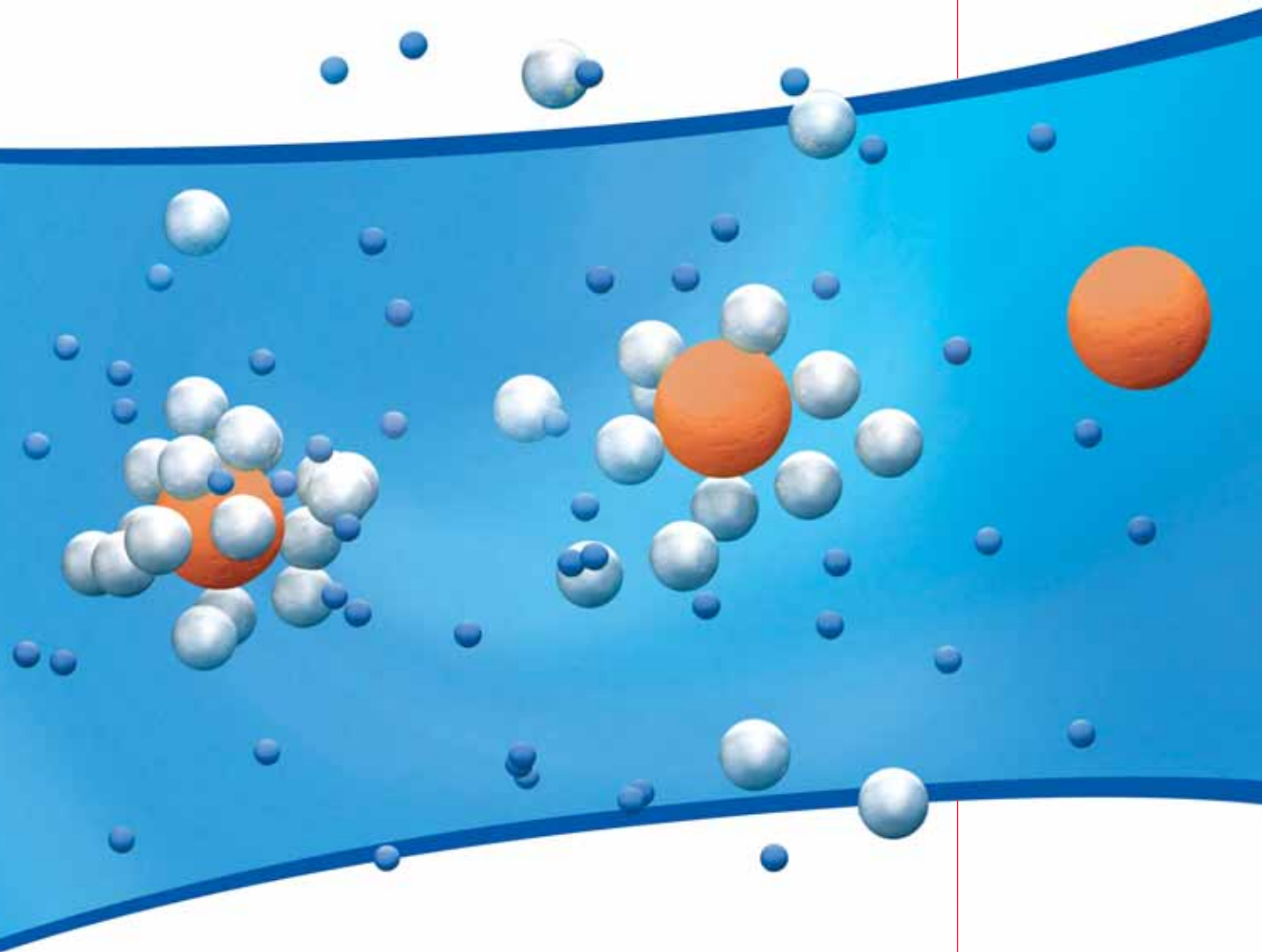


LC-MS

Mobile Phase Additives – Tips & Tricks



LC-MS Mobile Phase Additives – Tips & Tricks

LC-MS is fast becoming a routine fixture in today's well-equipped analytical laboratory. Along with the increased use of LC-MS have come instrumental, chemical and database methods aimed at increasing the sensitivity, specificity and speed of analysis of this invaluable technique. New ion sources, high-resolution LC systems and rapid mass spectrometers with enhanced ion optics and detectors have lowered the limits of detection, but have raised the bar on the purity expectations of chemicals used for sample preparation, mobile phases and post-column additives. Some notable examples of how the purity and composition of the chemicals used in LC-MS affect the analysis include the following:

- Polymers, including biopolymers like proteins and DNA, form adducts with inorganic salts which leads to complicated mass spectra and a broad distribution of multiply charged sodium, potassium and chloride adducts.
- Even with small molecules, salts can suppress ionization in ESI sources.
- Reagents, chemicals and devices used in sample preparation and post-column additives, like formic acid, always present the risk of contaminating the analysis.

Alkali ions, plasticizers and surfactants are particularly problematic as they are widespread and interfere strongly with LC-MS by causing higher background noise and the formation of adducts.

Because of the integral part that chemistry plays in a successful LC-MS analysis, Sigma-Aldrich has developed and introduced many solvents, additives and reagents that are specifically designed to meet the requirements of high-purity and consistency. This brochure contains a compilation of articles on LC-MS additives and the advantages of high purity solvents for both small and large molecule analysis.

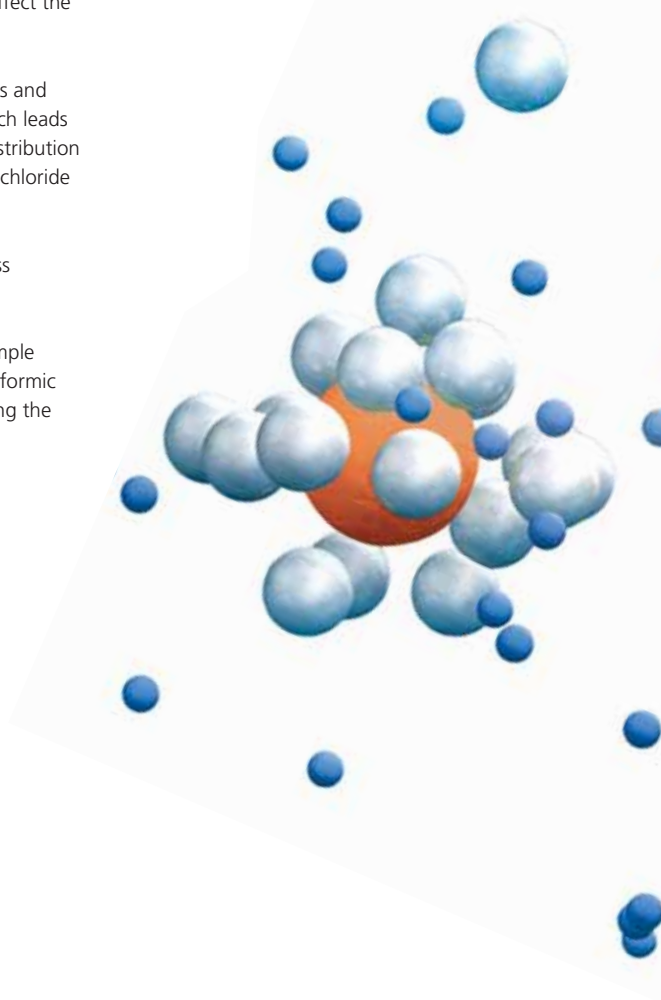


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Part 1 Mobile Phase Additives for LC-MS. Introduction

It is common practice in LC-MS to add certain chemicals to the mobile phase or introduce them post-column prior to the interface to influence analyte ionization. Most often, an improvement in the analyte signal is the goal. However, some additives may be used to suppress unwanted signals or selectively enhance the signal of particular compounds in a mixture, for example glycosidic species in a mixture of peptides.

Sigma-Aldrich, a leading supplier of chemicals for analytical applications, offers a wide range of high purity additives for LC-MS applications in addition to our pure CHROMASOLV® solvents and ready-to-use blends. Our offering includes the most commonly used acids, bases, volatile salts and a sodium source (see Table). All are of high purity, usually puriss p.a., and are tested for LC-MS application.

Product List LC-MS CHROMASOLV® Mobile Phase Additives

Cat. No.	Brand	Description*	Pack Size	Packaging
40967	Fluka	Trifluoroacetic acid, puriss p.a., eluent additive for LC-MS	50 mL 10 x 1 mL	HDPE bottle Glass ampoule
56302	Fluka	Formic acid, puriss p.a., eluent additive for LC-MS	50 mL 10 x 1 mL	HDPE bottle Glass ampoule
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 an homogeneous appearance. "p.a." or *pro analysi* denotes a product with guaranteed trace impurity levels and/or suitability for the indicated analytical application.

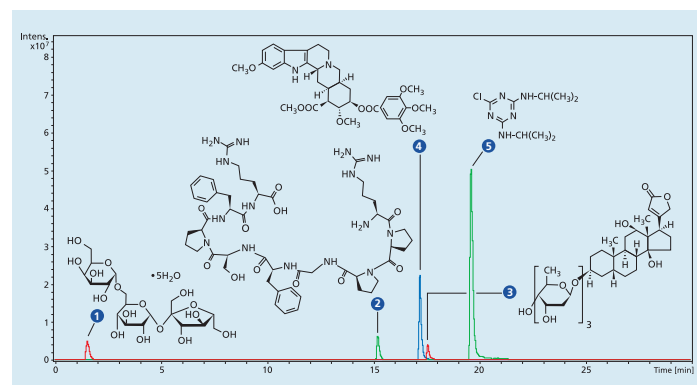


Figure 1 Extracted ion chromatogram of 5 test compounds with LC-MS CHROMASOLV 0.1% acetic acid as mobile phase additive; elution order: raffinose, bradykinin, reserpine, digoxin, propazine (For details, please see next issue of this newsletter)

The influence of these additives will be discussed and demonstrated in the next five articles in this brochure.

- Part 2: „Acids - The Most Common Choice“
- Part 3: „How to Overcome Suppression Effects of TFA“
- Part 4: „The Neutral Salts“
- Part 5: „Special Case - Sodium Adduct Formation“
- Part 6: „The Bases, Reverse Buffering, Negative and Reverse Ionization“

Product List Test compounds

Cat. No.	Brand	Compound	Class	Elution Order	Molecular Mass
83400	Fluka	Raffinose	Saccharides	1	504.2
15859	Fluka	Bradykinin	Peptides	2	1059.6
D6003	Sigma	Digoxin	Glycosides	3	780.4
13173	Fluka	Reserpine	Alkaloids	4	608.3
45640	Fluka	Propazine	Triazines	5	229.1

In each part of the series, we will demonstrate the effects and influences of additives belonging to a particular group on five model compounds which are representative of a typical class of analytes.

Part 2 Mobile Phase Additives for LC-MS. Acids – the Most Common Choice

In LC-MS certain chemicals are often added to the mobile phase or introduced post-column prior to the interface to influence analyte ionization. Small organic acids like formic and acetic acid are among the most commonly used additives (see **Table 1**). Their widespread use is derived from two fundamental reasons. First, many chromatographic separations benefit in terms of

retention and/or peak shape under acidic conditions, because any silanol activity is suppressed. Second, most mass spectrometric measurements are done in positive ion mode, which is accomplished by the addition of a proton to form the molecular ion $[M+H]^+$. The above mentioned organic acids have necessary acidity and volatility to provide an excess of cations for this purpose.

Table 1 Product List of LC-MS additives

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

*"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.

Test conditions

In this article, our aim is to demonstrate the effect on ionization, chromatographic and mass spectral behavior of some common acidic LC-MS mobile phase additives with five test analytes. **Table 2** lists the test compounds, their sum formula and mass in addition to the observed mass and its explanation. The additives were dissolved in both aqueous and organic mobile phase components at a concentration of 0.1%. The alternative method of adding them prior to the interface was not tested in this case. The MS was an ion trap (Bruker Esquire 3000+) operated in positive ion mode. Reliable MS-identification and quantification depends upon using MS-compatible HPLC columns and solvents to minimize background, reduce instrument fouling and maximize sensitivity. LC-MS CHROMASOLV® solvents and Supelco's Ascentis® and Discovery® HS columns meet these requirements.

HPLC Conditions

LC-MS column: Supelco Discovery HS C18, 15 cm x 2.1 mm, 5 µm particles (Cat. No. 568502-U)
 Mobile phase: A: Water (LC-MS CHROMASOLV, Cat. No. 39253), B: Acetonitrile (LC-MS CHROMASOLV, Cat. No. 34967)

Gradient profile:	Time (min.)	%A	%B
	0	100	0
	10	100	0
	20	0	100
	30	0	100

Flow rate: 0.4 mL/min
 Sample: Raffinose, bradykinin, digoxin, propazine each 10 ng/mL, reserpine, 5 ng/mL
 Injection volume: 5 µl

Table 2 Test compounds

Cat. No.	Brand	Compound	Formula	Molecular Mass (monoisotopic)	Observed Mass	Explanation
83400	Fluka	Raffinose	C ₁₈ H ₃₂ O ₁₆	504.2	527.2	[M+Na] ⁺
B3259	Sigma	Bradykinin	C ₅₀ H ₇₃ N ₁₅ O ₁₁	1059.6	530.8	[M+2H] ²⁺
37100	Fluka	Digoxin	C ₄₁ H ₆₄ O ₁₄	780.4	803.4 651.3	[M+Na] ⁺ [M-Digitoxose] ⁺
13173	Fluka	Reserpine	C ₃₃ H ₄₀ N ₂ O ₉	608.3	609.3	[M+H] ⁺
45640	Fluka	Propazine	C ₉ H ₁₆ N ₅ Cl	229.1	230.1	[M+H] ⁺

Figure 1 EIC without mobile phase additives

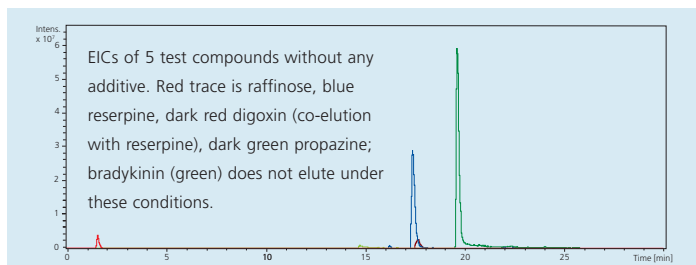


Figure 2 EIC with acetic acid additive

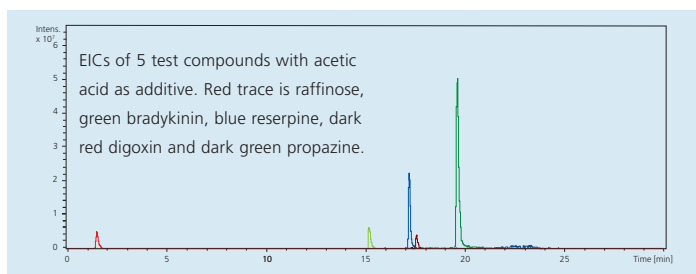


Figure 3 EIC with formic acid additive

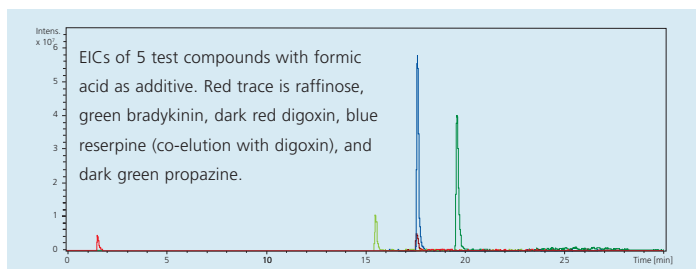


Figure 4 Mass spectrum of propazine with and without acidic additives

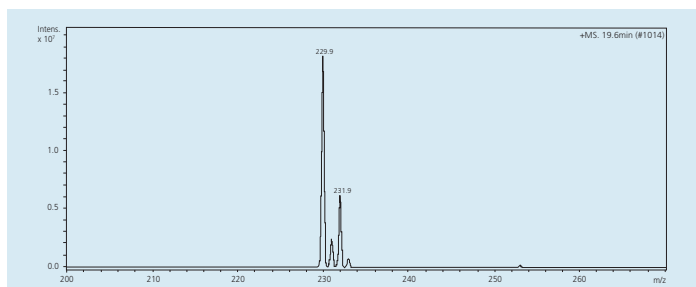
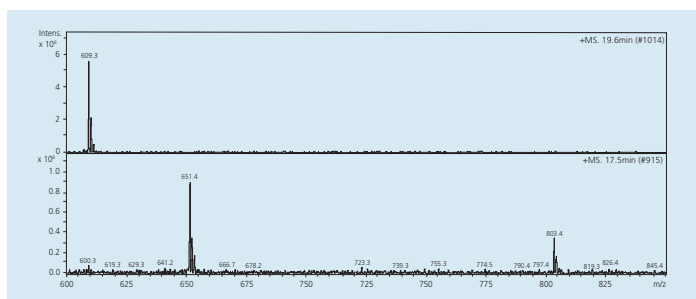


Figure 5 Mass spectra of reserpine (upper) and digoxin (lower) with acetic acid as additive



Effect of acidic additives on chromatography and sensitivity

When used as a mobile phase additive, acids impact the retention behavior of pH-sensitive compounds, especially reserpine where slight pH changes shift its position relative to digoxin and even cause co-elution. The chromatograms always consist of the extracted ion chromatograms (EIC) of the observed mass of each compound. If the observed mass changes due to conditions, the EIC is adjusted accordingly. The y-axis is a measure of the extent of ionization and the achieved sensitivity, and is therefore kept constant in most examples. **Figures 1 – 3** show the effect of acidic additives on the EIC of the test compounds.

Without acidic additives in the mobile phase (**Fig. 1**) elution and ionization of bradykinin and digoxin are insufficient. However, the ionization of propazine is slightly better than under acidic conditions. It will be shown later in the series that neutral conditions are preferred for the ionization of propazine.

Fig. 4 shows the mass spectrum of propazine with the typical chlorine isotopic pattern and the addition of one H⁺-ion; the theoretical observed mass is calculated with 230.1 Da. This spectral behavior does not change with the addition of acids, only the extent of ionization.

Resolution of reserpine and digoxin is accomplished by adding acetic acid (**Fig. 2**), which gives a pH of 3.3 to the aqueous portion of the mobile phase. Improved resolution results from a slight retention time shift of reserpine, sharper peaks and a change in the ionization of digoxin. In **Fig. 1** (no additives) the observed mass was the sodium adduct $M = 803.4 [M+Na]^+$, whereas with acetic acid as additive (**Fig. 2**) $M = 651.3 [M-Dig]^+$ is the most abundant mass and is actually a fragment, originating from the removal of one digitoxose unit. Reserpine shows the typical behavior of adding one H⁺-ion. **Fig. 5** shows the mass spectra of reserpine (upper) and digoxin (lower) when acetic acid is added to the mobile phase.

Effect of formic acid addition, which gives a pH of 2.7 to the aqueous mobile phase component, is shown in **Figure 3**. Adding formic acid increases the reserpine signal and changes the relative elution of digoxin and reserpine again. The effect on raffinose and bradykinin is only small.

Conclusions

Volatile, low molecular weight organic acids are commonly used as additives in LC-MS. Their primary advantage is that they improve ionization and resolution of a wide range of molecules.

Part 3 Mobile Phase Additives for LC-MS. How to Overcome Suppression Effects of TFA

Mobile phases for HPLC of proteins and peptides usually contain trifluoroacetic acid (TFA) to control the pH and improve peak shape and resolution. TFA enhances retention by ion pairing with the peptide and improves peak shape by reducing silanol interactions (1). However,

TFA has adverse effects on MS detection. Its high surface tension prevents efficient spray formation and TFA ions in the gas phase ion-pair with the peptide's basic groups suppressing their ionization and reducing the MS signal (2, 3, 4). When TFA cannot be avoided, its effects can be mitigated by additional use of other acids, like formic or propionic acid, either post-column or as so called triple blends (**Tables 1 and 2**).

Table 1 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 Ampoules
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
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 solvents and blends

Cat. No.	Brand	Solvent or Blend Description	Package Size	Packaging
34965	Fluka	2-Propanol Chromasolv LC-MS	1 L, 2.5 L	Amber glass bottle
34677	Fluka	Water with 0.1% formic acid and 0.01% TFA	2.5 L	Amber glass bottle
34676	Fluka	Acetonitrile with 0.1% formic acid and 0.01% TFA	2.5 L	Amber glass bottle

Table 3 Components of the peptide mixture

Cat. No.	Brand	Component	Molecular Mass	Mol. ion / Charge
B4181	Fluka	Bradykinin fragm. 1-7	756.4	[M+H] ⁺ / 1
A8846	Fluka	Angiotensin II	1045.5	[M+2H] ²⁺ / 2
P2613	Fluka	P ₁₄ R	1532.9	[M+2H] ²⁺ / 2
A8346	Fluka	ACTH fragm. 18-39	2464.2	[M+3H] ³⁺ / 3
I 6154	Fluka	Insulin oxid. B chain	3493.7	[M+3H] ³⁺ / 3

All analytical conditions and test compounds were the same as already described in the first article (5), using TFA as additive instead or the triple blends as solvents respectively. Propionic acid was added post column / pre electrospray via T-piece as a 10% solution in 2-propanol. For additional experiments, a peptide mixture (pepmix) was prepared to study the specific influence on this kind of separation. The test compounds and the pepmix were both separated on a Supelco Discovery HS C18 column, 15 cm x 2.1 mm ID, 5 μm particle size; the 5 components (peptides) of the pepmix are listed in **Table 3**. MS-EIC conditions are the same for all chromatograms.

Figure 1 shows the separation without any additive. Under these conditions, the basic peptide bradykinin is barely distinguishable from the baseline. Its mass spectrum can still be obtained (**Figure 2**, lower), showing the doubly-charged molecular ion [M+2H]²⁺ with m=1061.6 or m/z = 530.8. Raffinose is unaffected by adding TFA or other organic acids. Its spectrum (**Figure 2**, upper) shows the H⁺ (505 m/z) and NH₄⁺ adducts (522.1 m/z) and the high abundance Na⁺ adduct (527.1 m/z).

Addition of 0.1% TFA (**Figure 3**, top) causes all five test compounds to elute as well separated and sharp peaks. However, note that sensitivity drops almost 10-fold. The suppression effect is reduced by using 0.1% TFA and adding propionic acid (10% in 2-propanol) post-column (**Figure 3**, middle), an effect described in detail by Apffel et al. (2). Using the triple-blend of 0.1% formic acid/0.01% TFA (**Figure 3**, lower) greatly improves the signal, but with a compromise. Compared to TFA alone, resolution is poorer; and compared to formic acid alone (see previous part on acidic additives), sensitivity is poorer.

The three additives can be used in synergy, by balancing their benefits and limitations. Add small amounts of TFA to formic or propionic acid to improve peak shape; reduce TFA and add formic or propionic acid to improve the MS signal. Other MS and chromatographic para-

Figure 1

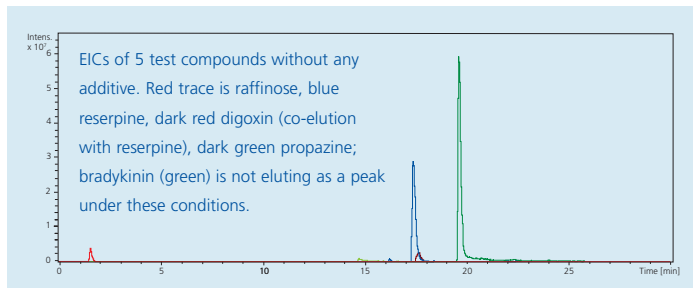


Figure 2

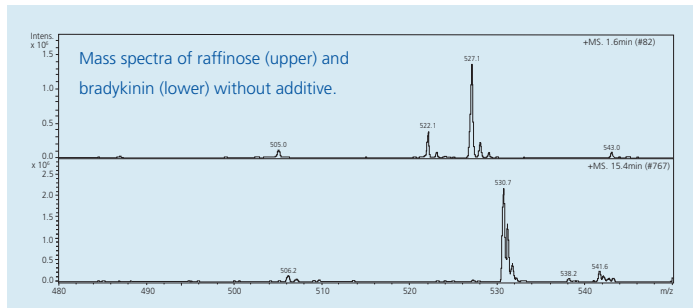
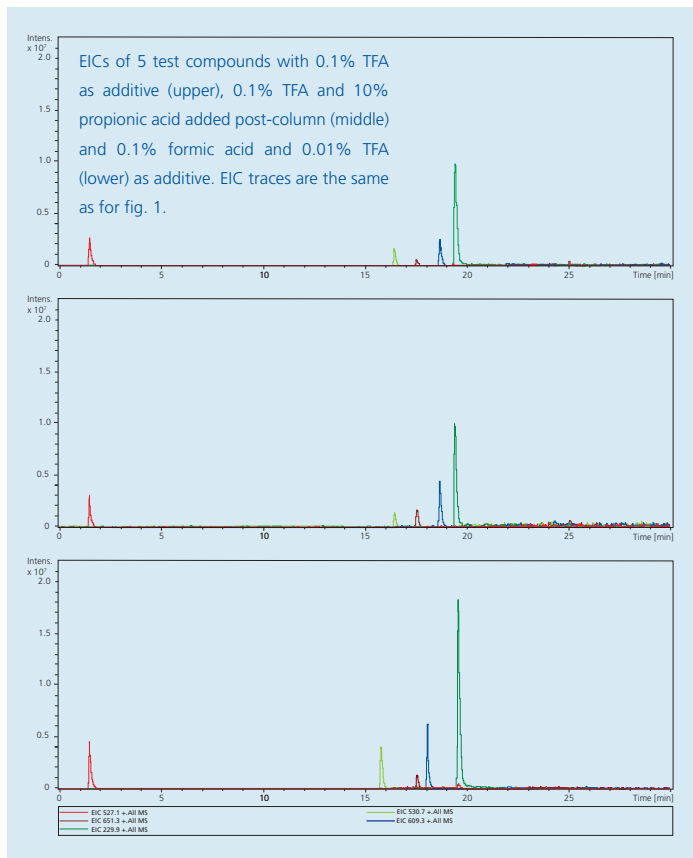


Figure 3

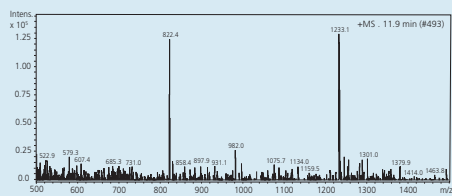


eters also influence this choice, including analyte type, column packing material and dimensions, length of mixing zone, flow rate, etc. (1, 2). This is especially true for peptide separations. The charge state of the molecular ion is not affected by this and varies in the pepmix between singly-charged (Bradykinin fragment 1-7) and triply-charged (insulin oxidized B chain) (Table 3). Depending on instrument and conditions it may be the case that one peptide appears in more than one charge state, i.e. doubly- and triply-charged in one spectrum (Figure 4).

In summary, the ionization-suppressing effects of TFA can be partly overcome by addition of other LC-MS compatible organic acids, like formic or propionic acid. For convenience and to guarantee reliable composition, Sigma-Aldrich offers pre-blended LC-MS mobile phases that contain acidic additives in high purity LC-MS CHROMASOLV® grade solvents. Our triple blends contain TFA with formic acid to provide both MS sensitivity and chromatographic performance.

Figure 4

Mass Spectrum of ACTH fragm. 18-39 with 0.1% TFA as additive and 10% propionic acid post-column. Doubly (m/z 1233.1) and triply (m/z 822.4) charged molecular ions.



References

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- [2] A. Apffel, A.; Fisher, S.; Goldberg, G.; Goodley, P. C.; Kuhlmann, F.E.; *J. Chromatogr. A*, 1995, 712, 177-190.
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- [4] Wang, G; Cole, R. B.; *J. Am. Soc. Mass Spectrom.*, 1996, 7(10), 1050-1058.
- [5] "Mobile Phase Additives for LC-MS. Part 1: Acids – The Most Common Choice" *Analytix* 2006/2, 8-9;
www.sigma-aldrich.com/analytix

Part 4 Mobile Phase Additives for LC-MS. The Neutral Salts

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 2 of this brochure on acid additives, 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 ampules
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
73594	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	Fluka	Water with 0.1% ammonium acetate LC-MS CHROMASOLV	2.5 L	amber bottle
34670	Fluka	Methanol with 0.1% ammonium acetate LC-MS CHROMASOLV	2.5 L	amber bottle
34669	Fluka	Acetonitrile with 0.1% ammonium acetate LC-MS CHROMASOLV	2.5 L	amber bottle
34668	Fluka	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.

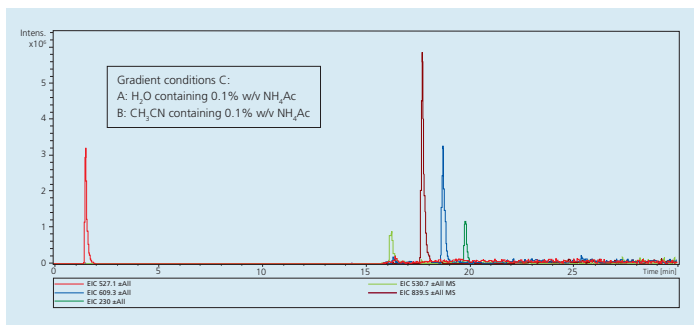
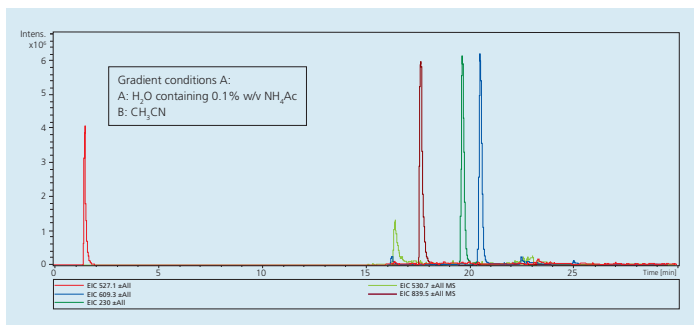


Figure 3 EIC (positive ion mode) of test compounds with ammonium acetate in the aqueous component only (conditions A in Figure 1)
Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine, and dark green is propazine.



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). Unfortunately, however, 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% ammonium formate in gradients with either pure

Figure 1 Apparent pH curves using buffered and unbuffered blends

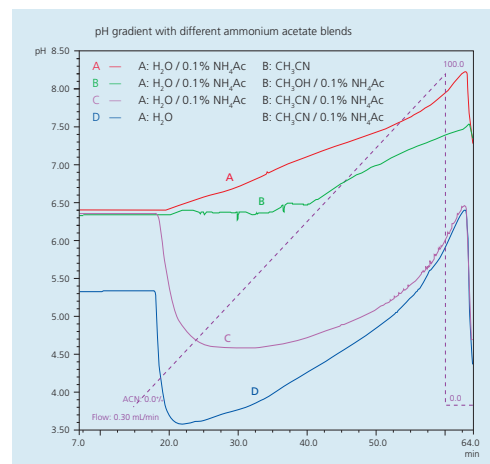


Figure 4 EIC (positive ion mode) of bradykinin analogues with both components containing buffered ammonium acetate (upper trace) and with 0.1% ammonium acetate / acetonitrile, not buffered (lower) (conditions C and A in Figure 1)

1 = bradykinin 1-6, 2 = Lys-Ala³-bradykinin, 3 = bradykinin, 4 = des-Arg¹-bradykinin, 5 = impurity

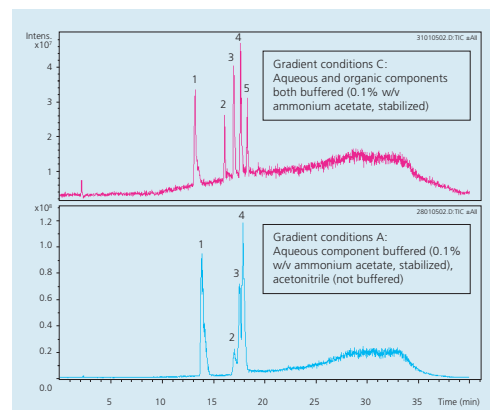


Figure 5 EIC (positive ion mode) of test compounds with ammonium formate in the aqueous component only

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

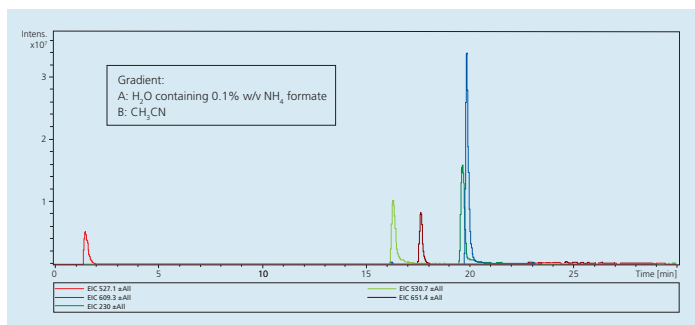


Figure 6 EIC (negative ion mode) of test compounds with ammonium formate in the aqueous component only Red trace is raffinose, green is bradykinin, dark red is digoxin, blue is reserpine; propazine is not detected in neg. ion mode.

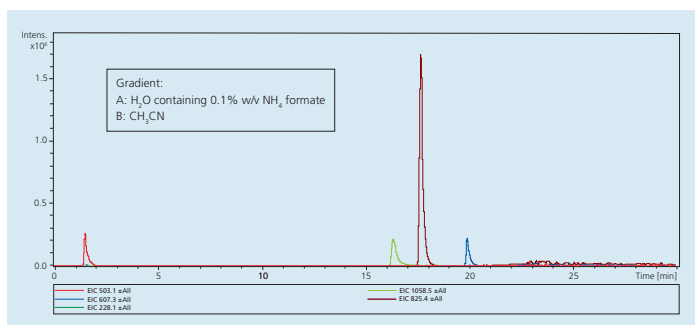


Figure 7 EIC (positive ion mode) of test compounds with ammonium formate in the aqueous component and formic acid in the organic component

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

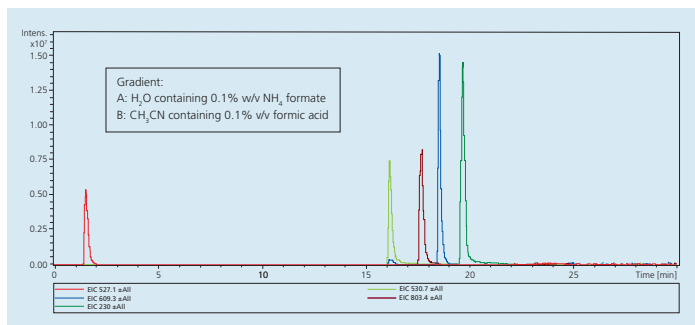
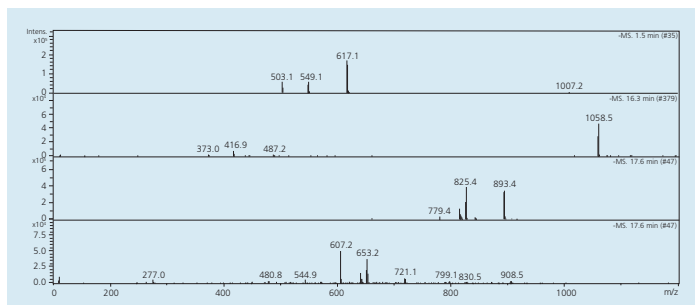


Figure 8 Mass spectra of test compounds in negative ion mode shown in Figure 6



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 ₃ CN	pH CH ₃ CN	pH CH ₃ CN / 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% w/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.

Part 5 Mobile Phase Additives for LC-MS. Special Case – Sodium Adduct Formation



Although sodium and other alkali metals are typically avoided in LC-MS, it can be useful to add sodium salts when the analyte ions have a very high tendency to form alkali adducts. This is especially true for the carbonyl group of sugars and glycosides, in addition to hydroxyl and carboxyl groups. These groups often form sodium adducts even under acidic conditions, e.g. with formic acid, because sodium is nearly always present at

trace levels. Adduct formation is associated with a decrease in sensitivity, and often simultaneous addition of H^+ , NH_4^+ and Na^+ is observed. When adduct formation tendency is strong, addition of small and defined amounts of sodium ions (mostly post-column) can help to obtain uniform and stable molecular ions for detection in LC-MS.

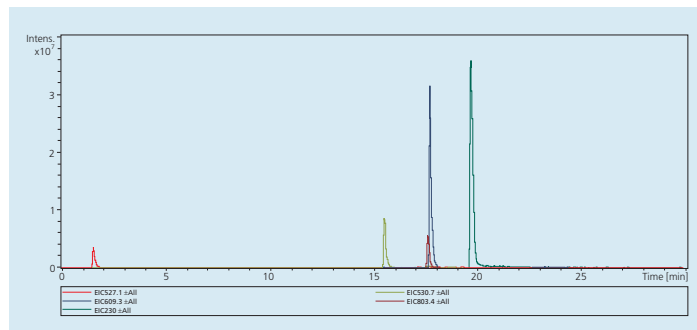
Table 1 List of Sigma-Aldrich LC-MS additives

Cat. No.	Brand	Description*	Package Size	Packaging
40967	Fluka	Trifluoroacetic acid, puriss p.a., eluent additive for LC-MS	50 mL 10 x 1 mL	HDPE bottle Glass ampuls
56302	Fluka	Formic acid, puriss p.a., eluent additive for LC-MS	50 mL 10 x 1 mL	HDPE bottle Glass ampuls
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.

Figure 1 EIC (positive ion mode) of test compounds with 0.1% w/v formic acid as mobile phase additive (no sodium added).

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



In this article, we will discuss the benefits and practical procedures for using sodium salts as LC-MS mobile phase additives. LC-MS CHROMASOLV® solvents and Supelco Discovery® HS C18 HPLC columns were used to meet the stringent requirements of LC-MS. These, and the MS conditions, test compounds and mobile phase (water-acetonitrile gradient with 0.1% formic acid) were as described earlier in this brochure (Part 2). In this study, an additional series of experiments was run with a concentration of 100 ng/μL raffinose. A 0.1% w/v aqueous solution of sodium, as acetate or citrate salt, was added post-column. The sodium citrate solutions were adjusted to pH 7.8, 5.0 or 3.1. Taking flow rate into account, the total concentration of sodium salt introduced into MS was 0.001% w/v (0.1 mM). It is important to keep the sodium concentration at 1 - 5mM or below, otherwise some MS instruments will be overloaded and show a dramatic loss of sensitivity caused by reversible suppression. The sodium citrate and other high-purity Fluka-brand LC-MS additives from Sigma-Aldrich appear in **Table 1**.

Figure 1 shows the initial separation of the five test compounds with formic acid as the sole additive. Co-elution of digoxin and reserpine is observed with reserpine having much higher intensity. Raffinose and digoxin exhibit formation of the sodium adduct of the molecular ion in the mass spectrum without any addition of sodium ions.

To observe the effect of addition of sodium ions, a 0.1% w/v aqueous solution of sodium acetate was added post-column. Results appear in **Figure 2**. By adding the sodium, the absolute intensity is reduced, and the relative intensity of digoxin and reserpine is reversed. The signal from the glycoside digoxin is boosted by addition of sodium, whereas the other compounds either lose intensity or stay constant.

The selective increase of the glycoside signal is also observed when sodium citrate is added. The influence of pH was also studied using sodium citrate. **Figure 3**

Figure 2 EIC (positive ion mode) of test compounds with 0.1% w/v formic acid in the mobile phase, and 0.1% w/v sodium acetate added post-column.

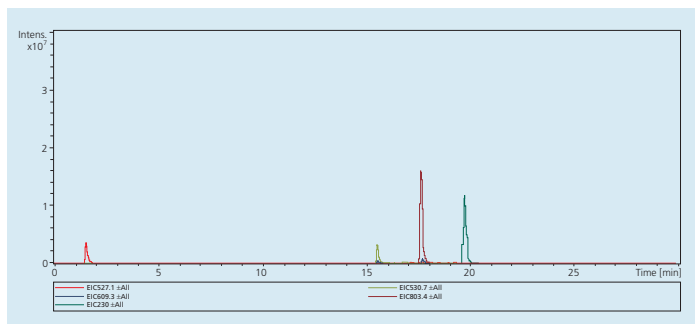


Figure 3 EIC (positive ion mode) of test compounds with 0.1% w/v formic acid in the mobile phase, and 0.1% w/v sodium citrate (pH 5.0) added post-column.

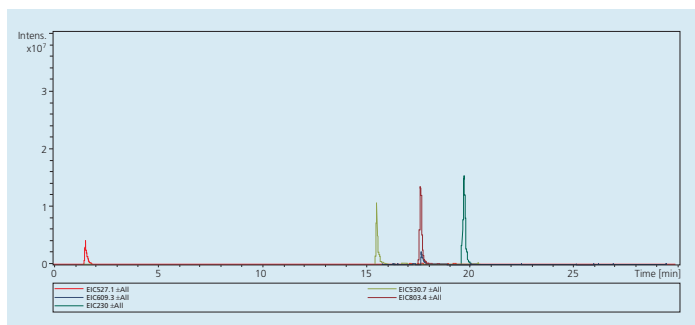


Figure 4 Signal intensities for the 5 test compounds with sodium addition under different conditions (anion and pH). Raffinose at 10 ng/μL.

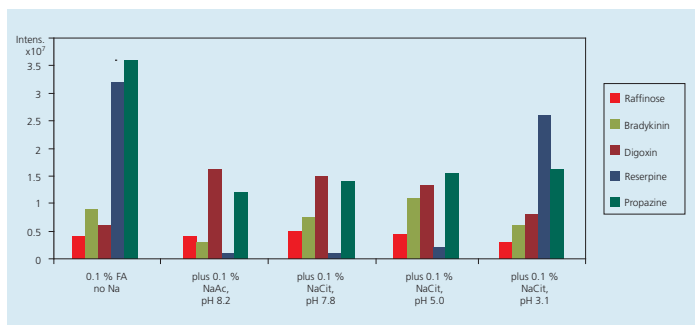
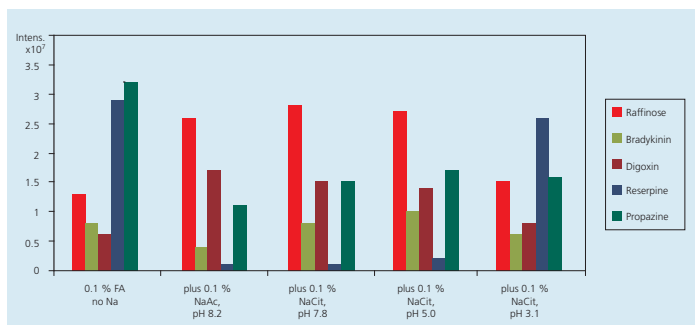


Figure 5 Signal intensities for the 5 test compounds with sodium addition under different conditions (anion and pH). Raffinose at 100 ng/μL.



shows the effect of addition of sodium citrate at pH 5.0. While the digoxin signal is reduced slightly, the bradykinin signal is increased significantly. This is only one example of how pH, together with addition of sodium ions, can influence analyte ionization. The phenomenon is attributed to a competition between the different ions within the electrospray interface.

Further experiments were carried out with sodium citrate solutions at different pH values and two different raffinose concentrations, 10 and 100 ng/μL. At low concentrations (10 ng/μL, **Figure 4**), the raffinose signal shows no dependence on pH. However, at higher raffinose concentrations (100 ng/μL, **Figure 5**), the signal is influenced by pH, exhibiting a maximum at pH 7.8. For digoxin, the maximum signal is obtained in sodium acetate at pH 8.2. Both raffinose and digoxin are highly susceptible to sodium adduct formation. Bradykinin is not susceptible to adduct formation, but its signal is also influenced by pH (hydrogen ion concentration) and the presence of sodium ions. Propazine and reserpine both go through a broad minimum when Na⁺ concentration is high and H⁺ concentration is low.

But sensitivity is only part of the story. Stability, and perhaps specificity, of the molecular ion are also considerations. The ability to form alkali adducts is useful for quantifying certain classes of molecules, e.g. formation of Cs⁺ adducts for digoxin and digitoxin or immunosuppressive drugs [2, 3] and the determination of ionophores by addition of Li⁺, Na⁺ or K⁺ [4]. It should also be possible to selectively enhance the LC-MS signals of glycopeptides within a peptide mixture with Na⁺. Truly, the use of alkali ions, particularly sodium ions, to enhance LC-MS sensitivity or selectivity has not been explored to the limits as yet.

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- [1] "Mobile Phase Additives for LC-MS. Part 1: Acids – The Most Common Choice", *Analytix* 2006/2, 8-9. Downloadable from: <http://www.sigma-aldrich.com/analytix>
- [2] Kaiser, P.; Kramer, U.; Meissner, D.; Kress, M.; Wood, W. G.; Reinauer, H. Determination of the Cardiac Glycosides Digoxin and Digitoxin by Liquid Chromatography Combined with Isotope-Dilution Mass Spectrometry (LC-IDMS) – a Candidate Reference Measurement Procedure, *Clin. Lab.* **2003**, *49(7/8)*, 329 – 343.
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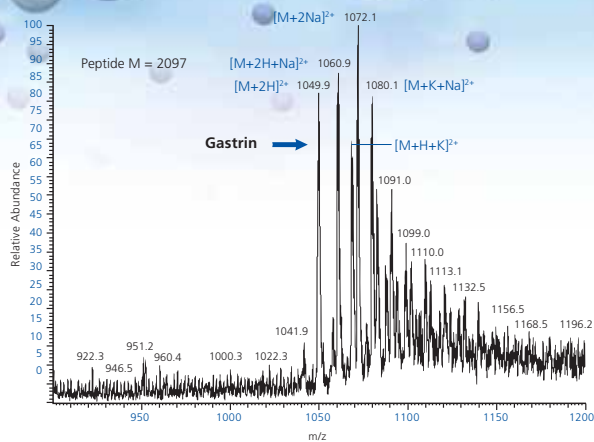
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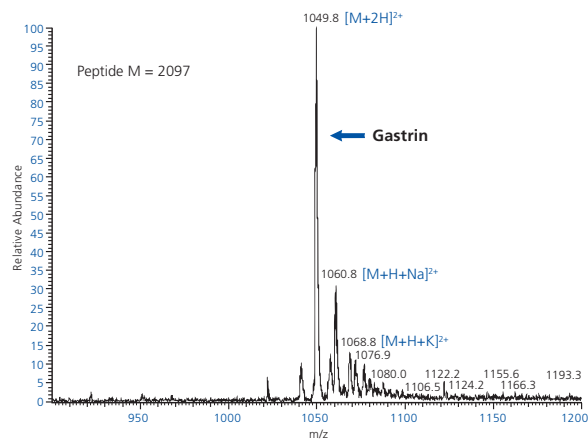
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Part 6 Mobile Phase Additives for LC-MS. The Bases, Reverse Buffering, Negative and Reverse Ionization



LC-MS analysis most often is run in positive ion mode using additives that support it, like organic acids and their ammonium salts. However, surprising possibilities exist outside this conventional approach. Basic additives are among the most interesting yet well-kept secrets in LC-MS. Basic pH is a good precondition for negative ionization (forming anions), but positive ionization (forming cations) is also possible. Under basic conditions, positive ionization would be the “reverse ionization,” whereas negative ionization here is the “straight ionization.” When the chromatographic resolution is best under acidic conditions, but sensitivity better in negative ion mode under basic conditions, the so-called “reverse buffering” can be useful, wherein a basic additive is added post-column via T-piece. Basic additives offer a much wider range of ionization capabilities than other additives, although they are somewhat compound-specific. Also, care must be taken in choosing an HPLC column that can withstand the high pH values that may occur when the basic additive is contained in the mobile phase.

For all figures
red trace is raffinose,
green is bradykinin,
dark red is digoxin, blue is
reserpine and dark
green is propazine.

Figure 1 EIC of test compounds with 0.1% w/v ammonium bicarbonate as mobile phase additive (positive ion mode = reverse ionization)

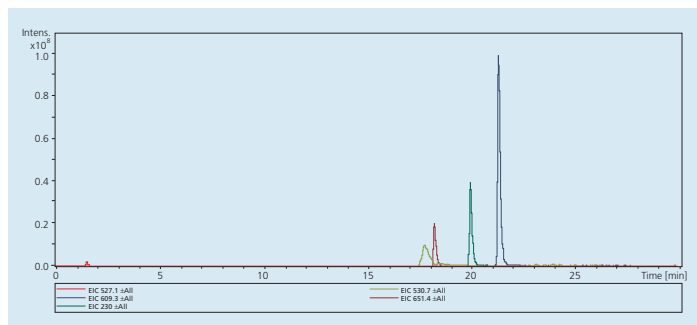


Table 1 List of Sigma-Aldrich LC-MS additives

Cat. No.	Brand	Description*	Package Size	Packaging
40967	Fluka	Trifluoroacetic acid, puriss p.a., eluent additive for LC-MS	50 mL 10 x 1 mL	HDPE bottle Glass ampuls
56302	Fluka	Formic acid, puriss p.a., eluent additive for LC-MS	50 mL 10 x 1 mL	HDPE bottle Glass ampuls
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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 pH values of basic (alkaline) blends

Solvent	additive	pH
Water	0.1% ammonium bicarbonate	8.0
Acetonitrile	0.1% ammonium bicarbonate (8% water)	10.2
Water	0.1% ammonia	11.2
Methanol	0.1% ammonia (0.3% water)	10.9
Acetonitrile	0.1% ammonia (0.3% water)	12.1
Water	0.1% triethylamine	11.4
Acetonitrile	0.1% triethylamine	12.4

Figure 2 EIC of test compounds with 0.1% w/v ammonium bicarbonate as mobile phase additive (negative ion mode = straight ionization)

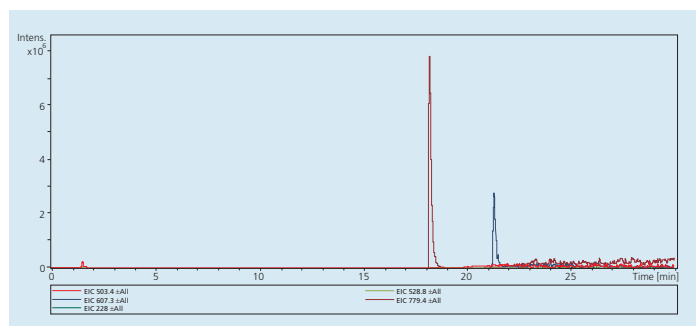


Figure 3 EIC of test compounds with 0.1% w/v ammonia as mobile phase additive (positive ion mode = reverse ionization)

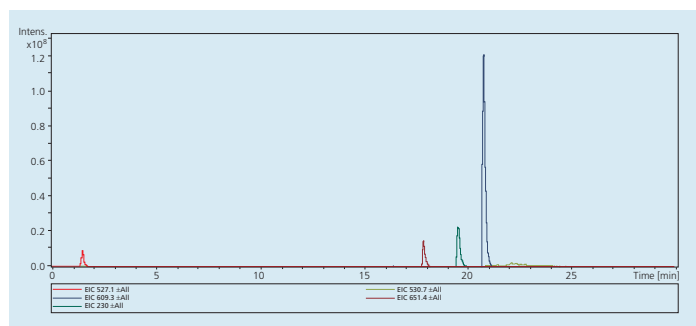


Figure 4 EIC of test compounds, no additive in HPLC, 10% w/v ammonia added via T-piece post-column (negative ion mode = straight ionization)

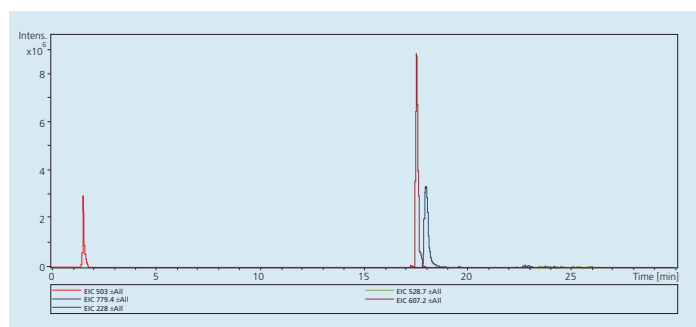
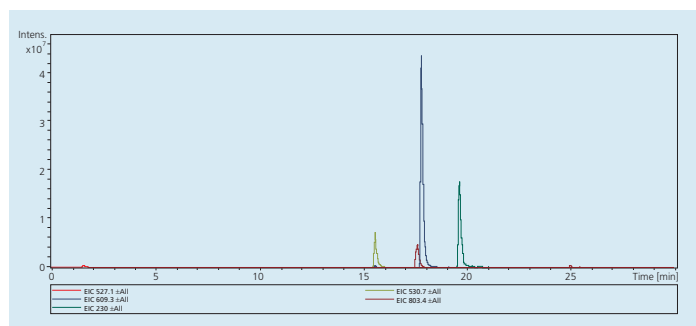


Figure 5 EIC of test compounds, 0.1% FA as additive in HPLC, 10% w/v ammonia added via T-piece post-column (reverse buffering, positive ion mode = reverse ionization)



In this section we will describe some basic additives and experimental setups for their introduction. LC-MS-quality grades of three basic (alkaline) additives are offered by Sigma-Aldrich: ammonium bicarbonate, ammonium hydroxide solution (ammonia) and triethylamine (**Table 1**), listed in order of increasing basic strength (**Table 2**). Ammonium bicarbonate is used for separation and detection of amines and polar compounds under mildly basic conditions. Ammonia solution (ammonium hydroxide) is used for alkaline separation and for post-column addition via T-piece. Triethylamine is mainly used to obtain alkaline conditions for lipophilic compounds. As in earlier sections [1-4], we used the test mixture of the five model compounds, the same HPLC instrument (Agilent 1100) and MS detector (Bruker Daltonics esquire3000plus ion trap), and an electrospray-compatible flow rate of 0.4 mL/min through the column. For separations under alkaline conditions we used high pH-stable columns with bridged C18 material, for separations under neutral or acidic conditions (with post-column addition of basic additives) a Supelco Discovery® C18, 15 cm x 2.1 mm I.D., 5 µm, the latter giving better selectivity and resolution. Additionally an accelerated separation of narcotic drugs (opiates) with ammonium bicarbonate as mobile phase analyzed on a Waters Quattro Micro API Triple Quad MS was evaluated.

Under the mildly-basic conditions of ammonium bicarbonate, the elution order of the test compounds is shifted (**Fig. 1**), but, surprisingly, the sensitivity in positive ion mode (reverse ionization) is one of the highest achieved in the entire series, especially for reserpine which is normally separated with addition of formic acid. Raffinose and bradykinin exhibit poor chromatography under these conditions (small or broad peak). In negative ion mode, only digoxin and reserpine are visible and raffinose is very small (**Fig. 2**). Similar results are obtained using ammonia as mobile phase additive, with reserpine giving once again a higher signal than ever achieved in positive ion mode (reverse ionization) and a good signal for raffinose in both modes (**Fig. 3**). Ammonia as an additive and negative ion mode (straight ionization) are the optimal conditions for raffinose and digoxin, especially, but not suitable for the other three test compounds. For these it may be useful to perform the separation under neutral conditions and to add the basic additive post-column via T-piece (**Fig. 4**). However, because the contribution of the flow from the column is much higher than that from the syringe, the additive has to be much more concentrated (here 10%). Under these negative ion mode conditions, raffinose exhibits an especially good signal.

Another interesting experimental setup is to perform

Figure 6 EIC of test compounds, 0.1% formic acid as additive in HPLC, 10% w/v ammonia added via T-piece post-column (reverse buffering, negative ion mode = straight ionization)

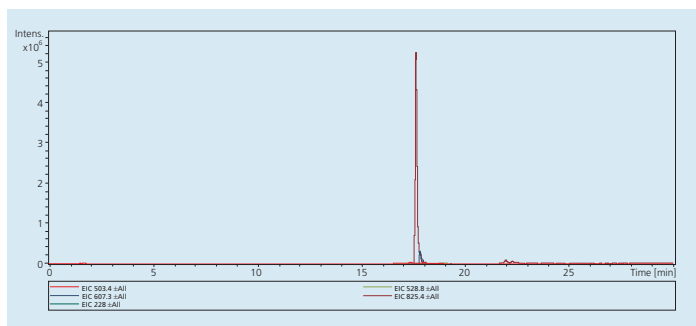


Figure 7 Mass spectra of bilirubin, 0.1% formic acid as additive in HPLC, 10% w/v TEA in acetonitrile added via T-piece post-column (reverse buffering, pos. and neg. mode)

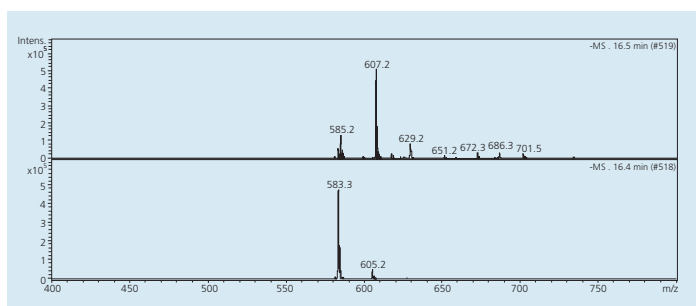
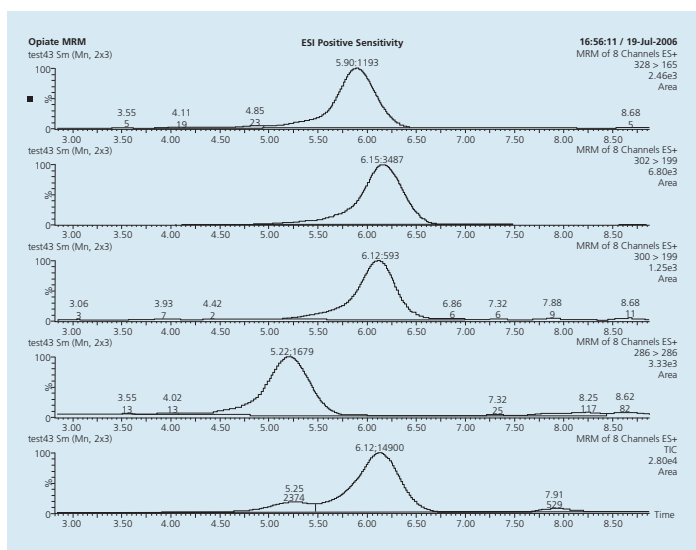


Figure 8 MRMs and TIC of opiates, 90% 10mM ammonium bicarbonate in water (pH=10), gradient to 90% methanol; heroin (328→165), dihydrocodeine (302→199), codeine (300→199) and morphine (286); last track is TIC (positive ion mode = reverse ionization)



the separation under acidic conditions, and change to alkaline conditions post-column before the flow enters the MS-interface (reverse buffering). This can be combined with negative ion (straight) or positive ion (reverse) ionization (**Figs. 5 and 6**). Care must be taken when choosing the right mass for monitoring in EIC. Under these conditions, digoxin forms the $[M+Na]^+$ ion in positive ion mode and the $[M+formate]^-$ ion in negative ion mode ($M+45!$).

Chromatographic separation of the different bilirubin isomers can be achieved only under acidic conditions, but the MS signal is poor. Bilirubin elutes with a high percentage acetonitrile, so the best way to increase the pH is to add triethylamine (TEA) dissolved in acetonitrile (10% v/v). With this setup, mass spectra can be obtained in both positive and negative ion modes (reverse buffering with reverse or straight ionization, **Fig. 7**). The mass of bilirubin is 694.2 Dalton.

The rapid measurement of narcotic drugs is essential in emergency overdose situations. An experimental setup that permits the simultaneous determination of opiates is shown in **Fig. 8**. The elution is very fast under mildly basic conditions with ammonium bicarbonate as additive. Although the compounds co-elute, quantification is achieved by use of the different MRM-tracks of the molecules. This is a typical triple quad application, where the capabilities of the instrument were combined with optimal chromatographic conditions, which also allow a sufficient ionization in MS.

These various examples show the broad applications for basic additives in LC-MS, which, although not so well known and often underestimated, offer a variety of possibilities over the more-commonly used acidic additives. With this article, we close our series on LC-MS additives. We sincerely hope that we have provided you with some practical hints for the use of these “little helpers” in your daily work with modern, high-tech LC-MS, and have demonstrated that advances in LC-MS are not only driven by the physics of the instrument, but also the chemistry of the column and the mobile phase.

References

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- [2] “Mobile Phase Additives for LC-MS. Part 2: How to Overcome Suppression Effects of TFA” **Analytix** 2006/3, 16-17.
- [3] “Mobile Phase Additives for LC-MS. Part 3: The Neutral Salts” **Analytix** 2006/4, 9-11.
- [4] “Mobile Phase Additives for LC-MS. Part 4: Special Case – Sodium Adduct Formation” **Analytix** 2006/5, 6-7.

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Part 7 Chiral HPLC Separations

Chiral chromatography requires chiral stationary phases (CSPs) with the power to resolve enantiomers, molecules that are non-superimposable mirror images that differ only in their molecular symmetry and, in most cases, their bioactivity. The powerful combination of chiral HPLC, both normal and reversed-phase modes, with MS detection offers both specificity and sensitivity. Sigma-Aldrich's analytical brands, Supelco and Fluka, offer chiral HPLC columns¹ and high-purity LC-MS CHROMASOLV[®] solvents and additives suitable for the most sensitive chiral LC-MS methods.

Normal phase with MS detection

Although reversed-phase is the dominant separation mode in HPLC, normal phase has three primary application areas: chiral HPLC separations, analysis of poorly water soluble compounds and preparative LC, the latter because normal phase solvents are easily removed from the purified fractions. Conventional normal phase mobile phases comprise binary or ternary mixtures of non-polar solvents with polar modifiers; hexane or heptane with aliphatic alcohols or ethylacetate are common examples.

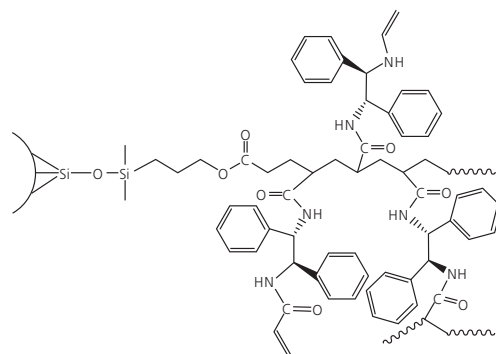
A significant drawback to using normal phase HPLC with MS detection is that normal phase solvents are typically not compatible with electrospray ion sources (ESI). High organic solvent flows prevent the ionization of dissolved solute molecules, as the solvent itself does not contain charge carriers like protons. However, other ion sources, like APCI (atmospheric pressure chemical ionization) and APPI (atmospheric pressure photoionization), do overcome this problem and produce detectable amounts of analyte ions under normal phase conditions. Importantly, APPI exhibits higher sensitivity when compared with all other MS sources.

Normal phase CSPs for LC-MS

The separation of warfarin enantiomers is an example of a normal phase chiral HPLC application with MS detection. For this analysis, we employed an Astec P-CAP-DPTM column with APPI detection. P-CAP-DP, based on the unique polycyclic amine polymer shown in **Figure 1**, was designed to separate a wide range of analytes under normal phase conditions.

A very versatile column, P-CAP-DP permits a wider choice of eluants, additives, flow rates and temperatures compared to many other CSPs. It is also available in two configurations, R,R and S,S, providing a predictable means of easily reversing elution order. Exceptional stationary phase stability, which is a result of covalent bonding of the chiral selector, makes it ideal for use with MS detection.

Figure 1 Molecular structure of Astec P-CAP-DP CSP, with covalent attachment to the underlying silica surface

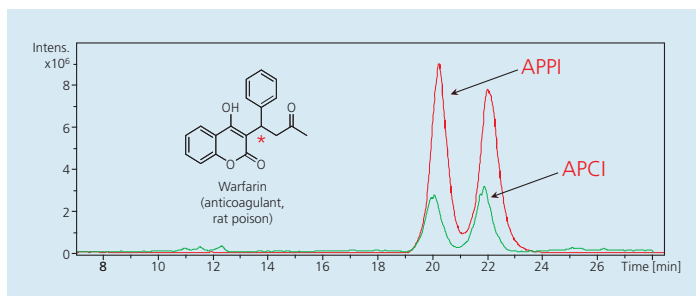


Choosing the ion source

Figure 2 shows the chromatograms of a racemic warfarin solution analyzed under normal phase conditions and two different ion sources: APCI and APPI. The APPI source (Syagen PhotoMate APPI source for Bruker/Agilent MS) gave the highest sensitivity, followed by APCI. Warfarin gave no ESI signal under these conditions. The APPI source outperforms the other sources since it uses photoionization that provides the benefit of selective analyte ionization. However, it is important to realize that very small amounts of impurities like DMSO or TEA in solvents, additives or dopants can dramatically suppress ionization. Solvents and additives in LC-MS CHROMASOLV quality guarantee the high purity necessary for this application.

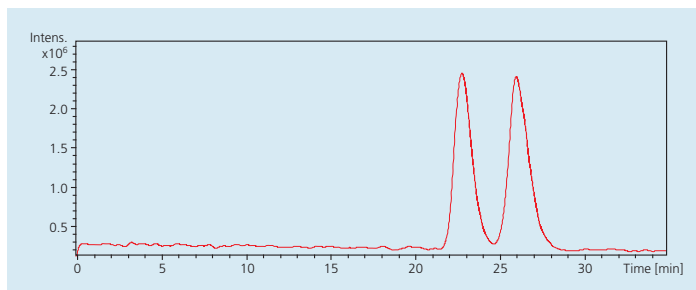
¹ Sigma-Aldrich products for chiral chromatography include Astec CSPs for HPLC, and chiral GC phases under both Supelco and Astec brands.

Figure 2 Normal phase chiral HPLC separation of warfarin enantiomers on an Astec P-CAP DP column with APPI and APCI detection



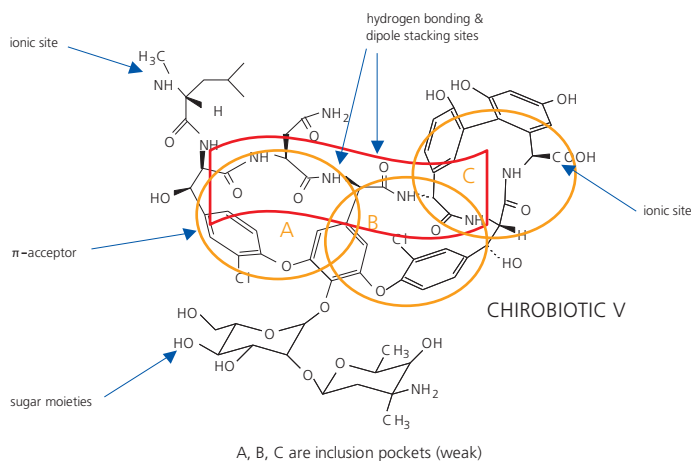
HPLC Conditions: column: Astec (R,R) P-CAP DP, 25 cm x 4.6 mm I.D., 5 μ m particles (35024AST); mobile phase: 0.1% acetic acid in 95:5, n-heptane:ethanol; flow rate: 0.8 mL/min.; temp.: 50 $^{\circ}$ C; det.: APPI (dry; gas flow: 4 L/min; dry gas; temp.: 350 $^{\circ}$ C; nebulizer press.: 50 psi; vaporizer temp.: 450 $^{\circ}$ C), APCI (same as APPI), ESI (nebulizer press.: 30 psi; dry gas: 8 L/min; dry gas temp.: 350 $^{\circ}$ C) (trace not shown); injection: 5 μ L; sample: racemic warfarin (Sigma A2250), 1 mg/mL in mobile phase

Figure 3 Chiral separation of warfarin enantiomers on an Astec P-CAP DP column with optimized conditions for APPI



HPLC Conditions: column: Astec (R,R) P-CAP DP, 25 cm x 4.6 mm I.D., 5 μ m particles (35024AST); mobile phase: 92:8, n-heptane with 0.1% formic acid:2-propanol; flow rate: 1.0 mL/min.; temp.: 55 $^{\circ}$ C; det.: APPI (dry gas flow: 4 L/min; dry gas temp.: 350 $^{\circ}$ C; nebulizer press.: 50 psi, vaporizer temp.: 450 $^{\circ}$ C); injection: 5 μ L; sample: racemic warfarin (Sigma A2250), 1 mg/mL in mobile phase

Figure 4 Molecular structure of vancomycin, the chiral selector of CHIROBIOTIC V, showing the different types of interactions that are possible



Optimizing the normal phase LC-MS separation

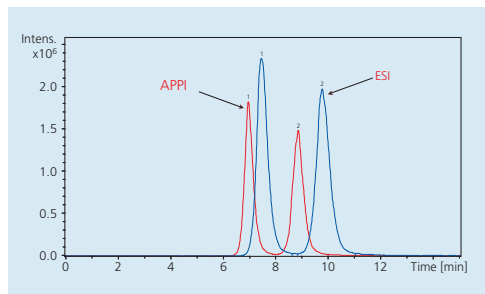
The use of additives in the mobile phase, like formic acid, acetic acid, or ammonium acetate, can positively influence peak shape and retention. In the case of the warfarin separation on the P-CAP-DP column, **Figure 3** shows that by adding formic acid to the heptane and replacing the ethanol with 2-propanol, resolution is significantly improved compared to the conditions in **Figure 2**.

Reversed-phase CSPs for LC-MS

Macrocyclic antibiotics, like vancomycin, are used as CSPs for HPLC in reversed-phase mode, in addition to polar and normal phase modes. The Astec CHIROBIOTIC™ V phase is one of three macrocyclic CSPs that feature a large number of side chains with differing chemistries and can therefore provide multiple types of interactions, including strong ionic interactions (**Figure 4**). The mobile phase solvents, pH and ionic strength all strongly affect retention by changing the interactions between the analyte and the various functional groups on the vancomycin molecule. The other retention modes that are possible with the macrocyclic CSPs depend on the solvent combination used. For example, polar ionic mode (PIM) comes into play with mobile phases of methanol containing triethylamine and acetic acid, or volatile salts, such as ammonium acetate.

The aqueous and polar organic solvent systems with ionic additives used for reversed-phase HPLC are also suitable for ESI detection. **Figure 5** compares APPI and ESI for the warfarin separation, this time under reversed-phase conditions. As expected, ESI gave greater sensitivity than APPI. However, the difference between these two ion sources is not as great as was observed under normal phase conditions.

Figure 5 Chromatograms of the chiral separation of warfarin with APPI and ESI on an Astec CHIROBIOTIC V column



HPLC Conditions: column: CHIROBIOTIC V, 15 cm x 2.1 mm I.D., 5 μ m particles (11019AST); mobile phase: 80:20, water with 5 mM ammonium acetate (pH 4); acetonitrile; flow rate: 0.4 mL/min.; temp.: 15 $^{\circ}$ C; det.: or ESI (MS conditions same as in Fig.2); injection: 5 μ L; sample: racemic warfarin (Sigma A2250), 1 mg/mL in mobile phase

Table 1 Selection of chiral HPLC columns featured in this article (see www.sigma-aldrich.com/astec for the complete offering. Other column sizes and guard columns also available)

Cat. No.	Brand	Description
35024AST	Supelco	Astec (R,R) P-CAP™ DP column (25 cm x 4.6 mm, 5 µm particles)
37024AST	Supelco	Astec (S,S) P-CAP™ DP column (25 cm x 4.6 mm, 5 µm particles)
11019AST	Supelco	Astec CHIROBIOTIC™ V column (15 cm x 2.1 mm, 5 µm particles)

Table 2 CHROMASOLV mobile phase solvents and additives used in this article (see www.sigma-aldrich.com/chromasolv for the entire list)

Cat. No.	Brand	Description	Package Size
34972	Fluka	Ethyl acetate, LC-MS CHROMASOLV	1 L, 2.5 L
34859	Sigma-Aldrich	n-Hexane CHROMASOLV	1 L, 2.5 L, 7 L, 45 L
34873	Sigma-Aldrich	n-Heptane CHROMASOLV	1 L, 2.5 L, 7 L, 45 L
39253	Fluka	Water, LC-MS CHROMASOLV	1 L
34967	Fluka	Acetonitrile, LC-MS CHROMASOLV	1 L, 2.5 L
34966	Fluka	Methanol, LC-MS CHROMASOLV	1 L, 2.5 L
34965	Fluka	2-Propanol, LC-MS CHROMASOLV	1 L, 2.5 L
56302	Fluka	Formic acid, puriss p.a., eluent additive for LC-MS	10 x 1 mL, 50 mL
49199	Fluka	Acetic acid, puriss p.a., eluent additive for LC-MS	50 mL
49638	Fluka	Ammonium acetate, puriss p.a., eluent additive for LC-MS	50 g

Comparing the two approaches, the reversed-phase method gave the best separation of the warfarin enantiomers, with baseline resolution and symmetrical peaks. The shorter run time is also amenable to processing more samples per unit time; a key consideration in clinical and forensic toxicology, and other high-throughput applications. Both ion sources (APPI and ESI) are sensitive enough to reach LLODs in the ppb range down to 1 ng/mL in blood plasma (ESI, negative ion mode, after SPE) [1]. To maximize sensitivity, it is strongly recommended to use solvents and additives specially tested for LC-MS, like the CHROMASOLV® and puriss p.a. LC-MS product line from Sigma-Aldrich. For a complete listing of our chiral HPLC columns, please visit the web site www.sigma-aldrich.com/astec. All LC-MS CHROMASOLV solvents, additives and blends can be accessed at www.sigma-aldrich.com/chromasolv

Reference

- [1] Čápková, V.; Viccarone, S.; Carter, S.; Nguyen, N.; Merkle, S. JASMS 2007, 18 (5), S26–S49.

Part 8 APPI Dopants – Solvents and post-column additives for photo-ionization

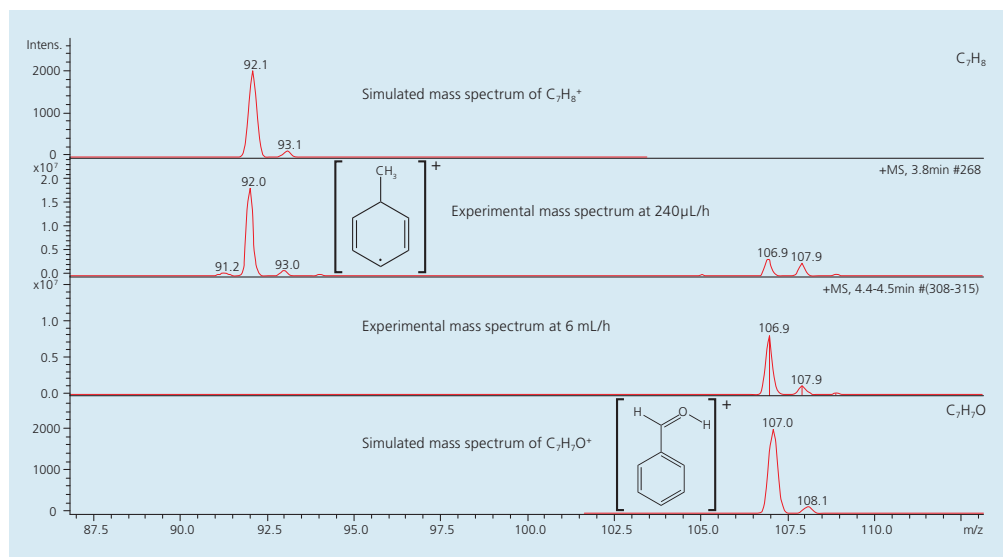
APPI (atmospheric pressure photoionization) dopants enable and enhance ionization in the APPI source. They include substances like toluene, acetone, anisole and chlorobenzene. High gas phase concentrations of the dopants are introduced into the ionization cavity of the APPI source. There, UV radiation readily ionizes the dopant molecules forming a large number of free radicals and molecular ions. Subsequently, other molecules are ionized by the dopants through electron or proton transfer.

Compounds with higher ionization energy than the energy level of the emitted photons from the UV lamp require dopants, otherwise charge is not generated and these compounds will not drift into the mass spectrometer. However, molecules that can be analyzed directly in the APPI source also benefit from the use of dopants. The intensity and sensitivity can be improved because the dopants increase the number of ionized analyte molecules [1– 4].

Using dopants to enhance APPI signal

A typical, yet impressive, application for dopants is the ionization of benzaldehyde dissolved in toluene. Benzaldehyde cannot be ionized directly by ESI and gives poor results with APCI and APPI without dopant. Thus, the use of a dopant in combination with an APPI source represents the only suitable way to analyze benzaldehyde. To demonstrate the improvement, a benzaldehyde solution was infused via syringe directly into the APPI source. At a low flow rate (240 $\mu\text{L}/\text{hour}$) positively charged toluene radicals ($[\text{M}(\text{C}_7\text{H}_8)]^{+\bullet} = 92.06 \text{ amu}$) are formed, as the upper calculated and observed mass spectra in **Figure 1** demonstrate. Increasing the flow rate to 6 mL/hour causes the formation of uncharged toluene radicals, which are not detectable by MS, and indirectly ionizing benzaldehyde through proton transfer. This in turn leads to the lower calculated and observed mass spectra in. APPI experiments are optimized in terms of dopant (single components or mixtures), percent dopant in the mobile phase and nebulizer gas (for direct injection into the APPI source). Specific requirements depend on the manufacturer of the mass spectrometer and the specific source design.

Figure 1
Mass spectra of an acetaldehyde solution in toluene at 2 different flow rates



Mass spectra of an acetaldehyde solution in toluene at 2 different flow rates. The solution is directly infused into the APPI source with a syringe pump. The upper spectrum was obtained at a flow rate of 240 $\mu\text{L}/\text{h}$. The primary mass of $m/z=92.0$ amu agrees with a charged toluene radical. Increasing the flow rate to the maximum of 6 mL/h changes the mass spectrum significantly as there is only the protonated benzaldehyde molecule observed. High flow rates support the proton transfer reaction of the charged toluene radical to benzaldehyde and other molecules decreasing the number of the charged toluene molecules nearly to zero.

Dopant purity requirements

Dopants can enhance signals of analytes in mass spectra, but they also intensify unwanted impurities when present. Small amounts of impurities in a dopant can result in excessive noise or a suppression of the analyte signal. **Figure 2** shows a benzaldehyde solution in suitably pure toluene and in contaminated anisole.

Benefits of dopants in normal phase LC-MS analysis

Electrospray ionization (ESI) is the method of choice for most polar compounds, like drugs and metabolites, and

is very sensitive under reversed-phase conditions. However, if normal phase is employed, the ion source must be switched from ESI to APCI or APPI because analyte ionization, a key requirement of ESI, does not occur in nonpolar organic solvents. Charge on the analyte must be generated by corona discharge or UV radiation. When normal phase in conjunction with MS detection is used, like in the separation of warfarin enantiomers on the P-CAP-DP chiral stationary phase shown in **Figure 3**, the combination of the APPI source with toluene dopant can significantly enhance the signal.

Figure 2 Spectra of benzaldehyde dissolved in toluene (top) and anisole (bottom)

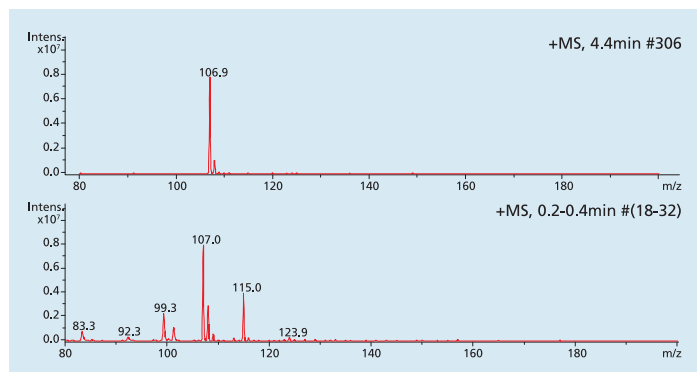
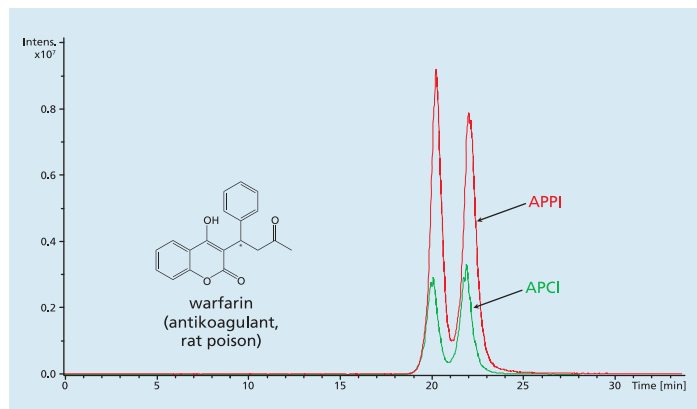


Figure 3 Separation of warfarin under normal phase conditions



Mass spectra of benzaldehyde dissolved in toluene (top) and anisole (bottom). The concentration is 100 µL/mL. In both cases the syringe pump operated at 6 mL/hour, resulting in comparable ionization of benzaldehyde. In the case of anisole, the mass spectrum shows additional signal from contaminants in the dopant.

Conditions:

Column: Astec P-CAP DP, 25 cm x 4.6 mm, 5 µm packing (Cat. No. 35024AST)
 Mobile phase: heptane:ethanol (0.1% ammonium acetate, formic acid), 95:5
 Flow rate: 0.8 mL/min.
 Temperature: 50°C
 Sample: 3 µL, warfarin 1 mg/mL

Method:

Astec P-CAP DP (4.6 –250 mm, 5 µm) heptane/ethanol (0.1% ammonium acetate, formic acid), 95:5, isocratic, T=50°C konz. 1 mg/mL, inject. vol. 3 µL

Cat. No.	Brand	Description	Package Size
650579	Sigma-Aldrich	Toluene CHROMASOLV® Plus, for HPLC, ≥ 99.9%	1 L, 4 L
650501	Sigma-Aldrich	Acetone CHROMASOLV Plus, for HPLC, ≥ 99.9%	1 L, 4 L
270644	Sigma-Aldrich	Chlorobenzene CHROMASOLV Plus, for HPLC, 99.9%	100 mL, 1 L, 2 L, 4 L
96109	Fluka	Anisole puriss. p.a., standard for GC, ≥ 99.9%	5 mL, 10 mL

References

- [1] Robb, D.; Covey, T.; Bruins, A. *Anal. Chem.* **2000**, 72 (15), 3653–3659.
- [2] Kauppila, T.; Kostainen, R.; Bruins, A. *Rapid Comm. Mass Spectrom.* **2004**, 18, 808–815.
- [3] Cai, S.; Hanold, K.; Syage, J. *Anal. Chem.*, **2007**, 79 (6), 2491–2498.
- [4] Syage, J.; Hanold, K.; Lynn, T.; Horner, J.; Thakur, R. *J. Chromatogr. A* **2004**, 1050, 137–149.

LC-MS Product Overview

LC-MS Solvents

Cat. No.	Brand	Description	Package Size
34967	Fluka	Acetonitrile LC-MS CHROMASOLV®, ≥99.9%	250 mL, 1 L, 2.5 L
34966	Fluka	Methanol LC-MS CHROMASOLV, ≥99.9%	1 L, 2.5 L
39253	Fluka	Water LC-MS CHROMASOLV	1 L
34965	Fluka	2-Propanol LC-MS CHROMASOLV, ≥99.9%	1 L, 2.5 L
34972	Fluka	Ethyl acetate LC-MS CHROMASOLV, ≥99.7%	1 L, 2.5 L
34986	Fluka	n-Hexane LC-MS CHROMASOLV, ≥97%	1 L, 2.5 L
34999	Fluka	n-Heptane LC-MS CHROMASOLV, ≥99%	1 L, 2.5 L

LC-MS Blends

34978	Fluka	Water with 0.1% trifluoroacetic acid LC-MS CHROMASOLV	2.5 L
34976	Fluka	Acetonitril with 0.1% trifluoroacetic acid LC-MS CHROMASOLV	2.5 L
34974	Fluka	Methanol with 0.1% trifluoroacetic acid LC-MS CHROMASOLV	2.5 L
34973	Sigma-Aldrich	Water with 0.1% formic acid LC-MS CHROMASOLV	2.5 L
34977	Sigma-Aldrich	Water with 0.1% formic acid and 0.01% trifluoroacetic acid LC-MS CHROMASOLV	2.5 L
34668	Fluka	Acetonitril with 0.1% formic acid LC-MS CHROMASOLV	2.5 L
34676	Fluka	Acetonitril with 0.1% formic acid and 0.01% trifluoroacetic acid LC-MS CHROMASOLV	2.5 L
34671	Fluka	Methanol with 0.1% formic acid LC-MS CHROMASOLV	2.5 L
34675	Fluka	Water with 0.1% acetic acid LC-MS CHROMASOLV	2.5 L
34678	Fluka	Acetonitril with 0.1% acetic acid LC-MS CHROMASOLV	2.5 L
34672	Fluka	Methanol with 0.1% acetic acid LC-MS CHROMASOLV	2.5 L
34674	Fluka	Water with 0.1% ammonium acetate LC-MS CHROMASOLV	2.5 L
34669	Fluka	Acetonitril with 0.1% ammonium acetate LC-MS CHROMASOLV	2.5 L
34670	Fluka	Methanol with 0.1% ammonium acetate LC-MS CHROMASOLV®	2.5 L

LC-MS Additives

40967	Fluka	Trifluoroacetic acid puriss p.a., eluent additive for LC-MS	10 x 1 mL, 50 mL
56302	Fluka	Formic acid puriss p.a., eluent additive for LC-MS	10 x 1 mL, 50 mL
49199	Fluka	Acetic acid puriss p.a., eluent additive for LC-MS	50 mL
49916	Fluka	Propionic acid puriss p.a., eluent additive for LC-MS	50 mL
55674	Fluka	Ammonium formate puriss p.a., eluent additive for LC-MS	50 g
49638	Fluka	Ammonium acetate puriss p.a., eluent additive for LC-MS	50 g
61333	Fluka	Sodium citrate puriss p.a., eluent additive for LC-MS	50 g
40867	Fluka	Ammonium bicarbonate puriss p.a., eluent additive for LC-MS	50 g
44273	Fluka	Ammonium hydroxide solution puriss p.a., eluent additive for LC-MS	10 x 1 mL, 100 mL
65897	Fluka	Triethylamine puriss p.a., eluent additive for LC-MS	500 mL

LC-MS Solutions & Standards

34689	Fluka	2-Propanol solution for LC-MS	1 L
34692	Fluka	Water with 8% formic acid for LC-MS	1 L
21004	Fluka	Cesium iodide standard for LC-MS	1 g

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