Chromatography Solutions

Knowledge note #0031

The use of Mobile Phase pH as a Method Development Tool

INTRODUCTION

In LC separations, the mobile phase pH determines the ionisation state of ionisable analytes. The mobile phase pH can therefore be varied and used as a powerful tool to control analyte retention, peak shape and selectivity. This Knowledge Note explains how retention of acidic, basic and neutral analytes is affected by mobile phase pH, as well as the requirements for carrying out separations at high and low pH. It also discusses how the chromatographer can utilise pH during the development of new LC methods.

The fundamental goal of any LC separation is to obtain suitable resolution of the key analytes of interest. The fundamental resolution equation states that the resolution between two analyte peaks is governed by the separation efficiency (N), analyte retention (k) and separation selectivity (α) [1]. Of these three parameters, α has the biggest impact on resolution and, therefore, it pays to invest method development time in optimising the separation selectivity. Many analytical parameters can be used to affect the selectivity, including optimisation of the column stationary phase, the organic modifier, percentage organic and temperature. For separations involving ionisable analytes, mobile phase pH can have a profound affect on analyte retention and selectivity. It is, therefore, an important parameter to investigate during method development.

ANALYTE RETENTION AT DIFFERENT MOBILE PHASE PH

To a large degree, analyte retention in reversed-phase is dictated by analyte hydrophobicity. For ionisable analytes, as the degree of ionisation increases, retention typically decreases (providing that no alternative modes of interaction such as ion-exchange are present). Figure 1 summarises how the mobile phase pH affects the degree of ionisation for simple acidic and basic compounds. For basic analytes, at mobile phase pH's below their pK_a, the analyte will primarily be positively charged. At high pH (above their pK_a), they will be in their neutral form and will be better retained by reversed-phase. Conversely, acidic species show their strongest retention with a mobile phase below their pK_a and are more weakly

retained at high pH, in their deprotonated form. To demonstrate the effect of mobile phase pH on analyte retention, a set of basic, acidic and neutral analytes were chromatographed at different pH's (Figure 2) on an alkali-stable Avantor® ACE® SuperC18 column. All other separation parameters such as gradient profile, buffer concentration and temperature, were kept constant and changes in analyte retention are therefore determined by the mobile phase pH.

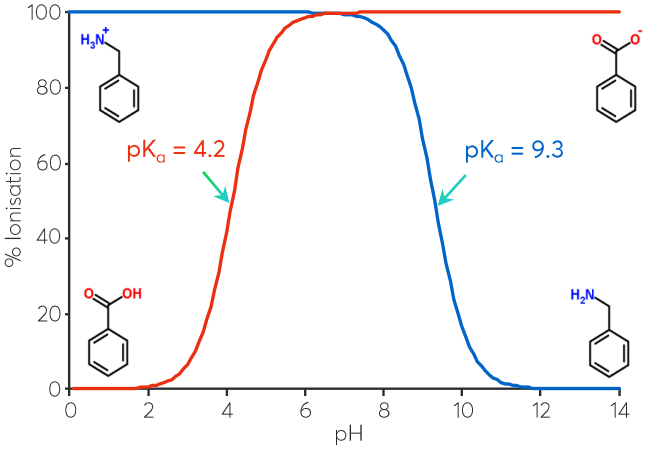

Figure 1: Percentage ionisation of acidic (red) and basic (blue) analytes at various pH values.

Toluene contains no ionisable functionality and is therefore neutral over the entire pH range. This means that mobile phase pH has no significant effect on the retention of toluene. At low pH, the acidic analytes (3,4-dichlorobenzoic acid and mefenamic acid) are present in their non-ionised, neutral form and therefore show their strongest retention. As the mobile phase pH is increased to the analytes pK$_a$ and above, the degree of ionisation increases and a gradual decrease in retention is observed. In contrast, the basic analytes (nortriptyline and carvedilol) are positively charged at low pH and consequently show shorter retention. As the pH increases, the ionisation is suppressed and analyte retention increases. Protonated basic analytes may exhibit low retention and/or poor peak shape when analysed at low pH. Performing the analysis at high pH (neutral form), is an approach which can dramatically improve both peak shape and retention and also provides gains in sensitivity (Figure 3).

It is important to note that, for maximum column lifetime, many silica based reversed-phase columns should be used within a limited pH range of approximately 2-8 and are therefore not suited to alkaline conditions.

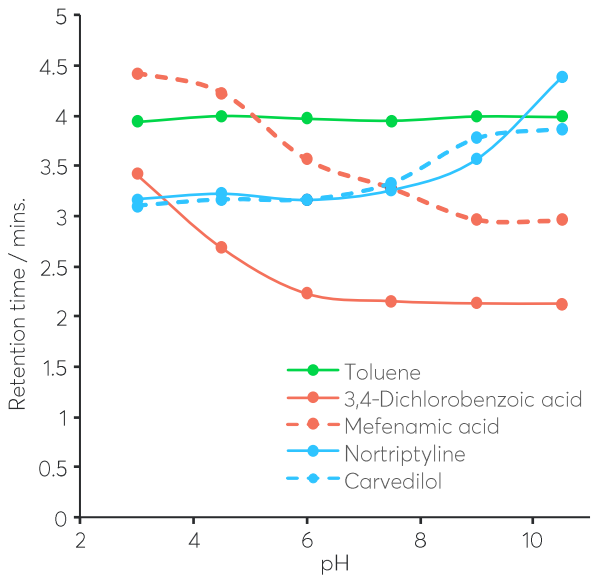

Figure 2: Effect of mobile phase pH on analyte retention. Column: Avantor® ACE® Excel 3 SuperC18, 50 x 2.1 mm; Mobile phase A: 20 mM ammonium formate pH 3.0, 4.5, 6.0, 7.5, 9.0 and 10.5 (aq), B: 20 mM ammonium formate pH 3.0, 4.5, 6.0, 7.5, 9.0 and 10.5 in MeCN/H_2O 9:1 v/v; Gradient: 3 to 100% B in 5 minutes; Flow Rate: 0.6 mL/min; Injection Volume: 1 µL; Temperature: 40 °C; Detection: UV, 214 nm.

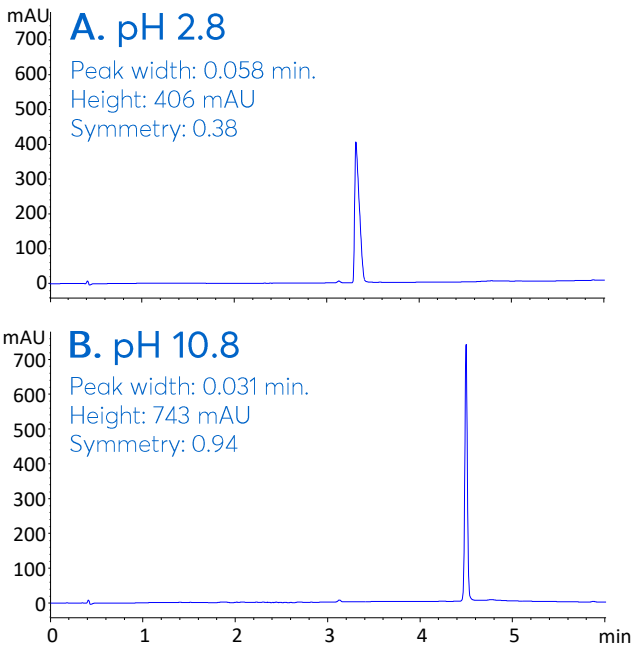

Figure 3: Chromatograms of carvedilol on an Avantor® ACE® Excel 3 SuperC18, 50 x 4.6 mm column with (A) low pH and (B) high pH mobile phases.

To perform a high pH separation, it is essential to use a stationary phase that is compatible with high pH. Columns that can tolerate alkali, such as the Avantor® ACE® SuperC18 and SuperPhenylHexyl, are typically manufactured from a hybrid organo-silica material, polymer based, or utilise modified bonding technology.

USING PH TO CONTROL ANALYTE SELECTIVITY

From Figures 2 and 3, it is clear that when ionisable analytes are present in a sample, the selectivity between analytes can vary significantly with mobile phase pH. For such samples, it is highly recommended that mobile phase pH is explored during initial method development, to determine the most suitable option for the sample. When beginning any method development, it is useful to consider analyte structures and properties, if known, to anticipate any acidic/basic behaviour. If unknown, screening the sample on a generic gradient with low and high pH mobile phases can be a productive starting point. Figure 4 shows an example gradient separation of a set of acidic, basic and neutral analytes at low and high pH; analysed on an Avantor® ACE® UltraCore SuperPhenylHexyl column (extended pH compatibility, pH 1.5-11.0). Neutral analytes, or analytes whose ionisation state remains unchanged within the pH range examined, show little change in retention with pH (e.g. peak #11). In contrast, all the acidic analytes show a sharp decrease in retention as mobile phase pH increases, whereas the opposite is observed for basic compounds. Clear differences in selectivity are observed: for example, peaks 5 and 12 show complete reversal in elution order. Mobile phase pH can also provide useful selectivity for samples containing just acidic or basic components: for example, the resolution between the bases carvedilol (9) and trimipramine (12) increases dramatically from low to high pH. Similarly, an increase in resolution between acids 4 and 5 is seen at low pH.

For method robustness, it is generally recommended to work at a mobile phase pH at least 2 pH units away from the analyte pK_a. Often this may not be possible, e.g. for complex samples or for multifunctional analytes containing moieties with overlapping pK_a values. In these situations, it is important to accurately control and document mobile phase preparation to ensure consistent pH between batches of mobile phase. Examining the effect of small changes in pH on the separation is also highly recommended to assess the method robustness.

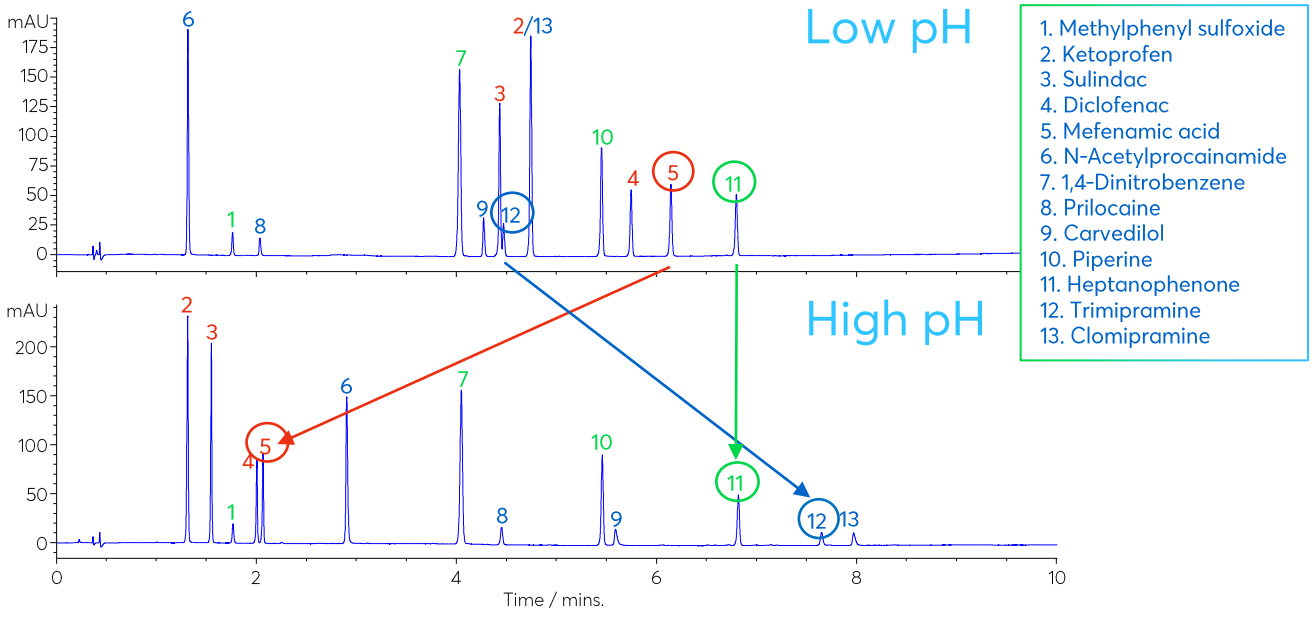

Figure 4: Separation of a range of acidic, basic and neutral analytes on an alkali-compatible solid-core column with novel encapsulated bonding.
Column: Avantor® ACE® UltraCore 2.5 SuperPhenylHexyl, 100 x 3.0 mm; Mobile phase (low pH) A: 15 mM ammonium formate pH 3.0 (aq), B: 15 mM ammonium formate pH 3.0 in MeCN/H_2O 9:1 v/v; Mobile phase (high pH) A: 0.1% NH_3 (aq) B: 0.1% NH_3 in MeCN/H_2O 9:1 v/v; Gradient: 5 to 100% B in 10 minutes; Flow Rate: 1.2 mL/min; Injection Volume: 1 µL; Temperature: 40 °C; Detection: UV, 260 nm.

ADDITIONAL BENEFITS OF CHANGING MOBILE PHASE PH

Careful selection of mobile phase pH can also provide several other benefits for the chromatographer. For LC/MS applications, pH can provide enhanced sensitivity in some cases. For example, using a high pH mobile phase to ionise acidic components may enhance detection in negative ion mode. For preparative applications and analyses involving one or more overloaded peaks (e.g. impurity testing) working at a pH where the analyte is neutral can be beneficial. Figure 5 summarises a loading study for amitriptyline at 2 different pH's. At low pH, amitriptyline is positively charged and shows peak tailing, whereas at high pH (non-ionised form), peak shape is vastly improved. As the sample load on column is increased, the peak width at low pH increases significantly. This could lead to loss of resolution or difficulty in observing/quantifying smaller impurity peaks. At high pH, however, the peak width is more constant as the mass of analyte on column is increased.

CONCLUSION

When working with ionisable analytes, the mobile phase pH can dramatically affect analyte retention behaviour. The mobile phase pH is therefore an important parameter to consider during method development. This article has shown how pH can be utilised during method development to alter selectivity and help optimise the separation. Appropriate pH selection also provides additional gains in retention, peak shape and sensitivity.

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REFERENCES

1. ACE Knowledge Note AKN0005: The Fundamental Resolution Equation and the Impact of k, N and α (accessed at https://uk.vwr.com/cms/ace_knowledge_notes)

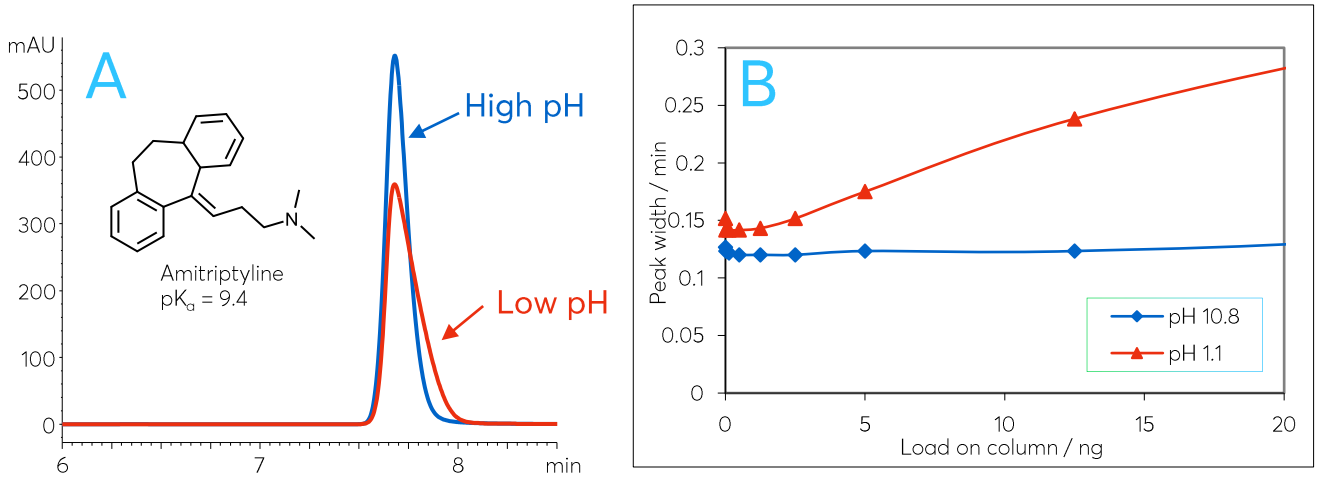

Figure 5: Loading study for amitriptyline at low and high pH. (A) Comparison of peak shape (normalised retention time) and (B) comparison of peak width as analyte load on column is increased. Note that mobile phase composition was set to obtain a retention factor (k) of approximately 5 at both pH values.
Column: Avantor® ACE® Excel 3 SuperC18, 150 x 4.6 mm; Mobile phase (low pH): 1% TFA in MeCN/H_2O 44:56 v/v; Mobile phase (high pH): 0.1% NH_3 in MeCN/H_2O 77:23 v/v; Flow Rate: 1.0 mL/min; Injection Volume: 5 µL; Temperature: 40 °C; Detection: UV, 254 nm.

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