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FOCUS: CHROMATOGRAPHY

Issue 1 2024

Welcome to FOCUS: Chromatography

Welcome to the first edition of the FOCUS: Chromatography magazine 2024

In this latest edition of our FOCUS: Chromatography magazine we consider the method development aspect and the tools available to improve your analytical workflows. Whether you are looking to perform in LC-MS or to learn more in Sample Preparation, you will find expert advice and detailed recomendations on a variety of technical issues.

When developing a new LC or LC-MS separation, careful consideration of mobile phase composition is essential for optimising peak shape, improving separation selectivity and ensuring method robustness. A variety of Tips and Tricks can be found on this subject. In this edition, you will discover also a simple and systematic protocol for screening new samples using reversed-phase conditions, which can help rationalise and streamline the development of new LC methods.

In addition, you will learn more about the Influence of HPLC-System dead volume on the performance of UHPLC Columns or in a novel concept for HPTLC suitability test.

To protect the planet and anticipate the future, we also look at ways to be more sustainable and discuss the use of returnable barrels for HPLC solvents.

Lastly a wide range of chromatography webinars are available on demand to supplement the magazine content at vwr.com/webinar and more information about products and services for your analytical lab at vwr.com/chromatography.

If you have any questions or need application advice, please contact our industry-leading technical support team at chromsupport@avantorsciences.com.

Enjoy reading and exploring!
Avantor Chromatography Workflow Team

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A practical guide to maximising sample peak capacity for complex low molecular mass molecule separations.

Arianne Soliven, Matt James and Tony Edge, Avantor

INTRODUCTION

Method development for complex low molecular mass (LMM) samples using reversed-phase (RP) separation conditions is a challenging problem that typically requires gradient separation conditions, especially when the sample matrix itself may contain many unknown analytes present over a wide dynamic concentration range. This short article presents guidance based on an established approach published in 2013 aimed at optimising the practical method parameters (column length *(L)*, temperature *(T)*, flow rate *(F)*, and final mobile phase conditions ($\varnothing_{final}$) to maximise the separation's peak capacity[1,2]. The robustness of the protocol was verified in a previous study, and applied to optimise a highly complex maize seed extract sample[1]. This protocol may benefit the analysis of challenging samples with complex matrices in metabolomics, natural products and contaminant screening laboratories to name a few.

SAMPLE PEAK CAPACITY (n_c)

There are numerous peak capacity descriptors and variations in the way they are calculated[3,4]. Essentially, it is a metric that represents 'how many peaks from my sample can fit in my separation space/window?' Hence, the mathematical variations are associated with how the two (peak width and separation window) are measured and calculated. The sample peak capacity (n_c) approach where a large number of peaks is being separated, is based on chromatographic data via equation 1[3]

$$\text{Equation 1. } n_c = \frac{t_{R,last} - t_{R,first}}{4\sigma_{avg}}$$

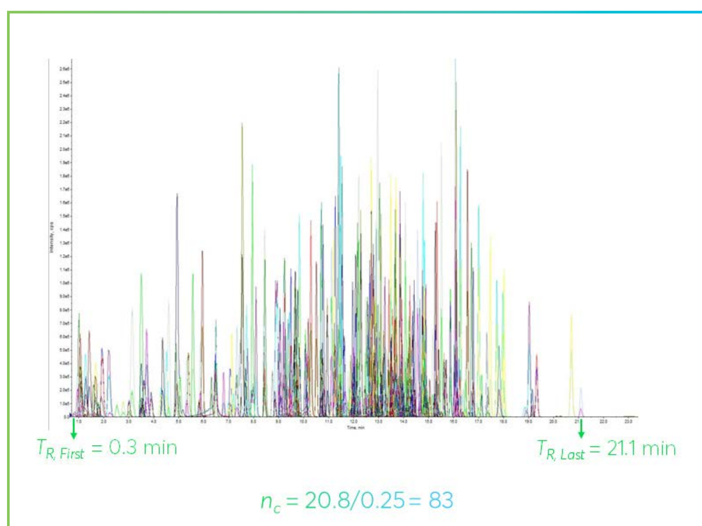

Figure 1: Calculating the sample peak capacity (n_c) based on a chromatogram (reproduced with permission of National Food Chain Safety Office, Directorate of Plant Protection, Soil Conservation and Agri-Environment, Hungary).[5]

Where the separation window is defined by the retention time difference between the last eluting ($t_{R,last}$) and the first eluting peaks ($t_{R,first}$). The maximum resolved number of peaks between them is defined by simply dividing the separation space or window by the average peak width measured within four standard deviations of the mean ($4\sigma_{avg}$); statistically $4\sigma_{avg}$ takes into account 99.9% of the population and therefore is a descriptor of peak shape and width.

Figure 1 shows how this is calculated from an actual chromatogram. The average peak width is 0.25 min, and the separation window/space was defined as 20.8 min, hence the sample peak capacity n_c equates to 83. Hence, a maximum of 83 baseline resolved peaks can fit within this defined separation space for this sample using this specific method.

The peak capacity can be used during method development to monitor method performance when changes are made, as well as to compare two methods to one another. Note that the sample peak capacity is representative of the LC separation strategy.

TRENDS IN n_c FOR LOW MOLECULAR MASS MOLECULES

The practical parameters and the complexity of the multivariate relationships associated with n_c has been previously studied with regards to relatively high molecular mass (HMM) analytes (peptides of a tryptic digest) and low molecular mass (LMM) species (representative set of indoles of a maize seed extract used for demonstrating the n_c for 2DLC studies)[6,7]. For further reading, please refer to the following references on this topic[1,2,8,9].

There are two main distinct differences between the HMM and LMM trends with regards to n_c[1,2,8,9]. One difference is related to the flow rate, which should be optimised for both HMM and LMM analytes. For HMM analytes, including peptides, the optimum peak capacity occurs at a lower flow rate that must be experimentally determined[8,9]. For LMM compounds, the increase in flow rate resulted in an increased n_c, and is related to the difference in the diffusion coefficients relative to larger peptides[1].

Another difference in trends between peptides and LMM species is that the column length is fixed and does not need optimisation for peptides. For LMM analytes on the other hand, column length must be optimised to maximise n_c. Hence, there is a difference in the practical strategy for maximising nc between peptides and LMW complex samples[1,8]. Furthermore, the success of the practical guide for maximising n_c for LMW complex samples, the Snyder-Dolan test is critical and is discussed in the next section of this communication[10].

The effect of temperature was also studied for three different search strategies utilising a free tool in Microsoft Excel 'Solver' to simulate method development experiments. Solver was instructed to maximise peak capacity, while simultaneously optimising (i) three practical variables (Φ_{final} , F, and L), (ii) two practical variables (Φ_{final} and F), and (iii) one practical variable only (Φ_{final}). All simulations had a fixed gradient time (t_G = 30 min). Temperature was also a fixed variable and set at 40, 60, 80, 100 and 120 °C for all three scenarios. Figure 2 highlights that an increase in temperature resulted in an increase in peak capacity and a maximum was reached near T = 80°C for optimisations (ii) and (iii). Figure 2 clearly shows that it is best to adopt search strategy (i) to optimise Φ_{final} , F, and L to achieve the best possible peak capacity. Hence, this strategy has been used to create the practical guide to maximise peak capacity for complex LMM samples. Not only does the column length and flow rate, as well as the final mobile phase composition (Φ_{final}), need to be optimised (so the last eluting species elutes at the end of the separation window), the temperature is also important and must be increased.

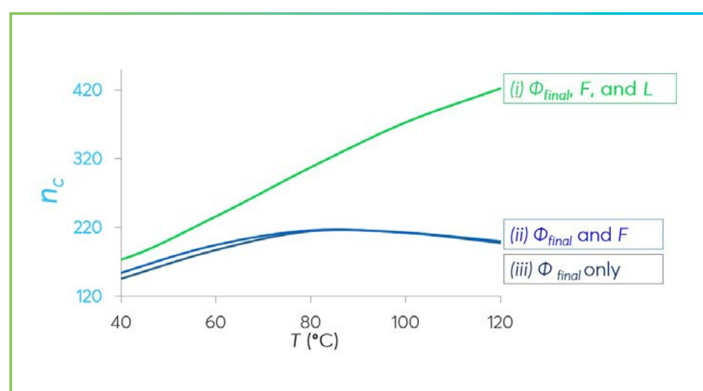

Figure 2: Effect of temperature for three simulated method development strategies where peak capacity was maximised: (i) three practical variables optimised (Φ_{final} F, and L), (ii) two practical variables optimised (Φ_{final} and F), and (iii) one practical variable optmised (Φ_{final})[1].

SNYDER-DOLAN TEST

The Snyder-Dolan (S-D) test is a critical aspect of this practical guide. An initial RP gradient screening run is performed that determines whether the complexity of the sample requires a gradient separation and additionally guides the column length choice[10]. The screen is conducted at 5-100% organic strength in 30 min, at 1 ml/min for a 4.6×100 mm column (dead time (t_0) of the column ≈ 1 min) at 30 °C. Adjustments can be made, for different column lengths or formats by multiplying t_G by the dead time of the column (i.e. 30×t_0). In addition, the flow rate

should be reduced for different column formats, as a basic guide approximately 1 column volume per minute (0.21 ml/min for a 2.1 mm ID column and 0.43 ml/min for a 3.0 mm ID column). To 'pass' the S-D test, the solutes must occupy more than 25-40% of the gradient time (t_G), where:

$$\text{Equation 3.}\ \frac{t_{R,last} - t_{R,first}}{t_G} \geq 0.25 - 0.40$$

Once the S-D passes the test to proceed with developing a gradient separation strategy, it is then recommended to follow the guide in the next section to maximise n_c. If the S-D test does not satisfy equation 2 (example in Figure 2b), isocratic conditions are recommended and the following protocol to maximise peak capacity may not be applicable.

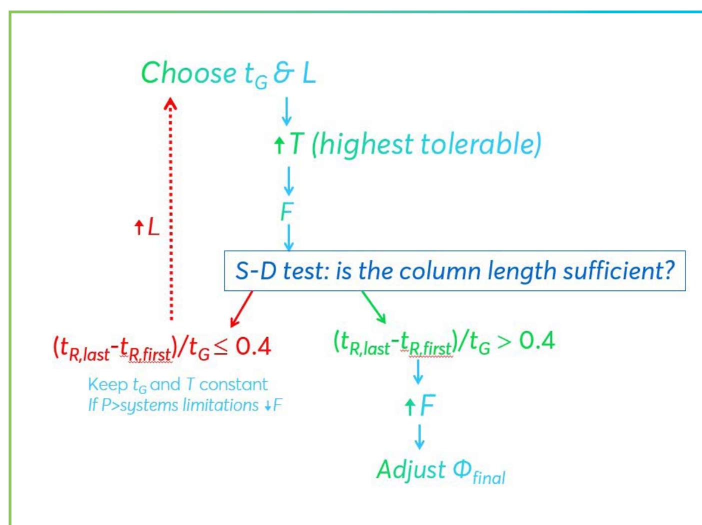

Figure 3: The decision tree aimed at maximising peak capacity for complex small molecule samples via RP gradient separation conditions [1].

A PRACTICAL GUIDE TO MAXIMISE n_C FOR COMPLEX LOW MOLECULAR MASS SEPARATIONS

Based on the previous study[1], the decision tree shown in Figure 3, is used to map out decisions and experiments in order of priority, based on the trends observed in the multivariate relationships between practical parameters when maximising peak capacity.

The first decision is intuitively related to the separation space, the longer the gradient time, the larger the peak capacity. **Note:** This is conducted after a column selectivity study and/or a final column selection decision is made. However, in the practical world, time is a constraint that is driven by the laboratory's productivity. Hence, while it may be an arbitrary decision, the gradient time is the first choice and must be guided by the priorities of the laboratory and how much time can be dedicated to analysing one sample. Hence, after setting t_G, the column length *(L)* must be selected. The most resourceful decision is to use what is readily accessible in the laboratory. Guidance on initial column lengths, based on different solute sets are provided in the previous study[1]. With regards to column selectivity and particle size selection - column selectivity and backpressure limitations must be considered and these must be fixed when conducting this protocol[11-13].

The next step is to select the highest temperature *(T)* possible that is compatible with the system, column and sample. If sample degradation is a concern during the analysis, a set of systematically different column temperatures can be tested to determine the highest temperature that is tolerable without compromising the integrity of the sample. If the temperature limits for the system and/or column are not clear - including the fittings and accessories - please refer to the care and use guidelines provided by the manufacturer.

The next step is to set the flow rate *(F)* at 1 ml/min (4.6 mm ID column) it can be scaled accordingly to other IDs and lengths (a free-method translator tool is available to **download** with a **how to use guide**). Next, the S-D experiment is repeated and if $(t_{R,last} - t_{R,first})/t_G \leq 0.4$ the column length must be increased while keeping the temperature and t_G constant, until $(t_{R,last} - t_{R,first})/t_G > 0.4$.

Once the correct column length is established, the flow rate is increased to the highest flow rate possible (compatible with the system and column), and the final mobile phase organic strength is adjusted so $t_{R,last}$ elutes $\leq t_G + t_0$. By following this guidance, all the available separation space is utilised, and both the separation space and peak width optimised for complex LMW samples separated via gradient RP conditions.

CONCLUSION

This short communication outlines how to maximise peak capacity for complex low molecular mass separations based on a previous study. This protocol is not presented to be used instead of computational optimisation strategies. It is aimed at providing a practical guide for analysts to follow to aid method development decisions for complex low molecular mass samples. We recommend using the presented decision tree that prioritises which variables to optimise first. This was developed based on the understanding of multivariate trends of practical parameters and peak capacity. Furthermore, the Snyder-Dolan test is critical for the success of this established protocol, to initially determine if the sample requires gradient or isocratic elution and to guide the column length decision.

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Tips & tricks: Sensitivity gains in LC-MS

Stephan Altmaier, Head of Instrumental Analytics R&D, Merck

INTRODUCTION

The prerequisite of any highly sensitive analysis via HPLC-MS (high performance liquid chromatography coupled to mass spectrometry) is the use of ultrapure solvents and reagents and careful handling of all associated materials, consumables and systems. This prevents any contamination throughout the entire sample handling process from preparation to MS detection, and improves sensitivity.

In the following sections, various measures and options for maximised LC-MS sensitivity and low limit of detection (LOD) are shown. Each and every tip avoids contaminations causing signal suppression, adduct formation, elevated background noise and increased spectrum complexity.

SOLVENTS & ADDITIVES - GENERAL

Typical solvents utilized in LC-MS include water, acetonitrile, methanol, isopropanol and n-propanol. Additives such as acids (e.g., formic acid), bases (e.g., ammonia) or buffers (e.g., ammonium acetate) are used to enable the protonation or deprotonation of the analytes.

The quality of the above-mentioned solvents and additives strongly influences the sensitivity of MS detection; therefore, utilisation of MS grade solvents and ultrapure additives is mandatory. Make sure that these reagents are labeled as LC-MS grade by the manufacturer.

Generally, organic solvents for HPLC, such as acetonitrile and methanol, are available in three qualities: Isocratic grade, gradient grade and hypergrade for LC-MS LiChrosolv®. For MS analysis, hypergrade quality solvents should be used to ensure best performance and reliable results.

With regard to water, bottled or Milli-Q® ultrapure water from water purification systems are suitable for use with MS instrumentation. In case of low water consumption, bottled water is preferable, whereas Milli-Q® water is suggested in an environment with higher consumption. Milli-Q® systems deliver type I water and are a perfect match with LC-MS analysis. They should be used/flushed regularly in order to maintain or even further improve water quality.

Buffers are utilised to set and control the pH of a specific chromatographic separation and to protonate or deprotonate analytes in solution, which can support the electrospray ionisation process. For LC-MS, only volatile buffers and additives such as ammonium formate or acetate or triethylamine should be utilised. The use of nonvolatile buffers (e.g., sulphates, phosphates, borates) will cause precipitation in the MS source and ultimately result in tedious cleaning procedures. High buffer concentrations might lead to signal suppression.

Buffers ionise an analyte molecule M, but the formation of adducts [M+buffer] with, e.g., ammonium, formate or acetate is possible. This causes additional signals with specific m/z values in a spectrum which may compromise quantitative analyses. Consequently, for samples with high salt load such as food, body fluids or tissue, a desalting step using solid phase extraction (SPE) (e.g., Supel™-Select HLB, or LiChrolut® and Supelclean® cartridges) is recommended.

Buffers should be prepared by titration of the respective acid and base, as their purity is normally higher than the related salts. If the use of salts is necessary, an MS analysis of those used should be performed prior to use application to determine if and what type of contaminant is present in the salts.

Impurities in or contaminants of solvents and additives can accumulate on the stationary phase and elute as ghost peaks in gradient runs Figure 1. This scenario may occur when the column is equilibrated under highly aqueous conditions prior to a gradient run. Ghost peaks can even appear without equilibration if the concentration and/or retentivity of contaminants is high and/or the starting conditions of a gradient are highly aqueous. To avoid ghost peaks in gradient runs, column equilibration time should be kept as short as possible and the flushing volume should not exceed ten column volumes.

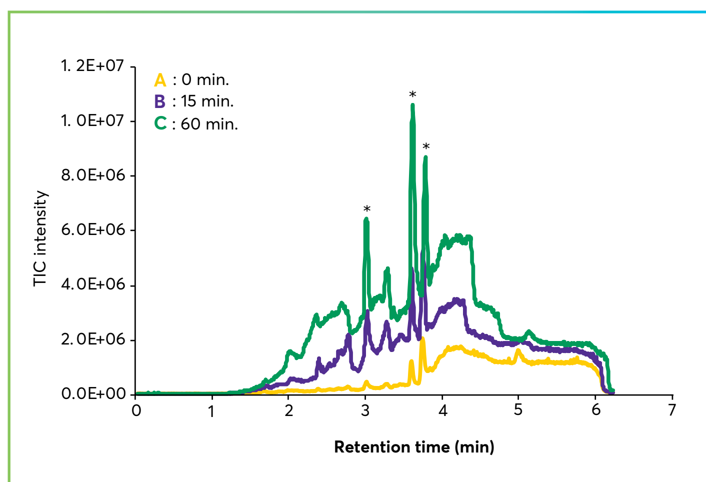


Figure 1: Accumulation of contaminants on an HPLC column during equilibration and elution via a gradient profile; peaks attributed to plasticisers are marked with an asterisk (*).

Conditions

Instrument	Bruker Esquire 6000plus
Mobile phase	A: water/acetonitrile 95/5 (v/v) + 0.1% formic acid B: acetonitrile + 0.1% formic acid
Gradient	0 min 100% A, 3 min 5% A, 5 min 5% A
Flow rate	0.4 ml/min
Temperature	25 °C
Detector	pos. ESI-MS (TICs)
Sample	Plasticisers added by the immersion of plastic tubing in aqueous solvent A

Strong acids such as hydrochloric acid, sulphuric acid or nitric acid should be avoided because they tend to form strong ion pairs with analytes and therefore make the analyte unsuitable for any type of ionisation.

Additionally, some of these strong acids have unfavorable oxidising properties.

Many laboratories use trifluoroacetic acid (TFA) in order to form ion pairs with peptides and proteins and to improve subsequent HPLC separation; however, TFA causes strong ion suppression of the analyte during MS detection and may as well contaminate the mass spectrometer. If the use of TFA is necessary, then a weak acid or isopropanol should be added to help decrease the signal suppression effect. Alternatively, difluoroacetic acid (DFA) is an option that decreases the signal suppression effect (as compared to using TFA).

SOLVENTS & ADDITIVES- STORAGE & HANDLING

Solvents should be stored in the original manufacturer's bottle; this can be either surface treated amber or borosilicate glass. Adjustment of the bottle size to specific needs is recommended, because decanting/transferring to a different container, a source of contamination, should be avoided whenever possible. Avoid standard clear or soda-lime glass bottles. Leaching alkalines and silica can form adducts with analytes.

Bottles have to be sealed and connected to the HPLC system using professional caps, adapters and tubing directly mounted to the solvent bottle. Any homemade solution will likely cause contamination of the solvent or eluent and could lead to the evaporation of organic solvents into the lab atmosphere.

Avoid plastic devices such as bottles, funnels, beakers, or gloves which can leach additives like plasticizers, anti-static agents, stabilisers or anti-slipping agents Figure 2. The only exceptions are devices that have been tested for leachables and extractables by the manufacturer, e.g., pipette tips or syringes.

LABORATORY EQUIPMENT

Cleaning of laboratory equipment and vessels can most simply be done by evaporation in a fume hood, as all reagents used in MS applications are volatile and of high purity. In cases where contamination is observed, flushing with MS grade solvents will be necessary in order to properly clean the equipment.

If a dishwasher needs to be used for any reason, it is critical that after washing the vessels are flushed/rinsed with an MS grade solvent multiple times.

HPLC COLUMN

The choice of an HPLC column dimension is guided not only by factors such as sample size, detection technique, and necessary loadability, but also by economic considerations such as reducing solvent consumption. A decrease in column internal diameter (i.d.), while geometrically scaling injection volume and flow rate accordingly, is a simple means of also improving sensitivity of a given separation.

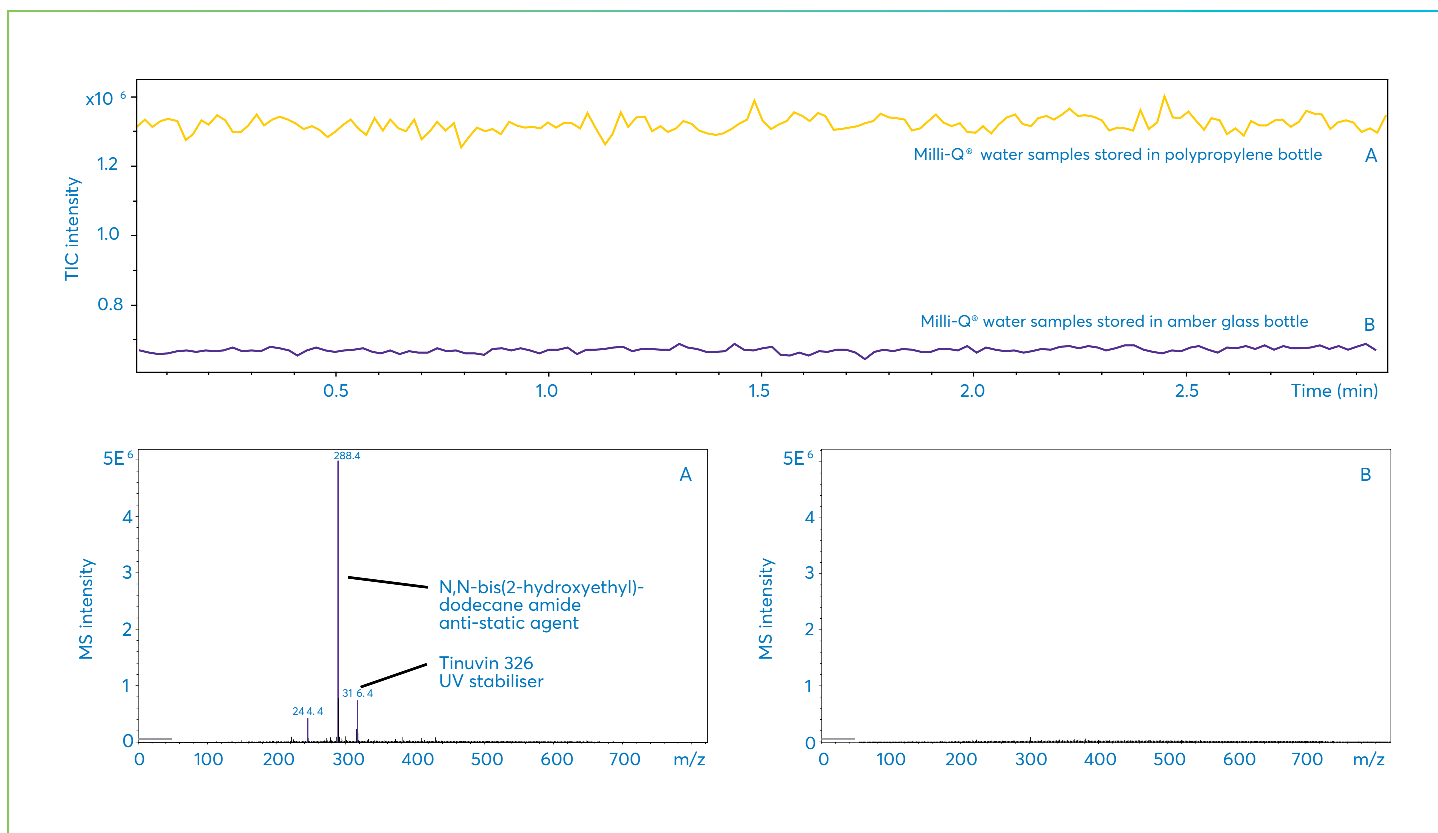


Figure 2: Mass spectra of two Milli-Q® water samples stored in polypropylene (A) and clean amber glass bottles (B), respectively (bottom), and TICs of the same samples (top). The analyses were performed via direct injection of the solvents into the MS operated in positive ESI mode.

A possible and frequent but often overlooked source of contamination in an LC-MS run is the chromatographic column itself. Many of the silica-based bonded phases are inherently prone towards bond/phase cleavage by hydrolysis, mainly at acidic pH (e.g., below pH 2), a phenomenon referred to as column bleeding Figure 3.

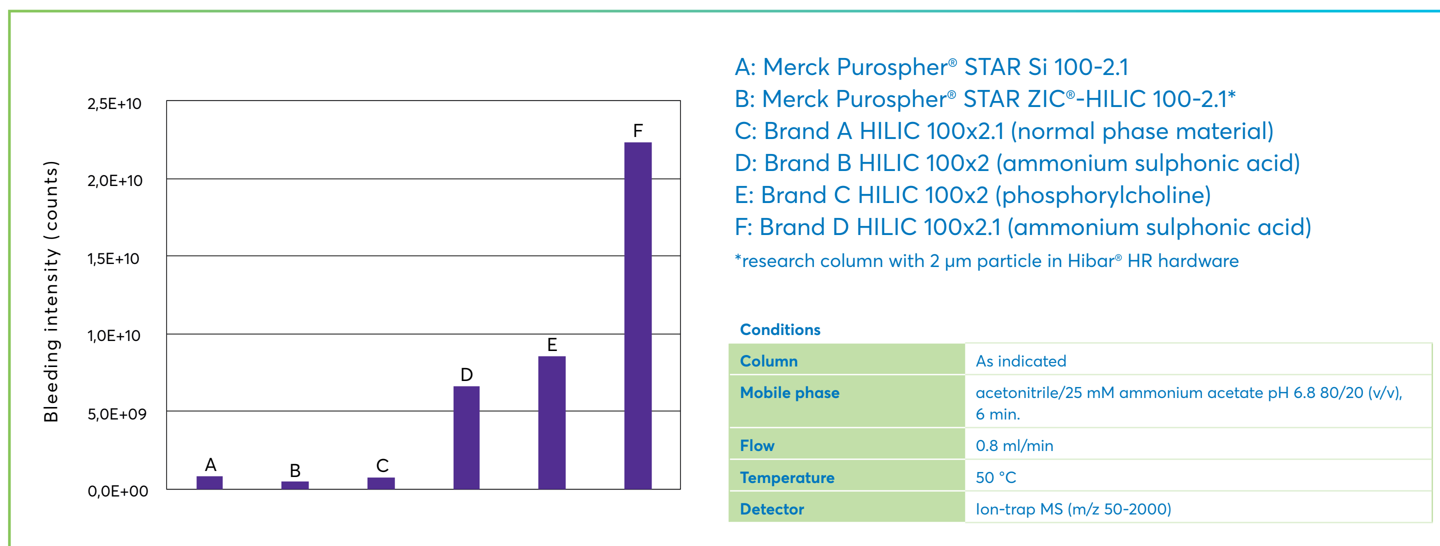
The use of a washing protocol can help to decrease the negative effect of column bleed. Alternatively, a column should undergo up to ten gradient runs from strongly aqueous to strongly organic before use with MS.

HPLC SYSTEM

A proper setup of the HPLC system itself can contribute to increased sensitivity as well. An important parameter is the minimisation of dead volume, i.e., the volume of all system parts from the injector to the detector cell, except for the HPLC column volume. Large dead volumes can cause peak broadening, tailing, or splitting and lead to poor resolution and decreased performance, and hence can decrease sensitivity and prevent detection of low abundant analytes. Consequently, all system parts (tubing, connectors, fittings) must contribute the smallest possible dead volumes.

Replace the pump inlet filter every 1 to 2 months or after changing from acetonitrile to methanol (or vice versa) as a solvent. This maintenance will lower the baseline noise and protect the system and column from pump debris.

Eluent filter frits (from solvent inlet filters) should be made out of stainless steel or PEEK rather than glass. Cleaning of the latter is tedious, as buffer residue is hard to remove, and silica and alkali might be leached out of the glass filter and form adducts.



Conditions

Column	As indicated
Mobile phase	acetonitrile/25 mM ammonium acetate pH 6.8 80/20 (v/v), 6 min.
Flow	0.8 ml/min
Temperature	50 °C
Detector	Ion-trap MS (m/z 50-2000)

Figure 3: Quantification of HILIC column bleeding of various Merck columns in comparison with alternative products measured by mass spectrometry.

GENERAL RECOMMENDATIONS

The specific requirements of different chromatographic problems might make the use of various mobile phase compositions necessary, ranging from aqueous to organic. As a general recommendation, the water content in an eluent used in LC-MS should be set to 5 to 80% in order to work trouble-free and with a stable spray.

If the water content is below 5%, buffers may precipitate in the eluent and the HPLC system. A countermeasure can be the use of a suitable organic solvent or a decrease of buffer concentration in the eluent. Buffer solubility in utilised solvents (and gradient range) should always be checked prior to analysis.

A water content of more than 80% might lead to a breakdown of the MS spray. Several options help to keep the MS spray working.

- Decrease in the surface tension of the eluent by addition of a volatile organic solvent such as acetonitrile or methanol to the mobile phase after the LC system and in front of the MS source
- Reduction of the flow delivered to the MS by means of a split or column exchange
- Manipulation of the MS source conditions (increase in dry gas temperature or flow)

In order to avoid microbial contamination of both system and mobile phase, and phase collapse, water content of the mobile phase should not be set above 95%. If a highly aqueous mobile phase is necessary, 0.05% sodium azide can be added to the eluent. Alternatively, regular flushing of the HPLC system with organic solvent, preferably isopropanol or methanol, prior to standby is mandatory. Do not use acetonitrile, because acetonitrile can polymerise and block system valves.

CONCLUSION

Mass spectrometry is a powerful technique for identification and quantification of molecules within complex mixtures. The success of mass spectrometry strongly depends on reducing contamination throughout the entire LC-MS workflo. From sample preparation to equipment cleaning. An important first step in this process is the exclusive use of highest quality materials for LC-MS, including solvents, buffers, reagents and columns. The combination of ultra-pure solvents and reagents with contamination-free handling ensures maximised LC-MS sensitivity and low LODs.

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HPLC Tips & tricks

Proper storage of HPLC/UHPLC columns

Dr. Egidijus Machtejevas, Lead Expert, Chromatography Product & Portfolio Management, Merck

The concept may seem simple. Once the last chromatogram of the day or the project is finished, we disconnect the column and put it into the drawer. However, what exactly should be done with the column before storing it? Does the procedure vary depending on the planned storage time? There is actually quite a lot to: Planned storage time, column modification (stationary phase), buffer concentration, pH, etc. In all the column storage scenarios, special care must be taken if buffers, which provide a microbe friendly environment, are used. In such cases, fresh buffers are to be prepared daily and filtered using 0.45 or 0.22 µm membrane filters. Also adding a small amount of organic solvent (~10%) or adding sodium azide (~0.05%) in the storage solvent - if buffers are used for storing e.g. needed for some HILIC columns - can be sufficient to prevent microbial growth. The easiest and safest way to store the column, however, is by using the same solvent in which it was delivered to you.

This applies in particular to the polymer-based stationary phases. Depending on their material properties, these might not be compatible with some organic solvents.

For silica-based normal phase columns, it is typically recommended that heptane or isopropanol are used. We have had good experience with dioxane, as it nicely removes residual water, but this cannot be generalised. Some stationary phases such as aminopropyl- or diol-modified stationary phases might be effectively stored in 2-propanol, which is in fact, compatible with both Reversed Phase and Normal Phase modes. Size exclusion columns should be stored in a solvent compatible with the swelling properties of the packing.

Column storage may be short, middle,and long term.
For short term storage, i.e., overnight, either the mobile phase used in the last analysis can remain in the column, or it is possible that the mobile phase passes at a very low flow rate (especially if the buffer concentration in the mobile phase is high, >50 mM). In these cases, column conditioning can potentially be skipped before continuing the analysis the next day. This option is particularly recommended for normal phase separations, where change in mobile phase composition can result in lengthy re-equilibration. However, if the buffer concentration in the mobile phase is very high (>0.5 M), then the lifetime of the pump parts (e.g. injector & switching valves) could depend on the length of time they are in contact with high concentration buffer. The same is true for the column if the pH is close to the limit of the column (for most silica-based columns - pH 2 to pH 7). Some salts, such as chloride salts used in ion chromatography in particular, are very corrosive to stainless steel and might attack the column wall as well as the inlet-outlet frits. In such cases, column (and all system) should be flushed with a less harsh mobile phase. In this case, I would recommend rinsing the column with a water-rich mobile phase (~90%) with about 10 column volumes (the approximate column volumes for some popular dimension are listed in Table 1).

Length (mm)	ID (mm)	Approximate column volume (ml)	10 Column volumes (ml)	15 Column volumes (ml)
250	4.6	4.15	41.5	62.3
250	2.0	0.79	7.9	11.8
150	4.6	2.49	24.9	37.4
150	2.0	0.47	4.7	7.1
100	4.6	1.66	16.6	24.9
100	2.0	0.31	3.1	4.7
50	4.6	0.83	8.3	12.5
50	2.0	0.16	1.6	2.4
25	4.6	0.42	4.2	6.2
25	2.0	0.08	0.8	1.2

Note: The volume of the recommended mobile phase as indicated in the table must actually pass the column. Be aware that, if you exchange the solvent bottle and remove tubing from one solvent and place it into another container, you have to consider the volume of the tubing (~2-3 ml), degasser (older degassers could be up to 15 ml, newer ~4 ml), pump (~1 ml), and injector until the new solvent reaches the column. Depending on the flow rate, additional time has to be added for the rinsing procedure.

Table 1: Approximate column volumes for some popular column dimensions and their multiples

If you disconnect a column from the instrument, end plugs should be tightly fitted to prevent solvent evaporation, otherwise a drying of the stationary phase could happen. The worst-case scenario is an improperly washed column previously used with a high salt concentration and allowed to dry over time, resulting in the formation of salt crystals. The column most likely will be irreversibly damaged. However, it might be permissible for some columns to be stored dry, others should not. Please check the manufacturer's column care guidelines. Standard HPLC columns should only be stored at room temperature and never in a freezer (exceptions are protein modified affinity or active enzyme reactor columns). These recommendations are also valid for mid- and long-term column storage.

Medium interval storage, i.e., 2 days or over the weekend. Columns should be flushed. Flush intensity or volume depends on the buffer concentration used during analysis. It is generally advisable to first flush buffering agents off the column with about 10 column volumes of mobile phase with 10% organic solvent in the water. In this case, washing will be effective, and we would also avoid buffer precipitation and possible column dewetting problems. When the buffer is washed out, pump 100% organic for 15 column volumes. The column could then be left connected to the instrument or disconnected and closed with end plugs. Please consider short-term column storage advice too, such as referencing column documentation for recommended storage solvent.

Storing a HILIC column in an acetonitrile water mixture may take a long time to re-equilibrate if a low ionic strength buffer such as 5 mM ammonium acetate is used for the analytical method. Therefore, for HILIC columns, it is recommended that they are stored in solvents containing 80–90% acetonitrile and buffers containing 5–10 mM ammonium acetate or ammonium formate. But for some HILIC phases this may differ, please check the column product information.

Ion-exchange and mixed-mode phases containing carboxylic acid functional groups (for example, weak cation-exchange phases) cannot be stored in solutions containing alcohols, because of a possible slow esterification and the resulting change in selectivity/capacity.

For long term storage (>2-3 days), silica based columns, after proper washing with a minimum of 15 column volumes Table 1 using ~ 10% organic solvent in water, should then be flushed with an organic-rich mobile phase for a minimum of 10 column volumes and should then be stored in an aprotic solvent. If water is also present, it should not be in higher concentrations (less than 50%). The best storing solvent recommended in the literature is acetonitrile or methanol (some exceptions exist, such as columns with amide modification, which should be stored in acetonitrile only). Some studies[1] also indicate that at RP conditions, rates of erosion and corrosion of the stainless steel components of the HPLC using pure acetonitrile or methanol were higher compared to when they were mixed with water. Therefore, 90% acetonitrile or methanol are perfect long-term storage agents for most reverse-phase columns. However, my personal favorite storage solution is a mixture of isopropanol and water (80/20), because of isopropanol's higher vapor pressure and the reduced chance for column dry-out, even if end fittings are not completely sealed. Isopropanol is also a stronger eluent, therefore, after storing in isopropanol, we can be sure that even more impurities will be removed than with acetonitrile or methanol gradients. Last but not least, isopropanol is also less toxic. It is also important to note that all mobile phases used for flushing, washing, or column storing must be of the same quality grade as the ones used for the analysis. Columns should be stored at room temperature (exceptions include affinity columns, as mentioned before) in their original box, with a copy of the certificate of analysis (CoA)/Column Report, and possibly with the column log book to show previous uses and to help the user evaluate the column prior to future use.

How long can columns be stored? This depends on many factors. Some columns do not change even after 5 or 10 years of storage. If you decide to use a column after such a long period of time, assume that the column most likely has dried out, and needs to be rewetted by first flushing with 100% acetonitrile (RP-phases), and then equilibrated in mobile phase for about 1 hour before making any selectivity measurements. Additionally, consider running a column test mix and compare the data to the CoA or previous column tests.

Correct column storage is essential for proper chromatography and a prolonged column life. In addition, always follow the manufacturer's guidelines for column operation details!

Reference

1. R.A. Mowery, Jr., J. Chromatogr. of Science, Volume 23, Issue 1, January 1985, Pages 22-29, https://doi.org/10.1093/chromsci/23.1.22

HPLC Tips & tricks: Getting greener in HPLC

Dr. Egidijus Machtejevas, Lead Expert, Chromatography Product & Portfolio Management, Merck

Liquid chromatography is a widely used analytical technique in various fields such as pharmaceuticals, biotechnology, food and beverage, environmental monitoring and more.

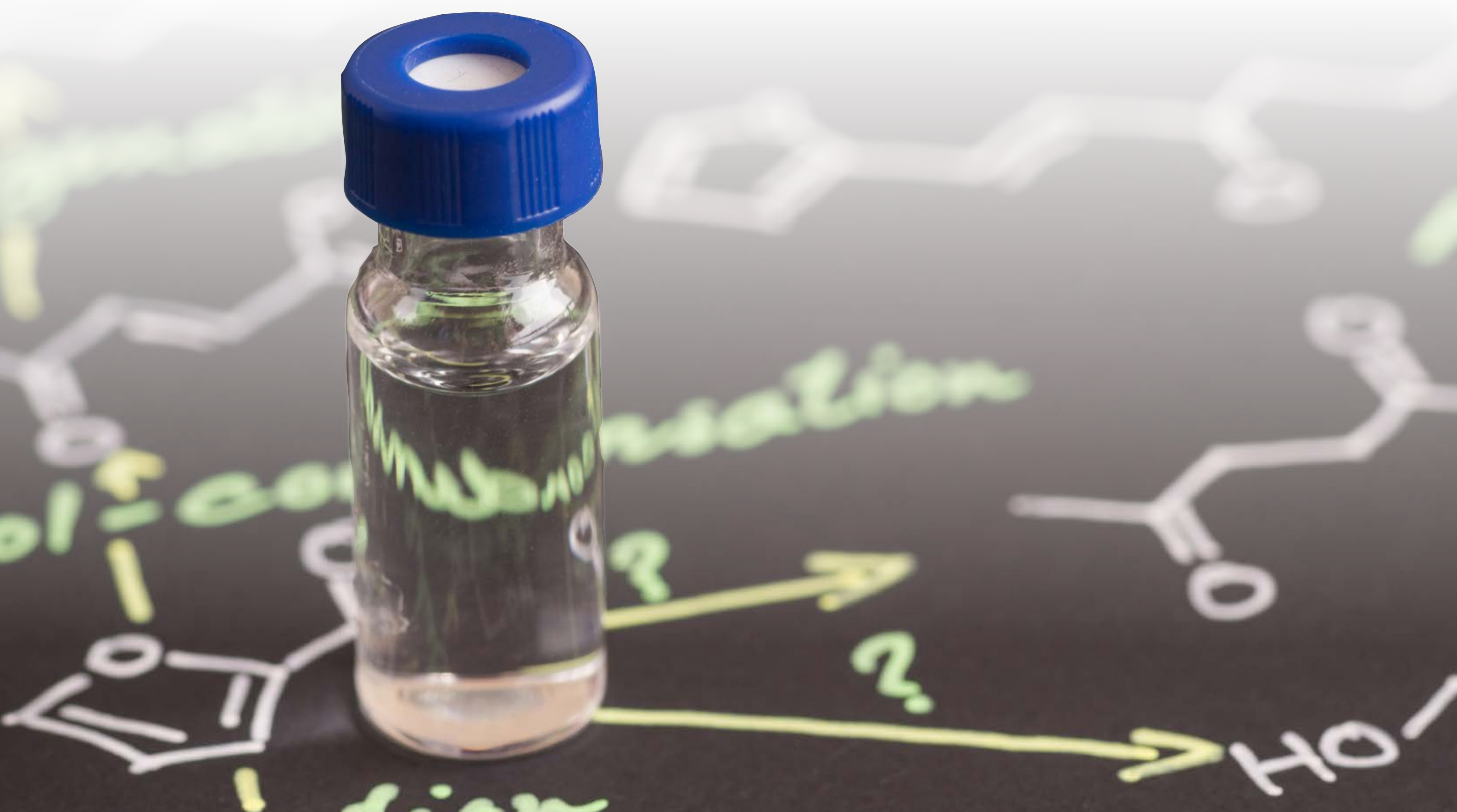
The most popular type of liquid chromatography is reversed phase (~>75%). Up until now, the technique often employs acetonitrile and relatively large columns (the most used column dimension still is 250x4.6 mm). However, there are a few options for adjusting the mobile phase to improve the sustainability of chromatography without compromising its performance.

Use eco-friendly solvents: One of the biggest environmental impacts of liquid chromatography is the use of solvents. Replacing hazardous solvents with more environmentally friendly options such as water, ethanol, or other organic solvents that are bio-renewable, safe, non-toxic and biodegradable can significantly reduce the environmental impact. Green solvents are an important component in making liquid chromatography more sustainable in general. However, higher viscosity/backpressure, UV cut-off and temperature limits of the used solvent system might need to be considered. Here are some examples of green solvents potentially to be used in liquid chromatography:
Water: Water is the most commonly used solvent in liquid chromatography, especially in reversed-phase chromatography. This solvent can be considered as one of the greenest solvents. Hot water (superheated water from 75 to 180 °C) has been already proven to have the potential to reduce organic solvent percentage in the mobile phase.[1]
Ethanol: Ethanol is typically a bio-based solvent that can be produced from renewable sources such as fermentation of bio-waste. This solvent is non-toxic, biodegradable and has a low environmental impact.
CO_2: Supercritical CO_2 is a green solvent that is used in supercritical fluid chromatography (SFC). This solvent is non-toxic, non-flammable and can be easily recycled.
Other bio-based solvents: Bio-based solvents such as terpenes or lactic acid for sample extraction, and glycerol[2] or dimethyl carbonate[3] are gaining attention in liquid chromatography workflows. These solvents are derived from renewable sources and have low toxicity and a low environmental impact.

However, it is important to note that not all green solvents are suitable for every chromatographic application, so users should carefully consider the specific properties and requirements of their method before selecting an alternative green solvent. Also, for validated methods, it is not allowed to make any changes in mobile phase composition according to Pharmacopoeias without full re-validation.

Another set of improvements is related to the method setup and different instrumental solutions:

Optimised methods: HPLC method optimisation can significantly reduce the consumption of solvents and the generation of waste. The environmental impact of an HPLC



method can be reduced by reducing the column dimensions, in particular the column inner diameter, reducing the injection volume, using different gradient conditions, and/or reducing the run time. The most significant reduction in solvent consumption can be achieved by using shorter columns with smaller inner diameters. The loss in separation efficiency of a shorter column can be compensated by more efficient smaller particles or superficially porous particles to still obtain accurate and reliable results.

Use "greener" equipment: Modern liquid chromatography equipment is designed to be more energy-efficient. Using systems that recycle solvents and/or using a lower flow split ratio can help to reduce solvent consumption and waste generation.

Recycle waste: Instead of discarding the waste generated during the chromatographic process, it could be (partly) recycled or reused for other applications, thus reducing the overall environmental impact. This approach so far is only applicable for isocratic runs.

Choose sustainable suppliers: It is important to select suppliers who prioritise sustainability and offer environmentally friendly products. This fact includes suppliers who use recycled materials, source raw materials sustainably, and prioritise energy- and raw material-efficient production methods. Look out for e.g. high EcoVadis rating or Environmental, Social, Governance (ESG) rating from MSCI.

Consider alternative methods: In some cases, alternative analytical techniques such as capillary electrophoresis, supercritical fluid chromatography or sensorics-based methods may be more sustainable and have a lower environmental impact than liquid chromatography while still providing the needed analytical answer.

In conclusion, by considering and adopting the above mentioned strategies, in particular, the reduction of column dimensions, liquid chromatography can be made more sustainable, reducing its environmental impact and contributing to a more sustainable future.

Reference

1. Huang G, Smith RM, Albishri HM, Lin J-M. 2010. Thermal stability of thiazide and related diuretics during superheated water chromatography. Chromatographia. 72(11–12):1177–1181. http://dx.doi.org/10.1365/s10337-010-1789-1.
2. Habib A, Mabrouk MM, Fekry M, Mansour FR. 2021. Glycerol as a novel green mobile phase modifier for reversed phase liquid chromatography. Microchem J. 169(106587):106587. http://dx.doi. org/10.1016/j.microc.2021.106587.
3. Lajin B, Goessler W, Introducing dimethyl carbonate as a new eluent in HPLC-ICPMS: stronger elution with less carbon, J. Anal. At. Spectrom. 36 (2021) 1272-1279, DOI https://doi.org/10.1039/D0JA00525H.

Allowable adjustments of chromatographic conditions

United States Pharmacopeia

Need to change your method?

This guide describes general procedures, definitions, and calculations of common parameters and applicable system suitability requirements. Follow the listing of suitable Thermo Scientific™ chromatography columns for LC chromatography according to USP 621.

- Changed LC column dimensions?
- Existing column or supplier not available anymore?
- Need to modernise existing methods to increase lab productivity?

Variable	Isocratic separations	Gradient separations
Stationary phases	No change in the physio-chemical characteristic of the stationary phase (Same L category)	
Particle size/column length	Per constant L/dp or N: -25% to +50%	
Flow rate	An additional change in flow rate of ±50% is permitted	After the flow rate calculation, change in flow rate is not permitted
Injection volume	Optional flexible	
Column temperature	± 10 °C	± 5 °C
Mobile phase pH	±0.2 pH units, unless otherwise prescribed	
Buffer concentration	The concentration of salts in the buffer component of a mobile phase: ±10%	
Dwell volume	N/A	If the configuration of the equipment is changed, t min should adjust in the gradient table
Changes from TPP columns to SPP columns	The plate number (N) is within –25% to +50%	

HPLC COLUMN SELECTION BY USP SPECIFICATIONS

USP code	Description	Recommended phase
L1	Octadecyl silane chemically bonded to porous or non-porous silica or ceramic micro-particles, 1.5 to 10 µm in diameter, or a monolithic rod	Thermo Scientific™ Acclaim™ 120 C18 Thermo Scientific™ Acclaim™ RSLC C18 Thermo Scientific™ Accucore™ C18 Thermo Scientific™ Accucore™ aQ Thermo Scientific™ Accucore™ 150-C18 Thermo Scientific™ Accucore™ XL C18 Thermo Scientific™ AQUASIL™ C18 Thermo Scientific™ Hyperprep™ HS C18 Thermo Scientific™ Hypersil™ 100 C18 Thermo Scientific™ Hypersil™ BDS C18 Thermo Scientific™ Hypersil™ GOLD Thermo Scientific™ Hypersil™ GOLD aQ Thermo Scientific™ Hypersil™ ODS Thermo Scientific™ Hypersil™ ODS-2 Thermo Scientific™ Syncronis™ C18 Thermo Scientific™ Syncronis™ aQ
L3	Porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod	Thermo Scientific™ Accucore™ HILIC Thermo Scientific™ Hypersil GOLD™ Silica Thermo Scientific™ Hypersil™ Silica Thermo Scientific™ HyperPrep™ HS Silica Thermo Scientific™ Syncronis™ Silica
L7	Octylsilane chemically bonded to totally or superficially porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod	Thermo Scientific™ Acclaim™ 120 C8 Thermo Scientific™ Accucore™ C8 Thermo Scientific™ Accucore™ XL C8 Thermo Scientific™ Hypersil™ BDS C8 Thermo Scientific™ Hypersil GOLD™ C8 Thermo Scientific™ Hypersil™ MOS Thermo Scientific™ Hypersil™ MOS-2 Thermo Scientific™ Hypersil™ HS C8
L8	An essentially monomolecular layer of aminopropylsilane chemically bonded to totally porous silica gel support, 1.5 to 10 µm in diameter, or a monolithic silica rod	Thermo Scientific™ Hypersil™ APS-2 Thermo Scientific™ Hypersil GOLD™ Amino Thermo Scientific™ Syncronis™ Amino
L10	Nitrile groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod	Thermo Scientific™ Hypersil™ BDS CN Thermo Scientific™ Hypersil™ CPS Thermo Scientific™ Hypersil™ CPS-2 Thermo Scientific™ Hyperprep™ GOLD CN
L11	Phenyl groups chemically bonded to porous silica particles, 1.5 to 10 µm in diameter, or a monolithic silica rod	Thermo Scientific™ Accucore™ Phenyl-Hexyl Thermo Scientific™ Accucore™ Biphenyl Thermo Scientific™ Hypersil™ BDS Phenyl Thermo Scientific™ Hypersil GOLD™ Phenyl Thermo Scientific™ Hypersil™ Phenyl Thermo Scientific™ Hypersil™ Phenyl-2
L13	Trimethylsilane chemically bonded to porous silica particles, 3 to 10 µm in diameter	Thermo Scientific™ Hypersil™ SAS (C1)
L14	Silica gel having a chemicallly bonded strongly basic quaternary ammonium anion-exchange coating, 5 to 10 µm in diameter	Thermo Scientific™ Hypersil GOLD™ SAX Thermo Scientific™ Hypersil™ SAX
L17	Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the hydrogen form, 6 to 12 µm in diameter	Thermo Scientific™ HyperREZ™ XP Carbohydrate H Thermo Scientific™ HyperREZ™ XP Organic Acids

USP code	Description	Recommended phase
L19	Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the calcium form, 5 to 15 µm in diameter	Thermo Scientific™ HyperREZ™ XP Carbohydrate Ca Thermo Scientific™ HyperREZ™ XP Sugar Alcohols
L21	A rigid, spherical styrene-divinylbenzene copolymer, 3 to 30 µm in diameter	Thermo Scientific™ HyperREZ™ XP RP 100 Thermo Scientific™ MAbPac™ RP
L22	A cation-exchange resin made of porous polystyrene gel with sulfonic acid groups, 5 to 15 µm in diameter	Thermo Scientific™ HyperREZ™ XP SCX
L26	Butyl silane chemically bonded to totally porous or superficially porous silica particles, 1.5 to 10 µm in diameter	Thermo Scientific™ Accucore™ 150-C4 Thermo Scientific™ Hypersil GOLD™ C4
L33	Packing having the capacity to separate dextrans by molecular size over a range of 4,000 to 500,000 Da. It is spherical, silica-based, and processed to provide pH stability	Thermo Scientific™ BioBasic™ SEC 120 Thermo Scientific™ BioBasic™ SEC 300 Thermo Scientific™ BioBasic™ SEC 1000
L34	Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the lead form, 7 to 9 µm in diameter	Thermo Scientific™ HyperREZ™ XP Carbohydrate Pb
L38	A methacrylate-based size-exclusion packing for water-soluble samples	Thermo Scientific™ Acclaim™ SEC-300 Thermo Scientific™ Acclaim™ SEC-1000
L40	Cellulose tris-3,5-dimethylphenylcarbamate coated porous silica particles, 3 µm to 20 µm in diameter	Thermo Scientific™ Hypersil™ Chiral OT
L43	Pentafluorophenyl groups chemically bonded to silica particles by a propyl spacer, 1.5 to 10 µm in diameter	Thermo Scientific™ Accucore™ PFP Thermo Scientific™ Hypersil GOLD™ PFP
L51	Amylose tris-3,5-dimethylphenylcarbamate-coated, porous, spherical, silica particles, 3 to 10 µm in diameter	Thermo Scientific™ Hypersil™ Chiral AT
L52	A strong cation exchange resin made of porous silica with sulfopropyl or sulfoethyl groups, 1 to 10 µm in diameter	Thermo Scientific™ BioBasic™ SCX
L58	Strong cation-exchange resin consisting of sulfonated cross-linked styrene-divinylbenzene copolymer in the sodium form, about 6 to 30 µm diameter	Thermo Scientific™ HyperREZ™ Carbohydrate XP Na
L59	Packing for the size-exclusion separations of proteins (separation by molecular weight) over the range of 5 to 7000 kDa. The packing is spherical 1.5 to 10 µm, silica or hybrid packing with a hydrophilic coating	Thermo Scientific™ MabPac™ SEC-1
L60	Spherical, porous silica gel, 10 µm or less in diameter, the surface of which has been covalently modified with alkyl amide groups and endcapped	Thermo Scientific™ Acclaim™ Polar Advantage (PA) Thermo Scientific™ Acclaim™ Polar Advantage II (PA2) Thermo Scientific™ Accucore™ Polar Premium
L62	C30 silane bonded phase on a fully porous spherical silica, 3 to 15 µm in diameter	Thermo Scientific™ Acclaim™ C30 Thermo Scientific™ Accucore™ C30
L78	A silane ligand that consists of both reversed-phase (an alkyl chain longer than C8) and anion-exchange (primary, secondary, or tertiary amino groups) functional groups chemically bonded to porous or non-porous or ceramic micro-particles, 1.0 to 50 µm in diameter or a monolithic rod	Thermo Scientific™ Acclaim™ Mixed-Mode WAX-1 Thermo Scientific™ Acclaim™ Surfactant Plus
L80	Cellulose tris(4-methylbenzoate)-coated, porous, spherical, silica particles, 5 to 20 µ in diameter	Thermo Scientific™ Hypersil™ Chiral JT
L89	Packing having the capacity to separate compounds with a molecular weight range from 100 to 3,000 (as determined by polyethylene oxide), applied to neutral and anionic water-soluble polymers; A polymethacrylate resin base, cross-linked with polyhydroxylate ether (surface contains some residual cationic functional groups)	Thermo Scientific™ Acclaim™ SEC-300
L96	Alkyl chain, reversed-phase bonded totatlly or superficially porous silica designed to retain hydrophilic and other oplar compounds when using highly aqueous mobile phases, including 100% aqueous, 1.5 µm to 10 µm in diameter	Thermo Scientific™ Acclaim™ C30 Thermo Scientific™ Accucore™ C30 Thermo Scientific™ Hypersil GOLD™ aQ Thermo Scientific™ Syncronis™ aQ
L109	Spherical particles of porous graphitic carbon, 3 to 30 µm in diameter	Thermo Scientific™ Hypercarb™
L111	Polyamine chemically bonded to porous spherical silica particles, 5 µm in diameter	Thermo Scientific™ Hypersil GOLD™ AX
L116	Sulfonated ethylvinylbenzene/divinylbenzene substrate agglomerated with hydrophilic quaternary amine functionalized glycidyl-derivative methacrylate microbeads, approximately 2 to 50 µm in diameter	Thermo Scientific™ DNAPac™ PA200
L##	(Polyethylene Glycol 3350, Aquagel OH 40) – Packing having the capacity to separate compounds with a molecular weight range from 10,000 to 200,000 g/mol (as determined by polyethylene oxide), applied to neutral, anionic, and cationic water-soluble polymers, composed of a rigid macroporous material with a hydrophilic surface	Thermo Scientific™ Acclaim™ SEC-300 Thermo Scientific™ Acclaim™ SEC-1000

*HPLC column modernisation according to new USP 621 is also applicable for the EU pharmacopeia update as well

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Universal HPTLC mix (UHM) for simplified system suitability tests

A novel concept for HPTLC suitability test

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Hanspeter Sprecher R&D Scientist; Matthias Nold Product Manager Reference Materials

ABSTRACT
The recently launched HPTLC calibration mix (Cat. No. SUPL91816-1ML) for use as a universal system suitability test (SST) solution, developed in collaboration with CAMAG, a leading manufacturer of HPTLC instrumentation.

INTRODUCTION
In HPTLC, the SST often qualifies only a limited region of the chromatogram (e.g., specific RF values or narrow RF ranges). If no deviation from the acceptance criteria is observed, the entire chromatographic system is typically considered compliant. However, in practice, the chromatographic quality of the other regions remains unknown. Additionally, HPTLC methods using developing solvents of different polarities resulting in different selectivities may require different sets of substances for different SST. Cost and stability are the other criteria to consider when selecting reference substances for a system suitability test. To offer convenience and reliability, a Universal HPTLC Mix (UHM) for use in SST was developed, that is applicable for use with a wide variety of solvents[1].

The idea for a universal system suitability test (SST) for HPTLC originated from the company Anchrom (India). Dr. Manjusha Phanse started the evaluation of this concept. Thinking about the practical aspects of qualifying an HPTLC analysis and the needs of clients for routine analysis, the laboratory teams of CAMAG and Anchrom worked together to create a new SST concept for HPTLC. This project was later supported by Sigma-Aldrich Chemie GmbH (subsidiary of Merck KGaA, Darmstadt, Germany). The outcome was a joint publication in the Journal of Chromatography A1 and the launch of the HPTLC calibration mix, a ready-to-use analytical standard solution, suitable for the CAMAG SST concept.

This mix is applicable for SST in a wide range of chromatographic systems, with different polarities and selectivities. The replacement of conventional substances for SST by the UHM will help laboratories to save time and money required for laborious in-house investigations of specific reference substances for each method to be qualified. Different fields of application can benefit from the UHM concept, such as herbal drugs, forensics, pharmaceuticals, cosmetics, etc.

DEFINITION OF MIX COMPOSITION
In the first step of the investigation, suitable substances for the UHM were selected. An initial list of 56 candidates was determined using the following criteria:

01. Low hazard (not harmful and non-toxic substances)
02. Detectability at UV 254 and 366 nm prior to derivatization
03. High stability in solution

The chromatographic behavior of those 56 compounds was evaluated with 20 developing solvents (8 are shown in Table 1), covering a wide range of polarities and

No.	Developing solvent	Polarity index	Selectivity groups
A	Ethyl acetate, formic acid, acetic acid, water 100:11:11:26 (V/V/V/V)	5.63	VI, IV, VIII
B	Ethyl acetate, formic acid, water 15:1:1 (V/V/V)	4.76	VI, VIII
C	Dichloromethane, methanol, water 14:6:1 (V/V/V)	4.01	V, I, VIII
D	Toluene, acetic acid 4:1 (V/V)	3.12	VII, IV
E	Toluene, ethyl acetate 3:1 (V/V)	2.90	VII, VI
F	Toluene, ethyl acetate 9:1 (V/V)	2.60	VII, VI
G	Toluene, methanol, diethylamine 8:1:1 (V/V/V)	2.58	VII, I
H	Cyclohexane, ethyl acetate 5:3 (V/V)	1.73	VI

TABLE 1. Examplary listing of developing solvents with their polarities and selectivity groups according to Snyder

CHROMATOGRAPHIC CONDITIONS:

Plate: HPTLC plates silica gel 60 F254, 20×10 cm (Cat. No. 1.05642.001).

Standard solutions: In the development phase, 2.0 µl of individual compound solutions were applied as bands with the Automatic TLC Sampler (ATS 4), band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm. For the HPTLC calibration mix, an application volume of 2.0 µl is recommended for best results.

Chromatography: Plates were developed to 70 mm (from the lower edge) in the ADC 2 with chamber saturation (20 min, with saturation pad) and after activation at 33% relative humidity for 10 min using a saturated aqueous solution of magnesium chloride. 20 different developing solvents (eight of them are listed in Table 1) were investigated, followed by drying for 5 min.

Documentation: Images of the plates were captured with the TLC Visualizer 2 at UV 254 nm and 366 nm.

Densitometry: Absorbance measurement at 254 nm and fluorescence measurement at 366 nm with TLC Scanner 4 and visionCATS, slit dimension 5.00 mm x 0.20 mm, scanning speed 20 mm/s. For the fluorescence measurement, a mercury lamp and a cut-off filter 400 nm were used.

The objective was to find the ideal set of substances that provides an even distribution of zones throughout the entire chromatogram for a maximum number of different developing solvents. Additionally, each developing solvent should achieve a baseline separation for at least 3–4 substances. The finally chosen substances and their chromatograms with eight different developing solvents are shown in Figure 1.

To evaluate, whether the proposed UHM responds to variations in the chromatographic conditions, three experiments were performed:
In the first, plates were conditioned to different relative humidities (from 0% to 90%) prior to development. As shown in Figure 2, the UHM is sensitive to variations in relative humidity, particularly to the higher ones. The differences were more pronounced for developing solvents containing no water.

In the second experiment, the individual proportion of the solvents in developing solvents B and F Table 1 was changed (±10%), and the effect on the chromatography was evaluated. A difference of up to 0.06 RF units could be observed from the mean RF values of the control track.

In the third experiment, different levels of chamber saturation were tested: unsaturated, partially saturated (20 min, no saturation pad), and saturated (20 min, with saturation pad). RF values increased with partial saturation, but then decreased with full saturation Figure 3, proving that the SST with the UHM may indicate chamber saturation problems.

The UHM performance was evaluated in intra- and inter-laboratory tests based on the ΔRF in developing solvents B, F and G. For the intra-laboratory test, the confidence interval ΔRF was 0.03, while for the inter-laboratory test, this value was 0.04.

Throughout the development of the final composition, we supported CAMAG with the individual components that were considered and at a later stage with several prototypes of the mix. The subsequent optimisation lead to the final composition Table 2.

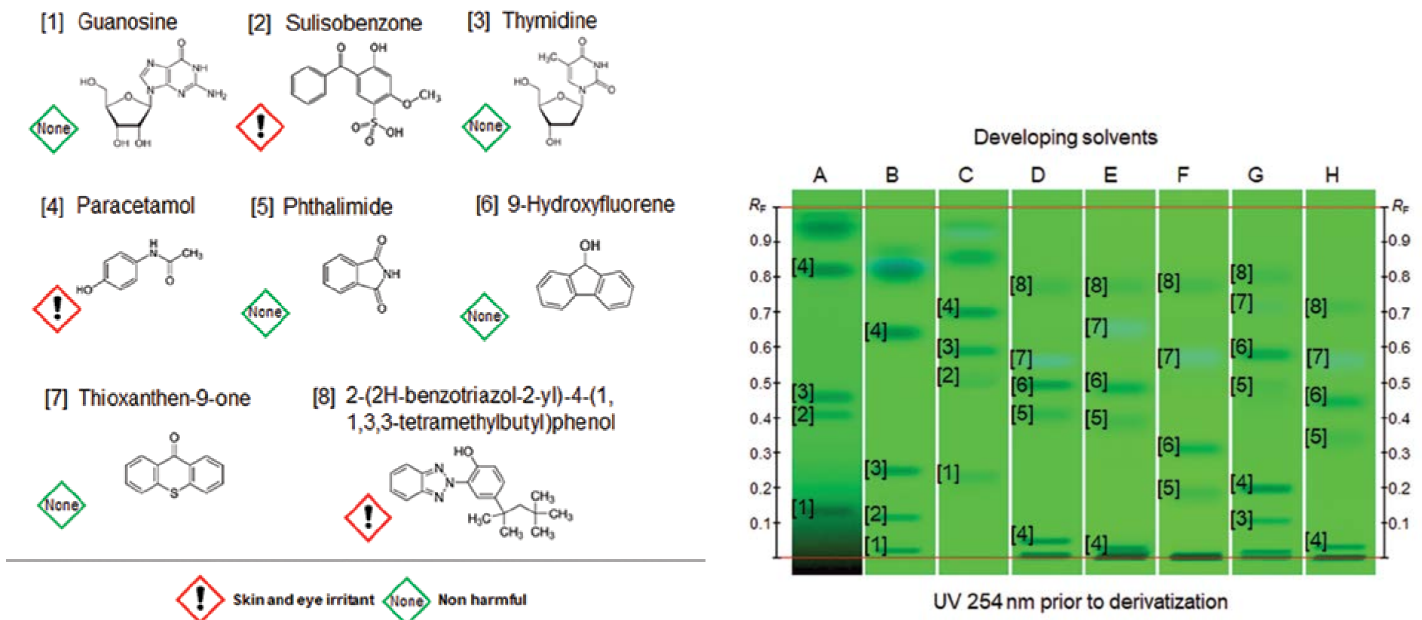


FIGURE 1. Substances selected for UHM and the HPTLC chromatograms of the UHM with eight different developing solvents Table 1. Bands: 1. Guanosine, 2. Sulisobenzone, 3. Thymidine, 4. Paracetamol, 5. Phthalimide, 6. 9-Fluorenol (9-Hydroxyfluorene), 7. Thioxanthone, 8. Octrizole (2-(2H-Benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol).

DEVELOPING SOLVENT G

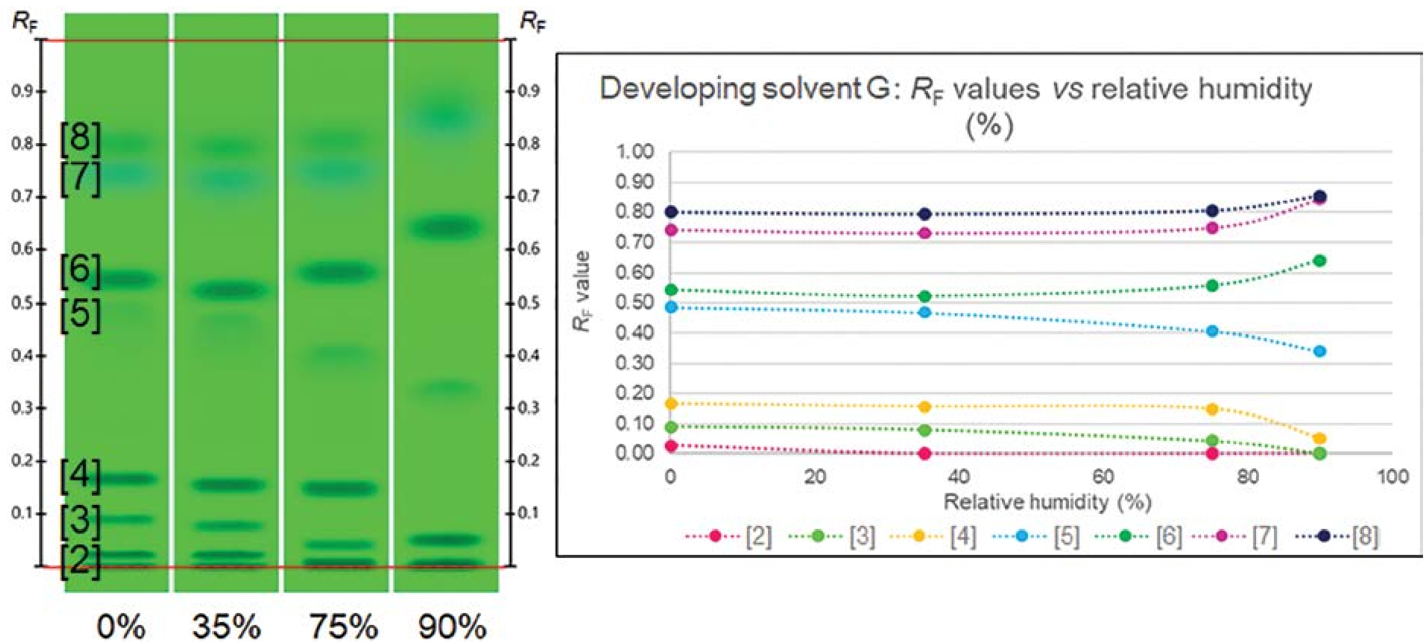


FIGURE 2. UHM evaluated with developing solvent G see Table 1 and conditioned with different relative humidities prior to development. (Bands: 1. Guanosine, 2. Sulisobenzone, 3. Thymidine, 4. Paracetamol, 5. Phthalimide, 6. 9-Fluorenol, 7. Thioxanthone, 8. Octrizole)

DEVELOPING SOLVENT G; CORRECTED FRONT

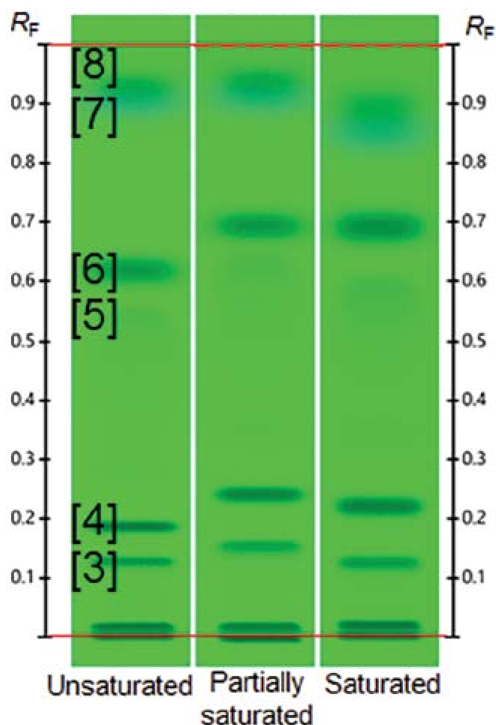


FIGURE 3. UHM evaluated with developing solvent G (Table 1) developed with different levels of chamber saturation (Bands: 1. Guanosine, 2. Sulisobenzone, 3. Thymidine, 4. Paracetamol, 5. Phthalimide, 6. 9-Fluorenol, 7. Thioxanthone, 8. Octrizole).

Compound	Concentration
9-Fluorenol	1000 mg/L
Guanosine	500 mg/L
Octrizole	1000 mg/L
Paracetamol	1000 mg/L
Phthalimide	2000 mg/L
Sulisobenzone	1000 mg/L
Thioxanthone	10 mg/L
Thymidine	1000 mg/L

TABLE 2. Final UHM components in methanol

The ready-to-use standard mix is available as Cat. No. SUPL91816-1ML. This product is manufactured under ISO 9001 management system as an analytical standard quality grade and is provided in a 1 ml amber glass ampoule. Stability checks were preformed to ensure that the mix is fit for purpose for the entire duration of the shelf life.

CONCLUSION

The newly developed universal HPTLC mix (UHM) enables HPTLC users to efficiently and reliably perform their system suitability testing (SST).

Reference

1. Do TKT, Schmid M, Phanse M, Charegaonkar A, Sprecher H, Obkircher M, Reich E. 2021. Development of the first universal mixture for use in system suitability tests for High-Performance Thin Layer Chromatography. J Chromatogr A. 1638(461830):461830. doi:10.1016/j.chroma.2020.461830.

FEATURED PRODUCTS

Description	Cat. No.
HPTLC calibration mix, 8 compounds in methanol, 1 ml	**SUPL91816-1ML**
HPTLC Silica gel 60 F_{254}, 20 cm × 10 cm, glass support, Pk.50	**1.05642.0001**

J.T.Baker® BAKERBOND® protein precipitation and supported liquid extraction plates for efficient LC-MS sample preparation

Matt James, Senior Research Scientist, and Tony Edge, R&D leader & Scientific Advisor, Avantor.

Liquid chromatography, coupled with mass spectrometry (LC-MS), is a highly specific, sensitive and rapid technique that can be used for the determination of low level target analytes in complex biological matrices, and is routinely applied for many bioanalytical analyses. Using this approach, analytes are separated by LC, prior to detection by MS. MS can be used to provide highly sensitive and selective identification and quantification of target analytes.

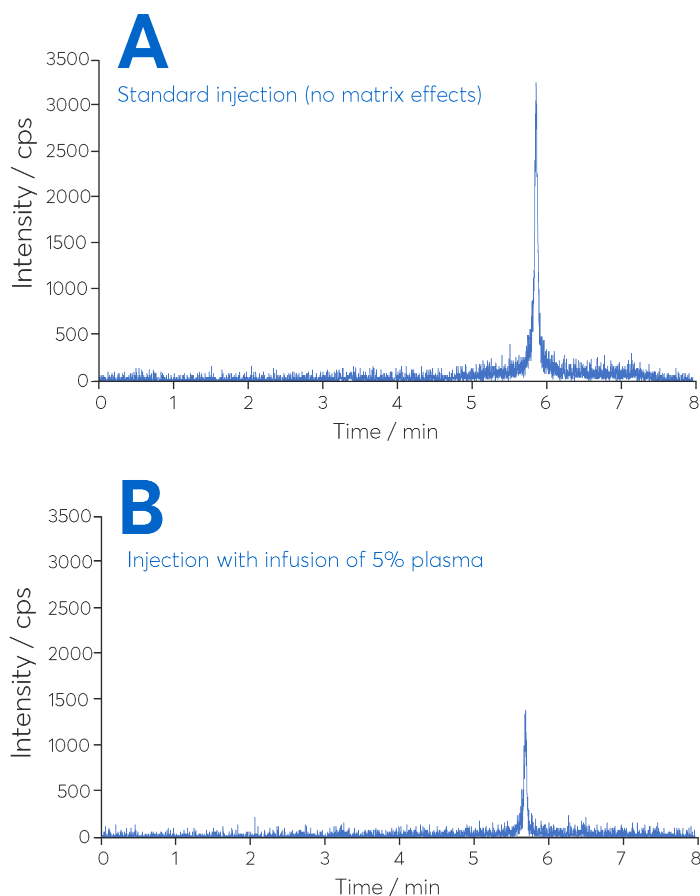
Biological matrices, such as plasma, serum and urine, are usually highly complex, comprising of high concentrations of endogenous components, alongside often low relative concentrations of the target analyte(s). Injection of neat biological fluids onto an LC-MS system is typically not practical, as this would result in a plethora of issues including, but not limited to:

- Particulate build-up on the column, resulting in increased back pressure
- Back pressure build up/blockage due to matrix components precipitated in the mobile phase
- Contamination of the column, resulting in poor peak shape and retention time shifts
- Contamination of LC-MS instrumentation, causing sensitivity and carry-over issues

These issues are highly undesirable, particularly in high throughput scenarios, as they result in significant reduction in chromatographic performance and column lifetime, more frequent instrument down time and increased instrument maintenance and cleaning requirements.

An additional and critical consideration is the effect that matrix components can have on the analytical data obtained from the assay. Despite chromatographic separation of the target analytes, the complexity of biological matrices and typically low relative abundance of target analytes, means that matrix components can interfere with analyte response, preventing accurate quantification[1]. If, as is likely in complex samples, more than one compound is eluted simultaneously from LC column, then there is potential for interference. These ultimately can result in either a decrease (suppression) or increase (enhancement) in signal response for the target analyte. The matrix can, therefore, have a significant impact on sensitivity and the accuracy of analyte quantification.

An example of the potential impact of interfering matrix components is demonstrated in Figure 1. In Figure 1A, a standard solution of estrone was injected and analysed on a reversed-phase gradient using an ACE® Excel® Super C18 column and the 269.1 → 145.0 MRM transition monitored. The experiment was then repeated with infusion of a 5% solution of protein precipitated plasma directly into the MS source. The presence of plasma components has a significant impact on the response obtained. A 48% reduction in MS detector response was obtained. This loss in sensitivity is a result of the effect on estrone ionisation by matrix components.



Column	Avantor® ACE® Excel® SuperC18
Particle size	2 µm
Dimensions	100x2.1 mm
Mobile phase	A: 20 mM ammonium acetate (aq) B: 20 mM ammonium acetate in MeOH/H_2O (90:10)
Gradient	

Time (min)	% B
0	2
0.1	60
2.1	60
2.2	98

Flow rate	0.4 ml/min
Injection	1.0 µl
Temperature	50 °C
Detection	Sciex QTRAP® 6500+ LC-MS/MS system
Ionisation mode	ESI, negative mode
Source temperature	350 °C
Curtain gas	38 psig
Source voltage	-3500 V
Ion source gas 1	40 psig
Ion source gas 2	70 psig

FIGURE 1 : Demonstration of the influence of matrix effects on the analysis of estrone (MRM 269.1→ 145.0) by LC-MS. A: injection of a 100 ng/ml estrone standard. B: Injection of a 100 ng/ml estrone standard with infusion of a 5% rat plasma solution into the MS source at a flow rate of 7 µl/min.

STRATEGIES TO REMOVE MATRIX COMPONENTS

It is clear that matrix components which may interfere with the analysis, should preferably be removed prior to analysis. The purpose of sample preparation is to remove these interferences in an efficient, reliable and reproducible manner. J.T.Baker® BAKERBOND® provide several approaches for sample preparation and removal of matrix components from biological samples, including protein precipitation (PPP), supported liquid extraction (SLE) and solid phase extraction (SPE)[2]. This article focusses on the application of PPP and SLE for sample clean-up. These approaches utilise a 96-well plate format Figure 2 to allow for simplified, high throughput application in analytical workflows.

FIGURE 2 : J.T.Baker® BAKERBOND® 96-well plate.

PROTEIN PRECIPITATION PLATES

Biological samples containing proteinaceous matrix components, that may interfere with downstream analytical methods, require clean-up prior to analysis to remove these substances. Additionally, such components can precipitate when introduced into the LC mobile phase, leading to column or system blockages. Isolation of high quality, protein-free samples is, therefore, a vital pre-requisite for successful LC-MS analysis. J.T.Baker® BAKERBOND® 96-well protein precipitation plates use a crash method to provide a simple and effective solution to endogenous protein removal. Proteins in the sample are precipitated using an organic solvent and removed by filtration through a hydrophobic frit.

The 96-well format allows for high throughput, reproducible protein removal. Precipitation of proteins is achieved by the addition of three volumes of acetonitrile, followed by gentle mixing and incubation for a short period, typically up to 3 minutes[3]. The sample is then recovered by application of a vacuum or centrifugation. The novel hydrophobic frit is

designed to retain the sample without it draining away under high organic conditions, whilst the low binding characteristics ensure maximum analyte recovery. These attributes and ease of automation make J.T.Baker® BAKERBOND® protein precipitation plates an ideal choice for high throughput protein precipitation.

SUPPORTED LIQUID EXTRACTION

Traditional liquid-liquid extraction has been used for centuries to enrich or purify solutes, based on differential solubility in aqueous and organic solvents. The dissolved analyte solution is combined with an appropriate immiscible aqueous or organic solvent and shaken. The analyte preferentially partitions into one of the phases, whilst other sample components ideally partition into the opposite phase. The phase containing the analyte is then removed, the extraction is then repeated, and the sample concentrated by evaporation prior to analysis. Although effective, this process is typically lengthy, requires considerable volumes of solvent, and is more suited to extraction of large volume samples. Smaller scale versions of liquid extraction have been commercialised, however, the process is highly dependent on the mixing of two immiscible liquids, which can be challenging when dealing with very small volumes.

SLE can overcome these issues and can be performed on a smaller scale, compatible with biological samples and can be applied using a high degree of automation. The technique is widely applied for the clean-up of complex matrices in a diverse range of analytical fields, including environmental, clinical, forensics, food and beverage also environmental. In SLE, the aqueous solvent, which is pH adjusted to ensure the non ionised analyte, is supported as a very thin layer on an inert support Figure 3, typically diatomaceous earth (a siliceous material composed of the fossilised exoskeletons of diatoms), whilst the immiscible organic solvent percolates over the support to facilitate analyte transfer[4]. The high surface area of the support, combined with the thin aqueous film, makes the transfer significantly more efficient compared to traditional liquid-liquid extraction, with dramatic reduction in solvent requirements with greater reproducibility and recoveries. Additionally, sample processing is more efficient as laborious mixing steps are eliminated, emulsion formation is avoided, and the process is readily automated for high throughput analysis. Targeted analyte extraction can also be achieved through judicious selection and optimisation of the aqueous phase. J.T.Baker® BAKERBOND® SLE plates incorporate a diatomaceous earth packing material into a high throughput 96-well plate format for quick, reproducible sample processing and excellent analyte recovery from biological matrices. This SLE solution is also available with two sorbent bed weights and a 3 ml cartridge format for larger volume samples.

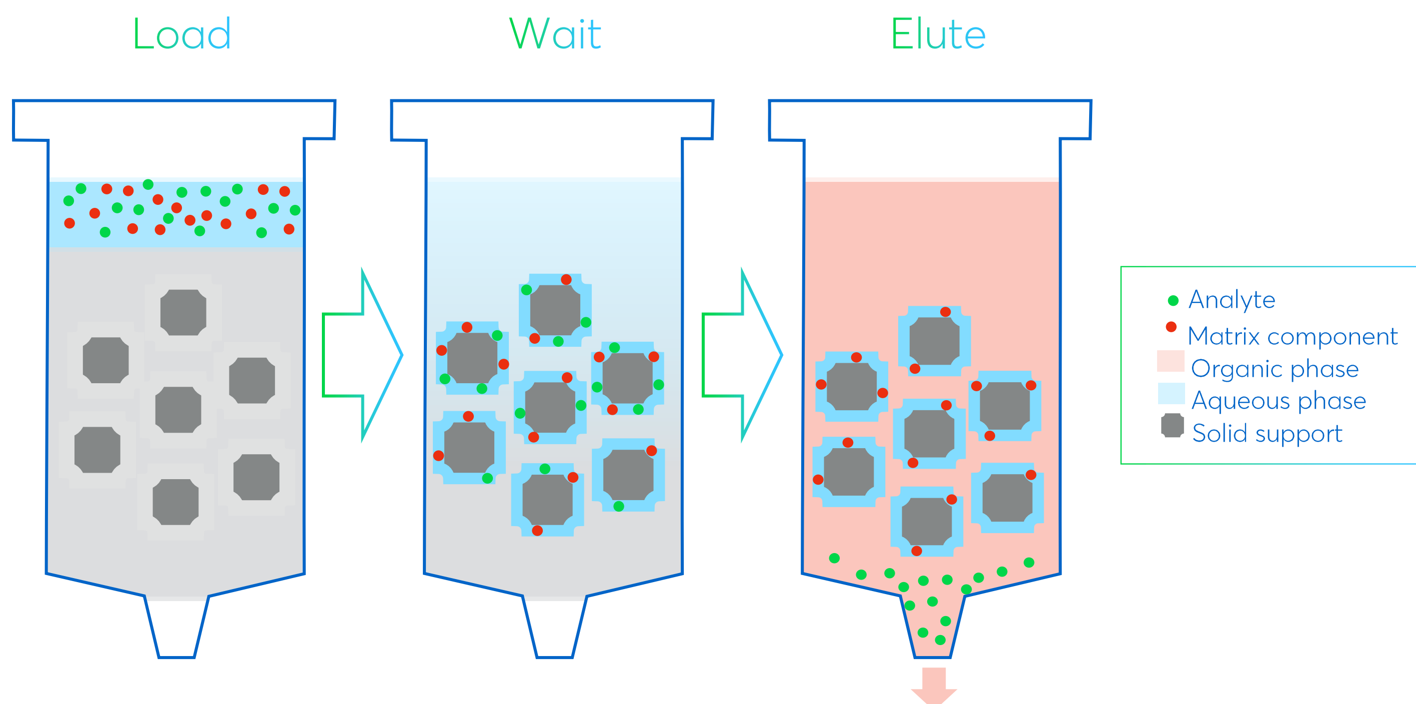


FIGURE 3 : Schematic representation of the extraction of an aqueous analyte using the SLE approach.

CONCLUSION

The use of appropriate sample preparation techniques prior to the analysis of biological samples is highly beneficial. Their use typically results in improvements to method reproducibility, sensitivity and quantification accuracy, along with reduced LC-MS system maintenance requirements and downtime. PPP and SLE are effective and widely utilised techniques for the removal of endogenous matrix components from complex matrices. In addition, regulatory considerations such as requirements for analyte resolution from interfering peaks that can potentially invalidate a method, variability in sample interference between samples, and an understanding of sample matrix effects as part of bioanalytical validation, support the use of sample reparation protocols.

J.T.Baker® BAKERBOND® PPP and SLE plates provide a high performance, reproducible and fully automatable solution for the quick and efficient removal of proteins using PPP and selective removal of matrix components with SLE prior to LC-MS analysis. The removal of interfering matrix components improves the reproducibility and sensitivity of bioanalytical assays, whilst also extending the lifetime of the LC column and reducing LC-MS maintenance and instrument downtime.

Protein precipitation 96-well plates

Description	Sorbent weight	Pk	Cat. No.
BAKERBOND® protein precipitation plate	200 mg	1	**8181-96**
BAKERBOND® protein precipitation plate	200 mg	5	**8181-596**

SLE 96-well plates and cartridges

Description	Sorbent weight	Pk	Cat. No.
BAKERBOND® SLE 96-well plate	200 mg	1	**8182-96**
BAKERBOND® SLE 96-well plate	400 mg	1	**8183-96**
BAKERBOND® SLE 3 ml cartridge	200 mg	50	**8182-02**
BAKERBOND® SLE 3 ml cartridge	400 mg	50	**8183-04**

Accessories

Description	Cat. No.
Mid gasket for vacuum manifold 8183-VM	**8190-RG**
Vacuum manifold for multi-well plates	**8183-VM**
Top gasket for vacuum manifold 8183-VM	**8185-RG**
Space insert for vacuum manifold	**8186-SI**
Disposable reservoir tray 25 pk	**8188-RT**

References

1. Annesley, T. M. Clinical Chemistry 49 (7), 1041–1044
2. J.T.Baker® BAKERBOND® spe 96-well plates
3. J.T.Baker® BAKERBOND® PPP protocol
4. J.T.Baker® BAKERBOND® SLE protocol

VWR® LC-MS solvents, additives, mixes and associated products

Perform LC-MS with a complete range of reagents, filters, vials and safety caps.

MORE INFORMATION

PB20067-EN

Enhanced LC sample preparation with new high performance syringe filters from J.T.Baker®

Matt James, Senior Research Scientist, Mark Fever, R&D Manager and
Tony Edge, R&D leader & Scientific Advisor, Avantor

INTRODUCTION

Many samples analysed by liquid chromatography (LC) contain particulates that can potentially damage both LC instrumentation and the analytical column. Particulates can range from large particles and microorganisms to fine, submicron suspended particles. Insufficient sample filtration can result in system blockages, increases in back pressure, reduced column lifetime and distortion of the analyte peak (e.g. increased peak tailing, split peaks etc). It is, therefore, important to remove particulates from the sample prior to analysis. Single-use, disposable syringe filters provide a quick and convenient approach, the sample is drawn into a syringe and then pushed through an attached syringe filter into a sample vial, ready for LC analysis.

To ensure effective particulate removal and to preserve the integrity of the sample, it is essential to use high quality syringe filters that provide reproducible and efficient filtration, and that maintain the sample integrity by not contaminating the sample by leaching extractable components during use. This short article introduces the new range of syringe filters from J.T.Baker® and demonstrates how they provide exceptional performance to ensure confidence in analytical data produced.

PREMIUM SYRINGE FILTERS

J.T.Baker® syringe filters are premium filters that have been specifically designed for the filtration of chromatography samples. They have been optimised to deliver the highest levels of performance and provide consistent and reproducible results with minimal extractables/leachables. These HPLC certified syringe filters are manufactured in ISO 9001 certified facilities to ensure the highest quality and reproducible batch performance.

Filtration media	Main features	Applications
Nylon*	Hydrophilic	Chemical filtration
	Robust	Beverage filtration
	Broad chemical compatibility	HPLC sample preparation
	pH range: 3 - 14	
PES*	Hydrophilic	Protein filtration
	High asymmetry; high flow rate	Buffer prep
	Low extractables	HPLC sample preparation
	Low protein binding	
	pH range: 3 - 12	
PTFE*	Hydrophobic	Organic solvents
	Broad chemical compatibility	Strong acids and alkalis
	pH range: 1 - 14	Gas filtration or air sampling
		HPLC sample preparation
H-PTFE*	Hydrophilic	Universal filtration
	Broad chemical compatibility	Organic solvents
	pH range: 1 - 14	Strong acid and alkaline resistance
		HPLC sample preparation
RC*	Broad chemical compatibility	Organic solvents
	pH range: 3 - 12	General aqueous
Glass fibre	Broad chemical compatibility	Clarification and pre-filtration
	High particulate capacity	DNA/RNA adsorption and purification
	pH range: 3 - 14	

* Also available with glass fibre pre-filter for applications involving high particle load samples

They are available with a wide range of membranes Table 1 and a range of filter diameters (13, 25 and 30 mm) and pore sizes (0.22; 0.45 and 1.00 µm) to suit every application. The housing is manufactured from polypropylene and the filters are batch tested and certified for housing burst pressure, bubble point, flow rate performance and extractables. J.T.Baker® syringe filters are also available with built-in glass fibre pre-filters (double-layer type, 100% binder-free borosilicate glass fibre), which is specially designed for high particle loading and is an ideal solution for difficult to filter liquids.

WHY USE SYRINGE FILTERS?

If a sample contains particulate matter and is analysed by LC without filtration, particulates may block the tubing within the LC system or may accumulate at the head of the LC column. This can result in an increase in back pressure, distortion of peak shape, loss of performance and a reduction in column lifetime, ultimately compromising the analytical data generated. Figure 1 demonstrates the impact that effective sample filtration can have on the LC column. In this experiment a sample containing a 5% latex solution of polystyrene beads (0.46 to 1 µm) was injected onto an Avantor® ACE® Excel® 3 C18 column (50x3.0 mm), with and without use of a syringe filter. When the sample is filtered using a 0.45 µm syringe filter prior to injection, the beads are successfully filtered from the sample and the column back pressure remains constant (103 ±2.5 bar) for 500 successive injections. When the sample is not filtered, an identical column back pressure is initially obtained for the first 10 injections. However, the pressure then begins to slowly increase, as the particulates accumulate at the head of the column, before increasing dramatically after 50 injections, as the column is blocked, and the back pressure exceeds the pressure limits of the LC column. This experiment demonstrates how the use of syringe filters can protect the column and dramatically extend the column lifetime.

J.T.BAKER® SYRINGE FILTERS FOR HIGH PERFORMANCE SAMPLE FILTRATION

To provide reliable protection for the LC instrumentation and column, it is important to use syringe filters with well defined, reproducible porosity to ensure that particulates are always removed from samples. J.T.Baker® syringe filters are manufactured to strict standards to ensure efficient and reproducible extraction of particulates. Figure 2 shows experimentally determined extraction efficiencies for the range of 0.22 µm syringe filters. The data bars represent triplicate filters for multiple batches (n = 2-6). All membranes tested showed excellent extraction efficiencies, demonstrating their high performance and excellent reproducibility.

In addition to providing reliable particulate removal, it is also important that syringe filters do not release chemical

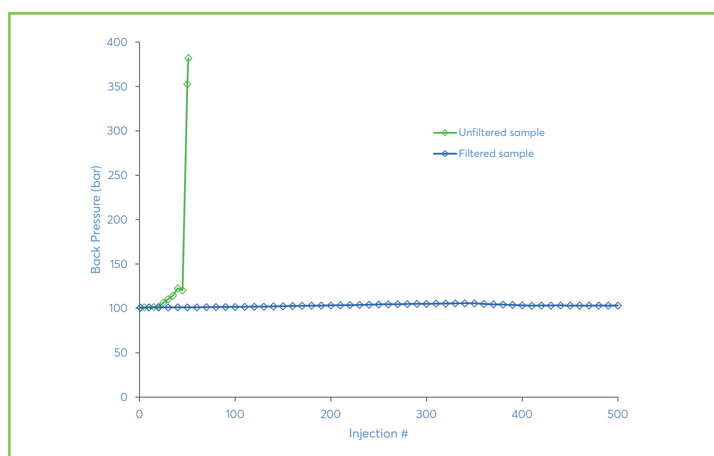


Figure 1: Plot of recorded column back pressure vs injection number for an experiment injecting a 5% latex solution of polystyrene beads (0.46 to 1 µm) onto a 3 µm C18 column using an LC system with a 400 bar pressure limit. The green trace shows the results obtained for injection of the sample without filtration, whilst the blue trace shows the results when the sample filtered using a J.T.Baker® H-PTFE, 0,45 µm syringe filter prior to injection. Column: Avantor® ACE® Excel 3 C18 column (50x3,0 mm), Mobile phase: MeOH:H_2O (70:30 v/v), Flow rate: 0.43 ml/min, Temperature: 22 °C, Injection volume: 5 µl. The plot shows every 5th injection for the unfiltered sample and every 10th injection for the filtered sample.

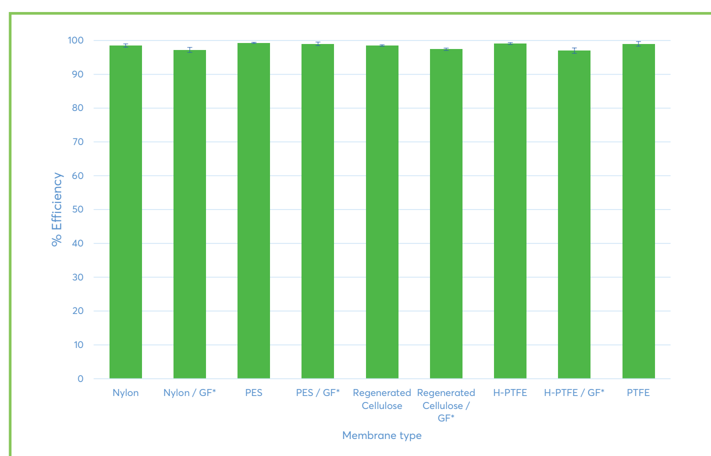


Figure 2: Extraction efficiency of J.T.Baker® 0.22 µm syringe filters by membrane type. Extraction efficiencies were experimentally determined by filtering a 0,01% latex of 0.3 µm polystyrene beads using syringe filters containing the various membranes, followed by determination of extraction efficiency against a set of prepared calibration standards by spectrophotometry (UV, 272 nm). Triplicate analyses were performed for multiple batches. Samples were prepared in water for hydrophilic membranes and in methanol for hydrophobic membranes. * Refers to filters with built-in glass fibre pre-filters.

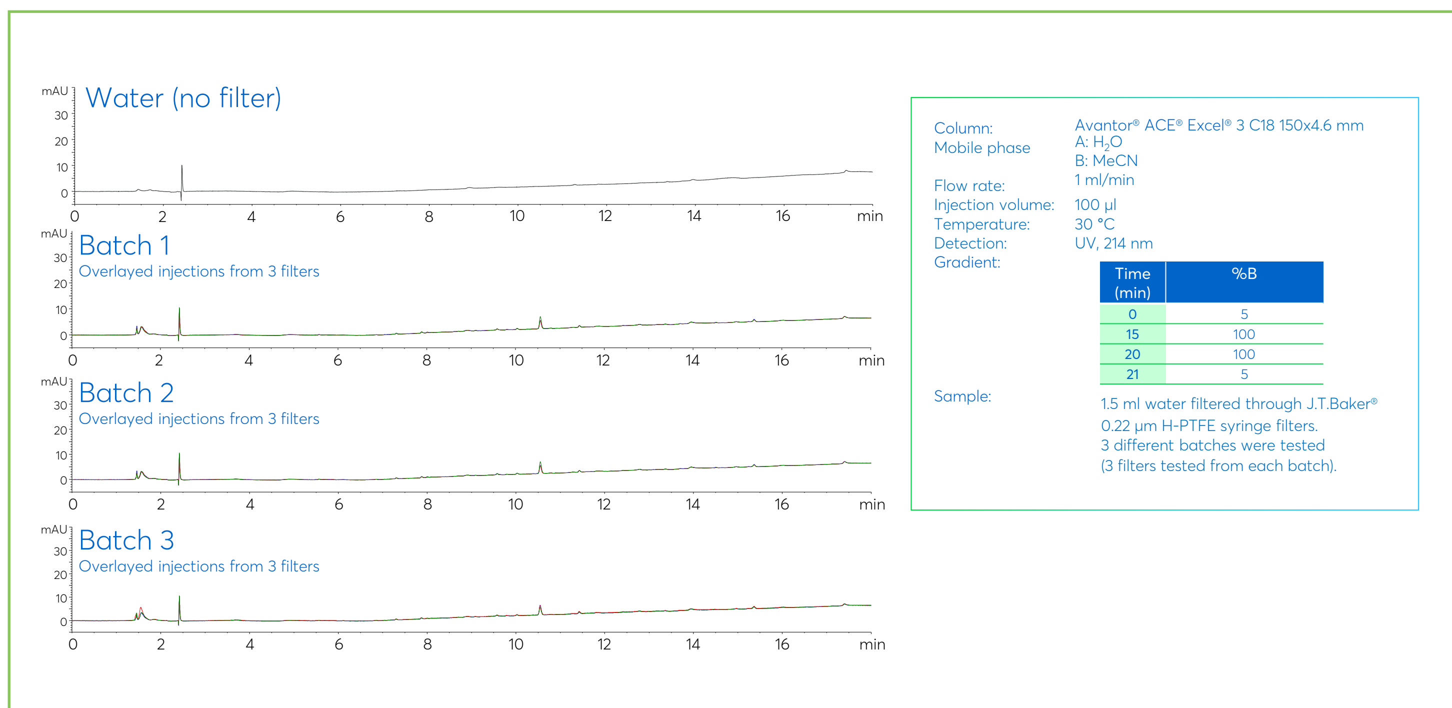


Figure 3: Chromatograms showing results of an extractables test performed on three batches of J.T.Baker® 0.22 µm H-PTFE syringe filters. Three filters were tested from each batch.

components that may contaminate the sample. In LC analysis, the release of such extractable components can result in the presence of contaminant or 'ghost' peaks in the resulting chromatogram. These peaks may interfere with the accurate identification and quantification of target analytes and potentially even be misidentified as analytes of interest. Ultimately, the use of poor quality filters can severely compromise the analytical results obtained.

The high performance materials used to manufacture J.T.Baker® syringe filters minimise the potential for leaching of extractable components, thereby ensuring the integrity of filtered samples is maintained. Figure 3 shows the chromatograms obtained from an extractables testing procedure carried out on three batches of 0.22 µm J.T.Baker® H-PTFE filters. In this test, 1.5 ml water were filtered using the H-PTFE filter and 100 µl of the resulting eluent injected onto an LC column and analysed by gradient elution. The injection volume used is much greater than typically used for LC analysis on this column dimension to ensure that this is a demanding test, capable of detecting trace level components extracted from the filters. All three batches showed consistently low levels of detectable extractable components, demonstrating the high performance of these filters.

To further demonstrate the importance of using high quality syringe filters for LC sample preparation, the performance of J.T.Baker® filters were compared to a competitor filter for the LC analysis of nitrosamines in active pharmaceutical ingredients (API). Nitrosamines are highly genotoxic compounds that require trace level determination to ensure the safety of pharmaceutical products, often by LC-MS/MS. In this example, a 67 mg/ml sample of valsartan was spiked with eight nitrosamines at 1 µg/ml. The sample preparation involves extraction in 1% formic acid to precipitate the valsartan, therefore, the samples were filtered using 0.45 µm nylon syringe filters prior to LC analysis. The competitor filter was found to contaminate the sample with several extractable components that could interfere with the analysis. An unknown component partially coelutes with peak 4 (NEIPA), compromising accurate integration, whilst another component elutes between peaks 6 (NMPA) and 7 (NDPA), completely obscuring peak 6 and preventing accurate integration and quantification. A major contaminant was also observed to elute at 3.8 minutes. The chromatograms obtained from the two samples clearly demonstrates the superior performance of the J.T.Baker® syringe filters.

CONCLUSION

Liquid chromatography columns and instrumentation can be damaged by particulate material originating from injected samples. It is, therefore, important to adequately filter samples to extend column lifetime and protect LC instrumentation. J.T.Baker® syringe filters provide a convenient, high performance solution to achieve this. The use of premium quality syringe filters is highly recommended to ensure that valuable samples are filtered efficiently, reproducibly and without introducing extractable components that could contaminate valuable samples and compromise the analytical results obtained. The data and applications outlined in this short article have demonstrated the high performance that can be expected from J.T.Baker® premium syringe filters and their suitability for use in demanding applications.

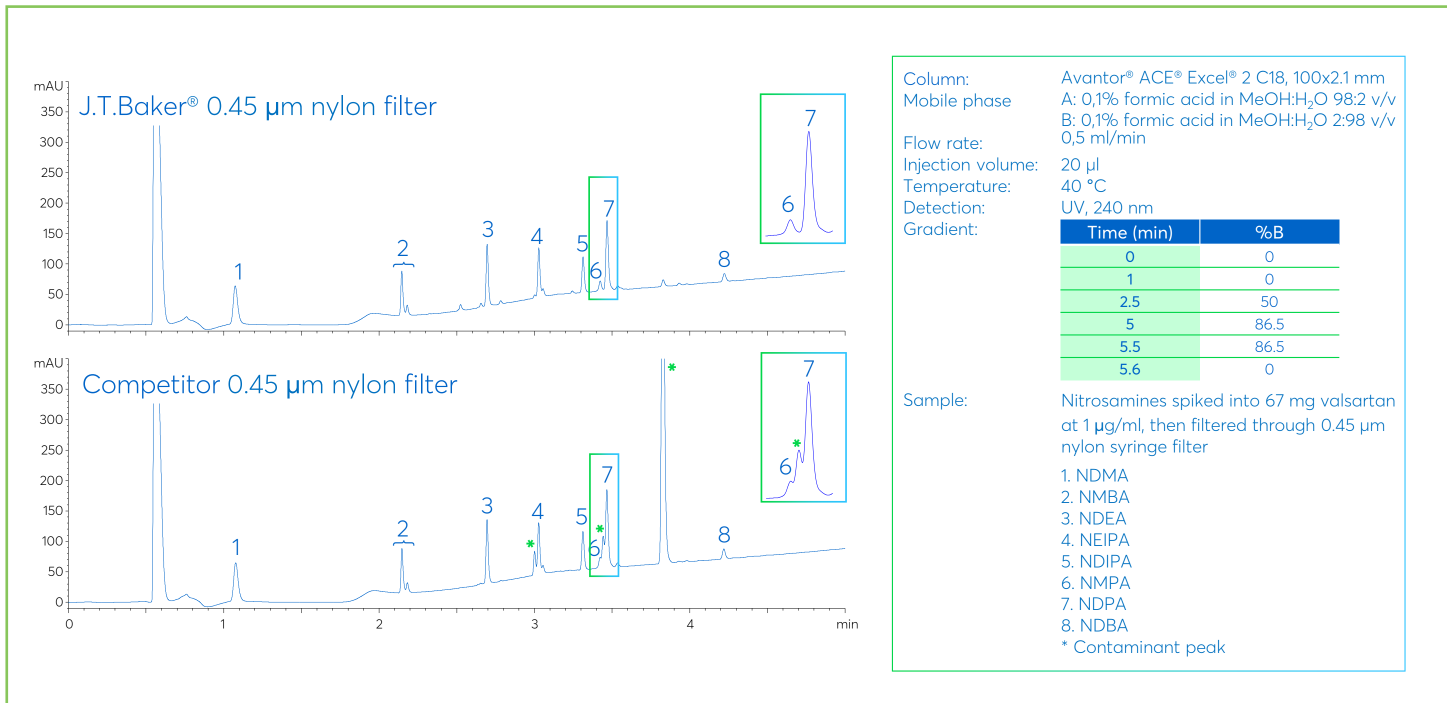


Figure 4: Comparison of chromatograms obtained from filtering a sample of valsartan spiked with eight nitrosamines with J.T.Baker® and competitor syringe filters.

Minimum extractables for maximum sample purity

NEW

J.T.BAKER® SYRINGE FILTERS OFFER QUALITY & PERFORMANCE YOU CAN TRUST

For over a century, professionals around the world have chosen the J.T.Baker® brand for quality and performance they can trust. J.T.Baker products consistently meet the needs of the most demanding applications, and their premium, high performance syringe filters are no exception, providing efficient filtration with minimal extractables to maximise sample purity.

Every batch is delivered with the assurance of a chromatogram and a certificate of quality and is rigorously tested for burst pressure of housing, bubble point, flow rate performance and extractables.

These syringe filters are specifically designed for chromatography sample preparation applications and are optimised to provide the most consistent results with minimal extractables.

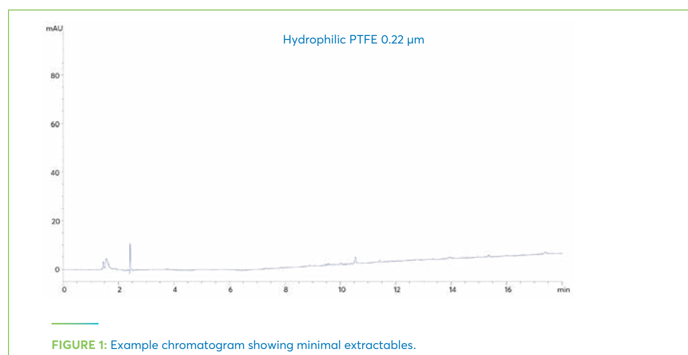


FIGURE 1: Example chromatogram showing minimal extractables.

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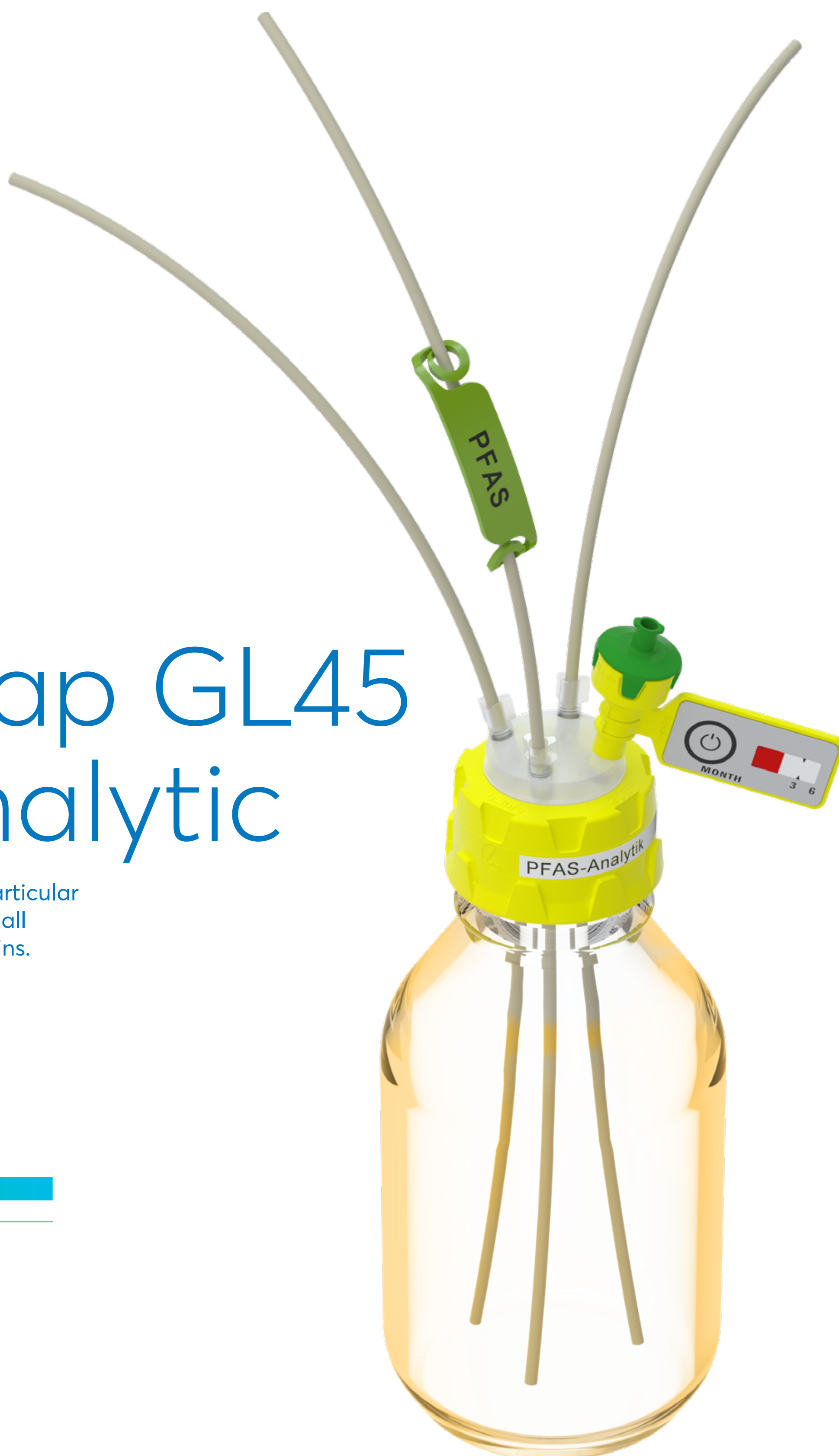
Diameter	Pore size	Membrane	Packaging	Cat. No.
Hydrophilic polytetrafluoroethylene (PTFE)				
25 mm	0.22 µm	Hydrophilic PTFE	2 jars of 100	**SF01-22**
Nylon				
25mm	0.22 µm	Nylon	2 jars of 100	**SF01-50**
Polyethersulfone (PES)				
25 mm	0.45 µm	PES	Bulk	**SF02-10**
25 mm	0.45 µm	PES, GF pre-filter	2 jars of 100	**SF02-06**
Hydrophobic polytetrafluoroethylene (PTFE)				
13 mm	0.45 µm		2 jars of 100	**SF02-36**
Regenerated cellulose (RC)				
30 mm	0.22 µm	RC	2 jars of 100	**SF02-88**



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b.safe Cap GL45 PFAS-Analytic

For the specific detection of PFAS, in particular in environmental and product samples, all components are free from polyhaloolefins.

- Free from polyhaloolefins
- Chemically highly resistant
- EU Guideline 2020/2184 conform
- DIN 38407-42 conform
- DIN 38414-14 conform

Description	Cat. No.
Cap GL45 PFAS-analytic 4X UNF 1/4"	BOHLM445-03

Step-by-step protocol for streamlined reversed-phase method development using Avantor® ACE® MDKs

Matt James, Senior Research Scientist

INTRODUCTION

The use of a systematic screening strategy to explore LC stationary phase selectivity for new samples is a well-established approach to method development and allows chromatographers to rapidly identify a suitable stationary phase and analytical conditions. Avantor® ACE® Method Development Kits (MDKs) contain three LC columns, each providing substantially different selectivity and are therefore ideally suited to this approach. This article outlines a simple and systematic protocol for screening new samples using reversed-phase conditions, that can help rationalise and streamline the development of new LC methods.

WHY USE COLUMN SCREENING?

Reversed-phase LC columns offering different selectivity to a standard C18 phase are widely available (e.g. PFP, phenyl, polar embedded phases etc.)[1,2]. Changing the column stationary phase can have a dramatic impact on selectivity Figure 1. As part of any method development strategy, it is therefore useful to assess the stationary phase chemistry to obtain a successful separation[3]. However, it is often difficult to predict which stationary phase will be the most suitable for a new separation.

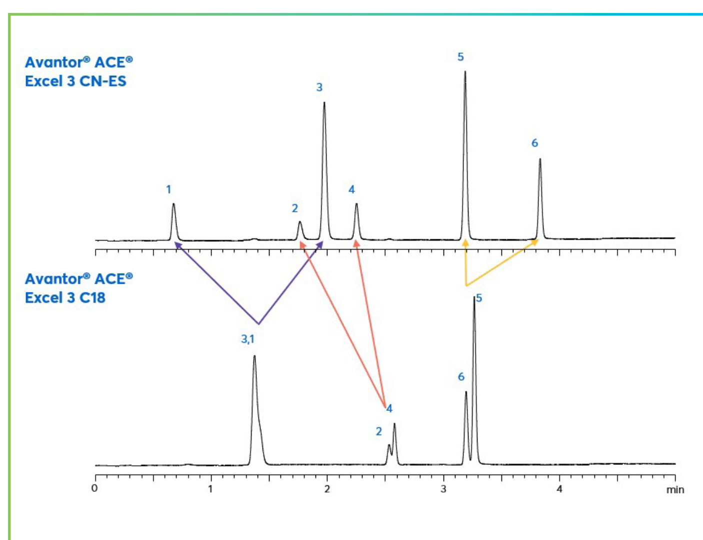

Figure 1: The effect of changing column stationary phase chemistry. Columns: 50x2.1 mm; Mobile phase A: 0.1% Formic acid in H_2O, B: 0.1% Formic acid in MeOH:H_2O (9:1 *v/v*); Gradient: 3 to 100% B in 5 minutes; Flow rate: 0.21 ml/min; Temperature: 40 °C; Detection: UV, 254 nm; Sample: 1) Metronidazole, 2) Benzyl alcohol, 3) Hydrochlorothiazide, 4) Vanillin, 5) Methyl Paraben, 6) 1,2-Dinitrobenzene.

Screening a defined selection of stationary phase chemistries at the beginning of method development, using identical mobile phase conditions, is an efficient way to assess the impact of stationary phase selectivity and can help achieve the desired separation quicker with better resolution. Avantor® ACE® MDKs group columns with different stationary phase chemistries (i.e. different mechanisms of analyte-stationary phase interaction) to maximise selectivity and increase the likelihood of separating challenging mixtures. These MDKs offer a cost effective solution for method development. The two most popular Avantor® ACE® reversed-phase (RP) MDKs (see Table 1) include unique phases engineered to exploit different retention mechanisms and maximise selectivity. All six phases can be used with standard RP conditions and are as robust as a C18 phase. Other Avantor® ACE® MDK's including HILIC, Bioanalytical 300 Å, UltraCore and Microbore are also available.

	Bonded phase	Separation mechanism and relative strength[1]				
		Hydrophobic binding	π-π Interaction	Dipole-Dipole	Hydrogen bonding	Shape selectivity
ACE Advanced Method Development Kit	ACE C18	****	-	-	*	**
	ACE C18-AR	****	*** (donor)	*	**	***
	ACE C18-PFP	****	*** (acceptor)	****	***	****
ACE Extended Method Development Kit	ACE SuperC18	****	-	-	-	**
	ACE C18-Amide	****	-	**	****	**/***
	ACE CN-ES	***	*	***	**	*

Table 1: Phase characteristics of columns included in Avantor® ACE® reversed-phase MDKs[1]. Weightings and/or by reference to other ACE phases using >100 characterising analytes.

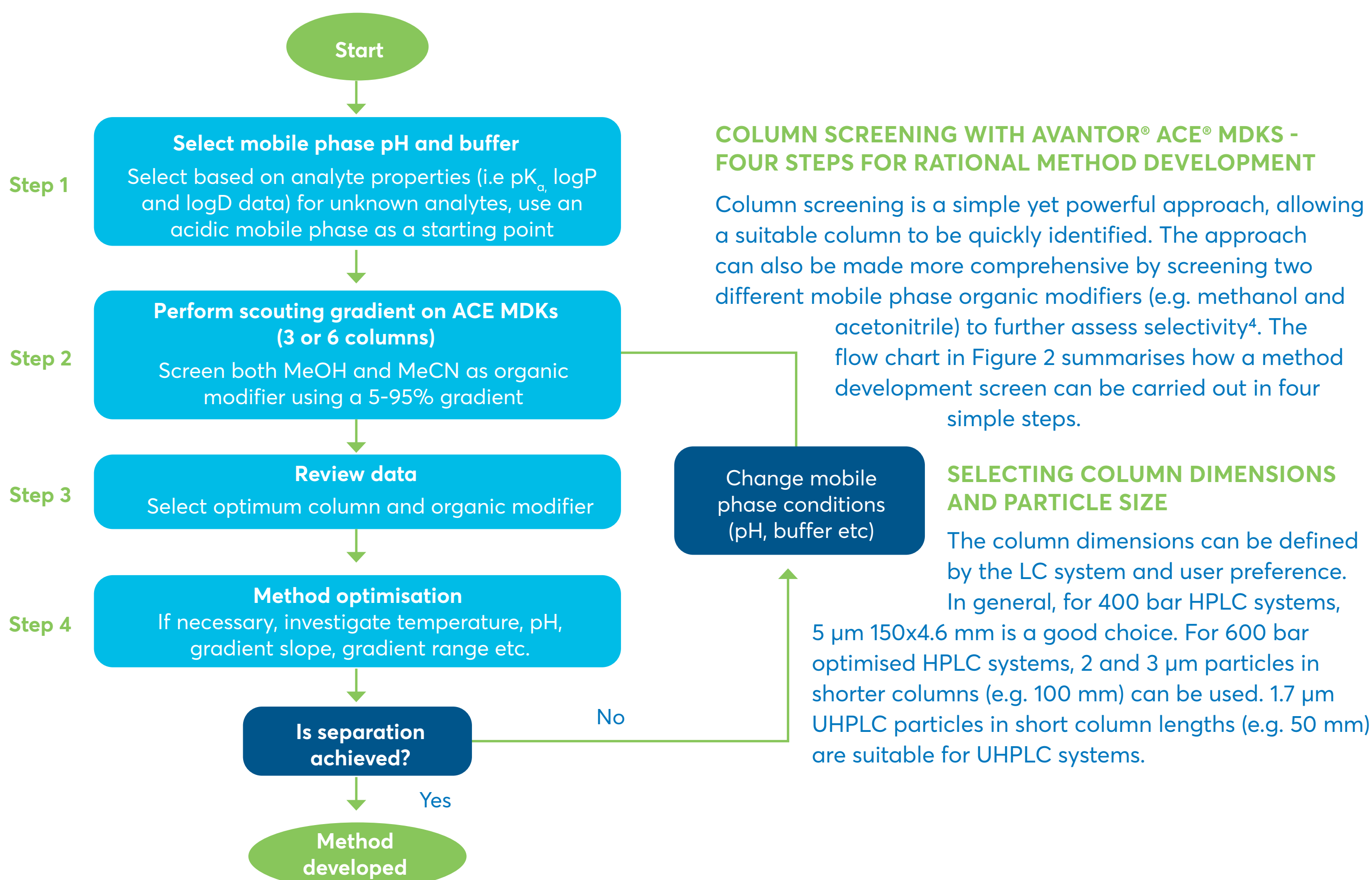

Figure 2: Step-by-step protocol for a streamlined reversed-phase method development strategy using column selectivity screening.

COLUMN SCREENING WITH AVANTOR® ACE® MDKS - FOUR STEPS FOR RATIONAL METHOD DEVELOPMENT

Column screening is a simple yet powerful approach, allowing a suitable column to be quickly identified. The approach can also be made more comprehensive by screening two different mobile phase organic modifiers (e.g. methanol and acetonitrile) to further assess selectivity[4]. The flow chart in Figure 2 summarises how a method development screen can be carried out in four simple steps.

SELECTING COLUMN DIMENSIONS AND PARTICLE SIZE

The column dimensions can be defined by the LC system and user preference. In general, for 400 bar HPLC systems, 5 µm 150x4.6 mm is a good choice. For 600 bar optimised HPLC systems, 2 and 3 µm particles in shorter columns (e.g. 100 mm) can be used. 1.7 µm UHPLC particles in short column lengths (e.g. 50 mm) are suitable for UHPLC systems.

HOW TO DETERMINE AN APPROPRIATE SCREENING GRADIENT TIME

A suitable gradient time for the screening experiments can be selected using equation 1. The column volume (V_M) can be estimated using equation 2. It is important to always include a post-gradient isocratic re-equilibration of at least 10 x V_M before the next injection.

$$t_G = \frac{k^* \times \Delta\varphi \times V_M \times S}{F} \quad (1)$$

$$V_M = \frac{0.5 \times L \times d_c^2}{1000} \quad (2)$$

t_G = Gradient time (mins.)
k^* = Gradient retention factor (typically set to approx. 5)
$\Delta\Phi$ = Gradient range (for a 5-95% B gradient, $\Delta\Phi$ = 0.9)
V_M = Column internal volume (ml)
S = 4 for small molecules
F = Flow rate (ml/min)
L = Column length (mm)
d_c = Column internal diameter (mm)

WORKED EXAMPLE

Figure 3 shows an example of the application of the column screening protocol to a pharmaceutical sample containing acetaminophen (paracetamol) and related substances. As per Figure 2, the mobile phase pH was selected based on logD and pK_a data for the 10 analytes. The sample was screened on the Avantor® ACE® Advanced and Extended MDKs (six ACE® stationary phase chemistries) detailed in Table 1.
The first six chromatograms (A) show the sample screened on the six Avantor® ACE® reversed-phase columns using methanol (MeOH) as the organic modifier in line B. In the second set of chromatograms (B), the experiment was repeated using acetonitrile (MeCN) as the organic modifier. As can be seen, clear differences in analyte selectivity are apparent on the six columns. Additionally, methanol and acetonitrile also provide different analyte spacing. The most common starting point for method development (C18) did not separate all the analytes using either methanol or acetonitrile. Further method development would be required. On the novel phases however, this six column/two mobile phase screening strategy immediately provided six solutions for the separation of all the sample components, meaning that no further method development was required.

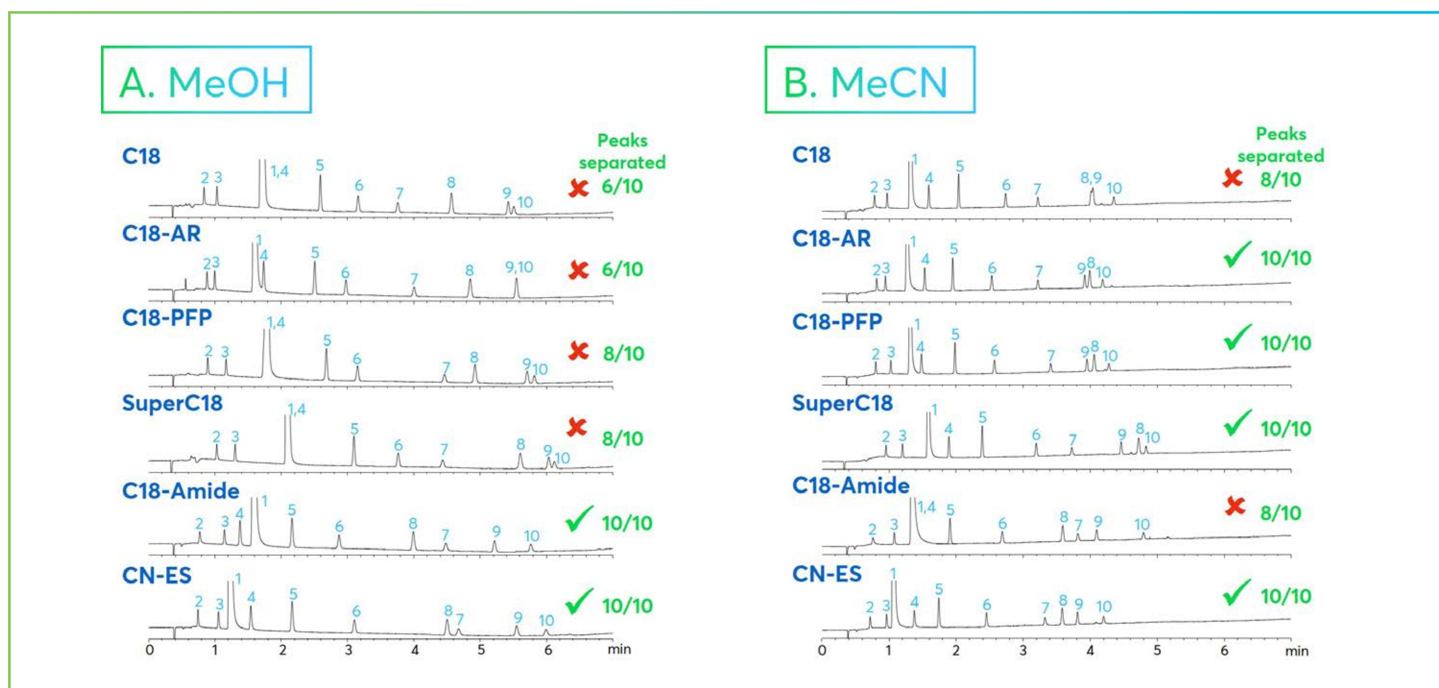

Figure 3: Worked example of a six-column screen using the protocol outlined in Figure 2. Columns: Avantor® ACE® Excel 2 µm, 100x3.0 mm; Mobile Phase A: 20 mM NH_4OAc pH 6.0 (aq); B: 20 mM NH_4OAc pH 6.0 in Organic:H_2O (9:1 *v/v*); Gradient: 5 to 95% B in 10 minutes; Flow rate: 1.2 ml/min; Temperature: 40 °C; Detection: UV, 210 nm; Sample: 1) Acetaminophen (paracetamol), 2) 4-Aminophenol, 3) Hydroquinone, 4) 2-Aminophenol, 5) 2-Acetamidophenol, 6) Phenol, 7) 4-Nitrophenol, 8) 2 Nitrophenol, 9) 4 Chloroacetanilide, 10) 4-Chlorophenol.

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CONCLUSION

This short article has outlined a simple and universal protocol for reversed-phase method development using Avantor® ACE® Method Development Kits. Screening a new sample on multiple stationary phase chemistries and multiple organic modifiers allows chromatographers to quickly identify a suitable stationary phase/mobile phase combination for the separation. This helps to streamline the method development process. In the worked example shown, the screening protocol produced six possible options for the full separation of all sample components, with no further method development required.

Reference

1. Avantor® ACE® Knowledge Note #0013 "The power of Stationary Phase Selectivity" (https://uk.vwr.com/cms/ace_knowledge_notes)
2. Euerby, M and Patersson, P J. Chromatogr A (2012) 994 13-36
3. Euerby, MR, Fever, M, Hulse, J, James, M, Petersson, P and Pipe, C LCGC Europe (2016) 29 8-21
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Influence of HPLC-system dead volume on the performance of (U)HPLC columns

UHPLC columns show worse results than HPLC columns on non-optimised HPLC Systems

INTRODUCTION

Recent trends in HPLC column and particle technology have facilitated faster, more efficient separations by utilising smaller particle size solid supports and reducing column geometry. Optimization of these column parameters yields improvements in sensitivity and chromatographic resolution, which results in more accurate quantitation, identification, and characterisation of analytes. However, to benefit from these optimised columns it is necessary to use a likewise optimised HPLC-System.

Size exclusion chromatography (SEC) is the standard method for aggregate and fragment analysis of monoclonal antibodies (mAbs) in biopharmaceutical quality control (QC). Ideally, in SEC there is no interaction of the sample with the column and the separation solely occurs by diffusion of the sample in and out of the pores. Because of the absence of interaction with the stationary phase, SEC methods are generally faster than adsorptive methods and are more sensitive to increased dead volume of the system.

This application note compares the performance of a TSKgel® UP-SW3000 and TSKgel SuperSW mAb HR column on a non-optimised HPLC system, an optimised HPLC system and a state-of-the-art UHPLC system. Comparisons between columns and instruments were made to isolate and understand the impact of each variable on the chromatographic separation.

TOSOH

The dead volume of the non-optimised HPLC was especially increased by using larger than normal tubings to emphasize the effect. But, due to the use of salt containing mobile phases, the inner diameter of the tubings is often chosen to be larger to counteract salt precipitation.

EXPERIMENTAL CONDITIONS

Columns	1. TSKgel® UP-SW3000, 2 µm, 4.6 mm ID×30 cm L 2. TSKgel® SuperSW mAb HR, 4 µm, 7.8 mm ID×30 cm L
Instruments	Thermo Fisher Dionex Ultimate® 3000 (Fitted with Peek Tubings)
	Thermo Fisher Dionex Ultimate 3000 (Fitted with Viper Tubings)
	Thermo Fisher Vanquish (Fitted with Viper Tubings)
Mobile phase	100 mmol/L $NaH_2PO_4Na_2HPO_4$, pH 6.7, 100 mmol/L, Na_2SO_4, 0.05 % NaN_3
Gradient	Isocratic
Flow rate	UHPLC: 0.35 ml/min; HPLC: 1.0 ml/min
Detection	UV @ 280 nm and 20 Hz, 2.5 µl flow cell, 7 mm pathlength
Temp.	25 °C
Injection vol.	UHPLC: 10 µl; HPLC: 20 µl
Samples	Protein Standard Mix 15 - 600 kDa (69385 Sigma-Aldrich)
	TBG mAb 01 (stressed), 3.8 mg/ml in mobile phase, 4 °C

(U)HPLC Instrument	Injector to column	Column to detector	Extra column dead volume
System 1	0.13 mm IDx350 mm 0.76 mm IDx240 mm	0.76 mm IDx240 mm 0.13 mm IDx600 mm	232 µl
System 2	0.1 mm IDx350 mm 0.1 mm IDx250 mm	0.1 mm IDx250 mm 0.13 mm IDx600 mm	15 µl
System 3	0.1 mm IDx350 mm	0.1 mm IDx440 mm	7 µl

Table 1: Extra column dead volume of the UHPLC and HPLC systems used.

RESULTS AND DISCUSSION

Figure 1 shows the resolution of the 15 kDa – 600 kDa Protein Standard with TSKgel® SuperSW mAb HR and TSKgel UP-SW3000 on the differently optimised systems. Table 2 shows the asymmetry and theoretical plate count for the last peak in the used standard mix. There is a drastic loss in column performance when using the TSKgel® UP-SW3000 on a the non-optimised HPLC system.

The amount of theoretical plates drops from 51.023 on System 3, to 44.853 on System 2 and to just 4.152 plates per column on System 1. Due to the larger inner diameter and higher flow rate when using the TSKgel® SuperSW mAb HR, the analyte spends less time in the extra column dead volume of the system. Therefore the peak broadening due to longitudinal diffusion in the capillaries is decreased and the number of theoretical plates of the 4 µm TSKgel® SuperSW mAb HR is greater compared to the TSKgel® UP-SW3000.

When comparing the stressed mAb injections Figure 2 all resolution of aggregates and fragments is lost when using the TSKgel UP-SW3000 on the non-optimised System 1.

By reducing the extra column dead volume, the resolution of monomer, dimer, trimer and fragments becomes better on the TSKgel UP-SW3000 compared to the TSKgel® SuperSW mAb HR due to the smaller particle size. Table 2 shows the results for the non-stressed mAb sample.

CONCLUSIONS

The data clearly shows, that the extra column dead volume drastically influences column performance. When using a not optimised system it is recommended to use a larger inner diameter column. This way the time the analyte spends in the extra column volume is decreased and the extra column volume compared to the column volume becomes smaller. Smaller particle size and narrower column ID increase efficiency values resulting in sharper, taller peaks, which translates to a better resolution for QC, but only if the correct HPLC system is chosen. Instrument dispersion volume has a direct effect on column performance in SEC; instrument optimisation is key to improving separation quality. An optimised UHPLC method provides the best quality separation, yielding to higher resolution and sensitivity.

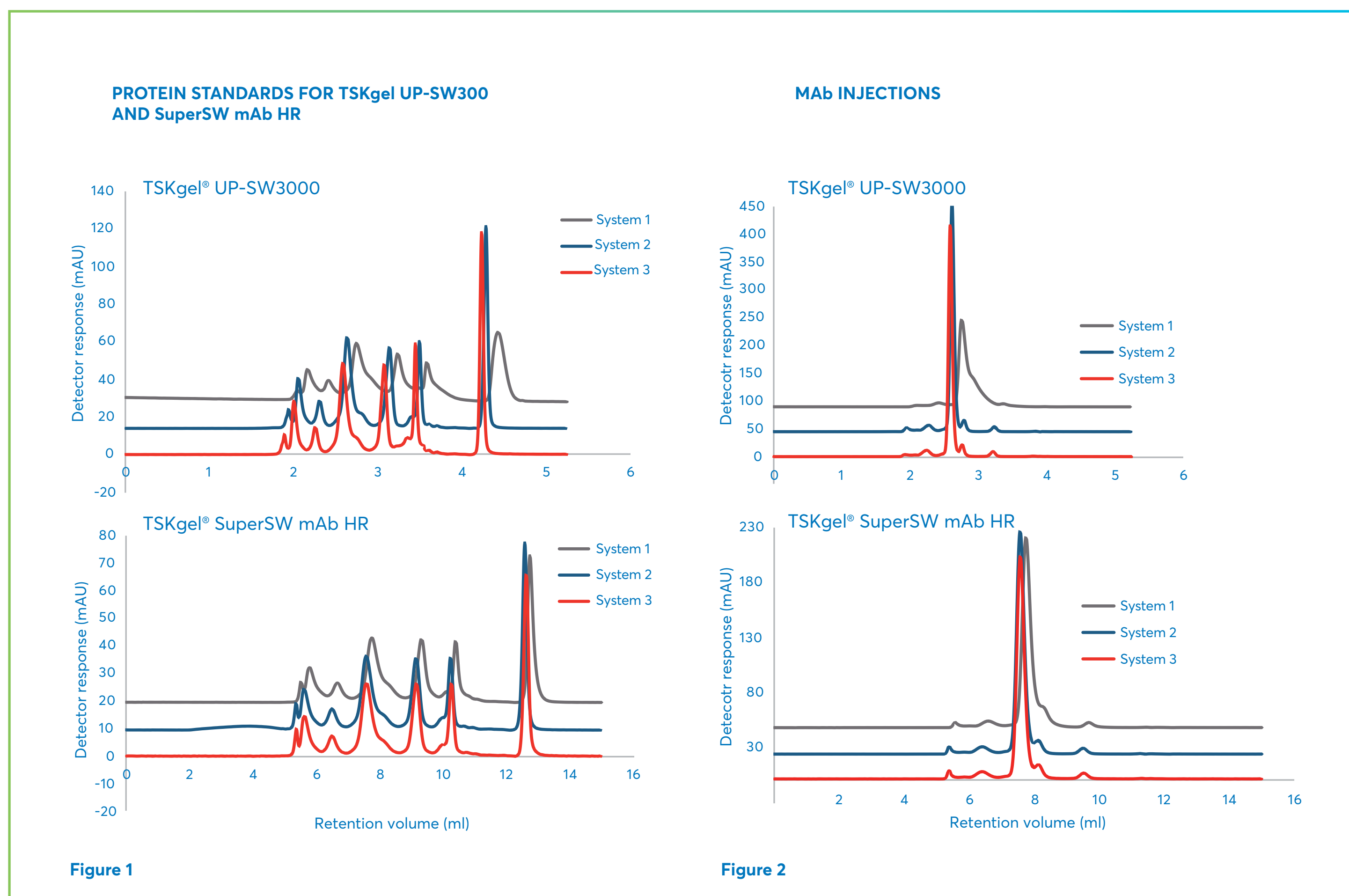

Figure 1

Figure 2

	UP-SW 3000 *	SuperSW mAb HR	UP-SW 3000	SuperSW mAb HR	UP-SW 3000	SuperSW mAb HR
(U)HPLC	System 1 (232 µl)		System 2 (15 µl)		System 3 (7 µl)	
N (pAba)	4.152	23.616	44.853	34.861	51.023	36.050
N (BI mAb)	3.220	5.604	13.752	6.410	16.327	6.731

* Due to pressure limit of fitting, flow was reduced to 0.15 ml/min

Considerations and best practices for mobile phase buffer selection and pH control for LC and LC-MS method development

By Dr Matt James, Senior Research Scientist, Avantor

When developing a new LC or LC-MS separation, careful consideration of mobile phase composition is essential for optimising peak shape, improving separation selectivity, and ensuring method robustness. For samples containing ionisable compounds, it is important to select an appropriate mobile phase pH to control the analyte ionisation state, in order to achieve reliable retention times. Buffers are commonly used in reversed-phase separations to tightly control the mobile phase pH, to improve method robustness, and to suppress undesirable analyte interactions with the silica surface.

For LC methods, buffer selection requires consideration of both the analyte and buffer salt properties. Incorrect buffer selection can result in problematic methods exhibiting poor peak shape, retention time shifts and reproducibility issues. Additionally, the analyst must ensure that the buffer is compatible with the detection mode and analytical column used for the separation. This white paper discusses the important aspects to consider when selecting mobile phase buffers to ensure the development of robust and reproducible LC separations.

WHY IS MOBILE PHASE PH IMPORTANT?

In reversed-phase separations, analyte retention is largely determined 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). The degree of analyte ionisation is determined by the mobile phase pH and analyte pK_a. This means that mobile phase pH can have a profound effect on how strongly some analytes are retained, and on the separation ultimately obtained. Selecting a suitable mobile phase pH is, therefore, vitally important when developing methods for samples containing ionisable analytes.

In many cases, small changes in the mobile phase pH can have a significant effect on a separation, as shown in Figure 1. In this example, a small change in the mobile phase pH of just 0.1 pH units results in complete loss of resolution between peaks 3 and 4. Clearly, this separation shows a lack of robustness and could prove to be problematic during the method lifetime due to inter- and intra-laboratory variations in mobile phase preparation. In cases like this, it is important to carefully consider and optimise the mobile phase pH during method development to, not only ensure full analyte separation, but also to provide a robust and usable method.

For methods that are highly sensitive to small changes in pH, the use of a correctly buffered mobile phase will help to minimise the potential impact of small variations in pH.

Another important consideration is the effect of mobile phase pH on unbonded acidic silanol groups at the silica surface. On older 'type-A' silicas, these silanol groups have pK_a values in the region of pH 4 to 5[1]. At mobile phase pH's above pH 6.0, significant silanol ionisation can occur. Interaction of these negatively charged silanol groups, with positively charged basic analytes, is a major historical cause of peak tailing for bases in reversed-phase LC. Higher purity 'type-B' stationary phases typically have less acidic silanol activity (pK_a approx. pH 7), leading to significantly improved peak shape and improved reproducibility. This is highlighted in Figure 2, which compares the peak shape for basic analytes on the Avantor® ACE® C18 and a column packed with 'type-A' silica. The Avantor® ACE® C18 shows vastly improved peak shape due to the highly inert, ultra-pure silica used in the column manufacturing process. It is highly recommended that any new method is developed using a modern 'type-B' column, such as the Avantor® ACE® reversed-phase HPLC and UHPLC columns.

UNDERSTANDING HOW MOBILE PHASE PH AFFECTS ANALYTE RETENTION

To understand how the mobile phase pH may affect analyte retention, it is good practice to assess known analyte properties prior to beginning method development. The analyte pK_a allows the chromatographer to assess how the ionisation state of an analyte varies as a function of pH. This information can be used to define the most appropriate mobile phase pH for the separation. Figure 3 demonstrates how the ionisation state of simple acidic and basic analytes varies according to pH. For basic analytes, at mobile phase pH's below their pK_a, the analyte will be predominantly 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.

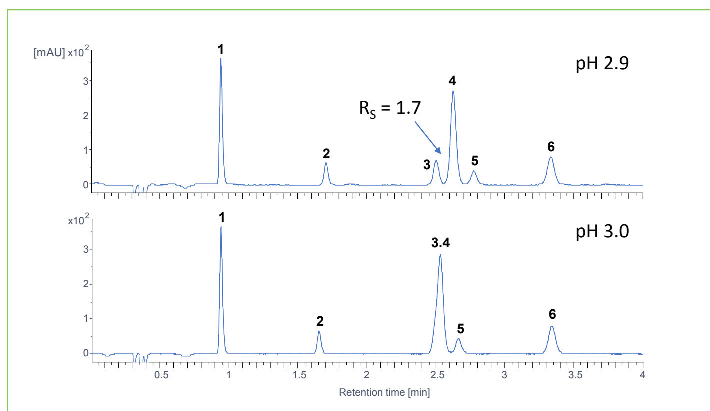

FIGURE 1 : Chromatograms showing the effect of a small change in pH on a benzoic acid derivative separation. Column Avantor® ACE® UltraCore 2.5 SuperC18, 100x3.0 mm; Mobile phase 20 mM ammonium formate in MeCN/H_2O 15:85 v/v; Flow rate 1.20 ml/min; Injection volume 5 µl; Temperature 40 °C; Detection UV, 214 nm. Sample 1. 3-Hydroxybenzoic acid, 2. 4-Cyanobenzoic acid, 3. Benzoic acid, 4. 3-Nitrobenzoic acid, 5. 4-Nitrobenzoic acid, 6. 4-Methoxybenzoic acid.

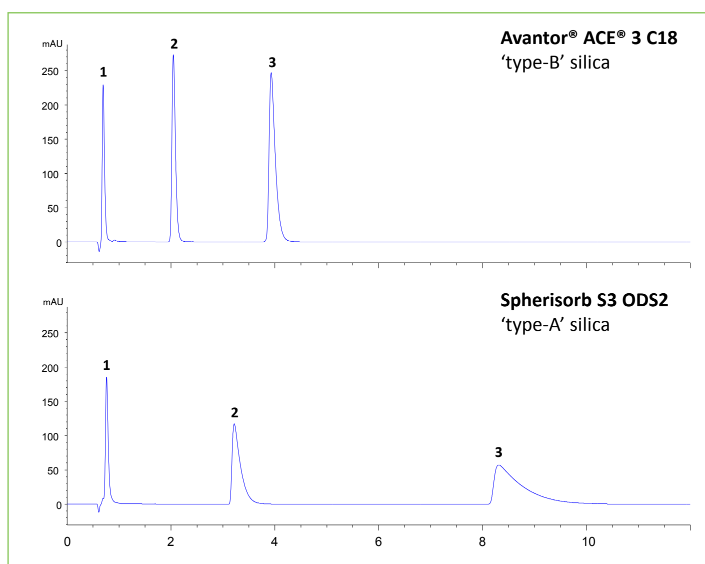

FIGURE 2 : Comparison of peak shape for basic analytes chromatographed at pH 7.6 on a modern, high purity 'type-B' silica column (Top) and an older, low purity silica (Bottom). Column dimensions 50x2.1 mm; Mobile phase 20 mM KH_2PO_4 pH 7.6 in MeOH/H_2O 75:25 v/v; Flow rate 0.2 ml/min; Injection volume 2 µl; Temperature 30 °C; Detection UV, 214 nm; Sample 1. Salbutamol, 2. Diphenhydramine, 3. Imipramine.

When the mobile phase pH equals the analyte pK_a, the analyte is considered to be 50% ionised and 50% unionised. From here, a small change in pH (for example 0.5 pH units) will result in a comparatively large change in the analyte's ionisation state and, therefore, retention time. This may result in a non robust method; any small change in pH, e.g., variations in mobile phase preparation, will result in a shift in the analyte's retention. A more robust method would be achieved at a pH well away from the analyte pK_a. As a general rule, it is recommended that, if possible, the mobile phase should have a pH of ±2 pH units from the critical analyte's pK_a.

Figure 4 demonstrates experimentally how the mobile phase pH affects the retention of a set of acidic, basic and neutral analytes. Toluene does not contain any ionisable functionality and is neutral across the entire pH range. Therefore, mobile phase pH has no significant effect on its retention. At low pH, acidic analytes (3.4-dichlorobenzoic acid and ketoprofen) are in their neutral, non ionised form and, therefore, show their strongest retention. As the pH is increased, the degree of ionisation increases, and retention gradually decreases. In contrast, basic analytes (propranolol 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.

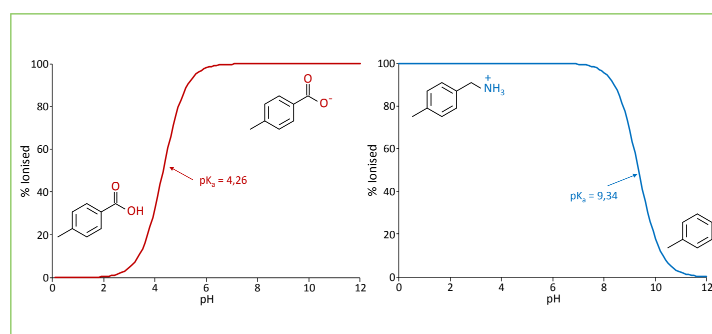

FIGURE 3 : Plot of percentage ionisation vs pH for a simple acidic analyte (red) and a simple basic analyte (blue).

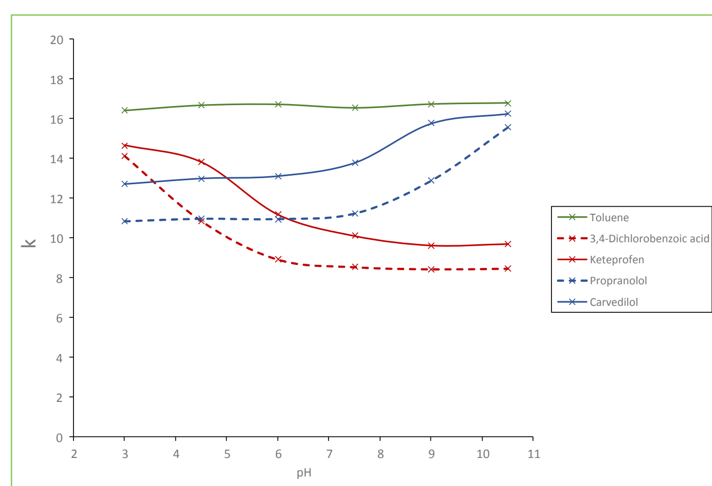

FIGURE 4 : Effect of mobile phase pH on analyte retention factor (k) on a high pH stable Avantor® ACE® SuperC18 column. Column Avantor® ACE® Excel® 3 SuperC18, 50x2.1 mm; Mobile phase A 20 mM ammonium formate pH 3.0; 4.5; 6.0; 7.5; 9.0 and 10.5 (aq), B 20 mM ammonium formate pH 3.0; 4.5; 6.0; 7.5; 9.0 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.

From Figure 4, it is clear when ionisable analytes are present in a sample, the selectivity between analytes can vary significantly with pH. For such samples, it is highly recommended that mobile phase pH is explored during initial method development, to determine the most suitable value. When beginning any method development, it is useful to consider analyte structures and properties, to anticipate any acidic/basic functionality with the ability to ionise. If these properties are unknown, screening the sample on a generic gradient using several mobile phase pH values can be a productive starting point.

HOW TO CONTROL MOBILE PHASE PH & FACTORS AFFECTING ADDITIVE/BUFFER CHOICE

A variety of mobile phase additives can be used to control the mobile phase pH. Table 1 lists some of the most commonly used additives. In many cases, where for example an acidic mobile phase is required at a pH well away from the analyte pK_a, a simple mobile phase containing a low percentage by volume of the additive may be sufficient. For example, mobile phases containing 0.1% v/v formic acid or phosphoric acid are commonly used. Similarly, for high pH work 0.1% v/v ammonium hydroxide is useful. For peptide and protein separations, 0.1% v/v TFA is a commonly used ion-pairing additive. For simple mobile phases containing only additives, it is important to use ultra-high purity LC columns, such as Avantor® ACE®, for optimum results.

	pK_a (25 °C)	LC-MS compatible	UV cut-off (nm)
Trifluoroacetic acid (TFA)	0.3	●	210 (0.1%)
Phosphoric acid	2.15; 7.20; 12.33		<200 (10 mM)
Citric acid	3.13; 4.76; 6.40		230 (10 mM)
Formic acid	3.75	●	210 (10 mM)
Acetic acid	4.76	●	210 (10 mM)
Ammonium hydroxide	9.3	●	<200 (10 mM)
Triethylamine	10.8	●	<200 (10 mM)

TABLE 1 : Common mobile phase additives[2,3,4].

Often, separations involving ionisable analytes can be highly sensitive to small variations in pH, and the use of a buffered mobile phase is required. A correctly buffered mobile phase will resist any small changes in pH, (e.g., through absorption of CO_2 or from errors in the mobile phase preparation). A buffered mobile phase consists of a dissolved buffer salt that is pH adjusted using an appropriate acid or base. Table 2 shows

some of the most commonly used reversed-phase buffer systems. When used within the stated pH range, 10 to 50 mM concentrations have a high buffering capacity and can be used for highly robust control of the pH.

For LC analyses with UV detection, phosphoric acid combined with its sodium or potassium salts are a common choice, as the multiple suppress cover a large pH range (although practical limits exclude the upper pK_a). The gap in buffering capacity between pH 3.1 to 6.2 is conveniently filled by ammonium acetate / acetic acid. Citrate also possesses three overlapping pK_as however, its use is somewhat limited by its comparatively high UV cut-off.

For analyses using detectors such as Mass Spectrometry (MS) and Evaporative Light Scattering Detectors (ELSD), it is essential to use volatile buffers; non volatile buffers such as phosphate will precipitate within the detector. In these cases, volatile additives such as formic acid, ammonium hydroxide, formate, acetate and ammonium bicarbonate buffers are commonly used.

MOBILE PHASE & BUFFER PREPARATION

A typical buffer can potentially be prepared using several different approaches, which are summarised in Table 3.

	pH range	LC-MS compatible	UV cut-off (nM)
Phosphate / phosphoric acid (pK_{a1})	1.1 – 3.1		<200 (10 mM)
Citrate / citric acid (pK_{a1})	2.1 - 4.1		230 (10 mM)
Ammonium formate / formic acid	2.7 - 4.7	●	210 (10 mM)
Citrate / citric acid (pK_{a2})	3.7 - 5.7		230 (10 mM)
Ammonium acetate / acetic acid	3.8 – 5.8	●	210 (10 mM)
Citrate / citric acid (pK_{a3})	4.4 – 6.4		230 (10 mM)
Ammonium bicarbonate (pK_{a1})	5.4 - 7.4	●	<200 (10 mM)
Phosphate / phosphoric acid (pK_{a2})	6.2 - 8.2		<200 (10 mM)
Ammonium bicarbonate (pK_{a2})	9.3 - 11.3	●	<200 (10 mM)
Phosphate / phosphoric acid (pK_{a3})	11.3 – 13.3		<200 (10 mM)

TABLE 2 : **Common reversed-phase mobile phase buffers[2,3,4].**

To obtain reproducible separations and minimise the potential for inter-lab variation, the mobile phase and buffer preparation should be accurately recorded in documented procedures.

Perhaps the most reproducible method to prepare buffers is to weigh the individual buffer components according to a prescribed recipe, and then dilute them to the required volume with water[1,5]. This gravimetric approach ensures that the resulting buffer has a fixed concentration and pH, although it is worthwhile checking the pH of the final buffer solution for accuracy. Alternatively, the buffer could be prepared by mixing separate equimolar solutions of the acid and buffer salt (e.g., 15 mM formic acid and 15 mM ammonium formate). The pH is monitored as the two solutions are mixed until the desired pH is reached. The resulting buffer solution will have the specified pH and concentration. One of the most widely used approaches is to dissolve the buffer salt in water (approximately 90% of the final volume required), and adjust the pH using concentrated acid or base to the desired pH value before making up to the final volume with water[5]. The final buffer will have a higher concentration than the equimolar approach, but will have the correct pH.

As a practical note, it is important to always make any pH measurement or adjustments at the appropriate stage of mobile phase preparation. The pH of the buffer / mobile phase should never be adjusted or measured after the organic modifier is added. Although a pH measurement can be made, it will not be numerically comparable to aqueous pH values.

A useful approach for preparing buffers, especially if a buffer is used frequently, is to prepare a concentrated stock buffer solution that can be diluted to formulate the required mobile phase. This approach has several advantages; the number of lengthy buffer preparations can be minimised and additionally, concentrated buffer solutions can typically be stored for longer periods of time under suitable conditions. Finally, the stock solution can be used to prepare mobile phases containing different organic modifier concentrations. For example, a mobile phase containing 10 mM ammonium formate pH 3.0 in 1 litre of MeOH:H_2O 50:50 v/v can be prepared by combining 50 ml of 200 mM ammonium formate pH 3.0 with 450 ml water and 500 ml of methanol. The same volume of stock buffer could also be

	Approach			
	1. Gravimetric	**2. Blending equimolar solutions**	**3. Titration with concentrated acid / base**	**4. Stock buffer**
Procedure	Weigh prescribed amount of buffer salt and acid / base. Dilute to specified volume with water.	Prepare separate solutions of the acidic and basic buffer components (e.g., formic acid and formate salt). Blend the solutions to achieve desired pH.	Weigh and dissolve the specified amount of buffer salt in ~900 ml of water. Adjust the pH to specified pH using concentrated acid / base. Make up to 1000 ml with water.	Prepare an aqueous stock buffer (e.g., 200 mM) at the specified pH using approach 1, 2 or 3. Dilute an aliquot of the stock solution with prescribed amount of water and organic solvent.
Benefits	Most accurate and reproducible preparation method. Final buffer has a fixed concentration and pH. The final pH should be measured for confirmation.	Provides buffer with correct concentration and accurate pH.	Straightforward and reliable. Buffer concentration is higher than equimolar approach. Accurate target pH.	Concentrated stock buffers tend to have longer shelf life. Fewer time-consuming buffer preparations. Can be used to prepare multiple mobile phases with different concentrations of organic solvent.

TABLE 3 : **Summary of some commonly used approaches to preparing mobile phase buffers.**

combined with, for example, 800 ml of water and 150 ml of methanol to produce a mobile phase of 10 mM ammonium formate in MeOH:H_2O 15:85 v/v. When using this approach, it is advisable to combine the aqueous portions of the mobile phase first, followed by addition of the organic modifier. Addition of concentrated buffer directly into the organic solvent can lead to buffer precipitation.

Precipitation of the buffer when preparing the mobile phase, or within the LC system, can cause blockages in the column or system, or potentially damage LC system components. It is, therefore, important to consider the solubility of buffer salts in the mobile phase as some buffers have greater solubility than others (for further details regarding buffer solubility in organic solvent, please refer to Table 4 in Reference 1). For example, potassium dihydrogen phosphate shows substantially reduced solubility in over 60% organic, whereas ammonium formate is readily soluble in 90% organic. The solubility of a buffer salt is dependent on the counterion, with inorganic counterions generally being less soluble than organic equivalents. In addition, buffers tend to be more soluble in methanol than acetonitrile. For isocratic methods using a high percentage of organic in the mobile phase, or gradient methods that finish at a high organic percentage, care should be taken.

These considerations are also important when separate preparations of the buffer and individual mobile phase components are mixed online. For example, 10 mM potassium phosphate in MeCN:water 50:50 v/v could be mixed online using 20 mM potassium phosphate on line A and 100% MeCN on line B. In this case, although 10 mM potassium phosphate is soluble in the final composition after mixing, buffer precipitation could occur at the point where the concentrated buffer meets the pure organic solvent. An alternative approach would be to use 10 mM potassium phosphate on line A and the same concentration of buffer dissolved in MeCN:water 60:40 v/v on line B. The two can then be blended to produce the desired mobile phase concentration.

In HILIC mode, the use of high concentrations of acetonitrile in the mobile phase (typically 70 to 90%) restricts the use of many buffer salts. Only buffers that are highly soluble in organic solvent are suitable for use. Ammonium formate and ammonium acetate both have good solubility in organic solvent and can be used successfully in HILIC separations[6]. The use of inorganic buffers, such as phosphate, is not recommended for HILIC separations.

BUFFER CONCENTRATION

Buffer concentrations in the range of 5 to 50 mM are common. In general, a buffer concentration of at least 5 to 10 mM is recommended to ensure sufficient buffering capacity to provide robust pH control. In addition to controlling pH, the buffer can also help to pK_as as ionic interactions between charged analytes and silanol groups on the silica surface and improve peak shape[7]. Modern columns manufactured from type-B silica contain fewer acidic silanol groups than older generation columns, and can typically be used successfully with lower buffer concentrations. Often, the ability to use lower additive concentrations is highly beneficial. For example, when analysing proteins and peptides by LC-MS, TFA is often utilised as an additive in the mobile phase to improve analyte peak shape and retention. However, TFA can suppress the MS signal and has a

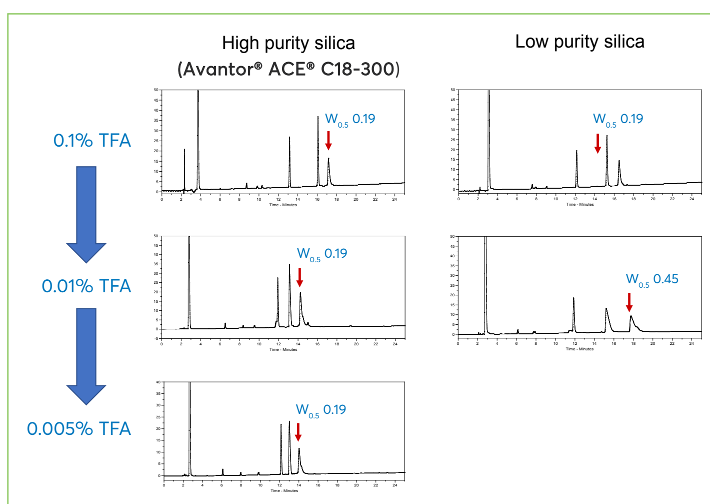

FIGURE 5 : Effect of TFA concentration on the peak shape of a peptide separation performed on a column manufactured using modern, high purity silica (Avantor® ACE® C18-300) and a low purity silica column. Column dimensions 250x4.6 mm, 5 µm, C18 300 Å; Mobile phase A TFA (aq), B TFA in MeCN (% TFA as specified above); Gradient 10 to 55% B in 37.5 minutes; Flow rate 1.5 ml/min; Detection UV, 200 nm; Sample 1. Gly-Tyr, 2. Oxytocin, 3. Angiotensin II, 4. Neurotensin.

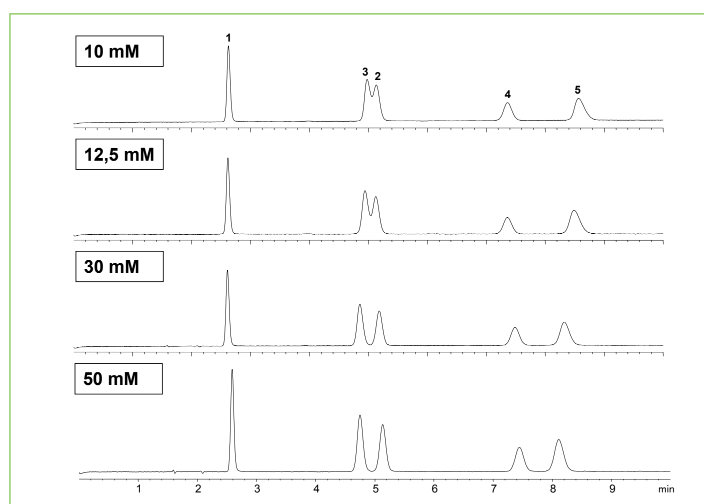

FIGURE 6 : Effect of buffer concentration on the separation of catecholamines on an Avantor® ACE® 5 C18-PFP column. Column dimensions 150x4.6 mm; Mobile phase Ammonium formate pH 3.0 (aq); Flow rate 1.0 ml/min; Injection volume 5 µl; Temperature 22 °C; Detection UV, 266 nm; Sample 1. Norepinephrine, 2. Levodopa, 3. Epinephrine, 4. Tyrosine, 5. Dopamine.

detrimental effect on sensitivity. Figure 5 shows how, by using an Avantor® ACE® column which is manufactured using ultra-inert base silica, the concentration of TFA can be drastically reduced whilst still maintaining excellent peak shape. In contrast, when an older, lower purity column is used, peptide peak shape rapidly deteriorates as the TFA concentration is reduced. This is another advantage provided by modern, ultra-high purity columns, such as Avantor® ACE® columns.

Although it may be tempting to minimise the buffer concentration wherever possible, it is worth considering that buffer concentration can be a useful parameter for fine tuning the selectivity of a separation during method development. Figure 6 shows the effect of buffer concentration on the separation of catecholamines on an Avantor® ACE® C18-PFP column using a 100% aqueous mobile phase. In this example, at low buffer concentrations, insufficient separation is obtained between levodopa and epinephrine. As the buffer concentration is increased, the separation is improved. This example demonstrates that, although not typically used as a primary method development parameter, assessing buffer concentration can be useful.

COLUMN CONSIDERATIONS

As discussed, it is highly recommended that columns manufactured from modern, high purity silica are used for the development of new methods to improve peak shape and reproducibility. Additionally, fresh columns should be used when beginning method development, to ensure that previous methods run on the column have not altered the characteristics of the stationary phase. This is especially important when ion-pairing additives, such as TFA have been used, as these can alter the selectivity of the column and may be impossible to fully remove. Not following this approach may lead to methods that were successfully developed on an old column but go on to fail when run on a fresh column.

When selecting a mobile phase pH and buffer for a new method, it is important to consider column stability. Generally, most silica columns should be used between a pH of 2 and 8 for optimum column lifetime. At low pH, hydrolysis can lead to stationary phase loss, whereas beyond pH 8, silica dissolution can occur. Both processes are accelerated at higher temperatures. Consequently, many reversed-phase columns cannot be used with high pH mobile phases. A number of columns that can tolerate high pH are commercially available and are typically manufactured from a hybrid organo-silica material, polymer-based, or utilise modified bonding technology. The Avantor® ACE® Excel® SuperC18, Avantor® ACE® UltraCore SuperC18 and Avantor® ACE® UltraCore SuperPhenylHexyl phases are novel stationary phases that have been developed for use over a wider pH range. The use of proprietary Encapsulated Bonding Technology (EBT) during the manufacturing process means that these phases can be used with mobile phases over an extended pH range of pH 1.5 to 11.0 (pH 1.5 to 11.5 for the Avantor® ACE® Excel® SuperC18). The ability to work with a wider range of mobile phase pH's provides the chromatographer with increased flexibility to fully explore pH as a method development tool when working with ionisable analytes[8]. Figure 7 demonstrates how the ability to investigate high pH mobile phases during method development can be a powerful approach when working with ionisable compounds. In this case, a high pH mobile phase provides, not only significantly better selectivity and analyte resolution, but also improved retention and peak shape for the basic analytes.

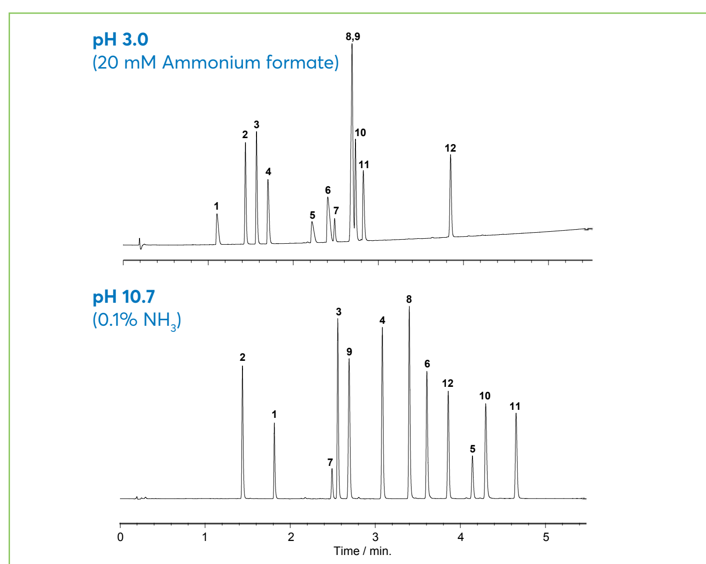

FIGURE 7 : Separation of a sample containing neutral and ionisable analytes using low and high pH mobile phases on an Avantor® ACE® UltraCore 2.5 SuperC18 column. Column Avantor® ACE® UltraCore 2.5 SuperC18, 50x2.1 mm; Mobile phase (low pH) A 20 mM ammonium formate pH 3.0 (aq), B 20 mM ammonium formate 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 3 to 100% B in 5 minutes; Flow rate 0.60 ml/min; Injection volume 1.5 µl; Temperature 40 °C; Detection UV, 254 nm.

ADDITIONAL TIPS & GOOD PRACTICES

It is important to always use high purity buffer salts, additives and solvents (HPLC grade or better) when making up LC mobile phases. Buffer purity tends to be more critical for gradient separations than isocratic separations, as any buffer impurities can build up on the column and are then eluted as impurity peaks as the gradient progresses. Buffer solutions and mobile phases can also be filtered to remove any dust or particulates.

For gradient methods using mobile phases containing buffers or additives, it is highly recommended that a buffer or additive is included in equal concentration in both mobile phases. Often, the buffer or additive is included in only the A line, with organic solvent on the B line. This approach creates a buffer concentration and/or pH gradient throughout the gradient program, which can cause issues with method reproducibility and robustness. In addition, for methods using UV detection, if the additive absorbs significantly at the detection wavelength, then a sloping baseline can result, as shown in Figure 8.

This situation is readily resolved by incorporating the additive into the B line.

Microbe growth is a potential issue for aqueous solutions of buffers. It is always important to set appropriate expiration dates for buffer preparations. In general, lower concentration buffers at neutral pH's will have shorter expiration times than concentrated stock solutions of acidified buffers.

As a final note, it is always good practice to remove buffers from the LC system after use to help reduce the risk of buffer precipitation and microbial growth. This can be accomplished using a 50:50 water organic mobile phase. Likewise, it is important to remove additives and buffers from the LC column before storage to help prolong column lifetime. For reversed-phase columns, this should not be performed with 100% water, as this could lead to de-wetting, or phase collapse, of the stationary phase.

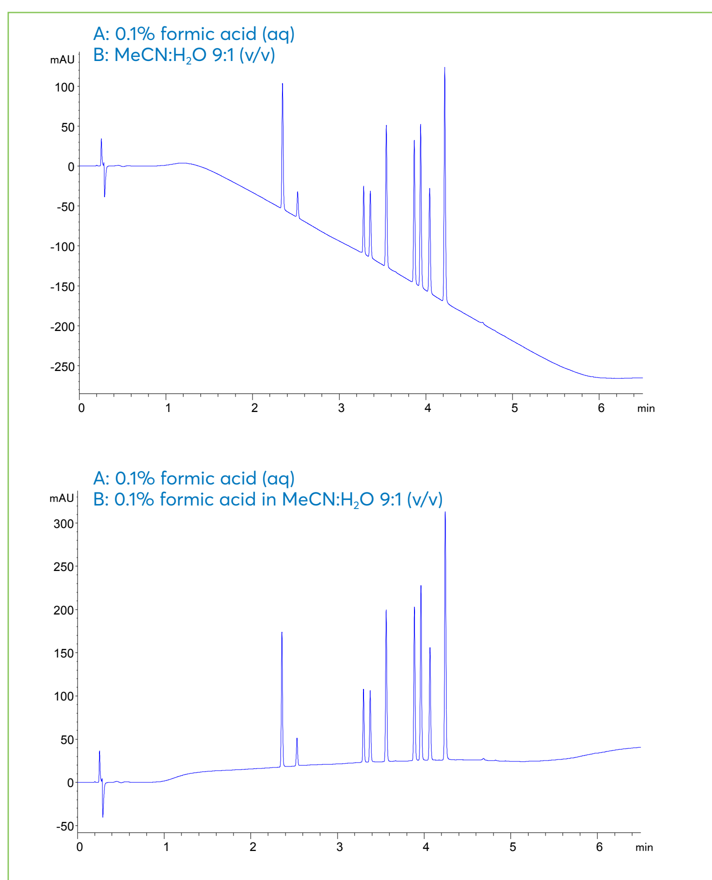

FIGURE 8 : Gradient separation of non steroidal anti-inflammatory drugs on an Avantor® ACE® Excel® C18-PFP column, showing the effect of including the mobile phase additive in the A line only (top) and both the A and B lines (bottom). Column Avantor® ACE® Excel® 2 C18-PFP, 50x3.0 mm; Gradient 5 to 100% B in 5 minutes; Flow rate 1.20 ml/min; Injection volume 1 µl; Temperature 40 °C; Detection UV, 214 nm. Sample (in order of elution) 1. 2-Acetoxybenzoic acid, 2. Phenacetin, 3. Sulindac, 4. Tolmetin, 5. Naproxen, 6. Flurbiprofen, 7. Diclofenac, 8. Phenylbutazone, 9. Meclofenamic acid.

CONCLUSIONS

When developing new LC and LC-MS separations, it is important to carefully consider the mobile phase composition to control and optimise analyte retention, selectivity and peak shape. For ionisable analytes, mobile phase pH is a critical parameter which can dramatically affect retention. Mobile phase buffers are commonly used to control mobile phase pH and help to achieve robust and reproducible separations. The wide range of buffers and additives that are available can make choosing the right mobile phase a complicated and daunting task. However, careful consideration of a few key concepts can help to ensure that the appropriate buffer is selected and can dramatically influence the success of method development.

This white paper has outlined how consideration of analyte properties can help the analyst to better understand how retention is affected by pH, and how to select the most suitable buffer. Important practical considerations, including buffer solubility, concentration, usable pH range and compatibility with different detection modes, have been additionally discussed. Importantly, several different approaches to mobile phase preparation are often encountered. This means that it is important to consider and accurately document procedures to ensure that methods can be reliably reproduced and operated by other laboratories and users. Finally, the use of modern, ultra-pure LC stationary phases, such as Avantor® ACE®, can help to improve method reproducibility and robustness, thereby minimising the risk of method failure throughout the method lifecycle.

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HiperSolv CHROMANORM® HPLC solvents in returnable barrels

Chromatography methods are continually improving to offer better sensitivity to meet today's demands. In the last 5 years, many improvements have been made to answer the strict requirements of the apparatus or detection methods, especially with impurities which can generate many issues. So, with their high degree of transmittance, low evaporation residue level, low particle count, low acidity and alkalinity, HiperSolv CHROMANORM® solvents are ideal for reproducible and accurate chromatographic results in HPLC. They are produced from specially selected raw materials that undergo a number of purification steps prior to final packaging.

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HPLC is now a key technique in research and development, quality control and purification used in different applications. Now, each of your demands can be covered with different pack sizes from 1 to 1400 L and materials from glass, aluminium and stainless steel. Depending on your annual consumption or application, choose the right packaging.

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- High resolution and sensitivity
- Excellent batch-to-batch reproducibility

VWR BDH CHEMICALS

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As one of the global market leaders in laboratory reagents and production, we still try to improve our quality and safety standards to our reagents themselves, and also to the containers we supply. That's why we continuously develop and improve packaging and tailor-made solvent withdrawal systems that meet the requirements of our customers. After the first launch of 185 L stainless steel barrels and 1000 and 1400 L containers for production, today we are able to provide the complete range with two additional sizes (10 and 30 L) with different withdrawal systems. These two new barrels sizes will certainly be used more often in analytical laboratories.

This new solvent management solutions will help to minimise the environmental impact of packaging waste and health risks for your employees.

BENEFITS

- Maximum user safety – minimise fire risks, incidents of spillage, and reduce the risk of staff being exposed to hazardous solvents
- Less storage space - our solvents take up to 50% less space than glass bottles, reducing storage costs by half
- Easy and contamination-free solvent handling – be confident in your results. Our barrels are filled with high quality solvents that have an excellent batch-to-batch reproducibility
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- Application-oriented solutions and individual installations
- Ecological and economic benefits - with virtually no packaging waste, no hazardous disposal costs, no rising done on site, your lab will be greener and more environmentally friendly
- Time savings - save time testing solvent batches, handling solvent bottles and connecting and replacing bottles with your instrument

Technical data for stainless steel barrels

Parameter	10 L	30 L	185 L
Height (mm)	315	435	1085
Diameter (mm)	278	363	550
Wall thickness (mm)	1.5	1.5	1.5
Volume (L)	12	32	205
Filling quantity (L)	10	30	185
Weight (empty) (kg)	5.5	9.6	38
Number per pallet	11	6	
Working pressure (bar)	Max. 5	Max. 5	
Material	Stainless steel 1.4301		
Openings	2" centrally with Tri-Sure screw cap		None - dry brake coupler Type M and K

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SPECIFICATIONS OF THE SOLVENTS AVAILABLE IN STAINLESS STEEL BARRELS

Description	Pk (L)	Assay min. %	Residue max. %	Water max.%	Min. % UV transmittance/wavelength (nm) (1 cm quartz cell, distilled water)	Cat. No.
Acetonitrile super gradient grade	10	99.95	0.0002	0.003	80/195 95/200 96/210 97/220 98/230 99/240 99/250	83639.9010
Acetonitrile super gradient grade	30	99.95	0.0002	0.003	80/195 95/200 96/210 97/220 98/230 99/240 99/250	83639.9030
Acetonitrile super gradient grade	185	99.95	0.0002	0.003	80/195 95/200 96/210 97/220 98/230 99/240 99/250	83639.500
Acetonitrile Isocratic grade	10	99.9	0.0005	0.03	80/200 85/210 90/220 98/230 99/250	20048.9010
Acetonitrile Isocratic grade	30	99.9	0.0005	0.03	80/200 85/210 90/220 98/230 99/250	20048.9030
Methanol super gradient grade	10	99.9	0.0001	0.02	45/210 65/220 70/225 85/235 90/240 95/250 98/260	85681.9010
Methanol super gradient grade	30	99.9	0.0001	0.02	45/210 65/220 70/225 85/235 90/240 95/250 98/260	85681.9030
Methanol isocratic grade	10	99.8	0.0005	0.05	60/210 80/220 90/230 92/235 95/240 98/250 98/260	20837.9010
Methanol isocratic grade	30	99.8	0.0005	0.05	60/210 80/220 90/230 92/235 95/240 98/250 98/260	20837.9030

MERCK

HPLC tips and tricks for choosing an LC buffer

Dr. Egidijus Machtejevas, Lead Expert, Analytical Science Liaison.

When analysing samples containing ionisable compounds, the buffer can be one of the most important variables controlling the retention in an HPLC separation. The pH of the mobile phase determines the presence of ionisable compounds (analytes and matrix) to be in either an ionised or non ionised state. Ionised species in reverse phase (RP) chromatography always elute from the column earlier than the non ionised species. Changing the pH can also increase the selectivity for effective separation of closely eluting or overlapping peaks. Run to run variability in pH results in a separation inconsistency. Buffers prevent pH variations. Therefore, the proper buffer choice, in terms of buffering species, ionic strength and pH, is the most critical step in HPLC method development when ionisable substances are analysed.

TIPS FOR CHOOSING AN LC BUFFER

BUFFER SELECTION

The choice of the appropriate buffer for an application is governed by the buffer characteristics such as pKa, pH range and UV cut-off. As a rule, buffers should be used for a pH within ±1 unit of their pKa value. Within this range, buffers resist any deliberate attempts of change in pH. The buffer's capacity is at its maximum when its pH is equal to its pKa. The UV cut off value also needs to be considered, as the detection wavelength should not interfere with the buffer absorbance (significant absorbance: trifluoroacetic acid <220 nm; formic acid, acetic acid <240 nm). For the best results with an ionisable analyte of interest, use a buffer with a pH at least 2 units away from the analyte's pKa. If the pH of the mobile phase is too close to the analyte's pKa, split peaks or shoulders might be observed due to the presence of both species in the sample. For several ionisable analytes of interest, it is preferable to choose a pH value wherein all the analytes exist in the same form, either ionised or non ionised.

MEASURING BUFFER pH

pH of the buffer is the pH of the aqueous portion before the organic mobile phase part is added. The addition of an organic solvent can shift the pH either up or down (pH shift should be consistent for the same buffer). It is not so important to know the exact pH value of the buffer in an organic medium, but it is important to have a consistent pH value (because pKa of your analytes is also determined in aqueous phase, and we do not know the individual pKa shifts either).

CHEMICAL PURITY

The quality/purity of mobile phase additives (buffers, salts, acids and bases) along with organic solvents utilised in an HPLC experiment must be adapted to the detector sensitivity and elution protocol.

CHEMICAL COMPATIBILITY

Buffer composition, along with mobile phase pH, must be chosen in agreement with column housing material and nature of the stationary as well as different parts of LC instrument (pump, tubing, etc) phase to prevent corrosion or degradation of either.

MS COMPATIBILITY

Introducing inorganic buffer salts into a mass spectrometer soon fouls the system. Examples of suitable volatile buffers are ammonium acetate, ammonium formate and ammonium citrate. pH modifiers like formic acid and acetic acid should be used to control pH and help ionisation for LC-MS.

BUFFER SOLUBILITY

Ideally, the buffer should be completely water soluble (RP methods) and should not precipitate during the analysis when mixed with a chosen organic solvent. Buffer concentration must, therefore, be carefully chosen to avoid precipitation at higher concentrations in the organic solvent. If neglected, this can create operational problems with the pump and instigate HPLC column blockage or back pressure rise.

BUFFER IONIC STRENGTH

In case of ionic interactions between analytes and stationary phase, the ionic strength of the buffer must be chosen in a way that compounds are eluted. The required ionic strength of the buffer depends on the stationary phase. Besides elution strength, the viscosity of the buffer plays an important role in terms of its suitability for use in HPLC analyses.

BUFFER CONCENTRATION

Ideally, the lowest concentration that gives reproducible results should be chosen. Higher concentrations lead to a faster elution of polar molecules. Generally, the buffer concentration should not be lower than 5 mM. Below this concentration, the solution may not perform as a buffer (depending on analyte concentration and buffering capability). Raising the buffer concentration can increase viscosity and the risk of buffer precipitation, which in turn can increase column back pressure. Commonly, the concentration should be kept in the 5 to 100 mM range. A concentration higher than 100 mM of mineral salt buffers wear out the pump's movable parts faster, therefore, a back-seal wash is recommended to be installed. It can be observed that buffers play a crucial role in a majority of HPLC separations. Method development often requires careful selection of buffers and adequate care in their preparation. So, the general rules to be kept in mind are - buffer solutions must be homogeneous, clear and free from any particles. If stored, please keep in mind that buffers have a limited lifetime, so consider their preparation daily.

Essentials and tips for choosing your mobile phase quickly in LC-MS

INTRODUCTION

Liquid Chromatography - Mass Spectrometry (LC-MS) is now the favourite tool of liquid chromatographers, combining the power of liquid chromatography with the detection specificity of mass spectrometry. But, it's not so easy to find the right mobile phases suitable for LC-MS analysis. The 'dream' mobile phase for all separations doesn't exist. If you want to check all the parameters and various conditions, the challenge is difficult and can take time. So, today, we would like to give some advice on how to determine the best conditions, as a general approach, to perform your analysis in a short time. Of course, it's not perfect but can be a good compromise between efficiency and time.

RECOMMENDED SOLVENTS & ADDITIVES

Electrospray ionisation (ESI) is surely the most common method. This technique is used to generate ions through the application of a high voltage to a flow of eluent. The charged eluent is then mixed with a nebulising gas to generate a charged aerosol. It is especially useful in producing ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionised. In the following, you will find the solvents and mobile phases we recommend. All of them can dissolve polar compounds (water and polar organic solvents).

RECOMMENDED SOLVENTS & MOBILE PHASES

Solvents

- Alcohols, such as methanol mainly and ethanol or 2-propanol
- Acetonitrile
- Acetone

Water or volatile aqueous solutions

- Acetic acid or ammonium acetate in water
- Formic acid or ammonium formate in water
- Trifluoroacetic acid or aqueous ammonia

Volatile ion-pair reagents

- Perfluorocarbonate (C2 to C8)
- Dibutyl ammonium acetate

Based on the recommended solvents and additives indicated previously firstly try these 'favourite' mobile phases (A and B) shown below.

01. Mobile phase A: 0,1% aqueous formic acid solution

- Small molecular weight (MW = 46)
- Adjustable to low pH levels
- Low contamination
- Odourless

Reasons for using this acid solution

- Formic acid has a lower molecular weight than acetic acid and does not ion pair as is the case with TFA.
- This kind of acid mobile phase is often used for analysing basic compounds, often analysed in pharmaceutical companies
- Low pH mobile phases reduce the amount of dissociated silanols, and so reduce the degree of tailing associated with basic compounds
- Formic acid is a stronger acid than acetic, so it means that the analysis is made at a lower pH. In ESI work, you might need to be slightly more sensitive regarding flavonoids with formic acid, but in general the performances could be similar
- In LC-MS analysis, the purity of additives and mobile phase is a key parameter to increase sensitivity. During mobile phase preparation, it is apparent that formic acid mobile phases are less contaminated compared to acetic acid mobile phases
- Compared to other acids like acetic acid, formic acid is odourless which can represent a big advantage for scientists

02. Mobile phase B: Acetonitrile

- Ionisation efficiency of ESI is higher than methanol

Reasons for using acetonitrile

- Acetonitrile presents a high ionisation efficiency of ESI compared to methanol
- Lower viscosity is better for producing fine droplets
- No acid is added to this mobile phase because it's easier to control the impact of the acid addition. It is also better to add acid even to organic solvents, but usually this action should verified beforehand

REMARKS

In mobile phase A, we've chosen acidic conditions. But, depending on the compounds, we could use other additives such as ammonium salts. So, it's mandatory to know exactly what the analytical conditions are before modifying the pH mobile phase. If you want to limit the number of tests with different mobile phase, avoid changing both the organic solvent and the aqueous mobile phase at the same time.

However, despite this advice, it is even more serious and more expensive to let high purity mobile phases gradually lose their purity in storage. So, try to follow these recommendations to avoid contamination.

ADVICE, TIPS & TRICKS

01. Use ultrapure water

Water is often a possible contamination source. Water can be used for many applications to wash glassware, prepare standards and blanks, and to be a component in the mobile phase. Two options can be chosen.

- Ultrapure water Grade LC-MS particle-free, chemically clean with a resistivity of 18 mΩ
- Purification systems that produce ultrapure water. These systems use reverse osmosis to remove most contaminants, ion exchange to remove ions, carbon filtration for the removal of organics, UV sterilisation to kill bacteria, and a pharmaceutical grade 0,2 µm membrane filter to remove particulates

02. Prevent microbial growth

Microbial growth can be particularly problematic for UHPLC systems, which can be much more sensitive to blockages due to smaller tubing diameters and column frit porosities. Aqueous mobile phases are prone to microbial growth (even over short time periods); this can cause extra peaks in gradient elution, and increase background absorbance during isocratic methods. Microbial growth behaves as a particulate and can block filters, frits and columns, as well as causing check valve malfunctions. All these problems will result in higher operating pressures which can damage columns and cause a system shut down.

- Microbial growth can be prevented by preparing mobile phases fresh each day by filtering and degassing as well as filtering the air before it goes into the mobile phase
- Regular replacement of the mobile phase, accompanied by flushing the LC system to ensure the removal of any residual solvent from the previous batch, will help to reduce the likelihood of microbial contamination

03. Additive quality

- Use the highest quality additives available (minimum, LC-MS grade). For example, formic acid LC-MS with low concentrations of iron and other metal ions

04. Be aware of possible contamination sources

- During mobile phase preparation, take care to not introduce contamination coming from plasticisers used in plastic gloves or pipettes

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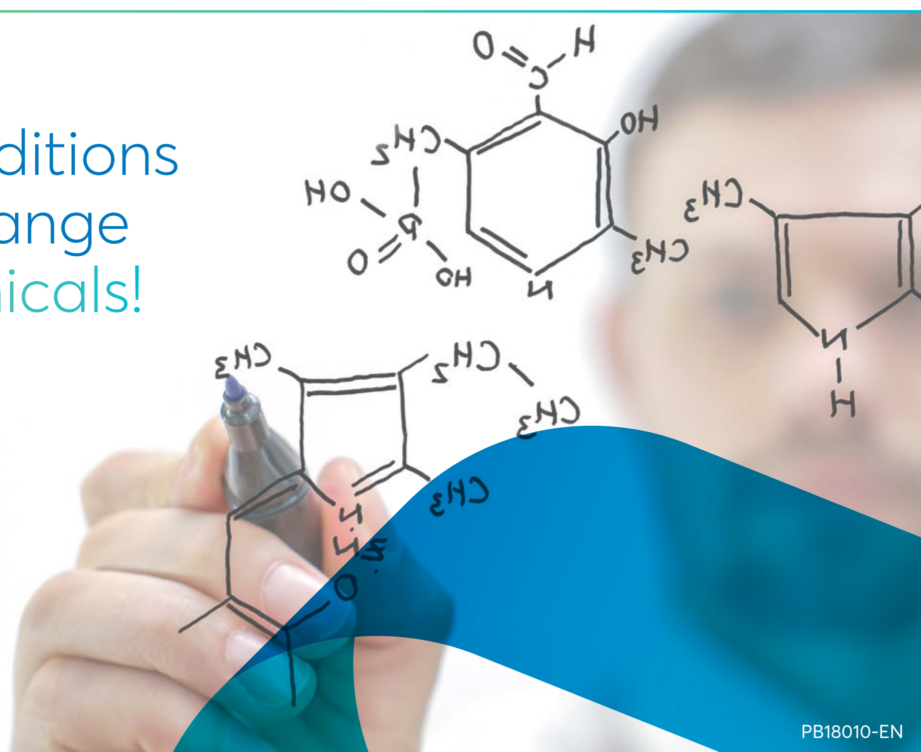
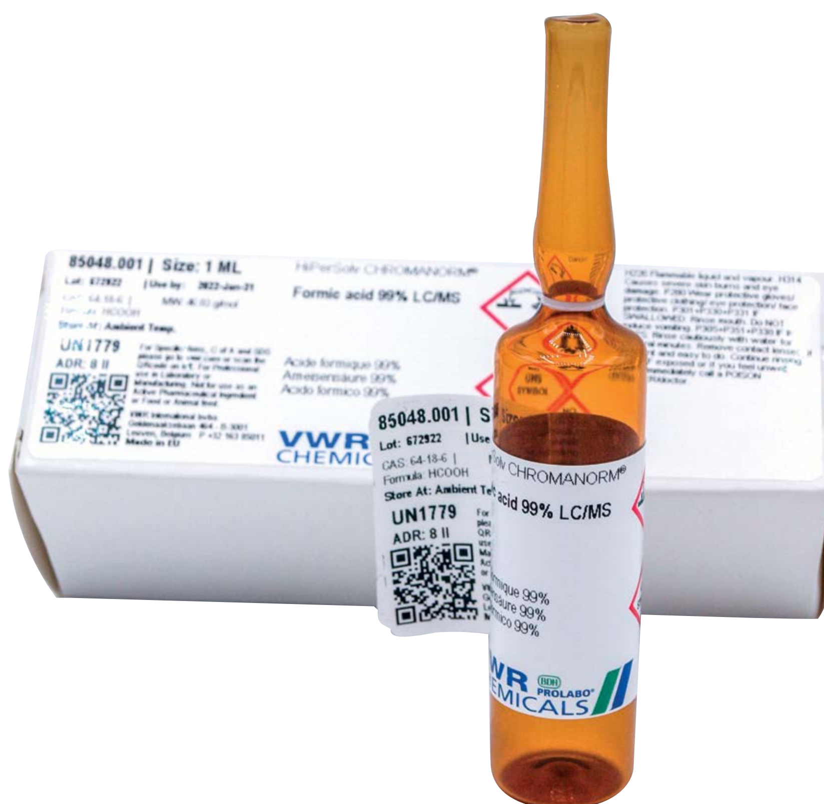




- In addition, using pipettes to add LC-MS additives (reagents such as TFA or formic acid to the mobile phase) can be a potential source of contamination. Ideally, different sets of pipettes should be used to prepare the mobile phase and stock solutions for analysis

05. Wear nitrile gloves

Gloves can represent a source of contamination. When handling instrument components, filling solvent bottles and preparing samples, some biomolecules and other contaminants on the skin can be transferred into the system (for example, by skin cells falling into sample vials or in the mobile phase solvent).

- Keep gloves dry and replace them if they become wet - wet gloves can introduce contamination
- Avoid incidental skin contact, do not wear finger cots as a substitute for gloves

06. Prepare and store solvents in clean glass reservoirs with covers

It is essential to maintain the quality of a solvent once it is in use, because it's easy to forget that contaminants can be absorbed from the environment.

- LC-MS mobile phases should be stored on the instrument for a minimum amount of time, and solvent reservoirs should always be capped. The choice of storage container is critical. Preferably, use a borosilicate glass bottle (type 1, class A, or type 3.3) instead of a plastic bottle able to leach plasticisers into the aqueous solvent over time
- Cover the reservoir to prevent airborne contaminants from entering the solvent
- To cover the reservoir, use aluminium foil or caps supplied with the system
- Use the smallest solvent reservoir appropriate for your analysis (this depends on your flow rate and the length of your analysis)
- Use dedicated bottles for a specific instrument and to specific solvents (for example, use one specific bottle for acetonitrile, one for formic acid in water, and so on), and don't wash them with detergent. The risk of contaminating mobile phases with residual detergent is huge. It is helpful to rinse bottles used for aqueous solvents with a small amount of high quality acetonitrile or methanol before filling with more aqueous solvent
- Any glass container used to prepare or store a mobile phase must be thoroughly cleaned with organic solvent or high purity water before use

LC-MS solvents

Description	1 L	2,5 L	4 L
Acetonitrile	83640.290	83640.320	83640.400
Acetonitrile Ultra	84642.290	83642.320	
Ethyl acetate		85481.320	
Methanol	83638.290	83638.320	
Methanol Ultra	85800.290	85800.320	
Methanol Ultra plus	85855.290	85855.320	
N Hexane	85799.290	85799.320	
N Heptane	84899.290	84899.320	
2-Propanol	84881.290	84881.320	
Tetrahydrofuran	84882.290	84882.320	
Water	83645.290	83645.320	83645.400

LC-MS additives

Description	10x1 ml	10 ml	5x10 ml	100 ml	500 ml	1 L
Acetic acid 99%				84874.180	84874.260	84874.290
Ammonium formate				84884.180	84884.260	
Ammonium acetate				84885.180	84886.260	
Formic acid 99%	85048.001	85048.010	85048.051	84865.180	84865.260	84865.290
Triethylamine				84883.180	84883.260	
Trifluoroacetic acid	85049.001	85049.010	89049.051	84868.180	84868.260	84868.290

How much ethanol is in my beer?

Analysed with Lucidity GC-FID. Smallest footprint of any fully functional GC-FID.

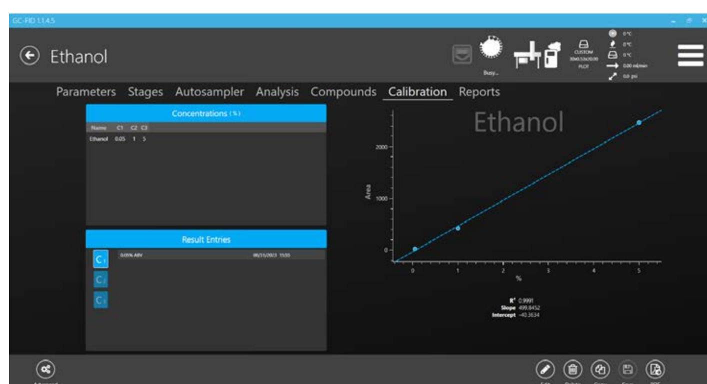
Whether you drink a beer from a major manufacturer, the local brewer down the street, or even brew the beer yourself, knowing the ethanol content of that beer is an important piece of information. Up until 1995 the alcohol content wasn't allowed to be on beer labels at all until the Coors Brewing Company successfully challenged the law in the Supreme Court. Now, brewers are free to either disclose that information on the labels or not.

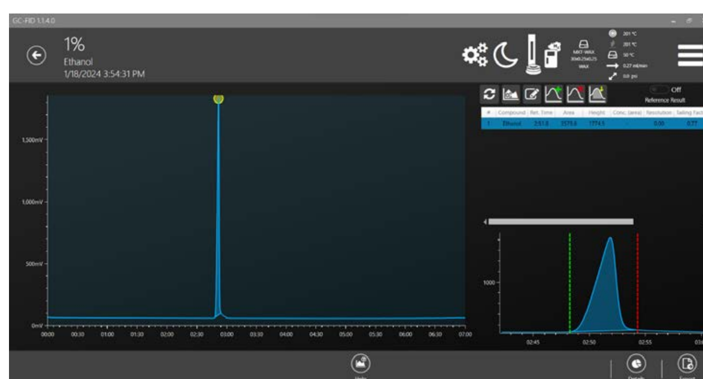
How is ethanol content, or Alcohol By Volume (ABV), measured? The old fashioned way is by what is called a hygrometer. A hygrometer is a sealed glass weighted tube that will float in liquids to give the specific gravity of the liquid. The brewer would take the measurement of the pre-brewed beer, called a wort, and then a second measurement after brewing and use a simple calculation to determine the ABV.

Another traditional technique would be a distillation, where the beer would be placed in special glassware and heated. The vapour would be cooled and collected into another vessel and the masses of each would be used to determine the ABV. Both of these techniques use a lot of the beer to run properly and are time consuming.

A more modern technique is to test by GC-FID. This technique uses less than a milliliter of the beer sample and once the GC and methods are set up, the method is very simple and quick.

The Lucidity team grabbed some beers from various vendors including a popular microbrew beer (1) and the light version of the microbrew (2)





Lucidity GC-FID Conditions

Carrier		Hydrogen
Control		Constant Pressure
Flow		1.2 ml/min
Split ratio		100:01:00
Column	**Rtx-WAX**	30 mx0.25 mm, 0.25 µm
Injector		300 ℃
FID		300 ℃

Oven Program

Rate	Temperature	Hold Time
	40 ℃	1.0 min
10 ℃/min	75 ℃	0.0 min
30 ℃/min	150 ℃	0.0 min

Using the conditions stated a Lucidity Chemist analysed the beer samples using the Lucidity-GC-FID and Lucidity GAS

Beer	Labeled Amount	Calculated Amount
A	6%	5.95%
C	4.2%	3.83%

Experience the versatility of Lucidity GC-FID and Lucidity GAS as an ideal combination for your analytical requirements. Lucidity offers many advantages such as:

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- User-Friendly
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Efficiency is enhanced with removable column cartridges saving time on column changes and simplifying the installation process.
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The CFR compliant Lucidity Software streamlines the process of starting a run, collecting data, and analysing results, offering user-friendly features with no licensing fees.

Explore intriguing studies on the website, such as the Lucidity R&D team's analysis of ethanol content in local beers, presented with engaging screenshots from previous experiments.

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