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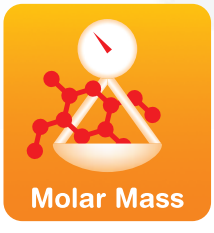

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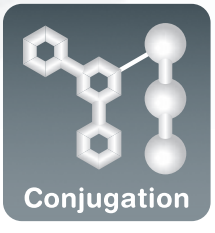

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A Q&A

Perchlorate Analysis by IC: Water Impact

Joseph Plurad
Head of Field Marketing – Milli-Q Lab Water North America
MilliporeSigma

Perchlorates are found in many commercially-available products and have been identified as an environmental pollutant. Since most perchlorates are water soluble, they enter potable water supplies quite easily. This Q&A session with Joseph Plurad, the North American Field Marketing Manager for Milli-Q Lab Water Systems at MilliporeSigma discusses the problems associated with measuring perchlorate levels and what water purification technologies are available to provide the highest quality water for ion chromatography.

Why is the analysis of perchlorates of interest to our audience today and why did Millipore Sigma choose to investigate this?

Over the last 20 years, perchlorates have received a lot of attention from regulatory agencies because they've been identified as an environmental pollutant with real impact on human health, which can interfere with iodide uptake by the thyroid gland. One of the most recognized sources of perchlorate contamination is from rocket fuels and other propellants. Other sources also include fireworks, matches, disinfectants and some bleaching agents and some fertilizers made from sodium nitrate deposits. And since most perchlorates are readily water soluble, they enter the groundwater water systems quite easily.

Additionally, back in the late 1990s, the EPA developed a detection method for perchlorates in drinking water using ion chromatography. However, ultrapure water is one of the most important reagents in ion chromatography, which is ultimately derived from local drinking water supplies, and as a result, is likely to already have detectable levels of perchlorate.

When considering reagent water for use in ion chromatography, which specific contaminants have the greatest impact on the analysis?

The water needs to be effectively ion-free. It's best to make sure that the reagents used don't have the ion you're trying to determine. So, ionic contaminants in the water are a potential problem and in particular anything else that would elute near the perchlorate peak.

However, we still need to be concerned about organic contaminants, because they can coat the active surface of the resins in the column. This can result in poor peak resolution, low intensity, and shifting baselines, affecting the ability to accurately identify and quantify the perchlorate ion. Some dissolved gasses are also known to cause problems with IC, but this is typically addressed by degassing the water and eluents prior to use.

Other contaminants that could impact the IC system's performance are particulates that could block columns, leading to an increase in pressure, and potentially damaging the system. And finally, bacteria and other microbials growing in the bottle could contribute other biological byproducts in the ultrapure water.

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What water purification technologies are used to provide the best water for ion chromatography and specifically perchlorate analysis by IC?

The best ultrapure water is made from the local municipal tap water. It's also important not to produce significantly more water than you plan to use in your experiments that day. And by owning the purification process from tap water to final use, lab professionals have full control of the water, resulting in better confidence in its quality.

Generally, we recommend purifying the tap water to an intermediate stage via our Elix electrode ionization process. Tap waters pass through pre-filters to remove large particles, some disinfectants and organics, and to sequester ions that cause hardness, which can generate other purification problems downstream. After pre-filtration, the water passes through a reverse osmosis (RO) membrane that will remove more than 95% of all the contaminants in the tap water. Our patented electrodeionization process will further remove any ionically-charged species from the water resulting in 1-50 megohm resistivity.

After deactivating bacteria with UV radiation, we'll store the water before final cleanup for use. And when the analyst is ready for the ultrapure water for IC, we'll pass it through purification steps embedded in our Milli-Q ultrapure water systems. The water will pass through a dual wavelength UV lamp not only to mitigate microbial risk, but to also photo-oxidize trace organics into free radicals that can be removed with ion exchange media. The ion exchange resin will further remove ions that may have passed through the Elix technology. And the final filtration step is to pass the water through a 0.2 micron filter to remove any remaining particles and microbes that might remain. Using these techniques, we produce 18.2 megohm water with a Total Organic Carbon (TOC) content of < 5 ppb.

What did Millipore Sigma do to test the efficacy of water purification to provide suitable water for perchlorate analysis?

Perchlorates exist in many municipal drinking water sources, so we evaluated the ability of four different Millipore Sigma water systems to remove perchlorate from water. First we tested a Direct-Q 3 UV system utilizing reverse osmosis, UV photo-oxidation, activated carbon and ion exchange. The second system was an Elix 5 system, which combines activated carbon, reverse osmosis and electrodeionization to produce water similar to distilled water. And finally, our two entry-level ultrapure systems, the Simplicity UV and Milli-Q Advantage A10 water systems that use UV photo-oxidation, activated carbon and ion exchange to produce ultrapure water from the Elix 5 water system.

Because the tap water did not have significant perchlorate content, we spiked the tap water fed to the Direct-Q and Elix systems with about 80 ppm of perchlorate. Then the Elix water used to feed the Simplicity and Milli-Q systems was also spiked with perchlorate at an order of magnitude lower than the tap water (80 and 120 ppb). It's important to emphasize that the level of perchlorate we spiked into each of these systems was significantly higher than you would normally expect to find in tap water, which is typically around 25 ppb. The final step was to measure the perchlorate content by IC at the inlet to each system and after each of the purification steps in the separate lab water systems.

So, can you share some of those results with us?

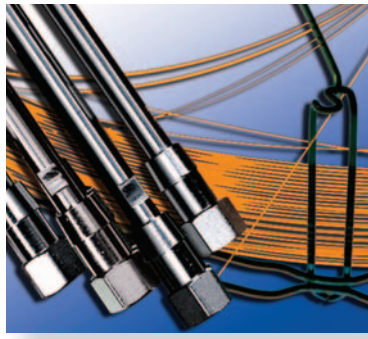
The reverse osmosis step removed 97% of the perchlorate that was in the tap water fed to it. When the RO water was then purified via traditional ion exchange or via electrodeionization in our Elix module, perchlorate levels already fell below the 5 ppt detection limit. When the RO and the Elix water was then passed through ion exchange resins in the Simplicity system and the Milli-Q system, perchlorate was not detected at the outlet of either system.

So basically, with the exception of reverse osmosis alone, any Millipore Sigma system with ion removal after the RO would be suitable to use. So even though Elix water can produce perchlorate-free water, we still have to be concerned about organic microbial and particular contaminants that could impact the analysis. So for that reason, we would recommend using an ultrapure system like a Milli-Q or Direct-Q system for IC analysis.

Is MilliporeSigma doing anything else to meet the needs of the laboratories requiring ultratrace analysis of ions?

In the last 20 years since the EPA methods first came out for perchlorate analysis, there have been continual advancements in detection technologies which are achieving lower detection limits allowing for smaller sample sizes, and even the elimination of some sample preparation steps. Additionally, from an analytical perspective, 18.2 megohm ultrapure water can still have ionic contaminants that cannot be quantified using resistivity measurement. For that reason, we have a responsibility to our customers to provide water that meets these strict requirements. Our ultrapure systems are highly customizable at the dispensing point to meet the demands of today's analytical scientists, whether they are ionic, organic or microbial in nature.

One example of this would be our Q-POD Element dispensing unit. It was originally intended for ICP-MS applications for use in a clean room and as a result uses semiconductor-grade materials in its construction. But inside this Q-POD Element is an additional ion exchange cartridge, as well as low-leeching materials that minimize recontamination along with a 0.01 micron semiconductor-grade filter for further particle removal. The original target of this Milli-Q accessory was for trace metals but we can take advantage of the design and the purification media to achieve low ppt ionic levels, even though resistivity meter will continue to report 18.2 megohm.



COLUMN WATCH

A Simple and Interactive Column Classification for Reversed-Phase Liquid Chromatography: The Carotenoid Test, Part I: Studied Properties, Probes, and Fundamentals

Carotenoid compounds can be used as probes for studying bonded stationary phases for reversed-phase liquid chromatography, such as C18, phenyl-hexyl, and cholesteryl. From one supercritical fluid chromatography (SFC) analysis that favors the chromatographic behaviors related to the stationary phase properties, bonding density, ligand type (monomeric or polymeric), and endcapping treatment, two separation factors are calculated allowing us to build a two-dimensional map. These two axes are related to either the shape selectivity or the polar surface activity (residual silanols). Each point on the map corresponds to a column. The retention factor of β-carotene, which describes the phase hydrophobicity, is indicated by the size of the point. More than 200 stationary phases were studied, including small particle sizes and superficially porous ones.

Eric Lesellier *is the guest author of this installment.*
David S. Bell *is the editor of Column Watch.*

Acquiring knowledge about the properties of the bonded stationary phases used in reversed-phase liquid chromatography, mainly for C18 bonded chains, has always been a great challenge for chromatographers and manufacturers (1). The challenge can arise either for the initial choice of the suitable phase for the replacement of one stationary phase by another (1,2), or for quality-by-design method development (3,4).

In recent years, the choice of column has been partly dictated by a desired particle size or type (fully or superficially porous), to allow for very high efficiencies (plate number) (5). However, the chemical properties of the stationary phase are not correlated to particle size or type, and different C18 columns may even display significantly different retention behavior, sometimes yielding reversal in elution orders, under identical operating conditions.

In the past, numerous attempts were made to provide chromatographic tests with the goal of achieving the evaluation of these properties (6–12). These tests did not provide any information related to the durability of the column when working with acidic or basic conditions—that is, phase aging. Instead, the tests provide information about the retention of hydrophobic (retention factor of a nonpolar aromatic compound) or hydrophilic compounds (polar and nonpolar selectivity), the ability of compounds to penetrate the C18 chain network (steric resistance, shape selectivity), the presence of acidic and ionized groups onto the silica surface, or of metallic traces (6–14). Simple tests are based on two datasets, either retention factor (k) or separation factor (α), meaning that a two-dimensional (2D) map can be sufficient to compare the phases (9). Hydrophobicity and polar surface activity are often used as axes for these maps, and the obtained classification is usually in agreement with the data provided by the manufacturers in terms of carbon content (%), specific surface area (m^2/g), and the presence of residual silanol groups or hydrophilic endcapping groups. From four to six properties (the maximum number of properties usually studied), results can be presented by radar plots (6), principal component analysis (PCA) (15–17), or ranking factor (2,13,14,18). Each of these presentations display advantages and drawbacks (1,18). Some of those drawbacks include

- the use of radar plots is not convenient for the comparison and classification of numerous stationary phases together;
- the clusters obtained from PCA cannot always be easily related to the retention properties; and
- the ranking is only relevant from a

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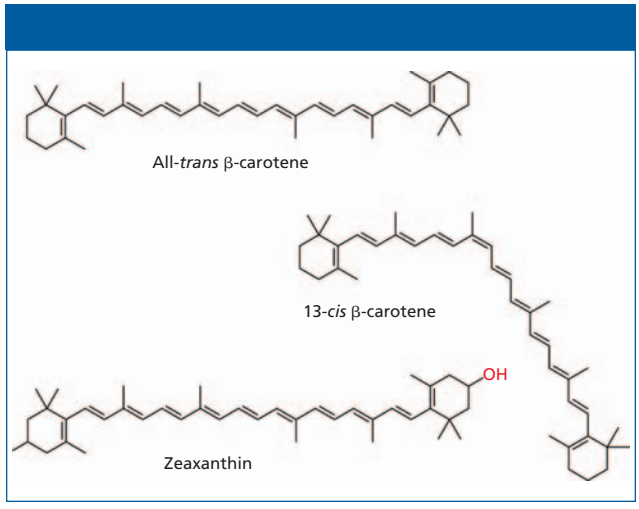

Figure 1: Structure of the probes used for the carotenoid test.

reference column, meaning that no simultaneous comparison of all stationary phases can be done.

Moreover, the use of numerous parameters can introduce a bias in the classification, either because of their lack of relevance or because of the correlation between some of them (1,19). For instance, *methylene selectivity*, the separation factor between two compounds differing by a CH_2 group, varies in relation to the carbon content of C18 bonded phases only up to 10% (1,6,20), which is a rather small value for modern bonded phases. The use of this factor reduces the ability of chemometric treatments to highlight column differences. Besides, the measurement of ionic interactions at two pH values, around 2 and 7, seems unnecessary because few silanols are protonated at pH 2, and it covers in part other hydrophilic interaction measurements with nonionizable compounds—for example, caffeine–phenol used in the Tanaka test to estimate hydrogen bonding (1). Finally, some of these tests required different mobile-phase compositions to measure all properties, further complicating the process.

Test Presentation

Taking into account the problem of the presentation of results, the relevance of the used data, and the mobile-phase conditions, more than 20 years ago we suggested the use of another compound family, the carotenoid pigments, with supercritical fluid chromatography (SFC) as a measurement technique, and limiting the studied parameters (properties) to three to avoid nonrelevant data (21–25).

As shown in Figure 1, three compounds are used to check the stationary-phase properties. All-*trans* β-carotene contains only hydrogen and carbon atoms, with two aliphatic rings at the extremities of a carbon chain with conjugated double bonds. Because this compound does not have any heteroatom, its retention factor (*k*) can be related to the phase *hydrophobicity*—that is, the ability of the compounds to be retained by dispersive interactions by the C18 bonded chains. The hydrophobic retention is related to the carbon content, which determines the bonding density by taking into account the specific surface area.

This compound is also linear and its retention can be compared to the retention of its main isomer, 13-*cis*-β-carotene. Because of the double-bond conformation, the cis isomer is bent. This structural difference between the all-*trans* and the 13-*cis* isomers can modify the interaction surface between the compounds and the C18 bonded chains. This is measured by the separation factor (α) between the two isomers (cis–trans). The obtained values can be related to the *shape selectivity*, also called *shape recognition*, which is the ability of the stationary phase to separate compounds on the basis of their structural organization, for instance, diastereoisomers.

The third compound is a *xanthophyll*, which is a carotenoid pigment with oxygen atoms. Compared to all-*trans* β-carotene, zeaxanthin has two more hydroxyl groups located on the rings. We can suppose that these additional groups are perfectly located to establish "polar interactions," mainly hydrogen bonding, with all polar groups located on or near the silica surface. The separation factor (α) between the hydrocarbon and oxygenated molecules (β/zea) could be related to the polar surface activity of the stationary phase: the higher this selectivity value, the lower the polar surface activity.

The retention of these three compounds is studied for each column in SFC, with a 85:15 v/v carbon dioxide–methanol mobile phase, a temperature of 25 °C, an outlet pressure of 15 MPa, a flow rate of 3 mL/min, and a UV detection wavelength of 440 nm.

These conditions have been optimized, mainly the

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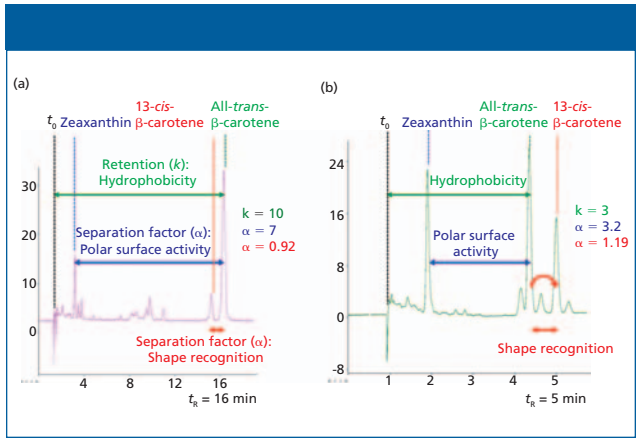

Figure 2: Chromatograms of the analyzed carotenoid mixture obtained with two stationary phases: (a) Atlantis dC18 (Waters); (b) Wakosil 5C18 AR (SGE). Analytical conditions are described in the text.

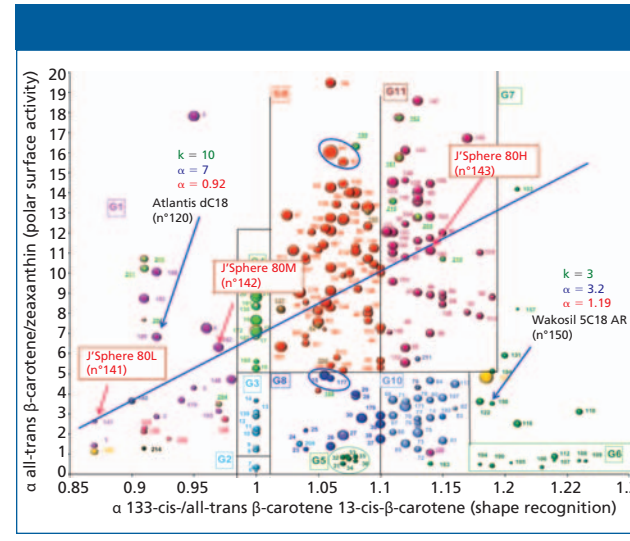

Figure 3: Classification map for the comparison of column performance. *x*-axis: separation factor 13-*cis*/all-*trans* β-carotene (shape recognition); *y*-axis: separation factor all-*trans* β-carotene/zeaxanthin (polar surface activity); point size: retention factor of all-*trans* β-carotene (hydrophobicity).

nature and the percentage of modifier (methanol) to ensure reliable results and large ranges in the scales of the studied parameters, with a reasonable duration time for the analysis.

The use of a supercritical fluid avoids the presence of water in the mobile phase, favoring the interactions between zeaxanthin and polar groups like residual silanol groups. Moreover, the separation of cis–trans isomers of β-carotene on C18-bonded phases is enhanced compared to liquid mobile phases. Such a separation in high performance liquid chromatography (HPLC) often requires the use of long carbon chains (C30 bonded phases). On the other hand, no C8 bonded phases can be characterized by the carotenoid test because of the lack of separation between cis and trans isomers. However, the test can be applied to phenyl-hexyl phases, which are slowly going to replace C8 phases for new method developments.

As shown in Figure 2, a single chromatogram allows the calculation of one retention factor (*k*) for hydrophobicity and two separation factors (α), for polar surface activity and shape recognition. One can see in Figure 2b that numerous other cis isomers can be separated with a short run time for the analysis, showing that SFC is rapid and can perform the separation for the studies of the stationary-phase properties.

When plotting those data on a map (Figure 3), with the 13-*cis*/all-*trans* β-carotene separation factors on the *x*-axis (scale from 0.85 to 1.3) and the all-*trans* β-carotene/zeaxanthin separation factors on the *y*-axis (scale from 0 to 20), one can locate each column depending on its properties related to the carotenoids chromatographic behavior. On the map, each point represents one column, and the third property, hydrophobicity, measured by the retention factor of all-*trans* β-carotene, is reported by the size of the point. The map was divided into 11 groups, related to the values of the two axes, and the properties of columns of each group will be explained in part II of this article series. The main idea is that columns included in one group would provide close elution order for most analytes in a sample, meaning that their chromatographic selectivity should be close. However, inside each group, depending on the size of the point, the phase hydrophobicity can be very different. For example, in group 9, phases n°63 and n°64 (or n°15 and n°177 in group 8) are close but the hydrophobicity of columns 64 and 15 are higher than that of columns 63 and 177. That means that the chromatogram would display a close retention order

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(separation factor) for the first and the second couple of columns, but with a longer analysis time for columns 64 and 15. This can be explained either by a different specific surface area or a different bonding density.

The results from the two stationary phases tested in Figure 2 indicate that they are located in two different groups, group 1 (G1) for Atlantis dC18 (n°120) (Waters), and group 7 (G7) for Wakosil 5C18 AR (n°150) (SGE), mainly because of different shape recognition, with a reversal of the retention order of the two β-carotene isomers and with shape recognition values of 0.92 and 1.19, respectively. The Atlantic dC18 column has lower polar surface activity ($\alpha = 7$ versus $\alpha = 3.2$) and a higher hydrophobicity ($k = 10$ versus $k = 3$).

Test Relevance

Before one can effectively use the map, the relevance of the discussed properties—that is, the measurements proposed—should be addressed.

First we studied the location of three J'Sphere 80 phases (YMC America), called L (low bonding density), M (medium bonding density), and H (high bonding density). These three stationary phases are made of the same silica (pore diameter = 80 Å), with different carbon content (9%, 14%, and 22%, respectively), and different bonding density (0.9, 1.6, and 2.9 μmol/m^2, respectively), and are often used as reference materials (9,26). Because the pore diameter is the same, the three phases display an identical specific surface area (500 m^2/g), and the increase in the carbon content explains the bonding density enhancement, meaning that the C18 chains become closer to each other from J'Sphere 80L to J'Sphere 80H. As shown in Figure 3, this bonding density increase leads to both an increase in the shape recognition and the polar surface activity values because the three points representing the three columns are located along a diagonal line from the bottom left to top right.

The measurement of shape recognition (along the abscissa) indicates that with very low bonding density, the bent isomer (13-*cis*) is more retained than the linear (all-*trans*) one. Then, when increasing the bonding density, the retention of the bent isomer increases compared to the linear isomer. Because the J'Sphere phases are *monomeric*, meaning that one C18 chain is bonded to one silanol group, this result underlines the relationship between the bonding density and the separation factor for the two isomers of β-carotene. Figure 4 shows the relationship between the 13-*cis*/*trans* β-carotene separation factor (*x*-axis), and the TbN/BaP (blue) (14–28), the Tri/o-Ter (red) (6,15,16) or the steric selectivity S^* one (green) (2,13,27) (*y*-axis), which are other measurements of the stationary phase organization. Tetrabenzonaphthalene (TbN), benzo[*a*]pyrene (BaP), triphenylene (Tri), and *o*-terphenyl (o-Ter) are polycyclic aromatic compounds, which also have linear or bent structures, whereas the steric selectivity S^*

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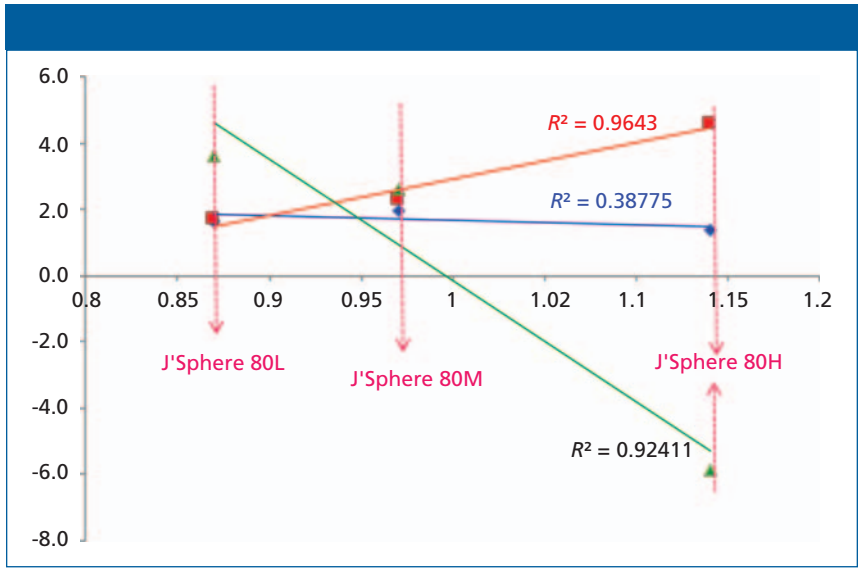

Figure 4: Relationship between 13-*cis*/all-*trans* β-carotene separation factor and TbN/BaP one (blue line); Tri/oTer one (red); steric resistance (*S**) (green). Data adapted with permission from references 20 and 26.

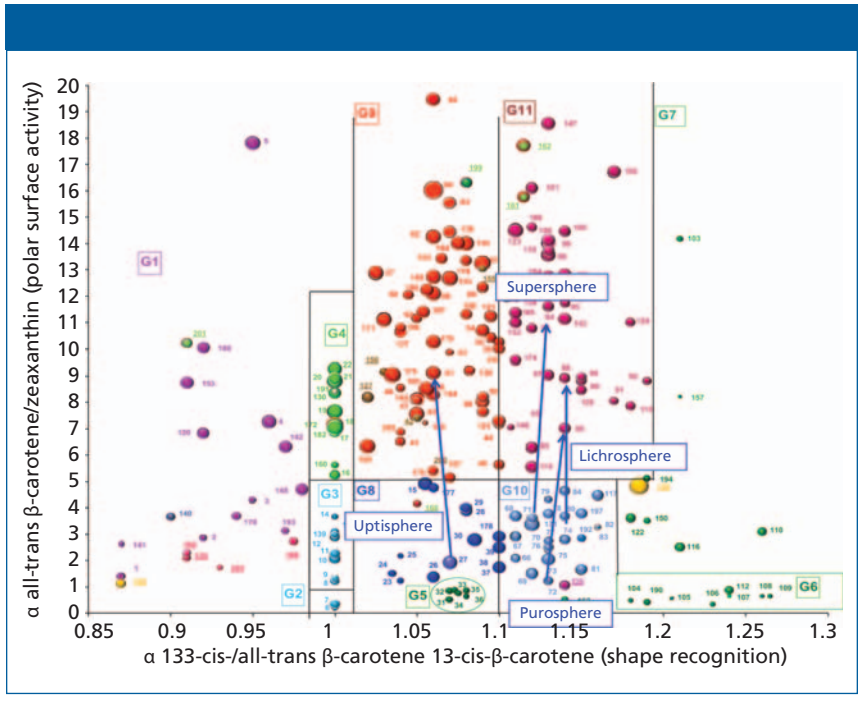

Figure 5: Classification map and effect of endcapping treatment

is related to the compound size. By using a linear relationship, a rather satisfactory correlation is observed between the studied values, except for TbN/BaP. However, this last point was not confirmed by using a greater number of stationary phases, including polymeric bonded phases (29). Nevertheless, these results indicate the relevance of the use of linear and bent carotenoid isomers to assess shape recognition properties.

Secondly, the increase in the separation factor between all-*trans* β-carotene and zeaxanthin indicate that the decreasing polar surface activity (along the ordinate axis) is because of the reducing retention of zeaxanthin (Figure 3). The two hydroxyl groups of zeaxanthin can interact with residual (free) silanol groups, or other polar groups. The increase in the carbon content—that is, the number of C18 chains bonded per surface unit—is related to a reduced amount of residual silanol groups, especially because no endcapping treatment is applied on these stationary phases.

Further evidence of the relevance of the polar surface activity is provided by plotting four pairs of stationary phases, with (n°51, n°86, n°88, and n°94) or without (n°27, n°71, n°72, and n°74) endcapping treatment. This treatment is an additional bonding of small hydrophobic compounds, for instance trimethylsilane, after the bonding of C18 chains, which reduces the amount of residual silanol groups. As shown in Figure 5, the endcapping treatment leads to a significant increase in the value of the all-*trans* β-carotene/zeaxanthin separation factors, meaning a strong decrease of polar surface activity because of the decrease of free residual silanols. Besides, one can see that the shape recognition, which is related to the bonding density of C18 chains, does not change with the endcapping treatment, whereas hydrophobicity of the phases is also almost constant, before and after endcapping treatment.

The effect of the change in the pore size (120 Å, 200 Å, and 300 Å) can also be observed with three phases bonded with an identical approach from the same manufacturer (n°144, n°145, and n°146). The increase in pore size from 120 Å to 300 Å causes decreased carbon content, from 17% to 12% and 7%. Figure 6 shows that the polar surface activity is very close for the three phases, indicating a similar proportion of residual silanol groups. Note that these phases are fully endcapped. In addition, shape recognition does not change from 120 Å to 200 Å, whereas it is strongly modified for the 300-Å phase. One can suppose that the curvature of the pore, related to the pore radius, can modify the three-dimensional (3D) organization of C18 bonded phases, inducing changes in the interaction surface of linear or bent compounds with the C18 chains. In relation to this observation, one can remark on the different location of groups 5

and 6. All phases included in these two groups are designed for polycyclic aromatic hydrocarbon (PAH) separation. They are called *polymeric*, meaning that after the first C18 chain is bonded onto the silica (and in the case of the use of di- or trichorosilane as bonding reactant), in the presence of traces of water, another C18 chain can be linked to the first one, finally producing a polymeric bonding (28). This type of polymerization is called *vertical polymerization* (28), as opposed to *horizontal polymerization* (30), which consists of binding the reactive silanes together to "protect" the silica surface. The main difference between these two groups is the pore size, which is around 100 Å for group 5 and 300 Å for group 6. Extensive studies of changes in shape selectivity of monomeric and polymeric phases were done by Sander and Wise with the PAH test (NIST 869a) (29). Besides, as expected, the decrease in the carbon content caused by the pore size increase reduces the phase hydrophobicity because of the lower specific surface area for larger pore phase.

Finally, Figure 7 shows the location of numerous stationary phases known to have a high carbon content. These phases are called HL for high loading, HD for high density, RS for recognition of stereoselectivity, or HS for high stability. All of these phases are located in a close area, with a low polar surface activity (the separation factor of all-*trans* β-carotene/zeaxanthin is high), and a rather similar shape recognition (most of the values are higher than 1.1). This shows that all these monomeric phases display a high bonding density, which reduces the amount of residual silanol groups, and provides a rather organized stationary phase with C18 chains very close to each other.

Conclusion

To summarize, the carotenoid test provides chromatographers with a simple classification map based on three main properties of the stationary phases designed for reversed-phase liquid chromatography. This classification can be done despite the use of a supercritical fluid as the mobile phase, which is supposed to enhance some behaviors because of the lack of water in the mobile phase.

Several studies using classical and known stationary phases showed the relevance of the measurements performed in relation to some chromatographic behaviors and stationary phase properties. Comparison with other tests also confirmed this point.

By using two separation factors on the two axes, the polar surface activity, which is related to the retention behavior of compounds having different polar groups, and the shape recognition, which is involved in separations of compounds having different spatial structure (for instance diastereoisomers), one can easily compare the stationary phases. This comparison can be done with no limit in the number of columns compared, and without the need for a reference column. Moreover, the location of the point and its size, which describes

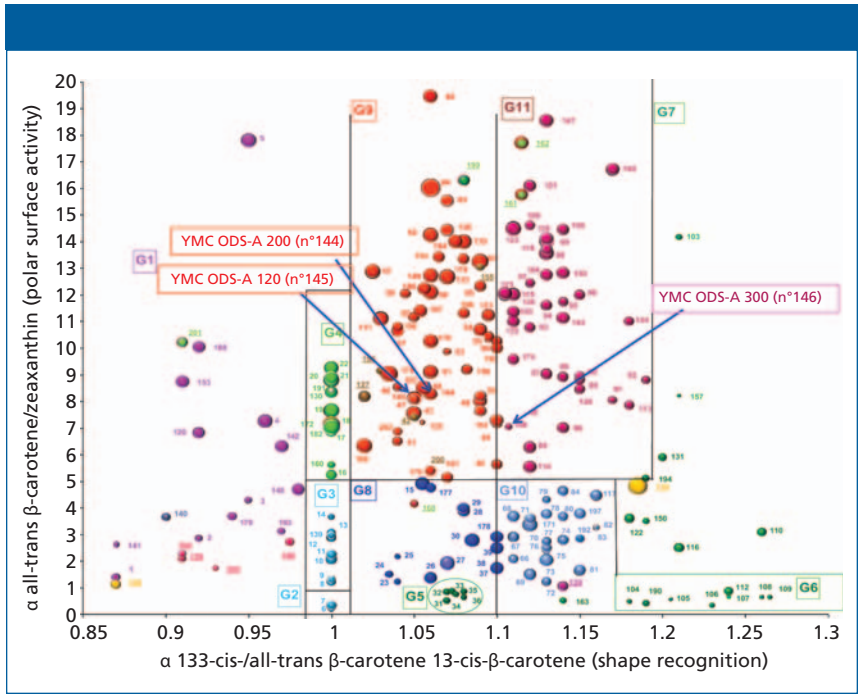

Figure 6: Classification map and effect of the pore size

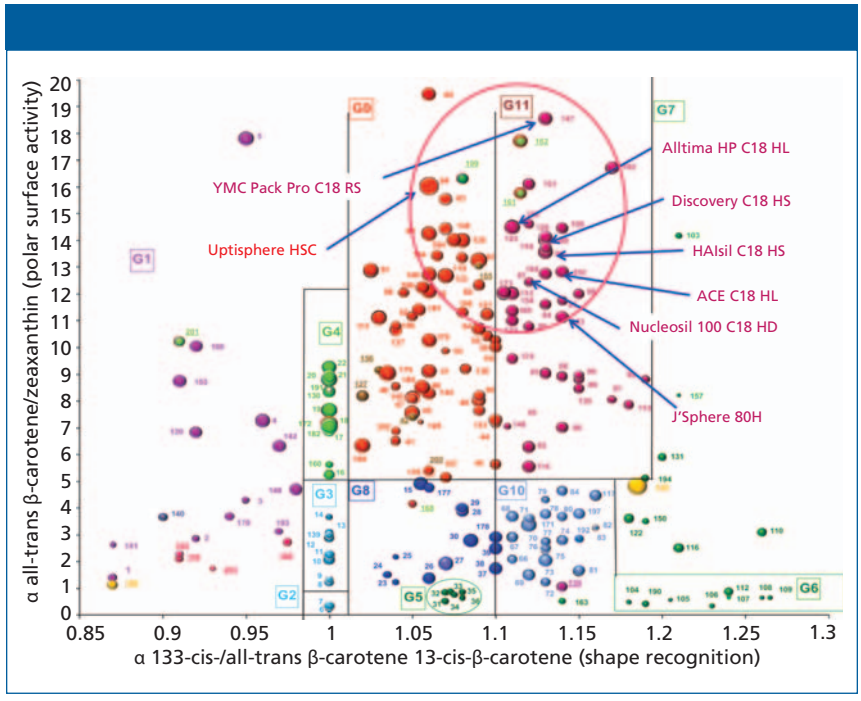

Figure 7: Classification map for high loading (HL), high stability (HS), high density (HD), and recognition of selectivity (RS) stationary phases.

the phase hydrophobicity, can easily be related to the phase properties. This approach could be an advantage to explain the choice of a phase in a quality-by-design approach.

In the second part of this series, we will describe the specificity of the 11 groups of stationary phases, and the following other uses for this map:

- the study of the change in phase properties when manufacturers provide phase evolution,
- the location of numerous phases made with superficially porous particles, or with hybrid silica (organic–inorganic), or with other ligands (phehyl-hexyl, cholesteryl), and
- the effect of the particle size on the studied phase properties, and the use of the map for phases devoted to ultrahigh-pressure liquid chromatography (UHPLC), which have sub-2-µm fully porous particles.

Finally, a presentation of a new web tool will be included. An interactive map is now available on-line as an open source, including the names of the columns that correspond to the numbers on the map (31). This tool allows users to have a look at each phase location, by using the column name, the column group, the column manufacturer, the ligand, or the particle type. Last, but not least, it allows users to compare two or more columns, or the portfolio of two manufacturers.

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LC TROUBLESHOOTING

Readers' Questions: Gradient Ghost Peaks

Interfering peaks or high baseline background can compromise the results of gradient liquid chromatography (LC) separations.

John W. Dolan
LC Troubleshooting Editor

I regularly receive questions via e-mail from readers of "LC Troubleshooting" (to send me a question, contact me at the e-mail shown below with my biographical summary). I try to answer these questions at the time I receive them, or if I cannot answer the question I try to send the reader to another source, such as a written reference, a web source, or another expert. From time to time, as I purge my mailbox, I pick several of these questions that are likely to be of general interest to the readership of *LCGC*. For this month's discussion, I've chosen two questions, centered around unwanted peaks in gradient methods.

Those Elusive Ghost Peaks in Gradients

Two readers had problems which require similar troubleshooting strategies. The first (R.H.) was running a reversed-phase gradient liquid chromatography (LC) method. He observed a peak that was eluted at a retention time that interfered with the active ingredient of a drug formulation being investigated. The peak was present in injections of blank sample diluent when no reference standard or sample was present. He also observed that the peak intensity varied between batches of mobile phase or when different grades of reagent were used. The second question (from S.K.) also involved a gradient LC method and was run at a detector wavelength of 210 nm. The mobile phase contained sodium lauryl sulfate (SLS), for which a solution was prepared, then filtered through a 0.2-µm porosity nylon-66 membrane filter before use. The SLS was "extra pure," with a stated purity of >99.0%. When a blank gradient was run, the baseline was not sufficiently stable at the retention time of interest to allow analysis. It was not clear in the e-mail interaction if the blank gradient was with or without injection of sample. Unfortunately, as is often the case for e-mail communications, after a few interactions with each reader, I never heard if my advice helped them identify the definitive problem source so that the problem could be eliminated. (Note to readers: Please "complete the loop" with me—after I've invested my time in such troubleshooting activities, I would really like to know the final outcome of a problem I've helped with.)

Divide and Conquer

As with most troubleshooting activities, I find that the "divide-and-conquer" strategy is a very useful approach to isolating the problem source for situations like these. This strategy is quite simple—just do a mental or physical experiment designed to eliminate as many potential problem sources as possible, thus reducing the number of possibilities that need more attention. In the present examples, I notice a few common threads. First, the problem peak or noisy baseline occurs when no sample or standard is injected. This allows me to eliminate the analyte or sample matrix as the likely problem source. Also, I know that both methods are gradients. In my experience, problems with extra peaks ("ghost peaks") in blank gradients usually are related to contaminants in the mobile phase or injection solvent.

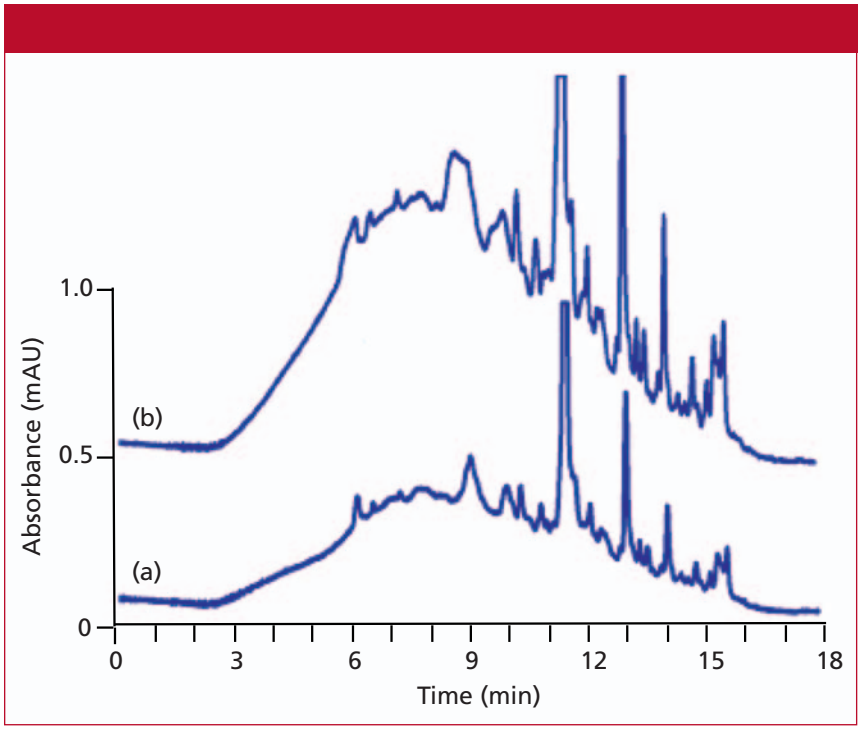

Figure 1: Comparison of gradient background peaks in no-injection blank gradients using the three-gradient test (runs 2 and 3 shown): (a) 10-min equilibration; (b) 30-min equilibration. Column: 150 mm × 4.6 mm C18; A-solvent: 0.1% trifluoroacetic acid in water; B-solvent: 0.1% trifuoroacetic acid in acetonitrile; gradient: equilibration followed by blank gradient of 5–83% B in 13 min plus a 5-min hold at 83% B; flow rate: 1.5 mL/min; column temperature: 35 °C; detection: UV absorbance at 255 nm. Adapted from Figure 1 in reference 1.

A few more mental experiments will help me save time and perhaps refine my approach. The first reader (R.H.) made blank injections in sample diluent. This means that the diluent could be the source of the problems, so it might be fruitful to run a no-injection, blank gradient just to verify that the problem still exists. If the problem disappeared, the diluent or injection process would be the source of the problem—it would be silly to go to the work of the gradient tests discussed below if the problem could be isolated so easily to another source.

In the second case, S.K. was using SLS. I'm not sure why it was added, but SLS can act as an ion-pairing reagent. Ion pairing and gradient elution generally are not a good combination because the slow equilibration of the ion-pairing reagent and the column means that the system is never fully equilibrated. I also note that the SLS is not 100% pure, so impurities from the SLS are possible sources of the observed problems. If

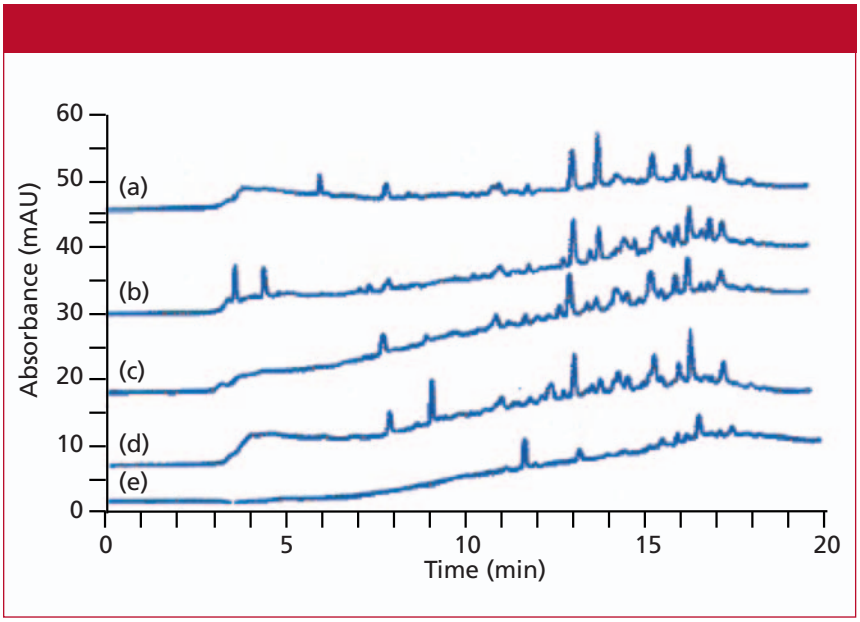

Figure 2: Comparison of gradient background peaks in no-injection gradients for different sources of buffer using the three-gradient test (run three of each sequence shown): (a–d) gradients with four different sources of phosphate reagents; (e) same as a–d, with no buffer, only HPLC-grade water as "buffer." A pH 7.0 10 mM phosphate buffer was prepared by blending 10 mM monobasic potassium phosphate with 10 mM dibasic potassium phosphate. Column: 150 mm × 4.6 mm C18 column; A-solvent: 5:95 (v/v) acetonitrile–buffer; B-solvent: 80:20 (v/v) acetonitrile–buffer: gradient: 30-min equilibration followed by blank gradient of 0–100% B in 15 min plus a 5-min hold at 100% B; flow rate: 1.5 mL/min; column temperature: 30 °C; detection: UV absorbance at 215 nm. Adapted from Figure 2 in reference 2.

it were my problem, I would keep this possibility in mind throughout the troubleshooting process.

Three-Gradient Test

The first step in the isolation of the source of ghost peaks in gradients is to run a series of three blank gradients using what my business partner, Tom Jupille, refers to as the three-gradient test. This test takes advantage of the tendency of reversed-phase gradients to concentrate nonpolar contaminants during the equilibration phase of a gradient and then release them during the actual gradient. An oversimplified description of gradient elution is that sample components stick at the inlet of the column until a strong enough solvent comes along to wash them through the column. This means that the column can concentrate impurities from the initial mobile phase during equilibration; these impurities will be released during the gradient and appear as peaks in the gradient.

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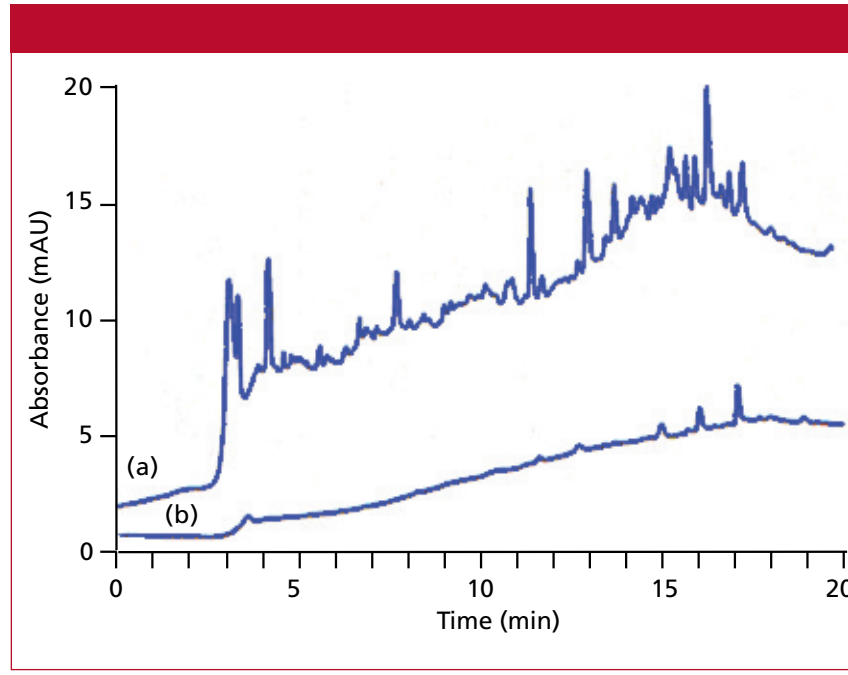

Figure 3: Comparison of chromatograms (general conditions as in Figures 2a–d) of (a) contaminated buffer; (b) buffer prepared in extra-clean glassware and without contacting the pH meter probe. Adapted from Figure 4 in reference 2.

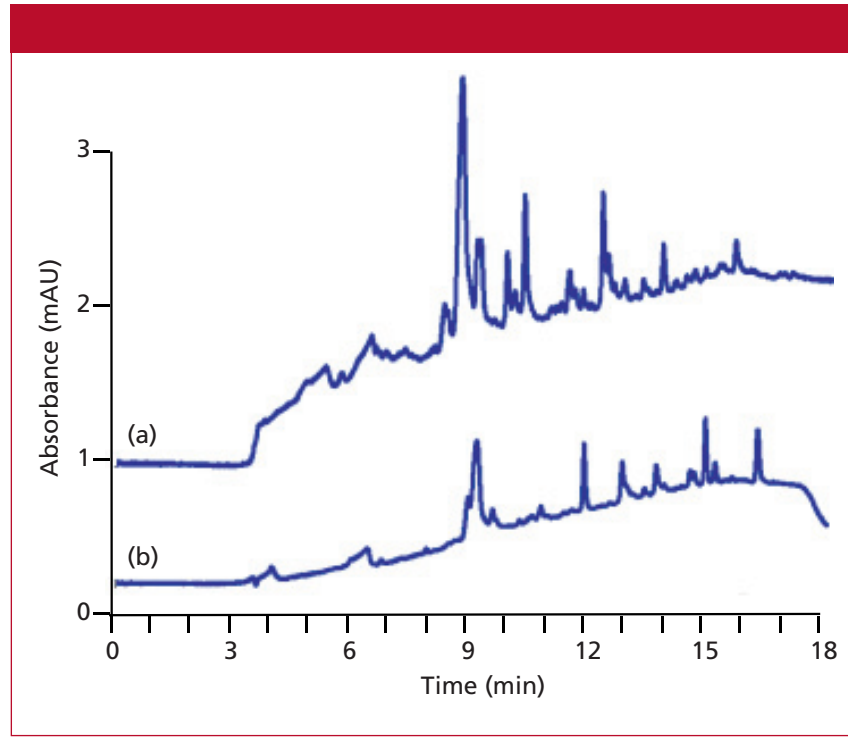

Figure 4: Comparison of gradient background peaks in no-injection gradients for different sources of water using the three-gradient test (run three of each sequence shown): (a) gradient using laboratory-prepared, contaminated HPLC-grade water; (b) steam-iron grade distilled water. A-solvent: water; B-solvent: HPLC-grade acetonitrile; gradient: 30-min equilibration followed by blank gradient of 0–83% B in 13 min plus 5-min hold at 83% B. Other conditions were as in Figure 1. Adapted from Figure 3 in reference 1.

That is, the gradient doesn't "know" if the peaks originate in the sample or in the weak mobile phase.

To perform this test, a series of three gradient programs is run. The first two programs are identical—for example, a 10-min equilibration followed by the normal gradient ramp.

For the third gradient program, increase the equilibration time by threefold—for example, to 30 min—followed by the normal gradient ramp. Run these three programs consecutively without injecting anything (zero-volume injection) and record the baselines. It is very difficult to control the true equilibration of the first run, because it includes the programmed equilibration time plus any time the system was running before starting the program. So discard the first run and compare the second two. If the background or problem peak in the chromatograms increases from the second to third blank run in the same proportion as the increase in equilibration, the source of the problem is the A-solvent.

An example of the three-gradient test is shown in Figure 1 (only the second two chromatograms are shown). The standard method comprises a 10 min equilibration at 5% B followed by injection and the start of a gradient of 5–83% B gradient in 13 min followed by a 5-min hold. The A-solvent is 0.1% trifluoroacetic acid; 0.1% trifluoroacetic acid in acetonitrile is the B-solvent. So the first two runs were programmed as a 10-min equilibration followed by a 13-min gradient and a 5-min hold. The third run was identical except that the equilibration between the second and third run was increased by threefold to 30 min. As you can see, the background peaks increase approximately threefold between the 10-min (Figure 1a) and 30-min (Figure 1b) runs. This increase tells us that the problem peaks are associated with the A-solvent.

Because the source of the ghost peaks as described by the two readers is almost always in the mobile-phase reagents, I've assumed that this is true in the present cases. The next step, then, is to further apply the divide-and-conquer strategy to further isolate the problem source. In the first case, R.H. indicated that the problem peak changed intensity with different batches of mobile phase and different sources of reagents. In the second case, S.K. indicated that he suspected the nylon mobile-phase filter, but also indicated that the mobile phase contained SLS with a purity of >99.0%. Note, however, that >99.0% impurity implies that the impurities can amount to up to 1%—plenty to cause chromatographic problems. Next, we have to systematically eliminate one potential source of the problem at the time. One approach would be to compare mobile phase prepared from different buffer sources. Or compare a SLS source of higher purity with the >99.0% one. Or we could eliminate a reagent or a process step, such as skipping filtration of the mobile phase through the nylon filter to eliminate the filter (and associated glassware). Be sure to follow the "Rule of One," which guides us to change just one thing at a time so that it is easy to identify the real problem source.

Often the source of ghost peaks

is one of the reagents, such as the buffer. This could be the result of contamination of a reagent in the laboratory because of a poor laboratory practice, such as dipping into a reagent container instead of pouring from it or using glassware that is not properly cleaned. In other cases, there may be inherent contamination in the buffer, such as the possible 1% contamination of the SLS mentioned above. An example of differences between buffer sources is shown in Figure 2 (1). The top four chromatograms (Figures 2a–d) show identical conditions (for example, run 2 or 3 of the three-gradient test) for four different sources of phosphate buffer. The bottom chromatogram (Figure 2e) is a modified mobile phase in which no buffer is used. Two observations are apparent in the data of Figure 2. First, all the buffers generate peaks that are not present in the buffer-free method. Second, all buffers show some common peaks (for example, at ~13 and ~17 min), although with different intensity, and some buffers contain peaks that are absent in other buffers.

To further isolate the source of the extra peaks in the buffers of Figure 2, we first had to determine what they had in common: all had been filtered, all had the pH adjusted, and all had been degassed by helium sparging. Each of these steps was eliminated or changed (divide-and-conquer) in a one-at-a-time process (rule of one). In this case, the extra peaks were present only when the pH meter probe was dipped in the buffer during pH adjustment. Figure 3a (1) shows the baseline when the pH meter was allowed to contact the bulk buffer. Notice the difference when this run is compared to the same conditions, except that an aliquot of the buffer was poured off to check the pH and then discarded, so the pH probe never contacted the buffer that would be used in the LC system (Figure 3b). This rather involved (and expensive) troubleshooting process caused us to change our laboratory practice to eliminate contact of the pH probe with any buffer to be used as mobile phase.

In my experience, gradient ghost peaks most commonly arise from contaminated reagents, either created (or not eliminated) during manufacturing or inadvertently added in the laboratory, as was the case for the problem shown in Figures 2 and 3. Usually we trust high performance LC grade (HPLC-grade) reagents because of the high standard of purity ascribed to such reagents. It is rare today to have HPLC-grade acetonitrile or methanol be the source of extra peaks, but small peaks occasionally originate from these reagents. However, it is more common that HPLC-grade water can be the problem source, especially if it is generated in the laboratory, as is the usual practice. A well-maintained

HPLC-grade water purification unit rarely fails, but problems can arise if the filter cartridges are installed in the wrong order or if the feed water is not of sufficient quality. Some laboratories use distilled or reverse-osmosis (RO) water or water from other sources as mobile phase water. This may not cause problems for isocratic methods or even gradient methods where detection limits are not of concern, such as content uniformity methods, but HPLC-grade water generally gives superior results for gradients. The simple way to check for potential water problems is to substitute one water source for another and compare them using the three-gradient test. For the problem in Figure 1, in a stepwise fashion we carefully eliminated the potential contributors to extra peaks. These included filtration, degassing, the column, the instrument, the operator, and glassware cleanliness (which was a minor source of ghost peaks, easily corrected by adding an additional solvent rinse before use). Finally, we tried a different water source—distilled water purchased at a local convenience store. In Figure 4, it is easy to see that the steam-iron grade distilled water (Figure 4b) was not perfect, but it was superior to the HPLC-grade water generated in the laboratory (Figure 4a)! This actually was not a surprise, because we knew we had a corroded pipe in our water supply system and suspected that it was the source of the ghost peaks. We did devise a work-around for the problem, but we ended up moving to a new laboratory building before the water problem was completely solved.

One final comment here is that we shouldn't automatically dismiss a particular reagent quality as unsuitable. I remember visiting China a few years ago and being startled that several laboratories we visited used bottled drinking water as their water source. I commented about this to my host from one of the instrument companies. He said this was a common practice and pulled out his laptop and showed me a comparison of blank gradients with several different brands of bottled water compared to one manufacturer's HPLC-grade water—several of the drinking water sources were better than the HPLC-grade water!

Conclusions

The three-gradient test is a powerful tool that can help to identify the source of ghost peaks in gradient LC methods. As LC methods continue to move to lower and lower detection limits, problems with ghost peaks in gradients will only become more common. Be sure to consider the absolute peak response of the blank gradient in the context of the solvent specifications. For example, a common specification for HPLC-grade acetonitrile is that a water–acetonitrile blank gradient monitored at 254 nm can have no peaks larger than 0.5 mAU, and at no larger than 1 mAU at 205 nm. Note that even through the problem baseline of Figure 4a looks very bad by visual examination, there are only three peaks (at ~9, ~10.5, and ~12.5 min) that exceed the 0.5 mAU at 254 nm specification.

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John W. Dolan

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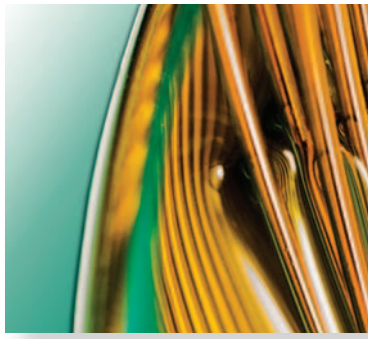

GC CONNECTIONS

Gas Cylinder Safety, Part I: Hazards and Precautions

Many gas chromatographers are not fully aware of safe practices for handling high-pressure gas cylinders. Gas chromatography (GC) operators should be trained to properly transport, install, connect, and maintain their gas supplies, as well to deal with emergencies. In the first of a two-part series, this month's "GC Connections" examines the principal hazards and safety issues surrounding the compressed gas cylinder. Next month's installment will present safe procedures for routine cylinder use.

John V. Hinshaw
GC Connections Editor

The following is a concept script for a gas safety video. Readers are encouraged to find as many safety violations or bad practices as they can. Monday morning, 10:02 am, in a small chromatography lab. While starting up the gas chromatographs and lighting their flame detectors, Sam finds that one of the helium cylinders in the laboratory has gone empty over the weekend. He reaches over the other gas cylinders, applies a large tank wrench, and accompanied by a loud hissing sound, detaches the regulator fitting from the tank. Letting the regulator hang by its plastic connecting tubing, he moves the hydrogen and air cylinders out of the way into the space between the laboratory benches, tilts the empty cylinder on its bottom edge, and rolls it into position near the door.

Sam leaves the laboratory and returns in a moment pushing a furniture dolly. With a grunt, he tilts the cylinder sideways onto the dolly and, pushing it along, saunters down the corridor whistling the "Heigh-ho, Heigh-ho" theme from Disney's 1950's *Snow White*. His coworker Amanda looks at him aghast as she heads into the laboratory.

Ten minutes later Sam returns with a new cylinder on the dolly. He lifts the tank up to a vertical position and the dolly rolls off, banging against the laboratory bench as Amanda jumps out of the way. Without bothering to strap any of the cylinders in place, Sam ducks down slightly and cracks open the new cylinder's stem valve. Amanda is startled by a 110-decibel roar as the escaping gas expresses its new freedom.

Satisfied with the demonstration, Sam rolls the tank into position and reattaches the regulator. Then he starts to secure the other tanks. Amanda calls his name out loudly, "Sam, what do you think you're doing?" As he spins around to deliver a clever reply, his belt buckle catches one of the dangling gas lines. In slow motion, the hydrogen tank starts to head for a horizontal position. Its valve and regulator glance off the bench top on the way down. The cylinder heads for the walls, and in a flash a bright orange-yellow light fills the laboratory . . .

Certainly, no one would take all of the wrong actions that this video dramatizes, but how many of us have done just one of them? I've witnessed them all, and I'm guilty of a few myself from time to time, especially in exceptional situations such as setting up a demonstration in a conference room. I sincerely hope that everyone in the laboratory treats flammable solvents and toxic chemicals with well-deserved respect and understands the short and long-term hazards involved with handling hazardous materials. So, what leads some of us to fall short of giving compressed gas cylinders the respect they deserve? In terms of stored potential kinetic energy, they are bombs waiting to explode; in terms of suffocation potential or flammability, they can be just as much a fire hazard and as potentially toxic as any number of solvents and solids.

Periodically, "GC Connections" reviews gas cylinder safety. It's been a while since the topic was last touched (1,2), so let's take another look at the hazards gas cylinders present and some procedures and practices that can maximize safety for those who must work with them.

Cylinder Hazards

Gas cylinders present several obvious and some less-familiar hazards, including sudden decompression that can propel a cylinder remarkably quickly across the laboratory and displace breathing air; the risk of explosion or reaction; possible acute toxicity; heavy-object hazards; and personal injury from high-pressure gas streams or

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How fast will a cylinder go?

Let's assume that helium is allowed to vent unobstructed through a 1.1-cm (0.5 in.) orifice, such as the cylinder valve stem, over a 10-s interval. That's just my guess at the time frame that seems reasonable. The thrust or force exerted on the cylinder at any moment will be the sum of two terms: the mass flow of the helium times its exit velocity through the orifice, and the pressure differential across the orifice times its area, as delineated in equation 1:

$$F = q \times V_e + (p_e - p_a) \times A_e \quad [1]$$

where q is the rate of helium mass flow, A_e is the orifice cross-sectional area, V_e is the exit velocity through the orifice, and p_e and p_a are the cylinder and ambient pressures respectively.

The helium will need to expand through the orifice—which has a diameter of 0.95 cm^2—into an 8.3-m^3 volume in 10 s, which gives an average exit velocity over the duration of the release of 87 m/s. That's approximately 314 km/h, 200 miles per hour, or 25% of the speed of sound, and these numbers certainly accentuate the hazards of rapid decompression. The exit velocity will be higher at first and then slow as the tank pressure decreases. This reaction mass of the helium will impart an average force of about 12 kg-m/s^2. Acting for 10 s against the mass of the cylinder—we'll ignore the loss of the helium's mass—this average force will impart a velocity change of around 4.8 km/h or 3 mph. That number is not very impressive, but it seems right for a relatively small mass of helium acting against a heavy cylinder.

The rocket effect primarily comes from the second term of equation 1, which involves the high pressure drop from the cylinder to the atmosphere. At the first instant of decompression from a full cylinder at 18.1 MPa, there will be a force of 1710 kg-m/s^2 exerted by the pressurized gas across the orifice. This is so much larger than the first term that we can ignore the helium reaction mass effect, as equation 2 shows below. As the remaining gas pressure drops off the force will decrease as well and reach zero after 10 s, for all practical purposes. Recalling that $F = ma$ (force equals mass times acceleration) and then integrating the decreasing acceleration across time, equation 2 describes the situation for an exponential decay in pressure:

$$v = (p_e - p_a)A_e \div m \cdot \int_0^{\infty} e^{-kt} dt = (p_e - p_a)A_e \div k \cdot m \quad [2]$$

where v is the cylinder velocity after the gas has escaped, k is the pressure decay constant, and t is the time interval. A pressure decay rate of 50% per second, where $k = 1 - 1/e = 0.632$, gets the pressure down to less than 0.2% after 10 s. With a 91-kg cylinder mass, the terminal velocity is approximately 30 m/s, 108 km/h, or 66 mph. Even if the pressure drop decreased more rapidly and approached zero after 5 s, the velocity would still be as high as 19 m/s, 68 km/h, or 42 mph.

cryogenic liquids. For reference, the Occupational Safety and Health Administration (OSHA) regulations 29CFR, Parts 1910.101–105 (3) provide specific guidelines for the use of compressed gases in the workplace that should be followed strictly. An excellent practical gas-safety document can be found on-line as well (4). These procedures and guidelines are discussed in more detail in the second part of this two-part series.

Table I lists hazard classes for commonly used gas chromatography (GC) gases. Gas chromatographers normally do not use some of the common hazardous gases in pure form such as acetylene, oxygen, nitrous oxide, or propane. These gases may be present in laboratories where other instrumentation is used, such as atomic absorption (AA) or atomic emission (AE) spectrometers. Everyone in the laboratory should be aware of the extra dangers posed by chemically reactive, fuel, or oxidizer gases.

Decompression

The first thought that comes to mind when discussing gas cylinders is their rocket potential. A 1-A size cylinder of helium contains 8.3 m^3 (293 ft^3) of room-pressure gas that's compressed into a space of less than 0.5 m^3 (2.0 ft^3) at a nominal fill pressure of 18.1 MPa (2640 psi). European "L" size cylinders contain slightly more compressed gas. These cylinders weigh approximately 91 kg (200 lb) when empty, and the weight of helium contained in a fully pressurized cylinder is around 1.4 kg (3 lb). When the gas pressure is released rapidly through an opening the size of the valve stem, the cylinder—if it accelerates in a straight line—can reach velocities of close to 30 m/s, 108 km/h, or 66 mph. A 91-kg metal cylinder hurtling at high velocity can do tremendous damage almost instantaneously, and there is nothing that a person can do to stop it once a decompression incident starts. See the sidebar "How Fast Will a Cylinder Go" for the calculations that produced this velocity figure.

The thought of a heavy cylinder careening through the laboratory walls gets the attention of most lab workers. This type of accident is easy to avoid by always restraining cylinders with appropriate chains or brackets, transporting them in cylinder carts, and keeping them capped at all times unless actually in use with a regulator or manifold attached. Any cylinder that is found to be damaged or has a stuck valve should be returned immediately to the supplier. If the damage is to the cylinder body the supplier should be notified to come and remove it. Never try to vent a damaged cylinder.

Asphyxiation

Even though the cylinder is restrained, another problem can occur when the contents of any large gas cylinder—other than an air cylinder—are vented rapidly. The sudden release of over 8 m^3 of unbreathable gas in the laboratory may reduce the level of oxygen in the air drastically and present a real suffocation hazard. Liquefied gases expand by as much as 1000-fold when vaporized and can present a much greater hazard. Liquid nitrogen Dewars contain enough nitrogen gas to make a room incapable of sustaining life if the gas is released rapidly. Carbon dioxide can cause immediate unconsciousness followed by death when breathed in any significant concentration. It is much denser than air and will settle in low unventilated areas. Liquid carbon dioxide tanks, such as used for GC oven cooling, can release especially large quantities of gas during a tank rupture.

If an event such as this happens, leave the area immediately, prevent others from entering the laboratory, and seek the assistance of personnel trained in the use of a

self-contained breathing apparatus. Without the proper breathing equipment, never try to re-enter a hazardous area to assist someone else. Some companies have such equipment on-site, but many rely upon emergency services to enter the affected area. Always make sure the area has been well ventilated before returning. Many unnecessary tragedies have occurred due to misguided rescue attempts.

Explosion and Fire Hazards

If a hydrogen cylinder vents into the laboratory in an uncontrolled manner, even if the leak is through the pressure-release disk on the cylinder or regulator, leave the area immediately, close the doors, pull the fire alarm to evacuate the building, and call emergency services. Don't try to extinguish flame detectors, or shut down anything else in the laboratory—just get out quickly. Hydrogen has a lower explosive limit (LEL) in air of 4%, so a venting cylinder easily can create an explosive concentration in moments. In its favor, hydrogen rapidly diffuses in air so that venting the flows encountered in flame detectors or when used for carrier gas present no significant hazard under normal conditions. However, hydrogen can accumulate in a closed GC oven in the event of a broken column. Most electronic pressure control (EPC) systems incorporate flow-monitoring safety features that will detect this condition and shut down the carrier-gas flow.

The same evacuation procedure is required with other flammable gases like propane and acetylene or reactive gases and oxidizers such as oxygen and nitrous oxide. Breathing air contains about 20% oxygen, but high oxidizer concentrations will accelerate combustion dangerously and can cause serious burn injuries. Remember that clothing, paper, paint, and plastic can all burn rapidly in the presence of high oxidizer concentrations.

If a gas fire starts and the gas leak cannot be stopped safely and positively, don't try to extinguish the flame. Unburned gas may accumulate and explode if an ignition source is present.

Hydrogen particularly presents a special hazard because it burns in air with an invisible flame. Never try to investigate a possible hydrogen fire by approaching the suspected flame area: leave it to the professionals. Although the combustion by-products of hydrogen are nontoxic, the fire may burn other nearby items such as plastics, which can produce toxic combustion by-products.

High-pressure gas cylinders can rupture explosively when heated in a fire. All cylinders include a thermal fuse that is supposed to melt and release the cylinder contents in a semicontrolled manner before the internal pressure exceeds a safe upper limit. However, if the cylinder has been mechanically stressed by falling over or from the impact of another cylinder, it can burst before the pressure release valve can act. A chain-reaction effect sometimes occurs in large fires in areas where many cylinders are stored.

Toxicity

GC gases generally aren't toxic. That is, after a victim has been removed from an accident area and has received first aid, the immediate effects of inert gas exposure, such as dizziness and difficulty breathing, will rapidly diminish. Chemically active sample or reaction gases, on the other hand, can present a real toxic health hazard and a significant disposal problem. If even a small

Table I: Hazard classes for commonly used GC gases and other gases that may be found in the laboratory

	Decompression	Flammability	Asphyxiation	Toxicity	Cryo-Hazard
Nitrogen	√		√		√ (liquefied)
Helium	√		√		√ (liquefied)
Argon	√		√		√ (liquefied)
Air	√				
Hydrogen	√	√	√		√ (liquefied)
Carbon dioxide	√		√	√	√ (liquefied)
Acetylene	√	√	√		
Propane	√	√	√		
Oxygen	√	√ (accelerates combustion)	√		√ (liquefied)
Chemical reagents (reactive compressed gases)	√	√	√	√	

leak of a toxic gas such as carbon monoxide or ammonia is detected, leave the area and call in trained personnel to remove the leaking cylinder to a safe place.

Each type of gas or gas blend has an associated material safety data sheet (MSDS) that must be sent in advance to the purchaser who must then keep the information on file for access by any employee or emergency response personnel. MSDSs contain extensive information about the use, storage, and disposal of chemicals—including compressed gases—their toxicity, and any other relevant information. Refer to the appropriate MSDS when you have any questions about a particular material.

Many years ago I saw lecture bottles of methyl bromide, hydrogen fluoride, carbon monoxide, and various highly reactive silanes—not all in the same laboratory, fortunately—carelessly stored on shelves above floor level with unprotected valves. No analytical or chemical laboratory can justify operation with such hazards present. Improperly stored or deployed toxic gas cylinders have no place in anyone's workplace. If any are found, it's good procedure to evacuate the area and call in a hazardous materials team to remove the danger. In any case, never try to move or dispose of hazardous or unknown chemicals in gaseous, liquid, or solid form yourself—it's not worth the risk.

Heavy Lifting

No one should try to lift a cylinder that weighs more than about 12 kg (26 lb). Heavy cylinders belong on the floor, restrained to a bench or a wall. Always use a cylinder cart to move cylinders around, even from one part of the laboratory to another. The practice of rolling a cylinder on its bottom edge, while prevalent, risks injury to feet—and the risk of the cylinder becoming unbalanced and falling over. Never place a cylinder on its side and roll it: the sidewalls are the thinnest parts and aren't designed to take any weight. You could be creating a dangerously weak cylinder that may explode the next time it's filled with gas.

Liquid carbon dioxide cylinders, used for cryogenically cooling GC ovens, weigh much more when full because of liquid carbon dioxide's density, and they can be deceptively heavy. Always pay special attention to these cylinders. In all cases, it's good practice to wear protective eye wear, shoes, gloves, and clothing when manipulating large gas cylinders.

Cryocooling

Cryogenic liquefied gases such as liquid nitrogen or carbon dioxide present additional hazards in the laboratory. Carbon dioxide, a liquid when stored under pressure at room temperature, cools to subzero temperatures when decompressed because of both expansive and evaporative cooling. Liquid nitrogen is stored under low positive pressure in

Omission

In our May review of new gas chromatography products (J.V. Hinshaw, *LCGC North Am.* **34**[5], 330–339 [2016]), the following product was inadvertently omitted:

Product
G908 GC system

Company
908 Devices

Product Description
The G908 GC system from 908 Devices is designed to be an all-in-one, multicolumn, plug-and-go system for in-line, at-line, laboratory, and remote field qualitative and quantitative analysis. The 28-lb system includes a microscale ion trap mass spectrometer and flame ionization and thermal conductivity detectors. Applications include detection of benzene, ethylbenzene, toluene, and xylene (BTEX) in crude oil and seawater; extended analysis of natural gas composition; determination of ethers, alcohols, aldehydes, and ketones in hydrocarbon process streams; and speciation of sulfur compounds in natural, refinery, landfill, sewage digester, and other fuel gases.

special Dewar tanks at around -195 °C. Both cryogenic gases can cause immediate frost burns on exposed skin. Liquid nitrogen also presents a cryogenic freezing hazard that embrittles almost any object it contacts in bulk, including fingers. Connecting tubing that conducts cryogenic liquids also presents a freezing hazard—the tubing should always be insulated or shielded to prevent accidental contact. Again, appropriate protective measures such as thermal gloves, eye wear, and skin-covering clothing help prevent accidents.

High Pressure

The hapless lab rat in the video liked to crack open the high-pressure valve with no regulator attached. I suppose the idea is to blow out any dust particles as well as see if the tank is pressurized, but this behavior never is a good idea. The force exerted by gas decompressing from high pressures is tremendous. If he happened to have part of his hand or arm in front of the cylinder fitting he could suffer a serious abrasion, deep cut, or worse. A much better way to clear the dust out is to spray the area with clean, dry compressed air from a good air source. Never spray a halocarbon-based material onto the cylinder fitting—the gas can get into the lines and cause problems with electron-capture and mass spectrometry detectors.

Conclusion

I've addressed many of the hazards associated with compressed and liquefied gases in this month's "GC Connections." The four most important considerations when dealing with compressed gas cylinders are proper physical restraint, personal protection, knowledge of potential hazards, and appropriate emergency procedures. After a cylinder is in place in the laboratory, the next step is to hook it up and put it in service. In the next installment I'll present some good procedures to follow when installing, using, and replacing gas cylinders and pressure regulators.

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For more information on this topic, please visit www.chromatographyonline.com/column-gc-connections

PERSPECTIVES IN MODERN HPLC

Quality Control Methodologies for Pharmaceutical Counterions

Quantitative determination of the counterions associated with pharmaceutical salts is a mandatory requirement for quality control. Although ion chromatography (IC) is the standard technique in most laboratories, capable of delivering excellent sensitivity, specificity, and flexibility, there are other simpler and quicker analytical methodologies that should be considered for this quality control application.

Ross M. Woods *is a guest coauthor of this installment.*
Michael W. Dong *is a coauthor of this installment and the editor of Perspectives in Modern HPLC.*

Accurate determinations of counterions are mandatory for the release testing and quality control (QC) of all pharmaceutical salts to confirm the identity of the salt form and mass balance of the active pharmaceutical ingredient (API) (1). A literature survey indicated that roughly half of the pharmaceuticals in use are salts with hydrochlorides, while sulfates and hydrobromides are the other common pharmaceutical counterions (2,3). While ion chromatography (IC) is the standard analytical technique used in most pharmaceutical laboratories capable of excellent accuracy, specificity, and sensitivity for both cations and anions (4,5), we have experienced frequent instrumental issues with our open-access IC systems in our QC laboratory when they are operated periodically for release testing. These issues of drifting response and long equilibration times were often associated with the extended instrumental idle time between uses. Since the majority of our API salts being tested were chlorides at high levels of 5–15 wt %, the use of high sensitivity IC instruments was not a requirement. This prompted us to investigate alternative methodologies for chlorides and sulfates for our QC laboratories.

In this study, we evaluated three analytical techniques for anion analysis and compared their performances to our standard reference methodology of IC with suppressed conductivity. The following analytical techniques were examined:

- Ion-exchange chromatography with indirect ultraviolet detection (IEC–UV)
- Mixed-mode chromatography with charged aerosol detection (MMC–CAD)
- Microtitration with potentiometric endpoint detection (MT)

In our investigation, we evaluated the method performance parameters accuracy, precision, specificity, linearity, dynamic range, and sensitivity as described in method validation guidelines proposed by The International Conference on Harmonization (ICH) in ICH Q2 (R1) (6). We also assessed other method characteristics such as ease of use, run time, and sample requirements. Evaluation samples included chloride and sulfate salts of commercial drugs and at least one in-house development drug candidate. Procedures, data summaries, and comparative assessments are described in the next sections.

Experimental Procedures and Evaluation Results

Ion Chromatography with Suppressed Conductivity Detection: The Reference Technique

The IC system used was a Thermo Scientific Dionex Reagent-Free ICS-2000 system controlled by the Thermo Scientific Chromeleon chromatography data system. The columns, IC operating conditions (7), and example chromatograms are shown in Figure 1. IC was the reference analytical technique in this comparative study. Not surprisingly, IC was found to have excellent accuracy (good correlation with theoretical salt equivalents), precision (<1% relative standard deviation [RSD]), specificity (very high with conductivity), linearity (coefficient of determination, R^2 > 0.999 in 10–100 µg/mL) and sensitivity (limit of quantitation [LOQ] < 100 ng/mL). Summary data are shown in Tables I and II. As previously mentioned, the major shortcomings of IC as experienced in our QC laboratory were response drift and long system equilibration time.

IEC–UV

A simple technique for the determination of many common anions using IEC or

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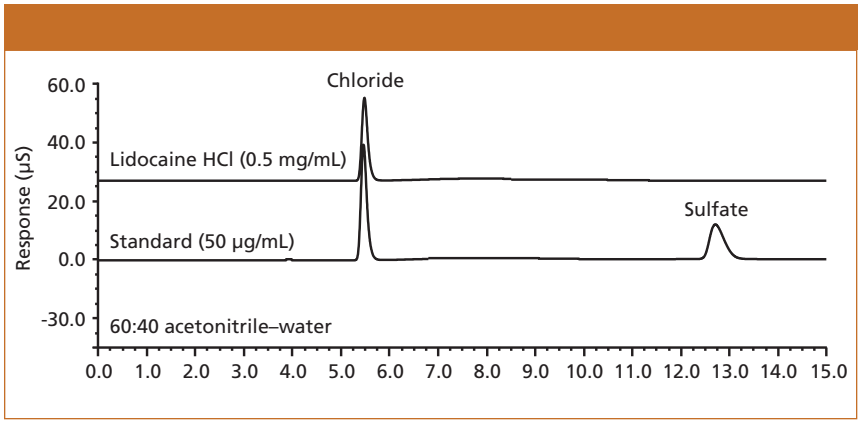

Figure 1: Example IC chromatograms of a blank, a standard, and a drug sample using operating conditions of a previously validated regulatory IC method (7). Column: 250 mm × 4.6 mm, 10-µm d_p Dionex AS18 SAX; mobile phase: 20 mM KOH; flow rate: 1.0 mL/min; temperature: 30 °C; detection: suppressed conductivity; sample: 10 µL of a chloride and sulfate standard at 50 µg/mL and a drug sample at 0.5 mg/mL; system: Dionex ICS-2000.

reversed-phase ion-pairing chromatography with indirect photometric detection has been described previously (8,9). This method is amenable to many common pharmaceutical anions (for example, chloride, bromide, and sulfate), and can be performed on any high performance liquid chromatography (HPLC) system with a UV detector, which is particularly attractive for laboratories without IC instrumentation. Indirect photometry detection is based on the change in absorbance that occurs when an eluent with high absorbance is used to elute anions with low absorbance, creating negative absorbance peaks in the chromatograms when equivalent amounts of the chromophoric components are displaced from the column.

In our study, we used a column and operating conditions described in a Hamilton application note (9) and produced the chromatograms shown in Figure 2. This IEC–UV method yielded reasonably accurate results for chloride ions (Table I); however, sensitivity for later eluted ions such as sulfate was found to be much lower because of broader peaks. We also observed long column equilibration times and substantial baseline disturbances for large-volume injections of sample diluents with a different composition than the mobile phase. This is a key concern because low-solubility drugs require a high concentration of organic solvents in the sample diluent.

MMC–CAD

A promising technique capable for simultaneous determinations of most common pharmaceutical cations and anions using a mixed-mode column with CAD was described in a recent publication (10) and was evaluated in our laboratory. This technique was successfully implemented in a validated method for the identification and quantitation of pharmaceutical counterions

Table I: Comparative summary data of the percent equivalents of theoretical salts for drug samples

Drug	IC	IEC–UV	MMC–CAD	MT
Lidocaine HCl	101	100	100	100
Amitriptyline HCl	100	100	100	100
GNE A HCl	98	103	100	97
Albuterol SO_4	100	99	98	—
Atropine SO_4	100	99	96	—

Table II: Summary of comparative method performance of the four techniques

	IC	IEC–UV	MMC–CAD	MT
Accuracy, % salt equiv. (lidocaine HCl)	101	100	100	100
Precision (% RSD, lidocaine HCl)	0.4%	0.4%	0.6%	0.4%
Linearity (R^2)	0.999	0.999	Nonlinear	1.00
Specificity	Very high	Low	Medium	Low
Sensitivity (LOQ Cl^-)	<100 ng/mL	5 µg/mL	~2 µg/mL	~2 mg
Ease of use under QC environment	Medium	Medium	Easy	Very easy

by a European Pharmacopeial laboratory (11). Example chromatograms of MMC–CAD from our own laboratory obtained using an Agilent 1200 HPLC system and a Thermo Scientific Dionex Corona charged aerosol detector are shown in Figure 3. We were able to implement the assay quickly and obtained results with reasonable method performance for both chloride and sulfate counterions. We did, however, observe a nonlinear calibration curve in our standard linearity range of 10–100 µg/mL, despite a linear response that was reported in a previous study at a lower concentration range of 1–24 µg/mL (11).

MT

Wet chemical techniques such as titration using visual or potentiometric endpoint detection have been used reliably for many years (12). Since approximately 50% of pharmaceutical salts are chlorides at high levels, we believed that titrimetry with an automated microtitrator would be a feasible QC technique. In this study, we evaluated a Metrohm Titrando 857 microtitration system equipped with a Silver Titrode electrode and a 2-mL burette. The automated equipment was controlled with Tiamo software and was used with a purchased precalibrated titrant of 0.1006 ± 0.0008 M silver nitrate. The initial evaluation was conducted for chloride salts at ~30 mg sample size dissolved in ~50 mL of water in a small beaker with a magnetic stirrer. The initial experiments yielded results in close agreement with those from IC as shown in Table I.

Next, we evaluated the performance with lower sample weights. Results at 10- and 5-mg sample sizes showed mean recoveries of 101.6% and 100.4%, respectively, with precision values of <2% RSD. Further lowering the sample amount to 2 mg showed a result of 106.1% recovery and a precision of >10% RSD. We also investigated the use of sample diluent as a 1:1 methanol–water mixture with a sample size of 10 mg and obtained excellent recoveries of 99% and 100% for sodium chloride and amitriptyline, respectively, with a precision around 2% RSD. The method was also evaluated for bromide with good recovery results.

We found excellent accuracy performance with acceptable precision and sensitivity for the microtitration methodology. However, MT is only amenable to chlorides and bromides, which constitute the majority of the pharmaceutical anion samples encountered in our laboratory.

Comparative Method Evaluation Data Summary

Table I shows the summary data of the percent equivalents of the theoretical salts of five drug samples yielding good correlation of chloride data of the three techniques with the reference IC methodology. The data for sulfates were less favorable because of the broader peak shapes of sulfate from IEC–UV and MMC–CAD methods. MT would not work for sulfates under the operating conditions for chloride.

Table II shows a data summary of comparative method performance of the four techniques evaluated. Using lidocaine HCl as a test sample, all four techniques yielded very comparable accuracy and precision data. Linearity in the range of 10–100 µg/mL was found to be excellent for IC and IEC–UV, and for MT in the range of 2–30 mg. Specificity was very high for IC, for which a variety of columns and mobile phase conditions for pharmaceutical anions are available. MMC–CD offers a reasonably good peak capacity under gradient conditions. Under isocratic conditions, IEC–UV was found to be significantly less specific with limited peak capacity. MT is only amendable to chlorides and bromides.

Sensitivity (LOQs) for chlorides was <100 ng/mL, 5 µg/mL, and 2 µg/mL for IC, IEC–UV, and MMC–CAD, respectively. The LOQ of MT for chloride was estimated to be ~2 mg. The sensitivity for sulfate was considerably lower with IEC–UV and MMC–CAD. We ranked MT highly for ease of use. MT is a non-chromatographic technique that does not require system suitability verification for QC testing. The use of relatively simple instrumentation and precalibrated reagents in MT makes it an attractive QC technique for release testing.

Other Alternate Analytical Techniques

Our preliminary investigation and literature search yielded several additional analytical techniques for pharmaceutical anions. One obvious choice was to use IEC with nonsuppressed conductivity detection, yielding adequate sensitivity with good specificity at parts-per-million (ppm) levels for both anions and cations (14). Unfortunately, we did not have such a conductivity detector in our laboratory and most HPLC manufacturers do not offer one. Adding such a detector using an analog or digital convertor to our chromatography data system network was possible, but would impose additional quality assurance documentation.

Mass spectrometry, prolific in most pharmaceutical laboratories, would be a flexible and sensitive platform technology for ion analysis. One pharmaceutical laboratory presented excellent liquid chro-

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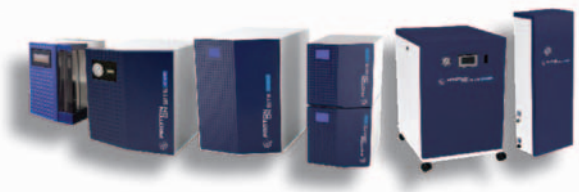

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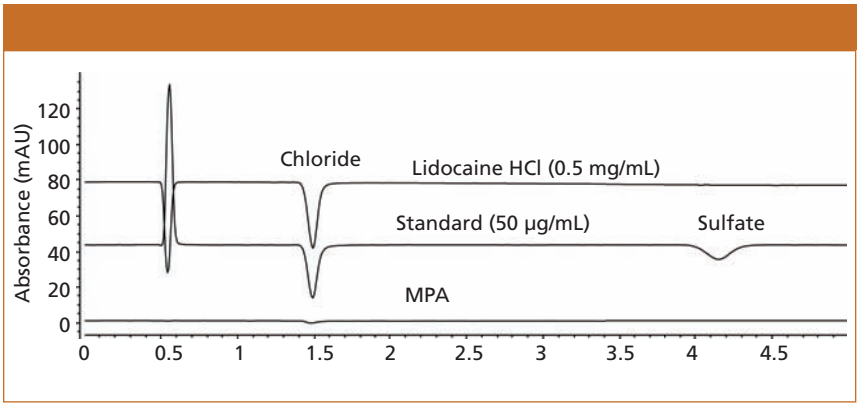

Figure 2: Example HPLC chromatograms of a blank, a standard, and a drug sample obtained using IEC–UV with indirect detection. Column: 150 mm × 4.6 mm, 10-µm d_p Hamilton PRP-X100 SAX; mobile phase: 3 mM sodium phthalate, pH 6.0; flow rate: 2.0 mL/min; temperature: 30 °C; detection: UV at 280 nm (indirect); sample: 10 µL of a chloride and sulfate standards at 50 µg/mL and a drug sample at 0.5 mg/mL; system: Dionex ICS-2000 with suppressed conductivity detection.

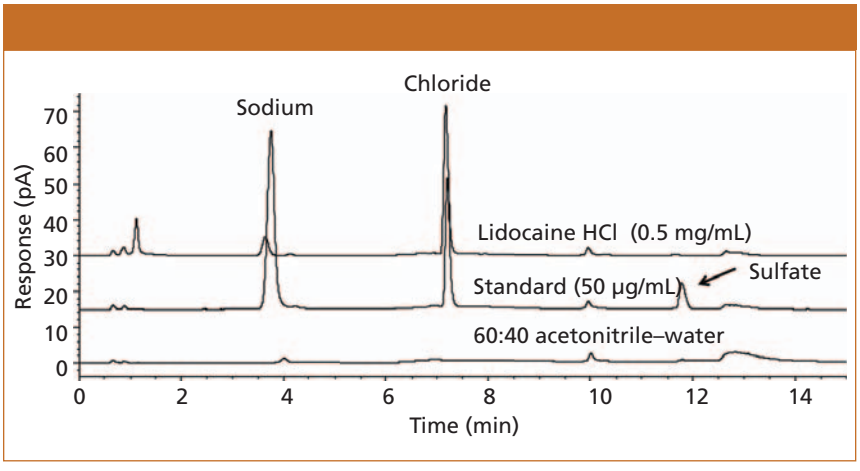

Figure 3: Example HPLC chromatograms of a blank, a standard, and a drug sample obtained using MMC–CAD. Column: 50 mm × 3.0 mm, 2.7-µm d_p Thermo Trinity P1; mobile-phase A: 200 mM ammonium formate, pH 4.0; mobile-phase B: distilled water; mobile-phase C: acetonitrile; flow rate: 2.0 mL/min; temperature: 30 °C; gradient program (time in min, ratio of A–B–C): 0 min, 2–38–60; 3.5 min, 5–35–60; 7 min, 90–5–5; 10 min, 90–5–5; 10.1 min, 2–38–60; 15 min, 2–38–60; detection: CAD; sample: 10 µL of a chloride and sulfate standards at 50 µg/mL and a drug sample at 0.5 mg/mL; system: Agilent 1200 quaternary HPLC system with a Dionex Corona charged aerosol detector.

matography–mass spectrometry (LC–MS) data for both anions and cations, and demonstrated its use in release testing of early drug development candidates (15). The laboratory's data showed comparable method performance to IC with a much shorter run time of 1.5 min. Our own investigation indicated that the feasibility of this LC–MS approach would be highly dependent on the model of single-quadrupole mass spectrometer used since not all brands could be tuned to low-molecular-weight analyses for anions such as chloride at a mass-to-charge ratio of 35.

Another separation technique would be the use of hydrophilic interaction chromatography (HILIC) with CAD, evaporative light scattering detection (ELSD) or refractive index detection (16). However, our preliminary investigation indicated that HILIC was less robust than MMC and thus offered no clear advantages for this technique.

Conclusions

Our evaluation data confirmed the excellent performance of IC, which delivered exceptional method specificity, sensitivity, and accuracy performance. In subsequent meetings with the manufacturer's representatives, several recommendations were made to improve system reliability and reduce equilibration times for our open-access equipment:

- upgrading the current IC system to a new capillary high-performance IC system that can be left running at low flow rates;
- employing a recommended shutdown procedure for both the column and the suppressor when the system is idle for more than one week; and
- implementing a weekly startup protocol of the IC system with blank injections to reduce equilibration time.

While all three alternate methodologies yielded accurate results for chloride samples, IEC–UV using indirect photometry delivered a lower performance in specificity and sensitivity. MMC–CAD is an attractive alternative to IC for the simultaneous analysis of many pharmaceutical anions and cations for both QC testing and research investigations of residual ions in process samples. The nonlinear response of the CAD system can be mitigated by a narrower calibration range in the actual method. MT was our first choice as an alternate technique for chloride and bromide samples. MT has exceptional reliability and excellent accuracy to a sample size of 5 mg in both aqueous or 50% methanol–water diluents.

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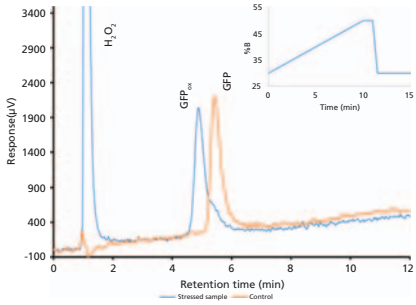
Development of a Stability Indicating Method for Green Fluorescent Protein by HPLC



This article describes the development of a high performance liquid chromatography (HPLC) method for the assay of green fluorescent protein (GFPuv) in-process samples from our model therapeutic protein production process. The specificity of the method is evaluated by demonstrating a suitable HPLC method to separate and detect closely related protein degradation species.

Green fluorescent protein (GFP) is a ubiquitous protein that has been used throughout the scientific community since the 1980s (1,2). The protein is typically used as a reporter protein whereby a recombinant protein expression is tied directly to that of GFP or to identify cellular translocation events. Our laboratory uses the GFPuv variant as a model therapeutic protein; GFPuv contains three amino acid substitutions (Phe-99 to Ser, Met-153 to Thr, and Val-163 to Ala) compared to GFPwt, and the substitutions enhance the levels of expression as well as fluorescence (3). Similar to a therapeutic protein produced in the biopharmaceutical industry, the GFPuv protein is processed throughout various large-scale (for example, 300-L) upstream and downstream operations. During these operations, GFPuv is exposed to various buffers, hold times, temperatures, and concentrations. For example, green fluorescent protein is recombinantly produced in *E. coli*, where the cells are grown in bacterial cell culture medium at 30 °C. After the protein induction is complete, the cells are harvested, washed, centrifuged, and resuspended in 50 mM Tris, pH 8.0. The washed cells are then subjected to high-pressure homogenization, and the homogenate is then centrifuged and the clarified lysate is collected for purification. At this stage, the bulk clarified lysate is held for up to 24 h at 4 °C to allow for further clarification. The solution is then subjected to anion-exchange chromatography, where elution occurs at approximately 250 mM sodium chloride, 50 mM Tris, pH 8.0. The appropriate fraction is then adjusted to 1 M ammonium sulfate for further purification by hydrophobic interaction chromatography. The final purified product is then subjected to an ultrafiltration–diafiltration step to buffer exchange, concentrate, and filter before bulk fill. GFPuv is assayed for concentration and purity at each processing point.

In addition to concentration and purity, we are also interested in protein degradation (that is, truncation, oxidation, and dimerization), which can affect biological function or decrease stability. Of the many types of possible degradation mechanisms, we are primarily focused on oxidation because of the susceptibility of GFPuv to oxidation. Methionine, cysteine, histidine, tryptophan, and tyrosine residues are most susceptible to oxidation by oxygen free radicals (4). Oxidative damage is an important quality attribute for biotherapeutics because it can ultimately lead to aggregation or eventually fragmentation. Oxidation can either directly affect potency or even induce immunogenicity, depending on the affected amino acid position (5). Based on previous mass spectral data obtained

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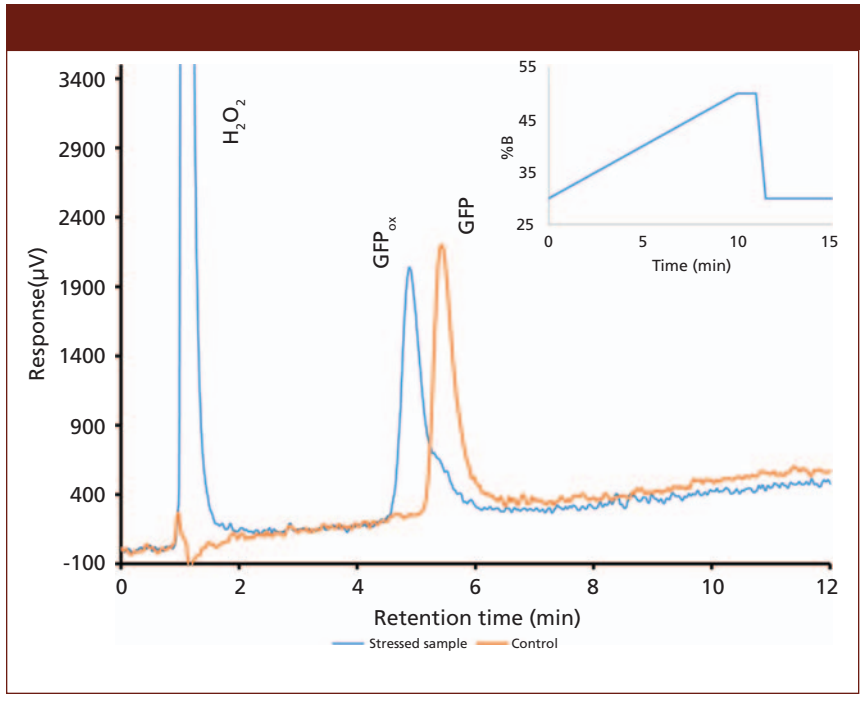

Figure 1: Chromatogram overlay of oxidized and control green fluorescent protein samples using the second optimized gradient using a Luna C18 column.

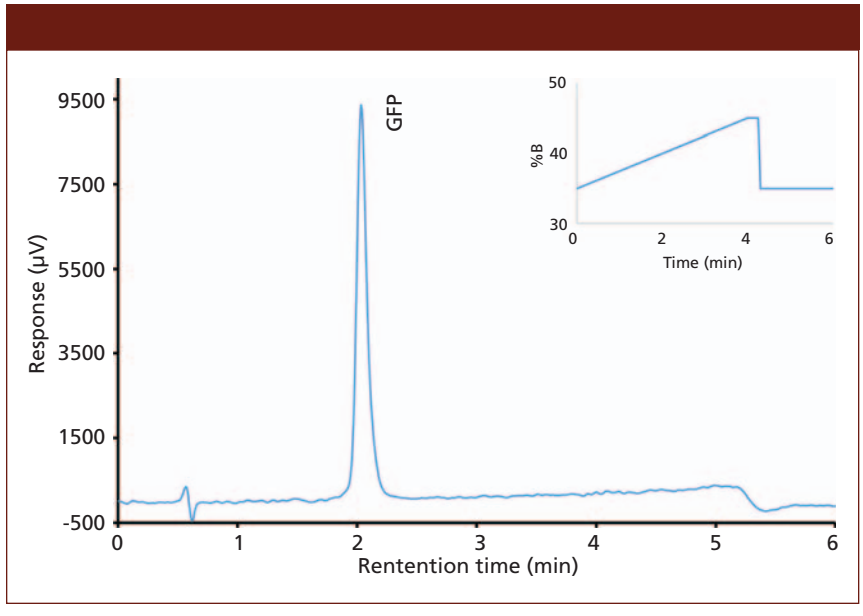

Figure 2: Chromatogram of the green fluorescent protein control sample after separation on a Kinetex C8 column.

in our laboratory, oxidation of methionine residues to methionine sulfoxide is the most common oxidative pathway for GFPuv during processing. Methionine oxidation has been implicated in many negative effects on biopharmaceuticals (6). Since methionine contains sulfur it is relatively easily oxidized, and oxidation can occur during processing or storage because of the varied conditions that the protein is exposed to, especially at elevated pHs. In the case of GFPuv, oxidation tends to increase the propensity of GFPuv to become dimerized or truncated (data not shown).

Green fluorescent protein consists of 238 amino acids, five of which are methionine residues. Previously reported GFP stability studies focused primarily on fluorescence and truncations. For example, the stability of GFP has been studied with respect to chlorine and pH (7), temperature and pH (8), and glucose (9) as well as heat and pH in the presence of sodium chloride (10). All studies published to date consist of monitoring fluorescence as the stability indicator. Although fluorescence is a good functional indicator, it does not necessarily provide information on structural changes. For example, it has been previously shown that GFP can tolerate truncation at the C- or N-terminus to a certain extent (11) as indicated by fluorescence. In these cases, fluorescence remains unchanged as amino acids are clipped off either termini.

To obtain insight on structural stability, we focused our efforts on methionine oxidation by developing a stability indicating method using reversed-phase high performance liquid chromatography (HPLC). Green fluorescent protein contains fairly hydrophobic regions and is well separated by reversed-phase HPLC. As the various methionines become oxidized, the overall protein polarity increases. By taking advantage of this property, we predicted that the various species with different levels of oxidations could be separated by reversed-phase chromatography. To date, separation of oxidized GFPuv species has not been reported in literature. This report examines the method development process to separate intact GFPuv from its oxidized derivatives.

Experimental

Chemicals and Reagents

Acetonitrile (HPLC-grade) and trifluoroacetic acid (Optima-grade) were purchased from Fisher Scientific. Deionized water (18-MΩ) was prepared through a MilliQ water purification system (EMD Millipore). Hydrogen peroxide (30% reagent grade) was purchased from EMD Chemicals and diluted just before the beginning of the experiment. The degradation experiments were initiated upon addition of dilute hydrogen peroxide to protein. GFPuv standard (1 mg/mL and >98% purity based on protein) was produced (based on CloneTech sequence and inserted into the pET17b vector expression system), purified, and characterized in house.

Instrumentation

A Shimadzu Prominence model HPLC system consisting of an autosampler, a

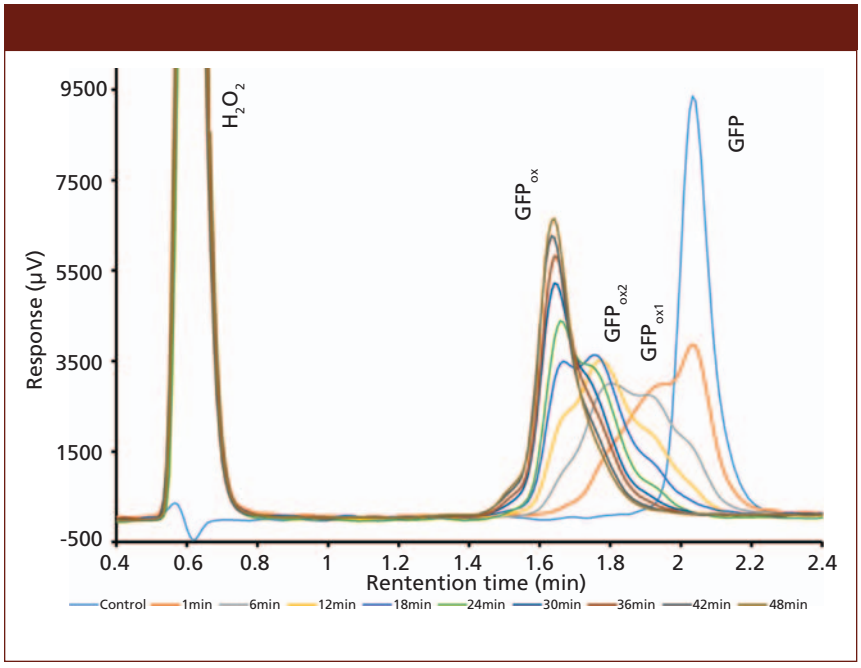

Figure 3: Chromatogram overlay of oxidized and control green fluorescent protein samples over time. Separations occurred on the Kinetex column with the final optimized gradient (expanded view).

degasser, binary pumps, a column oven, and a variable-wavelength ultraviolet (UV) detector was used. The system was interfaced with Shimadzu Labsolutions Version 5.54 for control and data acquisition. The column temperature was set to either 30 °C or 40 °C, depending on the method. The absorbance was monitored at 370 nm and the injection volume was set at 10 µL for all experiments. The mobile phases consisted of 0.1% trifluoroacetic acid in water for mobile-phase A and 0.1% trifluoroacetic acid in acetonitrile for mobile-phase B. The columns evaluated were 50 mm × 4.6 mm, 2.6-µm d_p, 100-Å Kinetex C8 and C18 (Phenomenex) or 125 mm × 4.0 mm 5-µm d_p, 100-Å Luna C18 (Phenomenex). The flow rate for all experiments was set to 1.0 mL/min.

Method Development

Initial development experiments utilized a 125 mm × 4.0 mm analytical column that was packed with 5-µm Luna C18 stationary phase. Preliminary scouting gradient experiments were performed using a 100-µg/mL GFPuv control sample diluted from a 1-mg/mL stock with a mixture of 65% mobile-phase A and 35% mobile-phase B, where the diluent represented the HPLC starting conditions. Chromatographic runs were monitored by UV absorbance at 370 nm. GFPuv has an absorption maximum at 395 nm, but 370 nm was chosen because of the upper limit of the deuterium lamp in the detector.

The primary goal was to separate GFPuv from the fully oxidized species, but during early experiments it was realized that separating GFPuv from partially oxidized species would add value to the analysis. Unpublished data indicate that partial oxidation eventually leads to full product oxidation if the conditions are not corrected in the production process. The stressed GFPuv sample was prepared by diluting stock GFPuv to 100 mg/mL in diluent containing 1% hydrogen peroxide and allowing the GFP to fully oxidize. Previous experiments demonstrated (data not shown) that the fully oxidized species occurred after 30 min of exposure.

Gradient optimization experiments were initially performed using the Luna C18 column to achieve optimal separation between GFPuv and oxidized species. When an ideal gradient could not be identified that would yield adequate separation, the stationary phase was changed in an attempt to improve selectivity. A 50 mm × 4.6 mm analytical column packed with 2.6-µm C18 Kinetex stationary phase was evaluated, but carryover of the protein was observed upon repeated injections. The carryover was alleviated by changing to a C8 Kinetex stationary phase, while keeping the column dimensions and particle size the same. It should be noted that only these columns were evaluated because they were readily available in our laboratory and they have been used for GFP analyses spanning several years. Furthermore, larger pore size stationary phases were not evaluated because green fluorescent protein has a relatively compact size of 4.2 nm × 2.4 nm (12) and effectively diffuses into the 100-Å pore size stationary phase. These 100-Å pore size columns also serve as a generic separation platform in our laboratory allowing us to conduct peptide mapping analysis or monitor other small molecule contaminants in our GFP process.

The gradient composition was optimized to achieve retention of GFPuv as well as separation of the resulting degradants. After adequate separation of GFPuv from the fully oxidized species was obtained, the oxidation degradants were profiled by performing repeated injections of the stressed sample immediately after preparation. The amount of exposure time was accurately accounted for as it was directly proportional to the analysis time of the HPLC method. For example, time points correlated to the span between each injection.

Results and Discussion

To ensure the pH of the mobile-phase system was well below the isoelectric point of GFPuv, both mobile phases were acidified with 0.1% trifluoroacetic acid. The acidic modifier ensured full protonation of the protein, as well as any free silanol groups, which minimized extra-column interactions. The Luna C18 column was initially chosen because of the wide pH stability and this type of C18 column has been successfully used in the separation of a variety of proteins (13,14).

The first scouting run used a shallow gradient, increasing the mobile-phase B concentration from 20% to 75% over a period of 20 min. The method gave a good, symmetrical peak shape for GFP, but the method run time of more than 25 min was longer than desired. The goal of the method was to use it for analysis of in-process GFPuv samples. It is common, during a manufacturing campaign,

Table I: Final HPLC method

Flow	1.0 mL/min	Detection	370 nm
Column temperature	40 °C	Injection volume	10 µL
Mobile-phase A	0.1% trifluoroacetic acid in deionized water		
Mobile-phase B	0.1% trifluoroacetic in acetonitrile		
Gradient	Time (min)	%A	%B
	0.00	65	35
	4.00	55	45
	4.25	55	45
	4.30	65	35
	6.00	65	35
Column	50 mm x 4.6 mm, 2.6-µm d_p, 100-Å Phenomenex Kinetex C8		

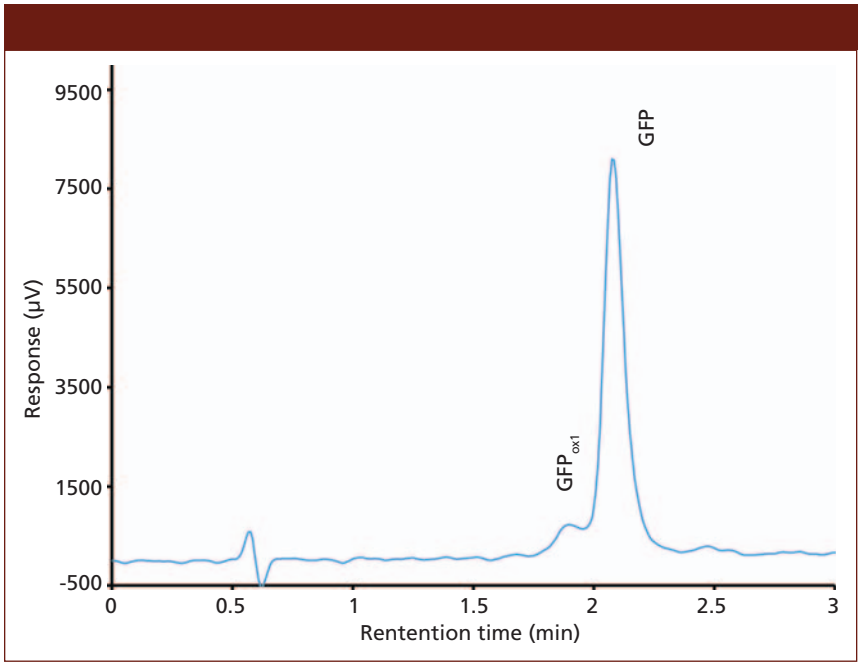

Figure 4: Chromatogram of a mixture of green fluorescent protein control (100 µg/mL) and oxidized green fluorescent protein (5 µg/mL).

for a production group to generate dozens of in-process samples a day. Results for these samples are usually needed as quickly as possible to ensure various quality attributes are achieved along each step of the manufacturing process before proceeding on to the next step.

To increase the elution power of the mobile phase, a second gradient was used where the initial concentration of mobile-phase B started at 40% and increased to 60% over 10 min. However, the initial concentration of mobile-phase B was too strong and resulted in no retention of GFPuv. A third gradient was attempted where mobile-phase B started at 30% to lower the mobile phase strength and increase the retention factor, k, of GFPuv. The steepness of the gradient, approximately 2% per minute to 50% over 10 min, was chosen to avoid band broadening yet increase selectivity between GFPuv and the resulting degradation products. The method with the new gradient provided good retention of GFPuv ($k \approx 4$) and acceptable peak shape with a peak tailing factor of 1.6.

After the basic method was established, a peroxide-stressed sample of GFPuv was analyzed along with a control sample. As GFPuv becomes oxidized, the resulting methionine sulfoxide groups decrease the overall hydrophobicity of the protein, and retention of the molecule decreases. Resulting chromatographic overlays of the stressed sample and control sample demonstrated this behavior. However, a peak resolution of 1.0 between GFPuv and the fully oxidized species (Figure 1) using the Luna C18 column was considered inadequate. This is especially important considering that the degradation products may be present at low concentrations. At this point it was determined that no further changes to the gradient composition or steepness would increase resolution, so a change in selectivity was performed by using a different stationary phase.

Solid core–shell particles provide a unique advantage in HPLC separations. Decreasing particle sizes result in decreased band broadening and increased chromatographic efficiency by minimizing the A and C terms as described by the van Deemter equation (equation 1).

$$H = A + B/\mu + C\mu \quad [1]$$

where H represents the overall separation efficiency, A is Eddy diffusion, B is longitudinal diffusion, C is the mass transfer coefficient, and µ is linear velocity (15).

Because the smaller particle size gives shorter diffusion paths for the analyte, the mass transfer between the mobile phase and stationary phase is more efficient. This extra efficiency is critical when attempting to separate closely eluting species. As chromatographic peaks become narrower, resolution increases. The increased efficiency can also lead to a decrease in analysis time, which is critical when analyzing in-process samples. The decrease in analysis time is inversely proportional to the amount of samples that can be analyzed. Halving the analysis time effectively doubles the number of samples that can be analyzed in a chromatographic run.

To take advantage of the improved separation efficiencies offered by the core-shell technology, a 50 mm × 4.6 mm, 2.6-µm d_p Kinetex C18 column was used. The flow rate remained unchanged, resulting in a 9-min decrease in total run time with very little change to the gradient profile. The column temperature was increased from 30 °C to 40 °C to further optimize the mass transfer of GFPuv and reduce the potential for band broadening (Table I). However, carryover of GFPuv was observed after repeated injections of the protein. The source of carryover was determined to be from the column and not the HPLC system, but further investigation into the cause was not

performed. It was hypothesized that the hydrophobic nature of the protein yielded a higher affinity for the C18 stationary phase. The mobile-phase gradient was not strong enough to elute the entirety of the protein from the stationary phase, resulting in carryover. Lowering the hydrophobicity of the stationary phase by switching to a C8 column with the same dimensions and particle size resulted in increased partitioning of the protein in the mobile phase, ensuring complete elution and alleviating the observed carryover.

Good retention of GFPuv was observed ($k \approx 3.5$) with a significant improvement in the peak tailing factor (1.2) (Figure 2). The total analysis time was therefore shortened to 6 min per injection. Subsequent experiments showed that decreasing the HPLC analysis time further was not realistic, because there was insufficient time to ensure the stationary phase was equilibrated back to initial mobile-phase conditions. An advantage of the reduced analysis time was the ability to monitor the oxidation reaction every 6 min. After adding the peroxide to the sample, it was injected immediately and re-injected every 6 min. An overlay of the repeated injections and the control sample showed the progression to the fully oxidized species, which was fully resolved from the parent peak (Figure 3). Although it was not baseline resolved, it was possible to identify the individual oxidation species as the various methionine groups were being oxidized over time. The intermediate oxidation species of GFPuv was detected down to a concentration of 5.3 µg/mL. While not optimal, the resolution of 0.7 was sufficient to achieve the goal of reaction monitoring (Figure 4). This result also provides an advantage for use of the method to evaluate the degree of oxidation of the GFPuv molecule for long term stability studies. It is important to note that the peaks are not indicative of each individual oxidized methionine residue, but an average of the oxidized species over time. As the protein undergoes oxidation, the change in hydrophobicity is proportional to the amount of oxidation across all methionine groups. Each of the peaks are representative of an average oxidized species at a given exposure time to the stressor. The final peak, the point at which retention stops decreasing and remains constant, is the fully oxidized GFP species.

Conclusion

Development of a stability indicating HPLC method requires adequate separation of the parent analyte peak from the resulting degradation products. This experiment demonstrated one aspect of development required to ensure an HPLC method that is specific to the desired analyte and potential oxidation products. Further development would be to expose the protein to other stressors (acid, base, UV light) to ascertain if the method was specific to degradation products generated from these other sources.

By simply switching to a stationary phase with a smaller particle size, we doubled potential sample throughput by reducing the overall run time by more than 50%. We were able to achieve separation between GFPuv and the fully oxidized species, as well as monitor the oxidation of GFP over time.

The development of a HPLC method should focus not only on accuracy and specificity, but the ultimate goal of the method should also be considered. The responsibility of the development scientist is also to develop methods that are applicable for the intended purposes. As in this experiment, the ultimate goal of the method is for monitoring in-process samples for oxidation. Although an acceptable HPLC method was developed for GFPuv using a conventional C18 stationary phase, the analysis time of 25 min was not optimal when looking ahead to the potential number of samples for analysis. It is critical to take these factors into consideration when developing HPLC methods for in-process samples. For this reason, it was important to proceed with further development to decrease the analysis time as much as possible without impacting the performance of the final method. Ultimately, the method described in this article was suitable for detecting fully oxidized GFPuv degradants as well as to track formation of oxidations as it occurred throughout the production process.

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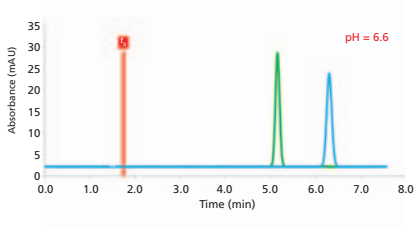
HPLC Teaching Assistant: A New Tool for Learning and Teaching Liquid Chromatography, Part I



The free spreadsheet-based program HPLC Teaching Assistant was developed for effective and innovative learning and teaching of liquid chromatography. This software allows teachers to illustrate the basic principles of high performance liquid chromatography (HPLC) using virtual chromatograms (simulated chromatograms) obtained under various analytical conditions. In the first installment of this series, we demonstrate the possibilities offered by this spreadsheet to illustrate the concept of chromatographic resolution, including the impact of retention, selectivity, and efficiency; understand the plate height (van Deemter) equation and kinetic performance in HPLC; recognize the importance of analyte lipophilicity (log *P*) on retention and selectivity in reversed-phase HPLC mode; and manipulate or adapt reversed-phase HPLC retention, taking into account the acido-basic properties (pK_a) of compounds and the mobile-phase pH.

Davy Guillarme and Jean-Luc Veuthey

High performance liquid chromatography (HPLC) remains one of the most widely used analytical techniques in industry and is taught at universities in analytical chemistry programs. Because HPLC separation is based on the partitioning of a solute between a mobile phase and a stationary phase, the technique is particularly difficult to master. There are a significant number of parameters (for example, physicochemical properties and molecular weight of the solutes; nature, composition, temperature, pH, and flow rate of the mobile phase; and chemical nature and dimensions of the stationary phase) that can influence the quality of the separation (retention time, selectivity, efficiency, pressure drop, peak area).

Several commercial HPLC simulators are available on the market, including Drylab (Molnar-Institute) (1), Chromsword (Iris Tech) (2), LC & GC Simulator (Advanced Chemistry Development) and Osiris (Datalys) (3). These simulators are particularly useful for efficiently developing HPLC methods based on a limited number of initial experiments and can also be used to better understand the principles of HPLC. However, these simulators remain difficult to use and expensive to purchase.

To easily understand the principles of chromatography in a relatively inexpensive way, some free or low-cost computer-based HPLC simulators have also been proposed (4–7). As reported by Boswell and colleagues (8), there are currently six HPLC simulators, but most are not available anymore or are not fully compatible with modern computers. To the best of our knowledge, the most interesting HPLC simulator was released in 2013 and is free (8). The software interface is relatively easy to use and produces a simulated chromatogram that is redrawn when the experimental parameters are changed.

In comparison to this software, the philosophy of our program, entitled "HPLC Teaching Assistant," is different but complementary. First, our program is a spreadsheet that can be easily used on any computer without the need to install a Java environment. Second, our spreadsheet allows making links between compounds' physicochemical properties (partitioning coefficient, pK_a) and chromatographic behavior.

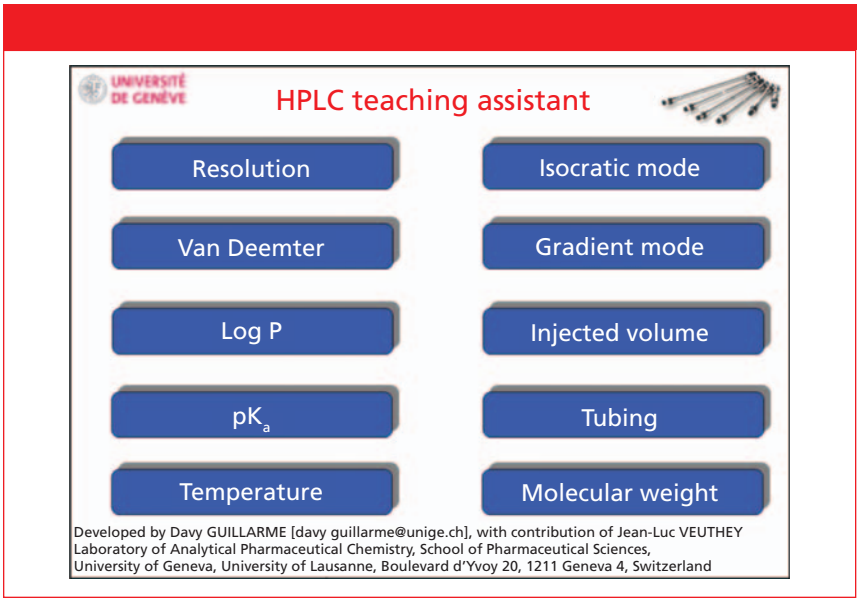


Figure 1: Main menu of HPLC Teaching Assistant.

Last but not least, each spreadsheet of our calculator provides an understanding of a single concept (for example, the effect of the mobile-phase pH on the chromatographic behavior). This series of two articles provides the theoretical background of the spreadsheet and gives some practical examples to illustrate the usefulness of this tool (see Figure 1).

Understanding the Concept of Chromatographic Resolution

Theoretical Background

In liquid chromatography (LC), the separation of two peaks is described by their resolution (R_s), which represents the difference in retention times (t_R) divided by the average peak widths at baseline (W), according to the following equation:

$$R_s = \frac{2\times(t_{R2}-t_{R1})}{W_1+W_2} \quad [1]$$

To better understand the impact of analytical conditions on the overall resolution, the fundamental equation of resolution can be used to interpret the chromatograms obtained during method development. R_s can also be expressed based on the retention factor (k), selectivity (α), and plate number (N):

$$R_s = \frac{\sqrt{N}}{4}\times\frac{k}{k+1}\times\frac{a-1}{\alpha} \quad [2]$$

Using the "Resolution" Spreadsheet

In the first spreadsheet, entitled "resolution," the impact of k, α, and N on R_s can be directly visualized. For this purpose, a chromatogram was simulated to show the chromatographic resolution of two molecules when modifying k, α, and N. For this example, the column had a void time of

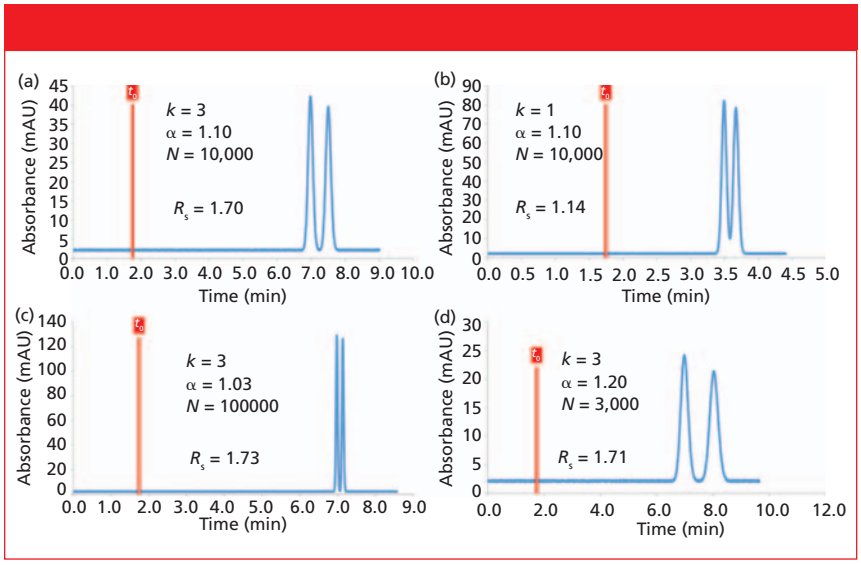


Figure 2: Simulated chromatograms to better understand the impact of retention, selectivity, and efficiency on the overall resolution.

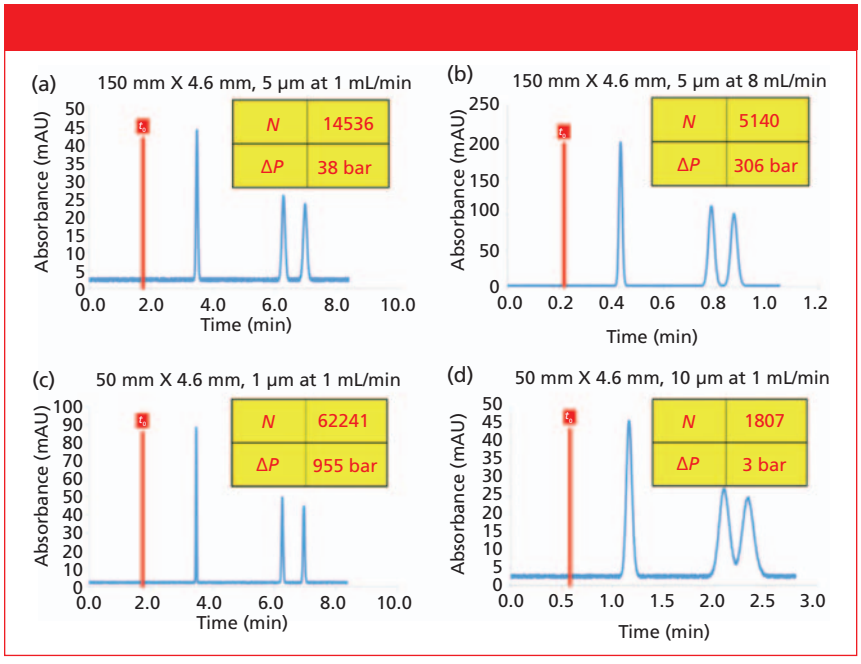


Figure 3: Simulated chromatograms to illustrate the impact of the column dimensions (length and particle size) and mobile-phase flow rate on the efficiency and column back pressure.

1.74 min (corresponding to a 150 mm × 4.6 mm column operated at 1 mL/min).

In addition to the simulated chromatogram, three graphics at the bottom of the spreadsheet illustrate the change in resolution with the three individual variables, namely, k, α, and N. The impact of α on the resolution is strong: For α values between 1 and 4, the resolution drastically increases, and a plateau is only attained for very high (inaccessible) α values (α > 4). The retention factor also has a significant impact on resolution for low k values, but its impact becomes moderate for k between 3 and 10 and low for k higher than 10. For k > 10, the analysis time becomes long, and the improvement in the resolution is minor (this can be easily verified by simulating a chromatogram obtained under conditions of very high k values). Finally, because efficiency is expressed as the square root in the resolution equation, its impact on resolution is moderate to low, and N must be dramatically increased to significantly improve R_s.

From this spreadsheet, four different conditions were simulated to better illustrate the influence of k, α, and N on R_s, as shown in Figure 2. In Figure 2a, the selectivity, retention factor, and efficiency were reasonable, leading to an overall resolution of 1.70. Figure 2b illustrates the conditions of an insufficient retention factor in LC (k = 1), thereby leading to a significant reduction in resolution (R_s = 1.14). Figure 2c shows that a very low selectivity (α = 1.03) can be compensated by a very high plate number (N = 100,000 plates). Finally, Figure 2d proves that selectivity is the primary driver for optimizing chromatographic separation in LC. As shown on this simulated chromatogram, when the selectivity is appropriate (α = 1.20), there is no need for a very high plate count (only 3000 plates in this case).

Based on these observations, it is easy to understand why method development follows three successive steps after selecting the appropriate stationary and mobile-phase conditions:

- Select a column with a sufficient plate number considering the complexity of the sample to be analyzed (usually a column generating 10,000 plates is a good starting point).
- Adjust the solvent strength to have a reasonable retention factor (k between 2 and 10).
- Optimize selectivity by tuning different chromatographic parameters.

Understanding the Kinetic Performance and van Deemter Curves

Theoretical Background

In LC, the column dimensions (length, internal diameter, and particle size), as well as the mobile-phase flow rate and viscosity, have an impact on the plate number (N) and column back pressure (ΔP). In the second spreadsheet, the kinetic performance is shown for a mixture of three compounds with a molecular weight of approximately 100 g/mol. All the calculations were made for a column with a total porosity (ε) of 0.7 and a flow resistance (Φ) of 500. The column temperature was 30 °C, and the mobile phase was a mixture of 30% acetonitrile and 70% aqueous buffer.

To calculate the plate number (N) based on the column dimensions, the following equation was used:

$$N = \frac{L}{H} \quad [3]$$

where L is the column length (in millime-

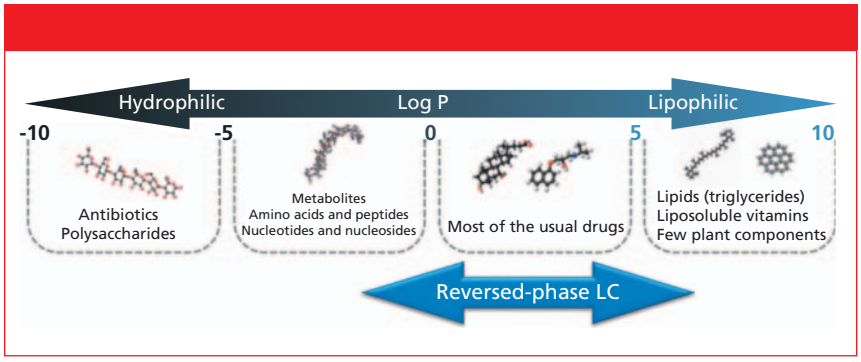


Figure 4: Log *P* values of common substances and applicability of the reversed-phase LC mode.

ters) and *H* is the height equivalent to a theoretical plate (in micrometers). To estimate the *H* value, the van Deemter equation was considered:

$$H = A + \frac{B}{u} + Cu \quad [4]$$

In this equation, *A, B,* and *C* correspond to eddy dispersion, longitudinal diffusion, and mass transfer, respectively. They represent a different set of constants for a particular solute, column, and mobile-phase conditions. The *u* value is the linear velocity, which is related to the mobile-phase flow rate (*F*), column porosity (ε), and column internal diameter (d_c) using the following relationship:

$$u = \frac{4 \times F}{\pi \times d_c^2 \times \varepsilon} \quad [5]$$

To use generic *a, b,* and *c* terms (*a* = 1, *b* = 4, *c* = 0.05 in our case), which are independent on the analytical conditions, the van Deemter equation was transformed into its reduced form:

$$h = a + \frac{b}{v} + cv \quad [6]$$

where *h* is the reduced height equivalent to a theoretical plate and *v* is the reduced linear velocity. The following two equations provide the definitions for these two reduced parameters.

$$h = \frac{H}{d_p} \quad [7]$$

$$v = \frac{u \times d_p}{D_m} \quad [8]$$

Here, d_p represents the column particle diameter (in micrometers) and D_m is the diffusion coefficient of the solute in the mobile phase, which can be estimated using the Wilke-Change equation (9).

Finally, the column pressure drop was calculated using Darcy's law, with η being the mobile-phase viscosity:

$$\Delta P = \frac{\eta \times L \times u \times \Phi}{d_p^2} \quad [9]$$

In the spreadsheet entitled "Efficiency," the impact of the column dimensions (L_{col}, d_{col}, and d_p) and the mobile-phase flow rate (*F*) on *N* and Δ*P* can be directly visualized. A simulated chromatogram with three compounds (*k* = 1.0, 2.6, and 3.0) shows the corresponding chromatogram when modifying the column dimensions and flow rate. In addition, the van Deemter curve, *H* = *f*(u),

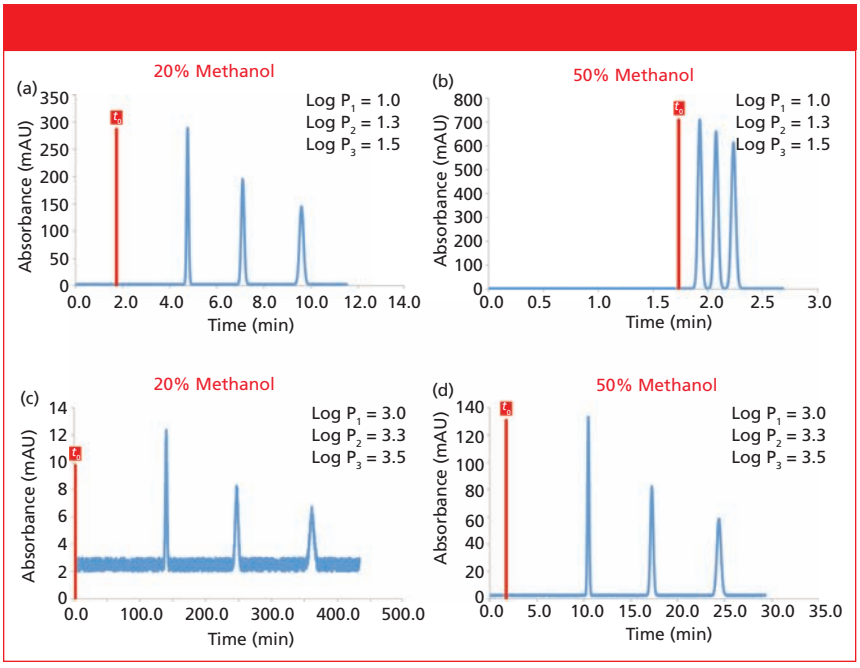


Figure 5: Practical chromatograms to demonstrate the impact of compound lipophilicity on retention in reversed-phase LC mode.

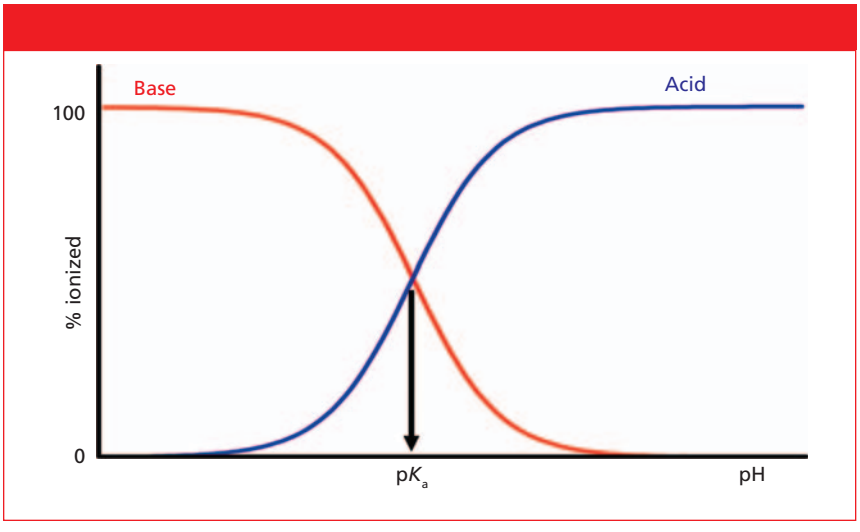


Figure 6: Impact of pH on the ionization state of acidic and basic substances.

was drawn, as well as the more practical curve representing $N = f(F)$.

Using the "Efficiency" Spreadsheet

The user can modify the column dimensions (L_{col}, d_{col}, and d_p), as well as mobile-phase flow rate, to see the impact on a simulated chromatogram at the bottom of the spreadsheet. The corresponding plate count and column pressure drop are also calculated. In addition, users can visualize the corresponding van Deemter curve and evaluate whether the conditions are far from the optimal linear velocity (or flow rate). This ability to visualize the curve could help users determine the maximum plate count achievable on the column under optimal flow rate conditions.

As shown in Figure 3a, an efficiency of 14,536 plates and a pressure drop of 38 bar were achieved on a 150 mm × 4.6 mm, 5-µm d_p column at 1 mL/min, leading to a resolution greater than 1.5 for the chromatogram reported. Increasing the mobile-phase flow rate to 8 mL/min decreased the efficiency to only 5140 plates, which is in agreement with the van Deemter curve (see Figure 3b). The pressure drop also increased to 306 bar, as expected from Darcy's law (equation 9). Next, the impact of particle size was illustrated in Figure 3c, where the efficiency was drastically improved (N = 62,241 plates) when the particle size was reduced from 5 to 1 µm, but the pressure became incompatible with regular HPLC systems (955 bar). In Figure 3d, the column length was 50 mm and the particle size was 10 µm. Compared to the chromatogram reported in Figure 3a, the last two peaks were not baseline resolved because of the poor efficiency (only 1807 plates), which is in agreement with equations 3 and 7. However, with such a short column, the pressure drop was only 3 bar.

Understanding Retention in Reversed-Phase LC

Theoretical Background

In reversed-phase LC mode, the retention at the surface of the alkyl stationary phase (for example, C18) is related to compound hydrophobicity and is often expressed as the partition coefficient (P). P can be defined as the ratio of the compound concentrations between two immiscible phases (1-octanol and water) at equilibrium. For reasonable partition coefficient values (within a limited range), the logarithm of P should be considered (log P), and its definition is provided below:

$$\log P = \log\left(\frac{C_{octanol}}{C_{aqueous}}\right) \quad [10]$$

As illustrated in Figure 4, when the log P value is less than 0, molecules are considered hydrophilic. Then, the molecule has greater affinity for the hydrophilic mobile phase versus the hydrophobic stationary phase and will therefore be poorly retained. In contrast, when the log P value is greater than 0, the molecule is lipophilic and preferably interacts with the hydrophobic stationary phase, leading to significant retention. A few examples of hydrophilic and lipophilic molecules are given in Figure 4. In regular reversed-phase LC mode (with a C18 stationary phase and acetonitrile–water mobile phase), only compounds with log P values between -1 and +6 can be adequately analyzed.

A simulated chromatogram with three compounds with different log P values (set by the user) illustrates the chromatographic behavior for any mobile-phase composition (methanol percentage). For this spreadsheet, the transformation of log P values into retention times was performed based on the work by Henchoz and colleagues (10), considering a Waters Acquity BEH Shield C18 column and a mobile phase containing methanol and water.

The following empirical equation was

used to calculate log k_w (the extrapolated retention factor to pure water, mimicking 1-octanol–water partitioning [11]) based on the log P value set by the user (10):

$$\log k_w = 0.83 \times \log P + 0.21 \quad [11]$$

Then, log k_w was transformed into log k at the mobile-phase composition set by the user using the following equation (12):

$$\log k = \log k_w - S\Phi \quad [12]$$

where Φ is the volume fraction of the organic solvent (value between 0 and 1) and S is a characteristic constant for each solute, which corresponds to the elution strength of the organic modifier (slope of the logarithmic plot: d(log k)/dΦ). In reversed-phase LC, the S value varies from approximately 3 (compounds of approximately 100 g/mol) to more than 100 for very large proteins. In this spreadsheet, a generic value of S = 4 was considered because it is representative of low-molecular-weight compounds (<300 g/mol). Finally, the log k values were transformed into t_R to construct the final chromatogram, considering the column dead time of a 150 mm × 4.6 mm, 5-µm column at a flow rate of 1 mL/min (column dead time of 1.74 min).

Using the log *P* Spreadsheet

The user can modify the log P values of three compounds to see the impact on the retention on a simulated chromatogram. In addition, users also have the ability to tune the mobile-phase composition by adjusting the methanol percentage.

As shown in Figure 5a, when the log P values of the three compounds are between 1 and 1.5, 20% methanol is sufficient to elute all the peaks from the column within a reasonable analysis time of less than 10 min (k values between 1.5 and 4.5). This is because such compounds are not highly lipophilic (intermediate log P values). To speed up the separation, Figure 5b shows that the same compounds can be eluted in less than 2.5 min when the mobile-phase composition is increased to 50% methanol. However, under these conditions, the retention was rather low because 50% methanol was too strong of an eluent (k values between 0.1 and 0.3). In Figure 5c, the compound lipophilicity (log P) was increased in the range of 3.0–3.5. When increasing the log P values by only 2 units, the retention became much higher than Figure 5a and the analysis time was 360 min. To achieve a more reasonable analysis time with these lipophilic compounds, the mobile-phase composition was changed to 50% methanol, and these conditions allow the elution of all three compounds within 25 min.

The examples reported in Figure 5 demonstrate the benefits of reversed-phase LC, which can address a wide range of compound lipophilicity using a generic C18 material and simply adjusting the proportion of organic modifier in the mobile phase. If compounds with more diverse lipophilicity have to be analyzed, the isocratic mode would not be valid and gradient elution is used.

Understanding the Impact of Compound Ionization in Reversed-Phase LC

Theoretical Background

An important parameter for tuning retention and selectivity in reversed-phase LC is the mobile-phase pH. When considering ionizable substances (either acidic or basic), the pH impacts the percentage of neutral and ionized forms. Figure 6 shows the per-

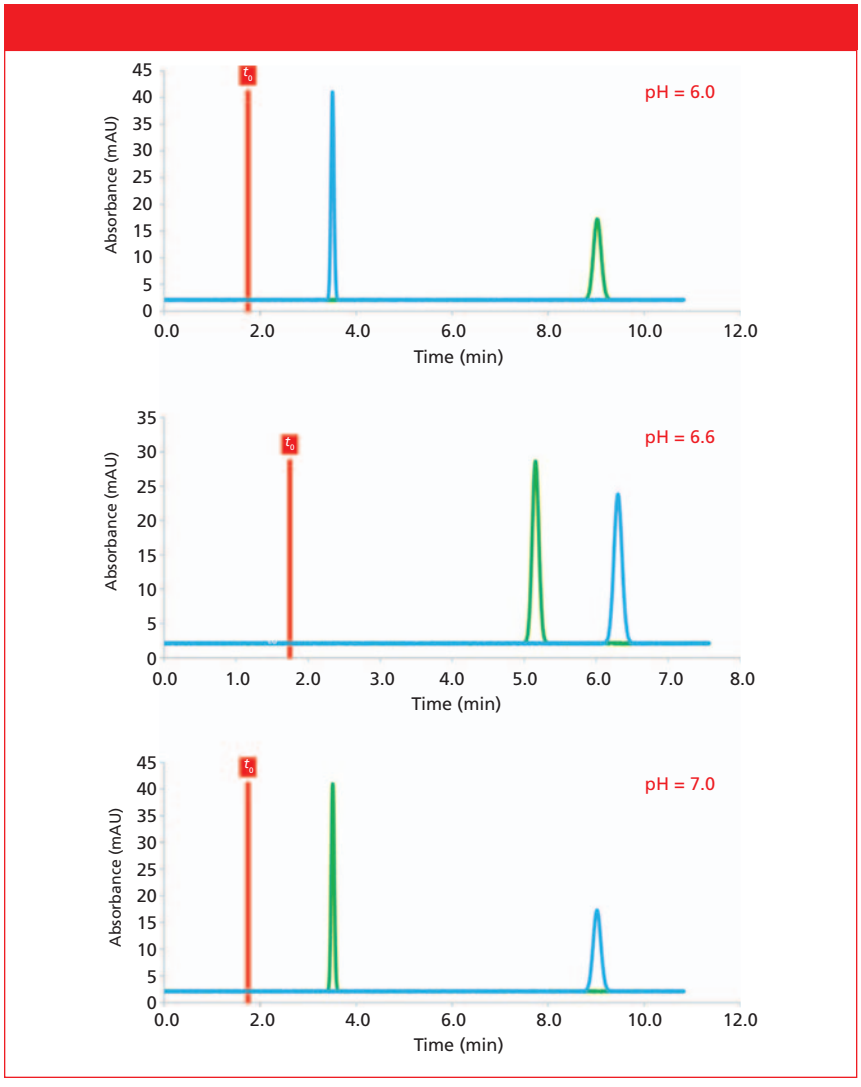


Figure 7: Impact of the mobile-phase pH and compound pK_a on retention in reversed-phase LC mode. The green peak corresponds to an acidic compound with a log *P* of 2 and pK_a of 6.0, while the blue peak corresponds to a basic compound with a log *P* of 2 and pK_a of 7.0. The experiments were performed at 25% methanol and only the pH was varied.

centages of the ionized form for an acidic (blue sigmoidal curve) and a basic substance (red sigmoidal curve) depending on the buffer pH and analyte pK_a.

Based on Figure 6, the retention of basic compounds decreases in reversed-phase LC at low pH (pH below the pK_a of the molecule) because of the presence of a high amount of ionized form. In contrast, the retention of acidic compounds increases in reversed-phase LC at low pH (pH below the pK_a of the molecule) because of the presence of a high proportion of neutral form. Ionized compounds are much more hydrophilic than neutral compounds; therefore, the retention and selectivity can be altered by tuning the mobile-phase pH. In the case of ionizable substances, the partition coefficient (log *P*) cannot be used and should be replaced by the distribution coefficient (log *D*) expressed using the following equation for an acid (13):

$$\log D = \log\left(\frac{[\mathrm{AH}]_{\mathrm{octanol}}}{[A^-]_{\mathrm{aqueous}} + [AH]_{\mathrm{aqueous}}}\right) \quad [13]$$

For an acid, the percentages of neutral and ionized forms at a given pH are given by the following equations (14):

$$\%ionized = \frac{100}{1+10^{\,\mathrm{pH}-\mathrm{p}K_a}} \quad [14]$$

$$\%neutral = \frac{100}{1+10^{\,\mathrm{p}K_a-\mathrm{pH}}} \quad [15]$$

For a base, the percentages of neutral and ionized forms at a given pH are given by the following equations (14):

$$\%ionized = \frac{100}{1+10^{\,\mathrm{p}K_a-\mathrm{pH}}} \quad [16]$$

$$\%neutral = \frac{100}{1+10^{\,\mathrm{pH}-\mathrm{p}K_a}} \quad [17]$$

All the calculations were made for a 150 mm × 4.6 mm, 5-µm column at a flow rate of 1 mL/min using methanol as the organic modifier. Only two compounds were selected for the simulation to limit the complexity of the sample, and a color code was used to distinguish the two substances. For these two compounds, the user can set the log *P* and pK_a values, as well as the nature of the compound (acidic or basic). In addition, the user also has to define the mobile-phase pH and the percentage of methanol in the mobile phase. Based on these inputs, the log *D* at the pH indicated by the user is calculated using the following two equations for an acid (equation 18) or a base (equation 19) (13):

$$\log D = \log P - \log\left(1+10^{\,\mathrm{pH}-\mathrm{p}K_a}\right) \quad [18]$$

$$\log D = \log P - \log\left(1+10^{\,\mathrm{p}K_a-\mathrm{pH}}\right) \quad [19]$$

For (pH – pK_a) values greater than |3.5|, log *D* was constant. The log *D* values were then transformed into retention factors using equations 11 and 12.

The impact of the mobile-phase pH and compound pK_a can be directly visualized. In addition to the simulated chromatogram, the ionization profiles of the two simulated compounds are presented in the upper part of the spreadsheet.

Using the pK_a Spreadsheet

Figure 7 illustrates the impact of the mobile-phase pH (modified in a very narrow range around the pK_a of the substances) on the chromatographic separation of two species (one acid and one base) with identical log *P* values. The retention of the acid (green peak) decreases with pH, which is logical because the acid is mostly deprotonated at higher pH (charged compounds are less retained in reversed-phase LC mode). In contrast, the retention of the base (blue peak) increases with pH because the molecules are less charged at a higher pH. Because the mobile-phase pH was very close to the pK_a of the two substances, the impact of a pH change on retention was significant for both compounds. In these three examples, the elution order was completely reversed between pH 6.0 and 7.0.

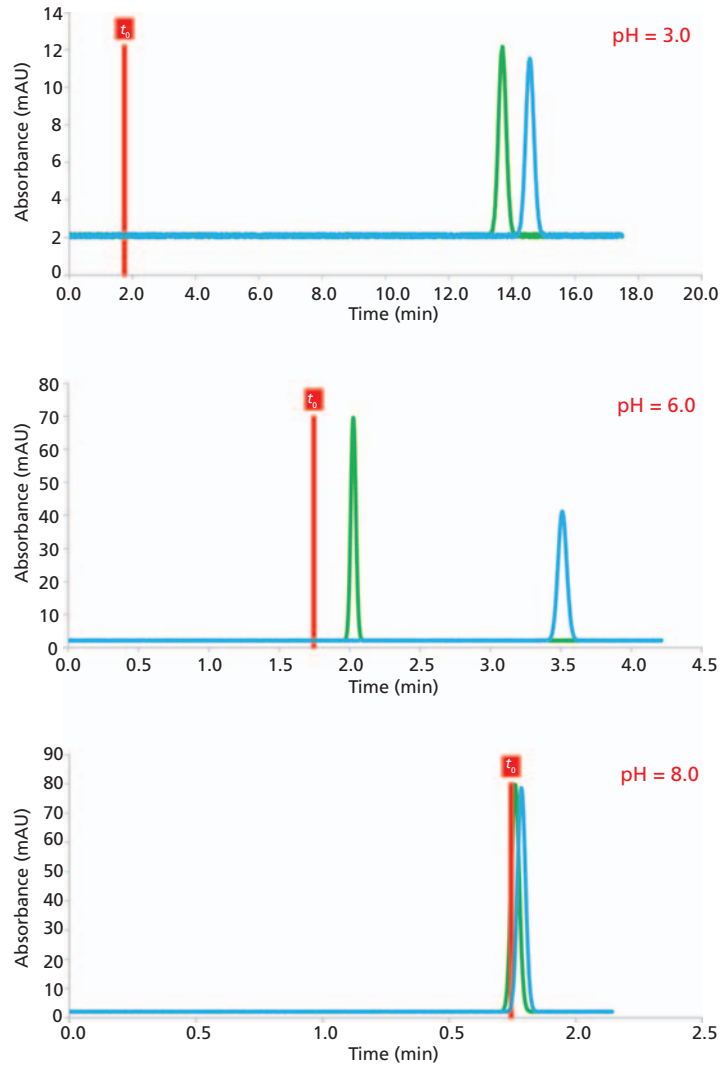


Figure 8: Impact of the mobile-phase pH and compound pK_a on retention in reversed-phase LC mode. The green peak corresponds to an acidic compound with a log *P* of 2 and pK_a of 4.0, while the blue peak corresponds to an acidic compound with a log *P* of 2 and pK_a of 5.0. The experiments were performed at 25% methanol and only the pH was varied.

In the second example (Figure 8), two acids were selected, and the pH was varied in a much wider range (from pH 3.0 to 8.0). The retention was significant at pH 3.0 for these two acidic molecules. The retention factor was approximately 8 on average, and the two peaks were just baseline resolved. When the pH was increased to 6.0, the retention decreased (*k* between 0.2 and 1.0), but the two peaks were much better resolved. Finally, at more basic pH (8.0), the peaks were not sufficiently retained and were not resolved. Under these conditions, the two acidic molecules were completely deprotonated and unsuitable for reversed-phase LC at 25% methanol. A lower proportion of methanol would improve the separation quality.

These examples illustrate the impact of the mobile-phase pH on retention, selectivity, and resolution in reversed-phase LC mode and the importance of adequately controlling this variable in reversed-phase LC.

Conclusion

In conclusion, the spreadsheet HPLC Teaching Assistant (15) helps in learning and teaching liquid chromatography in an innovative and efficient way, using virtual (simulated) chromatograms obtained under numerous analytical conditions. This tool can be used by academic teachers, as well as company training instructors, who are interested in using innovative technology to better convey information during their courses.

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(15) This spreadsheet can be downloaded for free at: https://epgl.unige.ch/labs/fanal/hplc_teaching:en.

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Direct correspondence to: davy.guillarme@unige.ch ■

PRODUCTS & RESOURCES

Vitamin analysis brochure

A brochure from Waters Corporation reportedly describes the company's sample preparation, separations, and mass spectrometry products and software designed for quantifying levels of vitamins in foods, beverages, and dietary supplements in a single analytical run.
Waters Corporation, Milford, MA.
www.waters.com/vitamin

Countercurrent chromatography system

The Quattro countercurrent chromatography and centrifugal partition chromatography system is designed for extraction applications using ionic liquids and liquid ion exchange or chelating agents for targets such as precious metals, lanthanides, actinides, and chiral compounds. According to the company, the system has remote electronics and touch screen computer control for use in extreme-hazards glovebox applications.
AECS-QuickPrep Ltd., London, England. www.quattroprep.com

HPLC column

The Hamilton PRP-C18 HPLC column is designed to provide high-efficiency, reversed-phase separations over an extended column life in nearly any mobile phase or pH. According to the company, the rigid stationary phase has mechanical and thermal stability (>100 °C), does not shrink or swell, and is inert to most conditions commonly encountered in reversed-phase chromatography.
Hamilton Company, Reno, NV.
www.hamiltoncompany.com

Chromatography pump

A pump designed for liquid chromatography, the Intelligent Pump UI-12 from JM Science, reportedly maintains a constant flow rate even with trapped air bubbles and allows for intake from containers placed below the pump. According to the company, the pump is available in two plunger type varieties: 3.2-mm analytical or 9.5-mm semi-prep.
JM Science, Inc.,
Grand Island, NY.
www.jmscience.com

HPLC tubing tool

The Beta Tool-2 tubing tool from MicroSolv is designed for use in HPLC laboratories to straighten or bend stainless steel tubing for a tight fit while keeping the integrity of the tubing's internal diameter, which minimizes band broadening that occurs from kinked tubing. According to the company, the tool's snub nose allows for greater access to tubing that is located in tight spaces, such as inside instruments.
MicroSolv Technology Corp., Eatontown, NJ. www.mtc-usa.com

GC system

OI Analytical's S-PRO 3200 gas chromatography system is designed for the analysis of speciated volatile sulfur compounds in gas-phase matrices. According to the company, the system is suitable for measuring sulfur in natural gas and liquefied petroleum gas; COS in ethylene and propylene feedstock; thiophene in benzene, semiconductors, and industrial gas purity testing; and quality control in gas production and blending operations.
OI Analytical, College Station, TX. www.oico.com

Nitrogen gas generator

The Solaris benchtop nitrogen gas generator from Peak Scientific is designed for laboratories using evaporative light scattering detection (ESLD) instruments and compact mass spectrometers. According to the company, the generator can supply nitrogen to one or two ESLD instruments, or a single compact mass spectrometer, and is capable of delivering up to 10 L/min at purity levels of up to 99.5% (at lower flow rates).
Peak Scientific, Billerica, MA.
www.peakscientific.com/solaris

Mass spectrometer

PerkinElmer's QSight Triple Quad LC–MS/MS mass spectrometer is designed for applications such as food safety, environmental testing, and industrial research. According to the company, the mass spectrometer provides high sensitivity and uptime, and offers remote support capabilities.
PerkinElmer,
Waltham, MA.
www.perkinelmer.com/qsight

Hydrogen gas generator

Proton OnSite's G600-HP high-purity hydrogen gas generator reportedly uses a proton exchange membrane cell stack and dessicant dryer to produce hydrogen at 99.99999% purity. According to the company, features include a touch-screen display, auto-fill water level control, a water monitoring system, a removable water tank, and an automatic leak detection system.
Proton OnSite, Wallingford, CT. www.protononsite.com

Coatings for LC and GC applications

SilcoTek's coatings for LC and GC applications are designed to make the surfaces of customer-supplied components suitable for analytical applications, especially when measuring trace levels of highly active compounds. According to the company, the coatings can prevent unwanted chemical reactions, corrosion, and particulate sticking within the flow path for lower detection and quantitation limits while increasing the lifetime of stainless steel, glass, and other materials.
SilcoTek Corporation, Bellefonte, PA. www.silcotek.com

GC–MS system

Thermo Fisher Scientific's DFS DualData XL Magnetic Sector GC-HRMS system is designed to increase throughput for dioxin analysis while ensuring compliance with existing regulations, including EPA 1613. According to the company, up to double productivity is possible compared to a standard GC–MS system.
Thermo Fisher Scientific, San Jose, CA. www.thermofisher.com/DFS

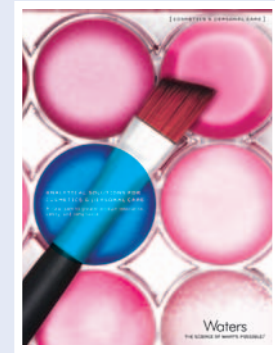
mAb analysis application note

An application note from Tosoh Bioscience titled "Fast Monoclonal Antibody Titer Determination with the TSKgel Protein A-5PW column" reportedly demonstrates the quick capture of human IgG monoclonal antibody and accurate titer analysis over a wide concentration range. According to the application note, the column used for the application is packed with 20-µm hydroxylated methacrylic polymer beads coupled with a recombinant protein A ligand. **Tosoh Bioscience, LLC,** King of Prussia, PA. www.tosohbioscience.com

Flash purification columns

UCT's Ultra-Flash purification columns are designed with three flash lines: standard silica, bonded-phase columns; ILOK format columns conductive for manual assembly and column stacking; and spherical high-pressure columns rated up to 400 psi. According to the company, the columns are leak-free, 100% compatible with all flash instrumentation currently in the marketplace, and available in a range of sizes and chemistries. **UCT, LLC,** Bristol, PA. www.sampleprep.unitedchem.com/products/flash-chromatography/ultra-flash-purification

Cosmetics and personal care products brochure



A brochure from Waters Corporation describes the company's sample preparation, separations, and mass spectrometry products and data management software for counterfeit detection, raw materials testing, formulation control, safety and regulatory testing, and end-product quality control.
Waters Corporation,
Milford, MA.
www.waters.com/cpc

LC–MS system

Shimadzu's Nexera MX ultrahigh-speed LC–MS system is designed for multiplex analysis. According to the company, the system provides continuous data analysis by incorporating a special dual analysis stream structure and instrument control system.
Shimadzu Scientific Instructions,
Columbia, MD.
www.ssi.shimadzu.com

Methods database

METHODSNOW™
A CAS SOLUTION

The MethodsNow database from CAS is designed to provide users with access to details from millions of scientific methods in table format. According to the company, the database has step-by-step instructions for analytical and synthetic methods in areas such as pharmacology, HPLC, and food analysis.
Chemical Abstracts Service,
Columbus, OH.
www.cas.org/MethodsNow

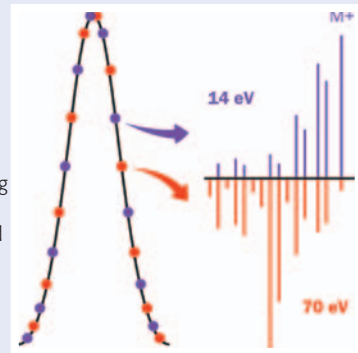
Neonicotinoid standards

Neonicotinoid standards are available from Chem Service. According to the company, the certified analytical reference standards range from acetamiprid to thiamethoxam and are available neat, in a solution, or in a mixture.
Chem Service, Inc.,
West Chester, PA.
www.chemservice.com

TOF-MS system

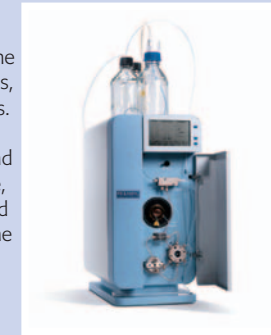
The *Pegasus* BT time-of-flight mass spectrometry (TOF-MS) system from LECO is designed for GC–MS applications. According to the company, the spectrometer's software can simplify quantitation while identifying more components, and its StayClean ion source eliminates the need for source cleaning.
LECO Corporation,
St. Joseph, MI.
www.leco.com

TOF-MS systems with tandem ionization



BenchTOF mass spectrometers from Markes International are designed with a tandem ionization feature that generates two spectra from a single peak by rapid switching between "soft" ionization (typically 10–16 eV) and conventional "hard" ionization (70 eV). According to the company, the technique is fully automated by its TOF-DS software.
Markes International,
Llantrisant, UK.
www.markes.com

Postcolumn derivatization system



Pickering's Pinnacle PCX Sigma Series is designed as an optimized HPLC postcolumn derivatization system for the analysis of samples such as amino acids, carbamates, mycotoxins, and antibiotics. According to the company, the system includes an electronic syringe pump and valves, a quick-change reactor cartridge, a column oven, inert flow paths, a liquid crystal display, and control software. The system reportedly works with all HPLC systems.
Pickering Laboratories,
Mountain View, CA.
www.pickeringlabs.com

HPLC performance maintenance kits

Sciencix's HPLC performance maintenance kits are designed to be equivalent to the corresponding OEM kits. According to the company, the kits contain essential parts to keep HPLC systems performing at an optimal level.

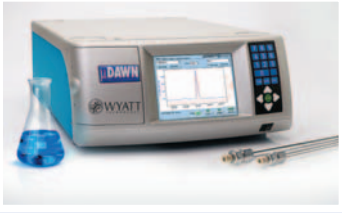
Sciencix,
Burnsville, MN.
www.sciencix.com

Gas purifiers

GasTrap purifiers, available from Quadrex, are designed to be self-regenerating, and reportedly can eliminate the need for replacing in-line gas filters. According to the company the purifiers are suitable for GC, GC–MS, and other laboratory applications.

Quadrex Corporation,
Woodbridge, CT.
www.quadrexcorp.com

SEC–MALS detector for UHPLC



The µDAWN multiangle light-scattering detector from Wyatt Technology is designed to be coupled to any UHPLC system to determine absolute molecular weights and sizes of polymers, peptides, and proteins or other biopolymers directly, without column calibration or reference standards. The detector reportedly connects to the company's Optilab UT-rEX differential refractive index detector.

Wyatt Technology Corp.,
Santa Barbara, CA.
www.wyatt.com

Mass spectrometer

The LCMS-8060 triple-quadrupole mass spectrometer from Shimadzu is designed to provide a scan speed of 30,000 u/s while maintaining mass accuracy and multiple reaction monitoring speeds of 555 ch/s. According to the company, the instrument has a polarity switching speed of 5 ms.

Shimadzu Scientific Instruments,
Columbia, MD.
www.shimadzu.com

GC–MS system

The Agilent 5977B high-efficiency source (HES) gas chromatography–mass selective detector (MSD) system is designed as a tandem gas chromatograph and mass spectrometer. According to the company, the detector allows scientists to use smaller sample volumes, spend less time on sample preparation, reduce instrument downtime, minimize solvent usage, and reduce the environmental impact of GC–MS analysis.

Agilent Technologies,
Santa Clara, CA. www.agilent.com

Automated SPE system for PFCs

The TurboTrace PFC Parallel SPE system from FMS is designed to automate existing manual SPE techniques for perfluorinated compound (PFC) analysis. According to the company, the system is closed, and delivers a low background and reproducible, consistent results.

FMS, Inc.,
Watertown, MA.
www.fms-inc.com

Chiral LC column

The CHIRALPAK IG column from Chiral Technologies is designed with an immobilized chiral stationary phase consisting of a meta-substituted phenyl carbamate of amylose. According to the company, the chiral column can be used with its CHIRALPAK IA, IB, and IC columns for primary screening and with its CHIRALPAK ID, IE, and IF columns for secondary screening.

Chiral Technologies,
West Chester, PA.
www.chiraltech.com

Combustion ion chromatograph

Metrohm's combustion ion chromatograph (CIC) is designed to automate the determination of halogens and sulfur. According to the company, the system's autosampler can run both solid and liquid samples, and flame sensor technology is used to measure the light intensity from the pyrolysis oven during combustion.

Metrohm USA,
Riverview, FL.
www.metrohmusa.com/CIC

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AD INDEX

THE ESSENTIALS

Excerpts from LCGC's professional development platform, CHROMacademy.com

GC Troubleshooting in Simple Pictures, Part I

A picture paints a thousand words. The art of gas chromatography (GC) troubleshooting often lies in being able to recognize a problem from the evidence presented in the chromatogram or baseline appearance. In this short series of two articles, we present 10 simple GC pictures that will show you how to recognize the issues, deal with the causes, and prevent them from happening again. Here, the first five pictures are presented in Figures 1a–1e.

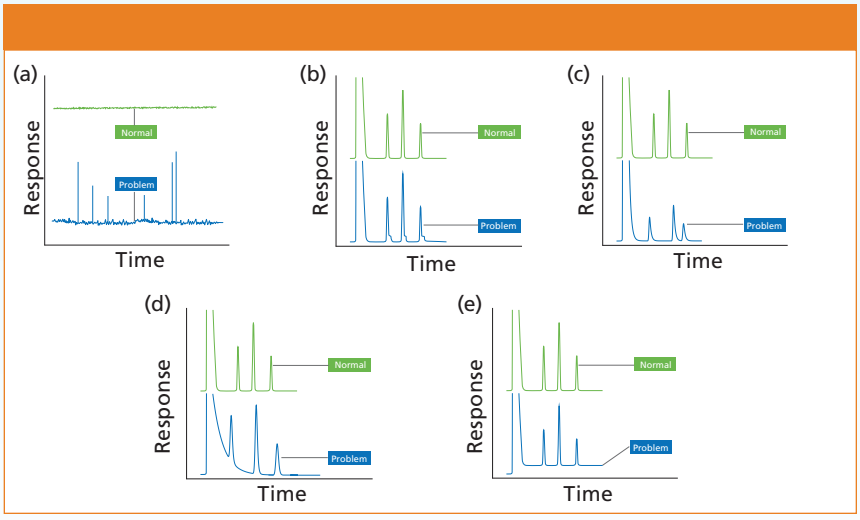

Figure 1: Common GC problems: (a) baseline spikes; (b) chair-shaped peaks; (c) tailing analyte peaks; (d) tailing solvent peak; and (e) shifting baseline.

Baseline Spikes

What type of spike do you have? Zoom into the chromatogram and look at the noise—do the peaks have Gaussian shape and a peak width? If not, then the likely cause is electrical noise. Electrical noise can be caused by a poorly smoothed electrical supply to nearby electrical equipment. This type of noise can be overcome by fitting a power scrubber to the supply, isolating any equipment giving rise to the problem, removing it to a different area of the laboratory, or placing it on a different ring circuit. If the peaks have width and occur in a homologous series, then column bleed is to blame. To fix column bleed, the column can be reconditioned; however, if the bleed profile does not improve you may need to trim the column to remove any contaminated or damaged stationary phase (approximately 5% of the column length) and then recondition. If the peaks have a Gaussian shape, no apparent homologous series, and random peak heights, the column may be mounted too high in the flame ionization detector and may be coming into contact with the flame, which causes the polyimide coating to flake and peel off into the detector resulting in baseline spikes.

Chair-Shaped Peaks

If all peaks are affected, a physical issue rather than a chemical one is to blame. Chair-shaped peaks can be caused by a poor column cut. If the chair-shaped peak is accompanied by a broad solvent peak that exhibits a sudden vertical cutoff, the column is installed too high in the inlet.

Tailing Analyte Peaks

If some, but not all, analyte peaks are tailing, a chemical problem is indicated. The most likely cause of chemical tailing is secondary interactions of polar analytes with active sites, typically silanol groups, in the inlet liner, on glass wool in the liner, or at the edges of a poor column cut. When all analyte peaks tail, a physical problem is indicated (unless all analytes have similar chemistries—that is, they are all basic). For example, a poor column cut can expose surface silanol species that basic analytes can interact with, which can cause tailing, or it can result in turbulent eddies at the column inlet that cause analytes to be held up before they enter the column, leading to tailing. Recutting the column and examining the cut under a microscope to ensure it is smooth and at 90° to the column wall will resolve this issue.

Tailing Solvent Peak

A tailing solvent peak during splitless injection indicates a poorly optimized splitless time. Too long a splitless time causes solvent peak tailing because not enough of the solvent is vented away and instead enters the column. Whereas too short a splitless time results in loss of early eluted analytes. Monitoring the peak area of an early eluted peak and plotting it against splitless time until a consistent peak area is observed will give the optimum splitless time (5–10 s can be added to further optimize this time).

Shifting Baseline Position

A shifting baseline position is different from baseline drift. Here a shift between the start and end of the solvent peak is seen, which suggests that the gas flow is changing during the injection cycle. This flow change could be due to leaks during, before, or after injection caused by a cored or leaking septum. Check your septum regularly for damage.

There may be other causes for these problems, so for further tips visit the CHROMacademy GC Troubleshooter at www.chromacademy.com/gc_troubleshooting.html.

More Online:

Get the full tutorial at
www.CHROMacademy.com/Essentials
(free until November 20).

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