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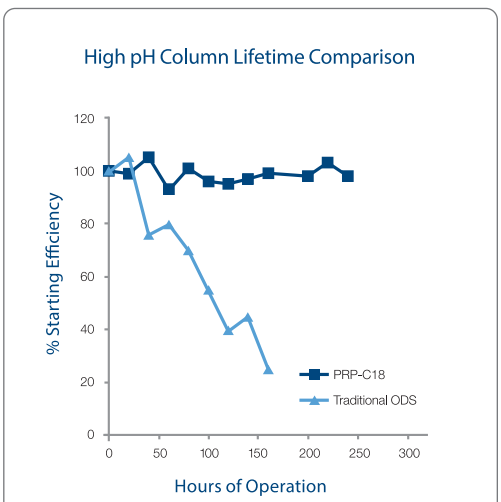

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Note from the CEO

Mike Hennessy, Jr.
President & CEO, MJH Life Sciences®

The dawn of a new year often spurs us to make improvements in our lives and work. But to improve something, you have to understand how it is currently functioning.

Those who want to know how liquid chromatography (LC) columns and systems work have surely taken note of the work of Fabrice Gritti. Throughout his career, Gritti has been exploring fundamental questions on topics like adsorption and retention mechanisms in reversed-phase LC, how superficially porous particles really work, and how better column packing procedures can improve column performance. Given this impressive body of work, Gritti was honored last fall at the Eastern Analytical Symposium (EAS) with the EAS Award for Outstanding Achievements in Separation Science. Bravo, Fabrice!

In this issue, Gritti is at it again, this time addressing the challenge of accurately measuring the column hold-up volume in LC. Since the very genesis of LC, this topic has been a subject of disagreement, and there are as many values of column hold-up volume as there are techniques used to measure it. This puts LC users in a difficult situation as they attempt to predict the retention behavior of LC columns. In this study, Gritti and his coauthor Kerri Smith propose a user-friendly, accurate, and precise method that harmonizes the results obtained regardless of column type and mobile-phase composition.

Understanding what is really going on in a separation is also the theme in this month's "GC Troubleshooting." Nick Snow observes that the concept of polarity is commonly misunderstood with respect to gas chromatography (GC) stationary phases, and the common measures of stationary phase polarity (McReynolds constants and the polarity scale) are not always accurate predictors of retentiveness or selectivity. As the saying goes, "It ain't what you don't know that gets you into trouble. It's what you know for sure that just ain't so." Snow shares advice about how to think more clearly about polarity in GC.

In "LC Troubleshooting," Dwight Stoll always reminds us that to be able to troubleshoot, we need a solid grasp of fundamental concepts. Here, he considers a user's question about how to improve upon an established method. The place to start, he notes, is to understand the primary levers available to adjust performance—which, of course, he explains with his usual clarity and just the right amount of detail.

This ongoing quest to deepen our knowledge of the mechanisms of chromatography and disseminate that information so it can be harnessed for practical use, is what drives the separations community—and *LCGC*. Celebrating this pursuit, and taking it on anew, is a great way to start this new year. Here's to a happy and productive 2023!

LC TROUBLESHOOTING

What Are My Options to Improve My Separation? Part I: Foundational Concepts

Many high performance liquid chromatography (HPLC) users are confronted with questions about how to improve the performance of an existing or recently developed method. These days, we have many technological options to consider (for example, using higher pressures or temperatures, or both), but how do we choose one (or a few) to try? A systematic approach to method improvement can save time and resources by using methods that are likely to be better, and more robust, than those developed using a trial-and-error approach.

Dwight R. Stoll

During a recent visit to a local laboratory here in Minnesota, I was asked a question that I initially thought would be easy to answer, but then it caused me to pause and think a lot. And the more I thought about it, a series of "LC Troubleshooting" articles unfolded in my mind, and this will be a frequent discussion topic here in 2023. The essence of the question was (paraphrasing) as follows: "I've got this validated method that isn't great, but it gets the job done. The method is approximately 20 years old, and I've heard and read that high performance liquid chromatography (HPLC) technology has changed a lot in 20 years, so it seems to me it must be possible to improve this separation a lot. What are my options for doing that?"

Of course, as someone deeply committed to all things chromatography, I immediately felt the urge to say, "Why yes, a lot has changed, and you can improve that separation. But…you have a lot of options, and…it's a little complicated." Nevertheless, I am certain there are many HPLC users that have this exact question on their minds, and feel unsure about where to start. In a series of "LC Troubleshooting" articles, we are going to confront this question directly and make it more manageable to think about how to proceed. Not all of the options we will discuss will be available to all users, but it is valuable to understand what the full array of options looks like, so that you can feel confident in making an informed decision about how to proceed.

Some Analytical Challenges Need To Be Treated Differently

HPLC users routinely face a staggering variety of analytical challenges ranging from relatively fast (tens of seconds) separations of just a few analytes to long separations (several hours) of complex mixtures that contain thousands of analytes. Figure 1 shows a comparison of chromatograms obtained for simple and complex mixtures using contemporary technologies. In the case of the simple mixture, there is a lot of empty space in the chromatogram, and one means of decreasing analysis time would be to reduce the amount of wasted, empty space. On the other hand, the entire chromatogram for the complex sample is full of peaks. In this case, improvement may mean decreasing the peak widths on average so that fewer analytes are coeluted, but it is very unlikely that all of the analytes in the mixture can be fully resolved, even if the analysis time is extended beyond one hour. These disparate challenges necessarily require different chromatographic solutions, and improving on old or poor separations in current use for these applications require different lines of thinking.

When dealing with relatively simple mixtures, we generally have more paths to improve the separation, including changing mobile-phase chemistry, the stationary-phase chemistry, or both, adjusting the retention and optimizing efficiency (that is, finding the most time efficient way to achieve the chromatographic efficiency needed to meet the chromatographic objectives). On the other hand, when dealing with complex samples, our options are more limited. A big difference is that changing the mobile-phase chemistry, the stationary-phase chemistry, or both, will move peaks around, but there will still be coelution, so a simple reordering of the peaks may not be all that helpful—and usually mass spectrometry (MS) is critically helpful for resolving compounds that are not separated chromatographically. In this case, the most effective way of improving the separation would be to improve the efficiency as a means of improving the peak capacity—roughly, the number of peaks that can be fit side-by-side into a separation space. We will discuss the concept of peak capacity in more detail in a future installment focused on improving separations of complex mixtures.

The Menu of Options

Relative to the situation 20 years ago, HPLC users currently have many options to consider as possible means to improve upon the performance of old methods. Some of the most prominent options currently are changes in particle morphology and size,

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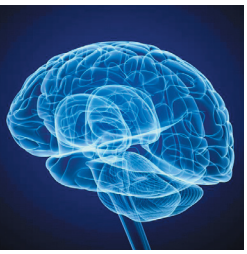

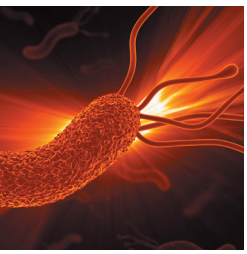

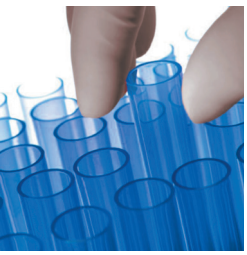

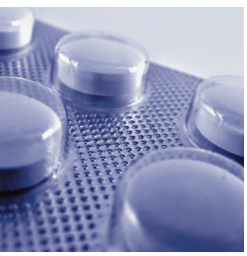

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operating pressure, column temperature, and an increasingly diverse array of stationary-phase chemistries (for example, consider the growing number of "mixed-mode" phases that contain both charged and lipophilic functional groups). We can roughly divide these options into two categories: those that affect peak width (kinetic adjustments), and those that affect peak spacing, or relative retention (thermodynamic adjustments). Here, I provide a full list of options simply to make the point that there is a lot to consider. We won't discuss any one of these in detail now, but we will discuss each of them in detail in future installments in this series.

- Kinetic adjustments—reducing peak width relative to analysis time, which include:
  - changing the particle size (usually decreasing it, but not always);
  - moving to a higher pressure range (allows increase in flow rate, increase in column length, decrease in particle size, or both);
  - moving to a higher temperature range (allows increase in flow rate, increase in column length, decrease in particle size, or both); and
  - changing the particle morphology (for example, moving from totally porous particles to superficially porous ones).
- Thermodynamic adjustments—improving peak spacing (that is reducing the fraction of the time axis that is empty), which can include:
  - finding a stationary-phase chemistry that provides more even distribution of peaks across the time window;
  - adjusting the mobile-phase chemistry in a way that moves toward more even distribution of peaks; and
  - adjusting the column temperature can also be considered here.

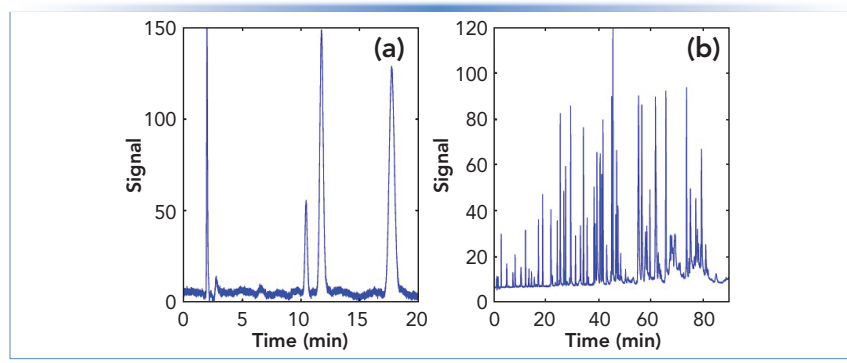

FIGURE 1: Comparison of (a) sparse and (b) crowded chromatograms.

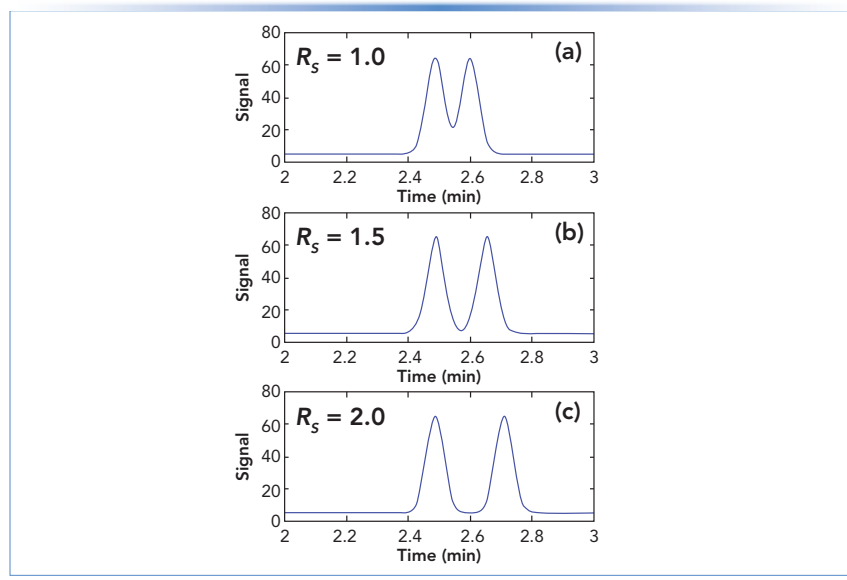

FIGURE 2: Examples of peak separation for different resolution values of R_S = 1.0, 1.5, and 2.0.

Resolution is a Key Metric of Separation Performance in This Context

There are many ways to measure separation performance, including analysis time, efficiency (that is, plate number), selectivity, retention, and resolution. Among these, resolution (R_S) is one of the more holistic measures of performance because it takes into account both the difference between retention times of adjacent peaks (which is related to selectivity), and their widths (which is related to plate number). This is shown in equation 1, where $t_{R,A}$ and $t_{R,B}$ are the widths of the first and last eluting members of a peak pair, and w_A and w_B are their 4·σ widths (measured at 13.4% of the peak height).

$$R_S = \frac{2 \cdot (t_{R,B} - t_{R,A})}{w_A + w_B} \quad [1]$$

Even if there is a good distance between the peaks as measured by their retention times (let's say 1 min), they will not be resolved if they are very wide (>>1 min). Conversely, even if the peaks are very narrow (let's say 1 s), they will not be resolved if there is no distance between them (<<1 s). Thus, successfully resolving two peaks requires at least some minimal distance between them and that they be reasonably narrow. Of course, there is some room for variation here—the narrower the peaks are, the smaller is the distance between them that is needed, and the more distance between the peaks there is, the wider the peaks can be while still actually resolving them.

Whenever discussing resolution, I think it is valuable to have in our minds a correspondence between the calculated resolution values, and what the separation looks like in a chromatogram. A few examples are shown in Figure 2 to reiterate this important point.

Effects of Efficiency, Selectivity, and Retention on Resolution

Given the importance of resolution as a metric of separation performance, discussions

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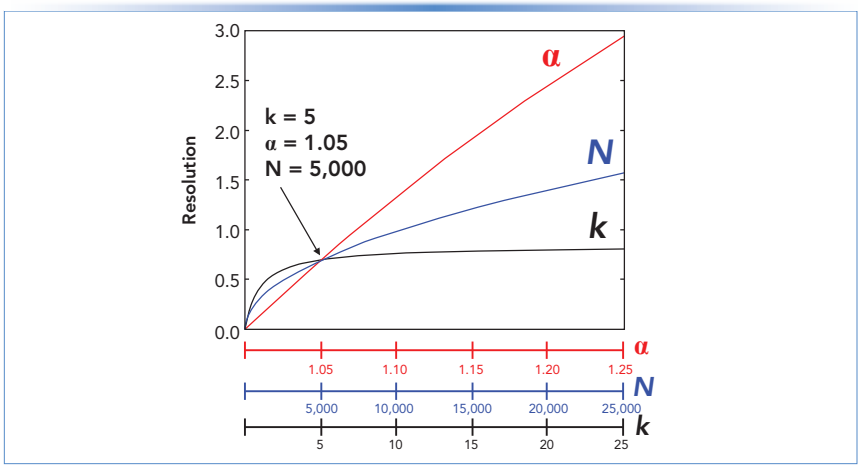

FIGURE 3: Effect of plate number (N), retention factor (k), and selectivity (α) on resolution (R_S) over similar ranges in the independent variables. For calculation of R_S with varying N, $\alpha = 1.05$ and $k = 5$. For calculation of R_S with varying k, $\alpha = 1.05$ and $N = 5{,}000$. For calculation of R_S with varying α, $N = 5000$ and $k = 5$. The positive impact of α on R_S is the most persistent over a chromatographically relevant range of values. Adapted from reference (3).

of the effects of different chromatographic variables on performance are often focused on their effects on resolution. Equation 2, sometimes referred to as the Purnell equation (1,2), shows an approximate expression for resolution that makes explicit the relationships between resolution and plate number (N), retention factor (k), and selectivity (α).

$$R_S = \left(\frac{\sqrt{N}}{4}\right)\cdot\left(\frac{k}{k+1}\right)\cdot\left(\frac{\alpha-1}{\alpha}\right) \qquad [2]$$

where α is defined as the ratio of the retention factors of the two peaks we are trying to resolve ($\alpha = \frac{k_B}{k_A}$). We might think about this expression as the three-legged stool of resolution—without any of the legs, the stool will fall over, and we will not have the resolution we need. From this follow some basic but essential tenets of method development:

- We must have some retention. If there is no retention ($k = 0$), there cannot be any resolution ($R_S = 0$), no matter how good the plate number and selectivity might be.
- We must have some selectivity. If there is no selectivity ($k_A = k_B$; $\alpha = 1$), there cannot be any resolution ($R_S = 0$), no matter how good the plate number and retention might be.
- We must have some chromatographic efficiency (N). If the plate number is very low, then resolution is likely to be low.

Now what is particularly interesting is that although each of the three legs of the stool is essential for success (N, k, and α), some of them are more impactful than others. Figure 3 shows the impact of each variable on resolution when the remaining two are held constant. First, we see that increasing N from zero has an immediate positive impact on resolution, but then the impact diminishes because of the square root dependence of R_S on N. Second, we see that increasing k from zero has an even larger positive effect on R_S initially, but then the effect diminishes quickly, because $\frac{k}{k+1} \sim 1$ when $k > 5$. Finally, we see that increasing the selectivity, α, has the most persistent effect on increasing resolution. Increasing α from 1.10 to 1.15 has almost the same degree of impact on R_S as increasing a from 1.00 to 1.05. Given the strength and persistence of this impact, we should always seriously consider how to change α in the process of trying to improve an existing separation, whether the desired improvement is focused on improving resolution in a given analysis time, or achieving the same resolution in a shorter analysis time. There are many possible ways to change selectivity, and we will discuss these in detail in future installments. For now, it is most important to embrace the relationships shown in Figure 3 and always have these in mind when considering how to improve an existing separation.

Summary

Many HPLC users are confronted with questions about how to improve upon the performance of an existing method. Fortunately, modern HPLC technologies (that is, instrumentation, columns, and software) provide many possible avenues to make improvements. However, the number of possibilities can be intimidating, making it difficult to decide upon a particular path to improvement. In this installment of "LC Troubleshooting," I have discussed the essential concepts to keep in mind when considering different ways to improve a method, including how to measure performance, a summary of different technology options that should be considered, and the impact of different chromatographic variables on resolution. In future installments in this series, we will look at each of these topics in detail, surveying contemporary technologies and their potential impact on performance, all with the goal of providing users with a foundation of knowledge to support confident decisions about how to proceed with method improvement.

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SAMPLE PREP PERSPECTIVES

Overview of Recent Development of Needle-Trap Devices for Analysis of Volatile Compounds

Needle-trap devices (NTDs) are another sorbent-based tool in the haystack of methods used in analytical extractions. Syringe needles, similar to those used for gas chromatography (GC) injection, can be partially filled with suitable sorbents and are used for extracting and collecting volatile organics, followed by injection into a GC instrument via thermal desorption. Although NTDs share many similarities and advantages of solid-phase microextraction (SPME), the larger sorbent bed provides robustness and offers potentially exhaustive extractions. This month, we take a look at the principles and applications of NTDs, and recent developments in their use.

Douglas E. Raynie

In the early 1990s, Janusz Pawliszyn and his research group at the University of Waterloo (Canada) introduced solid-phase microextraction (SPME), and a new era in sample preparation for gas chromatography (GC) began. With SPME, analytes could be sampled by adsorption onto a stationary phase via direct immersion into a liquid sample or via headspace sampling of volatiles. Sample collection, extraction, clean-up, and concentration are combined into a single operation prior to injection into a chromatograph. Although SPME has seen applications with liquid chromatography (LC), the technique really made its mark as a sample preparation device for GC. We've somewhat recently provided an update to developments in SPME (1).

Perhaps most impressive about the development of SPME is its versatility, evidenced by new embodiments of the technique and spurring other sorptive-based extraction methods, such as thin-film microextraction (TFME), coated-blade spray (CBS) for mass spectrometry (MS), or stir-bar sorptive extraction (SBSE). Another of these unique geometries for SPME is the concept of needle-trap devices (NTDs) introduced by Koziel, Odziemkowski, and Pawliszyn in 2001 (2). Although different geometries exist, NTDs typically use syringe needles, generally the 23-gauge needles common for GC injection, packed with solid sorbent particles. NTDs are not to be confused with other packed-needle or syringe-tip approaches, like disposable pipet tips (DPX) or microextraction by packed sorbent (MEPS), which bear greater resemblance to conventional solid-phase extraction (SPE). Although, in theory, an NTD could be used with liquid samples, in practice the flow resistance limits these applications. An NTD configuration is displayed in Figure 1.

Extraction with NTDs

With NTDs, given the greater amount of sorbent compared with conventional SPME approaches, diffusion of analytes into the needle and to the sorbent bed is not subject to equilibrium limitations. In fact, needle-trap extraction (NTE) is often considered exhaustive. The exhaustive nature of NTE removes the calibration considerations found with SPME and other non-exhaustive equilibrium extractions. The extraction mode typically occurs in conjunction with dynamic headspace sampling. This active sampling approach is possible because of the lack of diffusion limitations and serves to increase the speed of extraction. However, passive sampling approaches are also possible. Like SPME, NTE is a single-step procedure and solvent free (discounting the sorbent phase, which acts as the extracting solvent). Following extraction, the NTD can be stored for extended periods with little loss of analyte, then taken to the gas chromatograph, where, upon insertion into the injection port, analytes are thermally desorbed from the sorbent bed and move to the GC column. The needles packed as NTDs often have a side hole that provides three advantages: providing a portal for introducing the sorbent during the packing process; allowing for flow during dynamic headspace sampling; and, most importantly, facilitating flow of carrier gas in the GC inlet to stimulate quantitative transfer of desorbed analyte to the GC column. Some needle configurations feature a conical or extended tip to further direct analyte to the column.

Perhaps the biggest limitation of NTE is breakthrough volume: Once the sorbent bed becomes saturated with analyte, additional analyte is not collected in the needle trap. Breakthrough volume will primarily be a function of sorbent amount and surface area, and a plot of amount extracted versus analyte concentration in the sample is linear until breakthrough occurs. This allows quantitative extraction. Compared with standard SPME, NTE addresses concerns with fragility and adsorption capacity of the SPME fiber. Because there is more sorbent in an NTD, partition coefficient limitations are addressed, allowing extraction of analytes with smaller log P values. The time for an NTE is shorter than both SPME and traditional sorbent-based sampling tubes. All of the attri-

TABLE I: Summary of NTD applications using metal organic frameworks (MOFs) since 2020

MOF Phase	Target Compound	Limit of Detection	Storage Capacity	Notes	Reference
Ni-based	Chlorobenzenes	2–10 ng/L		Needle-to-needle reproducibility of 5–11%	9
Ti-based	Airborne phenolics	0.0001–0.12 ng/L	No observed differences after 60 days storage at 40 °C		10
Eleven Cr-, Fe-, and Zn-based	Polycyclic aromatic hydrocarbons in tea, coffee, and water	0.1–0.2 ng/L		Roles of metal, ligand, and modification process studied with ligand type greatly enhancing extraction efficiency	11
Zn-based with nitrogen-rich organic ligands	BTEX (benzene, toluene, ethylbenzene, xylene isomers)	20 ng/L			12
Ni/Zn-Benzene-1,3,5-tricarboxylic acid bimetallic	Non-metabolized halogenated hydrocarbons in urine	1.02–1.10 ng/L	95–97% efficiency after 10 d of storage at 4 °C		13
MIL-100(Fe)	BTEX in urine	0.0001–0.0005 µg/mL			14
Ni/Co-Benzene-1,3,5-tri-carboxylic acid bimetallic	BTEX in urine	0.2–1.1 ng/mL			15
MIL-100(Fe)	Airborne organochlorine pesticides	0.04–0.41 µg/m^3	No significant change after 6 d of storage at 4 °C		16
Molecular-im-printed polymer	Diazinon in air	0.02–0.1 µg/m^3	No significant change after 15 d at 25 °C or 50 d at 4 °C	Effects of temperature and humidity studied	17
Zr-based	Amphetamines in urine	---	Over 95% recovery after 10 d of storage at 4 °C	Effects of salt, pH, time, and temperature studied	18

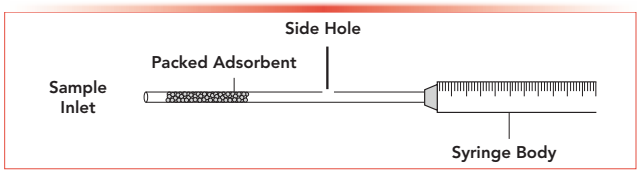

FIGURE 1: Schematic diagram of a needle-trap device (NTD) featuring an adsorbent packed inside a syringe needle, with an (optional) side hole in the syringe to facilitate air flow during sampling and carrier gas flow during desorption. Modified from reference (3) with the Creative Commons license.

butes of NTDs render them useful for on-site sampling or in-field analysis; for example, with occupational exposure studies. These features, combined with its solvent-free nature, give NTE serious green chemistry attributes. A recent review (4) provides a thorough discussion of the principles and practice of NTDs. NTDs have evolved to where they are now offered commercially by PAS Technology, Shinwa, PerkinElmer, and CTC Analytics.

Recent Developments with NTDs

Perusal of the NTD literature since 2020 reveals interesting trends. Particularly interesting are the extraction of aerosol droplets and particles, use of metal organic frameworks (MOFs) as sorbent phases, and breath analysis, including for disease diagnosis.

Extraction of Droplet-Bound and Gaseous Sample Components

Typically, the capture and analysis of droplet-bound and gaseous sample components, such as in breath analysis, are performed

separately. Zeinali and Pawliszyn (5–8) have reported on combining a filter in front of the sorbent bed for a unified approach to this analytical challenge. The filter (for example, divinylbenzene) collects particulate matter and aerosol droplets, while Carboxen or other sorbent phases trap the volatile, gaseous compounds. They studied the stability of the droplets and gases in the air-sampling bags and obtained detection limits down to 0.05 mg/mL for their analysis. The method was applied with great success to pesticide aerosols, air from (burning) scented candles, and in the breath of volunteers following exposure to air freshener spray, fragrance mists, cannabis smoking, and incense.

Metal Organic Frameworks (MOFs) as Sorptive Media in NTDs

Development of newer phases to comprise the sorbent bed in NTDs is of ongoing interest. One type of media seeing wide development are MOFs. Table I summarizes selected recent applications of this technology. As a general rule, this approach provides sub-ng/mL limits of detection and exhibit minimal loss of analyte when the NTDs are stored at sub-ambient temperatures.

Other Sorptive Media

In addition to MOFs, other sorptive phases have been recently presented for NTD applications. A reduced graphine oxide-melamine formaldehyde phase created a superhydrophobic sorbent (19) with a reported 0.2 µg/L detection limit for chlorobenzenes. The NTD was reused over 200 times. An NTD with a Schiff base network-1/single-walled carbon nanotube (SNW-1/SWCNT) was created for phenolics analysis with limits of detection down to 0.002 ng m/L (20).

Disease Diagnosis

NTE for bovine respiratory disease diagnosis via cattle breath analysis was developed, though not enough evidence was obtained to create a useful profile of volatile organic compounds (VOC) (21). VOC analysis via NTE of fecal and tissue samples from cattle and goats identified a bacterial culture (22). With optimization, VOC emissions could be monitored for diagnosis of paratuberculosis in livestock.

Conclusion

As a unique embodiment of SPME, NTE presents a versatile, exhaustive extraction device for the sample preparation of volatile analytes prior to GC analysis. Continued development of needle configurations and interfacing with a GC inlet and types of sorbent media, including filter-sorbent combinations, will advance NTE into the everyday realm for occupational exposure, disease diagnostics, aerosol-bound analytes, and breath analysis.

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GC CONNECTIONS

Does High Polarity Mean High Retention on Stationary Phases in Gas Chromatography?

Stationary phase chemistry, polarity, and selectivity have been of ongoing interest since the inception of gas chromatography (GC) in the 1950s. In the early days when most analyses were performed on packed columns, there were hundreds of stationary phase materials available. Today, with modern capillary columns, most GC analyses are performed with a few stationary phases, with a wide array of choices for specialty applications. Stationary phases are often classified using the broad term polarity, with polar stationary phases recommended for separating polar analytes and nonpolar stationary phases recommended for nonpolar analytes. In this installment, we examine the idea of stationary phase polarity. We examine the assumptions inherent in the most popular stationary phase polarity evaluating systems—McReynolds constants and the polarity scale. We see that high polarity does not always mean greater retention or higher selectivity.

Nicholas H. Snow and Hetal Rana

Nonpolar, moderately polar, and *polar* are probably the most used modifiers when describing stationary phases in gas chromatography (GC). We have all heard about stationary phases, such as polydimethylsiloxane (PDMS), being called nonpolar, cyano-modified PDMS being called moderately polar, and polyethylene glycol (PEG) being called polar. We also all remember the definitions of polar molecules and polar bonds from undergraduate chemistry, so we think about electronegativity differences and the presence of heteroatoms in the structure as a sign of a more polar compound. Translated to GC, we then use the old maxim "like dissolves like" to think about which stationary phase to choose to retain a given analyte or set of analytes. The stationary phase that seems to have a structure most similar to the analytes or the stationary phase that is on hand is often the eventual choice.

Whenever I am told that a compound is polar, I ask, "Compared to what?" As we are in winter here in the eastern United States, I am reminded of a common and often entertaining social situation—talking about the weather. Depending on who you talk with, a nice January day in New York City, with an average temperature of 39 °F or 4 °C, may be either hot or cold, depending on who is discussing it. My friend from Florida might think it is cold, whereas my other friend from Vermont might think it is warm. So which is it? Polarity must be thought of in a similar fashion in that the word by itself is meaningless without a standard for comparison.

In GC, there are two common measures for providing this comparison, both of which we discussed in an earlier column (1). These are Rohrschneider-McReynolds constants, developed in the 1960s, and a polarity scale developed by Mondello in 2011 (2–4). As discussed in the previous column, the Mondello polarity scale is based on a sum of McReynolds constants, so both measures have the same basis. Both methods can easily lead to the common assumption that higher numbers for the constants indicate greater retention and greater selectivity for compounds that have a similar structure to the test probes.

To examine this assumption, consider three stationary phases (PDMS, PEG, and an ionic liquid, SLB-IL-100) whose structures are shown in Figure 1. A quick examination of the structures shown in Figure 1 shows a different chemistry for each stationary phase. We can expect that PDMS retains analytes by dispersive interactions, indicating strong retention for analytes containing high hydrocarbon content. PEG, with a high hydroxy content, indicates strong retention of analytes capable of hydrogen bonding. Finally, SLB-IL-100, a molten salt, has complex retention properties, but because it is a salt, we can expect that it will not strongly retain hydrocarbons. Although a long hydrocarbon chain is seen in the structure, it seems likely that this chain, being flexible, bends to a conformation that exposes more of the polar and ionic character of the structure.

Table I shows McReynolds constants and the sum of the constants for the three stationary phases seen in Figure 1. The constants and the total are much higher for the PEG and ionic liquid stationary phases than for PDMS. Details on how the McReynolds constants are determined using Kovats retention indexes and how the sum of the McReynolds constants are used to determine polarity numbers are provided in a previous column (1).

Kovats retention indexes and McReynolds constants are determined isothermally, so the temperature also has a major impact on the result and must be known. Figure 2 shows a chromatogram of four of the test probes used for determining McReynolds constants plus the necessary alkanes on a

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PDMS stationary phase, simulated using ProEZ GC online software (5). To calculate the McReynolds constant for an analyte, such as benzene (peak 2), in the chromatogram, the adjusted retention times of hexane (peak 1), benzene, and heptane (peak 5) must be known. The actual Kovats retention index for benzene in this chromatogram is 655, indicating that it is eluted about halfway between hexane (600) and heptane (700). The McReynolds constant for benzene would then be determined by subtracting the Kovats retention index for benzene on squalene from the Kovats retention index on PDMS.

Benzene is an aromatic hydrocarbon that undergoes both dispersive and pi–pi interactions. McReynolds constants are determined by the difference between the Kovats retention indexes measured on the stationary phase of interest and on a classical nonpolar stationary phase, squalene, which is the liver oil from sharks. One challenge when working with McReynolds constants today is the availability of columns made using squalene, which were popular in packed columns but not used in capillary columns. Our nonpolar stationary phase in this discussion, PDMS, is considered the most nonpolar stationary phase in GC today.

Looking more closely at the constants for benzene shown in Table I, we see that the constants are much higher for PEG and SLB-IL-100, implying that the more polar stationary phases should retain benzene much more strongly. However, this is not the case.

Table II shows the retention factors, McReynolds constants, Kovats retention indexes, and polarity numbers for benzene on the three stationary phases. The retention factors were determined at 60 °C. With the thin film columns used, we see that the retention factors are low, indicating that retention is not strong. We quickly make a couple of interesting observations. First, benzene is more strongly retained, as seen by the higher retention factor on PDMS, the least polar column than on SLB-IL-100. This observation may not be too surprising as PDMS primarily exhibits dispersion forces which are prevalent in benzene while SLB-IL-100 does not.

If benzene is less retained on SLB-IL-100, then why are the McReynolds constants so much higher? Looking at the Kovats retention index data in Table II, we see that the hydrocarbons that are used as standards in the calculation must shift to much lower retention factors themselves as the stationary phase becomes more polar, whereas the retention factor of benzene remains roughly the same or decreases. On PDMS, benzene is eluted between hexane and heptane. On PEG, it is eluted between nonane and decane. On SLB-IL-100, it is eluted after undecane. In short, the n-alkanes have shifted to a much shorter retention, but retention of the analyte remains approximately the same. If this is the case, is it appropriate to then state that the stationary phase is more polar, more retentive of the analyte, or has higher selectivity for similar analytes?

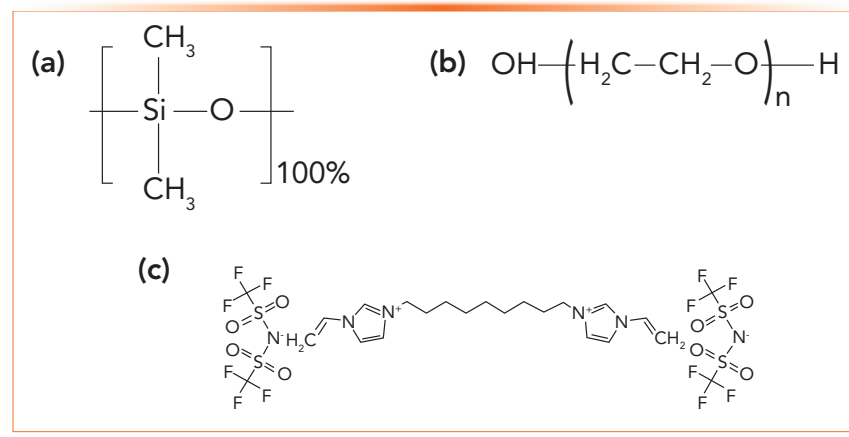

FIGURE 1: Structures of nonpolar, polar and very polar stationary phases. (a) poly-dimethylsiloxane, PDMS (nonpolar), (b) polyethylene glycol, PEG (polar), (c) SLB-IL-100 (very polar).

TABLE I: McReynolds constants for three stationary phases

Probe	Name	Interactions	PDMS	PEG	SLB-IL-100
X′	Benzene	Pi–pi interactions for aromatics and olefins	16	334	602
Y′	Ethanol	Proton donor and acceptor	55	509	853
Z′	2-pentanone	Proton acceptors	44	375	884
U′	Nitropropane	Dipole–dipole interactions	65	601	1017
S′	Pyridine	Strong proton acceptor	42	505	1081
	Total		222	2324	4437

TABLE II: Retention data for benzene at 60 °C on three stationary phases

Stationary phase	Polarity Number	Kovats Retention Index	McReynolds constant	Retention Factor (*k*)
PDMS	5	654.9	16	0.426
PEG	52	954	334	0.718
SLB-IL-100	100	1114.1	602	0.323

Does Polar Mean Retentive?

Does a high polarity, indicated either by large McReynolds constants or by a high polarity number, always indicate that a stationary phase will be retentive, giving long retention times? From the benzene example, a high polarity number or a high McReynolds constant does not always mean long retention times or high retention. Although the constants are a convenient and often illustrative example, we must consider how they are

generated and whether the constants change because of a shift in retention of the analyte or of the n-alkane standards.

Does Polar Mean Selective?

Does a high polarity number or a high McReynolds constant mean that a column will also be highly selective? Because the polarity number scale is derived from a combination of several McReynolds constants, it is not useful in determining selectivity. It is just an overall measure of polarity, and because it considers several intermolecular interactions, it cannot be used to determine the effectiveness of the stationary phase to separate based on any one of them. A high McReynolds constant can be used as an indicator of selectivity for compounds that exhibit similar properties as the test probe, but this should be used with caution. If the high McReynolds constant results from a shift in alkane retention, rather than analyte retention, then the stationary phase might not exhibit the expected selectivity.

What About the Use of n-Alkanes?

The use of n-alkanes as standards in the Kovats retention index calculation and by extension for the McReynolds constants and the polarity scale presents an interesting problem. Alkane retention provides a measure of dispersive interactions which are major components of retention in both gas chromatography and reversed-phase (RP) high performance liquid chromatography (HPLC). Because n-alkanes themselves exhibit high dispersive interactions, using them as standards effectively subtracts dispersive interactions out of the discussion of retention.

Dispersive interactions, also called *London dispersion forces*, are by far the most seen intermolecular interaction in GC. We remember from undergraduate general chemistry that they occur in nonpolar molecules or in the nonpolar portion of larger or polar molecules. They occur because electron clouds are rarely evenly distributed around the molecule, generating momentary dipoles. This is especially evident in aliphatic hydrocarbons, which contain only nonpolar carbon–hydrogen bonds, arranged in a tetrahedral orientation. As the hydrocarbon chains get longer, dispersive interactions between molecules of the hydrocarbon increase, generating an increased boiling point. Similarly, increased dispersive interactions between analyte molecules and the stationary phase lead to longer retention times in GC.

Looking at n-alkanes with our three stationary phases, we easily see that n-alkanes will be most strongly retained on PDMS, followed by PEG and then by SLB-IL-100, because of the strongest dispersion forces being with PDMS, followed by PEG and very little dispersion force with SLB-IL-100. In the case of SLB-IL-100, the stationary phase repels alkanes so strongly that undecane, with a normal boiling point of 384 °C, has a retention factor of approximately 0.3, with 70% of the undecane molecules in the vapor phase at 60 °C, indicating very little retention.

What is the Solution?

The first solution to this problem is to consider the actual structure of the analytes and the expected intermolecular interactions qualitatively and carefully with the stationary phase,

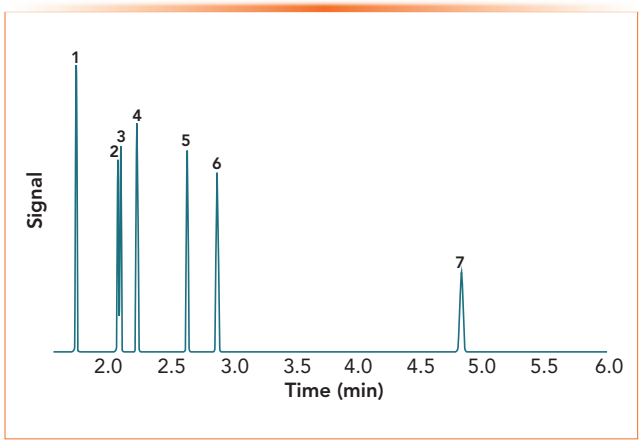

FIGURE 2: Pro EZGC chromatogram modeler for McReynolds probes and alkanes on PDMS. Column: PDMS, 30.00 m, 0.25 mm i.d., 0.25 µm, carrier gas: helium, constant flow @ 2.03 mL/min, Average velocity: 41.37 cm/sec, outlet pressure: 14.70 psi, oven temperature: 40 °C. Sample: 1) hexane (C6), 2) benzene, 3) 1-butanol, 4) 2-pentanone, 5) heptane (C7), 6) pyridine, and 7) octane (C8).

rather than simply looking at the McReynolds constant related to that interaction. Stronger retention can still be expected from stationary phases that exhibit similar intermolecular interactions, such as dispersion, pi–pi, or hydrogen bonding of the analytes. If a more rigorous analysis is needed, thermodynamic retention indexes, which are based on classical thermodynamics of separations and form the basis for chromatography modeling software, can be used, but these can also be tedious (5,6). To date, there is still no systematic way to fully predict separation power of a given stationary phase other than experimentation and classical method development.

Conclusions

Polarity and polar are common and easily misunderstood terms used to describe stationary phases in GC. When polar is used to describe a stationary phase or a solvent, the question, "Compared to what?" must be asked. PEG would be considered more polar compared to PDMS but less polar when compared to SLB-IL-100. The common measures of stationary phase polarity—McReynolds constants and the polarity scale—are not always accurate predictors of retentiveness or selectivity because of the use of n-alkanes as retention time standards. A truly systematic and easy-to-use means for predicting retentiveness and selectivity based on stationary phase chemistry remains elusive.

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FOCUS ON BIOPHARMACEUTICAL ANALYSIS

Analytical Methods to Determine the Stability of Biopharmaceutical Products

Analytical methods are used in the biopharmaceutical industry to ensure the quality, efficacy, and safety of drug substances and drug products. One of the fundamental measures of the quality of a drug substance or drug product, including biopharmaceuticals, is the stability of the active pharmaceutical ingredient (API). In fact, the first International Council for Harmonization (ICH) guideline on quality, *ICH Q1,* is for drug stability. In this column, we look at drug stability in general, differences between large- and small-molecule stability (*ICH Q1* and *ICH Q5*), as well as the analytical methods used to measure the stability of a product. Although there are several analytical methodologies that can be used, and we touch on those briefly, we focus mostly on the chromatography and mass spectrometry methods used to characterize drug stability.

Jared R. Auclair and Anurag S. Rathore

At the center of everything we do in the biopharmaceutical industry should be patients and ensuring that their medicines are high quality, safe, and effective. A cornerstone of any drug substance or drug product quality assessment is stability. Ensuring the stability of a biologic (or any pharmaceutical) gives patients confidence that their drugs are efficacious, perform as expected, and are available when needed. Stability ensures that patients receive the same high-quality product throughout the product's expiry period (2).

Drug stability, both pharmaceutical and biopharmaceutical, can be defined as the ability of a drug substance or product to retain the same properties and characteristics, within specified limits, that it had at the time of manufacture (3). Biopharmaceuticals are complex drugs that comprise thousands of molecules and a complex amino acid chain that is folded into complex structures. As a first-year biochemistry student can tell you, that complex structure directly impacts protein function. Thus, in biopharmaceutical stability, we are looking for the correct structure of a given protein therapeutic, such that its function, or its specific therapeutic effect, is achieved. Ensuring the stability of the drug substance or product ensures a high-quality product, accurate dosing, identification of degradants, mitigation of impurity-induced adverse events, and increased patient compliance.

In the case of a biopharmaceutical, instability is most commonly referring to degradants in terms of fragments or aggregation (protein aggregation was discussed in a previous set of columns in 2015 (4,5). Product instability can lead to a loss of potency or efficacy. Degradation products can lead to impurities and contaminants and safety and toxicity issues. They can also lead to problems with immunogenicity, an unwanted immune response against oneself (6), and alterations of bioavailability. In addition, product instability can affect appearance, smell, feel, taste, and precipitation, which may impact both product efficacy and safety but also patient compliance.

This all highlights the importance of understanding drug stability. How do we characterize drug stability? To determine the stability of a biopharmaceutical, a stability testing program is developed and implemented throughout the life cycle of the product. This stability program can comprise various components, which we will not go into in any great detail. However, briefly, one might consider long-term stability testing, accelerated stability testing, annual (follow-up) stability testing, and discrepancy testing as key components of a stability testing program. Long-term testing of the drug substance or drug product serves as the basis of the recommended storage conditions and shelf-life. Accelerated stability testing provides information on degradation pathways that the product is most vulnerable to. Annual testing provides additional information to guide long-term storage conditions. Discrepancy testing can confirm the stability of specific batches. In addition to these tests, other types of studies commonly seen in stability testing are cumulative expiry (for biologics, supports processing time, temperature, freeze, thaw, and refiltration), temperature cycling, photostability, reconstitution, comparability, and in-use studies (2,7). Thus, one of the major end goals of a stability testing program is determining shelf life or expiry under specific conditions, which are determined to support the maximum stability of the drug over time. The United States Food and Drug Administration (U.S. FDA) defines an expiration date as "the time period during which the product is known to remain stable, which means it retains its strength, quality, and purity when it is stored according to its labeled storage conditions" (8).

Stability Guidelines

With a general understanding of drug stability in hand now, we can briefly discuss interna-

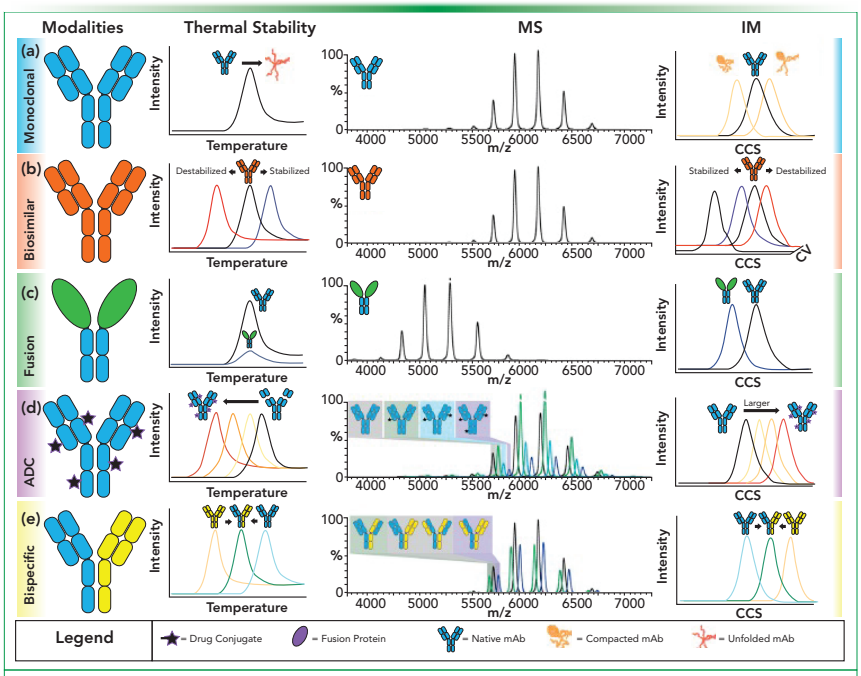

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FIGURE 1: Data and information content that can be expected from variable-temperature, MS, and IM data sets for the following biotherapeutic modalities: (a) mAbs, (b) biosimilars, (c) fusion proteins, (d) antibody–drug conjugates, and (e) bispecific antibodies. For variable temperature experiments, shifts to lower Tm values indicate a decrease in stability and higher values indicate an increase in stability. Changes in mass spectrometry indicate different structures or stoichiometries. For IM, shifts to lower CCS values indicate more compact structures while larger values indicate larger, often unfolded structures. By applying activation energy and monitoring unfolding, for example biosimilar IM, shifts in stability can be monitored by shifts in the IM peak relative to the activation energy.

TABLE I: ICH Guidelines: international guidelines for drug stability

ICH Code	Guideline Title
Q1A(R2)	Stability Testing of New Drug Substances and Products
Q1B	Stability Testing: Photostability Testing of New Drug Substances and Products
Q1C	Stability Testing for New Dosage Forms
Q1D	Bracketing and Matrixing Designs for Stability Testing of New Drug Substances and Products
Q1E	Evaluation of Stability Data
Q1F	Stability Data Package for Registration Applications in Climatic Zones III and IV*
Q5C	Stability testing of Biotechnological/Biological Products

*Q1F was withdrawn at the Yokohama June 2006 ICH meeting and definitions of Climatic Zones III and IV to the respective regions and the WHO guidelines (9).

tional guidelines for drug stability and how the differences between small (pharmaceuticals) and large (biopharmaceuticals) molecules are characterized. Over the years, there have been several guidelines developed by international consortia to individual drug authorities, World Health Organization (WHO) and others (2). Here, we focus on guidelines developed by one of those international consortia, the International Council for Harmonization (ICH), specifically the *ICH Q1* series (Table I).

The *ICH Q1* series of guidelines, like all ICH guidelines, promotes a science-based, risk-based evaluation of drug substances and products related to stability. In addition, they allow for flexibility in their implementation so that they can be broadly implemented across the world. *ICH-Q1A(R2)* (with the *R2* indicating revision 2 of the guideline) is the foundational ICH guideline related to drug stability. The purpose of *ICH Q1A(R2)* is to provide basic guidance on how to ensure the quality of a drug substance or product under four general key considerations: selection of batches; study design; evaluation and outcome; and stability commitment. For the selection of batches, *ICH Q1A(R2)* provides guidance on what is considered a primary batch and production batch, how to select batches for specific testing, and how to consider storage conditions such as container closure. It provides guidance on formal stability studies, supporting data to be considered, how to conduct stress studies, and storage condition studies (for example, temperature and humidity), including long-term, intermediate, and accelerated testing and frequency of testing. *ICH Q1A(R2)* provides guidance on how to evaluate stability data and its outcomes, including specifications and significant changes. *ICH Q1A(R2)* also provides guidance on the appropriate stability commitment to propose and implement. It is important to note, that *ICH Q1A(R2)* specifically addresses pharmaceutical products (small molecule drugs), but more on that later (7).

The remaining *ICH Q1* guidelines were developed and implemented in support of *ICH Q1A(R2)*. For example, *ICH Q1B* was developed to address the possibility that light exposure can affect product quality. Specifically, *ICH Q1B* provides guidance to characterize the intrinsic photostability of new drug substances and products. It also offers guidance on the evaluation of photostability data to ensure light exposure does not result in changes to the drug substance or product. *ICH Q1C*, one of the shortest ICH guidelines, reiterates the application of *ICH Q1A(R2)* for new dosage forms. *ICH Q1D* provides guidance for using bracketing and matrixing in stability studies. *Bracketing* is defined as evaluating samples on the extremes of certain design factors at all time points. *Matrixing* is defined as evaluating a subset of the total number of possible samples for all factor combinations tested at a specific time point. *ICH Q1E* provides guidance on how to perform stability data evaluation and on such topics as extrapolation (using a known data set to infer information about future data sets)

(10–13). And finally, *ICH Q1F* was withdrawn at the Yokohama June 2006 ICH meeting and definitions of Climatic Zones III and IV were left to the respective regions and the WHO guidelines (9). For reference, Table II provides the climatic zones used in temperature and humidity stability studies, including Zones III and IV from WHO.

Like *ICH Q1A(R2)*, *ICH Q1B* through *ICH Q1E* were written with pharmaceutical (small molecule) products in mind and are largely applicable to biopharmaceuticals. However, as we know, there are significant differences between large and small-molecule drugs. Briefly, more traditional pharmaceuticals are comprised of a defined chemical structure of identical copies and are synthesized chemically. There is rarely biological contamination, there are defined standards and specifications for impurities, there are sensitive, well-defined standards and discriminating analytical characterization methods, and stability programs that are well-defined and can be modeled. On the other hand, biopharmaceuticals are comprised of complex chemical structures

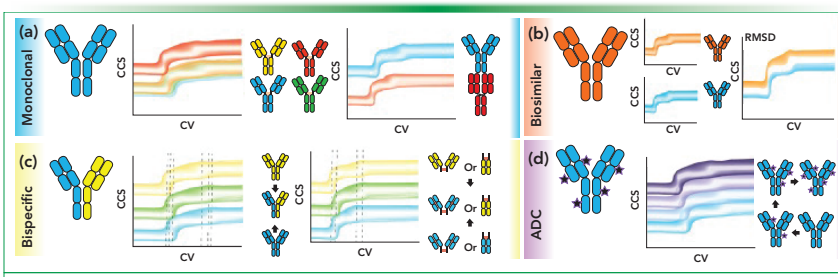

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FIGURE 2: CIU applications for biotherapeutic mAbs. (a) Differentiation of monoclonal IgG subclasses by disulfide bonding patterns and difference in CIU unfolding because of domain exchange. (b) Biosimilar antibodies have qualitatively similar fingerprints, but contemporary CIU analyses can quantitate subtle differences in stability. (c) Bispecific antibodies present CIU characteristics centered between the precursor structures. (d) Shifts in CCS and stability can be quantified as a function of increasing drug load in ADC biotherapeutics.

TABLE II: ICH climatic zones, including zones III and IV from WHO (2,7).

Climatic Zone	Type of Climate	Reference Condition
I	Temperate	21 °C, 45% RH
II	Subtropical and Mediterranean	25 °C, 60% RH
III	Hot and dry	30 °C, 35% RH
IVa	Hot and humid	30 °C, 65% RH
IVb	Hot and very humid	30 °C, 75% RH

that are comprised of thousands of atoms making up heterogeneous macromolecules made in living cells. Biopharmaceuticals are prone to biological contamination because of contamination for the production process using living cells. There are also process and product-related impurities with no fixed acceptable threshold and multiple orthogonal analytical methods used for characterization (see next section). And finally, biopharmaceuticals are susceptible to stability loss through temperature, shear, and light (14). These differences lead to the development of specific guidance, *ICH Q5C*, related to biopharmaceutical stability.

ICH Q5C provides guidance on stability testing of biotechnological and biological products and applies to well-characterized proteins and polypeptides isolated from tissues, body fluids, cell cultures, or if they are produced using recombinant deoxyribonucleic acid (DNA) technology. A well-characterized biologic is defined by its product quality attributes, such as appearance, purity, activity and quantity, and structure. Many aspects of stability testing contribute to elements of a well-characterized biologic, such as specifications, analytical characterization, product trending, process controls, and monitoring and adherence to current Good Manufacturing Practices (cGMP). Specifically, *ICH Q5C* provides specific guidance for biologics around a selection of batches, stability-indicating profile, storage conditions, test frequency, specification, and labeling. In addition, *ICH Q5C* in sections 5 and 8 provides guidance on test methods to detect stability-indicating profiles (15).

Analytical Characterization of Stability

To determine the stability of a biopharmaceutical, several orthogonal analytical techniques are employed for both the drug substance and the drug product. Generally speaking, these stability monitoring techniques must be able to detect changes in identity, purity, and potency. Methods are validated at the time of market approval.

For potency, *ICH Q5C* (15) recommends cell-based bioassays or using enzyme-linked immunosorbent assay (ELISA)-based binding assays for both drug substances and drug products. To monitor purity, *ICH Q5C* recommends monitoring size variants and charge variants. To monitor charge variants for both drug substance and drug product several methodologies are suggested including size exclusion chromatography-high performance liquid chromatography (SEC-HPLC), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and capillary electrophoresis (CE)-SDS. Of note, SEC-HPLC is particularly useful in monitoring stability because it can differentiate aggregates and high molecular weight species, which are indignant of protein instability (16). Ion exchange (IEX)-HPLC and isoelectric focusing are recommended for monitoring charge state variants for drug substances and products. *ICH Q5C* also recommends monitoring the appearance and protein concentration for drug substance stability. For drug product stability, *ICH Q5C* recommends monitoring the appearance, color, clarity, protein concentration, subvisible particles (aggregates), container closure integrity, stabilizers, and preservatives.

In addition to these analytical methods, liquid chromatography–mass spectrometry

(LC–MS) has become increasingly used in monitoring biopharmaceuticals and biopharmaceutical stability. A recent review by Vallejo and others (1) highlights the use of LC–MS as a tool to monitor biomolecular stability. This review gives a thorough look at MS-based techniques used to monitor protein stability from native MS to ion mobility–MS (IM–MS). However, for this column, let us review some of the trends in biotherapeutics.

For biopharmaceuticals, in particular monoclonal antibodies (mAbs), structurally sensitive MS methods have become more widely used, critical perhaps, to rapidly analyze high molecular weight species. Some of these trends include variable-temperature MS, footprinting MS, IM–MS, and collision-induced unfolding (CIU). Figure 1 provides representative thermal stability, MS, and IM–MS stability data for mAbs, biosimilars, fusion proteins, antibody–drug conjugates (ADCs), and bispecific antibodies. Variable-temperature experiments can monitor protein stability through monitoring melting temperatures whereas gas-phase technologies, such as MS, can be used to detect changes in gas-phase mAb structures in correlation with variable temperature experiments or changes in CIU mode. In variable temperatures, MS mAb samples are heated within the ESI source and the detection of degradation products and non-native disulfide bonds can be used to characterize the mAb stability (1,17). Footprinting MS techniques, such as hydrogen deuterium exchange (HDX)-MS or covalent labeling (CL)-MS, can provide information on structural integrity, changes in high molecular weight species, and antigen interactions.

IM–MS has been shown to be a powerful tool to characterize disulfide bonding patterns, structural differences between a biosimilar and its reference product, glycosylation patterns, and several other key structural elements linked to stability. Variable temperature IM–MS can also be used to monitor structural changes where the temperature of the IM-MS drift gas is adjusted. These techniques in parallel to CIU methods can help with monitoring domain structure and anion and cation adduction and binding. In addition, Figure 2 shows representative data of how CIU can monitor disulfide bonding, glycosylation patterns, ADC drug loading, domain exchange, HDX uptake, light-chain variants, bispecific stoichiometries, and differences between reference products and biosimilars (1).

Conclusion

Biopharmaceutical stability is a fundamental part of ensuring product quality, safety, and efficacy. It refers to the ability of a drug substance or product to retain the same properties and characteristics, within specified limits, that it had at the time of manufacture (3). Several different organizations, such as ICH, produce guidance on how to monitor drug stability throughout a product's life cycle. The monitoring of biopharmaceutical stability requires several orthogonal techniques that include LC (for example, SEC, reversed-phase LC) and MS. LC methodologies are well established for monitoring stability and MS methodologies are becoming increasingly used. Regardless of the stability program in place, and the analytical technologies used in that program, drug stability is a cornerstone of ensuring that patients get the drugs they need when they need them.

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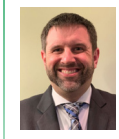
ABOUT THE AUTHOR



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Harmonization of Experimental Methods Used to Measure the True Hold-Up Volume of Liquid Chromatography Columns

There are as many measurement values of the true column hold-up volume, V_0, as techniques applied to evaluate this most important property in liquid chromatography (LC). The relative errors made on V_0 measurements using conventional "non-retained" markers—such as acetone, uracil, or thiourea in reversed-phase liquid chromatography (RPLC), or benzene or acenaphthene in hydrophilic interaction chromatography (HILIC)—can be as large as ±30%. This situation is extremely confusing for LC users who wish to classify and predict the retention behavior of LC columns. In this work, along with advances in mass spectrometry (MS) instrumentation, the hold-up volume of any LC column—including, but not limited to, RPLC, HILIC, ion exchange chromatography (IEX), and mixed-mode columns—is accurately measured by injecting labeled deuterated acetonitrile (CD_3CN) molecules and detecting them selectively by MS-single ion reaction (m/z = 45) using non-labeled and pure acetonitrile (CH_3CN) as the eluent. This proposed harmonization of all conventional V_0 measurement methods is illustrated and successfully applied to RPLC, HILIC, anion exchange (AEX), and RP-AEX mixed-mode chromatography, irrespective of the mobile phase composition selected.

Fabrice Gritti and Kerri Smith

Knowledge of the column hold-up volume in liquid chromatography (LC) is often required by users to accurately measure retention factors, to compare the retentivity of various manufactured columns and classify them, to control and assess the quality of the retention and selectivity properties of such columns, and to predict the retention times of analytes in both isocratic and gradient elution conditions. However, since the very birth of LC, accurate measurement of the column hold-up volume in LC has always been a subject of debates and controversy (1–3). The main reason is that, unlike in gas chromatography (GC), the delimitation between the mobile-phase and stationary-phase volumes is always ambiguous in LC. This is especially true when the mobile phase used is a solvent mixture where one solvent may interact with the stationary phase differently from the other solvents. As a result, a thick interfacial region with a solvent composition different from that in the bulk mobile phase is formed between the impermeable solid surface and the bulk eluent (4,5). Thus, it has become impossible to unambiguously delimitate the mobile and stationary phases in LC.

Current and routine methods adopted for the measurement of the column hold-up volume in LC abound. They have been reviewed extensively in the past (6–9); most are based on the measurement of the elution times of so-called "non-retained" markers—such as thiourea, uracil, or acetone for reversed-phase liquid chromatography (RPLC) columns; benzene for hydrophilic interaction chromatography (HILIC) columns, and small neutral markers in ion exchange chromatography (IEX). Some rely on the elution times of a series of homologous compounds (such as n-alkyl benzenes and phenones for RPLC columns) combined with an arbitrary linear free energy relationship (LFER) model of retention, while others are static methods, such as pycnometry, in which the column is weighed when filled with two distinct pure solvents having significantly different densities (for example, a methanol:dichloromethane pair). Some more tedious methods are based on minor disturbances of the solid–liquid equilibrium over the entire range of mobile phase composition for a binary eluent mixture. The minor disturbance method is somewhat cumbersome, because it relies on measurement of the wave speed of a non-labeled eluent perturbation for a series of equilibrium plateau concentrations ranging from pure water to pure organic solvent. A refractive index detector is usually needed, and, overall, this approach lacks simplicity. The adoption of non-retained markers is very convenient, but the measurement accuracy of the hold-up volume is often uncertain. In the end, each method carries its own advantages and downsides in terms of accuracy and precision, and there are as many observed hold-up volumes as methods used to measure this important column property. This situation can be very confusing for users because each method returns a V_0 value that is different from that obtained from another method. The selection of the most accurate method is then always ambiguous. Thus, we need to harmonize these different V_0 methods into a single method that accurately and precisely measures the unique hold-up volume of any chromatographic column, irrespective of its retention mode (RPLC, HILIC, SEC, ion exchange, mixed-mode, or other).

In this article, the problem faced by HPLC users when measuring the column hold-up volume is first clearly illustrated based on classical injections of assumed "non-retained" markers in RPLC and HILIC columns. A user-friendly, accurate, and precise method is then proposed to troubleshoot this problem based on the fundamentals of adsorption in solid-liquid adsorption and on recent advances in mass spectrometry (MS) detection. The method is directly applied for the determination of the hold-up column volume of RPLC, HILIC, IEX, and RP–anion exchange (AEX) mixed-mode columns. Finally, the relative errors made when the conventional use of "non-retained" markers are determined.

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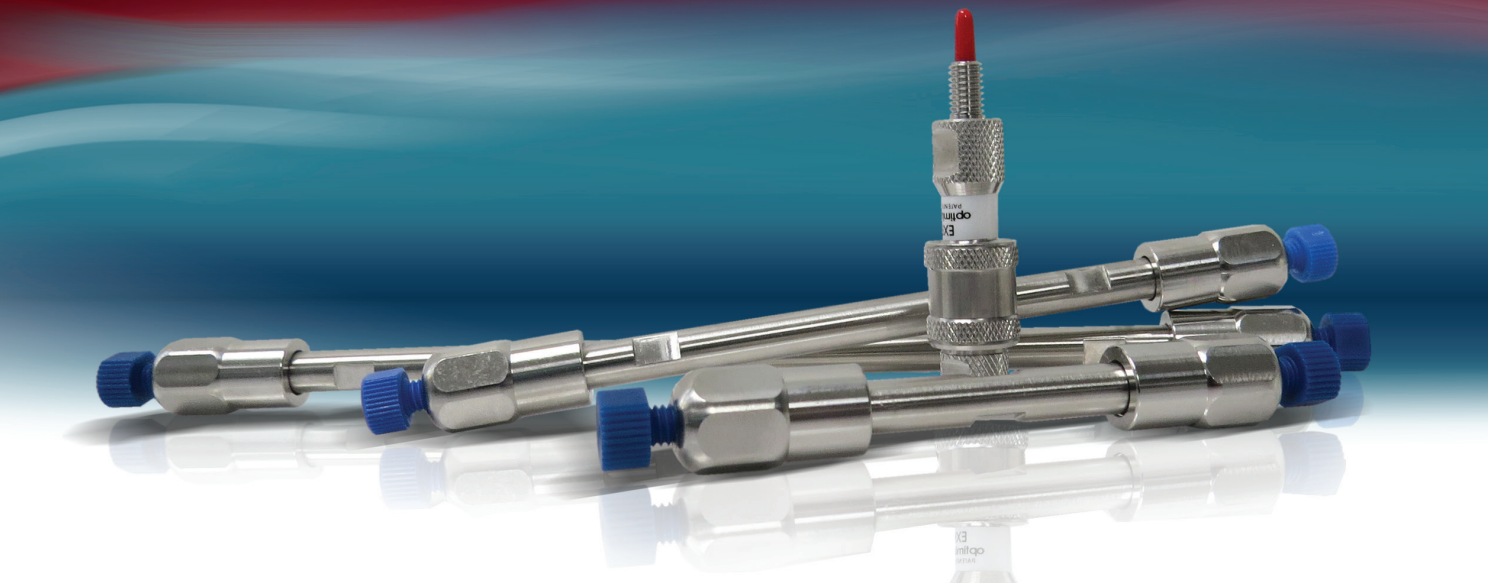

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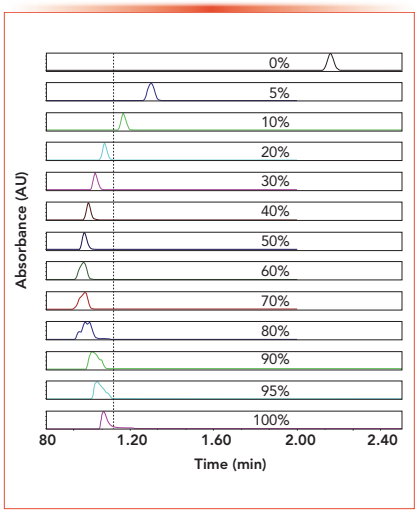

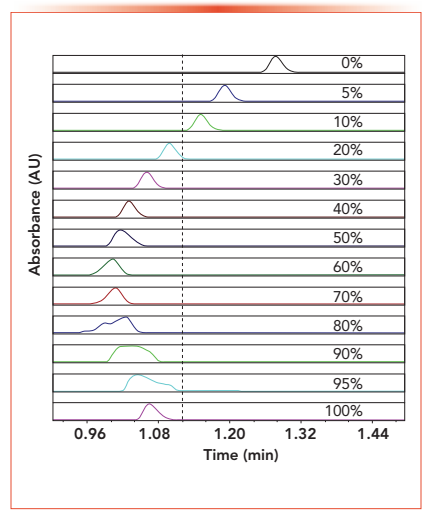

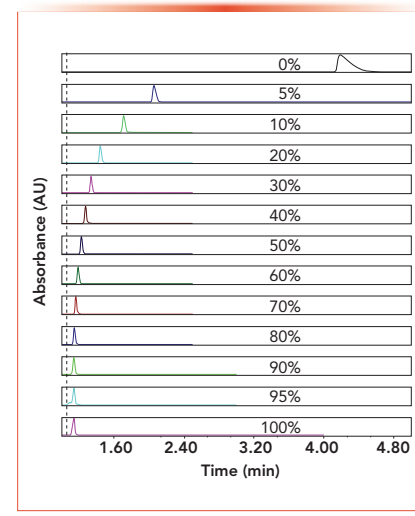

FIGURE 1 (LEFT): Evolution of the retention and peak shape of the assumed "non-retained" compound uracil in RPLC as a function of the volume fraction of acetonitrile increasing from 0 to 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100% as indicated in the graph. The sample uracil is dissolved in pure water. Injection volume: 1 µL; flow rate: 0.2 mL/min; T = 24 °C; 2.1 x 100 mm 1.7 µm BEH-C18 column. The vertical dashed line locates the elution time of the ideal t_0 marker.
FIGURE 2 (MIDDLE): Same as in Figure 1, except for the assumed "non-retained" compound thiourea.
FIGURE 3 (RIGHT): Same as in Figure 1, except for the assumed "non-retained" compound acetone.

Materials and Methods

Eight different HPLC columns (including RPLC, HILIC, RP-AEX mixed mode, and AEX columns) were used in this work for the measurement of their hold-up volumes:

- Two RPLC-C18 columns: a 2.1 x 100 mm column packed with 1.7 µm XBridge-C18 particles (Waters) and a 4.6 x 150 mm packed with 5 µm Sunfire-C18 particles (Waters).
- Three HILIC columns: a 2.1 x 150 mm column packed with 3.0 Atlantis HILIC silica particles (Waters), a 2.1 x 150 mm column packed with Acquity Premier 1.7 µm BEH amide particles (Waters), and a 4.6 x 150 mm column packed with 5.0 µm BEH amide particles (Waters).
- Two RP-AEX mixed mode columns: a 4.6 x 150 mm column packed with 5 µm charged surface hybrid (CSH) C18 particles (Waters) and a 4.6 x 150 mm column packed with 5 µm Atlantis Premier BEH-C18 AX particles (Waters).
- One AEX column: a 4.6 x 150 mm column packed with 5 µm Spherisorb SAX particles (Waters).

The measurements of the column hold-up volumes were performed on an Acquity H-class UPLC system (Waters). This LC system is equipped with a quaternary solvent delivery pump, a gradient proportioning valve, a 15 µL injection loop, a sample manager, a one-column oven compartment, and either an optical detector (500 nL optical cell, TUV monochromatic) or a Xevo TQ-S micro tandem quadrupole mass spectrometer (Waters). After replacing the LC column with a zero-dead-volume union connector, the total extra-column volume (from the LC injection valve to the electrospray ionization source) was measured by injecting 1 µL of CD_3CN and using pure acetonitrile as the eluent. Seven different flow rates increasing from 0.1 to 0.2, 0.4, 0.8, 1.0, 1.5, and 2.0 mL/min were applied. The *y*-intercept of the plot of the elution volume of CD_3CN as a function of the flow rate gives a total extracolumn volume of 27.2 µL and an offset delay time of 0.7 s between the moment the injection valve actuates (slight delay) and the moment the zero-start of MS signal is recorded. For the selective detection of deuterated acetonitrile, the electrospray ionization voltage was set at 3 kV, the cone voltage at 10 V, the desolvation temperature was fixed at 500 °C, the flow rate of the desolvation gas was fixed at 1200 L/h, and the cone gas flow rate was set at 50 L/h. The whole system was automated by either Empower version 3.0 software (Waters) for optical detection or MassLynx v4.2 (Waters) for single ion reaction (CD_3CN, $m/z = 45$) mass detection.

Water, acetonitrile, and acetone solvents were optima MS grade purchased from Fisher Scientific. Thiourea, uracil, benzene, deuterated acetonitrile (CD_3CN), phosphoric acid, mono- and disodium phosphate salts, and ammonium acetate salt were purchased from Sigma-Aldrich with 99%+ purity.

All the mobile phases were prepared by weight. For the five 4.6-mm i.d. columns, the flow rate was 1.0 mL/min, and the column temperature was set at 30 °C. For the three 2.1-mm i.d. columns, the flow rate was 0.2 mL/min, and the column temperature was room temperature (24±1 °C). The markers thiourea, uracil, and acetone were dissolved in pure water. The markers benzene and acenaphthene were dissolved in pure acetonitrile or an acetonitrile:water mixture (75:25, v/v), respectively. The injection volume was fixed at 1 µL (2.1 mm i.d. columns) and 5 µL (4.6 mm i.d. columns).

All the details concerning the methods applied in the molecular dynamics simulations are provided in reference 5. Briefly, the mesopore model consists of a three-layer silica slab (0.93 nm thick) bearing silanol groups at a surface coverage of 7.5 µmol/m^2. The silica surface is randomly grafted with octadecyl C18 (3.11 µmol/m^2) and trimethyl silane (0.93 µmol/m^2) groups. The validated force fields used to calculate the Brownian motions of all the atoms (Si, C, O, H, and N) involved in the equilibrium process are listed in reference (5). The reported density profiles were calculated from the atom number densities of the central C atom of acetoni-

trile and the center-of-mass of the analytes acetone and uracil.

Results

An Old Recurring Problem

RPLC

Figures 1–3 illustrate the old recurring problem faced by chromatographers when they select a hypothetically "non-retained" column dead time (t_0) marker (either uracil in Figure 1, thiourea in Figure 2, or acetone in Figure 3) for the V_0 measurement of the same RPLC column (a 2.1 x 150 mm 1.7 µm BEH-C18 column). These three figures show the retention and shape of the observed peak profiles of these three markers as a function of the mobile-phase composition. Acetonitrile is used as the organic solvent in the aqueous mobile phase. The volume fraction of the organic solvent in water is increased from 0 to 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100%, as indicated in the legend of the graphs. For the sake of reference, the expected elution time (eventually measured with the accurate t_0 method proposed in this work) of a truly non-retained marker is indicated by the vertical dashed line.

Remarkably, the retention times of these three conventional markers are strongly dependent on the mobile-phase composition. A U-shaped retention profile is clearly observed for both uracil and thiourea, suggesting the prevalence of RPLC-like interactions in water-rich mobile phases and of HILIC-like interactions in water-poor mobile phases (uracil and thiourea contains two HN-C=O and -NH_2 polar groups, respectively). Additionally, because these markers are typically either slightly retained onto the silica-C18 stationary phase or slightly excluded from the organic-rich pore volume, their peak shape is inevitably affected by the eluent (0 to 100% organic)-to-diluent (100% water) composition mismatch when the volume fraction of the organic solvent in the mobile phase exceeds ~50%. As far as the marker acetone is concerned, its retention time decreases evenly with the increase in organic solvent, suggesting that mostly hydrophobic interactions are involved in the retention mechanism of this marker, irrespective of the mobile-phase composition. Similar observations (not shown) and conclusions have been reported in this work when the organic solvent acetonitrile is replaced with methanol.

In the end, the main problem arising from the observations in Figures 1–3 (three non-retained markers, 13 mobile phase compositions) is that chromatographers are left with no clue about how to decide for the true value of the hold-up volume of this particular RPLC column. Should chromatographers consider as true the smallest retention time of uracil or thiourea, which is 0.96 min for 80% acetonitrile in the aqueous eluent? Or should they arbitrarily select the smallest retention time (1.16 min) of acetone in 100% organic solvent? To date, there is no scientific rationale enabling chromatographers to prefer one such t_0 marker over another for the accurate measurement of the hold-up volume of a RPLC column.

HILIC

Similar to Figures 1–3, Figure 4 (online) shows the peak profiles of the hypothetically non-retained marker benzene to measure the hold-up volumes of the 2.1 x 150 mm 1.7 µm BEH-amide HILIC column. The volume fraction of acetonitrile in water increases from 5 to 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, and 100%, as indicated in both graphs (pure water was not considered as an eluent because the retention time of the apolar compound benzene was too large, and its peak shape was severely distorted by excessively strong hydrophobic interactions). It is also remarkable that a U-shaped retention profile is clearly observed for benzene, its retention initially decreasing with the increasing volume fraction of acetonitrile from 5% to ~70% because RPLC-like hydrophobic interactions weaken as the organic content increases. As the acetonitrile content is further increased, the thickness and volume of the water-rich layer adsorbed onto the surface of the HILIC silica surface shrink. Therefore, benzene being quasi-insoluble (1.8 mg/mL) in water, it is less and less excluded from the internal pore volume, resulting in the slight increase of its retention times observed (10). The same observations (not shown) have been made in this work for a 2.1 x 150 mm 3.0 µm Atlantis HILIC column. Just as with RPLC markers, chromatographers can observe that the benzene t_0 marker generates as many V_0 values as the number of mobile-phase compositions applied experimentally. Should they consider the largest retention time of benzene in pure acetonitrile or its smallest retention time for the 70% volume fraction of acetonitrile in the eluent? Such a decision is impossible to make.

A clear answer is needed to solve this very general problem, which concerns not only RPLC and HILIC columns but also SEC, IEX, and RP-IEX mixed-mode LC columns. Below, the reasons why these markers are relatively poor t_0 markers are explained in terms of chromatography fundamentals; molecular dynamics simulations are performed to visualize the expected equilibrium density distributions of these marker molecules in the pore volume from the pore surface to bulk eluent.

Fundamental Insight

From a fundamental viewpoint, the solution to the problem pertaining to the accurate measurement of the column hold-up volume of a LC column is straightforward, and has been suggested since the early days of the LC technique (1,2,11–13). It consists of injecting and selectively detecting some solvent molecules while using the very same and pure solvent as the mobile phase. Therefore, if it exists, the ideal t_0 marker should have either the very same density profile from the surface of the LC stationary phase to the bulk mobile phase as that of the solvent molecule, or a different density profile but with the same overall amount in the entire pore volume when concentrations are normalized to the bulk concentration. Figure 5 (online) illustrates this point and compares the calculated (by molecular dynamics simulation) density profiles of pure acetonitrile (the ideal V_0 marker with itself as the eluent), infinitely diluted acetone in pure acetonitrile, and infinitely diluted uracil in a mixture of acetonitrile:water (80:20, v/v) across a silica-C18 mesopore. All three systems are in thermodynamic equilibrium at 27 °C with a conventional endcapped silica-C18 stationary phases (5). Clearly, none of the density profiles are closely similar to each other, and the three areas measured under these curves (that is, the overall amount of analyte present in the mesopore for the same bulk concentration) are also different. This reveals that the capacity factors and corresponding retention times of these three markers should be different, in complete agreement with the observations reported in Figures 1 (uracil marker) and 3 (acetone marker). Better, it is noteworthy that this area increases from uracil (0.81) to acetonitrile (reference area 1.00) and to acetone (1.05). In other words, the molecular dynamics calculations fully confirm that the acetone marker is still slightly retained onto endcapped silica-C18 stationary phases (even in pure acetonitrile used as the mobile phase), and the uracil marker is slightly excluded from the solid-to-liquid interfacial region of such adsorption systems. In conclusion, molecular dynamics simulations fully support the conclusion that neither acetone (in 100% acetonitrile) nor uracil (in an acetonitrile:water mixture, 80:20, v/v) can be considered as good "non-retained" markers in RPLC. The former marker leads to a slight overestimation of the column hold-up volume whereas the latter provides an underestimated value of this column property. Next, the solution to these inaccurate t_0 determinations and a harmonization of all these only approximate t_0 methods are proposed and tested experimentally for various LC columns characterized by different retention modes including RPLC, HILIC, RP-AEX, and AEX.

Harmonization of t_0 Methods

The harmonization of all the above-mentioned t_0 methods is based on the injection of any solvent molecules and using the same solvent as the mobile phase. However, the injected solvent molecules, being indistinguishable from those of the mobile phase, should be labeled and selectively detected by relevant detectors such as refractive index or MS detectors. Because triple-quadrupole MS is now commonly used in tandem with LC in most laboratories around the world, the simplest approach consists of injecting pure deuterated acetonitrile (CD_3CN), using pure acetonitrile (CH_3CN) as the mobile phase, and of selectively detecting CD_3CN from CH_3CN molecules by standard triple-quadrupole MS detection at m/z = 45. It is then assumed that the isotopic effects (stronger adsorption strength and possibly higher degree of exclusion of CD_3CN relative to CH_3CN) are negligible. At least, they are kept to a strict minimum because the benzene-d_6/1,3,5-benzene-d_3 and 1,3,5-benzene-d_3/benzene selectivity factors have been measured around 1.02 in RPLC (14–16). Therefore, it is assumed that the density profile of CD_3CN matches nearly exactly that of CH_3CN when in contact with silica-C18 adsorbents. This user-friendly method is applied next to the experimental determination of the true hold-up volume of 4.6 mm x 150 mm RPLC, HILIC, AEX, and RP-AEX mixed-mode columns.

RPLC

The true t_0 value of the Sunfire-C18 column was measured from the injection and detection of CD_3CN in pure acetonitrile. Figure 6a (online) confirms that acetone is definitely retained onto the Sunfire-C18 column regardless of the solvent composition from water-rich (95% in volume) to acetonitrile-rich (75% in volume) eluents. Uracil and thiourea are retained and excluded when eluted with the same water-rich and acetonitrile-rich eluent, respectively. Table I (online) summarizes the corresponding relative errors made (from –15% to >100%) when considering

these conventional markers to estimate the column hold-up time t_0 of the two RPLC columns used in this work.

HILIC

The true t_0 value of the BEH-amide column was measured from the injection and detection of CD_3CN in pure acetonitrile. Figure 6b (online) shows that the elution times of acenaphthene eluted by two buffered (10 mM ammonium acetate) acetonitrile:water mixtures (50:50 and 90:10, v/v) are smaller than that of CD_3CN in pure acetonitrile. The relative errors are about –17% and –6%, respectively (Table I, online). Similar to the benzene marker (Figure 4, online), acenaphthene can only provide an underestimated value of t_0 for HILIC columns. Thiourea should be avoided as a HILIC column hold-up marker because of its significant retention in acetonitrile-rich mobile phase. Interestingly, despite its polar nature, uracil is even excluded from the pore volume because of its poor solubility in acetonitrile-rich eluents. All the relative errors made on the true t_0 value of the three HILIC columns used in this work are summarized in Table I (online).

RP-AEX Mixed Mode

The true t_0 value of the Atlantis Premier BEH-C18 AX column was determined by the injection of CD_3CN in pure acetonitrile as shown in Figure 6c (online). RP-AEX columns are often used in pure water to promote retention of the most polar and charged (acids) analytes during the early stages of the gradient. Due to intense RPLC-like interactions in pure water, the acetone marker is strongly retained, and is not even a decent t_0 marker in RP-AEX. The same conclusion, but to a lesser extent, is true for thiourea and uracil markers, which are both retained on these mixed-mode columns. The need for the harmonization of t_0 methods is especially needed in mixed-mode chromatography, because several dominant interactions coexist and it is extremely challenging to find a priori a good and trustworthy t_0 marker for such columns. The relative errors made by the users when conventional marker methods are adopted are listed in Table I (online).

AEX

IEX columns are essentially used in pure water with variable contents of added salts. After measuring the true t_0 value of the Spherisorb SAX column, Figure 6d (online) shows the chromatograms recorded after injections of the neutral markers acetone, uracil, and thiourea at low (2 mM) and high (50 mM) ammonium acetate concentration in the aqueous mobile phase. Interestingly, uracil appears as an excellent t_0 marker, while both thiourea and acetone are slightly retained onto the Spherisorb and do not deliver accurate enough t_0 values for the Spherisorb SAX column. The relative errors when injecting these three usual markers are listed in Table I (online).

Conclusion

This work first demonstrates that there is no obvious and ideal t_0 marker that can always be injected in the mobile phase selected by the user and detected optically. This is true for all retention modes in liquid chromatography including those most commonly used, such as RPLC, HILIC, mixed-mode, and IEX. There are as many t_0 values measured for a given column as methods used to measure t_0. As advocated by the pioneers of fundamental LC, the most accurate method consists of injecting labeled solvent molecules in the same but non-labeled solvent and selectively detect the labeled molecules. Deuterated acetonitrile is an excellent solvent candidate that can be easily obtained from chemical suppliers and detected by conventional triple quadrupole MS at m/z = 45. This method enables users to be aware of the somewhat large relative errors made in the evaluation of the hold-up time t_0 of LC columns when conventional and assumed "non-retained" t_0 markers are considered.

Secondly, the impact of this universal t_0 method on fields of LC application is undeniably important: Harmonization of conventional t_0 methods and accurate determination of the unique hold-up time t_0 of LC columns will make it possible to precisely classify and compare column retentivity and selectivity across analytical laboratories worldwide, regardless of the experimental conditions applied (column chemistry, mobile-phase composition, temperature, ionic strength, and pH).

Finally, it is important to keep in mind that the claimed accuracy of this CD_3CN injection method relies on the assumption that the CD_3CN/CH_3CN isotopic effect is actually negligible. The extent to which this assumption can hold has yet to be determined by molecular dynamics; that work can be done once the force field of the deuterium atom in various adsorption systems (such as RPLC and HILIC) is known.

Additional Material Online

Figures 4–6, Table I, and the references can be found online using the QR code below.

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VIEWPOINTS

The Rise of Automation—Evolve or Die?

Tony Taylor

Recently, I read an article from *The Robot Report* website (1) contrasting the performance of robots in industry against those of humans. The article highlighted five areas where robots perform better than humans: a) handling tedium; b) extreme sensing; c) strength and speed; d) unwavering focus; and e) perfect, objective recall, as well as three areas where humans still outperform robots: a) empathy; b) flexibility; and c) acceptability and trust.

It strikes me that, considering these points within the laboratory context, we may not be taking full advantage of the positive aspects of robotics, or indeed working to address the negative aspects. This is especially true when considering sample preparation and sample manipulation.

Most laboratory workers with more than just a few samples to process will be familiar with the daily routine of preparing samples, setting up equipment, running a system suitability, checking fitness for purpose, and then starting the "batch" of samples just before leaving work for the evening. Modern instruments have very much reduced the amount of finger crossing required on the journey home, and that sense of anticipation as one enters the laboratory the next morning to see if the "run has worked" or not, but those feelings of anxiety have not been completely eliminated. Automated robotics have the power to change this paradigm, with samples being prepared in a "just-in-time" fashion so that the analysis can begin at the start of the day, and much of it will be done as we leave work-preparation and analysis times permitting. We could then even rack up enough samples to do the same thing overnight, hence improving our throughput, if required.

Of course, we need the automated solutions to be very robust and reliable, and this is perhaps where the industry needs to evolve, to ensure that our engineering is optimized to the point at which fallibility is not a consideration. Again, most of us are familiar with situations where campaigns have been halted overnight because of a vial not being gripped or picked up properly, or a vial tolerance that has caused the autosampler to reject a particular sample, or even simply to drop the vial. Here, evolution in the flexibility and "learning" of the instruments could be improved. Provided that the rejected vial does not lie in a dangerous or obstructive position, the robot should be able to move to the next operation and simply flag the failure in the batch report, provided the sample is not a key System Suitability or QC, which may render the remainder of the analyses invalid.

There are myriad situations in which robotics can be used for sample preparation in high performance liquid chromatography (HPLC) and produce a better result than the human laboratory worker might otherwise manage. Operations such as sample dilution (including serial dilution), filtration, and derivatization are all possible. There are sample robotics that can handle weighing, mixing, and even centrifugation, yet I don't see these solutions being employed in many HPLC laboratories, and I wonder why. I can state with certainty that the robot will follow a sample preparation or extraction protocol with much greater precision than a human if it has been properly "trained." It will also follow the standard operating procedure (SOP) every time. Can we honestly say that we follow the SOP for every sample preparation that we undertake? Always shaking for the precise time, using the same method of agitation, using the same spot in sonic bath, weighing or pipetting using exactly the correct technique... I could go on! Robots are infallible in terms of recall and repeatability; we are not.

Revisiting the title of this piece, do we need to evolve or die? Of course, it's a fact of every species on the planet. How true is this of automation of sample preparation for chromatographic applications? Well okay, I like to use a shocking title to draw in the reader; however, the flexibility of modern automated systems and the range of tasks they can complete has been revolutionized, even in the past decade. Their unfaltering accuracy and repeatability is proven, and their ability to reduce worker exposure to both tedium and hazardous reagents or operations can also be clearly demonstrated.

Do we then just fear the "rise of the robots"? Surely not in our modern society, and I encourage you to further investigate the upsides of automation that, hopefully, I've been able to point out in this article.

Reference

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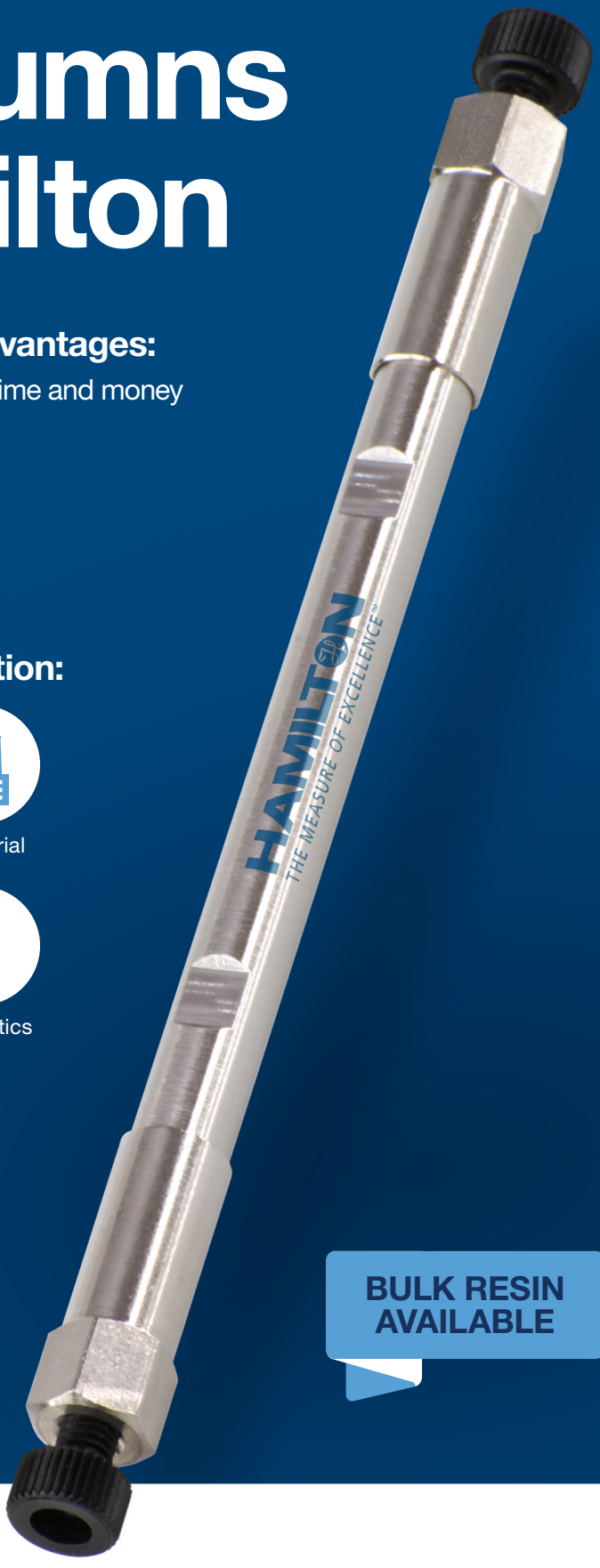

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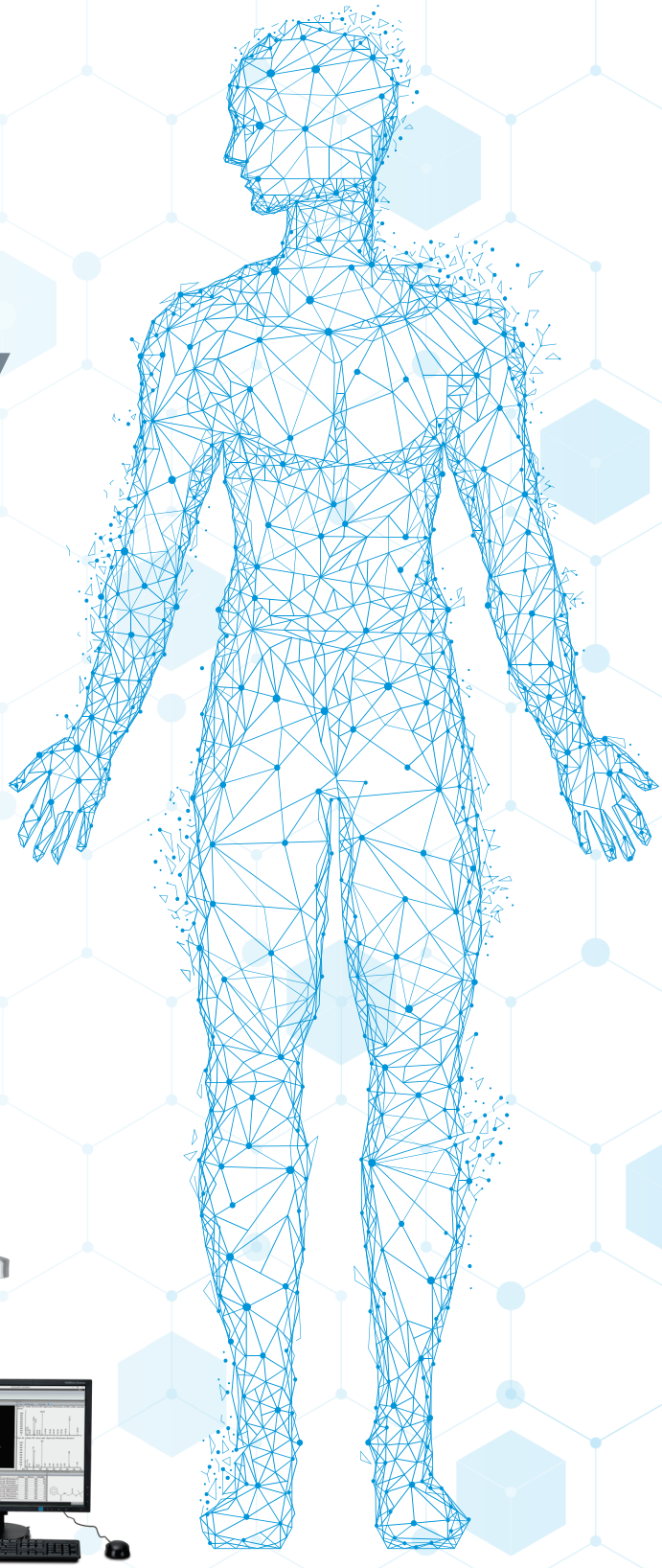

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