Volume 34 Number 12, 881–932
LCGC NORTH AMERICA
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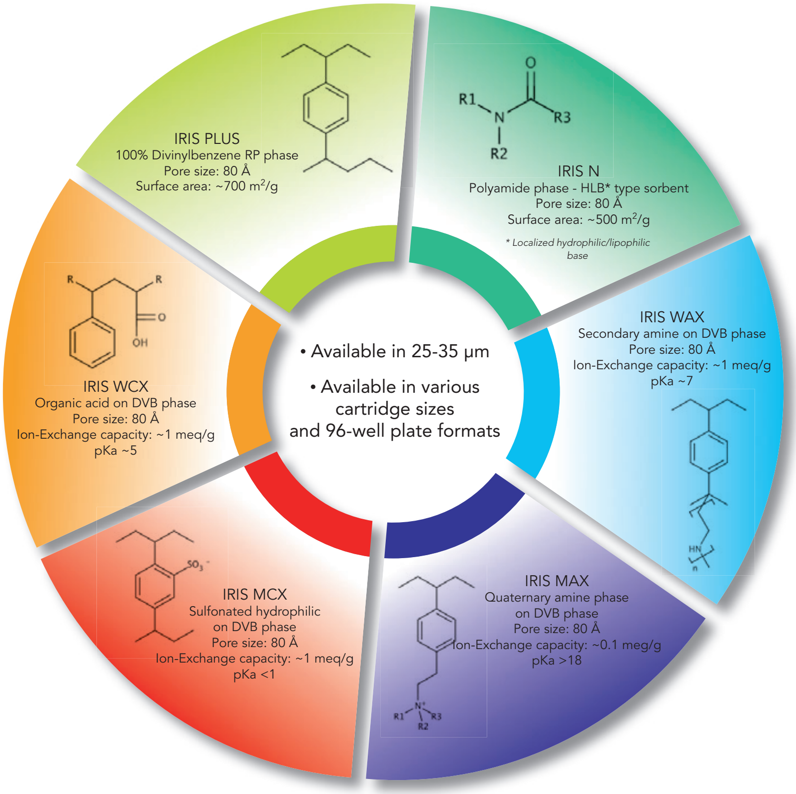

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LCGC North America (ISSN 1527-5949 print) (ISSN 1939-1889 digital) is published monthly by UBM Life Sciences, 131 West First Street, Duluth, MN 55802-2065, and is distributed free of charge to users and specifiers of chromatographic equipment in the United States and Canada. Single copies (prepaid only, including postage and handling): $15.50 in the United States, $17.50 in all other countries; back issues: $23 in the United States, $27 in all other countries. LCGC is available on a paid subscription basis to nonqualified readers in the United States and its possessions at the rate of: 1 year (13 issues), $74.95; 2 years (26 issues), $134.50; in Canada and Mexico: 1 year (13 issues), $95; 2 years (26 issues), $150; in all other countries: 1 year (13 issues), $140; 2 years (26 issues), $250. Periodicals postage paid at Duluth, MN 55806 and at additional mailing offices. POSTMASTER: Please send address changes to LCGC, P.O. Box 6168, Duluth, MN 55806-6168. PUBLICATIONS MAIL AGREEMENT NO. 40612608, Return Undeliverable Canadian Addresses to: IMEX Global Solutions, P. O. Box 25542, London, ON N6C 6B2, CANADA Canadian GST number: R-124213133RT001. Printed in the USA.

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PEAKS of Interest

Pioneering Chromatographer Jack Kirkland Dies at 91

Chromatography pioneer J.J. (Jack) Kirkland died on Sunday, October 30, after a short illness. He was 91 years old.

Kirkland was one of the original pioneers of modern liquid chromatography and made many substantial contributions to the development of high performance liquid chromatography (HPLC) since its inception in the 1960s. Highlights among his contributions include the development of the first spherical packing designed specifically for modern HPLC; the development of siloxane bonded phases; the establishment of processes for manufacturing spherical, small-particle (5-μm) totally porous packings that introduced a major leap forward in the performance of HPLC columns; the development of acid- and temperature-stable, sterically protected bonded phases; the development of the first small, superficially porous particles for separating high-molecular-weight molecules such as synthetic organic polymers and natural proteins; and the introduction of very small (2.7-μm) core–shell particles to enable ultrahigh-pressure liquid chromatography (UHPLC)-like performance of separations run at HPLC pressures.

Kirkland also developed, along with Jurgen Kohler, the first "type B" silica support, which vastly reduced the acidity of silica surfaces and thereby improved the HPLC of basic compounds. In collaboration with Wallace Yau, Kirkland developed the concept of using bimodal pore size packing materials for performing size-exclusion chromatography with solutes ranging over four orders of magnitude in size.

Kirkland was the vice-president of research and development at Advanced Materials Technology, Inc., of Wilmington, Delaware, and continued to be very active at the company until his recent illness. His latest project, 1000-Å pore-size superficially porous particles, will soon be commercialized. A manuscript describing this material, Kirkland's last technical contribution of many, has been submitted for publication in the *Journal of Chromatography A*.

Kirkland won numerous awards during his career, including the LCGC Lifetime Achievement in Chromatography Award, which he won in 2015. He published more than 160 peer-reviewed papers and wrote or edited eight books on chromatography, many of which were translated into several languages. He also received 32 patents.

Kirkland is survived by his wife, Karin, and his five children, Holly Kirkland Clouser, Mark Kirkland, Celeste Mozeik, Kent Kirkland, and Kerry Kirkland, and six grandchildren. ■

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GENE BARRY ON BRINGING A COLUMN BACK TO LIFE
Gene Barry from the University of Massachusetts Lowell discusses the three approaches that can help to bring a column back to life.

Other recent LCGC TV *interviews include:*

- Jared Anderson from Iowa State University explains how he has been working to address limitations within the analyses or ionic-liquid-based SPME coatings, which need to be capable of withstanding some harsh conditions in GC and GC–MS analyses.
- Alan G. Marshall from the National High Magnetic Field Laboratory at Florida State University explains how FT-ICR-MS enables the analysis of the smallest fullerenes and how these fullerenes are formed.

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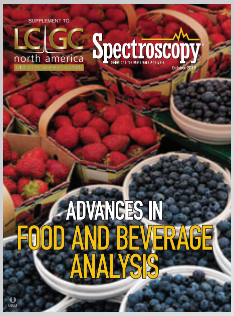

ADVANCES IN FOOD AND BEVERAGE ANALYSIS
New analytical methods and techniques are helping to ensure the safety and authenticity of the foods and beverages we consume. This special issue presents a look at advances in methods that make improvements in analysis time, sensitivity, precision, and simplicity. Articles include:

- Using Headspace Gas Chromatography for the Measurement of Water in Sugar and Sugar-Free Sweeteners and Products
- Determination of Contaminants in Beer Using LC–MS/MS and ICP-MS
- Determination of Cannabinoid Content and Pesticide Residues in Cannabis Edibles and Beverages
- LC–MS Characterization of Mesquite Flour Constituents
- Determination of α-Dicarbonyls in Wines Using Salting-Out Assisted Liquid–Liquid Extraction
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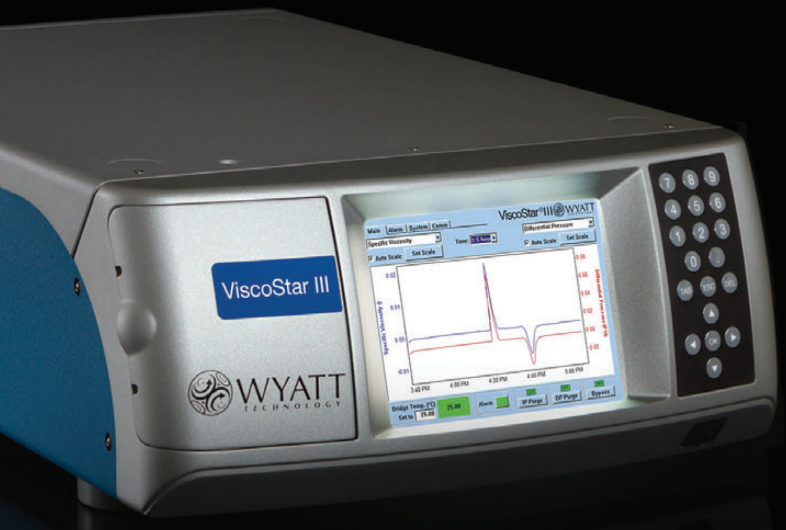
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How My Love Affair with Ion Chromatography Began, Part 1

Purnendu (Sandy) Dasgupta, Ph.D.
University of Texas

Professor Sandy Dasgupta recounts the history of his involvement in ion chromatography (IC) and his pioneering work in electrolytic eluent generation and electrolytic suppression in IC. He also discusses his current project, a NASA-sponsored undertaking to develop a miniature, low-power, low-eluent consumption IC instrument for the Mars exploration program and investigate the possible existence of life on this planet.

LCGC: Tell us about your contributions to key developments in ion chromatography, including electrolytic eluent generation and electrolytic suppression.

Dasgupta: In my late 20s in my first professional job at the University of California, Davis, our lab was among the first to acquire an ion chromatograph. My first chromatograph was the Dionex Model 10, with a serial number of 7. It was a very simple machine — I could tear it apart and build it back up, which I cannot do with modern instruments.

At that time, ion chromatography required stopping and regenerating the suppressor every so often. You had to take it offline, bring in another one, and regenerate the offline suppressor, which was a significant inconvenience.

A group of scientists at Dow Chemical — Hamish Small, Tim Stevens, and Ted Miller, and I independently — arrived at the same idea at about the same time: To use a membrane tube, bathe it with acid on the outside, and flow the eluent inside it. This device accomplished ion exchange in much the same way a packed column did except that it was never exhausted — it was continuously regenerated.

The Dow group published the first paper on the “continuous tubular suppressor,” but our group at Texas Tech developed a better design, which was later patented, in which filaments are put inside the membrane tubes and coiled.

I realized that there were two bottlenecks with the ion transport process using a membrane: transporting the ions to the membrane and transporting the ions through the membrane. Ideally the rate-limiting step should be the latter.

It occurred to me that I could augment ion transport if I applied an electric field. This process became the basis of electrodialytic suppression, which is in wide use today. Electrodialytic suppression allows continuous use, and it has the advantage of using water rather than chemicals to accomplish regeneration. For a KOH eluent, the process removes K^+ from the eluent to the negative electrode chamber where it reacts with water and makes KOH and hydrogen gas.

Early on I decided hydroxide was the best eluent because it can be suppressed to water. Any other eluent when suppressed will have a finite conductance that changes with the eluent concentration in a gradient. However, hydroxide in pure form is elusive because it immediately scavenges CO_2 from air and generates carbonate. Hydroxide and carbonate have very different eluent strengths, and you quickly find that the retention of all your ions is decreasing because the hydroxide eluent is changing to carbonate.

It occurred to me that the KOH I make on the cathode side of a suppressor is highly pure. Unlike a suppressor I do not have to remove all the K^+ from the donor side, the amount of K^+ transported and therefore the concentration of KOH generated is directly proportional to the current. Thus, electrolytic eluent generation became the best option — and it still is today — with the eluent or mobile phase being made in situ from pure water.

LCGC: Can you tell us about your work on the development of chip-scale instruments?

Dasgupta: My involvement in developing a miniaturized instrument derived from NASA’s desire to do ion chromatographic analysis in situ on Mars. The only wet experiment in Mars previously done was in 2008 with the Phoenix lander. Soil was put in a glorified beaker that had ion-selective electrodes embedded on the walls. After adding water and stirring, the electrodes took the reading and sent the results back to Earth.

Ion-selective electrodes are not specific. You may think you are measuring one thing and find out that you really measured something else. The experiment used 1.5 g of soil per beaker and the nitrate electrode reported back that there were 2 g of nitrate, which was clearly absurd!

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At this point, NASA scientists on earth quickly determined that the electrode responded much more to perchlorate than it did to nitrate. The eventual publication indicated that Martian soil may contain half a percent perchlorate, on average, which caused quite a stir. Perchlorate is a wonderful energy source. When heated, perchlorate decomposes, liberating chloride and oxygen. This means one has an easily tapped source of oxygen and heat. If I want to start a station on Mars, what could be better than that?

The other significance of perchlorate discovery is connected to the possibility of existence of organics. In the mid-70s, the Viking spacecraft was looking for organics on Mars. The idea was that, if organic matter existed, you could take dirt, heat it up, and use a gas chromatograph–mass spectrometer to determine the composition.

Viking 1 reported carbon dioxide, water, and traces of methyl chloride and dichloromethane. The carbon dioxide and water made sense, but people didn't understand the source of the methyl chloride and dichloromethane. They were originally attributed to terrestrial contamination, even though the Earth-bound blanks were clean. A few weeks later, Viking 2 landed on a different part of the planet and reported the exact same findings, suggesting that the methyl chloride and dichloromethane probably were not due to contamination.

Once this tentative discovery of perchlorate was made, somebody thought to take some good old Earth soil, add 2% perchlorate to it, heat it up, and to see what came off. And, what came off were carbon dioxide, water, and traces of methyl chloride and dichloromethane. Although the original data indicated no organic matter, the same results were now being interpreted that as there *are* organics on Mars.

To understand Martian soil chemistry and planetary evolution, it's vital that we understand the oxychlorine chemistry by using instruments more sophisticated than ion-selective electrodes — instruments that can look not only at perchlorate but at other chlorine-containing species, as well as other ions. An ion chromatograph is capable of directly measuring organic acids, so a miniature ion chromatograph would be ideal. This realization prompted our journey that began a few years ago.

Although the present rover, Curiosity, has a large suite of instruments, it cannot operate many at once due to power limitations. The ambient temperature on Mars is –15 °C to –100 °C; a normal ion chromatograph will require heating to keep the column from freezing even when the instrument is not used; this would consume too much power. We therefore decided to use an open tubular column.

Separation efficiency decreases indefinitely with decreasing column diameter, but below a certain diameter, it becomes extremely difficult to functionalize inside the column or to have enough detection sensitivity. We settled on working in the 10-30 μm region as an optimum compromise.

Let's discuss injection, separation and detection in that order. Today, we can reproducibly inject from sub-picoliter to 100 nanoliter quantities of sample (depending on the capillary bore) with high reproducibility, reasonably independent of sample viscosity. We can vary the injection volume without any need for sample loops.

If I was conducting capillary electrophoresis in a 20 μm capillary, I could use an off-the-shelf silica capillary, but for chromatography I must functionalize the walls. In addition, we ultimately wanted to use hydroxide eluents, silica was not a good material as it dissolves in base. We therefore had capillaries fabricated from poly (methyl methacrylate) (PMMA) and cyclic olefin polymer (COP) and copolymers.

PMMA capillaries already has negatively charge surface carboxylate groups. Borrowing from Hamish Small, I learned how to sulfonate the walls of these polyolefin capillaries so they have a permanent strong negative charge. Again following Small, it was easy to attach positively charged latex particles for anion separations and far simpler than creating polymeric layers within a small capillary!

Detection was the hard part. Because the individual band volumes are so small, one doesn't have the luxury of connecting external detectors. The on-capillary detection volume is so minute that the number of ions you are trying to detect is also equally small.

On-capillary detectors for ion analysis, called capacitively coupled contactless conductivity detectors (C4Ds) are commercially available. But they either didn't produce a signal or produced completely anomalous signals.

The best frequency for the measurement depends on the size of the capillary and the exact conductance/ composition of the solution. The commercial detectors simply could not operate at the frequency we needed. Once realized, we built simple detectors that are so sensitive that you can use capillaries as small as 2 μm in diameter and still sensitively monitor the ionic separations within them. But because of blockage, it's not practical to use capillaries this small.

We are currently working on developing a complete, portable unit. We carried out field testing in the Atacama Desert in Chile and conducted soil analysis in situ. We are in the process of perfecting our next generation of this miniature portable instrument, which eventually will be integrated into a single compact format.

My colleagues in NASA would like to see similar platforms developed for amino acid analyzers. Ultimately, people are fascinated by space exploration. We all want to know if we are here alone. Even if it seems certain that life isn't present on Mars currently, we wonder: Was there life on Mars? Were there at least the building blocks of life on Mars or on other planets?

For more on this subject, see "From Perchlorate in Breast Milk to Perchlorate on Mars," Sandy Dasgupta (SETI Talks), YouTube, **https://youtu.be/r-p55WLXAEI**.

COLUMN WATCH

Investigation of Reequilibration in Hydrophilic Interaction Liquid Chromatography

Interest in chromatography using hydrophilic interaction liquid chromatography (HILIC) has continued to build in recent years. Adoption of the technique has been slowed by experiences of poor reproducibility. In particular, reequilibration times in HILIC have been reported as being exceptionally long as compared to reversed-phase chromatography. In this study, reequilibration times in HILIC for both aqueous–organic gradients and buffer gradients are systematically explored. The results not only promise to improve method development practices, but also provide insight into HILIC retention mechanisms across mechanistically differing polar stationary phases.

Daniel L. Shollenberger *is a guest coauthor of this installment.* ***David S. Bell*** *is the editor of Column Watch and a coauthor of this installment.*

The interest in using hydrophilic interaction liquid chromatography (HILIC) has continued to build in recent years. The complementary nature to reversed-phase analysis, the chromatographic benefits conferred when analyzing polar and basic compounds, and the inherent compatibility with electrospray ionization (ESI) mass spectrometry (MS) are all driving forces toward greater adoption of the technique. Full acceptance, however, has been slowed by experiences of poor reproducibility. These issues arise both from the limitations of parameters chosen during method development and lack of system controls when designing a chromatographic experiment. Notably, reequilibration times in HILIC mode have been reported as being exceptionally long compared to those in reversed-phase mode. Although there are likely underlying fundamental differences in equilibration mechanisms that contribute to this phenomenon, the application of common reversed-phase operational practices to column equilibration could be a contributing factor for reproducibility issues.

This column installment describes a study of system controls for reequilibration times in HILIC with a systematic exploration of both aqueous–organic and buffer gradients. Reproducibility of retention and selectivity were observed as a function of equilibration times following runs in each type of gradient system. Two typical HILIC stationary phases, bare silica and pentahydroxyl bonded silica, were evaluated with different sets of molecular probes designed to suggest possible mechanisms driving the equilibration process as a function of time. It is hypothesized that both short equilibration times between analyses and run conditions that extend outside the normal HILIC range, representative of poor control of the chromatographic system, will show the greatest issues with repeatability. The results not only promise to improve method development practices, but also provide valuable insight into HILIC retention mechanisms across mechanistically differing polar stationary phases.

Experimental

Organic–Aqueous Gradient Study

Gradients were run on bare silica (10 cm × 3.0 mm, 2.7-µm d_p Ascentis Express HILIC, MilliporeSigma) and pentahydroxy stationary phases (10 cm × 3.0 mm, 2.7-µm d_p Ascentis Express OH5). A set of polar neutral molecules (adenosine and cytosine) and a set of polar basic molecules (metanephrine and normetanephrine) were studied for each column and gradient condition. Two gradients were evaluated: 5–50% aqueous (gradient 1) and 5–25% aqueous (gradient 2). Reequilibration times were set at 2, 5, 10, 15, and 20 min. Mobile-phase A was 5 mM ammonium acetate in 95% acetonitrile, mobile-phase B was 5 mM ammonium acetate in 50% acetonitrile, and mobile-phase C was 5 mM ammonium acetate in 75% acetonitrile. In each case, the pH of the mobile phase was unadjusted. Gradient 1 ran from A to B in 10 min. Gradient 2 ran from A to C in 10 min. In all experiments, column temperature was held at 35 °C, flow rate at 0.5 mL/min, injection volume at 2 µL, and detection by ultraviolet (UV) absorbance at 254 nm. All measurements were made in triplicate.

Buffer Gradient Study

The buffer gradient study was run on the same columns listed above using the same parameters for temperature, flow rate, injection volume, and UV detection. In addition to the polar neutral mix and the polar basic mix, a nonpolar basic mix using the tricyclic antidepressants amitriptyline and nortriptyline was included. A gradient of 2–10 mM ammonium acetate was run with constant organic–aqueous composition. Mobile-phase A consisted of 2 mM ammonium acetate in 90% acetonitrile and mobile-phase B consisted of 10 mM ammonium acetate in 90% acetonitrile. Both mobile phases were pH adjusted to 7.0 (±0.05) with acetic acid (pH measured in the presence of organic and calibrated using aqueous standards). The gradient was run from 100% A to 100% B in 10 min. All measurements were made in triplicate.

Results and Discussion

Figure 1 shows the structures of the test probes used throughout the study. Adenosine and cytosine represent neutral polar molecules that should only interact with the stationary phase via partition or polar (dipole type) interactions. The metanephrines are both polar and positively charged under the conditions of the study and thus can interact via partitioning, polar, and ionic mechanisms. The tricyclic antidepressants (TCAs), amitriptyline and nortriptyline, are likewise positively charged, but relatively nonpolar. The TCAs, then, are expected to retain via ion-exchange mechanisms and to some extent polar interactions, but not via partition mechanisms. Acenaphthene is included as a void volume marker.

Aqueous–Organic Gradients

Plots of retention time versus organic–aqueous reequilibration time for the neutral probes on the bare silica phase are shown in Figure 2. For the steeper gradient (to 50% aqueous, gradient 1, Figure 2a) there exists some slight irreproducibility when the reequilibration time was set to 2 min. At 5 min and above, highly reproducible results were obtained as noted by the barely discernable three overlaid symbols. For the shallower gradient (gradient 2, Figure 2b), 2 min appears to be sufficient to generate reproducible results. It was initially expected that short reequilibration times would result in greater variation in retention, but that was not the case. It was also expected that running the gradient to a higher level of the aqueous component would require additional time for reproducible retention times

to be measured. This effect was only slightly apparent at the 2-min time point. In both plots, the retention drifts upward as reequilibration times increase; however at each given time point, reproducible results are obtained. Increasing retention of neutral probes as a function of longer equilibration times may indicate that the preferential adsorption of water on the silica surface is slow. As more of the surface becomes solvated with the aqueous layer, polar neutral compounds such as adenosine and cytosine would be expected to partition more and thus show greater retention.

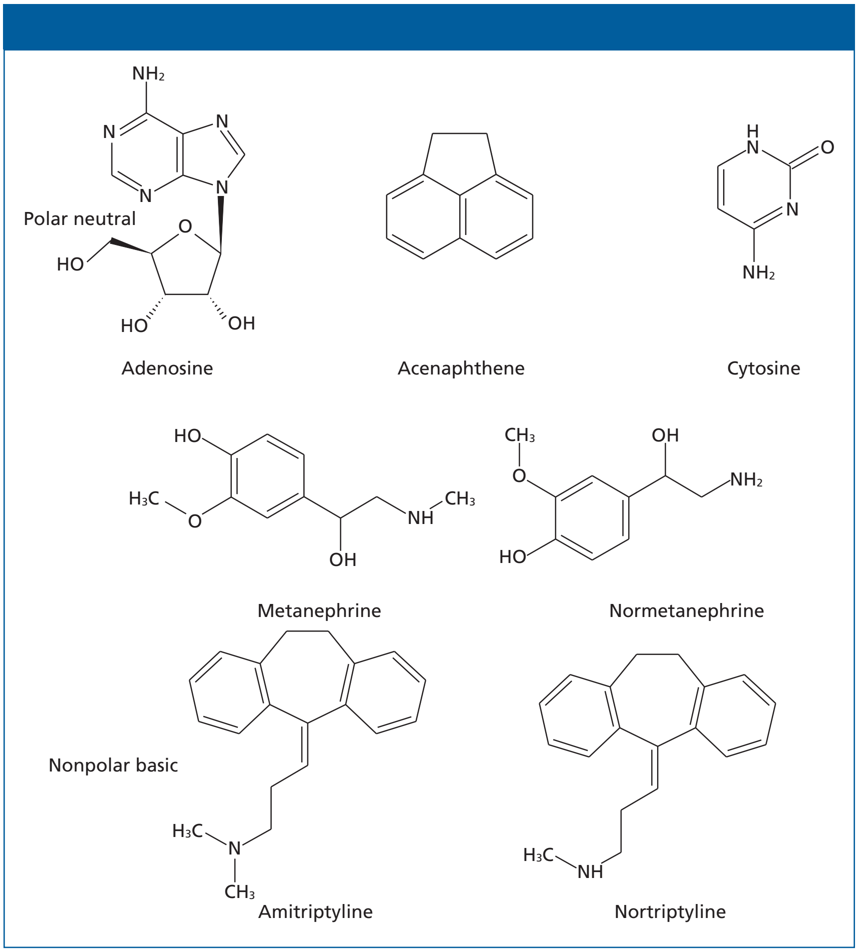

Figure 1: Structures of polar neutral, polar basic, and nonpolar basic analytes.

For the pentahydroxy phase, both gradient 1 and gradient 2 yielded highly reproducible results at each time point (data not shown). Retention variation as a function of reequilibration times were the same in terms of direction as compared to bare silica, but significantly attenuated.

Figure 3 shows the results from the polar basic probes using gradient 1 on the bare silica phase. Similar results were obtained on the bare silica phase for gradient 2. Again, the retention times are shown to be highly reproducible at each reequilibration time studied. In contrast to the neutral probes, the polar basic compounds show a downward drift in retention as a function of reequilibration time. Since the polar basic probes may retain by both ion-exchange and partitioning mechanisms, interpretation of this retention trend is difficult. If ion exchange is assumed to be the dominant mechanism of retention, it is conceivable that the slow preferential adsorption of water could potentially mask ionic interactions with the ionized surface silanol groups resulting in the observed reduction in retention with time.

For the neutral polar analytes, relative retention and peak shapes on both stationary phases remained highly consistent throughout the study and reproducible as a function of reequilibration time. Figure 4, however, shows significant changes in selectivity and peak shape of the polar bases when run on the

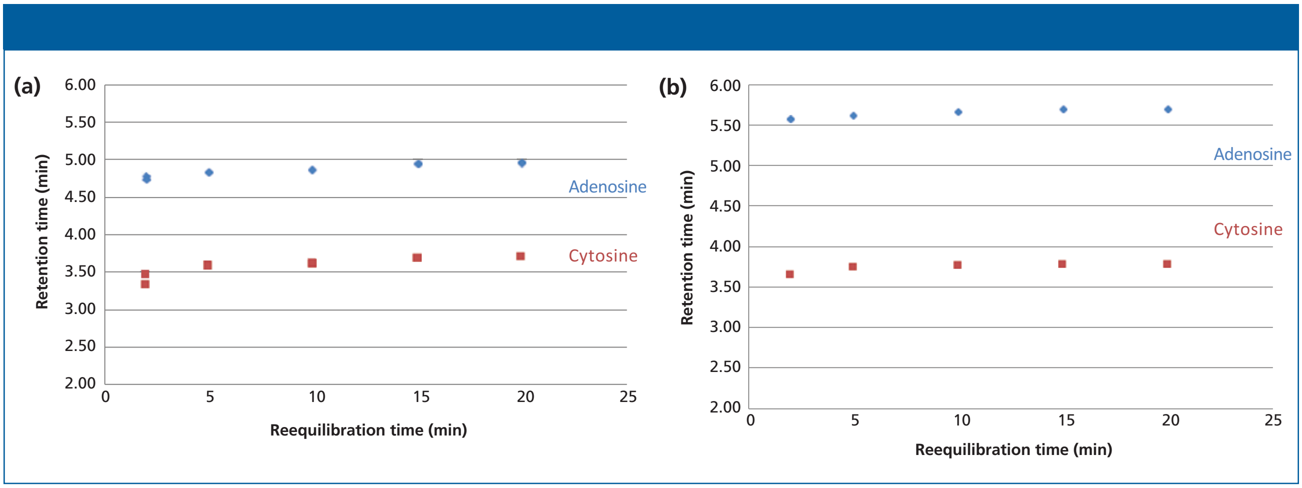

Figure 2: Retention as a function of reequilibration time: (a) bare silica phase, 5–50% aqueous mobile phase; (b) bare silica phase, 5–25% aqueous mobile phase.

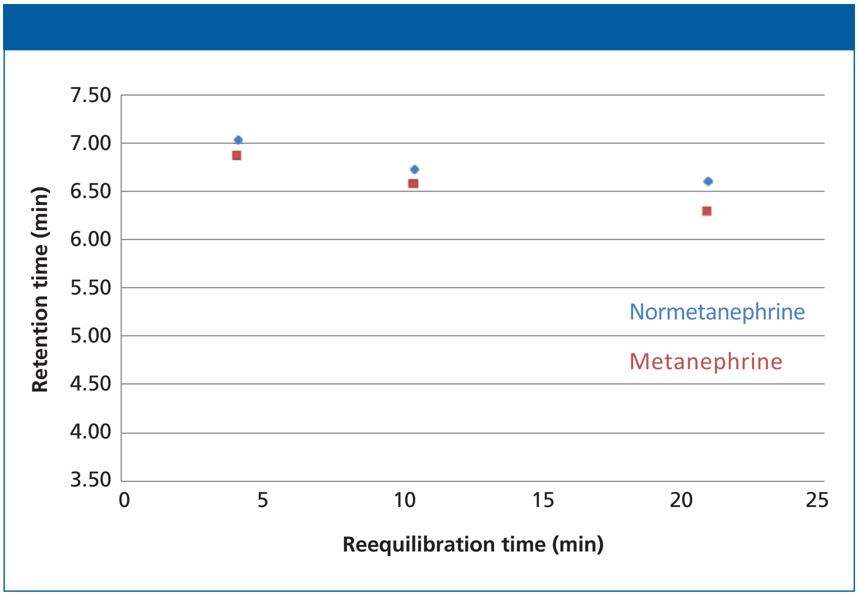

Figure 3: Retention as a function of reequilibration time for bare silica and polar bases with 5–50% aqueous mobile phase.

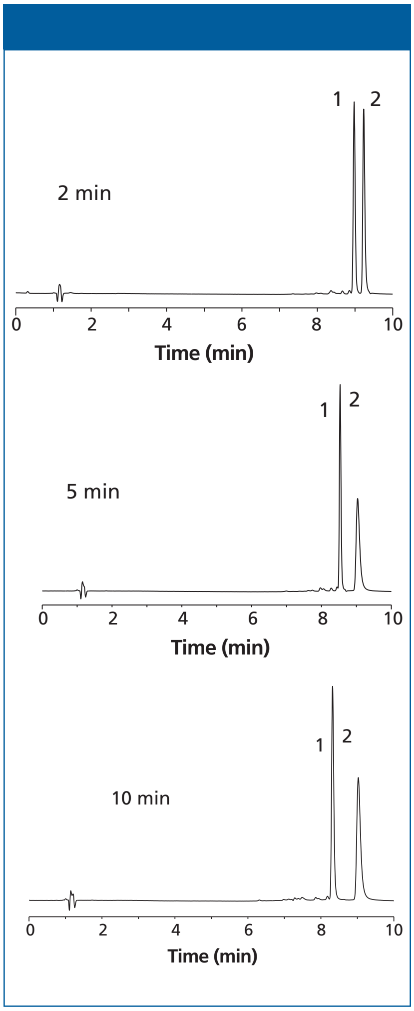

Figure 4: Chromatographic traces of basic analytes on bare silica phase at different reequilibration times. Peaks: 1 = metanephrine, 2 = normetanephrine.

bare silica phase as a function of reequilibration times. These variations in retention and peak shape were still highly reproducible at each discrete reequilibration time. These chromatographic changes imply the analytes experience a different interaction with the stationary phase as a function of reequilibration. The observation suggests consequences for standard method development practices common in reversed-phase chromatography when applied to HILIC systems. In reversed-phase mode it is often assumed that after the system is equilibrated "enough," that any time between injections beyond that time will result in reproducible data. The observations in this work demonstrate that care must be taken to ensure reequilibration times are considered as a significant parameter to be controlled during HILIC method development and expressly stated in the final method conditions.

In comparison, Figure 5 shows similar traces obtained for the polar bases on the pentahydroxyl phase, which indicates that the reequilibration time does not appear to significantly impact the retention, selectivity, or peak shape. In a previous study, bare silica phases were shown to exhibit moderate ion-exchange character and partitioning character whereas the pentahydroxy phase exhibited little ion-exchange and strong partition mechanisms (1). Comparison of the behavior of the polar bases on both stationary phases points to the presence of both ion-exchange and partitioning mechanisms available on the bare silica phase that affect the chromatographic profiles. Further effort is required to determine the exact dynamics driving this observation; however, it appears that the more polar pentahydroxy phase may facilitate preferential solvation of the water on the surface. The expedited coverage and likely extent of the water associated with the surface reduces change with time and is expected to mediate any ion-exchange interaction potential.

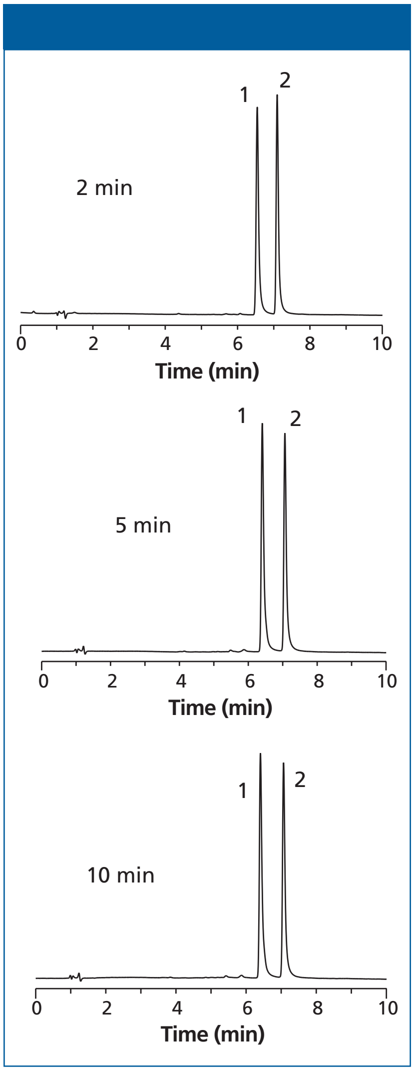

Figure 5: Chromatographic traces of basic analytes on pentahydroxy phase at different reequilibration times. Peaks: 1 = metanephrine, 2 = normetanephrine.

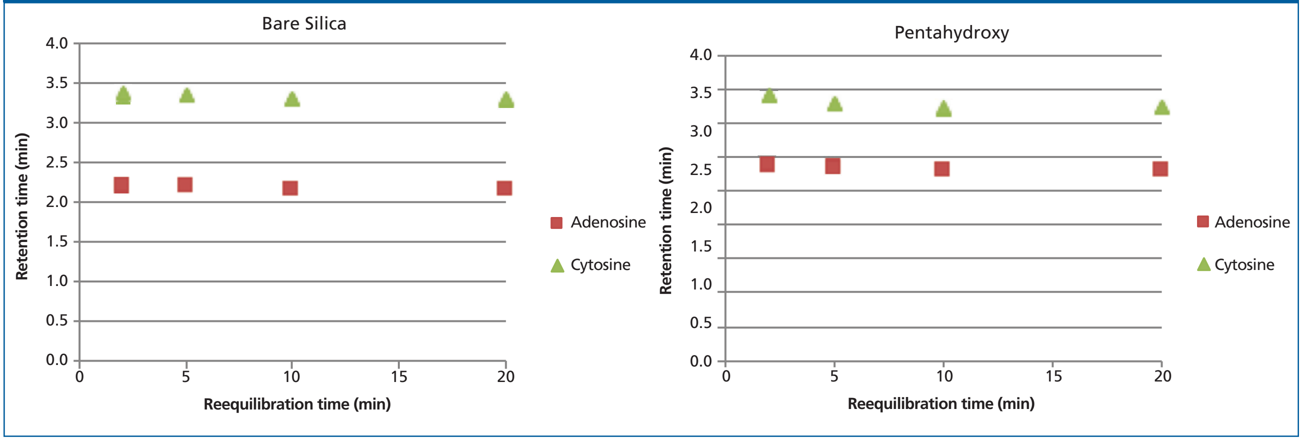

Figure 6: Retention as a function of reequilibration time for neutral polar analytes.

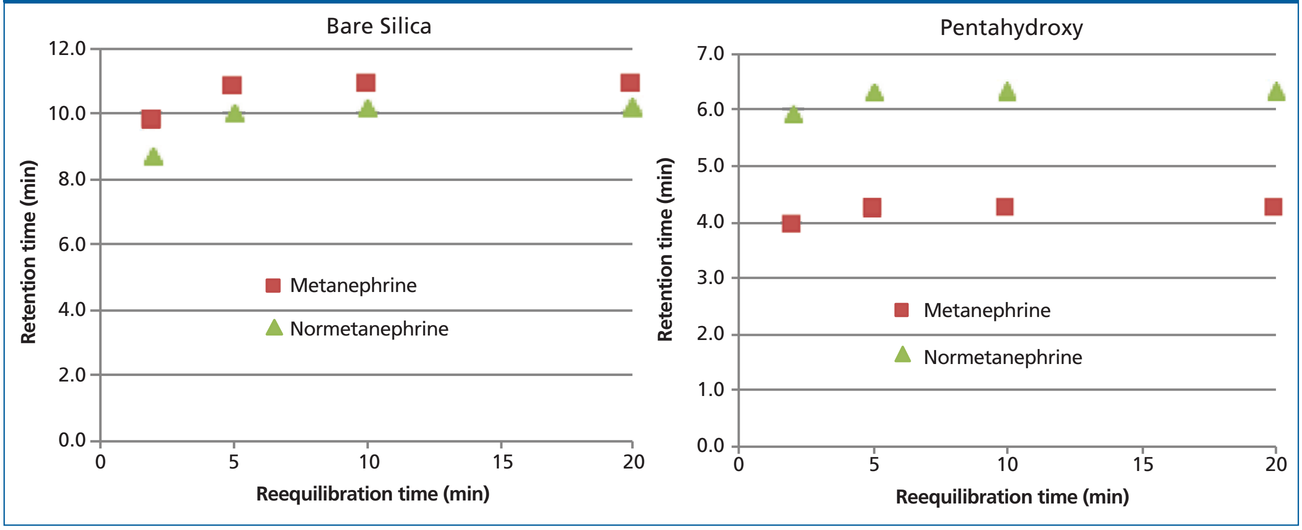

Figure 7: Retention as a function of reequilibration time for basic polar analytes.

Buffer Gradients

To investigate the role of ion-exchange mechanisms in the reequilibration processes, buffer gradients were run on both phases and an additional set of nonpolar basic probes was added. The buffer gradients were held at constant aqueous–organic ratios to minimize the impact of changing the preferential solvation of the surfaces with the aqueous component. The addition of the hydrophobic bases to the set of probes was intended to better interrogate the ion-exchange mechanisms, as partitioning of these probes should be minimal.

Figures 6–8 show plots of measured retention times versus buffer gradient reequilibration times on the bare silica and pentahydroxy phases. On both phases, the neutral polar molecules are barely affected by the buffer gradient. Retention times were again very consistent for each reequilibration time. Both phases showed a slight drift downward in retention time as reequilibration time increased. Peak shapes and selectivity were also highly consistent. For both the polar and nonpolar bases, retention increases as reequilibration time is extended. For each sets of basic probes, the magnitude of the increase on the pentahydroxy phase is greatly attenuated as compared to the bare silica phase. The hydrophobic probes show a greater variation in retention as a function of reequilibration time on the bare silica phase, consistent with the previous observations in the aqueous–organic gradients. This greater variation in retention supports the premise that relatively slow equilibration of the system is impacted by the ion-exchange mechanism and that the observed ion-exchange mechanism may be dependent on the slow adsorption of water on the surface. It is important to note that reproducibility at any given reequilibration time, on both phases, and with each set of probes, was again observed.

Conclusions

It was initially assumed that short reequilibration times in HILIC gradient methods would result in irreproducible chromatography. In addition, it was presumed that running gradients well outside the

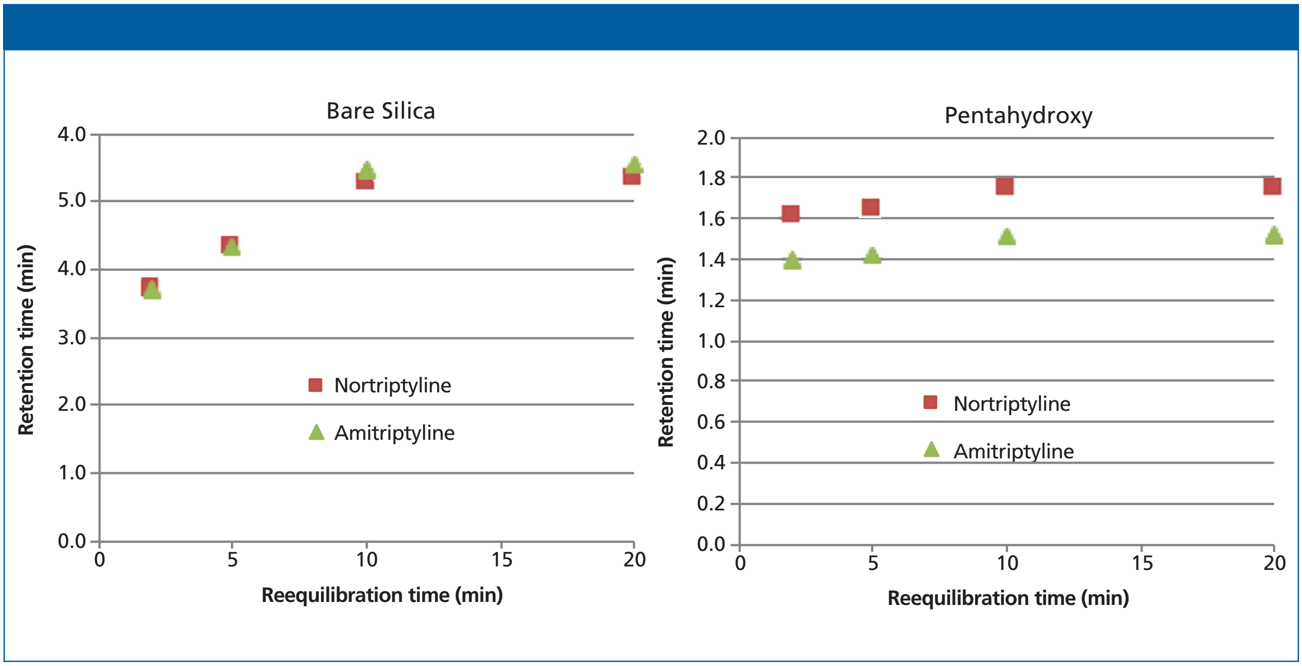

Figure 8: Retention as a function of reequilibration time for basic nonpolar analytes.

normal HILIC range would require additional reequilibration time to provide repeatable retention. Interestingly, the present study shows that consistent reequilibration times yield reproducible retention, selectivity, and peak shape. However, variability is observed as a function of reequilibration times, especially at extended times for basic analytes on the bare silica stationary phase. Each of the experiments hints that the presence of ion-exchange mechanisms results in slower overall equilibration and that the observed change in ion-exchange character may be the result of slow adsorption of water on the bare silica phase. The use of more polar HILIC stationary phases that exhibit less ion-exchange character may provide additional control of retention times when considering ionic analytes.

During the development of reversed-phase methods it is often assumed that if one allows the system to equilibrate for an extensive amount of time, the results would be the same as if the system were allowed to equilibrate for “just enough” time, typically equivalent to about 5–10 column volumes. The results of this study show that a gradient method in HILIC mode may appear equilibrated by virtue of reproducible retention times; however, with extended reequilibration time, the system is prone to change. It is therefore important that gradient HILIC methods are developed with reequilibration times in mind and that this parameter is specified in the final protocol to ensure reproducible and reliable results.

Reference

(1) D.S. Bell, *LCGC North Am.* **33**(2), 90–101 (2015).

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LC TROUBLESHOOTING

Failed System Suitability Test: A Case Study

A reader's problem of a method that fails the repeatability requirement of the system suitability test serves as an example of how to approach liquid chromatography (LC) method troubleshooting.

John W. Dolan
LC Troubleshooting Editor

I regularly receive questions from readers of "LC Troubleshooting." Many of these questions are simple and can be answered with a quick e-mail, and I often collect them to include in a discussion of readers' questions in this column. Others seem to stand alone as a good example of not only solving a specific problem, but also how to approach liquid chromatography (LC) problems in general. The present case study is a good example of the latter type of question.

In a somewhat edited version, the question went like this:

> I am using a pharmacopeial method for assay of a drug tablet. This is a normal-phase method with the following conditions. Column: unbonded silica, 10-µm diameter particles packed in a 300 mm × 4.6 mm column, operated at 25 °C [flow not specified]. Mobile phase: acetonitrile–ethyl acetate–tetrahydrofuran–*n*-hexane (1:1:1:7). Injection: 10 µL of sample dissolved in methanol + mobile phase. Samples were kept cool (6 °C) while on the autosampler.
>
> During verification of the method in the research and development (R&D) laboratory and initial transfer to the quality control (QC) laboratory, I did not observe any failure of the system suitability testing (SST) requirements (≤2% relative standard deviation [RSD]) for either the initial injection of replicate standards (n = 5) or bracketing standards.
>
> After six months of use in the QC lab, two types of SST failures were noticed:
> 1. Failure of %RSD for the initial n = 5 replicate standards. Sometimes SST passed, sometimes it failed. This occurred on two different LC systems.
> 2. If the initial n = 5 standards passed, sometimes the bracketing standards did not meet the requirements and other times they did.
>
> In addition to the failure of the standards, I've noticed a difference in peak area response that occurs occasionally and suddenly with multiple injections of either standard or sample.
>
> After observing the above problems, we have performed parallel analysis in the R&D lab using the same brand of LC system used in QC, but we have not observed any of the problems. I'm not sure what to do next.

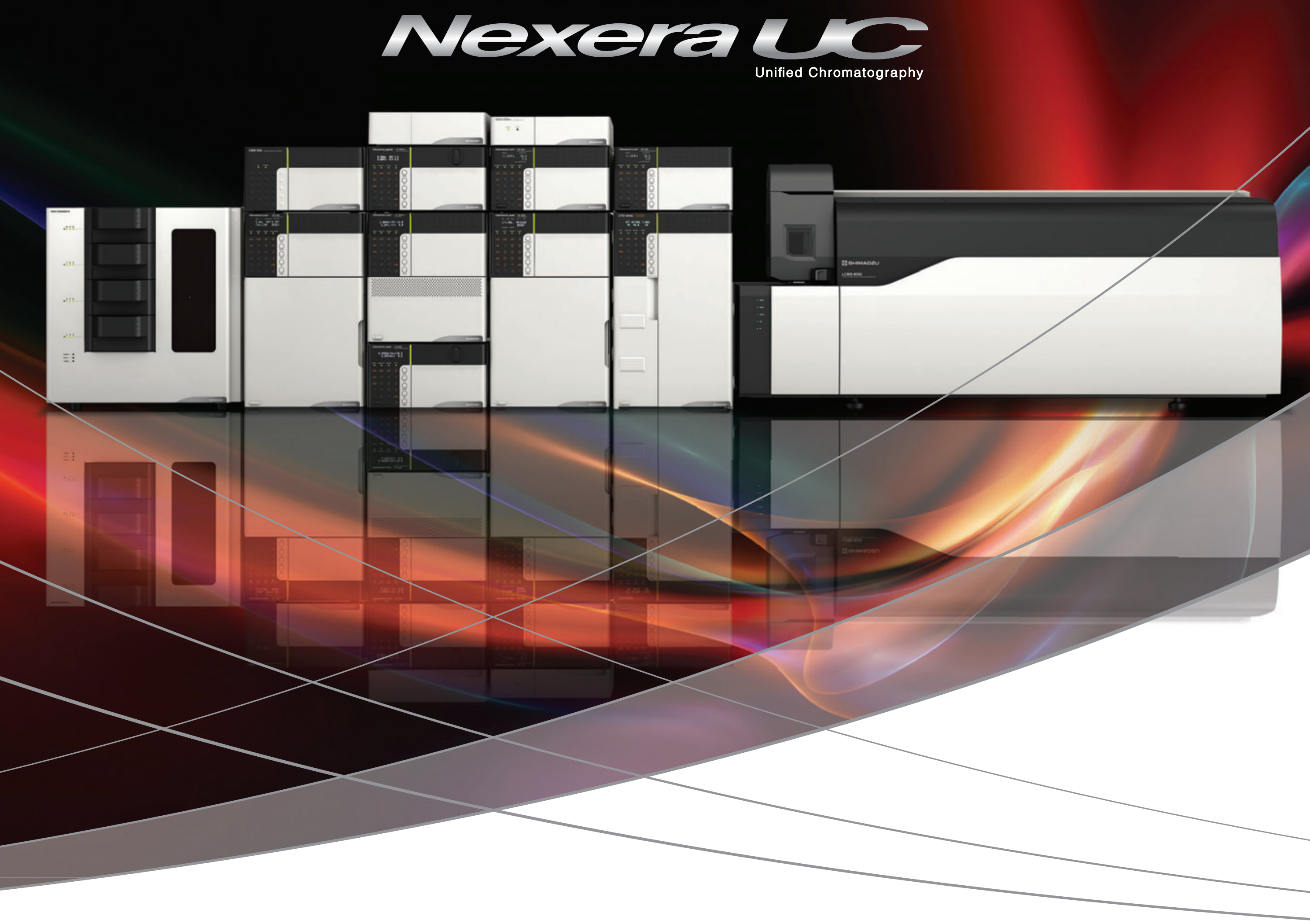
Mental Experiments First

As I read this question, my mind immediately pointed me to an injection problem. When I'm with a group and that happens, I'm often asked how I came to that conclusion so quickly. Here's the process that I use, either consciously or subconsciously.

I always like to begin troubleshooting by simplifying the workload as much as possible, so I do as many mental experiments as I can first. It is appropriate to apply the "divide and conquer" rule here. In this approach, an experiment (mental or physical) is chosen that divides the entire problem space into large pieces. Knowing the answer to the experiment, you can eliminate a large portion of the possible root causes. Repeat with the remaining potential problems to eliminate as many as possible.

When communicating by e-mail, I usually find that I don't have all the information that I would like when initially approaching an LC problem. For example, a few example chromatograms and a table of results for typical passing and failing runs would be nice—it is surprising how often a quick glance at a chromatogram or some data will help to spot the problem source quickly. I did not get this information for the present problem, so I'll have to try another approach.

Even without all the desired information (and this is usually the case for most problems you will encounter in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the laboratory), I think that I can safely make a few conclusions:

- The method is basically sound. If it came from one of the pharmacopeias (for example, the United States Pharmacopeia [USP] or European Pharmacopoeia [EP]), these methods have been thoroughly vetted before publication. When setting up such methods in the laboratory, they are sufficiently stable that revalidation usually is not necessary. Some kind of verification that the method works is required, usually defined by the receiving laboratory's standard operating procedures (SOPs) based on regulatory guidance. It appears to me from the reader's input that this process went smoothly. Divide and conquer result: It probably isn't the method itself.
- The method worked initially in the R&D laboratory on at least one instrument and later it continued to work in R&D when parallel analysis was done. Divide and conquer result: confirms the above assumption that the method is basically sound, and it might be instrument or operator dependent.
- The method worked initially in QC, but failed after approximately six months and continued to fail. It also failed on a second LC system, but it wasn't clear if it originally ran properly on this system. Divide and conquer result: Something has changed over six months and it isn't unique to a single LC system. Because it worked for six months, I am less likely to suspect operator problems than I would if the transfer to QC never worked.
- The comment that the areas appear to change suddenly from one injection to the next leads me to assume that this is not a gradual aging of the instrument or column. Or at least that this is not the most likely cause. Divide and conquer result: Don't focus first on the column.
- When the method was returned to R&D it worked as expected. Divide and conquer result: There's something different between the R&D and QC instruments that is causing the problem.

Is It Plugged In?

It is amazing how often the simplest things are overlooked, and I'm as guilty of this absentmindedness as the next person. We all know stories about some appliance that doesn't work and we fret and fuss trying to get it operational, but it never occurs to us that it might not be plugged in. So ask the obvious questions, even though you may be certain of the answers. Here are a couple:

Is retention reasonable? For consistent chromatography, including retention times and peak area or height, the first peak of interest should be retained so that it is well clear of the column dead-time peak (t_0). This means that the retention factor, k, should be at least 1 and preferably 2 or larger. A value of k = 0.5 may be acceptable in some cases, but smaller retention factors mean that the first peak is likely to run into interfering materials at the solvent front and will be less reproducible. You can calculate k as follows:

$$k = (t_R - t_0)/t_0 \qquad [1]$$

where t_R is the retention time. For the present purpose, you can visually estimate if the k-value is large enough by making sure the time (baseline distance) between injection and the solvent front is less than the time between the solvent front and the peak of interest. For the current setup, the column dead volume should be approximately 3 mL, so at 2 mL/min, $t_0 \approx$ 1.5 min. For $k \geq 1$, $t_R \geq 3$ min would be required. Because this is a pharmacopeial method, it is highly likely that the method is designed to satisfy this requirement, but the brand of column or accuracy of column oven calibration can change k significantly relative to the original method conditions. If I had been supplied a chromatogram of a good and failing run, I could check the k-value easily.

Another retention check to make is to make sure that the failed injections have the same retention times as the good ones. I have assumed that a change in retention between the two conditions would be so obvious, it was not overlooked, but it is good to ask. Again, a couple of chromatograms supplied with the question would allow me to evaluate the retention times.

I mentioned in the previous section that the symptom of variable peak size is very unlikely to be related to a problem with the column. However, replacing the column with a new one is such a simple check, it wouldn't hurt to try a new column before digging deeper. Another rule of thumb I often use is "easy over powerful." That is, it may be most efficient to try an easy experiment, such as column replacement, before executing a more complicated experiment, even if you don't expect it to succeed. Column replacement may have been done already, so it may not have to be repeated, but I don't have that information.

Reduce the Variables

Now that we've eliminated the easy possibilities with some mental experiments or by visual evaluation of existing chromatograms or data, it is time to roll up our sleeves and dig in deeper. Changes in peak area most commonly are the result of some change in the injection process, so that's where I'm going to concentrate.

I'm not sure how the sample sequence is setup, but for this type of method often a single injection is made from each vial. If replicate injections are made, they are made from two separate vials. Thus the sequence might be SST sample 1, SST2, SST3, SST4, SST5, then the bracketing standards (BS), BS1, BS2. This would be followed by several samples for analysis (SA), SA1, SA2, SA3, SA4, SA5, and a couple more bracketing standards BS3 and BS4, then more SA samples. Often all of the SST samples come from a common stock and the BS samples from another common stock. The %RSD calculation mentioned by the reader would then be made for SST1–SST5 (n = 5) and for BS1–BS4 (n = 4).

If the above sequence is correct, we can't be sure if the problem of differing areas is due to the injection process or something that happened to the samples before they were injected because a different vial was used for each injection. The first experiment that I would do is to eliminate this uncertainty. Fill two vials with sufficient SST or BS for a large number of injections. The method uses only 10 µL of sample, so 0.5–1.0 mL would allow 50–100 injections per vial. Set up the method sequence to make n = 10 injections of each vial, then repeat (10 × SST and 10 × BS), so the total number of injections is n = 20 for SST and n = 20 for BS.

After the run is complete, calculate the %RSD for each set of 10 and for the combined set of 20 for each sample. Compare these to the failed experimental results. If the %RSD is lower when the samples are from the same vial compared to when samples are in individual vials, this suggests that the problem is related to preparation of the vials or the position of the vial in the run sequence. If the variability is similar, the injection process itself is more likely the problem. Also, examine the data to look for any patterns in peak areas. Do peaks gradually get larger or smaller or are the changes random? A sequence of gradually changing peak sizes can occur if the sample vial is filled too full and poorly vented. It can also occur if sample or solvent evaporates as the sample sits on the sample tray. Evaporation of the sample solvent is more likely a problem with a normal-phase method, such as this one, than a reversed-phase method, where the sample solvent usually contains water, making it much less volatile.

If the %RSD is still high and the peak area changes are random, as I suspect they will be, I believe the problem is associated with the autosampler. Before going further, I would thoroughly check the autosampler for obvious problems. Is there enough wash solvent in the reservoir? Is the wash solvent compatible with the sample (for example, it can contain no water for a normal-phase method)? Is the correct syringe installed? Is it worn out? Is it tight in the mount? Is the sample needle clear of any septum debris or other material? Is the needle depth adjusted properly? Sometimes the tubing connecting the needle and syringe needs to be purged of any bubbles—check for this. If there is a needle-wash station in the autosampler, is it clean and working properly? Make sure that the draw or fill rate chosen for withdrawing sample from the sample vial isn't too large. Because of the volatility of the normal-phase sample solvent, more rapid fill conditions that may be satisfactory for reversed-phase methods can cause cavitation (bubble formation) during the sample transfer process, introducing error. If in doubt, reduce the fill rate to see if this improves things. Consult the operator's manual for other suggestions, as well as the earlier "LC Troubleshooting" column on autosamplers (1). If you find and correct any problems at this point, repeat the SST and BS injection series to see if the problem is solved.

Autosampler Performance Qualification

If the problem persists at this point, the autosampler needs to be evaluated independent of the method. Under test conditions, today's autosamplers should perform at %RSD ≤ 1% for n = 5 10 μL injections of a well behaved substance. In our laboratory we typically observed 0.3–0.5% RSD under such conditions.

I like to check the autosampler under reversed-phase conditions because typically there are fewer inherent problems with reversed phase than normal phase. I'll explain the reversed-phase test conditions here; if you want to stay in normal-phase mode, you can figure out comparable conditions. If you are working in the normal-phase mode, as is the present case, when you switch to reversed phase, be sure to use a compatible intermediate solvent. The simplest choice is propanol or isopropanol, either of which is fully soluble in aqueous and normal-phase solvents. In the present case, acetonitrile is a component of the mobile phase, so you can use acetonitrile as the changeover solvent. First, remove the column and replace it with a piece of connecting tubing. Then replace all solvents (wash solvents and any mobile-phase solvents) with acetonitrile. Flush 10–20 mL of acetonitrile through each line to ensure all the normal-phase solvents have been flushed from the system. Then switch to the desired reversed-phase mobile phase and flush with another 10–20 mL of mobile phase. Finally, install the reversed-phase column and equilibrate it.

The autosampler performance qualification test is described in an earlier "LC Troubleshooting" column (2), which can be found on the *LCGC* website. The test column is a C18 column of your choice. Choose a stable test compound that is easy to detect. Reference 2 recommends anthracene, but most low-volatility neutral aromatic compounds will do the job. Avoid volatile aromatics, such as toluene or benzene, because these can evaporate during the test and change the results. Select a concentration and detection wavelength that will give a detector signal of 0.2–0.8 absorbance units for a 10-µL injection; for anthracene, a solution of ~2 µg/mL and a wavelength of 260 nm works well. Choose methanol–water, methanol–buffer, acetonitrile–water, or acetonitrile–buffer at a ratio that gives good retention (for example, $k \approx 4$) for the test compound. A good starting place is 80% methanol–20% water or 70% acetonitrile–20% water. You may need to adjust the concentration to get $k \approx 4$, depending on the column brand you choose. For $n = 5$ injections of 10 µL each, you should see %RSD ≤ 1% for peak area under these conditions. If the variability exceeds this, there is something wrong with the autosampler. Review and double-check the component inspection mentioned above. If these items look OK, the autosampler is in need of more serious maintenance. Consult the service manual for more options or contact the manufacturer's technical support department for more help.

Summary

Based on the information I was supplied by the reader about the area variability he observed, I strongly suspect that the problem is related to the autosampler. If, after going through the method checks and autosampler checks, the problem persists, it's time to sit down and reevaluate the possibilities. For example, transfer the exact column and mobile phase that works in the R&D laboratory to QC and see if the method works now; this should help to eliminate problems with the mobile phase or column.

This case study serves as a good example about how to approach a method problem. We broke the problem down into its potential causes, then examined them with mental experiments first, because they are faster and can eliminate spending time on unproductive laboratory experiments. It is wise to double-check even the most obvious root causes (did you plug it in?) just to be sure you didn't overlook something. When tests under method conditions lead to a dead end, it is necessary to change to system or component qualification conditions that test the system or component independent of the method.

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(2) G. Hall and J.W. Dolan, *LCGC North Am.* **20**(9), 842–848 (2002).

John W. Dolan
"LC Troubleshooting" Editor John Dolan has been writing "LC Troubleshooting" for LCGC for more than 30 years. One of the industry's most respected professionals, John is currently a principal instructor for LC Resources in McMinnville, Oregon. He is also a member of LCGC's editorial advisory board. Direct correspondence about this column via e-mail to LCGCedit@ubm.com

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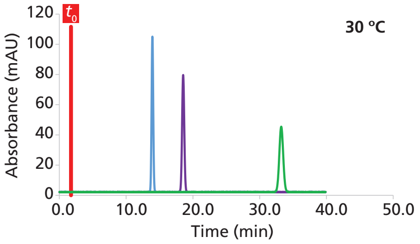

Part II of this series describes additional features of the HPLC Teaching Assistant software, including the capability to simulate the impact of the mobile-phase temperature on high performance liquid chromatography (HPLC) separations, understand the chromatographic behavior of a mixture of diverse compounds in both isocratic and gradient elution modes, show the influence of instrumentation (injected volume and tubing geometry) on the kinetic performance and sensitivity in HPLC, and demonstrate the impact of analyte molecular weight on thermodynamic (retention and selectivity) and kinetic (efficiency) performance.

Davy Guillarme and Jean-Luc Veuthey

In part I of this series (1), some of the capabilities offered by the high performance liquid chromatography (HPLC) spreadsheet were described, such as the opportunity to

- illustrate the concept of chromatographic resolution, including the impact of retention, selectivity and efficiency;
- understand the plate height (van Deemter) equation and kinetic performance in HPLC;
- recognize the importance of analyte lipophilicity (log *P*) on retention and selectivity in reversed-phase liquid chromatography (LC) mode; and
- handle reversed-phase LC retention, taking into account the acid–base properties (pK_a) of compounds and the mobile-phase pH.

Some additional features of this software are described here, including the capability to simulate the impact of the mobile-phase temperature on HPLC separations, understand the chromatographic behavior of a mixture of diverse compounds in both isocratic and gradient elution modes, show the influence of instrumentation (injected volume and tubing geometry) on the kinetic performance and sensitivity in HPLC, and demonstrate the impact of analyte molecular weight on thermodynamic (retention and selectivity) and kinetic (efficiency) performance.

Understanding the Impact of Mobile-Phase Temperature in Reversed-Phase LC

Theoretical Background

The mobile-phase temperature impacts both the kinetic (shape of the van Deemter curve, optimal linear velocity, and column pressure drop) and the thermodynamic performance (modification of the retention and selectivity).

In terms of kinetic performance, the optimal linear velocity (u_{opt}) of the van Deemter curve is given by the following equation:

$$u_{opt} = \frac{V_{opt} \times D_m}{d_p} \qquad [1]$$

The diffusion coefficient, D_m, of solute A in solvent B can be expressed using the Wilke-Chang equation:

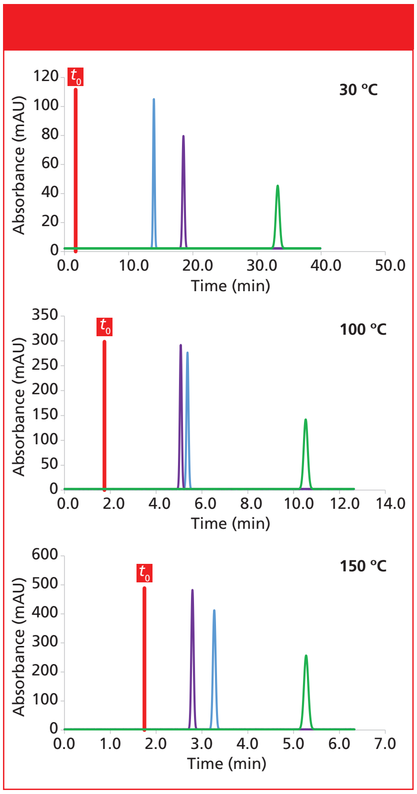

Figure 1: Impact of mobile phase temperature on the retention and selectivity in reversed-phase LC. The chromatograms were simulated at 20% acetonitrile. Column dimensions: 150 mm × 4.6 mm, 5 µm; flow rate: 1 mL/min; compound molecular weight: 100 g/mol.

$$D_m = 7.4 \times 10^{-8} \frac{(\Phi_B M_B)^{1/2} T}{\eta_B V_A^{0.6}} \quad [2]$$

where M_B is the molecular weight of solvent B, T is the absolute temperature (K), η_B is the viscosity of solvent B (cP) at T, V_A is the molar volume of solute A at its normal boiling temperature, and Φ_B is the association factor (the association parameter was introduced in the above equation to define the effective molecular weight of the solvent with respect to the diffusion process [2]) of solvent B (dimensionless). Wilke and Chang recommended a value of Φ at 2.6, 1.9, and 1.37 (3) when the solvent was water, methanol, and acetonitrile, respectively. The organic solvent selected for the calculation was acetonitrile. The mobile-phase viscosity was determined from reference 4 and depends on the nature of the organic solvent and the mobile-phase composition and temperature.

The column pressure drop was calculated using Darcy's law (see equation 9, in part I [1]). It depends on the mobile-phase viscosity and therefore also varies with temperature.

Log k increases linearly as a function of $1/T$ (K), according to the van't Hoff equation:

$$\log k = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \quad [3]$$

However, the slopes of the van't Hoff curves vary depending on the compound. On average, the retention factor is divided by a factor of 2 every 30 °C to 40 °C, depending on the compound (this is an empirical value, observed in reference 4). Three compounds were arbitrarily selected. For the first compound (log P of 2.0), the k value was divided by a factor of 2 every 40 °C, for the second compound (log P of 2.5), k was divided by a factor of 2 every 30 °C, and for the third compound (log P of 2.8), k was divided by a factor of 2 every 38 °C.

In the spreadsheet entitled "temperature," the impact of the column dimensions (L_{col}, d_{col}, and d_p), flow rate (F), percentage of organic solvent, compound molecular weight (MW), and mobile-phase temperature on the kinetic and thermodynamic performance can be directly visualized. The first graph shows the kinetic performance (N versus F), the second one illustrates the thermodynamic behavior (log k versus $1/T$) of three compounds, and a simulated chromatogram with the three compounds shows the chromatographic behavior when modifying the mobile-phase temperature.

Using the "Temperature" Spreadsheet

Figure 1 illustrates the impact of the mobile-phase temperature on the chromatographic separation of three species with different log P values. The retention decreases with increasing temperature from 30 °C to 150 °C because of the reduction of the mobile-phase polarity with temperature. The total analysis times were 33 min, 10 min, and 5.3 min at 30 °C, 100 °C, and 150 °C, respectively, and t_0 remained constant. This result confirms that the percentage of organic solvent has to be decreased to achieve similar retention factors at different temperatures. For example, at 1% acetonitrile, the retention at 150 °C was equivalent to the retention at 30 °C and 20% acetonitrile (data not shown). In addition to retention, the selectivity was also modified, and the elution order of the first two peaks was reversed at approximately 100 °C. This result proves that temperature is an effective parameter to tune selectivity, provided that a sufficiently wide range of temperatures is investigated and the solute structures vary significantly.

The plate count and column pressure drop were also calculated for the three conditions reported in Figure 1. The column pressure was reduced from 39 to 11 bar between 30 °C and 150 °C, and the plate count decreased from 14,276 to 12,817. This reduction in efficiency occurs because the van Deemter curve is shifted toward a higher linear velocity at an elevated temperature. Therefore, it would be useful to increase the flow rate to 5 mL/min at 150 °C to achieve the same plate count as observed at 30 °C and 1 mL/min. Under these conditions, the analysis time would be reduced to only 1 min at 150 °C, and the pressure would remain acceptable (53 bar).

Understanding Isocratic Mode in Reversed-Phase LC

Theoretical Background

Retention in LC can be described by linear solvent strength (LSS) theory (5), which shows that the log k value of a given compound is inversely proportional to the proportion of organic modifier in the mobile phase.

This linear behavior can be used to optimize the resolution and develop a reversed-phase LC method. Retention models (log k = f[%methanol]) can be drawn for several compounds contained within a mixture. Then, when considering a given methanol percentage, where the curves do not overlap, the corresponding selectivity is high. In this spreadsheet, the retention models of five compounds with log P values between 2.2 and 2.8 and S values between 4 and 6.3 are presented. Based on these val-

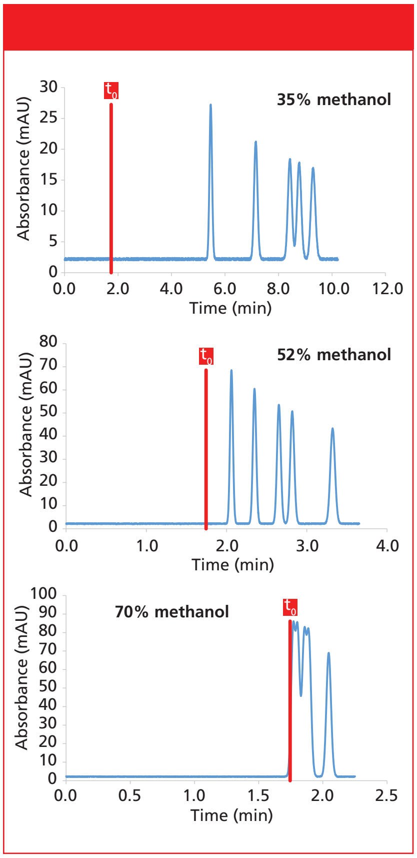

Figure 2: Impact of isocratic mobile phase composition on the retention and selectivity in reversed-phase LC mode. The chromatograms were simulated at 30 °C. Column dimensions: 150 mm × 4.6 mm, 5 µm; flow rate: 1 mL/min.

ues, the minimal resolution for each methanol percentage was plotted as a function of methanol percentage and the corresponding chromatogram was shown. This procedure can be used to optimize a reversed-phase LC method. Because the LSS curves are linear, only two experimental runs are required to determine the intercepts and slopes of the curves and optimize the chromatographic separation under isocratic conditions. This approach is how optimization software, such as Drylab (Molnar-Institute), Chromsword (Iris Tech), LC & GC simulator (Advanced Chemistry Development), and Osiris (Datalys), optimizes HPLC separations.

Using the "Isocratic Mode" Spreadsheet

A chromatographic separation of five substances can be simulated for any mobile-phase composition. As shown in Figure 2, the retention decreases at high percentages of methanol, which is in line with LSS theory. In addition, the selectivity is also modified. Even though the analysis time was longer at 35% methanol, the separation was not improved compared to 52% methanol. However, the separation obtained at 70% methanol was the worst in terms of selectivity because of the too low retention of the five substances under these conditions ($k < 1$).

Users can also set a minimal resolution value (for example, 1.5, which corresponds to a baseline separation of five compounds), and the optimal corresponding methanol percentage is calculated based on the graph representing the minimal resolution as a function of methanol percentage.

Understanding Gradient Mode in Reversed-Phase LC

Theoretical Background

Gradient elution mode is often used in reversed-phase LC to elute compounds of diverse hydrophobicity from a chro-

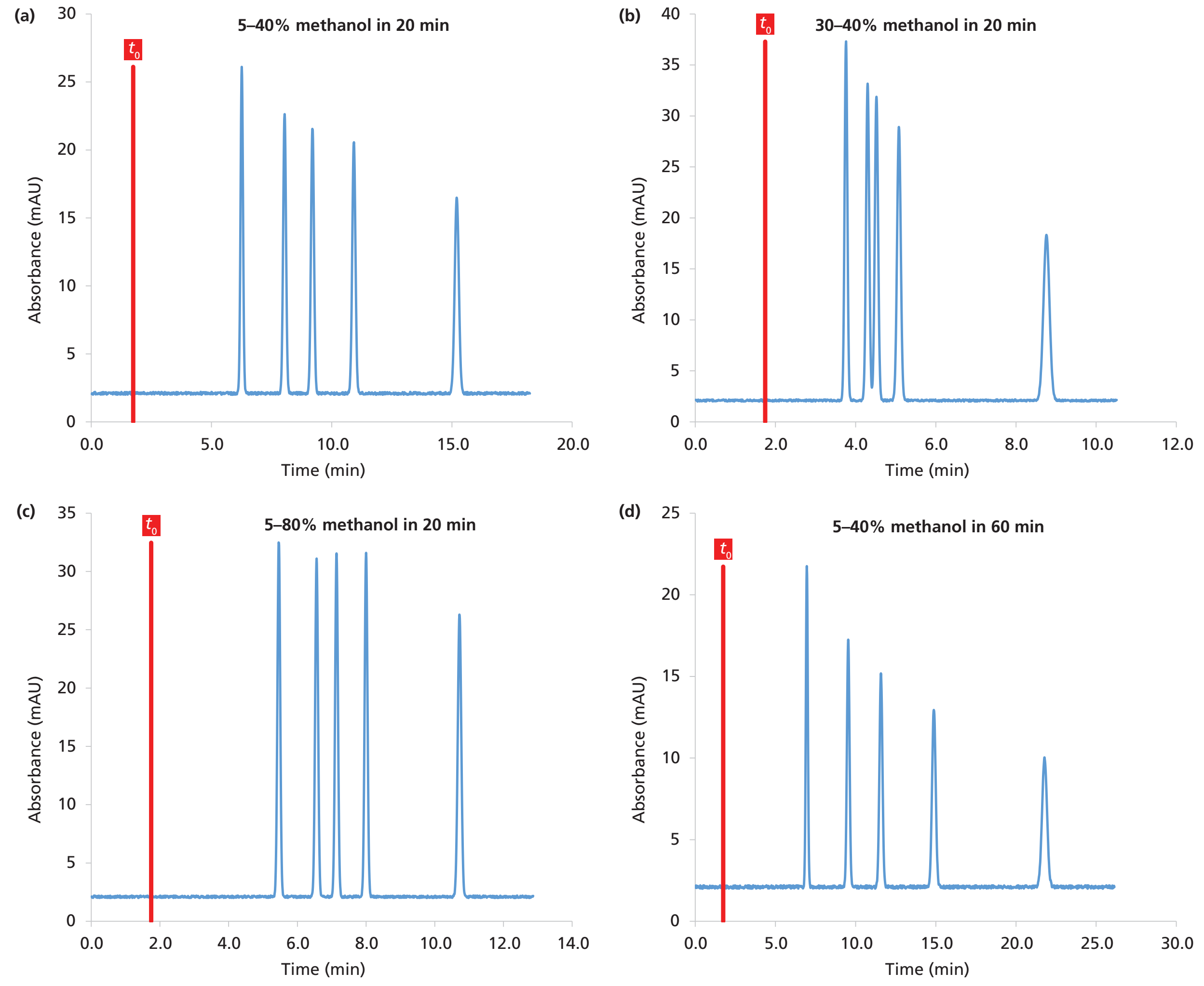

Figure 3: Relationship between the gradient conditions and the retention–selectivity in reversed-phase LC mode. The chromatograms were simulated at 30 °C. Column dimensions: 150 mm × 4.6 mm, 5 µm; flow rate: 1 mL/min.

matographic column. A chromatogram of five substances eluted under gradient conditions was simulated, and the following procedure was used to construct the chromatogram.

First, five substances of different lipophilicity (log P values ranging from 1.4 to 3.4) were selected. The S values of these five compounds were arbitrarily fixed between 4.0 and 6.3, respectively.

Second, based on the gradient profile (% initial, % final, and gradient time), column dimensions (L_{col}, d_{col}, and d_p) and mobile-phase flow rate (F) set by the user, the corresponding elution composition (C_e) of each compound was calculated. Then, the C_e values were transformed into t_R under the gradient conditions.

Third, the peak widths (W) in gradient mode were simulated using the following equation:

$$W = \frac{t_0 \times (1 + k_e)}{\sqrt{N}} \qquad [4]$$

where N is the plate count and t_0 is the column dead time (both calculated based on the column dimensions and mobile-phase flow rate), k_e is the elution retention factor, which is calculated from the elution composition, the log k_w value obtained from the log P value (using equation 11 from part I) and the S value, using the following equation:

$$\log k_e = \log k_w - S \times C_e \qquad [5]$$

Finally, the corresponding peak capacity (n_{peaks}) was calculated using the equation proposed by Neue (6):

$$n_{peaks} = 1 + \frac{\sqrt{N}}{4} \times \frac{1}{b+1} \ln\left(\left(\frac{b+1}{b} e^{S \times \Delta\Phi} - \frac{1}{b}\right)\right) \qquad [6]$$

where b is the gradient steepness, which can be expressed as follows:

$$b = \frac{t_0 \cdot \Delta\Phi \cdot S}{t_{grad}} \qquad [7]$$

where t_{grad} is the gradient time and $\Delta\Phi$ is the change in solvent composition during the gradient, ranging from 0 to 1.

Using the “Gradient Mode” Spreadsheet

This spreadsheet allows the simulation

of chromatograms for various column dimensions, mobile-phase flow rates, and gradient conditions. Figure 3 shows four simulated chromatograms, for which the column dimensions and mobile-phase flow rates were fixed and only the gradient conditions were modified (initial and final compositions, as well as gradient time). The first chromatogram shown in Figure 3a was obtained with a gradient from 5% to 40% methanol in 20 min and allows baseline separation of the five substances with a peak capacity of 63 and minimum resolution ($R_{s,min}$) of 3.3. For the second chromatogram, the initial methanol percentage was modified from 5% to 30%. As shown in Figure 3b, the separation was faster, which is logical because the initial composition of the mobile phase had more eluent, and the selectivity was also modified, which is in agreement with theory because the initial methanol percentage and gradient slope are the two most important parameters for tuning the selectivity under gradient conditions. In Figure 3b, the peak capacity was 63 and $R_{s,min}$ decreased to 1.3. Between Figures 3a and 3c, only the final methanol percentage was modified from 40% to 80%. Under these conditions, all the peaks were eluted in approximately 10 min, showing that there is no need to greatly increase the final methanol percentage. Indeed, the investigated molecules were not sufficiently lipophilic to require such an elevated methanol proportion. Finally, between Figures 3a and 3d, only the gradient time was modified, from 20 to 60 min. The increase of the gradient time improves the peak capacity (equal to 91) and $R_{s,min}$ (equal to 4.6). However, it could be beneficial to reduce the final methanol percentage in Figure 3d because all the peaks were eluted in only 22 min.

In this example, only the gradient profile was modified, but the user can also easily visualize the impact of the column dimensions and mobile-phase flow rate on the chromatogram obtained under gradient conditions. For example, for a constant gradient time, a higher mobile-phase flow rate often improves the resolution and peak capacity while simultaneously reducing the elution time. This improvement occurs because the column dead time is lower at elevated mobile-phase flow rates; therefore, the k_e values are enhanced, leading to better overall performance (7).

Understanding the Impact of the Injected Volume in Reversed-Phase LC

Theoretical Background

The injected volume in reversed-phase LC has two effects on the separation, which are simulated under isocratic conditions in the spreadsheet entitled "injected volume." First, a higher sensitivity (C_{max}) is expected when increasing the injected volume (V_{inj}) based on equation (8):

$$C_{max} \propto \frac{\sqrt{N} \times V_{inj}}{L \times d_c^2 \times (1+k)} \qquad [8]$$

However, it is not possible to inject a volume as large as is possible in reversed-phase LC, so a compromise

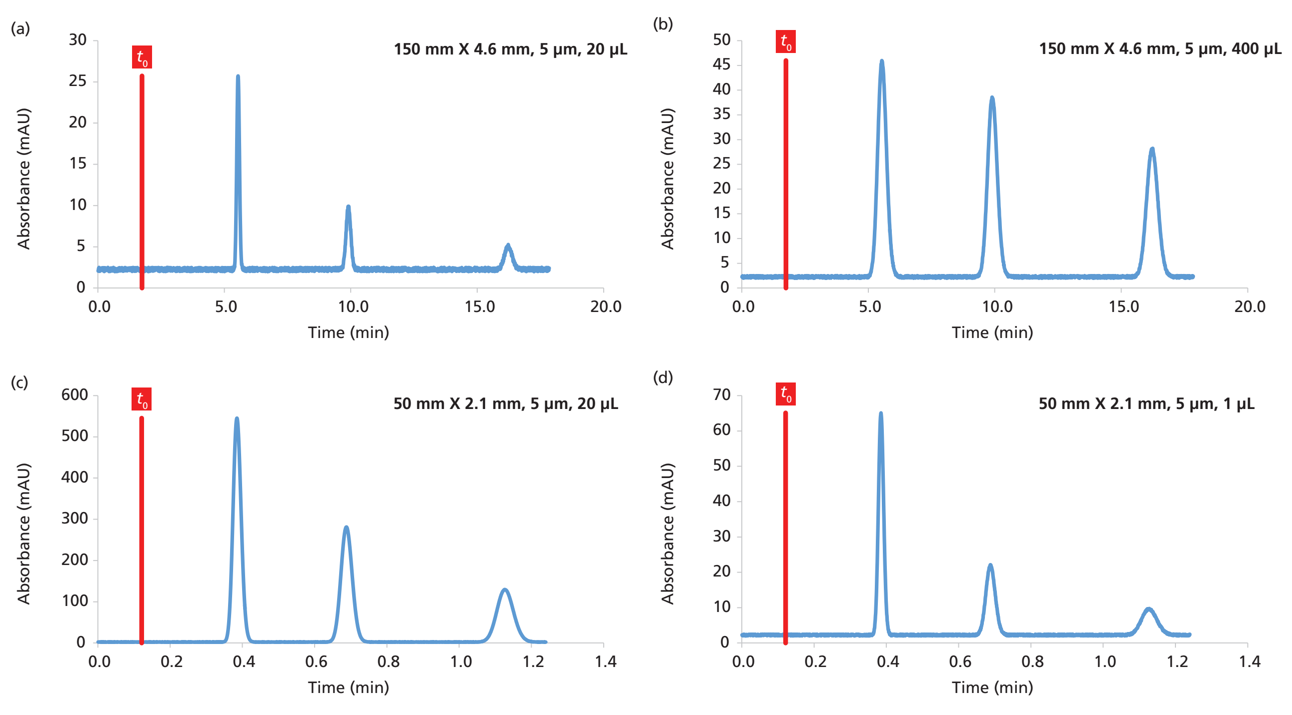

Figure 4: Impact of the injected volume on the sensitivity and kinetic performance in reversed-phase LC conditions. The chromatograms were simulated at 30 °C. Mobile phase: 30 methanol; flow rate: 1 mL/min; injected concentration (C_{inj}): 20 mg/L; compound log *P* values: 1.6, 2.0, and 2.3.

has to be found. Peaks become much broader and the plate count is reduced at high V_{inj} values for the following reason: In HPLC, the observed peak variance (σ^2_{tot}) is the sum of the chromatographic column dispersion (σ^2_{col}), the dispersion related to the injection system (σ^2_{inj}) and the dispersion related to the rest of the equipment (tubing and detector, σ^2_{ext}). It can be expressed as follows:

$$\sigma^2_{tot} = \sigma^2_{col} + \sigma^2_{inj} + \sigma^2_{ext} \qquad [9]$$

For the sake of simplicity, σ^2_{ext} was neglected.

Dispersion linked to the chromatographic column itself (σ^2_{col}) can be obtained by the following equation, when a very small volume of sample is injected:

$$\sigma^2_{col} = \frac{V_R}{\sqrt{N}} = \frac{V_0 \cdot (1 + k)}{\sqrt{N}} \qquad [10]$$

where σ^2_{col} is the column variance (in units of μL^2), N is the number of plates, and V_R is the retention volume, which is a function of the column dead volume V_0 and retention factor k.

The dispersion related to the injection (σ^2_{inj}) can be expressed as shown in equation 11 (9):

$$\sigma^2_{inj} = K_{inj} \cdot \frac{V^2_{inj}}{12} \qquad [11]$$

where K_{inj} is a constant (generally between 1 and 3) that depends on the injection mode. In our case, this value was set to 2.

The observed plate number (N_{obs}) could then be estimated by the equation below:

$$N_{obs} = N_{col} \cdot \frac{1}{1 + \dfrac{\sigma^2_{inj}}{\sigma^2_{col} + \sigma^2_{inj}}} \qquad [12]$$

where N_{col} is the theoretical number of plates of the chromatographic support.

The impact of V_{inj} on chromatographic performance (sensitivity and plate count) was assessed. Users can set the column dimensions (L_{col}, d_{col}, and d_p), mobile-phase flow rate, log *P* values of three model compounds, percentage of methanol in the mobile phase, injected volume, and compound concentration. Then, a chromatogram is simulated under the conditions set by the user, and a graph representing the column volume versus the injected volume is shown. All the chromatographic calculations and simulations were made for a sample diluted in a mixture of solvent strictly equivalent to the mobile phase itself.

Using the "Injected Volume" Spreadsheet

In reversed-phase LC, the injected volume should be between 0.5% and 5% of the column volume to achieve a good compromise between sensitivity and peak broadening. Figure 4 shows the effect of the injected volume on the chromatographic performance for three compounds with *k* values between 2 and 8. In Figure 4a, a chromatogram was simulated for an injected volume of 20 µL on a 150 mm × 4.6 mm, 5-µm column (V_{inj} equal to 1.1% of V_{col}). Under these conditions, the peaks were narrow (average efficiency of 11,700 plates), but the sensitivity was also poor, particularly for the last eluted peak, because of the dilution effect in the mobile phase. Therefore, the injected volume was increased 20-fold in Figure 4b (400 µL), and better

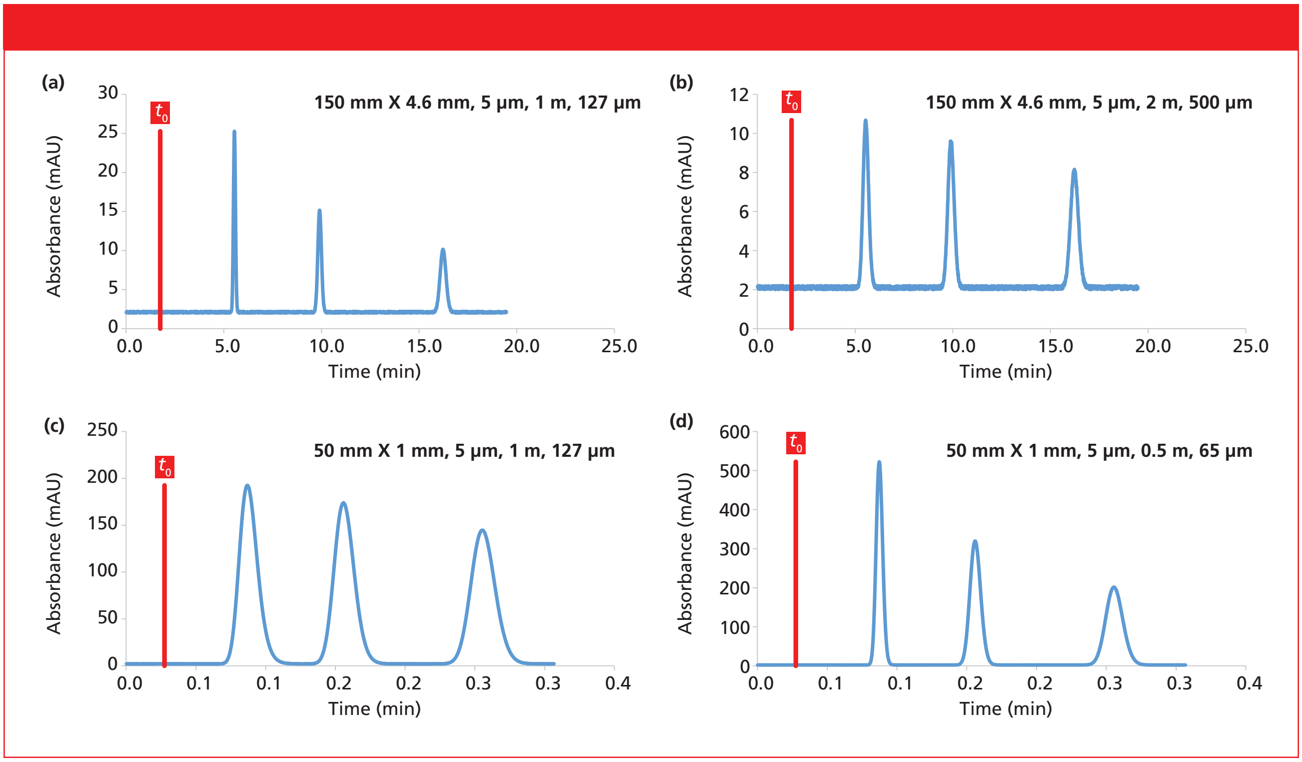

Figure 5: Impact of the injected volume on the sensitivity and kinetic performance in reversed-phase LC conditions. The chromatograms were simulated at 30 °C. Mobile phase: 30 methanol; flow rate: 1 mL/min; injected concentration (C_{inj}): 20 mg/L; compound log *P* values: 1.6, 2.0, and 2.3.

sensitivity was achieved. However, the peaks were also much broader in this chromatogram (*N* = 2500 plates, compared to a theoretical *N* value of 11,700), and strong differences were observed between the first eluted peak (*N* = 1050 plates) and the last one (*N* = 5300 plates), according to equations 10 and 12. Under these new conditions, V_{inj} is equal to 23% of V_{col}, which is too high. In Figures 4c and 4d, a column of reduced volume was selected (50 × 2.1 mm, 5 µm). A volume of 20 µL was injected in Figure 4c (V_{inj} equal to 16% of V_{col}), which is relatively high. The efficiency was poor (1200–2500 plates), but the sensitivity was relatively good. When decreasing V_{inj} to only 1 µL (V_{inj} equal to 0.8% of V_{col}), no loss in plate count was observed for the column (approximately 3000 plates), but the sensitivity was too low for the last eluted peaks.

These examples illustrate the impact of the injected volume on the chromatographic performance and the compromise that needs to be made to achieve sufficient sensitivity and reasonable band broadening.

Understanding the Impact of the Tubing Geometry in Reversed-Phase LC

Theoretical background

In HPLC, the volume between the injector and detector contributes to band broadening. Therefore, the tubing between the injector and the column inlet, as well as the tubing between the column outlet and the detector, should be optimized in terms of dimensions to limit peak broadening and efficiency loss under isocratic conditions.

Similar to what was previously described for the injected volume, the dispersion related to the tubing (σ^2_{tube}) can be expressed as a function of the tubing radius, r_{tube}, and its length, l_{tube} (9):

$$\sigma^2_{tube} = \frac{r^4_{tube} \cdot l_{tube} \cdot F}{7.6 \cdot D_m} \qquad [13]$$

Here, the dispersion related to the injection and detector was neglected for simplicity.

The tubing volume affects the plate count according to the following relationship:

$$N_{obs} = N_{col} \cdot \frac{1}{1 + \dfrac{\sigma^2_{tube}}{\sigma^2_{col} + \sigma^2_{tube}}} \qquad [14]$$

The pressure generated by the tubing itself is also modified by changing the tubing diameter (d_{tube}) or length (L_{tube}), according to the Hagen-Poiseuille equation:

$$\Delta P = 128 \frac{\eta \times L_{tube} \times F}{\pi \times d^4_{tube}} \qquad [15]$$

The impact of the tubing geometry (d_{tube} and L_{tube}) on chromatographic performance (generated pressure and chromatographic dispersion) was assessed. Users can set the column dimensions (L_{col}, d_{col}, and d_p), mobile-phase flow rate, log *P* values of the three model compounds, the methanol percentage in the mobile phase, and the tubing geometry (L_{tube} and d_{tube}). Users have a choice of four conventional tubing diameters that are widely used in HPLC, namely 65, 127, 250, and 500 µm. A chromatogram is simulated under the selected conditions, and a graph representing the column volume versus tubing volume

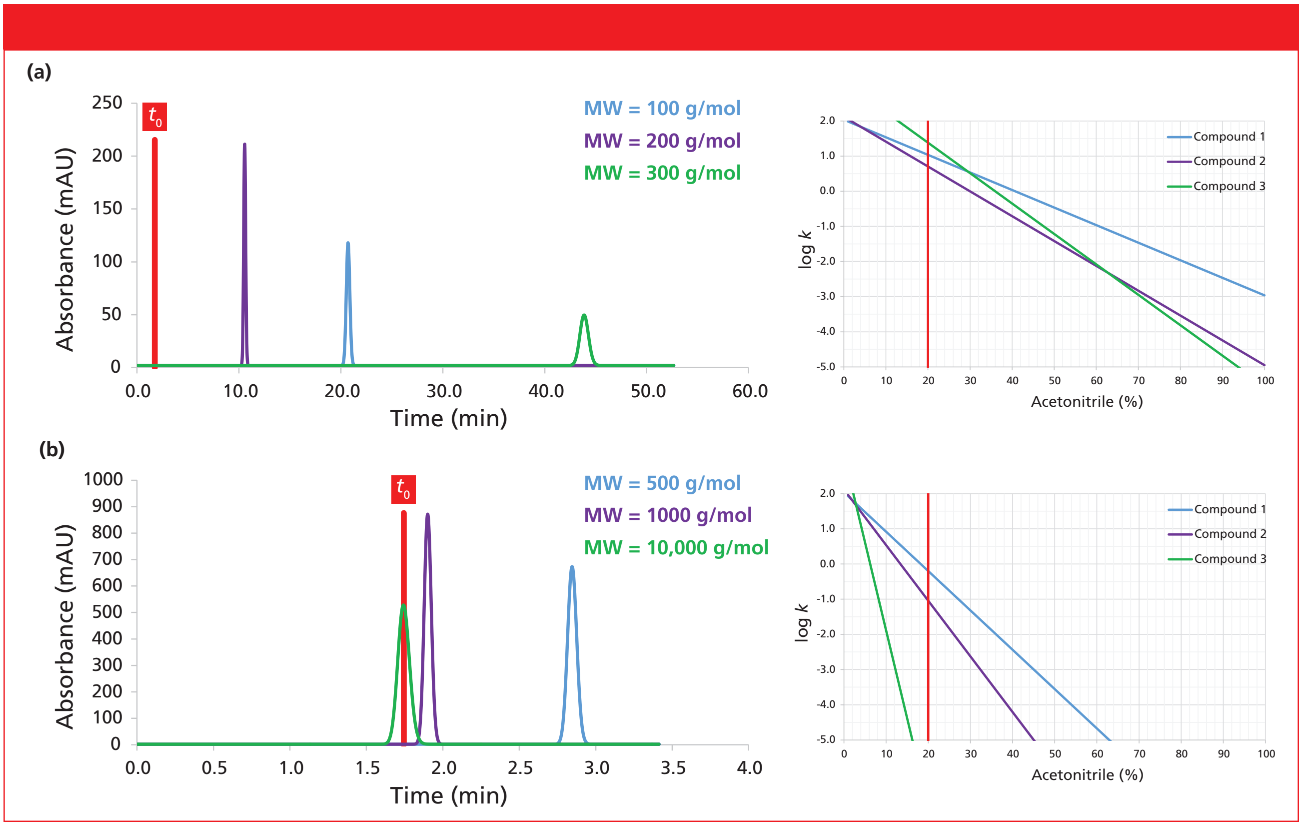

Figure 6: Impact of the compound molecular weight in reversed-phase LC mode. The chromatograms were simulated at 30 °C. Column dimensions: 150 mm × 4.6 mm, 5 µm; mobile phase: 20% acetonitrile; flow rate: 1 mL/min.

is shown. The pressure generated by the tubing is also provided to determine the best compromise between pressure and band broadening.

Using the "Tubing Geometry" spreadsheet

Figure 5 shows the impact of the tubing geometry on chromatograms simulated under various analytical conditions. In Figure 5a, the column dimensions were standard (150 mm × 4.6 mm, 5 µm) and the tubing had a relatively low volume corresponding to only 1% of the column volume. Under these conditions, the impact of tubing on the efficiency was negligible (theoretical efficiency of 11,700 plates and observed efficiency for compounds with *k* values between 2 and 8, equal to 11,500–11,600 plates), and the pressure generated by the tubing itself was reasonable (37 bar). After changing the tubing geometry to a length of 2 m and a diameter of 500 µm, the impact on the kinetic performance was drastic. In Figure 5b, the peaks were much broader and the efficiency was in the range of 1500–6600 plates. The efficiency was strongly decreased because of the modification of the tubing geometry, in agreement with equations 13 and 14. In Figures 5c and 5d, the column dimensions were modified and the volume was strongly decreased (50 mm × 1 mm, 5 µm). The impact of the tubing volume was obviously more critical, and the peaks were particularly broad when using this column on a system with approximately 1 m of tubing with a 127-µm diameter. The tubing volume represented 46% of the column volume, and efficiency was in the range of 190–930, and the column should produce 1870 plates at this flow rate. Based on this observation, the tubing diameter was reduced to 65 µm and the length was reduced to 50 cm. Under these conditions, the efficiency was much better because the tubing represented only 6% of the column volume, but the tubing pressure was too large (270 bar), in agreement with equation 15.

In conclusion, a compromise has to be made between plate count and pressure when selecting the optimal tubing geometry for plumbing a HPLC system. A chromatographic system cannot be easily used with a wide range of column internal diameters. Currently, even the best HPLC and ultrahigh-pressure liquid chromatography (UHPLC) systems on the market are not adapted to 1 mm i.d. columns, and the performance reported in Figure 5d cannot be achieved. Therefore, it would be relevant to develop a technical solution to limit the use of tubing in isocratic mode.

Understanding the Impact of Compound Molecular Weight in Reversed-Phase LC

Theoretical Background

As shown in equation 2, the diffusion coefficient (D_m) of an analyzed compound is directly proportional to the analyte molecular weight (MW). In addition, there is a direct relationship between the optimal linear velocity (u_{opt}) and D_m, according to equation 1. Therefore, the kinetic performance, and particularly the van Deemter curve shapes, is modified when analyzing compounds of different molecular weights. The achieved plate count at a given flow rate might change depending on the compound molecular weight. In most cases, a decrease in performance (broader peaks) is observed in reversed-phase LC when increasing the molecular weight of the analyzed substances because experiments are often conducted at flow rates higher than the van Deemter optimum.

In addition to the kinetic performance, the size of the analyzed compound also impacts the slope of the LSS curve (log *k* versus % organic solvent), which corresponds to the *S* parameter in equation 12 of part I (1). It has been empirically demonstrated that the relationship between *S* and MW can be expressed by the following empirical equation (5):

$$S = 0.5 \times MW^{1/2} \qquad [16]$$

Therefore, for a high *S* value (large molecules), the retention factor may vary strongly with the mobile-phase composition (methanol percentage). In some cases (for example, with large peptides and proteins), it is impossible to find isocratic conditions to analyze several large molecules, and gradient elution is mandatory.

In the spreadsheet, the user can set the column dimensions (L_{col}, d_{col}, and d_p), mobile-phase flow rate, acetonitrile percentage in the mobile phase and the molecular weight of the three model compounds. Using these values, kinetic curves for the three different model compounds are drawn, showing the efficiency versus mobile-phase flow rate. In addition, the LSS curves (log *k* versus %acetonitrile) are provided to illustrate the slopes of the curves and how the retention factors varies with %acetonitrile. Finally, a chromatogram is also simulated for the three model compounds. Here, a color code (blue, purple, or green) was used to distinguish between the three analytes. In this spreadsheet, the three selected compounds have log *P* values of 2.2, 2.3, and 3.5, respectively, and the *S* values were estimated using equation 16 based on the compound molecular weights. Equation 12 of part I (1) was used to calculate the log *k* of each substance and their corresponding retention times.

Using the "Molecular Weight" Spreadsheet

Figure 6 shows the impact of the compound molecular weight on the chromatographic performance, considering three compounds with constant log *P* values of 2.2 (blue trace), 2.3 (purple trace), and 3.5 (green trace). Between Figures 6a and 6b, only the molecular weights were changed, leading to modification of the *S* values.

In Figure 6a, the molecular weights were comparable (between 100 and 300 g/mol); therefore, the three compounds can be eluted under isocratic conditions with sufficient retention (*k* values between 5 and 24). This behavior is in line with the three LSS curves (log *k* versus acetonitrile percentage) because the slopes were comparable. From a kinetic point of view, the observed plate count varies from 14,700 (MW of 100 g/mol) to 11,300 (MW of 300 g/mol) when using a 150 mm × 4.6 mm, 5-µm column at a constant flow rate of 1 mL/min. Therefore, the flow rate should be decreased to 0.45 mL/min to attain a plate count of approximately 15,000 plates for a 300 g/mol molecule.

In Figure 6b, the three model compounds have molecular weights ranging from 500 to 10,000 g/mol with the same log *P* values as in Figure 6a. This example illustrates that molecules with very different molecular weights cannot be easily eluted under isocratic conditions at 20% acetonitrile with a reasonable retention. In this example, the retention factors were between 0 and 0.6. This chromatographic behavior can be explained by the LSS curves on the right side of Figure 6b. The curves have relatively different (but also very steep) slopes, which makes the separation incompatible with isocratic conditions. In addition to the retention behavior, the plate count was quite low for these larger molecules (2200–9500 plates) because the flow rate was much higher than the optimal flow rate. For the largest molecule of 10,000 g/mol, the flow rate should be 60 µL/min to achieve a plate count of approximately 15,000 plates. However, these conditions are impractical because the column dead time would be 29 min.

Conclusion

This spreadsheet provides an understanding of the basic principles of liquid chromatography using virtual (simulated) chromatograms obtained under various analytical conditions.

Acknowledgments

The authors wish to thank Dr. Szabolcs Fekete from the University of Geneva for his critical review of the manuscript and his suggestions to improve the spreadsheet.

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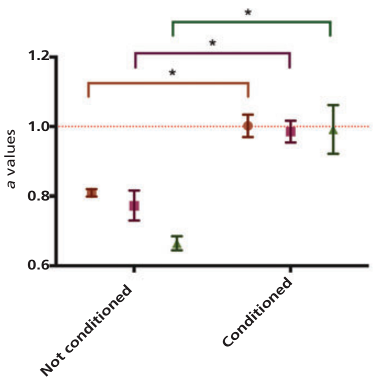
Methodology for Removing Dihalomethane Carryover from Solid-Phase Microextraction Fibers



Solid-phase microextraction (SPME) in conjunction with gas chromatography–mass spectrometry (GC–MS) is a simple and effective way to sample analytes. Ordinarily the coated fiber is rid of compounds during desorption in the GC system, allowing for the analysis of a new sample. Carryover of the analyte between samples, however, is a problem with many chemicals. Our data show that heating the fiber in a high-temperature injection port for only 2 min between runs prevents carryover. The short heating between samples improves the linearity of the peak area versus concentration relationship over four orders of magnitude of concentration, with a limit of detection below 10^{-7} M in every case. Although carryover is an acknowledged problem with SPME fibers, such short conditioning steps are rarely considered as a means to eliminate it. This study suggests that they should be evaluated as an option.

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Solid-phase microextraction (SPME) is a rapid and solvent-free extraction technique for concentrating a volatile or semivolatile analyte. Developed in the 1990s (1,2), this technique has seen rapid adoption and widespread use. The analyte in the headspace of a closed and equilibrated vial is allowed to equilibrate with a coated silica fiber. The sample is then thermally desorbed from the SPME fiber while in the injection port. For certain compounds, carryover on the SPME fiber is a problem that increases the limit of detection. The issue of carryover is discussed in major texts on the SPME technique (3–5), but the approaches taken to eliminate it in the literature are varied and usually do not include validation data. Frequently carryover is eliminated by blank runs in between samples or an extension of the desorption step, but these approaches are not always sufficient to remove sticky compounds. Previous work has established that a long (30-min or 10-min) high-temperature conditioning of the fiber between samples can remove carryover for certain analytes (6,7). Shorter (5-min) runs in a separately operated conditioning instrument have also proven to be effective (8). This work explores the effect of an even shorter (2-min) high-temperature conditioning in a second injection port. This approach is effective in removing persistent carryover of iodinated dihalomethanes. The short length of the conditioning run is an asset in large batch processing of these compounds, which are not stable over long time periods.

Halogens play an important role in the oxidative capacity of the atmosphere, so quantifying atmospherically abundant halogenated compounds and their precursors in seawater and marine aerosols is of significant interest (9–13). Wastewater treatment from nuclear power plants produces halogenated compounds, also necessitating measurements in aqueous solutions (14). The concentration ranges in which diiodomethane (CH_2I_2) and chloroiodomethane (CH_2ICl) are found in these venues tend to be in the parts-per-billion (ppb) (~10^{-9} M) range for seawater and the parts-per-trillion (ppt) (~10^{-12} M) range for wastewater.

These dihalogenated methane compounds are difficult to measure over a large range of concentrations using standard spectroscopic techniques. Although some studies have successfully used atomic emission spectrometry (15), most research groups have settled upon various types of chromatography and mass spectrometry (MS) to quantify CH_2ICl and CH_2I_2 levels. Most of these methods require a preconcentration or extraction step from aqueous samples to increase sensitivity enough to test field samples and avoid saturating the mass spectrometer with water signal. These methods include adsorbent cold traps coupled with gas chromatography–mass spectrometry (GC–MS) (16,17), purge-and-trap methods followed by GC with electron-capture detection (ECD) (14,17), as well as GC–MS (10), and cryogen-free cooling traps linked to GC with various detection methods (18). The equipment needed to perform several of these preconcentration steps is not widespread commercially.

SPME, coupled to both MS and ECD, has also been used with success to measure dihalomethanes (19–21) as well as trihalomethanes (22,23). This simple and widely accessible technique can be effectively used for laboratory studies of these compounds. It can also be combined with preconcentration techniques to test very low concentration field samples. However, iodine-containing compounds exhibit a significant problem with carryover on the SPME fiber. Iodinated compounds are generally acknowledged to adsorb to surfaces and be difficult to remove from materials (24,25), but there is little literature data that explicitly studies relative rates of adsorption and desorption under realistic conditions (26). A method is presented here for eliminating carryover on the fiber during GC–MS measurements of iodinated dihalomethanes with SPME sample preparation that is shorter than previous literature methods used with commercial fibers.

Experimental

Instrumentation and Reagents

The concentrations studied ranged from 1×10^{-4}–1×10^{-9} M chloroiodomethane in either water or aqueous 0.5 M sodium chloride solutions, and 1×10^{-4}–1×10^{-9} M diiodomethane in aqueous 0.5 M sodium chloride solutions. These concentration ranges equate to 17.6 mg/L–1.76 µg/L for chloroiodomethane and 26.8 mg/L–2.68 µg/L for diiodomethane.

Samples were prepared through serial dilutions of 1×10^{-3} M stock solutions. Sodium chloride (99+%), chloroiodomethane (98%), and diiodomethane (99+%) were all purchased from ACROS. All solutions used 18

Table I: Instrumental parameters for the headspace SPME GC–MS experiments

		SSL Injection Port Only	PTV and SSL Ports
SPME parameters	SPME fiber	75 µm Carboxen–PDMS	Same
	Incubation	40 °C, 10 min with agitation	Same
	Extraction	40 °C, 10 min	Same
	Desorption	7 min, 220 °C inlet, split flow of 50 mL/min for 1 min (split ratio 10), constant purge, glass liner with 0.75 mm i.d.	7 min, 250 °C, split flow of 10 mL/min (split ratio 10) and constant purge, Siltek SilcoSteel liner with 2 mm i.d.
	Fiber cleaning	None	SSL, 300 °C, 2 min, 50 mL/min constant flow, glass liner with 5 mm i.d.
GC parameters	Analytical column	30 m TR-FAME, 0.25 mm i.d., 0.25 µm film	Same
	Carrier gas	UHP helium (minimum purity 99.999%)	Same
	Injector	SSL	PTV
	Oven program	40 °C for 2 min, 10 °C/min ramp to 200 °C, 200 °C for 1 min	Same
Fitting parameters	Peak fitting	Genesis algorithm	Same
	CH_2ICl ion	$m/z = 176$ (molecular ion)	Same
	CH_2I_2 ion	$m/z = 141$ (fragment ion)	Same

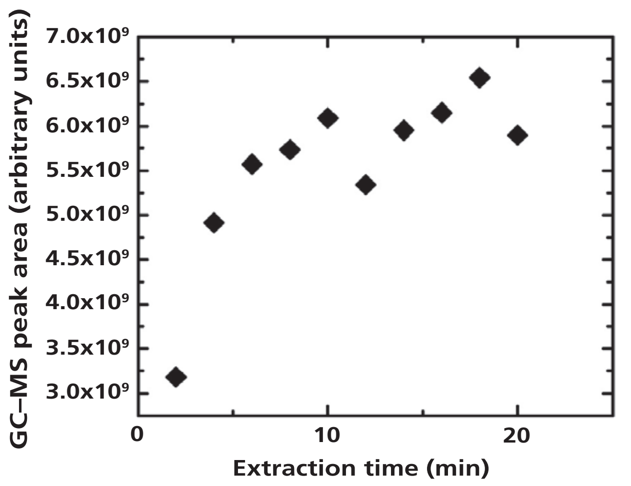

Figure 1: Extraction time variation for 1×10^{-4} M CH_2ICl in water. A 10-min extraction time was used for the experiments. Only signal from the molecular ion of CH_2ICl at m/z = 176 is used.

mΩ Barnstead E-Pure water (Thermo Fisher Scientific). Sodium chloride was dissolved, then filtered through sterile Millex syringe driven filters with a 0.45-µm pore size and a membrane of mixed cellulose esters from EMD Millipore. Samples, in 0.50-mL aliquots, were stored in amber glass sample vials with silicone–polytetrafluoroethylene (PTFE) septa (Fisher Scientific). The halogenated organics are light sensitive, so amber glass vials were necessary. Each concentration series was run in triplicate during every headspace SPME GC–MS sequence. Solutions were always run on the same day they were made because it was determined that the samples degraded significantly over longer periods of time. Each bottle took approximately 25 min to analyze and an entire run took about 15 h to complete, including blanks. Samples were run from lowest to highest concentration in multiple replicate sets, with two blank runs between samples. One blank held the temperature at the highest point of the ramp (200 °C) for 11 min, and the other replicated the normal temperature ramp.

Measurements were performed on a Thermo Fisher Trace GC Ultra gas chromatograph and DSQ II mass spectrometer with samples introduced via a Triplus autosampler (Thermo Electron North American LLC). The ability to do a conditioning run was obtained when an additional programmable temperature vaporizing injector (PTV) port was added to the system, changing the instrument configuration. Thus, measurements were done under two conditions: early measurements used a constant temperature split-splitless (SSL) injection port and later measurements used a programmable variable temperature (PTV) port for injection and the SSL port for cleaning between runs. Parameters for both sets of runs are listed in Table I. In all cases, new SPME fibers underwent a 1-h factory-recommended initial conditioning run at 300 °C before the first use.

Data for the full concentration ranges of chloroiodomethane in water, chloroiodomethane in 0.5 M sodium chloride, and diiodomethane in 0.5 M sodium chloride were collected with and without the conditioning runs between each sample. Peak area at selected masses was determined using the genesis function of the XCalibur Qual Browser program. The molecular

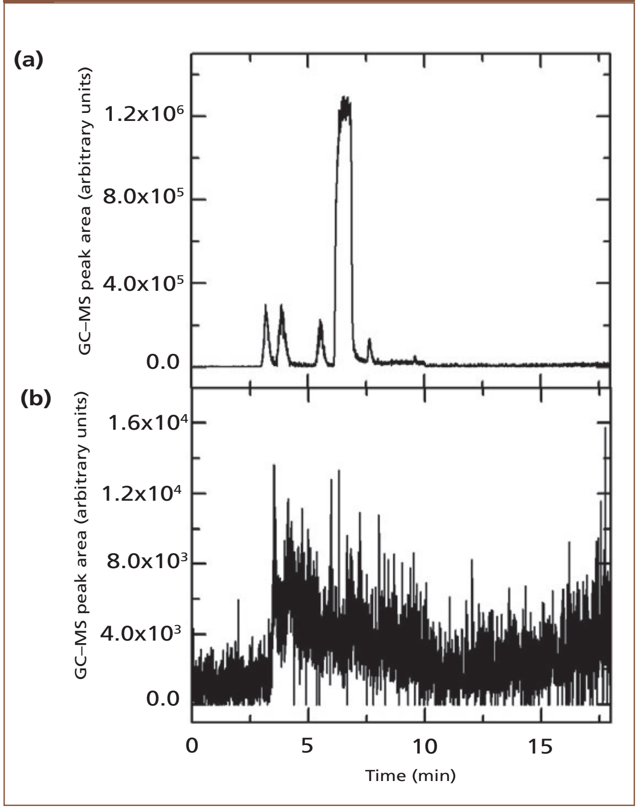

Figure 2: (a) Gas chromatogram of blank sample run after a 1×10^{-4} M CH_2ICl sample in water without a conditioning run. Only signal from the molecular ion of CH_2ICl at *m/z* = 176 is shown. The largest peak at 6.5 min is consistent with the retention time of CH_2ICl, indicating that there is carryover on the fiber. (b) Gas chromatogram of blank sample run after a 1×10^{-4} M CH_2ICl in water sample is followed by the conditioning run. There is no distinct peak at 6.5 min, signifying that carryover has been eliminated. Note that the scale is changed from (a) to show the lack of signal clearly.

ion peak at 176 *m/z* was used to quantify chloroiodomethane content in samples and the fragment ion peak at 141 *m/z* was used to determine diiodomethane concentration in samples. Retention times were about 6.5 min and 10.5 min for chloroiodomethane and diiodomethane, respectively. Samples at 1×10^{-8} M and 1×10^{-9} M concentration were collected, but are not included in the fits because they were under the statistical limit of detection. Peak identities were regularly checked through spectral matching with the National Institute of Standards and Technology (NIST) database. These matches always confirmed that the peaks under examination belonged to chloroiodomethane and diiodomethane.

The GC–MS signal was plotted versus concentration and fit to the power law $y = mx^a$ in Microsoft Excel. Hierarchical mixed model analysis using random intercepts was performed on the data to determine significance using SPSS Premium Campus Edition software.

Methods

Several parameters were examined to arrive at the method listed above. Both 100-μm polydimethylsiloxane (PDMS)

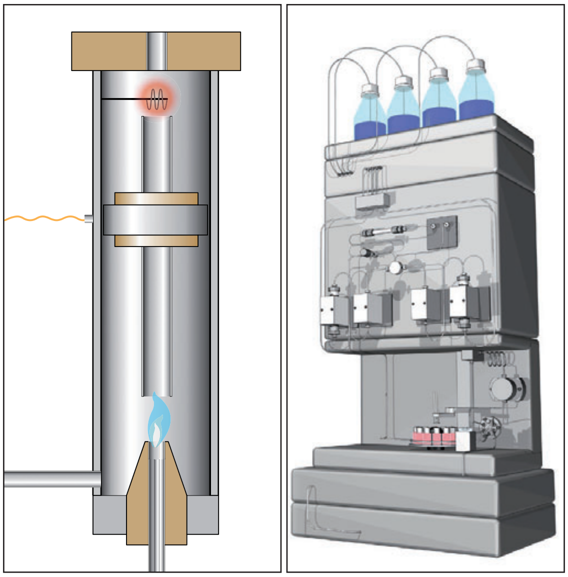

Table II: Parameters for fits to the equation $y = mx^a$ for plots of peak area versus concentration. Values are given for the a parameter for each individual trial replicate. Averages and standard deviations taking into account the replicates for each trial are also given. Values of a close to 1 indicate strong linearity, while m corresponds to the linear slope.

		Without Conditioning				With Conditioning			
		Average m*	Average R^2*	Average a	Individual a	Average m	Average R^2	Average a	Individual a
CH_2ICl in water	Trial 1	5 (± 3) × 10^{+13}	0.994 ± 0.001	0.81 ± 0.01	0.82025 0.8004 0.8055	1.5 (± 0.1) × 10^{+13}	0.9997 ± 0.0002	0.988 ± 0.008	0.9800 0.9872 0.9958
	Trial 2	2.079 × 10^{+13} 2.523 × 10^{+13}	0.9921 0.9932		0.8012 0.8195	2.4 (± 0.6) × 10^{+13}	0.9992 ± 0.0007	1.02 ± 0.02	0.997 1.026 1.024
	Trial 3					3 (± 3) × 10^{+13}	0.997 ± 0.003	1.00 ± 0.07	1.071 0.9410 0.9831
	Trial 4					2.1 (± 0.4) × 10^{+13}	0.9998 ± 0.0002	1.01 ± 0.02	1.022 0.9838 1.010
CH_2ICl in 0.5 M NaCl	Trial 1	2.5 (± 0.1) × 10^{+13}	0.991 ± 0.001	0.74 ± 0.01	0.7506 0.7322 0.7363	1.4 (± 0.6) × 10^{+13}	0.998 ± 0.003	1.02 ± 0.03	1.046 0.9973 1.010
	Trial 2	1.860 × 10^{+13} 1.800 × 10^{+13}	0.9913 0.9931		0.7519 0.7488	2.5 (± 0.4) × 10^{+13}	0.9987 ± 0.0005	0.97 ± 0.01	0.9849 0.9643 0.9571
	Trial 3	8 (± 1) × 10^{+12}	0.9738 ± 0.0007	0.82 ± 0.02	0.7973 0.8237 0.8428	2.2 (± 0.7) × 10^{+13}	0.9992 ± 0.0004	0.97 ± 0.03	0.9957 0.9655 0.9455
CH_2I_2 in 0.5 M NaCl	Trial 1	7 (± 1) × 10^{+10}	0.94 ± 0.01	0.67 ± 0.02	0.674 0.679 0.6424	1.9 (± 0.3) × 10^{+13}	0.9956 ± 0.0005	0.94 ± 0.01	0.9549 0.9423 0.9319
	Trial 2					8.8 (± 0.5) × 10^{+12}	0.980 ± 0.003	0.982 ± 0.004	0.9786 0.9863 0.9808
	Trial 3					2 (± 2) × 10^{+13}	0.995 ± 0.005	1.0 ± 0.1	1.170 0.9816 0.9979

*In the two cases for which only two replicates are present, individual values are given in lieu of averages.

and 75-µm Carboxen–PDMS fibers from Supelco (Sigma-Aldrich Corp.) were tested, and the latter were found to consistently give at least double the signal over the entire concentration range presented here. This agrees with a study of trihalomethanes that compared the same fibers (20). Cancho and colleagues found even higher signal for trihalomethanes from Carbowax–divinylbenzene fibers, which were not tested here (23). The TR–FAME column was found to give a higher signal of CH_2ICl and CH_2I_2 than the TR–5 column.

Extraction temperatures of 40 °C and 70 °C were tested, and the 40 °C temperature was found to give approximately seven times higher signal at the lower end of the concentration range. This temperature is similar to the optimized temperature of 37.5 °C used for trihalomethanes from a test range of 30–45 °C (20), but in contrast to the results of Yoshizawa (21), who found that higher temperatures, up to 80 °C, gave better signal for the same dihalogenated compounds examined here in measurements using a PDMS fiber. Here, 12 extraction times between 1 and 30 min were tested in multiple runs, and an extraction time of 10 min was chosen. The extraction time was not fully maximized for the highest signal because of the need for a quick method that would be applicable to a large numbers of samples. The compounds were observed to experience a slight decay over time, limiting the number of samples possible for longer run times. The decay was negligible on the time scale of the experiments presented here. An extraction time of 10 min yielded 89–99% of the equilibrium signal, depending on the concentration tested. To consistently improve the fraction of the equilibrium signal captured, the extraction time would have needed to be doubled because of an unexplained but reproducible dip in the signal at intermediate extraction times above 10 min (see Figure 1 for a representative run). This longer extraction time would substantially slow the measurement, thus limiting the number of samples possible before decay becomes an issue. Yoshizawa tested extraction times ranging from 15 to 60 min and also got variable results, observing a decrease in the CH_2I_2 signal with increased extraction time (21).

Results and Discussion

Automated GC–MS of CH_2I_2 and

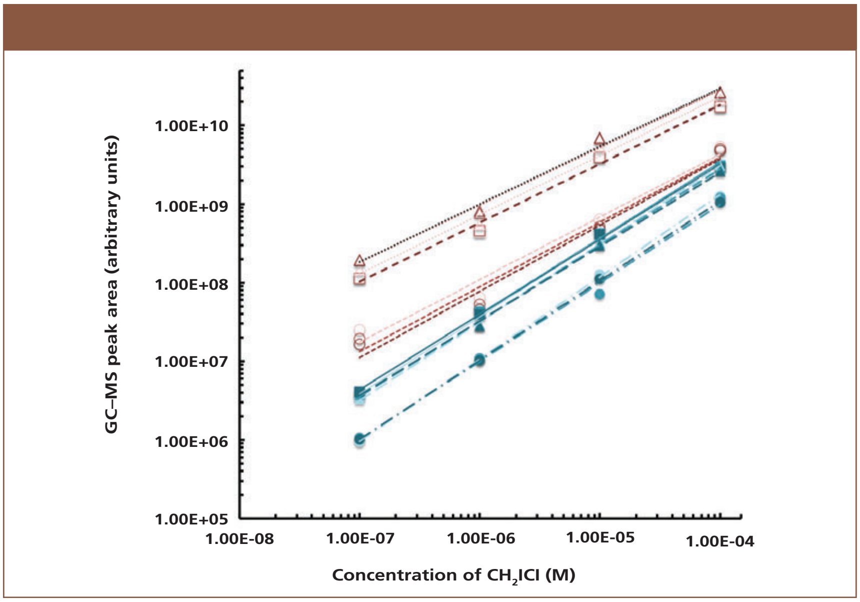

Figure 3: Standard curves of 1 × 10^{-4} to 1 × 10^{-7} M CH_2ICl in 0.5 M NaCl shown on a log–log scale. Peak area is from signal at m/z = 176. Trials with no conditioning of the fiber are shown in red open symbols and lines while trials with conditioning runs are shown in blue solid symbols and lines. Different replicates of the same trial are distinguished by shading of the markers and lines, although in some cases they overlap. Lines show fits to the equation $y = mx^a$ for each trial and replicate, for which the corresponding fit parameters are given in Table II. Specifically, for the fiber without conditioning, trial 1 is given by open triangles and dotted lines, trial 2 is given by open squares and medium dashed lines, and trial 3 is given by open circles and short dashed lines. For the fiber with conditioning, trial 1 is given by solid circles and dash-dotted lines, trial 2 is given by solid squares and thin solid lines, and trial 3 is given by solid triangles and long dashed lines.

CH_2ICl with SPME sample preparation was observed to produce carryover on the sample fiber following the highest concentration runs of 1 × 10^{-4} M dihalomethane (Figure 2a). This carryover persisted despite the addition of two blank runs between samples, one in which the temperature was held at the highest point of the ramp (200 °C) for 11 min. It was determined that a higher temperature conditioning of the fiber was necessary to eliminate the carryover signal. The installation of a new PVT injector, used as the analytical inlet, allowed for a 2 min conditioning run at 300 °C in the second, split–splitless, injector following each sample. The split–splitless injector was connected to a short piece of open-ended column ending in the oven and was maintained with a constant helium flow. Since the conditioning was done in a separate port, it did not add additional analyte into the column. In situations where contamination of the oven and outer column surface is a concern, the conditioning port could be directly

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connected to the detector vent outlet. Performing the 2-min conditioning step was successful in eliminating the carryover problem (Figure 2b). It is common to retain both ports after upgrades and this approach is practical for any instrument with two injection ports. A similar conditioning step could also be performed in the main injection port if only one port is available. This approach is less desirable because it would add to the run time and would also put unwanted material into the analytical column.

To gauge the effectiveness of the cleaning step, standard curves were compared with and without the fiber conditioning step for three types of systems: CH_2ICl in water, CH_2ICl in 0.5 M NaCl solution, and CH_2I_2 in 0.5 M NaCl solution (see example in Figure 3). Dihalomethane concentrations varied from 1×10^{-4} to 1×10^{-7} M in all runs. The different calibration curves are denoted as different trials with "no conditioning" and "conditioning." As shown in Table II, 14 solutions had three replicates taken on the same day, while two solutions had only two replicates taken. Different solutions were tested as separate trials on different days. All data shown for the standard calibration curves were collected within a 14-month period. The temperature runs described later were performed an additional 12 months after the initial data.

Fits to the power law $y = mx^a$ were performed in Microsoft Excel on plots of peak area versus concentration for each individual replicate. A representative graph of these fits is shown in Figure 3 for CH_2ICl in 0.5 M NaCl solution. The wide data range was conducive to a power law fit to fully investigate the behavior of the low concentration data points. A basic linear fit of the data gave an R^2 value very close to 1 (0.99 or higher) in all cases since the higher concentration data were very linear even without the conditioning step. The resultant fit parameters are found in Table II. For each solution tested, including CH_2ICl in water, CH_2ICl in 0.5 M NaCl, and CH_2I_2 in 0.5 M NaCl, values of the parameter a were plotted using the mean and standard deviation of each sample set (Figure 4).

The exponents of the power law fit were analyzed using hierarchical mixed model analysis using random intercepts to determine significant differences between runs with and without the conditioning step. This analysis places trial replicates from an individual experiment into a hierarchy, which is visualized for our study in Figure 5. Level one designates whether or not the fiber conditioning took place, level two separates the independent trials, and level three consists of the trial replicates. The hierarchical mixed model was chosen because of the limited number of independent trials for some chemical mixtures. The analysis can be used in cases where there is missing data because parameters can be successfully estimated using the available data. Thus the

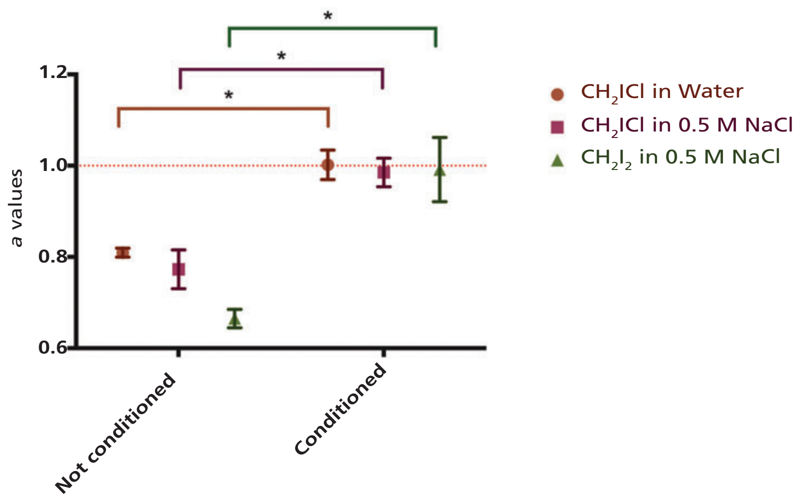

Figure 4: Mean and standard deviation values for the parameter a from fits of calibration data to the equation $y = mx^a$ are shown with and without the 2-min conditioning step. Values are given for the three different sets of solutions tested: CH_2ICl in water, CH_2ICl in 0.5 M NaCl, and CH_2I_2 in 0.5 M NaCl. Organic concentrations varied from 1×10^{-4} to 1×10^{-7} M in all runs. Stars indicate that the conditioned data was significantly different from the unconditioned data in all cases ($p < 0.05$) when compared with a hierarchical mixed model analysis. The dotted lined indicates an a value of 1, which corresponds to perfect linearity between peak area and concentration.

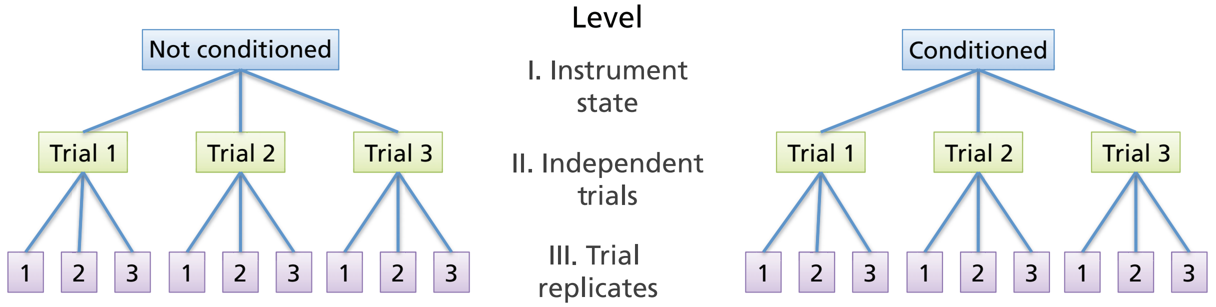

Figure 5: A schematic showing the design of the hierarchical mixed model.

trial replicates from a single trial can be used in a "weighted manner" (the replicates are nested within the trial) to determine significance between a conditioned and not conditioned fiber. The Wald score between trial replicates and independent trials was <0.05 for all solutions tested, indicating that the hierarchical model was necessary to analyze the data.

The model showed that, in each case, the runs using the conditioning step had an a value that was significantly higher than those without a conditioning step ($p < 0.05$). The p values were $p = 1.7 \times 10^{-9}$ for CH_2ICl in water, $p = 0.002$ for CH_2ICl in 0.5 M NaCl, and $p = 0.035$ for CH_2I_2 in 0.5 M NaCl. The average exponent was well within the standard deviation of 1 (indicative of perfect linearity) for each trial in which the extra cleaning step was included. In contrast, the average exponent was not within the standard deviation of 1 in any of the cases without a conditioning step (Table II and Figure 4). This clearly demonstrates increased linearity of the standard curves with conditioning. Trials containing no conditioning step showed a lower average a value for CH_2I_2 than for CH_2ICl. This result is likely caused by the larger number of iodine atoms in CH_2I_2, since iodine is the portion of the molecule most associated with carryover. The slope of the linear relationship (corresponding to the m parameter) does vary depending on instrumental conditions as can been seen in Table II. This behavior was expected. The m value relates to the absolute signal strength, which is affected by conditions that vary over time such as the filament age and cleanliness of the ion source. As with many GC–MS experiments, calibration curves for this system need to be done close in time to the measurement for which they will be used. The consistency within a run can be seen in the lack of variation in peak area at a given concentration between replicates of a single trial. The peak area, when averaged over replicates within a run, had a relative standard deviation that was never more than 0.4% and was usually less than 0.2%.

The two injection ports have different designs and geometries, requiring some injection parameters to be changed (Table I). In the process of making these changes, the inlet temperature for desorption was changed from 220 °C to 250 °C. Because this change could affect the signal, checks were run to make sure that the degree of linearity was not attributable to the increase in temperature. Control experiments were performed in which consecutive replicates of the same sample alternated between 220 °C and 250 °C while including the conditioning step. The 220 °C runs and the 250 °C runs did not exhibit any statistically significant difference in the a values after hierarchical mixed model analysis using random intercepts ($p = 0.081$). The average a values for the different temperatures agreed within their standard deviations.

The limit of detection (LOD) for the method including the cleaning step was determined through measurement of blank runs ($n = 17$) coupled with the measured calibration curves. It was calculated assuming a signal-to-noise ratio of 3. For CH_2ICl in water the LOD was 2.3×10^{-8} M. For CH_2ICl in 0.5 M aqueous NaCl the LOD was 6.2×10^{-8} M. For CH_2I_2 in water the LOD was 9.2×10^{-8} M. As mentioned previously, the calibration curves do vary with instrumental conditions, which in turn affect the exact value of the limit of the detection. The LOD values given here are averages determined from the multiple calibration curves listed as different trials in Table II.

The result found here is of particular interest because of the short time used for conditioning. There is precedent in the literature for a longer (30- or 10-min) conditioning step used with other compounds (6,7). A medium length (5-min) conditioning step in a free-standing conditioner has also been examined in the literature (8), but that instrument was also shown to be more effective than an injection port so the times may not be comparable. The example shown here with dihalogenated methanes is effective even at very short times. In automated runs, the autosampler begins incubation of one sample while the previous one is still moving through the GC system. The 2-min conditioning step used here was sufficiently short so as to finish before the incubation step began and did not increase the total run time. Thus, it was possible to eliminate carryover without adding any additional analysis time. As mentioned before, this possibility is advantageous with samples where degradation over time is a concern.

There is another very recent example in the literature exploring a short conditioning step that complements our study. Attari and colleagues (27) used a homemade sol-gel single-walled carbon nanotube–silica composite instead of a commercial fiber for the SPME. The focus of that study was to test the new fiber, so the temperature used and validation data for the cleaning step are not explicitly given. However, the paper confirms that a 2-min conditioning step cleaned the fiber of benzotrichloride, chloromethyl methyl ether, and trichloroethylene (27). These data suggest that the result described here is more generally applicable to halogenated compounds, and not limited to dihalogenated methanes.

Even with a conditioning step, the limits of detection are determined here to be in the 10^{-8} M range, and lie above the concentration range at which these dihalogenated methanes occur in natural systems. Seawater concentrations, as mentioned previously, tend to be in the 10^{-9} M range for these compounds, and wastewater from nuclear power plants shows concentrations in the 10^{-12} M range. The improvement discussed here, however, could be coupled with a preconcentration step for improved analysis of environmental samples of dihalogenated methanes and other similar compounds. Headspace SPME GC–MS analysis has been used to examine broader classes of halogenated organic compounds for a wide variety of applications. For example, a recent study used headspace SPME GC–MS analysis to look for several halogenated compounds (including the two studied here and many others) that function as potential allelopathic metabolites of a marine benthic diatom (28). The method has also been used in a medical application to track halogenated organic compounds used as inhalation anesthetics in urine samples (29). Many chemically complex halogenated pesticides exhibit severe carryover on SPME fibers and are good candidates for a conditioning step (30). More generally, our result suggests that very short conditioning steps should be evaluated as a method of decreasing the limit of detection in SPME sample preparation of other, nonhalogenated compounds exhibiting carryover.

Conclusion

The addition of a short, high temperature conditioning step was sufficient to eliminate carryover of CH_2I_2 and CH_2ICl, two environmentally important compounds, on a Carboxen–PDMS SPME fiber. SPME GC–MS coupled with short conditioning times was an effective way to quantify dihalomethanes over a concentration range spanning four orders of magnitude. Calibration curves that included a conditioning step showed significantly more linearity than those performed without this step. Because of the short length (2 min) of the conditioning step and the presence of a second injector, this change added no additional analysis time to automated sample processing. The new methodology allowed for carryover reduction similar to that seen in longer conditioning steps, while also facilitating rapid sample throughput.

Acknowledgments

The authors are grateful for a Camille and Henry Dreyfus Foundation Faculty Start-up grant, and a Research Corporation Cottrell College Science Award for equipment funding. We acknowledge student funding from the Trinity College Student Research Program and the Frank Fellowship at Trinity College.

The authors thank Lisa Yamada for her translation of the article by T. Yoshizawa (reference 21) from Japanese to English and Andres Delgadillo for his assistance with some of the sample preparation. We are grateful to Dr. Dan Cox of the Sackler School of Biomedical Sciences for his assistance in choosing the most appropriate statistical test for our data.

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For more information on this topic, please visit www.chromatographyonline.com

PRODUCTS & RESOURCES

Consumer products testing applications notebook

A consumer products testing applications notebook from Waters highlights LC and MS methods for ensuring food safety, maintaining product quality, and protecting brand names. According to the company, the notebook contains applications for the separation and quantification of biocides, disperse dyes, flame retardants, phthalates, primary aromatic amines, and azo dyes.

Waters Corporation,
Milford, MA.
www.waters.com/consumerproductsnotebook

Automated SPE system

Horizon Technology's SmartPrep Cartridge Extractor II solid-phase extraction system is designed for method development and automation of previously developed manual solid-phase extraction methods. According to the company, the system is suitable for special environmental applications such as 1,4 dioxane, nitrosamines, and PFOAs; food applications such as aflatoxins in peanut paste; and energy-related applications such as furans in transformer oils.

Horizon Technology, Salem, NH. www.horizontechinc.com

Countercurrent chromatography system

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

AECS-QuickPrep Ltd., London, England. www.quattroprep.com

Automated pressurized liquid extraction

The PLE automated pressurized liquid extraction system from FMS is designed for the rapid extraction of a variety of compounds from environmental, biological, and food samples. According to the company, the system is a replacement for Soxhlet extractions, and applications include a range of EPA methods and testing for semivolatile organic compounds in soils, foods, pharmaceuticals, and polymeric products.

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

Diluter–dispenser

The Hamilton Microlab 600 semi-automated liquid handling system is designed for tasks too small to automate or too large to reliably accomplish by hand. According to the company, the instrument is available as a single- or dual-syringe pump with a touchscreen interface.

Hamilton Company,
Reno, NV
www.hamiltoncompany.com

Viscosity detector

The ViscoStar III viscosity detector from Wyatt Technology is designed for use in conjunction with gel permeation chromatography systems and multiangle light scattering detectors. According to the company, the detector can determine the molar mass, size, and conformation of all types of macromolecules, including polymers and proteins.

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

GC system

The Agilent Intuvo 9000 GC system is designed with features such as direct column heating, no-trim columns, ferrule-free connections, and real-time instrument status information. According to the company, the system includes the instrument, consumables, and software and support.

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

Static mixers

PerfectPeak HPLC and UHPLC static mixers from Mott Corporation are designed to provide up to 98% noise reduction, with 75% less volume than comparable mixers. The mixers reportedly incorporate unique pathways obtained through 3D printing technology. According to the company, the mixers are available in 90-, 55-, and 35-µL versions.

Mott Corporation,
Farmington, CT.
www.mottcorp.com

Active inlet valve for Agilent systems

The Opti-Max 600 bar active inlet replacement cartridge from Optimize is designed with 316 stainless steel, PEEK, and zirconia for compatibility in 400-bar and 600-bar applications. According to the company, the cartridge's zirconia ball travel is minimized, allowing the cartridge to exhibit low pulsation.

Optimize Technologies, Inc.,
Oregon City, OR.
www.optimizetech.com

Mass spectrometer

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

PerkinElmer,
Waltham, MA.
www.perkinelmer.com/qsight

GC–MS system

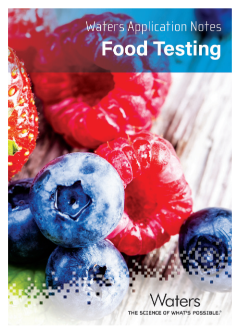
Shimadzu's GCMS-TQ8050 triple-quadrupole GC–MS system is designed for the detection of femtogram-level amounts of trace components. According to the company, the instrument is suitable for the analysis of extremely small quantities of dioxin in foods and the environment, persistent organic pollutant components, endocrine-disrupting chemicals, impurities in pharmaceuticals, and banned drugs in hair samples.

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

Food testing application notes

A booklet from Waters Corporation includes application notes that describe techniques for screening, profiling, quantifying, and authenticating food products. According to the company, the booklet can be downloaded for free at waters.com/foodtestingbooklet

Waters Corporation,
Milford, MA.
www.waters.com



Waters Application Notes
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Intact Proteoform Characterization Using CESI-MS

ON-DEMAND WEBCAST Originally aired Nov. 18, 2016

30 MIN. FORMAT

Register for free at www.chromatographyonline.com/lcgc/sciex_series4

EVENT OVERVIEW

Despite recent advances in mass spectrometry (MS) mass resolution and accuracy, sample complexity remains a challenge when analyzing intact proteins. Separation before MS analysis is needed to achieve reliable and sensitive characterization of minor protein variants and modifications. Integrating capillary electrophoresis separation with electrospray ionization (CESI) offers the selectivity and sensitivity required to distinguish closely related intact protein variants and isoforms by mass spectrometric detection. Examples will focus on the intact and middle-up analysis of proteins of pharmaceutical and biomedical interest. These include monoclonal antibodies (mAbs), single-domain antibodies (nanobodies) targeting GPCR receptors and amyloidogenic protein beta-2-microglobulin.

Join this presentation from the research group of Professor Govert Somsen at Vrije Universiteit Amsterdam to find out how.

Who Should Attend

- Scientists in academia, government and the pharmaceutical industry involved in proteomics research.
- R&D and analytical development directors, laboratory managers and scientists at biopharmaceutical companies.
- Academics collaborating with the biopharmaceutical industry.

For questions, contact Kristen Moore at kristen.moore@ubm.com

Presenters

Dr. Elena Dominguez Vega
Post-Doctoral Researcher, Biomolecular Analysis Group, Vrije Universiteit Amsterdam

Joining us for Live Q&A:
Dr. Rob Haselberg
Post-Doctoral Researcher, Biomolecular Analysis Group, Vrije Universiteit Amsterdam

Moderator: Laura Bush, Editorial Director, LCGC

Key Learning Objectives

- Learn about the analysis of protein conformational changes under native conditions.
- Understand how CESI-MS was used to reveal detailed protein difference, such as deamidation and truncation.
- Discover new intact and middle-up approaches for studying protein glycoforms.

Sponsored by SCIEX

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CALENDAR

24–25 January 2017
20th Annual Meeting of the Israel Analytical Chemistry Society
Tel-Aviv, Israel
bioforumconf.com/isranalytica17

24–26 January 2017
WCBP 2017 21st Symposium on the Interface of Regulatory & Analytical Sciences for Biotechnology Health Products
Washington D.C.
casss.site-ym.com/page/WCBP1700

4–8 February 2017
Conference and Exhibition of the Society for Laboratory Automation and Screening (SLAS 2017)
Washington D.C.
www.slas2017.org

5–9 March 2017
Pittcon 2017
Chicago, IL
pittcon.org

29–31 March 2017
Analytica Vietnam 2017
Hanoi, Vietnam
www.analyticavietnam.com

2–6 April 2017
253rd American Chemical Society National Meeting & Exposition
San Francisco, CA
www.acs.org/content/acs/en/meetings/spring-2017.html

3–4 April 2017
ICSST 2017: 19th International Conference on Separation Science and Techniques
Brisbane, Australia
waset.eu/conference/2017/04/brisbane/ICSST/program?forceTentative=1

18–22 June 2017
45th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2017)
Prague, Czech Republic
www.hplc2017-prague.org

Analysis of Antibody–Drug Conjugates by Hydrophobic Interaction and Size Exclusion Chromatography: An Orthogonal Approach

ON-DEMAND WEBCAST Aired November 29, 2016

Register for free at www.chromatographyonline.com/lcgc/Orthogonal

Antibodies, when conjugated with cell-killing cytotoxic drugs, are called *antibody drug conjugates* (ADCs). ADCs have a structure in which a low-molecular-weight cytotoxic drug is chemically bonded to an antibody. Because there are numerous binding sites for a low-molecular-weight drug on the antibody (Cys, Lys residues, etc.), heterogeneity arises with respect to the number of bonds and binding sites. It is necessary to study in detail the effect that this heterogeneity has on the medicinal effects and safety of drugs containing ADCs.

Size-exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) are used as routine techniques for analyzing ADCs. The SEC analysis focuses on the separation of heterogeneity in an ADC monomer from its dimer and other high- and low-molecular-weight variants. Since low-molecular-weight drugs are strongly hydrophobic, differences in hydrophobicity of antibodies arise when there are differences in the number of drug molecules bound to them. Hydrophobic interaction chromatography (HIC) is used to identify the drug-to-antibody ratio (DAR) of an ADC.

This presentation demonstrates the utility of combining SEC and HIC for the analysis of ADCs. These methods are used as an orthogonal approach for analyzing ADCs. This presentation will include tricks and tips of method development for both modes of separations.

Who Should Attend: Analytical chemists and chromatographers using HPLC for the analysis of antibody–drug conjugates

For questions, contact Kristen Moore at kristen.moore@ubm.com

Key Learning Objectives:

- Learn how to optimize methods for the analysis of antibody–drug conjugates
- Understand the benefits of size exclusion and hydrophobic interaction chromatography for the analysis of antibody–drug conjugates

Presenter
PHU DUONG, Ph.D.
Product Manager II, HPLC Columns
Tosoh Bioscience LLC

Moderator
LAURA BUSH
Editorial Director, LCGC

Sponsored by TOSOH BIOSCIENCE

Presented by LCGC

Book Review: *Chromatography: Principles and Instrumentation*

Lawrence W. Potts, Emeritus Professor of Chemistry at Gustavus Adolphus College, reviews Mark F. Vitha's new book from Wiley, *Chromatography: Principles and Instrumentation*.

Mark Vitha has written a book that will appeal to students, teachers, and perhaps professional analysts who need a refresher in the fundamentals of chromatography. The book consists of three sections of about equal length dealing with separation theory, gas chromatography (GC), and liquid chromatography (LC). The section on theory is especially strong. Vitha is an experienced educator who understands the undergraduate audience and explains concepts clearly. He uses analogies to help students with abstract ideas, something I have seen little of in the sciences. He also freely uses ideas and terms from thermodynamics that can be grasped by students who have studied physical chemistry. An example is his clear development of retention indices in GC. Discussions are comfortably worded and not at all pedantic in tone. There are many example problems and excellent end-of-chapter problems with solutions.

Graduate students might want to use this book, with additional depth provided by their instructors and current and classic papers (many are referenced). Graduate students need more depth in areas such as solvent theory and the selection of solvents, for example, than is given in this book.

The sections of the book dealing with specifics of hardware, stationary phases, and solvents are an easy read for a practicing chromatographer but might be harder for today's students, who seem to have shorter attention spans than ever before. I very much like the practical way Vitha justifies hardware choices based on performance and cost-effectiveness.

There is a lot that's new in chromatography and in the teaching of instrumentation. Vitha's new book gives teachers some new opportunities for dealing with both. First, in my opinion, chromatography is so important in chemical analysis that a modern undergraduate instrumentation course should devote at least a half semester to chromatographic separations. Vitha's book would be perfect for such a course. It would be valuable for students to do coursework in data handling, optical spectroscopy, mass spectrometry, and electrochemistry, perhaps using modular books, before using Vitha's book to bring those concepts together in chromatography. While most modern course-flippers use video lectures for students to watch and then use class time for problem-solving, they could flip back with Vitha's book as a substitute for video lectures in chromatography theory.

I taught instrumental methods to undergraduate students for many years using encyclopedic full-course texts. I wish there had been as fine a pedagogical tool as this more-focused new textbook at that time.

Professor Lawrence W. Potts *is an Emeritus Professor of Chemistry at Gustavus Adolphus College in St. Peter, Minnesota. He received the 2010 J. Calvin Giddings Award for Excellence in Education from the Analytical Division of the ACS. Direct correspondence to: potts@gustavus.edu* ■

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THE ESSENTIALS

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

Tips for Optimizing Key Parameters in LC–MS

Table I: LC–MS source parameter settings

	Nonassisted ESI	Assisted ESI	APCI
Eluent flow rate	1–5 µL/min	0.1–1 mL/min	0.5–2.5 mL/min
Nebulizing gas (N_2, L/min)	0	1–10	1–10
Desolvation gas (N_2, L/min)	1–1.5	3–9	2–4
Ion source temperature (°C)	100–150	100–400	150–200
Capillary (ionization) voltage (kV)	4–5	4–5	2.5–3
Cone voltages (kV)	10–60	10–60	10–60

The most significant choice in many liquid chromatography–mass spectrometry (LC–MS) methods is the ionization mode. The generally accepted rule is that electrospray ionization (ESI) works best for higher-molecular-weight compounds that are more polar or ionizable, and atmospheric pressure chemical ionization (APCI) is best for lower-molecular-weight, less-polar compounds. Atmospheric pressure photoionization (APPI) was originally designed to work with less-polar analytes; however, the careful choice of dopant or the use of direct ionization sources can significantly extend the capability of APPI techniques. Although these are good rules to help guide you toward the correct ionization technique, you should always treat each analyte independently and (where practical) perform an infusion of your analytes to select the correct ionization technique and polarity (positive or negative ion mode), and then optimize the critical parameters.

To perform this step this you need a 10 mM ammonium formate buffer adjusted to both pH 2.8 and 8.2. Carry out an infusion of your standard or sample through a tee piece at the analytical flow rate, with a 50:50 mix of organic–buffer at pH 8.2 and 2.8, using both negative and positive ionization modes. Use the instrument autotune routine first and then carry out a manual tune on the key parameters—that is, adjust voltages, temperatures, and gas flows to give you the optimum signals under each set of conditions (Table I). From these spectra you will be able to select the optimum ionization mode and eluent composition.

When tuning key parameters that can generate a response curve (for example, ESI source voltages, flow rates, and temperature), setting the value to a maximum may not give you the most robust method. Instead, you should set values on a maximum plateau where small changes in that particular variable will not produce a large change in instrument response.

More Online:

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

If you are carrying out a selected reaction monitoring (SRM) experiment, you can now optimize the SRM conditions. Using the optimum ionization mode and eluent composition, adjust the collision energy (CE) voltage to give the product ions; you should be left with 10–15% of the parent ion. You will want to use the product ions that give you the highest response.

Now to optimize the high performance liquid chromatography (HPLC)–MS method. Start with a high concentration of your standard (for example, 1 µg/mL). Run a 5–100% B gradient using the optimized mobile phase (that is, acetonitrile–ammonium formate buffer at the optimum pH, either 2.8 or 8.2), ionization mode, and SRM transitions. At this point, you should hopefully get a good total ion chromatogram (TIC) and optimum MS spectra for your analytes. The method can be further optimized to reduce analysis time by calculating values for initial %B, final %B, gradient time (t_g), and reequilibration time (see equations 1–4).

$$\%\mathrm{B}_{\mathrm{initial\ (gradient)}} = (\%\mathrm{B}_{\mathrm{initial}} + (t_{\mathrm{i}}\Delta\%\mathrm{B/min}))\cdot - \left(\frac{V_{\mathrm{D}}}{F}\Delta\%\mathrm{B/min}\right) - 10\ \%\mathrm{B} \quad [1]$$

$$\%\mathrm{B}_{\mathrm{final(gradient)}} = (\%\mathrm{B}_{\mathrm{initial}} + (t_{\mathrm{f}}\Delta\frac{\%\mathrm{B}}{\mathrm{min}})) - \left(\frac{V_{\mathrm{D}}}{F}\Delta\frac{\%\mathrm{B}}{\mathrm{min}}\right) - 10\ \%\mathrm{B} \quad [2]$$

$$t_{\mathrm{g}} = \frac{k^{*}1.15S\Delta\Phi V_{\mathrm{M}}}{F} \quad [3]$$

$$\text{Required reequilibration time} = \frac{2(V_{\mathrm{D}}+V_{\mathrm{M}})}{F} \quad [4]$$

where t_i and t_f are the elution time of the initial and final peaks (min), Δ%B/min is the rate of change of the mobile phase during the gradient, V_D is the dwell volume (mL), F is the flow rate (mL/min), t_g is the gradient time (min), k^* is the gradient retention factor (use 5 as a starting point), S is the shape selectivity factor (for small molecules use 5 or calculate using the molecular weight $S = 0.25\mathrm{MW}^{0.25}$), Δφ is the change in %B expressed as a decimal, and V_M is the column interstitial volume (mL).

LC–MS still relies on a good chromatographic separation before MS detection. When using single-ion monitoring (SIM) or SRM many quantitative problems are caused by coeluted substances entering the ion source with the analyte of interest, causing ionization efficiency issues. Therefore, it is a good idea to run a full scan acquisition on a representative sample to visualize any potential coelution problems. Quantitation issues do not solely lie with the mass spectrometer but often result from ineffective sample preparation or chromatographic separation.


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GC-2014