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Volume 39 Number 8 August 2021
www.chromatographyonline.com

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Separation of 12 Nucleosides on Hamilton PRP-1 Column

The primary nucleosides encompass a broad array of biological functions associated with RNA, particularly after configuration into multidimensional conformations. Nevertheless, researchers have found over 100 natural modifications to the native nucleosides that have led to a sea change in RNA design and discovery. These novel nucleosides provide increased chemical and thermal stability. Additionally, the nucleosides’ adaptations help to mediate various RNA functions within the cell. In addition to the natural modifications, a wide variety of non-natural nucleosides can be incorporated into RNA by natural or synthetic routes. These modifications often provide RNA with distinctive properties, such as expanded or altered hydrogen-bonding via van der Waals, base-stacking, or electrostatic interactions. Or by incorporating unique metal-binding sites, chemical reactivity, or fluorescent characteristics!

One of the more exciting applications with modified nucleosides is in the realm of disease markers, most notably, metastatic tumors and some viral infections. Modified nucleosides are primarily observed in both messenger (mRNA) and transfer RNA (tRNA). When mechanical or chemical stresses occur, catabolism of RNA material leads to inflammation and hypoxia. The removal of the non-essential components is facilitated through urine excretion and produces an increase in the concentration of pseudouridine, 1-methyladenosine, 7-methylguanosine, 2’-O-methylcytidine, and 1-methylinosine to name a few. The level of each metabolite reflects the quantity of RNA degradation in the organism. Moreover, urinary excretion of methylated nucleosides, resulting from increased turnover and degradation of RNA, especially from tRNA, is excreted in statistically increased amounts in the urine of patients with different types of malignant tumors and/or HIV.

Hamilton’s PRP-1 (5 µm) reversed-phase HPLC column demonstrated enhanced resolution of 12 common modified and non-modified nucleosides. The polymeric particle found in nearly all of Hamilton Company’s columns exhibit decreased pump pressure while still achieving the separation of that of small particle sorbents. Ammonium formate was used as the mobile phase eluent and allowed for satisfactory resolution of all the analytes. Similarly, using a more volatile mobile phase like ammonium formate allows for analysis with mass spectroscopy detectors if so desired by the analyst. The use of isocratic methodology enabled the suitable separation of the first nine nucleosides. The last three compounds eluted with a slight gradient of acetonitrile to facilitate better peak shape for the more lipophilic nucleosides.

Compounds:
- Cytidine 50 μg/mL
- Guanosine 25 μg/mL
- Inosine 25 μg/mL
- 1-Methyladenosine 25 μg/mL
- 5-Methylcytidine 100 μg/mL
- 2’-O-Methylcytidine 20 μg/mL
- 7-Methylguanosine 25 μg/mL
- 5-Methyluridine 50 μg/mL
- Pseudouridine 25 μg/mL
- 2-Thiocytidine dihydrate 10 μg/mL
- Uridine 25 μg/mL

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Author: Adam L. Moore, PhD
Detailed LNP-RNA analysis with FFF-MALS-DLS

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To learn more about Wyatt’s unique solutions for LNP-RNA characterization, visit www.wyatt.com/LNP
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GC and TLC methods are demonstrated for quantification of stigmasterol 3-O-β-D-glucopyranoside (S3G), the main active component in the herbal nutraceutical Balanites aegyptiaca.

Headspace Solid-Phase Microextraction Coupled with Gas Chromatography–Mass Spectrometry (HS-SPME-GC–MS) for the Characterization of Cigar Leaves
Hongfei Zhang, Juan Yang, Fengpeng Zhu, Cheng Luo, Yongqiang Pang, Beibei Zhu, Yanbo Luo, Xiyang Li, Xingyi Jiang, and Dongliang Li
HS-SPME-GC–MS was combined with OPLS-DA data analysis to tentatively identify eight chemical markers to differentiate the geographical origins of cigar leaf samples.
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From the Chairman

Mike Hennessy, Sr.
Chairman & Founder, MJH Life Sciences

Herbal medicines have been used worldwide for centuries, and their use has been growing steadily in Western countries, too. With that growth, the analytical science community is working to characterize and quantify the active compounds in these treatments for quality control or for further research. The peer-reviewed study featured on this month’s cover addresses the analysis of extract of the fruit of Balanites aegyptiaca, a tree native to Africa and much of the Middle East, which is used as an antihyperglycemic in Egyptian folk medicine. Ola M. Abdallah of Al-Azhar University in Cairo, Egypt, and colleagues have developed and validated gas chromatography (GC) and thin layer chromatography (TLC) methods to quantify stigmasterol 3-O-β-D-glucopyranoside (S3G), the main component of the extracts. The methods show excellent precision and a wide linear range, and are applicable for the determination of S3G in pure form and in pharmaceutical formulations.

Other peer-reviewed research in this issue looks at the products of a different growing market: high-quality cigars, where geographical origin is a critical factor in consumer value. In this work, conducted jointly between the China National Tobacco Quality Supervision and Test Center and the Technology Center at the China Tobacco Sichuan Industrial Co., Ltd., the authors characterized the chemical profiles of cigar leaf samples from 16 different geographical regions using headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS), combined with chemometrics.

As in every issue, we complement peer-reviewed research with practical help for everyday analytical challenges. This month’s “GC Connections” column considers causes of and solutions for peak tailing. “GC Connections,” explains simple steps to optimize a GC separation without changing the column. “Perspectives in Modern HPLC” focuses on sample preparation for bioanalysis of small-molecule drugs and metabolites by liquid chromatography–mass spectrometry (LC-MS). This comprehensive article explains fundamental considerations, key techniques, automation, and modern trends.

We close out the issue with a short piece from Kevin Schug and Zac Hildenbrand examining the arsenal of analytical methods for a challenging issue: point source attribution for environmental contamination.

Happy reading!

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But My Peaks Are Not Gaussian! Part I: Basic Concepts in Peak Shape, and the Effect of Extracolumn Connections

Although symmetric peaks with Gaussian shapes are predicted by models of the chromatographic process, “perfect peaks” are not observed very often outside of textbooks. Tailing peaks—the most common type of asymmetric peak shape—can negatively affect both the qualitative and quantitative performance of liquid chromatography (LC) methods. In this first installment of a multi-part series focused on the causes of peak asymmetry, I will discuss basic concepts in peak shape, and the potential for poor fluidic connections to cause peak tailing in a separation where the peak shape would otherwise be excellent.

Dwight R. Stoll

Introduction

Well established models of the chromatographic process predict peak shapes that can be very closely approximated by the Gaussian distribution. There is a long list of reasons why the Gaussian peak shape might not be observed, but the Gaussian is very convenient, particularly for use in chromatography simulation tools, and development of theory that relates important parameters (such as, for example, peak width to the retention characteristics of a given molecule). In this context, one important property of the Gaussian is that it is a perfectly symmetric function. In practice, however, perfectly symmetric peaks are not observed very often, and the degree of deviation from perfect symmetry can vary widely, depending on a large number of physical and chemical factors specific to particular instruments, applications, and operating conditions. These deviations from symmetric peak shapes (that is, asymmetry) are important in practice, because the asymmetry can negatively impact both the qualitative character of a chromatogram and the quantitative performance of a method. The problems are not unique to liquid chromatography, nor are they new. Indeed, these issues have been discussed many times by many authors in LCGC in the past. For example, I would point readers interested in learning much more about this area to an excellent series of articles by John Hinshaw (2–4), several articles by John Dolan (5), and two recent entries in the “LCGC Blog” by Tony Taylor (6,7). Nevertheless, given the importance and potential impact of peak asymmetry on the quality of separations and chromatographic results in day-to-day work, I think it is useful to draw several relevant ideas together in one place and discuss them in some detail, all with the aim of providing a robust resource to troubleshoot problems with peak asymmetry when they do arise. In this first installment on the topic, I will review some basic concepts in peak shape and asymmetry, and then go on to start discussing some of the root causes of asymmetry, as well as potential solutions.

Quantifying Peak Asymmetry

The equation used to model a chromatographic peak using the Gauss-
The Critical Role of CE-MS Methods for Biotherapeutic Characterization

Capillary electrophoresis (CE) methods play an important role in biomolecular characterization while microfluidic CE-mass spectrometry (MS) accelerates analytical workflows and improves development/commercialization timelines.

BIOPHARM/LCGC: Why is CE such an important technology for biomolecules?
MELLORS: Most of the developments in separations that have happened in recent history have focused on liquid chromatography (LC), which is great for many applications, but biomolecules often aren’t the best fit for chromatography. The need to optimize a separation to work with a stationary phase and deal with the range of properties of the molecules makes it difficult—plus, there is also a lot of column chemistry and other things like that.

Whereas CE works in an open-channel capillary, and it has no interactions with a surface—it simplifies the problem. You can optimize conditions to make the molecules happy, and you can separate on properties that are simpler than they would be for chromatography. Also, critical for biomolecules, a bigger molecular size isn’t a handicap for CE separations. The band broadening in CE: faster diffusion leads to wider peaks. Whereas in chromatography, when you’re pushing things with pressure, slow diffusion hurts you. So, big molecules are great candidates for CE separations.

BIOPHARM/LCGC: What are the limitations of traditional CE?
MELLORS: When you say traditional CE, the technology was originally developed in capillary tubes, traditionally with optical detection. You can do an amazingly good separation in a silica capillary tube. With optical detection, you don’t have to worry about transferring those peaks out of the tube, which is good, but you don’t have the mass spec information. The biggest limitation of traditional CE is it’s stuck inside of a capillary tube and the methods used to couple capillary tubes to MS are difficult to make work well.

A single tube is hard to couple with an electrospray emitter without introducing dead volume. Moving that into microfluidics gives you the ability to make a fluid network while keeping the volumes very small without introducing any dead volume. You have the freedom now to do the high-quality, low-volume separations that CE was born to do. You can couple that with MS and get so much information out of it that, for many applications, is crucial.
**BIOPHARM/LCGC:** Tell us about ZipChip and why it was developed?

**MELLORS:** What is today called ZipChip started in the academic lab with Mike Ramsey at the University of North Carolina as microchip CE-MS. The original problem was they were doing amazing separations in microchips, but with optical detection—mostly fluorescence detection—and it was obvious that if they could get those separations into a mass spectrometer, it would be powerful. But there was no good way to do it, and people were struggling with the connection between a microchip and some sort of electrospray emitter.

The key development was to figure out how to electrospray right off the corner of the microfluidic device. We don’t have to connect anything else; we just have a single piece of glass with channels etched in it, and the corner of the chip is the electrospray emitter. That allows you to maintain the speed and efficiency of a microchip separation but with the most sensitive and stable electrospray directly into the mass spectrometer—a combination of microfluidic separations and top-of-the-line MS technology.

**BIOPHARM/LCGC:** What are the benefits of coupling the chip to MS?

**MELLORS:** There is so much information that you get from MS. I come from the separations world where making as many peaks separate as possible is the best thing. But MS allows you to see and do so much: high-resolution accurate mass, fragmentation, characterization at a deep level, high sensitivity, etc. From that standpoint, coupling the separation to the MS is crucial to the problem.

From the MS side, ZipChip is a very easy-to-use source. It’s a single box, and the microchip is parked right in front of the inlet. There are no fittings to adjust, no voltages or gases to adjust. It performs fast runs with a simple workflow. It’s a nice, easy way to get data out of your MS, and on top of that, you can separate things that are very difficult to work with when using other technology.

**BIOPHARM/LCGC:** Can you describe the typical workflow?

**MELLORS:** Most of the time, it’s very simple. Diluting the sample into the background electrolyte (the CE equivalent of a mobile phase). Sometimes you add standards or something else to it, but it’s simply dilute and shoot. In our case, we work with an autosampler. We prep the samples and put them in LC vials or a 96-well plate. And if there is any sample prep necessary, we only need to desalt. And that’s because CE is driven by an electric field, and huge mismatches in conductivity can cause problems. So, it’s the same workflow as with chromatography; you may want to add standards or make a calibration curve, but the rest is familiar to anyone who works in the LC-MS world.

**BIOPHARM/LCGC:** Can you explain how ZipChip CE-MS offers an advantage in critical quality attributes (CQA) monitoring over alternative methods?

**MELLORS:** It’s uniquely versatile in the level of different applications it can do in that space. With the same system, using the same chips, you can look at everything from individual amino acids to intact native proteins and protein complexes because it’s not based on retention on a stationary phase; it’s just analytes moving through an open tube in an electric field. You can do a charge variant separation of an intact protein and get a separation that’s as good as or better than all the other charge variant separation techniques but with MS data directly coupled onto it. As such, from a single, 10-minute experiment, you get a huge amount of information. And, on that same system, you can do a peptide mapping experiment or a middle-up type analysis where you break a protein into its subunits and analyze those.

**BIOPHARM/LCGC:** What’s next for ZipChip?

**MELLORS:** We are only beginning to take advantage of this technology. We recently introduced our system to the market, and we’re trying to catch up on many decades of LC method development. There are new applications such as nucleic acids and broader classes of molecules that were challenging for the initial development. We’re also focusing on complete end-to-end workflows. These separations have the potential to be extremely fast. The speed of a microfluidic separation is largely only limited by the speed of the detector. The faster and more powerful MS gets, the faster we can speed up the separation. But to take advantage of that means speeding up the autosampler and the automation. There is a lot of room to take full advantage of analyzing small samples and optimizing the process of getting samples into the chip. We can drive the sensitivity and improve every other aspect of the technology.
A number of metrics to quantify the extent of peak asymmetry have been developed over the years. Two of the more commonly used metrics are the peak asymmetry factor \( A_S \) and tailing factor \( TF \). Figure 2 shows the measurements needed to calculate these values using equations 3 \((A_S)\) and 4 \((TF)\). Both metrics rely on measures of parts of the peak width, with \( A_S \) reliant on the widths at 10% of the peak height, and \( TF \) reliant on widths measured at 5% of the peak height. Generally speaking, \( TF \) values below 2 are preferred whenever possible.

I’ll also note here that recent work by Wahab and associates provides other approaches to quantifying char-

\[
C_{A,t} = \frac{m_A}{\sigma_t^2} \exp\left[-\frac{(t-t_{R,A})^2}{2\sigma_t^2}\right] \tag{1}
\]

\[
C_{A,t} = m_A \exp\left[\frac{\sigma_t^2}{2\tau} \frac{(t-t_{R,A})^2}{\tau^2} \right] \left[1 - \text{erf}\left(\frac{\sigma_t}{\sqrt{2\tau}} \left[\frac{t-t_{R,A}}{\tau}\right]\right)\right] \tag{2}
\]

Many different models can be used to simulate tailed chromatographic peaks (1). One of the most commonly used models is the exponentially-modified Gaussian (EMG) function, shown in equation 2, where \( \tau \) is the relaxation time associated with the exponential, tailing part of the peak, and \( \text{erf} \) is the mathematical error function.

Figure 1 shows an overlay of four peaks. The black peak is perfectly symmetric, simulation using the Gaussian function in equation 1. The other three peaks were simulated using the EMG function in equation 2, with increasing \( \tau \) values.

Figure 3 shows the effect of peak tailing on the ability to detect a small peak in the tail of a large peak. Peaks were simulated using equation 2, assuming a plate number before tailing of 5000 for each peak. Retention times for the first and second peaks are 5.00 and 5.44 min, and the height of the second peak is 1/10th of that for the first peak.
acteristics of peak shapes that take the entire peak shape into account, as opposed to measures of the peak width at specific heights (8).

\[ A_s = \frac{h_{\text{res}}}{T_{\text{res}}} \]  \hspace{1cm} \text{[3]}

\[ TF = \frac{f_{5\%} + h_{5\%}}{2f_{5\%}} \]  \hspace{1cm} \text{[4]}

**Why Peak Shape Matters**

Asymmetric peaks can negatively impact both qualitative and quantitative aspects of HPLC results. Figure 3 shows an example of how peak tailing can obscure the presence of a small peak in the tail of a much larger peak. When both peaks are symmetric, it is easy to see the small second peak on the right side of the larger peak. However, as the extent of tailing increases, it becomes more and more difficult to see the small second peak, and eventually it disappears into the tail of the large peak altogether.

**FIGURE 4:** Illustration of the effect of peak tailing on quantitative accuracy for poorly resolved peaks. Adapted from reference (4). Peaks were simulated using equation 2 and parameters similar to those used in Figure 3, except that the retention time of peak 2 was adjusted to obtain the desired resolution. Percent error was calculated using the observed peak area (left or right of the drop line for peak 1 or 2, respectively) relative to the expected areas of 100 and 10 for peaks 1 and 2, respectively.

Peak asymmetry can also affect both the accuracy and the precision of quantitative results. Figure 4 shows how more resolution is needed to obtain accurate estimates of peak area than that needed (typically a resolution of
1.5 is sufficient with highly symmetric peaks) with symmetric peaks. In the example shown here in the left panel, the peak area for peak 2 is overestimated because the tail of the larger peak 1 runs into the integration area when the drop method of integration is used. Of course, this can be improved in some cases using other more sophisticated integration methods, but other methods may not be as robust as the drop method. The magnitude of this type of error can range from less than 1% to more than 10%, and generally increases as resolution decreases, the ratio of peak heights increases, and the extent of tailing increases.

Common Causes of Peak Asymmetry in Liquid Chromatography

The common causes of peak asymmetry in liquid chromatography can be roughly grouped by whether they have a primarily physical or chemical basis.

Physical Causes

- Poorly packed particle bed at the time of manufacture
- Rearrangement of the particle bed during use because of physical stress (for example, repeated pressure cycles)
- Contamination of the particle bed or column inlet/outlet frits with physical debris (for example, particles shed from valve seals)
- Mismatch between the solvent composition of the injected sample and the mobile phase
- Extracolumn peak broadening and distortion outside the column because of poor connections or inappropriately large connecting tubing

Chemical Causes

- Rearrangement of the particle bed during use due to chemical stress (for example, dissolution of silica particles at high pH)
- Overloading of analyte adsorption sites on the stationary phase when there are multiple types of sites with different populations
- Slow kinetics of analyte desorption from the stationary phase

This list is obviously too long to talk about each case in any detail; these will be the focus of future installments of “LC Troubleshooting.”

Impact of Poor Fluidic Connections Outside the Column on Peak Shape

Of all the possible causes of peak tailing listed above, I think one of the most common—but easy to solve—causes is related to poor fluidic connections outside of the column. Tony Taylor has written about this recently in the “LCGC Blog” as it relates to gas chromatography (6).
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Overcoming Challenges with Aging Instrumentation.

Older instrumentation presents a host of challenges, including decreased sensitivity and longer run times. Plus, they often need more repairs. Newer technologies and robust maintenance plans are critical factors that enable organizations to increase laboratory efficiency and productivity by preventing unexpected downtime, supporting regulatory compliance, and enhancing data quality.

**LCGC:** What are some of the challenges of working with older technologies?

**RENNIE:** There are quite a few challenges with older technologies. Older instruments are not as sensitive as newer technologies, so you can miss a peak on an impurity run, for example. Older technologies also have longer run times, use more mobile phase, and have more waste disposal, which occupy expensive lab resources longer, i.e., people, consumables, and real estate.

Another aspect associated with older instruments or those approaching end-of-life from the vendor is that the expertise needed to repair those instruments diminishes as vendors focus on newer technologies. Additionally, parts might not be available to complete a repair if there is a failure. If this happens, the lab may need to scramble to replace a system that’s not in the budget.

**LCGC:** What about the maintenance needed for older technologies?

**RENNIE:** While our instruments are built to last, as instruments age, the possibility of repairs increases. Think of a car: As it ages, parts start to wear down, ultimately leading to the timing belt, fuel injection, etc., needing to be replaced. The same is true of laboratory instruments: The older they get, the higher the possibility of a major failure.

**LCGC:** What are the benefits of updating to new technologies?

**RENNIE:** One of the main benefits of updating to new technologies is taking advantage of smaller particle-sized columns, which allow you to run the same methods with the same chromatographic characteristics, in a shorter amount of time. A 45-minute run, for example, can potentially be cut in half, so you can run more samples in a day, increasing your overall productivity and efficiencies in the laboratory. Newer technologies are also often more sensitive than their predecessors, allowing you to evolve your analytical methodology to not only take advantage of the particle size but also...
make your methods better and more robust than some of your older methods. Investing in newer technology means more accurate results, leading to increased productivity and possibly bringing your product to market quicker.

LCGC: Let’s say I have updated my lab to newer technology, do I need to have maintenance included in that purchase?
RENNIE: It’s always good to include maintenance as part of instrument purchase. Ofentimes, you get a better deal by purchasing the maintenance upfront. Plus, it helps to ensure you are covered for years to come. If we keep with the car analogy, it will need oil changes and tire rotations as it’s used, so purchasing those upfront saves money in the long run.

LCGC: Are there additional aspects to service coverage that help customers realize the full benefit of the system, especially those in the regulated environment?
RENNIE: Yes, if you are working in a GxP environment, ensuring data quality is extremely important. At the foundation of data quality is system and software qualification. Performing routine maintenance and system qualification ensures the instrument is still working properly and can help ensure the user is meeting regulatory requirements, both internally from its quality assurance department as well as industry regulations, whether those are ISO regulations, FDA, or EMA regulations.

LCGC: What if I’m not yet ready to replace my aging technology?
RENNIE: You may need to work with older instruments while you budget for new ones. I recommend you have a robust maintenance plan around these instruments to help keep them running at their optimal performance.

LCGC: Can you explain how Waters’ philosophy on maintenance is different from other companies?
RENNIE: Conducting annual maintenance only means that a level of maintenance has been performed; it does not guarantee the maintenance conducted will be effective in optimizing instrument uptime. The Waters philosophy on maintenance—we call it Performance Maintenance (PM)—proactively replaces all normal wear-and-tear parts before they fail to help reduce the probability of unscheduled downtime. We believe that robust maintenance is a critical aspect to keeping instruments up and running and keeping our customer productive.

Other companies apply an inspect-and-replace preventative maintenance methodology, which means technicians will only replace parts that are either broken or visibly worn. The trouble with an inspect-and-replace maintenance approach is that most damage incurred by parts during normal operation cannot be seen with the naked eye. For example, micro-abrasions in a plunger cause microscopic leaks that create small variabilities in mobile-phase composition—these aren’t detectable by taking the pump apart and examining the parts with the naked eye. Even the act of taking the pump apart can introduce future issues with the system—it might not cause a breakdown right away, but it could cause OOS or other method-related issues due to a poorly performing pump. Inspect-and-replace maintenance is also subjective, which leads to inconsistencies.

LCGC: How have you made it more flexible for customers to purchase service coverage for their instruments?
RENNIE: Waters has a new way to offer service coverage called FlexCHOICE: mySystem Coverage. FlexCHOICE is designed to be customizable and tailorable to meet customers’ specific laboratory requirements and budgets. We use simple building blocks to assemble the coverage based on conversations and understanding the needs of the lab. We are big proponents of PM and recommend annual PM; however, if you are a high-throughput lab, you may need to have your system PM more often. We can build in additional PMs and can create multi-year coverage.

For those in a regulated environment, we can include our qualification with the coverage offering. One advantage of including our qualification as part of the coverage is that if any repairs are made on the covered instrument, the re-qualification after the repair is completed is already included. If you have a mass spectrometry (MS) system, and you’re not using clean samples, you may need the front end cleaned more often than a full PM. In that case, you can add ion-source cleanings to your coverage. We are always talking with customers and will continue to develop new service offerings to help meet their evolving needs.
In that context, there are multiple ways a “bad connection” can arise, including improper positioning of a column (for example, in a mass spectrometer interface), a poor cut on the end of a column (that is, a rough cut, or one that is not perpendicular to the long axis of the column), or use of the wrong ferrules. In HPLC, similar pitfalls exist. From the point of view of effects on peak shape, the only connections that matter are those between the sample injector and the detector. Typically, this flow path will involve four to eight connections, but could include more, depending on the complexity of the system (for example, injector -> capillary, capillary -> heat exchanger, and so on). Many laboratories use “pre-cut” connecting capillaries, where the ends of the tubing are prepared by the tubing manufacturer to give clean, square ends. Although I cut my own stainless steel tubing in graduate school, I am not aware of any laboratories that cut their own metal tubing these days. However, in my laboratory we routinely cut polyetheretherketone (PEEK) tubing to length for specific purposes. One can cut PEEK tubing using something as simple as a sharp razor blade, but this is really not recommended, because it is very difficult to obtain a very square end on the tube with this approach. Most chromatography suppliers sell cutters for both plastic and metal (and even PEEK-clad fused silica) tubing that are specifically designed to give clean, square cuts on the ends of these tubes. Using a tube with an end that is not square will lead to a significant void space at the bottom of a capillary connection port like that shown in Figure 5b, except that the shape of the void will look like a trapezoid rather than a rectangle. This extra void space will, in turn, lead to mixing of the analyte band as it moves across the gap, which manifests in chromatograms as peak tailing like that shown in Figure 6. The good news is that this particular problem can be avoided easily by either using pre-cut capillaries, or using the proper tools to cut your own tubing and ensure clean, square cuts.

The other major problem with connections that can lead to peak tailing in HPLC is clearly illustrated in Figure 5b. Here, the fitting has been tightened (referred to as swaging) when the end of the capillary was not seated properly at the bottom of the connection port. With a compression-style connection that uses a metal ferrule, once the ferrule position is set, it cannot be moved, and every time this capillary is connected to a fitting port, there will be a void space like that highlighted in green, which again will lead to peak tailing like that shown in Figure 6. There are multiple potential solutions to this problem. If a situation like that shown in Figure 5b exists with a metal ferrule, then the only solution is to throw the capillary away and start over. When making the new connection for the first time, be careful to make sure the tubing end is seated at the bottom of the fitting port before swaging the ferrule. Alternatively, one can use a fitting that does not involve a metal ferrule (usually PEEK, or a graphite-filled polymer) so that the position of the ferrule relative to the capillary end can be adjusted each time it is connected to a fitting port. Several manufacturers now sell very high quality fittings of this type. Readers interested in learning more about these options are referred to a previous installment of “LC Troubleshooting” focused on this topic (9).

Summary
In this installment of “LC Troubleshooting,” we have reviewed some basic concepts in peak asymmetry and how asymmetric peaks can affect the quality of HPLC results. There are many physical and chemical causes of peak tailing in particular—too many to discuss here. In this installment, we’ve discussed how poor fluidic connections in the flow path between the sample injector and detector can lead to peak tailing. The extent of tailing resulting from this particular cause can be serious, but the good news is that it can be addressed quite easily by fixing poor connections with new capillaries or fittings. In future installments, we will continue discussing some of the other causes of peak asymmetry in detail, all with the aim of adding to your personal knowledge base that can help you troubleshoot poor peak shapes when they arise.

References

About the Column Editor
Dwight R. Stoll is the editor of “LC Troubleshooting.” Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 75 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC’s editorial advisory board. Direct correspondence to: LCGCedit@mmhgroup.com
Capillary Gas Chromatography (GC): Getting the Best Separation without Turning (Too Many) Wrenches

It is often a good idea to look at our methods and see how we can make them more efficient and effective without doing a major overhaul. In this month’s column, I examine simple steps we can take to optimize a separation without changing the column. I discuss adjusting temperature, flow, and sampling with an eye toward the classical method optimization goals of high resolution, high speed, high sample capacity, and ease of use. I examine optimizing as much as possible without getting the wrenches out to install a different dimension or stationary phase column.

Nicholas H. Snow

One of the first and most important lessons I learned about optimizing and troubleshooting chromatographic problems is to try the simple fixes first and turn wrenches last. Changing a column in a gas chromatography (GC) instrument usually requires additional downtime to cool the instrument, install the new column, heat it back up, condition or bake out the new column, and then think about a change in peak elution order. Because these steps can be very time-consuming, I always taught my students to get as far as they can with optimizing the separation on the current column before changing it. Let us consider some optimizations that can be attempted without changing column dimensions or the stationary phase.

Method Optimization Goals

Before considering optimization steps, it is important to consider the goals of the optimization process. Many short course instructors (including me) use a simple triangle approach to assist students in thinking about optimization. A classical optimization triangle is shown in Figure 1. Each corner of the triangle represents an optimization goal—high resolution, high speed, or high capacity (ease of use). In method development and optimization, we can generally obtain any two of the three goals simultaneously. Obtaining all three often requires difficult compromises. A high-resolution, high-speed method will be more difficult to run and will likely have a lower sample capacity or require a small sample. A high capacity (easy) method that is also fast will likely be low resolution. Finally, a high-resolution method that has high capacity and is easy to run will generally be slow.

Because the goal of a chromatographic separation is resolution, the classical resolution equation, shown in equation 1, can also be helpful in thinking about the chromatographic challenges in method optimization.

\[
R_s = \frac{k}{1+k} \left( \frac{a-1}{a} \right) \left( \frac{N}{4} \right)
\]  

Resolution is seen as a combination of retention factor (the “k” term), selectivity (the “a” term) and efficiency (the “N” term). In these examples, we look at the terms in this equation broadly. Resolution can be thought of as involving long retention, as seen in the term involving k. If k is too small (less than 2), k/(1+k) is also small, so retention does not contribute to resolution. If k is too large (approximately 20), then there is little resolution gained by increasing k by lowering the temperature. Selectivity, a, is a measure of the relative strength of intermolecular interactions between an analyte pair and the stationary phase. We see several cases where a lack of selectivity hinders our ability to achieve resolution while maintaining our goal in this exercise of not turning wrenches. Finally, resolution is dependent on N, the number of theoretical plates, or efficiency. We would like the sharpest possible peaks, and because retention and efficiency are most affected by temperature and carrier gas flow rate, these are the variables that most influence resolution without turning wrenches. In our discussion, we use these three ideas of retention, selectivity, and efficiency in a broad framework, rather than dissecting the equation,
as our examples are temperature programmed and equation 1 assumes constant temperature.

With the first step being to set the goals of the optimization, we now examine each of the three scenarios and see how each can be optimized without changing the column. We assume temperature-programmed separations and discuss optimizing for each case. In my own laboratory, we do most of our initial method development work on 15 or 30 m x 0.25 mm x 0.25 µm 5% polydimethylsiloxane columns, so these dimensions will be used as examples throughout the discussion. Chromatograms were simulated using Pro-EZGC online software from Restek (1). To get started, Figure 2 shows a sample chromatogram of n-alkanes separated on a 15 m column as described above with a 10 °C/min temperature program, starting at 40 °C and a column flow rate of 1.5 mL/min. These simple conditions are typical of scouting runs that might be performed at the outset of method development for a new separation problem. For a simple comparison between runs, note the retention time and elution temperature of the last peak (dodecane) are 7 min and 110 °C, respectively, and the resolution between the last two eluted peaks is 37.

High Resolution and High Speed
The combination of high resolution and high speed is often the most challenging scenario. In Figure 2, there is already high resolution, so we can optimize for speed by increasing the temperature program rate. For most temperature-programmed separations, increasing the speed of separation, effectively reducing retention (k) by increasing the temperature programming rate, will reduce the resolution. In this case, this is fine because baseline resolution is 1.5 and our current resolution is 37. The injection technique and speed of injection can also impact the maximum speed

FIGURE 1: Method optimization triangle. Method development and optimization can lead to two out of the three options.

FIGURE 2: Simulated temperature programmed (40 °C initial, 10 °C/min program rate) separation of five alkanes (1 = octane, 2 = nonane, 3 = decane, 4 = undecane, 5 = dodecane) on a 5% phenyl polydimethylsiloxane column (30 m x 0.25 mm x 0.25 µm), with a flow rate of 1.5 mL/min.

FIGURE 3: Simulated chromatograms of the alkane mixture with further optimization. (a) Temperature program rate increased to 20 °C/m, (b) flow rate increased to 3 mL/min.
of the separation. In fast GC, a split injection with a high split ratio is used to ensure the narrowest bands entering the column, ensuring a high number of theoretical plates. However, a split injection with a high split ratio increases the limit of detection (LOD) as more of the sample is ejected from the split vent instead of entering the column. Additionally, the column flow rate can be increased to drive analytes in the vapor phase more rapidly through the column, causing a potential reduction in the number of theoretical plates that is mitigated by temperature programming, as discussed in a previous column (2).

Figure 3 shows two further optimized chromatograms of the alkane mixture. Figure 3a shows the temperature programming rate increased to 20 °C/min and Figure 3b shows the separation in Figure 3a with the flow rate increased to 3 mL/min. In Figure 3a, note that the retention time for dodecane has reduced to approximately 4.4 min, reducing the run time by approximately 35%. The resolution of the last two peaks is slightly lower, still approximately 35, because of the peak widths becoming smaller as the retention time is decreased. However, the elution temperature of dodecane increases to 128 °C, an increase of nearly 20 °C. A higher elution temperature increases down time between runs, reducing the speed benefit. Extremely high elution temperatures that approach the maximum column temperature can reduce column lifetime over time. In Figure 3b, the added increase to the flow rate further reduces the retention time of the dodecane peak to approximately 3.7 min. and its elution temperature to 114 °C, which is almost back to the original. Resolution of the last two peaks is still quite high (approximately 30). By increasing both the temperature program rate and the flow rate, we were able to reduce analysis time by nearly 50% with a similar elution temperature and a small cost in resolution.

High Speed and Ease of Use
A requirement for both high speed and ease of use or high capacity also presents special challenges. This combination usually requires that the problem involve few analytes (and few interferences!), so that there is not a great need for high resolution. High speed usually means low retention, high selectivity, and high efficiency. Resolution is provided by high efficiency or selectivity and not by long retention. Figure 4 shows the separation of the five components
with further optimizations for speed. The initial temperature is raised to 80 °C, the temperature programming rate is increased to 30 °C/min, and the flow rate to 5 mL/min, with a column head pressure of about 26 psig and a gas hold-up time (\( t_{h,0} \)) of about 15 s. The retention time of the final peak is now about 1.4 min with an elution temperature about 120 °C. The faster programming rate and higher initial temperature have combined to raise the elution temperature. However, this final temperature is only approximately 40 °C higher than the initial temperature, so downtime between runs would be significantly reduced when compared to the separations in Figures 2 and 3. Resolution between the last two peaks has reduced to approximately 25 but is still very high. This separation could be further optimized for speed, but the differences seen between Figure 4 and Figures 2 and 3 are dramatic. Further optimization can be tried using this online simulator or by method translation (3). With three simple changes, increased temperature programming rate, increased initial temperature, and increased flow rate, we have reduced the run time by 80% and the downtime between runs by approximately 50%, with a resolution loss of about 30%.

**High Resolution and Ease of Use**

Ease of use or high capacity and high resolution require a slower analysis; these are at the apex of the triangle in Figure 1 farthest from speed. In this case, high resolution comes from selectivity. The column with the most separating power will also likely have high retention and require less efficiency. Classical separations of petroleum-related samples on nonpolar stationary phases such as the one shown in Figure 5 illustrate this problem. This sample of brown mousse from the surface of the Gulf of Mexico during the Deepwater Horizon oil spill in 2010 was prepared by placing about 100 mg of the brown mousse in a vial and diluting with pentane, so the sample preparation was very simple. The conditions, shown in the caption, are very common and simple conditions. As seen from this figure, while the separation efficiency is quite high and the retention is strong, there is not enough selectivity to achieve a full separation of the many components of this mixture in a simple analysis. This sample could be better separated by a lower temperature programming rate, but this 15 m, thin-film column alone lacks the separation power to fully separate this mixture.

Although this is a much slower analysis than those presented in the simulated chromatograms in Figures 2–4, it is not slow enough to fully separate this highly complex mixture. Samples like this one led to the rapid development of multidimensional techniques, such as comprehensive two-dimensional gas chromatography.
(GCxGC), over the past decade (4). In analyses like this, sample preparation and injection are often quite simple, with high split ratio split injection employed for both simplicity and to ensure narrow peak width.

**Summary**

There is much method optimization that can be done without turning any wrenches. Looking back at the analysis goals, broadly at equation 1 and thinking about capillary columns in general, we see that even relatively short columns can have high plate numbers, with the 15 m columns discussed here having plate numbers around 50,000. Retention is controlled using temperature, with lower temperatures and lower temperature program rates leading to longer retention. Flow rate can be used as a final optimization to speed up the analysis with a higher flow rate. In temperature programming, higher flow rates lead to lower elution temperatures with surprisingly small effect on resolution; lower elution temperatures can increase column lifetime. In my own laboratory, we start with a column like the 15 m column described here and optimize the separation as far as we can before getting out the wrenches and changing the column.

**Remembering Harold McNair**

Finally, I close with some sad news. My research, career, and life mentor, Professor Harold M. McNair, passed away on June 27, 2021, at peace and surrounded by his family, in Blacksburg, Virginia. He was 88 years old. Harold was a man of the world whose work in research, teaching, and being a great mentor and friend touched everyone who practices GC. His career as a chromatographer spanned over 60 years, including publications in every decade from the 1950s to the 2010s. In 1959, he authored the first PhD thesis on GC in the United States. His most recent paper was published in 2019. If you took a webinar or short course online over the past year, or ever took a short course, you have Harold McNair to thank for the idea of short courses in chromatography, which he started in the 1960s. His classic book, Basic Gas Chromatography, has been published all over the world since the first edition in 1965 and set the original standard for training books and manuals (5). Basic GC brought GC into the mainstream and made GC accessible to all scientists, not just chemists and chromatography specialists. I plan on writing more about Harold, lessons from his work, and his impact on chromatography and analytical science in an upcoming column.

**References**


**ABOUT THE AUTHOR**

Nicholas H. Snow is the Founding Endowed Professor in the Department of Chemistry and Biochemistry at Seton Hall University, and an Adjunct Professor of Medical Science. During his 30 years as a chromatographer, he has published more than 70 refereed articles and book chapters and has given more than 200 presentations and short courses. He is interested in the fundamentals and applications of separation science, especially gas chromatography, sampling, and sample preparation for chemical analysis. His research group is very active, with ongoing projects using GC, GC–MS, two-dimensional GC, and extraction methods including headspace, liquid–liquid extraction, and solid-phase microextraction. Direct correspondence to: LCGCedit@mmhgroup.com
**VENDOR TIPS & TRICKS**

**Turn Down the Volume**

Conner McHale, Technical Support Specialist, Advanced Materials Technology

Extra column volume is the volume in an HPLC system external to the column that contributes to the total peak volume, which will impact peak width, resolution, and overall efficiency. Most new HPLC systems have been optimized for volume, and therefore are able to use high-efficiency superficially porous particles (SPP) and fused-core® columns, but many older chromatographs are still in active use. Older chromatographs are traditionally used with longer columns packed with larger particle sizes where extra column volume in the injector and connecting tubing lines have less of an effect on resolution and peak width. Now, when switching to shorter, highly efficient SPP columns, this extra column volume needs to be reduced in order to avoid peak broadening that can reduce resolution and reduce the accuracy of quantitative results.

**How do we minimize our extra column volume?**

1. Keep your injection volume to a minimum
2. Keep your sample solvent strength equal to or less than initial mobile phase if possible. If a 100% organic must be used as the sample solvent, use a minimum injection volume possible
3. Use minimal lengths of i.d. tubing from the injector to the column. Using multiple tubing i.d. in the sample flow path can cause additional dispersion
4. Heat exchanger volume (if used) should use the smallest volume consistent with flow rates
5. Reduce your post column tubing volume to a minimum
6. Reduce the detector flow cell volume

Loss in efficiency and peak broadening can be an issue for many reasons. This is a function of both external column effects and internal. These quick and simple external column changes can significantly improve your chromatographic separations and should be considered for every chromatographer. Extra column volume also needs to be considered when performing method scaling (moving to a different column dimension/ particle size) or transfer to another instrument that may have a different hardware configuration. For more information view our video: https://halocolumns.com/videos/halo-extra-column-volume/

**VENDOR TIPS & TRICKS**

**Efficient Protein Purification Strategies Using Multi-Well Filter Plates**

Lori Euler, Global Product Manager, Pall Corporation

Combined with chromatography media, 96-well filter plates have emerged as an efficient tool for the fractionation of small-volume protein samples. This format can be used to develop protein purification strategies and/or as a platform for moderate- to high-throughput protein isolation. In either case, purified sample can be used for further analysis and/or downstream applications.

**High-Throughput Screening on 96-Well Filter Plates**

Filter plates have the capability to quickly screen various conditions. The analyst can quickly optimize the chromatography resin and purification conditions with minimal sample consumption. Once the selected sorbent is equilibrated and resuspended as a 50% slurry in the equilibration buffer, the desired amount is dispensed into the wells to a final volume of 50 μL per well. After the slurry has been dispensed into the plate’s wells, the equilibration buffer is aspirated using a multi-well plate vacuum manifold. Next, a sequence mimicking a chromatographic run is performed on the plates. For each sequence step, the corresponding solution is pipetted into the wells. Once filled, the filter plate is covered with sealing tape and incubated while shaken. The liquid is then drained from the wells using the vacuum manifold and collected in a 96-well receiver plate. The individual fractions are then analyzed by HPLC, ELISA, or other analytical methods. The flexible 96-well plate format can be used with liquid-handling robotic systems or manual multi-channel pipettes.

**Membrane-Based Protein Purification**

If resin packing is inappropriate for your application, consider using ion exchange on 96-well filter plates with Mustang IEX membranes. To overcome mass transfer limitations associated with conventional resin-based chromatography, membrane chromatography was developed to obtain better flow distribution, fast flow rates, and increased accessibility for rapid purification of target proteins or removal of contaminants.
Five Frequently Asked Questions about Thermal Desorption
Laura Miles, Senior Applications Chemist, Markes International Ltd.

Thermal desorption (TD) is a versatile preconcentration technique for gas chromatography (GC) that is used to analyze volatile and semi-volatile organic compounds in air, materials or liquids. It arose out of the need to improve upon conventional sample preparation techniques for GC. Thermal desorption can be used for a wide range of applications such as environmental monitoring, materials testing, and food and beverage analysis.

I’m not working with air samples, so why would I need thermal desorption?
Thermal desorption is most widely known as a preconcentration technique for ambient air, but the simplicity and automation of the technique has led to its widespread use for a wide range of other matrices that are not amenable to direct GC analysis. This versatility is illustrated by its use for direct desorption (dynamic headspace) of solids and liquids and the field sampling of soil, water and breath using dedicated accessories.

Can I analyze target and non-target compounds?
Yes. The majority of sorbents are not compound-selective and can capture analytes over a broad range. They work by adsorbing suites of compounds based primarily on their boiling point and physical properties. The use of multiple sorbents increases the volatility range that can be analyzed in a single sample.

Is TD suitable for semi-volatile organic compounds (SVOCs)?
Yes. Markes’ TD-specific heated valve provides an inert, low-volume, consistently heated flow path, ensuring fast, efficient transfer of high-boiling compounds up to C44, including PAHs, PCBs and phthalates. Other thermal desorbers, without an optimized valve, can suffer from cold spots. This results in loss of high-boiling compounds, and such instruments therefore require much higher temperatures to analyze SVOCs.

I have volatile analytes and very humid samples. Can I still use TD tubes?
Yes. By optimizing sorbent selection, temperature, purging, and split ratios it’s possible to quantitatively retain very volatile compounds such as chloromethane and propylene (US EPA Method TO-17), and reduce water sufficiently for GC or GC–MS analysis.

Is thermal desorption a “one-shot” technique?
No. This is a common misconception. Markes’ instruments allow samples to be split during the tube desorption and/or trap desorption stages. The split portions can then be “re-collected” onto a sorbent tube to achieve reliable repeat analysis of a single sample multiple times. Re-collection can also be used to simplify method validation, enable confirmatory identification using a different detector, and even extend the storage stability of many sample types. In addition, re-collection can be used to extend the dynamic range of a method by automatically analyzing a sample at two different split ratios (“Hi/Lo” analysis).
A key advantage of core-shell (also called superficially porous, SPP) columns, such as Ascentis® Express or BIOshell™, is that their performance can rival that of the traditional fully porous particle columns, but at somewhat larger particle sizes and at significantly lower backpressures. Therefore, core-shell columns can often be used on conventional HPLC instruments, while providing performance similar to ≤2 µm totally porous particle columns.

The extent to which a column’s optimum performance can be achieved is limited by the relative magnitude of system dispersion (instrument bandwidth), which can be qualitatively approximated by the system extracolumn volume (also known as system dead volume). However, it is not the absolute system extra-column itself, but the magnitude of the system extracolumn volume relative to the column volume that is key. A larger system extracolumn volume means that the analyte peak (or band) has greater time and volume to diffuse and thus become more diluted, resulting to a broader analyte peak. Therefore, reducing the system extra column volume will virtually always result in improved measured column performance.

To achieve the optimum performance of core-shell columns, consider the following steps to reduce extra column volume:

1. **Minimize column inlet (injector to column) and outlet (column to detector) tubing lengths.** Minimizing the tubing lengths will reduce the system extracolumn volume will virtually always result in improved measured column performance.

2. **Column inlet and outlet tubing ID should be a maximum of 0.005” (0.125 mm).** Using smaller ID tubing is most often beneficial, but be aware that backpressure will increase as a function of the square of the ID. Therefore, for applications that are not particularly high flow, going to 0.004” ID can be a good compromise between performance and backpressure.

3. **For detectors that utilize a flow cell, keep the flow cell volume to ≤5 µL, and preferably to ≤3 µL.** Larger flow cells can cause extra-column dispersion, which results in an apparent decrease in column efficiency.

There are also instrumental considerations related to the detector that can be optimized to help achieve optimum core-shell column performance.

1. **Make sure your sampling rate is high enough.** You should be recording at least 10 points across the narrowest peak. Doing >20 points across a peak is excessive and only creates a larger file size. Also, the higher the sampling rate, the higher the noise.

2. **Most optical detectors (UV, DAD, fluorescence) include a parameter often referred to “response time” (or something similar). A recommended setting is 0.1 s.** A faster sampling rate is generally not necessary and only creates more noise.

All else being equal, optimum column performance will be achieved with larger columns. Use of smaller columns has its advantages but realized performance will be compromised. For many HPLC systems in which the configuration has been optimized as previously described, a 3 mm ID column is often a good compromise between the advantages of a small column, such as shorter run time and less consumption of mobile phase, and of realized performance.

As for gradient methods, be aware that a high-pressure mixing system will generally permit additional control of the retention behavior of early-eluting peaks and, therefore, can also allow for shorter run times.

Explore our HPLC columns—visit our website: sigmaaldrich.com/hplc
Using Data Pretreatment to Improve Resolution

Metrohm USA

Did you know that applying the second derivative as a pretreatment to your near-infrared spectral data can help resolve overlapped peaks?

NIR absorption spectra are often complex being comprised of overtone and combination bands. This leads to broad overlapping absorption bands that are virtually impossible to identify in raw data. Moreover, chemical, physical, and structural properties of all species present in a sample influence the measured spectra causing baseline shifts and spectral backgrounds.

Raw data contains information from one or multiple overlapped bands and quite often the information of interest is even hidden. Pretreatment of NIR raw data is the first step of model development and optimization to correct for baseline shifts, backgrounds and to uncover hidden information. The use of a second derivative will allow the position of nearby peaks to be identified serving to virtually increase the spectral resolution. Therefore, the second derivative spectrum will show the information of all overlapped bands from the original spectrum.
Bioanalytical Analysis of Small-Molecule Drugs and Metabolites in Physiological Samples by LC–MS, Part 2: Sample Preparation

This article is the second of a series of four white papers on the bioanalysis of small-molecule drugs and metabolites by liquid chromatography–mass spectrometry (LC–MS). Part 1 was an overview of the fundamentals and regulations. This installment, part 2, is on sample preparation. Part 3 will cover method development, optimization, and best practices, and part 4 discusses method validation for regulated bioanalysis. This section provides an overview of sample preparation fundamentals, best practices, and modern trends. A sample preparation method development case study illustrates these practices and concepts.

Stephanie J. Marin, Jeremy Smith, Jillian Neifeld, and Elena Gairloch

Many factors need to be considered when developing sample preparation methods for bioanalysis. For a more in-depth discussion on sample preparation techniques, the reader is referred to textbooks on general applications in chromatography (1), pharmaceutical assays (2,3), and bioanalysis (4). Modern trends and practical aspects in sample preparation can also be found in numerous articles on “Sample Prep Perspectives” in the LCGC North America column by Douglas Raynie (5).

Sample matrix, compounds, chemical properties, instrumentation, and desired limits of quantitation (LOQs) all affect method development decisions for liquid chromatography–mass spectrometry (LC–MS) analysis of complex physiological samples. Multiple options exist for sample cleanup, from more simple methods like filtration, pass-through solid-phase extraction (SPE), and liquid-liquid or supported liquid extraction (LLE and SLE), to more complex methods like hydrophobic interaction and mixed-mode ion-exchange SPE with bonded silica or polymeric sorbents.

This paper aims to review emerging trends in sample preparation of biological samples such as blood, urine, and tissues, and sample preparation options based on their characteristics. Chemical properties such as the octanol-water partition coefficient (log P) and the acid dissociation constant (pKₐ) that affect sample preparation and method development are discussed. Automation strategies are summarized. A case study on sample preparation method development and experimental design for a drug in plasma is presented.

Method Development Considerations

First, the desired compounds, their required LOQs, and the instrumentation used for analysis should be determined. Will the method be a sensitive, quantitative method or a qualitative screen? Are there any relevant metabolites that need to be included? How much of the specimen is available? What is the stability of the analytes in and out of the matrix?

Knowing the properties of the biological matrix is important to determine the type of sample preparation required. For example, urine, blood, or tissues all have components that can cause ion suppression or enhancement, and can foul the LC column and LC–MS instrumentation (4).

Matrices

Whole blood is a commonly analyzed matrix. It is about 80% water. The remaining 20% is primarily red blood cells (43%), plasma (50%), proteins, fats, and sugars. Plasma is mainly composed of proteins like albumin, lipids, and globulins. Lipids and phospholipids in plasma can cause ion suppression and build-up on LC columns, resulting in clogging and reduced column lifetimes. Analytes can be bound to red blood cells or plasma proteins. Many analytes are bound to red blood cells that must be lysed during sample preparation for accurate quantitation. Water, acidic or basic buffers (ammonium chloride, trichloroacetic acid, zinc sulfate, perchloric acid), freezing, or centrifuging can lyse red blood cells. Acidic buffers or organic solvents (trichloroacetic acid, phosphoric acid, formic acid, zinc sulfate, methanol, ethanol, isopropanol) can disrupt plasma proteins (4,6).

Urine, another commonly analyzed matrix, is about 95% water. The main interferences that should be removed from urine during extraction are salts, urea, pigments, and creatinine. Many drugs and metabolites are enzymatically glucuronidated to aid in excretion from the body. These glucuronides can be measured directly, but they usually do not ionize well, and are more polar than their non-conjugated “free” drug or metabolite counterparts. Hydrolyzing conjugated metabolites to the “free” drug or metabolite
increases sensitivity and simplifies method development. Hydrolysis can be done with acid or base, but these are not very selective, and usually require a pH adjustment prior to extraction. Enzyme hydrolysis with a natural or recombinant beta-glucuronidase enzyme is more selective. However, using an enzyme introduces proteins to your urine sample that should be removed before analysis (7).

Tissues require extensive sample clean-up. Commonly analyzed tissues include liver, brain, heart, and umbilical cord. Tissues contain blood vessels, arteries, veins, muscles, and other solid components. Tissues cannot be analyzed directly. They need to be homogenized and the analytes of interest extracted followed by sample preparation to remove inferences. Homogenization can be done with a blender, bead mill, tissue grinder, or bead ruptor. Samples can be homogenized in the presence of aqueous or organic solvent until they are liquified. The homogenate is usually centrifuged, and the supernatant is extracted for additional clean-up prior to LC–MS analysis (8).

**Figure 1:** Selectivity vs. sample cleanliness using different sample preparation techniques.

**Analyte Properties**

Knowing the physicochemical properties of the target analytes can help determine the sample preparation procedure. Information on the structure, functional groups, polarity, pK_a, log P, and stability is beneficial for selecting the appropriate approach and extraction parameters.

The log P indicates an analyte’s hydrophobicity. Analytes with a negative log P are more soluble in an aqueous solvent or more hydrophilic. Conversely, analytes with a positive log P are more soluble in an organic solvent or more hydrophobic. This is an important consideration when developing LLE, SLE, or hydrophobic interaction SPE methods. The distribution coefficient, log D, provides information on the solubility and hydrophobicity of ionized compounds (9).

Knowing the functional groups of the molecule can help determine whether it is an acid or a base. For instance, acidic drugs might have a sulfonic acid, carboxylic acid, or aro-
This phenomenon is defined by the Henderson-Hasselback equation (2). For example, for a weak base with a \( pK_a \) of 8, the analyte would be 100\% positively charged two units below the \( pK_a \) (pH 6 or lower) and 100\% neutral two units above the \( pK_a \) (pH 10 or higher). Similarly, for a weak acid with a \( pK_a \) of 6, the analyte would be fully ionized and negatively charged two units above the \( pK_a \) (pH 8 or higher) and neutral two units below the \( pK_a \) (pH 4 or lower). For LLE, SLE, and hydrophobic interaction SPE, compounds should be neutral. For mixed-mode ion-exchange SPE, compounds should be charged (10).

Many resources are available to research compound properties. Chemicalize (11) is a website that provides compound information like \( \log P \), \( pK_a \), \( \log D \), chemical structure, solubility, and other properties. It does require a paid subscription. The Human Metabolome Database (12) is a free service with information for many drugs and metabolites. PubMed and Google Scholar are resources for peer-reviewed publications. Multiple reference books can also be used to research compound properties, for example, *Disposition of Toxic Drugs and Chemicals in Man* (13).

**Sample Preparation Options**

Figure 1 shows sample preparation techniques organized by increasing selectivity and increasing sample cleanliness. Sample preparation options can be selected based on assay requirements and analyte properties. Table I summarizes SPE product offerings from several manufacturers categorized by sample preparation options and sorbent chemistries. This is not meant to be an all-inclusive list, merely a sampling of what is available. The reader is encouraged to visit the company’s website for updated and detailed information. An overview of sample preparation options is described briefly below.

Dilute and shoot methods, simply diluting the sample and centrifuging prior to analysis, yield the dirtiest samples. There is no clean-up involved, and all matrix components are still present in the sample, which can cause ion suppression or enhancement. Particulates can clog the LC column and contaminate the MS source.

Filtration of dilute and shoot samples can remove particulates from the sample. Samples can be passed through a syringe or vial filter or a filter plate for particulate removal prior to LC–MS analysis. These are usually 0.2 \( \mu m \) filters made of polytetrafluoroethylene (PTFE) or polyvinylidene fluoride (PVDF).

An inline filter is usually placed pre-column, and can capture any particulates that would otherwise build up in the LC system. These filters should be changed frequently, as retained particulates accumulate and can leach out due to increased backpressure. LC guard columns can be used before the analytical column to retain components and increase LC column lifetime.

Protein removal is used for blood or plasma samples. This can be done manually or in a 96-well plate or cartridge. A crash solvent (such as acetonitrile or methanol) is added to the sample for a manual crash. Samples are mixed and centrifuged, and the supernatant is collected. A protein removal plate or cartridge removes proteins and filters the sample. The crash solvent is usually added first, then the sample is added, mixed, and vacuum or positive pressure is applied to collect the crashed sample. The precipitated proteins are retained while the rest of the sample flows through. Protein removal, using manual, plate, or cartridge protocols, only removes proteins, which are not the only endogenous component that cause matrix effects or interferences when performing LC–MS/MS quantitation (4, 14).

Phospholipid depletion plates or cartridges are a simple pass-through SPE for plasma samples. This technique removes both proteins and phospholipids using a protocol similar to protein removal (add crash solvent, add sample, mix, and pass through sorbent). Precipitated proteins are retained by a filter, and phospholipids are retained by scavenger chemistry.

Dual mode extraction is a pass-through SPE technique that uses a hygroscopic salt layer and a scavenger chemistry to retain salts, urea, creatinine, and pigments from urine samples. Residual hydrolysis enzyme is also retained.

QuEChERS, a dispersive SPE, stands for “Quick, Easy, Cheap, Effective, Rugged, and Safe.” The extraction is done in a conical tube. Sample and acetonitrile are added to the tube. The tubes are then shaken, buffering salts are added, and the tube is centrifuged. The supernatant is transferred to a separate tube for evaporation, reconstitution, and analysis. This technique is popular for food and environmental analysis, but is sparingly used with biological samples. It is
A laborious, manual process that cannot be easily automated (15).

LLE involves adding a water-immiscible organic solvent to an aqueous sample. The two phases are mixed and separated, usually by centrifugation. Analytes of interest partition into the organic phase, while most matrix components remain in the aqueous phase. Analytes should be hydrophobic, with a log $P > 1$, and neutral for optimal results. This is a selective, cost-effective technique that yields clean extracts. However, emulsions may be present that make separating the layers difficult. Increased variability can result as it is difficult to collect the entire organic layer. This is also a labor-intensive technique (16). LLE can also be used to partition interfering materials into the organic layer, while the aqueous layer is collected for analysis.

SLE is analogous to LLE but with significant workflow advantages. Aqueous samples are loaded onto the sorbent (commonly refined, modified diatomaceous earth), and a water-immiscible solvent is added to elute the analytes of interest. The aqueous layer remains absorbed on the sorbent while the organic solvent flows through. No emulsions are present, and SLE can easily be automated. Like LLE, this technique works best when analytes have a log $P > 1$ and are neutral (17). The aqueous layer cannot be collected, as it remains absorbed on the SLE material.

Solid-Phase Extraction (SPE)

SPE methods are the most selective and yield the cleanest extracts. However, they are the most expensive and labor-intensive. A typical method includes conditioning and equilibrating the sorbent, usually with an organic solvent, followed by an aqueous buffer, to prepare the sorbent to receive the aqueous sample. Next, a pretreated sample is loaded, followed by wash steps. An aqueous wash removes water-soluble interferences. A second wash using an organic solvent or an aqueous-organic solvent solution (for example, 50:50 water:methanol or water:acetonitrile) can be added to remove more non-polar interferences. Controlling the pH and optimizing solvent strength ensures the analytes of interest remain bound to the sorbent while interferences are removed. Lastly, compounds are eluted from the sorbent, and the eluent is usually evaporated and reconstituted before analysis. Sorbents can be silica or polymer-based. Polystyrene-divinylbenzene (PSDVB) polymeric sorbents are stable across the entire pH range. Many manufacturers modify the hydrophobic polymer with hydrophilic functional groups to make it water-wettable, so the conditioning and equilibration steps can be eliminated.

Hydrophobic interaction (reversed-phase) SPE methods rely on the partitioning of the analytes between the hydrophobic sorbent and extraction solvent for retention and elution, similar to reversed-phase LC separations. The non-polar interactions are not as strong as ionic retention mechanisms, so the strength of any wash step containing an organic solvent must be carefully controlled, so that target analytes are not eluted during sample clean-up. Washes with only 5-10% organic solvent are common for more hydrophilic analytes. Stronger organic wash solutions can be used for more hydrophobic compounds. This technique can be used for non-ionized, hydrophobic analytes like steroids, or acidic and basic analytes at a pH where they are neutral. Very hydrophobic charged compounds may also be extracted.

Mixed-mode ion-exchange SPE products are reversed-phase sorbents modified with an ionic functional group. Analytes are retained by non-polar and ion-exchange interactions. Ion-exchange is a stronger retention mechanism than hydrophobic interaction, so a stronger organic wash can be used to produce cleaner extracts for ionized compounds. Strong cation- or anion-exchange sorbents have a strong acid or base (such as sulfonic acid or quaternary ammonium) as functional groups. These are charged over the entire pH range. These mixed-mode sorbents work well for weak bases or weak acids, respectively. Analytes of interest should be charged during sample load by pretreating the sample using the pH rule of 2 described previously. The charge is then “turned off” during elution. Retention and elution are controlled by modifying the pH of the sample. Weak cation- or anion-exchange chem-
Plate map to evaluate four different elution solvents using supported liquid extraction (SLE) to extract a weakly basic drug in plasma. Load and elution volumes are dependent upon SLE capacity. This experiment uses a 400 µL SLE+ plate.

**FIGURE 4:** Plate map to evaluate four different elution solvents using supported liquid extraction (SLE) to extract a weakly basic drug in plasma. Volumes are dependent upon sorbent bed mass. This experiment uses a 400 µL SLE+ plate.

The ultimate goal of most bioanalytical assays is the delivery of accurate and reliable quantitative results. While no two samples are exactly alike, optimized sample preparation procedures can minimize sample variability and produce cleaner extracts for more robust bioanalysis assays. Given the inherent variability with manual sample preparation procedures compounded by operator skill level, many laboratories have embraced automation and robotics to enhance laboratory productivity, reduce labor costs, and increase assay reliability.

Over the last twenty years, advancements in technology have resulted in the development of reliable automated instruments for the bioanalytical laboratory. Automation has quickly become the foundation of the modern laboratory, including highly customizable multifunctional liquid handlers to smaller task-focused products. Automated workflows translate to less manual intervention, higher reproducibility, and labor savings.

Robotic platforms with multifunctional, fully automated liquid handling abilities are prevalent in larger, high-throughput laboratories with heavy workloads. They are highly customizable for simple transfer steps, and may be programmed for entirely hands-free sample preparation procedures. Most are fitted with multichannel pipetting arms, which are adaptable to both individual test tube workflows and high-throughput assays in 96- or 384-well-plates. Table II describes some common automation platforms categorized by instrument, complexity, functionality, and vendor. The Fluent platform by Tecan and the Biomek i-Series systems from Beckman Coulter are some of the more complex yet versatile instruments on the market today. Integration with an end-user database or laboratory information management system (LIMS) allows these devices to provide added benefits beyond just sample handling. For example, analytical results can be linked to sample handling procedures, providing a clear, reliable chain of custody. This is important in regulated laboratories, or when bioanalytical testing bears legal implications.

Robotic automation platforms by instrument, complexity, functionality, and vendor. Fully automated systems provide high reproducibility and limited analyst intervention, but these conveniences do carry a considerable price tag. Besides the initial capital expense, these devices may require the use of vendor-specific consumables, specialized training to operate, and costly service contracts to maintain the investment.

Another practical concern with fully automated liquid handling systems is the use of vendor-specific consumables. While some automation platforms are customizably for simple transfer steps, and may be programmed for entirely hands-free sample preparation procedures. Most are fitted with multichannel pipetting arms, which are adaptable to both individual test tube workflows and high-throughput assays in 96- or 384-well-plates. Table II describes some common automation platforms categorized by instrument, complexity, functionality, and vendor. The Fluent platform by Tecan and the Biomek i-Series systems from Beckman Coulter are some of the more complex yet versatile instruments on the market today. Integration with an end-user database or laboratory information management system (LIMS) allows these devices to provide added benefits beyond just sample handling. For example, analytical results can be linked to sample handling procedures, providing a clear, reliable chain of custody. This is important in regulated laboratories, or when bioanalytical testing bears legal implications.

Table II lists common sample preparation automation platforms by instrument, complexity, functionality, and vendor.
Modern Trends for Sample Preparation in Bioanalysis

Bioanalytical assay development has evolved with the development of new chemistries and polymeric SPE sorbents. While traditional sample types and extraction methods exist, novel products and approaches are being introduced to streamline and enhance routine analysis. Some new products focus on handling complex chemical extractions, while others aim to simplify analyses using low specimen volumes. Bioanalytical assays are trending towards less invasive collection devices, and alternate matrices, like tissue extracts.

The most common and traditional technology for the clean-up of bioanalytical samples involves chemically modified silica products. Common issues with these products include channeling in the silica bed, premature drying, and moderate retention. In addition, bonded silica phases are often not stable at high and low pH, running the risk of hydrolysis of the bonded phase at low pH and dissolution of the silica backbone at high pH. The solution has been the introduction of mixed-mode polymer extraction chemistries. Most of these products are manufactured using a PSDVB backbone, which may be modified with various functional groups to facilitate ion-exchange and hydrophobic interactions. As a result, the retention mechanism is typically stronger than silica particles, and the polymer backbone is more resistant to channeling and premature drying during extraction procedures. This translates to simpler and easily automated extractions, capable of delivering rapid and cleaner eluates for analysis.

Limited specimen volume is a commonly encountered issue for many bioanalytical assays. For example, clinical neonatal tests for acylcarnitine may be supplied with as little as 50 to 100 µL of the sample, because collecting specimens from newborns is a delicate task (19). Additionally, some laboratories may need to perform several different types of analysis on a single sample, such as serum for steroids, xenobiotics, and electrolytes. In many cases, specimen...
### TABLE I: Solid phase extraction (SPE) products categorized by bonding chemistry, product, and vendor

<table>
<thead>
<tr>
<th>Technique</th>
<th>Chemistry</th>
<th>Biotage</th>
<th>TECAN</th>
<th>Phenomenex</th>
<th>UCT</th>
<th>Agilent</th>
<th>Waters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supported liquid extraction (SLE)</td>
<td>Diatomaceous earth</td>
<td>ISOLUTE SLE+</td>
<td></td>
<td>Novum (synthetic)</td>
<td>Strata DE</td>
<td>Chemo Elut</td>
<td>Chem Elut S (synthetic)</td>
</tr>
<tr>
<td>Hydrophobic interaction reversed-phase SPE</td>
<td>Polymeric reversed phase</td>
<td>EVOLUTE EXPRESS ABN</td>
<td>WWP2</td>
<td>&quot;Strata X Strata X Drug N&quot;</td>
<td>STYRE SCREEN C18</td>
<td>Bond Elut Plexa, Bond Elute PBA</td>
<td>Oasis HLB Oasis PRIME HLB</td>
</tr>
<tr>
<td>Mixed mode strong cation-exchange SPE</td>
<td>Polymeric mixed mode</td>
<td>EVOLUTE EXPRESS CX</td>
<td>PSCX</td>
<td>&quot;Strata X-C Strata X Drug B&quot;</td>
<td>STYRE SCREEN DBX/BCX</td>
<td>Bond Elut Plexa PCX Bond Elut Certify</td>
<td>Oasis MCX Oasis PRIME MCX</td>
</tr>
<tr>
<td>Mixed mode strong anion-exchange SPE</td>
<td>Polymeric mixed mode</td>
<td>EVOLUTE EXPRESS AX</td>
<td>PSAX</td>
<td>Strata X-A</td>
<td>STYRE SCREEN QAX</td>
<td>Bond Elut Plexa PAX Bond Elut Certify II</td>
<td>Oasis MAX</td>
</tr>
<tr>
<td>Mixed mode weak cation-exchange SPE</td>
<td>Polymeric mixed mode</td>
<td>EVOLUTE EXPRESS WCX</td>
<td>Strata X-CW</td>
<td>STYRE SCREEN CCX</td>
<td>Bond Elut CBA</td>
<td>Oasis WCX</td>
<td></td>
</tr>
<tr>
<td>Mixed mode weak anion-exchange SPE</td>
<td>Polymeric mixed mode</td>
<td>EVOLUTE EXPRESS WAX</td>
<td>Strata X-AW</td>
<td>STYRE SCREEN THC</td>
<td>Bond Elut DEA</td>
<td>Oasis WAX</td>
<td></td>
</tr>
<tr>
<td>Hydrophobic interaction reversed-phase SPE</td>
<td>Silica reversed phase</td>
<td>ISOLUTE C18</td>
<td>TRACE-N</td>
<td>Clean-Up C18</td>
<td></td>
<td></td>
<td>Sep-Pak C18</td>
</tr>
<tr>
<td>Mixed mode strong cation-exchange SPE</td>
<td>Silica mixed mode</td>
<td>ISOLUTE HCX/SCX</td>
<td></td>
<td>Clean-Up Benzene-sulfonic Acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed mode strong anion-exchange SPE</td>
<td>silica mixed mode</td>
<td>ISOLUTE HAX/SAX</td>
<td></td>
<td>Clean-Up Amino-propyl</td>
<td></td>
<td></td>
<td>Accell Plus QMA</td>
</tr>
<tr>
<td>Mixed mode weak cation-exchange SPE</td>
<td>Silica mixed mode</td>
<td>ISOLUTE CBA</td>
<td></td>
<td>Clean-Up Diethyl-amino</td>
<td></td>
<td></td>
<td>Accell Plus CM</td>
</tr>
<tr>
<td>Mixed mode weak anion-exchange SPE</td>
<td>Silica mixed mode</td>
<td>ISOLUTE PSA</td>
<td></td>
<td>Clean-Up Quaternary Amine</td>
<td></td>
<td></td>
<td>Sep-Pak PSA</td>
</tr>
<tr>
<td>Hydrophobic interaction SPE in-well hydrolysis</td>
<td>Polymeric reversed phase</td>
<td>EVOLUTE HYDRO ABN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed mode strong cation-exchange SPE</td>
<td>Polymeric mixed mode</td>
<td>EVOLUTE HYDRO CX</td>
<td>Strata X Drug B</td>
<td>&quot;XtractT DAU (CB)&quot;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microelution SPE mixed mode strong cation-exchange</td>
<td>Polymeric</td>
<td>MIKRO CX</td>
<td>NBE</td>
<td>&quot;Strata-X-C µ-Elution&quot;</td>
<td></td>
<td></td>
<td>Oasis PRIME MCX µElution</td>
</tr>
<tr>
<td>Sample filtration</td>
<td>PP and/or PVFD filters</td>
<td>ISOLUTE Filter+</td>
<td>0.45 um polypropylene filter plate</td>
<td>Refine Ultra-Filtration</td>
<td>CAPTIVA 96-well filter plate, Mini-UniPrep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine clean up</td>
<td>Pass thru SPE</td>
<td>ISOLUTE HYDRO DME+</td>
<td></td>
<td>β-Gone</td>
<td>Clean Screen FAS</td>
<td>CAPTIVA ND</td>
<td></td>
</tr>
<tr>
<td>Protein precipitation</td>
<td>Pass thru SPE</td>
<td>ISOLUTE PPT+</td>
<td>IMPACT</td>
<td></td>
<td></td>
<td>CAPTIVA ND</td>
<td></td>
</tr>
<tr>
<td>Phospholipid depletion/protein removal</td>
<td>Pass thru SPE</td>
<td>ISOLUTE PLD+</td>
<td>PHREE</td>
<td></td>
<td></td>
<td>CAPTIVA ND LIPID, CAPTIVA EMR LIPID Ostro Pass-Through</td>
<td></td>
</tr>
</tbody>
</table>
One trending solution to these problems is the use of micro-elution SPE products. The first option is loosely packed sorbents with selective chemistries fitted into narrow but hollow plastic tips. The typical bed volume is 0.5 to 5 mg. These are then fit
ted into a 96-well-plate for high throughput workflows. The resulting extraction protocol requires lower specimen volume and reduced solvent use, typically 50–100 µL for washes, and 30–50 µL for elution. Eluates can be concentrated for increased sensitivity or diluted, eliminating the evaporation step.

A second micro-elution format uses a dispersive pipet extraction (DPX), a technology approach consisting of loosely packed SPE resins inside individual pipet tips. These tips may be fitted to manual pipets or fully automated liquid handlers. The extraction process involves introducing samples, retaining analytes, and removing interferences by multiple aspirate-and-dispense steps to utilize a dispersive SPE technique. Compounds of interest are eluted with conventional solvents.

**TABLE II: Common automated sample preparation platforms categorized by instrument, complexity, functionality and vendor**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Complexity</th>
<th>Primary Functionality</th>
<th>Full Automation</th>
<th>Associated Consumables</th>
<th>Vendor</th>
<th>Additional Key Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomek Automated Liquid Handlers (4000, i5,i7)</td>
<td>High</td>
<td>Liquid handling, sample transfers</td>
<td>Yes</td>
<td>Tips, reagent reservoirs, sample trays</td>
<td>Beckman</td>
<td>Variable-channel liquid handler heads, independent arms</td>
</tr>
<tr>
<td>Fluent</td>
<td>Very High</td>
<td>Liquid Handling, complete sample processing</td>
<td>Yes</td>
<td>Tips, reservoirs, plates</td>
<td>Tecan</td>
<td>Complete laboratory automation, high-throughput start to finish</td>
</tr>
<tr>
<td>Freedom EVO</td>
<td>High</td>
<td>Liquid handling, sample preparation</td>
<td>Yes</td>
<td>Tips, plates, extraction media, reservoirs</td>
<td>Tecan</td>
<td>Highly customizable, delivered as “Packaged” Workflow Solutions</td>
</tr>
<tr>
<td>Microlab (VANTAGE, STAR)</td>
<td>High</td>
<td>Liquid handling, sample transfers</td>
<td>Yes</td>
<td>Tips, reagent reservoirs, extraction media</td>
<td>Hamilton</td>
<td>Method optimization, sample storage, integration of other devices</td>
</tr>
<tr>
<td>Microlab Nimbus</td>
<td>Moderate</td>
<td>Sample transfers, sample processing</td>
<td>Yes</td>
<td>Tips, extraction media, reservoirs</td>
<td>Hamilton</td>
<td>Small footprint, 8-channel head for high throughput</td>
</tr>
<tr>
<td>Resolvex (A100, A200)</td>
<td>Moderate</td>
<td>Sample preparation</td>
<td>Yes</td>
<td>Extraction media, reservoirs, seals</td>
<td>Tecan</td>
<td>Dispensing lines, touchscreen operation, small footprint</td>
</tr>
<tr>
<td>Exutraera</td>
<td>Moderate</td>
<td>Sample preparation</td>
<td>Yes</td>
<td>Tips, extraction media, reservoirs</td>
<td>Biotage</td>
<td>Small footprint, touchscreen programming, variety of sample prep workflows</td>
</tr>
<tr>
<td>Microlab Prep</td>
<td>Moderate</td>
<td>Sample preparation</td>
<td>Yes</td>
<td>Tips, extraction media, reservoirs</td>
<td>Hamilton</td>
<td>Small footprint, high customization, two independent channels</td>
</tr>
<tr>
<td>Resolvex (M10)</td>
<td>Low</td>
<td>Sample preparation</td>
<td>No</td>
<td>Manifold sealing mats</td>
<td>Tecan</td>
<td>Positive-pressure manifold, cartridge or 96-well compatible</td>
</tr>
<tr>
<td>[MPE]²</td>
<td>Low</td>
<td>Sample preparation</td>
<td>Yes, offline programming</td>
<td>Dispenser lines</td>
<td>Hamilton</td>
<td>Positive-pressure and evaporation, integration to other Hamilton products or stand alone device</td>
</tr>
<tr>
<td>Positive Pressure Manifold 2.0</td>
<td>Low</td>
<td>Sample preparation</td>
<td>No</td>
<td>Manifold sealing mats</td>
<td>UCT</td>
<td>Manual device, easy to operate, 48-sample capacity only</td>
</tr>
<tr>
<td>PPM+ (48 and 96)</td>
<td>Low</td>
<td>Sample preparation</td>
<td>No</td>
<td>Manifold sealing mats</td>
<td>Biotage</td>
<td>Manual devices, simple operation, cartridge or plate compatibility</td>
</tr>
<tr>
<td>SPEVAP Solvent Evaporator</td>
<td>Low</td>
<td>Evaporation/concentration</td>
<td>No, programmable</td>
<td>Sealing mats, various collection racks</td>
<td>UCT</td>
<td>Touchscreen, small footprint, PTFE nozzles available, 48 samples only</td>
</tr>
<tr>
<td>TurboVap LV</td>
<td>Low</td>
<td>Evaporation/concentration</td>
<td>No, programmable</td>
<td>Various collection racks, manifolds</td>
<td>Biotage</td>
<td>Touchscreen, small footprint, adaptable to low and high volume applications</td>
</tr>
<tr>
<td>SPE Dry</td>
<td>Low</td>
<td>Evaporation/Concentration</td>
<td>No</td>
<td>Needle heads</td>
<td>Biotage</td>
<td>Dual 96-well capability, temperature setting, compatible with anti-cross talk (ACT) plate</td>
</tr>
</tbody>
</table>
This approach is highly adaptable and yields reasonable recoveries (20), yet samples with higher viscosities, such as blood and tissue, may prove challenging to manage with dispersive pipet extractions.

Solid-phase microextraction (SPME) uses a fiber-coated frit incorporating an SPE extraction phase with an affinity for the analytes of interest. Here, exposing the fiber to liquidified samples results in a partitioning of analytes from the sample matrix to the extraction phase. The extraction requires no solvent, and analytes are concentrated on the fiber. The fiber can be placed in a small amount of solvent for desorption and LC–MS analysis. This technology is ideal for gaseous, liquid, and solid samples stemming from field-based applications, such as environmental studies. Applications of SPME in high-throughput bioanalysis with moderate recovery and reproducible data have been published (21).

In addition to novel chemistries and products, the use of alternate matrices and collection devices have altered approaches to bioanalytical method development. One highly intriguing approach involves bloodspot analysis. Small drops of blood may be collected onto thin, paper-like sampling devices with a mere prick of the finger. Once at the laboratory, these blood spots may be subjected to further chemical extractions, digestions, or washes targeting the analysis of numerous xenobiotics or endogenous biomolecules. Volumetric absorptive microsampling (VAMS) is a novel approach that permits collecting a fixed volume of whole blood using a sampling device with a porous hydrophilic tip to collect small, accurate, and precise blood volumes. The blood in the device is dried for storage or transport. These samples can be extracted with an appropriate solvent, which can undergo further sample clean-up by LLE, SLE, or SPE (22).

The advancement of technology improves the quality of bioanalytical analysis while reshaping applications by introducing new products and novel sampling techniques. Choosing an automated platform is no simple task. Throughput needs, budgetary restrictions, and LIMS compatibility are the primary considerations. Regardless of the workflow design, an automated or manual solution is available for sample preparation to ensure accurate and reproducible results.

### Method Development Case Study for a Weakly Basic Drug in Plasma

Sample preparation method development begins by researching analytic properties. Our hypothetical drug is a weak base with a primary amine. A deuterated internal standard (IS) is available. Using calculated values from Chemicalize.com, the molecule has a log P of 2.1 and a pKₐ of 8.2. The desired LOQ is 10 ng/mL, and the desired plasma volume is 200 μL. Once our LC–MS/MS method is optimized and sensitivity is established, we can begin our extraction experiments.

Based on the matrix and analytic properties, sample preparation options are phospholipid depletion/protein removal (because the matrix is plasma), LLE or SLE (since the analyte is hydrophobic), and mixed-mode strong cation-exchange SPE (as the analyte is a weak base). Sample preparation method development should not be done at the LOD or LOQ or using a single data point. Instead, spiked samples should be prepared and extracted in duplicate or triplicate at a single concentration 5 to 10 times the LOQ, in this case, 50 to 100 ng/mL. Include an extraction blank (EB, reagents with no specimen) and negative control (NC, analyte-free matrix with only IS added). Averaged area counts from triplicate extracts are compared to a no matrix, “unextracted” standard (UNX, analyte, and internal standard in reconstitution solvent) at the same concentration as the extracted sample to calculate the process efficiency. Once the best sample preparation method is determined, linearity, external QC, positive and negative specimens, recovery, matrix effect, and process efficiency (23) are evaluated using the final, optimized method conditions.

Phospholipid depletion involves minimal method development. Different ratios of the specimen to crash solvent can be scouted. Some products recommend the addition of an acid to increase the recovery of more polar analytes. Acetonitrile usually provides cleaner extracts than methanol, but either solvent can be used. Figure 3 shows an example of a 96-well plate (a “plate map”) configured to evaluate different conditions for phospholipid depletion using acetonitrile. Variables are three different crash solvent to plasma ratios (3:1, 4:1, and 6:1) and the addition of 1% formic acid to the crash solvent. Samples are extracted and analyzed in triplicate at 50 ng/mL (5x the desired LOQ) with an EB, NC, and UNX.

LLE or SLE works best if the analyte is neutral. The primary amine would be neutral 2 units above its pKₐ (pH 10.2 or higher). Pretreating plasma 1:1 with 0.5 M ammonium hydroxide will neutralize the primary amine for partitioning into the water-immiscible solvent. Figure 4 shows a plate map to scout four different elution solvents using SLE. Spiked samples at 50 ng/mL are analyzed in triplicate with an EB, NC, and UNX.

The analyte must be positively charged for polymeric mixed-mode strong cation-exchange SPE. The weakly basic drug would be charged at a pH <6.2. Pretreating plasma 1:1 with 0.1 M pH 5 ammonium acetate buffer or 2% formic acid will accomplish this. However, 2% formic acid will also disrupt plasma proteins if protein binding is a concern.

Figure 5 shows a plate map for triplicate samples pretreated with 2% formic acid. Our analyte should be retained by cation exchange which means a 100% methanol or acetonitrile wash will provide the best clean-up. In this case, we will scout a 50% methanol and a 100% methanol wash and three different elution solvents: 88:10:2 dichloromethane:2-propanol:ammonium hydroxide (DCM:IPA:NH₄OH), 98:2 methanol:NH₄OH, or 98:2 acetonitrile:NH₄OH.

The load and wash steps are collected to analyze for troubleshooting if low or no recovery of the target analyte is observed. If the analyte is in the load step, it was never retained. If the analyte is in a wash step, the wash was too aggressive and became an elution solvent. If it is not in the load, wash, or elution fractions, it was retained on the sorbent and never eluted, possibly decomposed, evaporated or was “stuck” to the elution vial or collection plate due to non-specific binding.

Elution can be done with a single fraction of solvent or two smaller aliquots. Improved recovery and better precision are usually seen with two fractions of elution solvent (2 x 0.5 mL compared to a single application (1 mL)). Scouting sample preparation options using plate maps can generate simple exper-
ments that investigate the effects of a few variables, or use more of the plate to evaluate more options. The plate map in Figure 4, for example, could be modified to evaluate the ammonium acetate pretreatment, an additional wash (like acetonitrile in addition to methanol), or additional elution solvents.

Process efficiencies for each sample preparation option are reviewed, and the best option is selected for further development. Calibrators, patient specimens, casework samples, proficiency testing samples, or other external QC are analyzed, and then recovery, matrix effect, and process efficiency are determined prior to full method validation (23).

Developing a new extraction method can seem daunting at first. However, knowing about the compounds in the panel, the matrix, the specifics about the extraction technique, and the use of plate maps can make the method development process faster and more efficient.

Summary and Conclusions

This article provided an overview of sample preparation fundamentals for the bioanalysis of physiological samples for the novice and updated guidance in selecting media and the best operating practices for the experienced practitioner. Modern trends summarizing newer SPE media, approaches, and automation platforms were discussed. A case study for sample preparation method development for a weak base in plasma and the optimization process demonstrated the recommended procedures for using various sample preparation approaches.

Acknowledgments

The authors would like to thank the following colleagues for the timely review of the manuscript to improve its accuracy and clarity: Stephen Merrigan, MS, R&D Scientist, ARUP Institute for Clinical and Experimental Pathology, Doug Raynie, Ph.D., Department Head, and Associate Professor, South Dakota State University, Jinlan Dong, Ph.D., Bioanalytical Research Scientist, Corteva Agriscience, Naidong Weng, Ph.D., Scientific Director and Head of Bioanalytical and Pharmacokinetics US East Coast, Discovery Sciences, Janssen and Johnson and Johnson, and Perry Weng, Ph.D., Research Chemist, USFDA.

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ABOUT THE COLUMN EDITOR

Michael W. Dong is a principal of MWD Consulting, which provides training and consulting services in HPLC and UHPLC, method improvement, pharmaceutical analysis, and drug quality. He was formerly a Senior Scientist at Genentech, a Research Fellow at Purdue Pharma, and a Senior Staff Scientist at Applied Biosystems/PerkinElmer. He holds a PhD in Analytical Chemistry from City University of New York. He has more than 130 publications and a best-selling book in chromatography. He is an editorial advisory board member of LCGC North America and the Chinese American Chromatography Association. Direct correspondence to: LCGCedit@mmhgroup.com.

ABOUT THE AUTHORS

Stephanie J Marin is a Senior Applications Chemist supporting the Analytical Life Sciences markets at Biotage in Salt Lake City, UT. She received her PhD in chemistry from Arizona State University and has expertise in sample preparation, LC-MS, and the development and validation of clinical assays. She is the author of over 30 peer-reviewed publications and book chapters.

Jeremy Smith is an Applications Chemist supporting the Analytical Life Sciences markets at Biotage in Charlotte, NC. He holds a BS in Biology from the University of Kentucky.

Jillian Neifeld is an applications chemist at Biotage in Fairfield, CA. She has a MS from Virginia Commonwealth University.

Elena Gairloch is a Science Support Manager at Biotage in Bellefonte, PA. She has a BS in Chemistry and Biology from Lynchburg College and has worked in the HPLC/Sample Prep industry for over 30 years, innovating in the Separation Sciences.
Industry Insights: Chromatography

Ensuring Your Pharmaceutical Processes are GMP Compliant

The regulation of product quality is an extremely important topic in the pharmaceutical industry. Pharmaceutical manufacturers must ensure that all products made for human consumption are produced to rigorously high standards, ensuring the formulation is consistent and free from any other, potentially harmful, compounds that could be introduced during the manufacturing process.

Good Manufacturing Practices (GMPs) are the regulations used to ensure pharmaceutical quality. GMP is considered when designing, monitoring, implementing, and controlling pharmaceutical purification and manufacturing processes. The regulations are extremely important because failures in adhering to them can result in contaminated medicines and, ultimately, human harm.

A GMP-controlled production process ensures high product quality and reduces the risk of harm toward consumers or the general public. The US Food and Drug Administration (FDA) is one of the world’s leading pharmaceuticals regulatory bodies, with the main regulatory standard for pharmaceutical quality being the Current Good Manufacturing Practices (CGMPs).

As suppliers of chromatographic systems for mRNA purification or continuous chromatography as well as systems for downstream processing in the biopharmaceutical industry, such as skids for lipid nanoparticle formulation, KNAUER is well-versed in GMP requirements. We provide a wide range of services to our customers to support them and to help ensure that their pharmaceutical purification and production processes adhere to GMP regulations.

Support for Manufacturers in Reaching GMP Compliance

Pharmaceutical manufacturers have an important responsibility to implement GMP-compliant pharmaceutical production. KNAUER’s customers often come to us with requirements relating to their specific manufacturing process that are usually defined in their User Requirement Specifications (URS). The URS defines the product specifications as well as all other topics relating to GMP compliance, such as product safety, the correct training of personnel, and quality control.

KNAUER welcomes every opportunity to share our expertise and resources with our customers in the pharmaceutical industry.

KNAUER’s GMP Services are based on our hardware and software solutions. Our services cover three key areas of GMP compliance: product safety, quality control, and personnel training. The remaining key element of GMP, risk management, is managed by our customers.

What does this mean in practice? Let’s look at KNAUER’s GMP services in closer detail:

Product safety:
• Documentation on product conformity
• Documentation on the compliance of materials used for wetted parts
  ○ Care must be taken to ensure that potentially harmful substances are not introduced to liquid products during the production process. This covers liquids for use in clinical, cosmetic, or food applications. Therefore, any materials used to construct the liquid flow path that come into contact with the final product must meet certain criteria.
  ○ According to our end user’s requirements, KNAUER can provide compliance with the order (EN 10204-2.1), certificates of compliance on the materials used for wetted parts, and further documentation from the supplier such as 2.1 certificates.

Quality control:
• Installation and operation qualification, performance verification
  ○ Factory acceptance test (FAT) at the KNAUER facility
  ○ A FAT is a functional test performed upon completion of the manufacturing process to prove that the equipment has the same specification and functionality as indicated in the datasheet, specification, and purchase order.
  ○ KNAUER is experienced in establishing such test procedures together with our customers before the delivery of our equipment.
• A test carried out on reception of the equipment at your site (known as a site acceptance test [SAT])
Here, a KNAUER technician visits your site to ensure that your new KNAUER system works to your utmost satisfaction.

We can also integrate the system into our customer’s existing production environment when required.

**Personnel training:**
- Software training and product training
- Service and maintenance training

**Engineering Support at KNAUER is Always Available**

At KNAUER, we love to work alongside our partners in biopharma to design the right system solution for their needs. In most instances our customers already know their process well and can provide us with the required specification for their desired system.

KNAUER provides engineering services to develop the tools that match the needs and requirements of our end users.

**KNAUER’s Impingement Jets Mixing Skid Technology—From Pharmaceutical R&D to Manufacturing**

KNAUER’s impingement mixing skids can be used in the production of liquid nanoparticles (LNP) for the encapsulation of an Active Pharmaceutical Ingredient (API). The number of parallel units, pumps, flow meters, jet mixers, and pipes used to construct a skid is dependent on the application for which it will be used for. All KNAUER skids are built into a stainless-steel frame that is suitable for the clean-in-place procedures required in pharmaceutical production environments.

The Small Production Scale Unit Impingement Jets Mixing (IJM) skid contains two parallel mixing units, with each unit containing four pumps. Here, a concentrated solution of the API is first diluted into an aqueous phase and then mixed via jet mixing with the organic phase, which results in API encapsulation. The mixture is then quenched in a second mixer to stop further growth of LNPs.

The Large Production Scale Unit IJM skid can be configured with up to eight parallel mixing units depending on the customer requirements. Each unit consists of two pumps to deliver lipid and API streams, two flow meters for flow control, and one jet mixer. Depending on the configuration, the predilution of the API and quenching can be performed for the combined flow of all units in one process step that takes place outside of the IJM skid.
Development and Validation of Novel Gas Chromatography (GC) and Thin Layer Chromatography (TLC) Densitometry Methods for the Quantification of Stigmasterol 3-O-β-D-Glucopyranoside (S3G) in Balanites Aegyptiaca Extract: Application to Newly Formulated Balanites Capsules

Balanites aegyptiaca is a commonly used antihyperglycemic in Egyptian folk medicine. The objective of the present study is to develop two chromatographic methods for standardizing the Balanites extract by quantification of its main component, stigmasterol 3-O-β-D-glucopyranoside (S3G). The first method is gas chromatography (GC) where nitrogen was adopted as the carrier gas. The second method was thin layer chromatography (TLC) densitometry where chromatographic separation was established on aluminum plates using chloroform; methanol was used as the mobile phase followed by a densitometric measurement at 254 nm. Validation of the proposed methods was applied per International Council for Harmonization (ICH) guidelines. Both methods were adopted for quantification of S3G in Balanites capsules. The standard calibration curve was established for S3G in the concentration range of 10–130 μg/mL and 0.5–7.5 μg/mL for the GC and TLC methods, respectively. A good linearity with a correlation coefficient of 0.999 was obtained for both methods. Furthermore, the mean percentage recovery was found to be 99.46% for GC and 100.67% for TLC. Good precision was achieved, with relative standard deviation values <1%, and finally, the limits of detection and quantification were 2.51 and 7.59 μg/mL for GC and 0.13 and 0.40 μg/mL for TLC, respectively.

Sara A.A. Elbahrawy, Amira Abdel Motaal, and Ola M. Abdallah

Experimental Instrumentation
An Agilent 7890A series GC apparatus (Agilent Technologies) connected with an autosampler was used in this experiment. The output signal was monitored and processed using Chemstation software version B.04.03. An Agilent HP-5 (30 m length × 0.32 mm i.d., 0.25-μm film thickness) column and flame ionization detector (FID) were used. A Camag TLC scanner with winCATS computer software was used in the densitometric method. The TLC plates were pre-coated with silica gel 60 F254 (20 × 20 cm), and a Hamilton 100-μL microsyringe, an ultraviolet (UV) lamp with a short wavelength of 254 nm, and a chromatographic tank (25 cm × 25 cm × 9 cm) were also used.
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TABLE I: Validation and regression parameters of the GC and TLC methods for the determination of S3G

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TLC</th>
<th>GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (μg/mL)</td>
<td>0.5–7.5</td>
<td>10–130</td>
</tr>
<tr>
<td>LOD (μg/mL)</td>
<td>0.13</td>
<td>2.51</td>
</tr>
<tr>
<td>LOQ (μg/mL)</td>
<td>0.40</td>
<td>7.59</td>
</tr>
<tr>
<td>Accuracy (mean ± %RSD)</td>
<td>100.67 ± 0.95</td>
<td>99.46 ± 0.81</td>
</tr>
</tbody>
</table>

**Precision:**
- Repeatability (%RSD): 1.23 ± 0.85
- Intermediate precision (%RSD): 1.32 ± 0.72

**Regression Parameters**
- Slope ± SD (y) | 0.0061 ± 0.000048
- Intercept ± SD (y) | 0.2151 ± 0.003
- Correlation coefficient (r) | 0.999

**Materials and Reagents**

**Pure Samples**
Pure S3G was kindly supplied by the Pharmacognosy Department at Cairo University, and the S3G was previously isolated from *Balanites aegyptiaca* extract with 99.9% purity, assessed by a high performance liquid chromatography (HPLC) method.

**Extracts and Market Samples**
A *Balanites* dry extract, in addition to the hard gelatin *Balanites* capsules (Batch No. Bal0009), were manufactured by Atos for production of medicinal herbs. Each capsule was labeled to contain 400 mg of the *Balanites* extract (standardized to contain 8.63% of S3G).

**Chemicals**
For the GC method, chromatographic HPLC grade methanol and acetonitrile (Fischer Scientific) and analytical grade chloroform (Honeywell) were purchased from the local market. An Agilent PTFE 0.45 μm syringe filter was also used.

For the TLC method, analytical grade methanol and ethanol (Adwic), chloroform (Honeywell), sulfuric acid (Sigma Aldrich), and vanillin powder (El-Nasr) were purchased from the local market. Pure S3G was kindly supplied by the Pharmacognosy Department at Cairo University.

A vanillin-sulfuric acid spraying reagent was prepared from 1% vanillin in ethanol (solution I) and 10% sulfuric acid in ethanol (solution II) (12). The plate was sprayed with 10 mL of solution I, followed immediately by spraying it with 10 mL of solution II. After heating the plate at 110 °C for 5–10 min under observation, it was evaluated in visible light or daylight.

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**Chemical structure of stigmasterol 3-O-β-D-glucopyranoside (stigmast-5, 22-dien-3-O-β-D-glucopyranoside).**

**Gas Chromatographic Quantization of S3G**

**Chromatographic Conditions**
The column oven was programmed as follows: The initial column oven temperature was 600 °C and increased to 2800 °C at the rate of 500 °C/min. The injector and detector temperature was kept at 2500 °C and 3000 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 3.35 mL/min and a split ratio at 1:10. The injection volume was 2.0 μL. Methanol was used as the syringe cleaning solvent between injections.

**Construction of Calibration Curve**
Aliquots of stock solutions of the standard drug (1.0 mg/mL) equivalent to 10.0–130.0 μg/mL of S3G were introduced into a series of 10-mL volumetric flasks. The volume was adjusted using 20% methanol in chloroform, and 2 μL were injected in triplicate for each concentration then chromatographed under the specified chromatographic conditions described previously. The calibration graph of S3G was obtained by plotting the peak area of each concentration against the corresponding concentration.

**Application to the Pharmaceutical Dosage Form**
The contents of 10 *Balanites* capsules were mixed well. Then, an aliquot of 593 mg was accurately weighed, dissolved in 100 mL of methanol-chloroform mixture in a ratio of 20:80 by volume, and sonicated for 30 min, after which 1 mL from the stock solution was transferred into a 10 mL volumetric flask then the volume was adjusted with the same solvent. The obtained solution was filtered via the syringe filter, and then injected following the specified chromatographic conditions previously described. Three replicate determinations were then made; the corresponding drug concentrations were calculated from the appropriate regression parameters.

The recovery of the procedure was assessed by applying the standard addition technique and the mean percentage recovery (% RSD) was calculated.

**Densitometry Quantization of S3G**

**Chromatographic Conditions**
Analysis was performed on precoated 20 × 20 cm TLC aluminum silica gel 60 F254 plates.
Samples were applied to the plates using a Hamilton micro syringe (100 µL). The plates were placed 2 cm apart from each other and 2 cm apart from the bottom edge. The chromatographic tank was presaturated with the mobile phase for 15 min before developing by ascending chromatography using chloroform with methanol (50:50, by volume) as a mobile phase. The plates were then air-dried and sprayed with the vanillin-sulfuric spraying reagent before being heated in the oven at 1100 °C for 5 min. Finally, once detected at 565 nm, all plates were scanned under the following conditions:

- Silt dimensions: 6.0 × 0.3 µm
- Wavelength: 565 nm after spraying
- Scanning speed: 60 mm/s
- Data resolution: 100 nm/step
- Measurement mode: absorption
- Result output: chromatogram and integrated peak area.

Construction of Calibration Curve
Aliquots of drug stock solutions (1.0 mg/mL) equivalent to 0.5–7.5 µg of S3G were introduced into a series of 10 mL volumetric flasks and the volume was adjusted using acetonitrile. Then, 20 µL of each solution were applied to the TLC plates following the specified chromatographic conditions described previously.

Calibration graph of S3G was constructed by plotting the peak area against the corresponding drug concentration in µg/mL.

The Application to the Pharmaceutical Dosage Form
The contents of 10 hard gelatin Balanites capsules were mixed well, and an aliquot of 100 mg of the mixed sample was accurately weighed before being dissolved in 100 mL of 20% methanol in chloroform; this stock solution was sonicated for 30 min. Next, 1 mL of the stock solution was transferred into a 25 mL volumetric flask and completed to volume with acetonitrile This solution was filtered via syringe filter polytetrafluoroethylene (PTFE) 0.45 µm, and the obtained solution was analyzed following the specified chromatographic conditions described previously.

Three replicate determinations were made for each sample. The corresponding drug concentrations were calculated from the appropriate regression model parameters.

The recovery of the procedure was assessed by applying the standard addition technique and the mean percentage recovery (±%RSD) of the added solution was calculated.

Results and Discussion
Method Development and Optimization
In the present study, a simple, rapid, and accurate GC method in addition to a sensitive and validated TLC method were developed for the determination of S3G.

The literature revealed that there is actually no validated GC or densitometric method concerning the estimation of S3G or any other quantification methods. Only a structure elucidation spectroscopic method has been reported.

The described methods in the study could be successfully applied for the analysis of S3G in crude plant sample and
The proposed methods required minimal sample preparation and an uncomplicated mobile phase. For the GC method, during the method development, different chromatographic parameters were optimized to obtain an acceptable peak shape and resolution between the peak of solvent and peak of drug with acceptable recoveries to satisfy the GC system suitability.

By trying different flow rates (1–5 mL/min, at constant pressure) it was found that a flow rate less than 3 mL/min causes significant observed peak tailing and that more than 4 mL/min leads to bad resolution between the peaks of the solvent and drug, so a flow rate of 3.35 mL/min was determined to be the best choice.

The split ratio factor was also studied, and it was observed that a high split ratio leads to poor sensitivity, so variable ratios were tried. A 1:10 ratio was selected because it gave good sensitivity and acceptable peak shape.

One of the most important studied factors in this research was the change in column oven heating rate because it was found that low heating rates lead to a peak tailing problem and higher rates (more than 500 °C/min) lead to bad resolution between the peaks of solvent and drug. After studying different heating rates, it was found that 500 °C/min was the best choice.

Satisfactory selectivity, sensitivity, resolution, and speed of chromatographic separations with stable baselines were only achieved on a HP-5 column (30 m length × 0.32 mm i.d., 0.25 µm film thickness) using nitrogen as a carrier gas before following the procedure mentioned previously. Applied analysis time was 3 min, as shown in Figures 2 and 3.

For the TLC method, different mobile phases in different ratios were tried, namely hexane:ethyl acetate in a 83:17 ratio, hexane:acetone in a 3.1 ratio (13,14), chloroform:ethanol in a 9.8:0.2 ratio (15), and chloroform:methanol in a 8:0.6 ratio (16).

The best peak shape of S3G was obtained upon using a mobile phase consisting of chloroform:methanol (50:50 by volume).

Different scanning wavelengths were tried, including 205, 254, 274, 365, and 565 nm. It was found that the most suitable detection wavelength for S3G was 565 nm after spraying and the spots appeared at Rf 0.78 (Figure 4).

### Method Validation

The developed method was validated according to the guidelines of the International Council for Harmonization (ICH) for validation of analytical procedures (17).

### Linearity and Range

Under the optimized conditions described previously, the analytical curve was obtained by plotting the peak area and the corresponding S3G concentrations over the range of 10–130 µg/mL for GC and 0.5–7.5 µg/mL for TLC. It showed a significant linear relationship and yielded correlation coefficient, r, of 0.999; the regression parameters were computed and presented in Table I.

### Limits of Detection (LOD) and Limits of Quantification (LOQ)

The limits of detection (LOD) and limits of quantification (LOQ) were determined based on the standard deviation of the response (σ) and the slope of the calibration curve (S) according to the ICH guidelines, as follows: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S). The LOD and LOQ for GC and TLC were 2.51 and 7.59 µg/mL for the GC method and 0.13 and 0.40 µg/mL for TLC method, respectively, as shown in Table I.
Accuracy and Precision
The accuracy and precision of the procedure was determined using three different concentrations of pure samples of the drug covering the specified range, each in triplicate, within one day for intraday analysis and three days for interday analysis.

To determine the intra- and interday precision of the method, S3G was assayed at three different concentrations: (20, 60, and 120 μg/mL) for GC and (1.5, 3.5, and 6.5 μg/mL) for TLC, respectively. The experiment was performed using nine replicates (n = 9) for intra-day and on three separate days in triplicates (n = 9) for inter-day. The %RSD was taken as a measure of precision, and the accuracy of the method was expressed as R%. The results were summarized in Table I.

Repeatability intraday precision of the proposed method for S3G was found to be reliable based on %RSD (<2%).

Intermediate precision (interday precision) was demonstrated on three successive days by two analysts. The %RSD values were less than 2%, confirming that the method is sufficiently precise, as shown in Table I.

Selectivity
The selectivity was assured by studying the possibility of interference of the inactive ingredients in Balanites capsules (placebo) with S3G standard by GC and TLC method. The obtained chromatograms of standard S3G and placebo did not show any peak interference which revealed high selectivity of the proposed method.

Robustness
For the GC method, it was evaluated by studying the effect of changing the injector and detector temperature by ±2 °C. It was found that these deliberate variations did not affect the system suitability parameters, confirming robustness of the method and the %RSD found to be <2%, which was also in favor of the developed GC method, as shown in Table II.

For the TLC method, it was evaluated by studying the influence of small variations in the mobile phase ratios (49:51 mL by volume) then observing Rf values upon introduction of these deliberate variations in chloroform and methanol volume.

It was found that these variations cause no significant difference in Rf values. Also, neither tailing of the separated bands or interference were observed, confirming robustness of the method, and the %RSD was found to be 1.41 and 1.53 (<2%) which was also in favor of the developed TLC method.

Stability of Standard Solutions
The stability of the S3G solution in 80% chloroform and 20% methanol was evaluated by analyzing two different solutions: One of them was kept at room temperature, and the other was kept in the refrigerator at 40 °C and compared against the freshly prepared standards. It was found that the drug solution was stable for one week at room temperature and for two weeks in a refrigerator.

Application to Pharmaceutical Dosage Form
The proposed GC and TLC methods were applied for determining S3G in newly formulated Balanites capsules. Three replicate determinations were performed; the obtained results were in good agreement with the label claim, where no interference from excipients and additives was observed, as demonstrated in Tables III and IV.

The recovery of the procedure was assessed by applying standard addition technique and the mean percentage recovery ±%RSD of pure added was shown in Tables III and IV.

Conclusion
A novel, simple, rapid and accurate GC method with minimum sample preparation and a wide linearity range, in addition to a newly developed, cost-effective densitometric method, were developed and validated for the estimation of S3G in extracts of Balanites aegyptiaca.

The newly proposed methods are applicable for the determination of S3G in pure form and in pharmaceutical formulations.

References

Sara A.A. Elbahrwany is with the Pharmaceutical Research and Development department at Helioptolis University for Sustainable Development in Cairo, Egypt. Amira Abdel Motaal is with the Pharmacognosy Department at Cairo University in Cairo, Egypt, and the Pharmacognosy Department at King Khalid University in Abha, Saudi Arabia. Ola M. Abdallah is with the Analytical Chemistry Department at Al-Azhar University in Cairo, Egypt, and the Pharmaceutical Chemistry Department at Egyptian Russian University (ERU), in Badr City, Egypt. Direct correspondence to: olamody@yahoo.com
Headspace Solid-Phase Microextraction Coupled with Gas Chromatography–Mass Spectrometry (HS-SPME-GC–MS) for the Characterization of Cigar Leaves

Manufacturers and consumers are increasingly concerned about high-quality cigar products with clear geographical sources. To characterize the chemical profiles of different geographical origins of cigar leaf samples, headspace solid phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS) combined with a chemometrics method was used in this study. The chromatography and chemical composition of cigar leaf samples from 16 geographical origins were acquired and profiled. The chemical markers contributing to the differentiation of cigar leaf samples were observed and characterized by a supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) method of chemometrics. Eight chemical markers were tentatively identified to be used as specific chemical markers for the differentiation of geographical cigar leaf samples. HS-SPME-GC–MS method coupled with chemometrics analysis has potential to be used for discriminating different geographical origins for cigar leaf samples.

Hongfei Zhang, Juan Yang, Fengpeng Zhu, Cheng Luo, Yongqiang Pang, Beibei Zhu, Yanbo Luo, Xiangyu Li, Xingyi Jiang, and Dongliang Li

The tobacco plant (Nicotiana tabacum) is widely planted and consumed all over the world. By manipulating its leaves, different products can be prepared for smoking or chewing. Today, cigars and rope tobacco (tobacco mincing and rolling) are popular.

The quality of smoke produced in the normal combustion process of cigars depends largely on the chemical composition of tobacco (1–3). The chemical composition of tobacco is affected by many factors, among which the origin of the tobacco is the most important. To obtain the best quality cigar tobacco, we must recognize the source of cigar tobacco. Cuba, Indonesia, Dominica, Brazil, Nicaragua, and China all have famous cigar leaf planting locations.

Nowadays, manufacturers and consumers are increasingly concerned about high-quality cigar products with clear geographical sources. Flavor is one of the important distinguishing feature of cigar quality and consumer affection. The taste of a cigar is very complex, depending on the compounds it contains, such as ketones, esters, alcohols, nitrogen compounds, acids, aldehydes, and lactones. More than 1000 aromatic compounds have been funded, covering a wide range of polarity and volatility (4–7).

Analytical techniques and separation techniques, such as high performance liquid chromatography (HPLC) and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOF-MS), have been used to determine the geographic origin of food (8–10). This multifactor method requires chemometric analysis of the data provided by analytical instruments.

Headspace solid phase microextraction (HS-SPME) is particularly beneficial for the detection of volatile and flavor substances because of the low chromatographic background. This method is especially suitable for analyzing aroma in cigar leaves (12–14).

Traditional methods to separate aroma and flavor from tobacco, such as solvent extraction, adsorbent capture, simultaneous distillation extraction, or the combination of these methods, is labor-intensive because of the very low concentration of relevant compounds in a complex matrix, and often the sensitivity is not sufficient (15,16).

Vas and Vekey (17) recently published a comprehensive review on the subject of SPME. HS-SPME is particularly suitable for the analysis of tobacco leaf components because most of these compounds are volatile and can be adsorbed by SPME fiber materials. Therefore, this technology has been used to analyze tobacco constituents many times. In most cases, these methods have been limited to substances such as organic acids like alkenylbenzene (18–22).

HS-SPME and gas chromatography (GC) are the most suitable techniques for determining the origins of tobacco leaves used for cigar-production. In this study, a quick and simple method of cigar leaf screening is introduced, and some of the cigar leaves are quantitatively analyzed by HS-SPME-GC–MS before principal compo-
Principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) is used to analyze the aroma spectrum of 16 cigar leaves.

**Materials and Methods**

**Reference Substances and Reagents**
Phenylethyl acetate, which served as the internal standard, was obtained from Sigma–Aldrich. The chemicals and reagents used in analytical grade purity were obtained from Merck; ultrapure water was purified by a Milli-Q system.

**Cigar Leaf Samples**
For this study, 16 species of cigar leaf samples were collected from five countries. Packs of 16 different common cigar leaves were provided by the Sichuan tobacco company (Sichuan, China), the information of which is displayed in Table I. The 3R4F reference cigarette was purchased from the University of Kentucky. The cigar leaf samples were stored at −40 °C prior to analysis.

**Instruments and Accessories**
For GC–MS and HS-SPME
An Agilent gas chromatograph coupled to a mass selective detector (MSD) (7890-5977B) was used for the GC–MS analysis. A multipurpose sampler called MPS2 (Gerstel) for automatic performance of the HS-SPME measurements was also used.

A DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) from Agilent was used for the gas chromatographic analysis with helium as the carrier gas at a flow rate of 1.0 mL/min. The SPME fibers tested in this work were 100 μm of polydimethylsiloxane (PDMS), 85 μm carboxen/
polydimethylsiloxane (CAR/PDMS), 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB), and 50 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). All SPME fibers were purchased from Supelco.

The optimal analyses were performed with the 50 µm DVB/CAR/PDMS fiber. All GC–MS analysis were performed using the enhanced ChemStation G1701 DA, version F.01.01.2317 software from Agilent Technologies.

Sample Preparation For Qualitative Screening Method and HS-SPME
In the optimized qualitative screening method, each cigar sample was analyzed under three different conditions. An 0.1 g sample of the cigar leaf was placed in a 22 mL headspace vial. Then, 50 µL of the phenylethyl acetate methanol solvent with a concentration of 100 µg/mL was added as the internal standard. Then, the 22 mL headspace vials were tightly closed and placed into the vial rack of the sampler. The samples were extracted for 40 min at 80 °C and desorpted at 280 °C for 3 min.

Gas Chromatography–Mass Spectrometry (GC–MS)
Using helium as the carrier gas, the flow rate was 1.0 mL/min, and the injection mode was splitless at 280 °C. The optimized method was as follows: 2 min at 60 °C, then the temperature was increased 10 °C/min up to 280 °C, and then it remained for 10 min at 280 °C. The temperature of quadrupole and the ion source were 150 °C and 230 °C, respectively. Analytes were detected in scan mode from m/z = 31 to 550. Peak recognition in scanning mode was performed using the software MS Spectral Library NIST17 (National Institute of Standards and Technology).

Qualitative and Quantitative Analyses
By comparing the retention time and mass spectra of the samples with the mass spectra present in the National Institute of Standards and Technology (NIST) MS 17 spectral database, the compounds were qualitative. Quantitative analysis was carried out by the internal standard method.

Statistical Analyses
All data of the peaks were exported into the Simca software (v. 13.0) for OPLS-DA analysis. One-way analysis of variance (ANOVA) was used to analyze the significant difference of markers in different samples. The differences were considered statistically significant with the analysis of variance, $P \leq 0.05$, maximum folding change $\geq 2$, and the valuable importance in the projection (VIP) value was $>1$ as the limiting conditions.
Results
HS-SPME-GC–MS Optimization
Volatiles present in cigar leaves were extracted using a 50 µm DVB/CAR/PDMS fiber at 80 °C for 40 min. For this study, 95 compounds that were identified and quantified are presented. Because of the different structure, polarity, and volatility of the target compounds, a medium polarity fiber material was needed. Four fibers (PDMS, PDMS/DVB, CAR/PDMS, DVB/CAR/PDMS) were compared for extracting volatile compounds. The experiment was performed using a China cigar leaf that contained the volatile compounds of esters, acids, and alcohols (23–25). The performance of each fiber was compared by the number of compounds identified and the response area, in which a group of 50 volatile compounds were considered (25 esters, 14 terpenes, 5 alcohols, and 6 acids).

Compared with other fibers, the extraction rate of DVB/CAR/PDMS and PDMS/DVB coatings is higher for volatiles. The results of the DVB/CAR/PDMS fibers were significantly different from the other three fibers, and showed that the total area of esters, acids, and acids was the highest, and that more volatile compounds (95) were detected in these functional groups. In addition, the repeatability of DVB/CAR/PDMS was less than 14%. The extraction efficiency of benzyl alcohol, pyridine, and benzaldehyde was slightly better than that of PDMS/DVB fiber, as shown in Table II. This is the most commonly used fiber in the literature (26–29).

Although DVB/CAR/PDMS and PDMS/DVB are bipolar fibers covered with porous solid coating, which indicates that the extraction of analytes is carried out by adsorption, the qualitative and quantitative behavior of PDMS/DVB fiber is worse than that of DVB/CAR/PDMS, and 63 compounds are detected. The extraction efficiency of the PDMS/DVB fiber for neophytadiene, one of the most important compounds in cigar leaves, is also very low. This choice could be confirmed by comparing the peak areas of alkaline, neutral, and acidic compounds in SIM mode.

Nonpolar-coating PDMS fibers could only identify 39 volatile compounds. However, the peak area of these compounds was low, which indicates that PDMS fibers are not suitable for quantitative analysis. The combination of DVB and CAR increases the porosity distribution and polarity of the fiber while improving the retention rate of analyte on the fiber compared with the coating only composed of PDMS.

The extraction efficiency of DVB/CAR/PDMS fiber for volatile components from cigar leaves were the highest, and the repeatability was better (RSD 10%). Previous studies indicated that the DVB/CAR/PDMS fiber is the most suitable because of its extraction ability for compounds (molecular weight 40–275) (30–34). As an example, the total ion chromatograms obtained after basic preparation are shown in Figure 1.

OPLS-DA Analysis
All results of 16 cigar leaf samples were exported into the Simca software (v 13.0) for OPLS-DA analysis. Outliers and classification trends of 16 cigar leaf samples were observed in the OPLS-DA results (Figure 2). The score plot obtained by OPLS-DA showed that there was a clear distinguish between the S1 and S2 groups and the S3 to S16 group, indicating that the cigar leaf sample from China Sichuan was very different from other samples in Indonesia, Dominica, Brazil, and Nicaragua. Cigar leaf samples from China Sichuan (S1 and S2 groups) clustered together and separated from other samples in Indonesia, Dominica, Brazil, and Nicaragua (Figure 3). R2X of the OPLS-DA model (0.554) showed that the model had good adaptability and predictability. S1 and S2 samples from China Sichuan province clustered together respectively, which shows that there are significant differences between domestic and foreign samples; the separation between S3–S10, S11 and S12, S13–S15, and S16 indicated that samples in Indonesia, Dominica, Brazil, and Nicaragua also had significant differences.

Identification of Chemical Markers
Based on the retention behavior and mass distribution, potential chemical markers in cigar leaves from differ-
ent geographical origins were identified. First, the OPLS-DA model was constructed from the Simca software because potential biomarkers of interest can be extracted from loading plots and VIP plots of that model.

In addition, with the analysis of variance of $P \leq 0.05$, maximum folding change of ≥2, and the VIP value >1 as the limiting conditions, the compounds with significant changes were screened out to reduce the “false discovery rate (FDR)”. According to the condition detailed above, eight chemical markers (1-hexanol, 2-ethyl-; megastigmatrienone; 2-pyrrolidinone, 1-methyl-; 3-hexene, 3-methyl-, (Z)-; ethanone,1-(3,4,5-trimethoxyphenyl)-; 1-hexene-3,5-dione; cyclopropane, 1-methyl-2-pentyl-; and 1H-naphtho[2,1-b]pyran, 4a,5,6a,7,8,9, structure was identified. Scientists related to the differences were made above, eight chemical markers (1-hexanol, 2-ethyl-; megastigmatrienone; 2-pyrrolidinone, 1-methyl-; 3-hexene, 3-methyl-, (Z)-; ethanone,1-(3,4,5-trimethoxyphenyl)-; 1-hexene-3,5-dione; cyclopropane, 1-methyl-2-pentyl-; and 1H-naphtho[2,1-b]pyran, 4a,5,6a,7,8,9, short named H1–H8) in cigar leafs from different geographical origins. Therefore, the research of the source of cigar leaves is an important subject. Flavor is one of the important indicators of cigar quality and consumer acceptance. In this study, 16 cigar leaf samples were collected from five countries.

Eight representative chemical markers related to the differences were made on 16 cigar leaf samples. The contents of H1 and H5 were the highest in S1 and S2. The results showed that H1 and H5 could be used as specific chemical markers of S1 and S2; H3 could be used as specific chemical markers of S16; H4 and H6 could be used as specific chemical markers of S6–S8; and H8 could be used as a unique chemical marker of S13. The OPLS-DA results of different samples showed that the cigar leaf from the Sichuan provinces in China were different from the foreign samples, indicating that the China samples from the Sichuan province had some similarity. The samples from Indonesia, Dominica, Brazil, and Nicaragua were also clustered together, respectively.

Cigar leaves contain a variety of alcohols, terpenes, ketones, esters, acids, aldehydes. 1-hexanol 2-ethyl- (H1) contains a slight floral fragrance, which increases the floral scent when smoking cigars. Megastigmatrienone (H2) is a key flavor compound in tobacco. As the most abundant substance in neutral aroma components, megastigmatrienone was found and its chemical structure was identified. Scientists also found that burley tobacco concentrate contains nearly 10% of the megastigmatrienone chemicals, and

**TABLE II:** The extraction efficiency (compound area) of four fibers

<table>
<thead>
<tr>
<th>Compound</th>
<th>PDMS</th>
<th>PDMS/DVB</th>
<th>CAR/PDMS</th>
<th>DVB/CAR/PDMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl alcohol area</td>
<td>23,182</td>
<td>18,536</td>
<td>16,724</td>
<td>38,684</td>
</tr>
<tr>
<td>Pyridine area</td>
<td>248,213</td>
<td>129,250</td>
<td>195,841</td>
<td>313,268</td>
</tr>
<tr>
<td>Benzaldehyde area</td>
<td>14,702</td>
<td>32,521</td>
<td>40,340</td>
<td>43,860</td>
</tr>
</tbody>
</table>

**TABLE III:** Identification of chemical markers useful to discriminate cigar samples belonging to different geographical origins

<table>
<thead>
<tr>
<th>Var ID (Primary)</th>
<th>No.</th>
<th>Compound</th>
<th>VIP</th>
<th>Fold Change</th>
<th>P-value</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>H1</td>
<td>1-Hexanol, 2-ethyl-</td>
<td>4.08</td>
<td>8.65</td>
<td>0.000005</td>
<td>1.43</td>
</tr>
<tr>
<td>47</td>
<td>H2</td>
<td>Megastigmatrienone</td>
<td>2.98</td>
<td>3.13</td>
<td>0.006794</td>
<td>0.80</td>
</tr>
<tr>
<td>9</td>
<td>H3</td>
<td>2-Pyrrolidinone, 1-methyl-</td>
<td>2.67</td>
<td>2.25</td>
<td>0.018283</td>
<td>0.83</td>
</tr>
<tr>
<td>64</td>
<td>H4</td>
<td>3-Hexene, 3-methyl-, (Z)-</td>
<td>2.50</td>
<td>8.99</td>
<td>0.000004</td>
<td>0.58</td>
</tr>
<tr>
<td>59</td>
<td>H5</td>
<td>Ethanone,1-(3,4,5-trimethoxyphenyl)-</td>
<td>2.19</td>
<td>5.63</td>
<td>0.000002</td>
<td>0.50</td>
</tr>
<tr>
<td>54</td>
<td>H6</td>
<td>1-Hexene-3,5-dione</td>
<td>2.00</td>
<td>2.35</td>
<td>0.010523</td>
<td>0.39</td>
</tr>
<tr>
<td>63</td>
<td>H7</td>
<td>Cyclopropane, 1-methyl-2-pentyl-</td>
<td>1.34</td>
<td>3.38</td>
<td>0.000614</td>
<td>0.41</td>
</tr>
<tr>
<td>74</td>
<td>H8</td>
<td>1H-Naphtho[2,1-b]pyran, 4a,5,6,6a,7,8,9,</td>
<td>1.20</td>
<td>3.65</td>
<td>0.025816</td>
<td>0.28</td>
</tr>
</tbody>
</table>

The content of H6 in S16 was the highest; the content of S2 and S13 were higher than S1, S3–S12, S14, and S15. The content of H7 in S1 and S2 was similar, which was higher than the other 14 samples.

**Discussion**

Manufacturers and consumers are increasingly focused on high-quality cigar products with well-defined geographical origins. Therefore, the research of the source of cigar leaves is an important subject. Flavor is one of the important indicators of cigar quality and consumer acceptance. In this study, 16 cigar leaf samples were collected from five countries.

Eight representative chemical markers related to the differences were made on 16 cigar leaf samples. The contents of H1 and H5 were the highest in S1 and S2. The results showed that H1 and H5 could be used as specific chemical markers of S1 and S2; H3 could be used as specific chemical markers of S16; H4 and H6 could be used as specific chemical markers of S6–S8; and H8 could be used as a unique chemical marker of S13.
it exists more or less in flue-cured tobacco. It is a degradation product of carotenoids and formed by the degradation of lutein, which has a very important contribution to the aroma of tobacco leaves.

The 16 cigar leaf samples from different geographical origins were identified by HS-SPME-GC–MS and chemometrics. These chemical markers with significant aroma can be used to discriminate the geographical origin of cigar leaf samples. Chemometrics technology has potential application prospects in the discovery and quality evaluation of flavor components in different geographical environment, which helps us to find natural substitutes for geographical origins more quickly.

**Conclusion**

Classification and differentiation of cigar leaves according to their producing areas is an important basis to ensure the quality of tobacco leaves. The chemical constituents of cigar leaves from different areas were characterized by HS-SPME-GC–MS. HS-SPME combined with GC–MS is a fast, simple and solvent-free method. The chromatographic results of adsorption with PDMS, PDMS/DVB, DVB/CAR/PDMS, and CAR/PDMS coated fibers showed that DVB/CAR/PDMS was the best one for analyzing alcohols, esters and acids in cigar leaves.

The OPLS-DA method was used to distinguish the cigar leaf samples from different geographical origins. The marker compounds used to differentiate cigar leaves were discovered. There were eight chemical markers that were preliminarily identified and semi-quantitated in cigar leaf samples from several countries. The chemical markers results showed that there were significant differences between different regions. These chemical markers can be used to distinguish the origin of samples from different countries. The results show that chemometrics technology may be used to find aroma components related to complex components and different geographical environments of cigar leaves, which will help us find natural substances for geographical sources quicker.

**Funding Sources**

The study was supported by Key Laboratory of Chinese cigar fermentation of China Tobacco Sichuan Industrial Co., Ltd. (ctx201901).

**References**


Hongfei Zhang, Fengpeng Zhu, Yongqiang Pang, Yanbo Luo, Xiangyu Li, and Xingyi Jiang are with the China National Tobacco Quality Supervision and Test Center in Zhengzhou, China. Juan Yang, Cheng Luo, Beibei Zhu, and Dongliang Li are with the Technology Center at the China Tobacco Sichuan Industrial Co., Ltd., in Chengdu, China. Direct correspondence to Dongliang Li at: 360188228@qq.com or Hongfei Zhang at: hfzhang1983@126.com
Comprehensive Analysis of C2–C8 PFAS Using a Novel LC Column

Restek Corporation

While not currently regulated, ultrashort-chain (C2–C3) per- and polyfluoroalkyl substances (PFAS) are of great interest. Current testing methodologies using reversed-phase liquid chromatography (LC) columns cannot be used because of a lack of retention, so either a separate method or a different column is required.

A unique, hybrid ion-exchange/HILIC column (Raptor Polar X) was used to develop a comprehensive LC–MS/MS method for the analysis of ultrashort-chain through long-chain, and alternative PFAS in water sources (tap, river, groundwater, and sewage effluent). The Raptor Polar X’s multimode retention mechanisms allow for retention with a single isocratic run.

Experimental

Chromatographic conditions are reported in Figure 1.

To avoid introducing background contamination, polypropylene vials and caps were used during sample preparation.

Each water sample of 250 μL was mixed with 250 μL of methanol and 5 μL of internal standard solution (10 ng/mL of 13C2-PFHxA, 13C2-PFOA, 13C3-PFBS, 13C4-PFOS in methanol).

Calibration standards were prepared by using deionized water and fortified with 14 analytes (see Figure 1) at a range of 10–800 ng/L. The calibration standard solutions were diluted 1:1 as above.

A Restek tap water sample, along with three water samples (river, ground, and sewage effluent) supplied by the United States Environmental Protection Agency were fortified at 40 and 160 ppt. Blank and fortified water samples were diluted 1:1 in methanol as above for chromatographic analysis. For TFA measurement in groundwater, the sample was diluted fivefold with deionized water before fortification due to its high TFA concentration.

Results and Discussion

All analytes were eluted in 4 min with good peak shapes (Figure 1). The overall analytical cycle time was 8 min to ensure no matrix-related interferences.

![Figure 1: Chromatogram of a 400 ng/L standard](image-url)
Method linearity from 20–800 ppt for trifluoroacetic acid (TFA) and 10–800 ppt for all other analytes provided $r^2$ values >0.996 and deviations <20% using a 1/x weighted quadratic regression.

Samples were fortified at the low and high concentrations of their calibration ranges and run in duplicate for each analytical batch. A total of three batches were measured on different days. Concentrations of fortified samples were adjusted to account for any observed background contamination in sample blanks. Results are presented in Table I.

Conclusions
These results demonstrate that switching to a mixed-mode LC column provides the capability to analyze currently monitored and emerging PFAS contaminants in a single, short, isocratic run, preparing laboratories for the future of PFAS testing.
The past year has been a remarkable ride for the oil and gas industry, and for the entire energy sector in general. Extreme volatility in commodity pricing, an oil contango, and an apparent paradigm shift away from fossil fuel-powered vehicles has all been "par for the course" in the past 12 months. Fortunately, the petroleum industry appears to be making significant strides to improve the environmental stewardship and operational efficiency of energy production by implementing new technologies and capitalizing on previously wasted resources like produced water and flare gas. Nonetheless, many questions still remain about the management of aging or abandoned infrastructure, specifically, "How can we monitor the environmental impacts of these neglected sites, and will these impacted sites grow in magnitude over time?" To answer this question, we must first understand what is required to perform such analyses.

In many cases, a relatively straightforward analysis of the environment can provide sufficient insight into the extent of environmental damage from a specific source (1). In the case of alleged groundwater contamination, using ion chromatography and inductively coupled plasma–mass spectrometry (ICP-MS), we can characterize various brine elements (chloride and bromide) and pertinent metal ions species, respectively, to better understand subsurface fluid migration from flowback and produced water. Concurrently, gas and liquid chromatography–mass spectrometry (GC–MS and LC–MS) can be utilized to characterize the presence of volatile and semi-volatile organic compounds, which collectively can be representative of chemical additives used in the energy production process (that is, hydraulic fracturing) and be a reflection of other natural gas constituents (C1-C5 hydrocarbon gases) being present. In the event that natural gas is present, as may be evidenced by a strong sulfur-bearing aroma or water effervescence, the characterization of methane, ethane, and propane, and their respective carbon isotopes, can provide insight into the origin of the rogue gas and help differentiate between naturally occurring biogenic (microbial) gas and thermogenic gas that may have made its way into the water via anthropogenic activities.

In numerous cases, we have seen that the isotopic signature of the rogue natural gas in a water source is a match with the equivalent signature collected from a nearby production well. Case closed, right? Not exactly. In some instances, there may also be a naturally occurring source of shallow gas that is not economically viable to extract, and that has a comparable isotopic signature to the two other thermogenic gases. In situations like these, higher-resolution analytical tools are required to parse out the differences. For example, the relatively recent emergence of noble gas and noble gas isotopic analyses has allowed investigators the opportunity to better perform point source attribution, and simultaneously characterize the migration pathway from the contamination source to the contamination site (2,3). Because noble gases are chemically inert and are not subject to oxidation and microbial degradation, they are ideal tracer elements that can be used to better understand subsurface fluid and gas migration. Using the aforementioned case with three matching methane isotope signatures, the characterization of noble gas analytes allowed us to differentiate water wells that had been inadvertently drilled directly into the intermediate gas layer from those that has been impacted by the activities of a nearby production well (4).

Unconventional oil and gas extraction is a multi-faceted landscape. From assessment of air, soil, and water quality to oilfield waste treatment and re-use, there are many places where both routine and advanced chemical analysis can provide insight. We will continue to be faced with situations where environmental forensics and point source attribution are needed to understand the cause of various contamination events. Even if fossil fuel extraction declines significantly over the next 20 years, we will still be left with the need to ensure abandoned well sites are properly closed and pose no threat to the environment. We have the technology and the know-how, but of course, there are still real limits in the research that can be performed with limited to no investment in such studies from traditional funding agencies.

References

Zacariah L. Hildenbrand is a Research Professor at the University of Texas at El Paso, Texas. Kevin A. Schug is a Full Professor and the Shimadzu Distinguished Professor of Analytical Chemistry in the Department of Chemistry & Biochemistry at The University of Texas at Arlington. Direct correspondence to: kschug@uta.edu
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