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THEORY & FUNDAMENTALS
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DATA HANDLING
Data Integrity and the Lifecycle Approach for Analytical Procedures

SAMPLE PREP
Methods and Considerations for Complex Samples

GC
A Definitive Checklist for GC Analysis
Most natural waters contain natural organic matter (NOM), which is primarily composed of humic and fulvic acids. Natural waters are used both as potable and non-potable sources and both need to be disinfected with an oxidant to deactivate pathogens from either use. The disinfection of NOM with an oxidant produces disinfection by-products (DBP). The disinfection of NOM can be achieved through the use of various oxidation sources, such as: UV, ozone, chlorine, or chloramination. Depending on the oxidant and the source water, various halo DBPs can be formed. Each source generates multiple DBPs. One of the main components of the nearly 600 identified DBP’s are haloacetic acids (HAA) which have been detected in our ecosystem and affect overall human health. As utility companies utilize more influent waters containing higher salinity or desalinated sea/brackish groundwater, a growing concern has mounted for HAA’s. The higher concentrations of bromide and iodide converted in these waters change the speciation of DBP’s toward their brominated and iodinated analogues rather than their more recognized chlorinated species. These species have been documented as more toxic than the chlorinated analogs and are not routinely tested for by regulatory administrations.

NOM in general contains healing properties, however, oxidation of NOM generates toxicity when the HAA’s react with themselves to form halocitric acids. When ingested, halocitric acids bind calcium in the citric acid cycle. The sequestered calcium leads to hypocalcemia through the inhibition of the citric acid cycle. Accurate detection of HAA’s is paramount for the prevention of over oxidation, while still managing enough water sanitation to eliminate water borne pathogens. As such, a need for a fast and repeatable method for the quantification of HAA’s is of great demand for sanitation departments to accurately and quickly determine if their eluent is environmentally friendly. This method isolates fluoro, chloro, bromo, and iodoacetic acids from water samples which highlights the effectiveness and robustness of the Hamilton PRP-X300, 7 µm ion exclusion column (150 x 4.1 mm). The simple isocratic method utilizes a mobile phase of 3 mN H2SO4 and acetonitrile (95:5) to make reproducibility fast and easy.

**Column Information**

<table>
<thead>
<tr>
<th>Packing Material</th>
<th>Dimensions</th>
<th>P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP-X300 (7 µm)</td>
<td>150 x 4.1 mm</td>
<td>79464</td>
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</tbody>
</table>

**Chromatographic Conditions**

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<thead>
<tr>
<th>Gradient</th>
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</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Injection Volume</td>
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<tr>
<td>Detection</td>
<td>Refractive Index</td>
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<tr>
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<td>Sulfuric Acid</td>
</tr>
<tr>
<td>Eluent A Conc</td>
<td>3.0 mN</td>
</tr>
<tr>
<td>Eluent B</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.0 mL/min</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>95:5</td>
</tr>
</tbody>
</table>

![Time vs. Intensity Graph](image)

**Compounds:**
1. Fluoroacetic acid
2. Chloroacetic acid
3. Bromoacetic acid
4. Iodoacetic acid

Author: Adam L. Moore, PhD, Hamilton Company

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Selectivity in Reversed-Phase Liquid Chromatography: 20 Years of the Hydrophobic Subtraction Model

How can I use the hydrophobic subtraction model of reversed-phase selectivity to help me in method development? A recent Pittcon symposium discussed the history and practical use of the model, as well as insights into recent research that may enable expanded use of the model in the future.

Dwight R. Stoll

For the 2020 edition of the Pittsburgh Conference (Pittcon) held in early March in Chicago, Professor Joe Foley of Drexel University organized a symposium entitled “To Selectivity and Beyond: Celebrating 18 Years of the Hydrophobic Subtraction Model.” It has been roughly 20 years since Lloyd Snyder, John Dolan, Peter Carr, and coworkers began work on what has become known as the hydrophobic subtraction (HS) model of selectivity for reversed-phase liquid chromatography columns. In my view, this model, and the accompanying public database of parameters for 750 commercially available columns, has been remarkably successful, and this Pittcon symposium aimed to both discuss the impact of the model on contemporary method development, and provide some insights from recent research on the use of the model going forward. Choosing a column is an important decision, not only at the beginning of method development, but also for the analytical life cycle of a method. In this installment of “LC Troubleshooting,” I first review the basic premise and features of the HS model and the column database, and then touch on highlights of the talks presented as part of the Pittcon symposium. From my point of view, and I think the other contributors to the symposium would agree, the HS model database is a column characterization tool that is underutilized by the LC community, in spite of the fact that it is a free resource. Users can leverage the database to supplement information available from suppliers, and become more familiar with their columns. Separation performance can improve by locating and evaluating more than one column to optimize selectivity, and one can quickly locate replacement columns if problems develop. I hope that symposia like the one at Pittcon can both help other users understand how the model can benefit their work, and promote discussion about how we can improve this and other resources as we work into the future.

Basics of the Hydrophobic Subtraction Model of Reversed-Phase Selectivity

The basic principle of the HS model was first described in a journal article by Snyder and coworkers in 2002 (1). Since then, many articles have been published on the topic, but two resources are particularly noteworthy for readers interested in learning more about the model. First, in 2012, Snyder and coworkers published a book chapter in Advances in Chromatography that is still the most comprehensive discussion of the model and its application that has been published to date (2). Second, a more recent article in LCGC provides more of an overview of the model and its application that may be an easier place to start for those that are completely new to the idea (3). The model, which was originally developed using retention data from alkyl phases (such as C4, C8, and C18) bonded to high purity type B silicas, assumes that reversed-phase selectivity (defined here as the ratio of retention factors for a compound of interest, and ethylbenzene) can be described using the sum of five pairs of column and solute parameters that are related to different physicochemical interactions between solutes and the reversed-phase stationary phase. A view of the nature of each of these interactions is shown in Figure 1.

The mathematical expression of the model is shown in equation 1, where the capital Roman letters \( H \), \( S^* \), \( A \), \( B \), and \( C \) are column parameters, and \( \eta \), \( \sigma \), \( \beta \), \( \alpha \), and \( \kappa \) are solute parameters:

\[
\log \left( \frac{k}{k_{EB}} \right) = \eta H + \sigma S^* + \beta A + \alpha B + \kappa C \tag{1}
\]
The column parameters are determined experimentally by measuring the retention times of 16 carefully chosen probe solutes in a mobile phase composed of acetonitrile and potassium phosphate buffer at pH 2.8, calculating the selectivity value for each compound ($k/k_{EB}$), and regressing those selectivities against the known solute parameters for the probe compounds (2). To date, parameters for 750 commercially available columns have been determined, and are publicly available for free through two websites: 1) a site maintained by the U.S. Pharmacopoeia (https://apps.usp.org/app/USPNF/columnsDB.html); and 2) a site maintained by my research group (www.hplccolumns.org). The two primary uses of this database are finding columns that have similar selectivities (for example, for identifying a set of columns to screen during method development). These applications will be discussed in more detail in the next sections.

**Overview of the Symposium**

The Pittcon symposium was composed of five podium presentations:
- Prof. Dwight Stoll (Gustavus Adolphus College) reviewed the early history of the development of the HS model, its physico-chemical basis, and shared some insights from analysis of the selectivities of reversed-phase columns recently introduced to database.
- Dr. Richard Henry (independent consultant) discussed use of the column parameters database from a practical perspective, with an emphasis on tools that can leverage the database to identify equivalent columns.
- Dr. John Dolan (LC Resources) discussed recent efforts by himself, Paul Haddad, and coworkers to predict retention times in reversed-phase LC (and other modes) starting from physicochemical descriptors of solutes of interest.
- Dr. Tony Taylor (Arch Sciences Group) discussed a recent study aimed at a retrospective refinement of the HS model, and the potential for a refined model to be used for retention prediction in reversed-phase LC.
- Prof. Joe Foley (Drexel University) described recent efforts by his research group to use the HS model to demonstrate the advantages of serially coupled columns from the HS model database, and ultimately predict which pairwise combinations of columns might yield the best separations of different mixtures of real or synthetic solutes.

**Use of the HS Model for Column Selection During Method Development**

In the early stages of method development, it is common practice to choose a diverse set of stationary...
phases that can be screened to identify candidate phases that exhibit the selectivity needed to separate the analyte mixture at hand (4). In this case, the HS model database can be used to identify a set of columns with sufficient diversity to be useful in this regard. Later in method development, when a leading candidate column has been identified and the method is refined prior to validation, it is also common to try to identify other columns with very similar, even “equivalent,” selectivity such that this column can be used as a drop-in replacement in the event that the manufacturer of the primary column stops making it, or has trouble manufacturing the column reproducibly.

Here again, the HS model database can be used for this purpose, by generating a short list of columns to evaluate experimentally and assess their similarity to the primary column. For both of these purposes Snyder and coworkers advocated for the use of a “similarity factor, $F_S$,” which is a weighted distance between two columns in five-dimensional column parameter space. While this might sound complicated, calculating $F_S$ is very straightforward, as shown in equation 2, where $H_1$ and $H_2$ are the $H$ parameters for the first and second columns in the comparison, and so on.

$$F_S = S_1 - S_2 + \chi_{H_1}H_1 - H_2 + \chi_{S^*_1}S^*_1 - S^*_2 + \chi_{A_1}A_1 - A_2 + \chi_{B_1}B_1 - B_2 + \chi_{C_1}C_1 - C_2.$$

(2)

The weighting factors $\chi_{H_i}$, $\chi_{S^*_i}$, and so on are user-adjustable parameters in the calculation, but usually taken as 12.5, 100, 30, 143, and 83 for $H$, $S^*_i$, $A$, $B$, and $C$, respectively (2). Both of the web-based tools cited above do this $F_S$ calculation for you, and facilitate sorting the database to identify columns that are similar, or different, compared to a target column you specify.

In his presentation, Henry emphasized the point that the task of identifying columns with similar selectivities has become more difficult over the past two decades, as the stationary phase offerings from manufacturers have become more diverse. The L-code system of the United States Pharmacopoeia is based on stationary phase types such as “C18” and “phenyl,” and compendial methods specify columns from a particular phase type. However, stationary phases with mixed chemistries are becoming more and more common (for example, phenyl-hexyl is a mixed chemistry phase with both aromatic and alkyl components), and increasingly we are coming to understand that the properties of the underlying silica substrate (for example, metal impurities, and specific synthetic methods) can have a significant effect on selectivity, particularly for complex solutes with many different types of functional groups. The net effect is that, when exploring the HS model database, it is possible to find that the column most similar to a target column of interest as measured by $F_S$ belongs to a different phase type. On one hand, this means that we should be somewhat open-minded when scouting for similar columns, but, on the other hand, this creates challenges when identifying columns that can be used as replacements in a regulated environment.

Recent Efforts to Predict Reversed-Phase Retention Using the HS Model

In the process of establishing the HS model 20 years ago, it was demonstrated that the model described by equation 1 could accurately reproduce the retention factors of about 90 solutes obtained for 10 different alkyl stationary phases based on high purity type B silicon with an accuracy of about 2% (1). Since the large database of column parameters already exists, it is logical to think about using the model and the database to predict retention for new analytes that are not represented in the set of solutes used to establish the model initially. However, this requires determination of the solute parameters to use the model (equation 1) for this purpose, and doing so experimentally is currently quite time- and resource-intensive. In his presentation, Dolan gave a summary of work by Paul Haddad, his group at the Australian Centre for Research on Separation Science (ACROSS), and other collaborators in recent years to predict HS model solute parameters from chemical structure that could in turn be used to predict reversed-phase reten-

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tion via the HS model. Readers interested in learning more about these efforts are referred to recent articles in LCGC and other publications where the details of the work are discussed (5,6). Briefly, Haddad and coworkers explored the use of computationally derived molecular descriptors produced by programs such as VolSurf+, and different approaches to constructing local and global models, and evaluated their effects on reversed-phase retention prediction. Again, the basic premise here is that one would take the chemical structures for analytes they are trying to separate, calculate molecular descriptors based on those structures (for example, log D, polar surface area, and so forth), then calculate HS model solute parameters based on those descriptors, and finally calculate retention factors for those compounds using equation 1 and the column parameters from the HS model database. In principle, all of this could be done without doing a single laboratory experiment. Dolan reported that using molecular descriptors to calculate the \( \eta' \) parameter alone (equation 1) for each solute was sufficient to make retention time predictions that were accurate to within 30 s for 70% of the 146 solutes evaluated in their study (5). Although this obviously leaves room for improvement in the future, this kind of work should capture the imagination of research groups around the globe to improve these modeling and retention prediction efforts.

Recent Additions to the Public Database of Reversed-Phase Column Parameters

In March of this year, we added 13 new columns (from three different vendors) to the HS model database, bringing the total number of columns in the dataset to 750. Whenever we make these additions, I am always curious to know what is being added; redundancy in selectivity, or unique selectivities? I don’t know the motivations of the manufacturers of these new columns, but, of course, there is value in both types of additions. It is helpful to have the redundancy in the selectivities of column offerings to have a backup column to use, in case there is some problem with the supply of the column of first choice. On the other hand, LC users are always interested in new selectivities that might be able to solve their challenging separation problems. Figure 2 shows the \( F_S \) values obtained from a comparison of each new column to its nearest neighbor (in selectivity space) in the database prior to the most recent additions. From these values, we see that 6 of the 13 new additions are equivalent to some other column already in the database (\( F_S < 3 \)). Of the other seven, two of them are very similar to existing phases (3 < \( F_S < 5 \)), and only one of them has a nearest neighbor with an \( F_S > 10 \), which we might consider somewhat unique. Here again, the
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HS model is useful for assessing the characteristics of commercial offerings as these continue to grow. New additions to the database have grown at a remarkably consistent pace of about two to three columns per month over the last ten years!

Summary
In this installment of “LC Troubleshooting,” I have reviewed the basic concept of the hydrophobic subtraction model of reversed-phase selectivity for liquid chromatography, and summarized some highlights from a recent Pittcon symposium organized to celebrate the success of the model over the last two decades. I encourage readers who are unfamiliar with the model to consider how it might benefit method development work, and I think we can all look forward to further development as research groups continue to refine the model and expand its scope of application.

Acknowledgement
I want to thank Dick Henry, Joe Foley, John Dolan, and Tony Taylor for reviewing the manuscript for this article and providing helpful feedback.

References
This article covers liquid chromatography columns and accessories commercially released after Pittcon 2019 through this year’s conference held in Chicago, Illinois. LCGC sent out a survey in late 2019 and early 2020, asking vendors to supply information on products launched after Pittcon 2019. Other areas of interest such as gas chromatography, chromatographic instrumentation, and sample preparation will be covered in other articles published in this and next month’s issues. Information for this article is obtained over the course of many months, and thus it is possible that some information has been missed or misinterpreted. The reader is encouraged to check with specific vendor sites for additional products, as well as more detailed information on product usage and attributes.

The vendors that responded to the survey with high performance liquid chromatography (HPLC) or ultra-high pressure liquid chromatography (UHPLC) columns and liquid chromatography (LC) accessories are listed in Table I. The products launched over the past year vary in targeted analyte type, as well as mode of operation. The entries can be initially categorized as addressing small molecule or large molecule needs. Within these categories, the products can be further separated based on the specific modes of separation they employ, including reversed-phase, hydrophilic-interaction chromatography (HILIC), chiral, and ion-exchange (IEC) chromatography. In addition to new chromatography columns, several vendors have released accessories, as well as new column formats, to address the needs of separation scientists. Trends noted throughout the article are based on comparisons to yearly reports since 2016 (1–4).

Upon examination of Table I, it is immediately apparent that there was a low number of respondents in 2020 compared to previous years. Table II shows this more clearly by providing a listing of the number of companies and the products they launched over the past five years aimed at specific categories. Please note that many products can be categorized in multiple areas, depending on usage. This is meant to be a loose interpretation to highlight trends only. The number of companies that provided new product information dropped from a high of 27 in 2017 to just 11 in 2020. Even more striking is that the number of total new column phases launched dropped from an average of about 60 columns per year over the past four years to just 22 this year. It will be interesting to see if these trends continue in the future.

Columns for Small Molecules
Reversed-Phase, HILIC, and IEC
The product offerings assigned to the small molecule category intended for reversed-phase, HILIC, and IEC are listed in Table III. A total of 11 new entries are shown. Within this broad category, ChromaNik Technologies has launched the most new products. SunArmor NH2 is described by the company as a highly stable and retentive aminopropyl phase with hydrophilic endcapping. The column is suggested for the analysis of sugars and other polar compounds. The company also released SunShell HILIC-S, described as a bare silica phase built on a superficially porous particle (SPP) that is suitable for polar compound analysis. Agilent Technologies also released a HILIC phase. The company applied their zwitterionic surface chemistry previously built on a 2.6-µm SPP to their 1.9 and 4 µm SPP sizes. These three phases constitute all of the HILIC phases released this past year. As noted in the 2019 edition of this article series, HILIC phase development slowed significantly. This trend continued in 2020.

ChromaNik Technologies also developed two new columns based on a mix of octadecyl (C18) and perfluorophenyl (FFP) ligands. The SunShell FPF&C18 is constructed on a 2.6-µm SPP particle, whereas the Sunniest FPF&C18 column uses a 5-µm fully porous particle (FPP) architecture. The company reports that the combination of ligands provides increased retention and stability,
while maintaining much of the selectivity of a PFP phase. In addition to the new phases noted, ChromaNik Technologies also released many of its SunShell line of phases in capillary dimensions. The phases include C18, RP-Aqua, phenyl, C8-30HT, and C4-100 and are available in dimensions from 50 x 0.075 mm to 150 x 0.5 mm. Particle sizes of 2.0-, 2.6- and 5-µm may be acquired.

Phenomenex launched an alkyl C18 phase with a positive surface charge based on its 2.6-µm SPP particle. The Kinetex PS C18 is suggested for reversed-phase analysis of basic compounds. The company notes that the phase exhibits multi-interaction selectivity and improved peak shape for bases. The phase is also purported to provide unique selectivity. Continuing the trend of modified C18 phases, Waters Corporation released the Atlantis Premier BEH C18 AX phase. The stationary phase is described as a “C18 and alkylamine,” and is suggested for separations containing polar acids or where alternative selectivity to other C18 phases is required. The phase exhibits increased retention for polar acidic compounds as a result of a mixed mode (reversed-phase/anion exchange) mechanism. The company also claims high batch-to-batch reproducibility and a wide (2–10) pH stability range.

**TABLE I:** Vendors responding to the 2020 LCGC new product survey

<table>
<thead>
<tr>
<th>Company</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Technologies, Inc.</td>
<td>Poroshell 120 SB-C18</td>
</tr>
<tr>
<td></td>
<td>Poroshell 120 SB-Aq</td>
</tr>
<tr>
<td></td>
<td>Poroshell 120 HILIC-Z</td>
</tr>
<tr>
<td>ChromaNik Technologies</td>
<td>SunShell Micro/Nano Columns</td>
</tr>
<tr>
<td></td>
<td>SunShell HILIC-S</td>
</tr>
<tr>
<td></td>
<td>Sunniest PFP&amp;C18</td>
</tr>
<tr>
<td></td>
<td>SunShell PFP&amp;C18</td>
</tr>
<tr>
<td></td>
<td>SunArmor NH2</td>
</tr>
<tr>
<td>ColumnTek</td>
<td>Enantiocel A6</td>
</tr>
<tr>
<td></td>
<td>Enantiocel C9</td>
</tr>
<tr>
<td>Developsil USA</td>
<td>FlexFire Series</td>
</tr>
<tr>
<td>DWK Life Sciences</td>
<td>Chromaflex</td>
</tr>
<tr>
<td>Optimize</td>
<td>EXP 2 filters, fittings and adapters</td>
</tr>
<tr>
<td>Phenomenex</td>
<td>LUX-i-Amylose-3 (3 and 5 µm)</td>
</tr>
<tr>
<td></td>
<td>Kinetex PS C18</td>
</tr>
<tr>
<td>Regis Technologies</td>
<td>Reflect I-Cellulose J Polysaccharide Chiral Column</td>
</tr>
<tr>
<td>Shodex</td>
<td>SI-36 4D</td>
</tr>
<tr>
<td>Waters Corporation</td>
<td>Mobile phase additive - IonHance DFA</td>
</tr>
<tr>
<td></td>
<td>Atlantis Premier BEH C18 AX</td>
</tr>
<tr>
<td>YMC</td>
<td>YMC-Triart Bio C18</td>
</tr>
</tbody>
</table>

**TABLE II:** Trending data in the numbers and types of liquid chromatography columns launched in the period 2016–2020

<table>
<thead>
<tr>
<th>Year</th>
<th>Companies (Count)</th>
<th>Small-Molecule Columns (Count)</th>
<th>Large-Molecule Columns (Count)</th>
<th>Total (Count)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Reversed-Phase</td>
<td>HILIC</td>
<td>Chiral</td>
</tr>
<tr>
<td>2020</td>
<td>11</td>
<td>25</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>2019</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>2018</td>
<td>17</td>
<td>17</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>2017</td>
<td>27</td>
<td>17</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>2016</td>
<td>18</td>
<td>25</td>
<td>18</td>
<td>3</td>
</tr>
</tbody>
</table>
### TABLE III: Small-molecule reversed-phase, ion-exchange (IEC) and hydrophilic interaction liquid chromatography (HILIC) columns

<table>
<thead>
<tr>
<th>Company</th>
<th>Product Name</th>
<th>Stationary Phases</th>
<th>Chromatographic Mode</th>
<th>Particle Sizes (µm)</th>
<th>Particle Type*</th>
<th>Dimensions (mm)</th>
<th>Comments**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agilent Technologies, Inc.</td>
<td>Poroshell 120 SB-C18</td>
<td>C18</td>
<td>Reversed-Phase</td>
<td>1.9, 4</td>
<td>SPP</td>
<td>50 x 2.1 to 250 x 4.6</td>
<td>Proprietary bonding technique allows operation in low pH (1–8) and at high temperatures (80 °C)</td>
</tr>
<tr>
<td></td>
<td>Poroshell 120 SB-Aq</td>
<td>not disclosed</td>
<td>Reversed-Phase</td>
<td>1.9, 4</td>
<td>SPP</td>
<td>50 x 2.1 to 150 x 2.1 (1.9 µm) and 50 x 4.6 to 250 x 4.6 (4 µm)</td>
<td>Designed for the retention of polar molecules in reversed-phase</td>
</tr>
<tr>
<td></td>
<td>Poroshell 120 HILIC-Z</td>
<td>Zwitter-ionic</td>
<td>HILIC/SFC</td>
<td>1.9, 4</td>
<td>SPP</td>
<td>50 x 2.1 to 150 x 2.1 (1.9 µm) and 50 x 4.6 to 250 x 4.6 (4 µm)</td>
<td>Unique selectivity for HILIC separations, outstanding pH stability range (pH 2–12)</td>
</tr>
<tr>
<td>ChromaNik Technologies</td>
<td>SunArmor NH2</td>
<td>Amino-propyl</td>
<td>HILIC/IEC</td>
<td>3, 5</td>
<td>FPP</td>
<td>150 x 2.0 to 250 x 20</td>
<td>Hydrophilic endcapping. Suggested for sugars and polar compounds. High stability and high retention.</td>
</tr>
<tr>
<td></td>
<td>Sunniest PFP&amp;C18</td>
<td>Penta-fluoro-phenyl (PFP) and C18</td>
<td>Reversed-phase</td>
<td>5</td>
<td>FPP</td>
<td>50 x 2.0 to 250 x 20</td>
<td>Recommended for polar compounds and isomers. Increased stability and hydrophobicity. Long column life.</td>
</tr>
<tr>
<td></td>
<td>SunShell HILIC-S</td>
<td>Bare silica</td>
<td>HILIC</td>
<td>2.6</td>
<td>SPP</td>
<td>50 x 2.1 to 150 x 2.1</td>
<td>Suggested for polar compounds. Suitable for LC–MS.</td>
</tr>
<tr>
<td></td>
<td>SunShell PFP&amp;C18</td>
<td>PFP and C18</td>
<td>Reversed-phase</td>
<td>2.6</td>
<td>SPP</td>
<td>30 x 2.1 to 150 x 4.6</td>
<td>Recommended for polar compounds and isomers. Increased stability and hydrophobicity. Long column life.</td>
</tr>
<tr>
<td>Phenomenex, Inc.</td>
<td>Kinetex PS C18</td>
<td>C18 with a positive surface charge</td>
<td>Reversed-phase</td>
<td>2.6</td>
<td>SPP</td>
<td>not disclosed</td>
<td>Multi-interaction selectivity and improved peak shape for basic compounds.</td>
</tr>
<tr>
<td>Shodex</td>
<td>SI-36 4D</td>
<td>Quaternary ammonium</td>
<td>Ion-exchange (IEC)</td>
<td>3.5</td>
<td>Polymeric-polyvinyl alcohol</td>
<td>4.0 x 150</td>
<td>Anion suppressor column</td>
</tr>
<tr>
<td>Thermo Fisher Scientific</td>
<td>Accucore Biphenyl</td>
<td>Biphenyl</td>
<td>Reversed-phase</td>
<td>2.6</td>
<td>SPP</td>
<td>10 x 2.1 to 100 x 2.1</td>
<td>For use in the separation of critical pairs and isomers.</td>
</tr>
<tr>
<td>Waters Corporation</td>
<td>Atlantis Premier BEH C18 AX</td>
<td>C18 and alkyamine</td>
<td>Mixed mode reversed-phase/anion-exchange</td>
<td>1.7, 2.5, 5</td>
<td>Hybrid</td>
<td>30 x 2.1 to 250 x 4.6</td>
<td>Reversed-phase separation of mixtures containing polar acids; alternative selectivity vs. conventional C18 columns; outstanding peak shapes for bases.</td>
</tr>
</tbody>
</table>

* FPP = fully porous (totally porous) particle; SPP = superficially porous particle; **Comments supplied by vendors
GCxGC adds chromatographic resolution for complex matrices and allows analysts to broadly expand their knowledge of the chemical compounds that make up cannabis.

By Alissa Marrapodi

As the cannabis industry continues to grow, purity, quality, quantification, and additional research into therapeutic potential are fundamental to creating a credible market. Analytical testing is of critical importance for driving continued research and for compliance with regulatory and safety standards that continue to evolve with the legalization of medicinal and recreational marijuana.

The analytical needs of the cannabis market are broken into two major parts, Joe Binkley, director of domestic applications for separation sciences at LECO, explained: routine targeted tests and research-oriented non-targeted discovery work related to the profiling of various compound classes found in the cannabis plant (think cannabinoids and terpenes).

Routine testing is important for safety and regulatory compliance and includes potency, quantitation of pesticides, and screening for residual solvents. Research-oriented discovery work has broad objectives and includes product development, working toward a better understanding of the physiological effects, connecting the chemical profile to therapeutic efficiency, and improving the ability to differentiate and classify cannabis types.

Classification and differentiation are important for predictive purposes. “Classically, cannabis has been characterized into three predominant strains: indica, sativa, and ruderalis, based on plant morphology and place of origin,” Binkley said. “These strains have been associated with various physiological effects, but this classification hasn’t met the needs of the medical industry.”

To provide better predictive information, the industry is moving toward using the chemical information of the plant for classification. Chemical information is crucial to meet all of these targeted and non-targeted analytical testing needs.

The Challenges with Routine Targeted and Research Non-Targeted Analysis

The chemical complexity of cannabis creates challenges for both targeted and non-targeted analytical testing.
When it comes to routine pesticide screening and quantification, the cannabis matrix is a big challenge because it’s extremely dirty and there is a lot of background. In fact, according to Binkley, it’s one of the most complicated matrices that LECO has screened for pesticides. “The cleanup process is very difficult,” he explained.

LECO’s Pegasus® BT GC-MS is well-suited to address these challenges. One reason is that LECO designed it to have a very robust, low-maintenance ion source that requires little-to-no maintenance other than filament changes. This is a unique distinction between the Pegasus BT and other GC-MS ion sources. “If systems are less robust and require frequent cleaning, there will be a lot more downtime,” Binkley said. This time savings translates to more information in less time.

Another distinction is the GC-MS system’s time of flight mass spectrometer (TOF-MS) gives the analyst the ability to mathematically separate analytes from each other and from interferences in a complex matrix. These deconvolution algorithms are built into LECO’s ChromaTOF® software that was developed over 20 years ago and continues to evolve.

“With time of flight, the ion ratios don’t change as you go across the chromatographic peak; they stay consistent all the way across, so if you have more than one analyte eluting at the same time, the deconvolution algorithms can separate them and get two unique peak profiles,” Binkley said. “It allows for a very consistent mass spectrum across the chromatographic peak, which is different from quadrupoles because they are a scanning instrument. What happens with a scanning instrument is as you go across the chromatographic peak, the ion ratios for a single analyte can change—this makes it more difficult for spectral deconvolution algorithms to properly assign which ion is eluting with which peak profile in the event of co-elutions.”

The advantage here is that analysts can learn more about what’s in a given sample by unraveling chromatographic co-elutions. Target analytes can be separated from the matrix and non-target analytes can be discovered. The amount of confidently identified compounds within a given sample is increased.

LECO takes these capabilities a step further with its Pegasus BT 4D GCxGC system by separating complex samples even more. GCxGC adds chromatographic resolution, which is really important for complicated matrices like cannabis. “Mass-spectral deconvolution reaches its limit when compounds are co-eluting and they’re at the apex of their peaks—meaning there is no chromatographic separation. The math can’t separate it; it has to become a physical separation,” Binkley explained. “We see these examples almost every time we run something with a complex matrix. There are times you can separate things with deconvolution, and there are times when you find additional compounds because you did the GCxGC separation.”

For targeted screening, GCxGC offers increased selectivity to help cut through the matrix to identify the presence of pesticides, herbicides, and other possible contaminants and to effectively perform quantitative determinations. For non-targeted analytical testing, hidden analytes can be uncovered and better coverage of the chemical profile can be determined.

Some analytical testing focuses solely on cannabinoids for classification, but it is thought that the interactions of cannabinoids with terpenes and other chemical constituents are important for predictive potential. “If you’re not focusing on just a small group of cannabinoids, such as THC and CBD, to differentiate the cannabis types, then these instruments will allow you to broadly expand the chemical knowledge through non-targeted analyses,” Binkley said.

Both the Pegasus BT GC-MS and BT 4D GCxGC expand on the chemical coverage and chemical profile compared to what is standard from these types of analyses and may lead to these improved classifications.

Conclusion

Given the complex matrix of cannabis, adding a second dimension of chromatographic separation uncovers even more compounds that could have been missed by systems not well-suited for non-targeted analyses.

“If you have hundreds of analytes, it’s impossible to separate everything on one stationary phase,” Binkley concluded. “With GCxGC, you choose a second column that has a separation mechanism different from that of the primary column. This gives you the ability to have separation on two independent stationary phases, on the X and Y axis.”

GCxGC and TOF-MS combine to provide a more complete chemical picture of cannabis—improving targeted and non-targeted analytical testing.

FOR MORE INFORMATION: leco.com/product/pegasus-bt-4d
Agilent Technologies also released two new phases on its 1.9- and 4-µm SPP for reversed-phase operation. The SB-C18, according to the company, possesses a proprietary bonding chemistry that allows for operation down to pH 1 and is stable to temperatures as high as 80 °C. Another phase denoted as SB-Aq is claimed by the organization to provide superior retention and peak shape for polar compounds that are poorly retained on traditional reversed-phase materials. The SB-Aq phase is also touted as being stable at low pH and high temperatures.

To round out the reversed-phase, small-molecule offerings from this past year, Thermo Fisher Scientific introduced a biphenyl chemistry to its Accucore line of stationary phases. The company recommends the biphenyl phase for use in the separation of critical pairs and isomers. The phase is touted as being stable at low pH and high temperatures.

For the analysis of small ionic compounds, polymeric supports are often modified to carry permanent (strong cation- or anion-exchange) or variable (weak cation- or anion-exchange) charge that can be used to interact with and separate analytes with the opposite charge. Only a single product was released this year in this category, the Shodex SI-36 4D column. The column is described as a quaternary ammonium functional group attached to a polyvinyl alcohol particle of 3.5-µm, and is suggested for use as an anion suppressor device. Suppressor columns act to lower the conductivity of the eluent in an IEC system to enhance the signal from the conductivity of the analytes reaching the detector.

Chiral Chromatography
Table IV provides information on columns introduced this year intended for chiral separations. As shown in Table II, the number of chiral stationary phases (CSPs) is similar to those released in the previous year, but substantially less than the number reported in 2018. Similar to last year, all newly released chiral phases were based on polysaccharide stationary phases. In addition, like in 2019, all of the chiral phases released this past year were developed on fully porous particles, reversing the trend noted in 2018 of CSPs based on SPP architecture.

A company new to the article series, ColumnTek, introduced two new CSPs. Enantiocel A6 is an amylose tris(3-chloro-5-methylphenylcarbamate) phase built on 3-, 5-, and 10-µm FPP particles. The Enantiocel C9 offering is the identical ligand attached to a cellulose structure with the same particle size availability. Both phases are supplied from analytical to preparative

**TABLE IV: Chiral stationary phases**

<table>
<thead>
<tr>
<th>Company</th>
<th>Product Name</th>
<th>Stationary Phases</th>
<th>Particle Sizes (µm)</th>
<th>Particle Type*</th>
<th>Dimensions (mm)</th>
<th>Comments**</th>
</tr>
</thead>
<tbody>
<tr>
<td>ColumnTek</td>
<td>Enantiocel A6</td>
<td>Amylose tris(3-chloro-5-methylphenylcarbamate)</td>
<td>3, 5, 10</td>
<td>FPP</td>
<td></td>
<td>Unique enantioselectivity, high column efficiency, and excellent peak shape.</td>
</tr>
<tr>
<td></td>
<td>Enantiocel C9</td>
<td>Cellulose tris(3-chloro-5-methylphenylcarbamate)</td>
<td>3, 5, 10</td>
<td>FPP</td>
<td></td>
<td>Unique enantioselectivity, high column efficiency, and excellent peak shape.</td>
</tr>
<tr>
<td>Regis Technologies</td>
<td>Reflec I-Cellulose J</td>
<td>Immobilized tris(4-methylbenzoate)</td>
<td>3, 5, 10</td>
<td>FPP</td>
<td></td>
<td>Unique, immobilized &quot;J&quot; type selector, proprietary phase coverage provides excellent peak shape and improved resolution.</td>
</tr>
<tr>
<td>Phenomenex Inc.</td>
<td>Lux i-Amylose-3</td>
<td>Amylose tris(3-chloro-5-methylphenylcarbamate)</td>
<td>3, 5</td>
<td>FPP</td>
<td></td>
<td>Strong solvent stability, broad enantioselectivity and robust reproducibility.</td>
</tr>
</tbody>
</table>

* FPP = fully porous (totally porous) particle; SPP = superficially porous particle
**Comments supplied by vendors
dimensions, and are purported to provide unique selectivity, high column efficiency, and excellent peak shapes.

Phenomenex launched Lux i-Amylose-3 CSP. The column is based on amylose tris(3-chloro-5-methylphenylcarbamate) modification and is noted as having strong solvent stability, broad enantioselectivity, and robust reproducibility.

Regis Technologies released a new line of CSPs called Reflect Polysaccharide Chiral columns that was reported in the 2019 article. Adding to that line, the company launched Reflect I-Cellulose J Polysaccharide Chiral Column this past year. The phase is described as a tris(4-methylbenzoate) ligand attached to a cellulose backbone. The immobilized stationary phase is noted to be a “J” type selector. The columns

<table>
<thead>
<tr>
<th>Company</th>
<th>Product Name</th>
<th>Stationary Phases</th>
<th>Chromatographic Mode</th>
<th>Particle Sizes (µm)</th>
<th>Particle Type*</th>
<th>Dimensions (mm)</th>
<th>Comments**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Develosil USA</td>
<td>FlexFire Series</td>
<td>C1, C4, C8, C18, C30, HILIC</td>
<td>Reversed-phase and HILIC</td>
<td>1.6, 2.6, 5</td>
<td>FPP</td>
<td>not disclosed</td>
<td>Suggested for oligonucleotide, insulin and antibody separations. Scalability from analytical to semi-prep, HPLC to UHPLC, high temperature, high pH functionality.</td>
</tr>
<tr>
<td>YMC Co., Ltd.</td>
<td>YMC-Triart Bio C18</td>
<td>C18</td>
<td>Reversed-phase</td>
<td>1.9, 3, 5</td>
<td>Hybrid, Polymerically bonded</td>
<td>not disclosed</td>
<td>YMC-Triart Bio C18 is a novel, organic-inorganic hybrid silica derivatized with C18 and based on wide-pore particles. The phase is designed for separations of proteins, peptides and oligonucleotides.</td>
</tr>
</tbody>
</table>

* FPP = fully porous (totally porous); SPP = superficially porous
**Comments supplied by vendors
are available in analytical and preparative dimensions and particle sizes. The company notes that a unique, proprietary, phase coverage provides excellent peak shape and improved resolution versus leading chiral phases.

**Columns for Large Molecules**

New columns introduced since Pittcon 2019 intended for large-molecule separations are provided in Table V. The categorization was done loosely based on the pore size of the base particles employed. Columns using particles with pore sizes of 300 Å and greater are often constructed to enable large molecules to easily access the internal structure of the particles. The data in Table II show that only phases in the reversed-phase category of large-molecule separations were released this past year (included in this was one unique 300 Å HILIC phase). In previous reports, columns utilizing different modes of separation such as hydrophobic interaction chromatography (HIC), size-exclusion chromatography (SEC), IEC, and affinity have been noted. The overall yearly number of large molecule offerings is down substantially from a height of 22 reported in 2017.

Develosil released a new line of columns branded as FlexFire. The series includes bonded phases of C1, C4, C8, C18, C30, and HILIC based on fully porous silica particles ranging from 1.6 to 5 µm with 300 Å pores. The company states that its value and performance lies in its high quality silica and 40 years of manufacturing experience. The new columns are suggested for oligonucleotide, insulin, and antibody separations. Develosil claims scalability from analytical to semipreparative dimensions, as well as high temperature and high pH functionality for its phases.

The other entry for this report is the YMC-Triart Bio C18. The column is based on a C18 polymerically bonded to a hybrid silica with a pore size of 300 Å. The new phase complements a 300 Å C4 surface chemistry already introduced. The company states that the column is designed to separate proteins, peptides, and oligonucleotides, and is particularly suited for biomolecules with a molecular weight >10,000 Da. The unique bonding and surface chemistry provides a wide usable pH range and high temperature durability.

**Accessories**

Accessories are important products that facilitate, and often enable, liquid separations. Products introduced in the past year that fit this category are listed in Table VI. DWK Life Sciences released a line of user-filled gel filtration columns under the brand name Chromflex. The reusable standard or jacketed columns range in volumes from 12 mL to 2 L. The borosilicate glass barrel offers purity and strength with flangeless high-density polyethylene (HDPE) end fittings. Accessories such as flow adapters, bed support frits, valves, fittings, and tubing adapters are available according to the company.

Optimize technologies launched several additions to its prefilter and connection lines of products. The EXP2 Stem Filter is described as a slim device ideally suited for the protection of expensive UHPLC columns, injectors, autosamplers, and mass spectrometer (MS) electrospray tips, without added extracolumn effects. The device may be hand-tightened, which seals up to 8700 psi, or wrench-tightened, to withstand pressures up to 20,000 psi. The company’s new EXP2 Filters provide added protection at back pressures up to 20,000 psi, with a redesign that offers more cost-efficient cartridges. In the category of connection devices, the company released EXP2 Ti-Lok and EXP2 Ti-Lok All-In-One (AIO) fittings.

### TABLE VI: Accessories for liquid chromatography

<table>
<thead>
<tr>
<th>Company</th>
<th>Product Name</th>
<th>Product Description</th>
<th>Comments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DWK Life Sciences</td>
<td>Chromaflex</td>
<td>User-filled gel filtration columns</td>
<td>Reusable standard or jacketed user-packed gel filtration columns for manual or automation-compatible applications.</td>
</tr>
<tr>
<td>Optimize Technologies, Inc.</td>
<td>EXP 2 Stem Filter, EXP 2 Filter, EXP 2 Ti-Lok fittings and adapters, EXP 2 AIO fittings</td>
<td>Filter systems and connection devices for HPLC and UHPLC equipment</td>
<td>Slim architecture, hand-tight or wrench tight fittings, filters and adapters.</td>
</tr>
<tr>
<td>Waters Corporation</td>
<td>IonHance Difluoracetic Acid</td>
<td>MS-grade mobile phase additive</td>
<td>MS-grade mobile phase additive that can be used for LC and LC-MS techniques, purified to achieve low metal content and high-quality standards.</td>
</tr>
</tbody>
</table>

*Comments supplied by vendors
The first mentioned product is noted by the company as ideal for making HPLC and UHPLC connections that easily fit into tight spaces. The fittings feature built-in PEEK ferrules and threaded nuts precision machined from titanium. The device allows for repeated use, adjustment of the tubing to different port depths, and includes a removable, knurled torque driver for hand tightening and loosening. When more room is available for making connections, the company recommends the all-in-one (AIO) format, where the driver is permanently fixed.

Finally, Waters Corporation released IonHance Difluoroacetic Acid mobile phase additive. The MS-grade mobile phase additive can be used for liquid chromatography (LC) and LC–MS techniques. The product is reported to be purified to achieve low metal content and high-quality standards. The company notes that the product improves sensitivity, retention, and peak shapes, and is quality-control tested, to ensure low metal content and high purity. The material is available in approximately 1 mL quantities in chemically resistant vials to maintain high levels of purity.

**Conclusions**

Liquid chromatography columns that were introduced this past year were largely product line extensions or alternatives to offerings existing on the market. One trend of introducing alkyl phases with alternative selectivity that has been noted in the past several yearly reports continued in 2020 (C18/PFP mixed ligands and charge-modified C18 phases). The most striking learning from the effort this year, however, was the drastically reduced number of products released. The decline was observed in the number of companies that reported product launches, as well as in all categories generally used to parse and discuss interesting aspects of the new releases. It will be interesting to see if the trend continues. What it may take is a breakthrough technology or a new market trend to emerge. The development of SPP architecture, for example, drove LC product development for several years following its introduction. Another recent product driver was the migration of the pharmaceutical market toward biotechnology. This resulted in many new product lines based on larger pore particle technology, as well as the resurgence of techniques like IEC and SEC. Is there another innovation or market driver on the horizon?

**Acknowledgments**

Products reviews such as the present work would not be possible without the contributions and cooperation of the manufacturers that have responded to the LCGC survey. Your effort is greatly appreciated. Although LCGC has made every attempt to include every submission in the series of review articles, it is possible that some have been missed. If there have been omissions, or if you want to be sure to be included in the 2021 review series, please contact Laura Bush, Editorial Director, LCGC North America at lbush@mjlifesciences.com.

**References**


**ABOUT THE AUTHOR**

David S. Bell is a director of Research and Development at Restek. He also serves on the Editorial Advisory Board for LCGC and is the Editor of “Column Watch.” Over the past 20 years, he has worked directly in the chromatography industry, focusing his efforts on the design, development, and application of chromatographic stationary phases to advance gas chromatography, liquid chromatography, and related hyphenated techniques. His main objectives have been to create and promote novel separation technologies and to conduct research on molecular interactions that contribute to retention and selectivity in an array of chromatographic processes. His research results have been presented in symposia worldwide, and have resulted in numerous peer-reviewed journal and trade magazine articles. Direct correspondence to: LCGCEdit@mmhgroup.com

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New HPLC Systems and Related Products Introduced in 2019–2020: A Brief Review

This installment describes new high performance liquid chromatography (HPLC) and mass spectrometry (MS) instruments, chromatography data systems (CDS), and related products introduced at Pittcon 2020 or during the prior year. It summarizes their significant features and user benefits.

Michael W. Dong

The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon) is one of the world’s largest conferences on laboratory science. The 71st Pittcon was held at McCormick Place in Chicago from February 29 to March 5, 2020, a week before the semi-shutdowns of Europe and North America from the spreading Covid-19 virus. Chicago has been the most frequent venue for Pittcons held in Chicago in 2014 and 2017, in a three-year rotation. Chicago is an industrial, agricultural, financial, transportation, and communication center, as well as the third largest city in the United States. The city has 2.7 million residents, and the Greater Chicago area boasts a population of 9.5 million. Chicago is home to 69 of the 1000 most prominent companies in the United States, which include Boeing, Abbott Laboratories, Caterpillar, and Kraft Foods. Nevertheless, one potential issue for this location is the unpredictable weather of this midwestern city near the Great Lakes in early March. We were lucky this year with moderate temperatures and no precipitation, though the meeting attendance was reduced because of travel bans related to Covid.

The technical program remained strong this year with more than 200 technical sessions, plenary lectures, and invited, contributed, or award symposia, as well as workshops, posters, and networking sessions, and approximately 90 short courses. The 3-day exposition, however, was visibly smaller than in the past, with the number of exhibitors from the United States dropping from 630+ in prior years to about 400. The number of international exhibitors was also dramatically reduced due to the travel bans from China and many European countries (1).

The technical program remained strong this year with more than 200 technical sessions, plenary lectures, and invited, contributed, or award symposia, as well as workshops, posters, and networking sessions, and approximately 90 short courses. The 3-day exposition, however, was visibly smaller than in the past, with the number of exhibitors from the United States dropping from 630+ in prior years to about 400. The number of international exhibitors was also dramatically reduced due to the travel bans from China and many European countries (1).

Table I lists new products, ordered alphabetically by vendor, that were introduced at Pittcon 2020 or in the prior year, followed by descriptions and commentaries of each product, categorized as systems, modules, CDS, MS, software, or accessories.

HPLC and UHPLC Systems and Line Extensions
New UHPLC systems introductions have slowed in the last couple of years as manufacturers have appeared to focus more on HPLC line extensions and customized systems for specific applications (1).

Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector
Agilent offers a new extension to its InfinityLab Purification Solutions with the Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector. This instrument combines analytical and preparative tasks in one instrument, to allow analytical-scale compound isolation through preparative-scale purification from a few milligrams to multiple grams of material. It has the unique capability to sample from any position of the fraction collector and allow purification and fraction reanalysis to be combined and homogenized before rejections.

Knauer Automated Quality Control of LC Columns
Knauer introduced a dedicated HPLC system capable of generating test certificates automatically for HPLC and fast protein LC (FPLC) columns at a column manufacturing facility.
### TABLE I: New HPLC and MS instruments, chromatography data systems, and related products for 2019–2020

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<td>An integrated online MS analyzer for fresh and spent media analysis in the biopharma industry</td>
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<td>ACD/Labs</td>
<td>ACD/method selection suite</td>
<td>New features include the generation of robust methods in silico and capability to predict the impact of method changes.</td>
</tr>
<tr>
<td>Agilent</td>
<td>1290 Infinity II preparative open-bed sampler/collector</td>
<td>An extension to InfinityLab Purification Solutions to allow analytical-scale compound isolation to multiple grams of material</td>
</tr>
<tr>
<td></td>
<td>InfinityLab LC companion</td>
<td>A mobile LC user interface to allow for remote control, monitoring, signal plotting, and diagnostics of Agilent 1260 and 1290 Infinity II LC systems</td>
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<td>InfinityLab LC–MS iQ</td>
<td>A compact mass selective detector (MSD) for HPLC with auto data acquisition and maintenance to track instrumental health</td>
</tr>
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<td>6495C TQ LC–MS</td>
<td>Agilent’s most sensitive triple-quadrupole MS system with easy maintenance to reduce downtime</td>
</tr>
<tr>
<td></td>
<td>6546 LC/Q-TOF</td>
<td>This quadrupole-time-of-flight MS system has an accelerated workflow with high resolution and wide dynamic range to deliver data for quick review.</td>
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<td>Antec Scientific</td>
<td>Decade elite electrochemical detector (ECD)</td>
<td>This ECD is compatible with UHPLC and micro-LC systems for sensitive detection of neurotransmitters, carbohydrates, and pharmaceutical compounds.</td>
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<td>ARC (Activated Research Company)</td>
<td>Solvere carbon selective detector</td>
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<td>Clarity</td>
<td>DataApex 8.2</td>
<td>A new version of web-based and 21 CFR part 11 compliant CDS used by many smaller instrument manufacturers</td>
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<tr>
<td>Cornerstone Scientific</td>
<td>SolvFil disposable filter degassers</td>
<td>A disposable, polypropylene device with 90-mm membranes for filtering HPLC mobile phases directly into solvent reservoirs</td>
</tr>
<tr>
<td>Exum Instruments</td>
<td>Massbox</td>
<td>The Massbox couples TOF MS with solid sampling using laser ablation</td>
</tr>
<tr>
<td>IDEX</td>
<td>Film degasser</td>
<td>A flat film Teflon AF-based membrane online degasser for removal of dissolved air from typical HPLC mobile phases</td>
</tr>
<tr>
<td>Elga LabWater</td>
<td>PureLab Quest</td>
<td>The Quest is a water purification unit that produces Type I, II, and III water with a small footprint and low lifecycle costs.</td>
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<td>Knauer</td>
<td>Automated quality control of LC columns</td>
<td>A dedicated HPLC system capable of generating test certificates for LC and PPLC columns at a column manufacturing facility.</td>
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<td>Optimized Technologies</td>
<td>EXP2 All-in-One Ti-Lok</td>
<td>A hand-tight UHPLC fitting with integral ferrule capable of connections up to 18,000 psi</td>
</tr>
<tr>
<td>Pickering Laboratories</td>
<td>Onyx PCX</td>
<td>An optimized post-column derivatization system with pulseless dual-syringe reagent pumps for many regulated analyses</td>
</tr>
<tr>
<td>S-Matrix</td>
<td>Fusion QbD 9.9.0</td>
<td>This new version offers features in PeakTracker with MS, an enhanced resolution response map, and support for forced degradation studies.</td>
</tr>
</tbody>
</table>

Table I Continued on Page 224
A crucial role for any laboratory is ensuring data quality and data integrity. To discuss the complexities of data integrity and the principles of good data process design, *LCG* sat down with Monica Cahilly, president and founder of Green Mountain Quality Assurance.

**LCG**: In your opinion, how much have data integrity requirements changed over the past five years, and what was the focus before compared to what it is now?

**Cahilly**: Data integrity requirements have not changed; they have remained the same for as long as regulations for medical devices, finished pharmaceuticals, biopharmaceuticals, and all other commodities regulated by health authorities have been in place. Fundamentally, per regulations, we must be able to fully reconstruct and evaluate data to provide objective evidence of the conduct of scientific work. What has changed is our understanding of how to apply those requirements to the choices we currently make.

For example, people understood that original paper records needed to be reviewed. And that, if someone made a cross-out or data correction on the paper, the reviewer also had to examine the cross-out to evaluate whether the change had an impact on the overall decision-making for that data set. What wasn’t well-understood when computers were implemented in the GxP-regulated industry, was that they generated electronic data sets that were, in fact, original records, and the same requirements for paper records applied to those original electronic records—i.e., they must be retained in a complete manner and are subject to review by a second person. The reviewer must also be able to examine any “cross outs” in electronic data sets.

Regulators and many system developers refer to these as computer-generated audit trails that help reconstruct who did what, when, and why.

The majority of the current focus on data integrity has to do with these historical “blind spots,” where an outdated control strategy did not help govern the choices we made. Now we are trying to undo these blind spots to gain some of the efficiencies and effectiveness in regard to the choices we are making, such as the use of computerized systems. At the end of the day, what matters most are the subjects on the receiving end of our actions. We want to make better and better decisions, because those decisions affect our subjects.

**LCG**: With respect to a vendor’s data system, where does the responsibility for data integrity end for the vendor and start for the customer?

**Cahilly**: First, it depends on the type of vendor. A cloud service provider is doing GxP-regulated work and has to follow GxP regulations. Working with a cloud provider is a different type of vendor-customer relationship. It is similar to the relationship between a sponsor company and a contract research organization. Both parties are, in fact, governed by GxP regulations and each must adhere to those regulations with the sponsor having ultimate accountability. Suppose instead a customer purchased a vendor data system, such a chromatography data system, brought it in-house and “validated the
system for its intended use.” This is an expression used by regulators to draw the line between vendor responsibilities and intended-user responsibilities with an emphasis on the latter. In that scenario, a company would verify a vendor’s qualifications, i.e., its software quality-assurance lifecycle.

A company must ensure that a vendor adheres to good design control principles and provides the features necessary for good documentation practices for electronic data, such as those expressed in FDA Regulation 21 CFR Part 11 for electronic records and signatures. Making sure the vendor has implemented those technical features is the first step. Then, after purchasing and installing the software, a company must confirm those features are configured appropriately—that is not the vendor’s responsibility. A company must also make sure that SOPs and training programs are in place to govern the work at their site.

LCGC: As a laboratory or business updates its software, what must it be aware of in terms of data integrity?

Cahilly: That is an important question, particularly in light of current cybersecurity risks. Historically, in GxP environments, people have been hesitant to implement software patches or updates because of implications to their validation package. People are concerned about incurring additional validation work through change control, affecting other software that is interfaced with a particular software system, or consequences in terms of how data are stored, given the way the update will behave with the operating system.

Those are real and legitimate concerns, but at the same time, if we are not vigilant about security patches and keeping them updated, our systems may be vulnerable to cyberattack. There have been recent challenges in the GxP-regulated industry with ransomware attacks that hold data hostage, which no laboratory wants to suffer.

It is important to step back, take a hard look at our quality systems, and ask ourselves if they are too burdensome. Do they allow for agile and flexible change management? If not, how can we modernize quality systems to make them more agile, flexible, and scalable? Is there a way to test these updates? In an ideal world, we would do as much testing as possible. However, we have to strike a balance between how much testing is done before implementing an update and the posed risks if we choose not to implement it, assuming the update is to patch a critical vulnerability in the system.

LCGC: Why is it important to have multiple reviewers to ensure data integrity?

Cahilly: This is a data entry requirement going back to the inception of GxP regulations. It is based on the principle that humans are prone to errors. The work we do in the GxP-regulated industry is so important; it potentially affects the lives of consumers. So, as humans, we must have a risk-reduction strategy such as having a second person review our work.

Historically, when completing a laboratory worksheet on paper, a second reviewer checked for any mistakes and ensured that proper procedures or analytical methods had been followed. They also looked for anything requiring additional investigation, e.g., a deviation from procedures or analytical methods or an outlier in the data set. In some instances, such deviations could signal falsification of data.

In any case, a second person is needed to review data for mistakes, intentional or not. In my experience, the vast majority of data integrity issues are not intentional. Most people try to do the best they can. But we are all human, and we all make mistakes. It is nice having a second pair of eyes to look over our work and help catch mistakes before they could potentially have a negative impact.

The majority of the current focus on data integrity has to do with historical ‘blind spots’, where an outdated control strategy did not help govern the choices we made.
### TABLE I: New HPLC and MS instruments, chromatography data systems, and related products for 2019–2020 (continued)

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</tr>
</thead>
<tbody>
<tr>
<td>Sciex</td>
<td>Triple Q-TOF 6600+ system</td>
<td>A low-flow LC-MS instrument with ultrafast scanning acquisition and high-resolution data</td>
</tr>
<tr>
<td>Shimadzu</td>
<td>Nexera series LC-40</td>
<td>The ultracompact system incorporates innovative features of many auto functions, mobile phase and maintenance monitoring, and remote user control and service diagnostics.</td>
</tr>
<tr>
<td></td>
<td>Anion suppressor ion chromatograph (IC)</td>
<td>A compact 3000-psi PEEK-based IC system for quantitative analysis of anions</td>
</tr>
<tr>
<td></td>
<td>LCMS-9030 Q-TOF</td>
<td>A new high-resolution Q-TOF MS system with a 3-m internal flight path that supports many ionization sources</td>
</tr>
<tr>
<td></td>
<td>MALDImini-1 digital ion trap (DIT) MS.</td>
<td>This compact bench-top ion trap MS allows the user to check MS results right next to the sample preparation area.</td>
</tr>
<tr>
<td>Thermo Scientific</td>
<td>Vanquish Core HPLC</td>
<td>A new 700-bar HPLC system for routine analysis with features to enhance productivity, including fully automated solvent monitoring and integrated health checks</td>
</tr>
<tr>
<td></td>
<td>Vanquish UHPLC online 2D-LC</td>
<td>Now supports two independent workflows and several standardized 2D-LC configurations</td>
</tr>
<tr>
<td></td>
<td>An automated peptide mapping system</td>
<td>A smart digest automation system capable of reproducibility of ~3%</td>
</tr>
<tr>
<td></td>
<td>Orbitrap Exploris 480 MS</td>
<td>Includes advanced capabilities, day-to-day reliability, and a compact footprint for high-throughput protein identification, quantitation, and structural characterization</td>
</tr>
<tr>
<td></td>
<td>Orbitrap Eclipse Tribrid MS</td>
<td>Designed for a wide range of applications from small molecules to intact proteins for performing qualitative and quantitative analyses at low to high flow rates</td>
</tr>
<tr>
<td></td>
<td>Chromeleon 7.3 CDS</td>
<td>This new version offers enhanced features for the laboratory and the information technology department.</td>
</tr>
<tr>
<td>Waters</td>
<td>Select series cyclic ion mobility separation (IMS)</td>
<td>This IMS combines acyclic flight path design and a TOF MS that supports resolution &gt;100,000.</td>
</tr>
<tr>
<td></td>
<td>Synapt XS</td>
<td>A tribrid (Q-IMS-TOF–MS) system with extended pathlength and improved sensitivity and flexibility.</td>
</tr>
<tr>
<td>Wyatt Technology</td>
<td>Dawn, miniDAWN and microDAWN multi-angle light scattering (MALS) systems</td>
<td>A new generation of MALS with updated optical, electrical, and mechanical components and enhanced interfacing for ease-of-use.</td>
</tr>
<tr>
<td>Young In Chromass (YL Instruments)</td>
<td>ChroZen UHPLC</td>
<td>A slimline UHPLC system with a binary pump (18,800 psi), equipped with UV-vis or PDA detector (2.4-µL flow cell), autosampler, and column oven (4 to 90°C, three 15-cm columns</td>
</tr>
</tbody>
</table>

**Shimadzu Nexera Series LC-40**

Shimadzu Nexera Series LC-40 employs concepts of artificial intelligence (AI) and the internet of things (IoT) to incorporate innovative features such as auto-start, auto-purge, auto-shutdown, flow pilot, mobile phase and maintenance monitoring, multiplexing with dual injectors, and remote user control and service diagnostics. The ultracompact system consists of these modules: a system controller SCL-40, CBM-40; an absorbance detector, SPD-40 / SPD-40V; and a photodiode detector, SPD-M40; a solvent delivery unit LC-40 series (XS, XR or X3, with pressure rating of 80, 105, and 130 MPa respectively); an autosampler SIL-40 series/plate changer; and a column oven, CTO-40 series.

**Shimadzu Anion Suppressor Ion Chromatograph (IC)**

Shimadzu’s entry into the ion chromatography market is a compact and low-dispersion 3000-psi PEEK IC system designed for quantitative analysis of anions. It is controlled by the LabSolutions CDS with auto-shutdown, data processing, and report generation.
Thermo Scientific Vanquish Core HPLC System
Thermo Scientific introduced the new 700-bar HPLC system to complement its family of Vanquish HPLC instruments (Flex, Duo, and Horizon with pressure ratings ranging from 1000 to 1500 bar). The Vanquish Core HPLC system targets routine analysis and quality control laboratories with a selection of quaternary, binary, dual-gradient, or isocratic pumps paired with a full line of detectors including UV-vis, diode array, fluorescence, charged aerosol, and MS detectors. The Vanquish Core HPLC system integrates SmartInject with additional intelligent features such as a fully automated solvent monitor and integrated automatic system health checks. Transitioning to this system from other HPLC systems is made simple with customizable injection programs, a fully tunable gradient delay volume, and enhanced method translation or transfer tools.

Thermo Scientific Vanquish UHPLC Online 2D-LC
This customizable 2D-LC system supports two independent workflows and several standardized 2D-LC configurations. It can also function as two independent HPLC without manual replumbing.

Thermo Scientific Automated Peptide Mapping
Thermo Scientific introduced an automated peptide mapping system capable of performing online digestion of a protein sample with reproducibility of ~3 %.

Young In Chromass ChroZen UHPLC System
Young In Chromass (YL Instruments from Korea) introduced a slimline ChroZen UHPLC system with a binary pump (18,800 psi), equipped with UV-vis or photodiode array detector (2.4 µL flow cell), autosampler (injection volumes up to 10 µL), and column oven (4–90 °C which accommodates up to three 15-cm columns). It can be controlled by Clarity’s DataApex CDS.

HPLC Modules
Antec Scientific Decade Elite Electrochemical Detector (ECD)
Antec Scientific introduced an electrochemical detector (ECD) that is compatible with UHPLC and micro-LC for the selective and sensitive detection of neurotransmitters, (poly)phenols, carbohydrates, and many pharmaceutical compounds. It is capable of a wide linear dynamic range of six orders of magnitude.

ARC (Activated Research Company) Solvete Carbon Selective Detector
The Solvete carbon selective detector from Activated Research Company (ARC) is a universal detector using a flame ionization detector that converts compounds with carbon atoms to methane for HPLC or UHPLC applications. It is capable of a linear dynamic range of 5 orders of mag-
The marketing landscape for mass spectrometry (MS) instruments has been described elsewhere (1). There is no slowing down of new MS introductions this year as manufacturers continued to upgrade their product offerings in high-resolution, hybrid, and trypsin MS—such as time-of-flight (TOF), quadrupole-TOF (Q-TOF), and orbital trap systems. Also, there are more offerings in compact MS instruments with unit resolution such as single-quadrupole (SQ), triple-quadrupole (TQ), and ion mobility MS (IMS) (1,3). There are also newer startup companies focusing on unique application systems such as analyzers for fermentation media or solid sampling.

**908 Devices Rebel**
The Rebel from 908 Devices is an integrated online MS analyzer for fresh and spent media analysis in the biopharmaceutical industry. It is capable of a 7-min assay of 32 analytes (amino acids, bioamines, vitamins, dipeptides) with a 10-µL sample volume. The system is designed for good manufacturing practices (GMP) compliant and can include automated sample preparation steps such as filtration and dilution.

**Agilent InfinityLab LC–MSD iQ**
One of three new MS systems introduced by Agilent Technologies in 2019–2020, the iQ is a compact single-quadrupole mass selective detector (MSD) for HPLC with auto data acquisition and reporting in auto-acquire mode, which automatically establishes optimal MS parameters with automatic tuning. The instrument can also help with maintenance tasks through its ability to track instrument health. It can be controlled by OpenLab CDS or a simpler open-access software. It is designed for small-molecule drug development and quality control, food and materials laboratories, and academic, chemical, or food industries. The iQ has a mass range of 2 to 1450 m/z, ~1 pg sensitivity, a maximum scan speed of 10,400 Da/s, unit resolution, and mass accuracy of 0.13 Da.

**Agilent 6495C TQ LC/MS**
The 6495C triple quadrupole (TQ) is a third-generation design as Agilent’s most sensitive TQ-MS instrument with easy maintenance to reduce downtime. The system is ideally suited for peptide quantitation as well as applications that require part-per-trillion sensitivity. It has a mass range to 3,000 m/z allowing flexibility to handle any MRM transition.

**Agilent 6546 LC/Q-TOF**
This quadrupole time-of-flight (Q-TOF) MS instrument has an accelerated workflow with high mass resolution and wide dynamic range to deliver high-quality data for quick and simplified data review. With a mass resolution over 60,000 (for high masses) and over 30,000 (for low masses), sub-ppm mass accuracy, and isotope fidelity within 5%, it can provide quick answers for complex samples in metabolomics research, food safety, food authenticity, and environmental screening.

**Shimadzu MALDImini-1 Digital Ion Trap (DIT) MS**
The MALDImini-1 is a compact benchtop ion trap MS system that allows the user to check MS results right next to the sample preparation area. The system’s digital ion trap uses rectangular wave radio frequency to enable ion trapping up to 70,000 Da. Furthermore, the MS/MS and MS3 functionality of the DIT allows researchers to perform comprehensive structural analysis.
Thermo Scientific Orbitrap Exploris 480
The new Orbitrap Exploris 480 is designed to ensure sound data with high-resolution accurate-mass (HRAM) selectivity (resolution up to 480,000), high scan speed (up to 40 Hz), and high mass spectral quality, all within a compact footprint. The standard mass range for the instrument is m/z 40–6000 and up to m/z 8000 with the BioPharma option.

Thermo Scientific Orbitrap Eclipse Tribrid
This newest Orbitrap Tribrid MS platform includes advanced ion management technology (AIM+) with the new QR5 segmented quadrupole mass filter, real-time search, enhanced vacuum technology, optional proton transfer charge reduction (PTCR), and optional high mass range MS^n (HMR^n) mode. Collectively, these features make this instrument uniquely suited for accurate and high-throughput full-proteome quantitation, characterization of complex mixtures of protein or small-molecule drugs, and deciphering higher-order protein structures.

SCIEX Triple TOF 6600+ System
This TQ-TOF system supports low-flow applications with ultra-fast scanning acquisition and high-resolution MS data.

Waters Select Series Cyclic Ion Mobility Separation (IMS)
This IMS system combines innovative design with a circular 98-cm flight path capable of multiple recycling, and a TOF-MS that supports resolution >100,000 for lipids, oligosaccharides, and other isobaric compounds.

Waters Synapt XS
The Synapt XS is an extended platform of the Synapt line of MS, which a tribrid (Q-IMS-TOF-MS) system with an extended pathlength, improved sensitivity, and flexibility in supporting multiple ion sources and acquisition modes for solving challenging analytical problems.

Chromatography Data System (CDS) and Software Products
The marketing landscape of chromatography data systems (CDS) has been described in an article published in LCGC North America’s December 2019 issue (4). The four leading chromatography manufacturers dominate the global CDS market with their current CDS product offerings of workstations and client-server systems (Waters Empower 3 feature release 5, Thermo Fisher Scientific Chromeleon 7.3, Agilent OpenLab versions 2.4, and Shimadzu LabSolutions version 5.3).

ACD/Method Selection Suite
The new version of this software from ACD/Labs for HPLC method development offers improvements such as the generation of robust methods in silico and the capability of predicting the impact of method changes. The software is based on principles of quality by design (QbD) with multivariate analysis and can utilize databases of archived physicochemical properties.

Agilent InfinityLab LC Companion
This software platform is a mobile LC user interface which allows for remote control, monitoring, signal plotting, and diagnostics of the Agilent 1260 and 1290 Infinity II LC systems. It resides on any mobile device (tablet or smartphone) using a compatible web browser.

Clarity DataApex 8.2 CDS
Clarity introduced a new version of its web-based and 21 CFR Part 11 compliant CDS used by many instrument manufacturers. The CDS offers an improved user interfaces with many new instrument control drivers (up to 600), MS extension, compound search, and options for good laboratory practices (GLP) environments. It provides control and data handling for Advion MS and speciation analysis by PerkinElmer NexIon inductively coupled plasma–MS (ICP-MS) instruments.

S-Matrix Fusion QbD 9.9.0
S-Matrix introduced a new version of its popular HPLC method development software based on the principles of QbD and design of experiments (DoE) as well as new features of PeakTracker (with Waters QDa SQ-MS), an enhanced resolution response map (vs. overlaid graph) for method robustness evaluation, and automation support for forced degradation studies.
Thermo Fisher Scientific Chromeleon 7.3 CDS
Thermo Scientific introduced Chromeleon 7.3 CDS which offers enhanced features for both the laboratory (streamlined user interface with up to 33% higher performance, system health diagnostics for the new Vanquish Core HPLC system, superior auditing, review, query, and support, and improved e-workflow procedures), and the information technology (IT) department (scalable enterprise solutions for global and in-cloud deployment with improved data security and stability) (4).

Other Accessories
Cornerstone Scientific SolvFil Disposable Filter Degassers
A disposable, polypropylene device with 90-mm membranes for filtering HPLC mobile phases directly into solvent reservoirs.

IDEX Film Degasser
IDEX introduced a flat film Teflon AF-based membrane on-line degasser for removal of dissolved air from HPLC mobile phases. It is adaptable to any HPLC system up to 10 mL/min and has either 2 or 4 channels.

Elga LabWater PureLab Quest
Elga LabWater introduced PureLab Quest, a diverse water purification unit that can produce Type I (HPLC), II (reagent preparation), or III (rinsing) water with a small footprint and low lifecycle cost.

Optimized Technologies
EXP2 All-in-One Ti-Lok
The EXP2 is a hand-tight UHPLC fitting with an integral ferrule capable of connections up to 18,000 psi. It has a slim-fit nut and a wing nut for hand tightening.

Summary and Commentary
Pittcon 2020 will be remembered as the chemistry conference which squeaked in just before more severe measures were taken in North America and Europe to combat the spread of the devastating coronavirus Covid-19. Less than a week later, the World Health Organization declared the spreading virus a global pandemic, and U.S. President Trump announced a state of national emergency for the United States. The country was in a semi-shutdown with travel bans, school closings, and indefinitely cancellation of all major public gatherings. The American Chemical Society (ACS) National Meeting in Philadelphia for late March was canceled, and the Analytica Conference in Munich was postponed from March to October. Still, Pittcon did suffer significantly from many cancellations of exhibitors and conferences, resulting in lower attendance and fewer exhibitors.

One disturbing trend unrelated to the coronavirus was the skipping of Pittcon this year by two major manufacturers (Waters and Thermo Fisher Scientific). Although Pittcon, as a premier event for new product introductions, has been declining in size in recent years as a result of competing conferences and other communication channels, it remains the largest general analytical chemistry conference in North America with global draws for buyers and sellers alike. It is my opinion that skipping Pittcon is a drastic and unpopular move in the eyes of many conferees who wish to compare new products on the exhibition floor. The opportunities to meet with friends and colleagues, learn new technologies, and see new instrumentation on the exhibition floor are what inspire many analytical chemists to come back to Pittcon year after year.

This installment summarizes new HPLC and MS products introduced at Pittcon 2020 and during the prior year and describes the pertinent features of these products. Personally, this is my 19th consecutive year of giving Pittcon HPLC short courses, which had a record attendance of 44 this year. My busy schedule included attending symposia, networking sessions, and the exhibition, interspersed with many meetings and social events, such as board or dinner meetings of LCGC and the Chinese American Chromatography Association (CACA), the Separation Community Mixer of the Chromatography Forum of the Delaware Valley, and the Pittcon Party at the Museum of Science and Technology.

In this unusual time of severe disruptions of conferences and travels, we are all hopeful that the world will survive the calamity and that our analytical chemistry community will return to some normalcy for Pittcon 2021 in New Orleans.

Acknowledgments
The author thanks the marketing staff of all manufacturers who provided timely responses to the LCGC questionnaires. The author is grateful for M. Farooq Wahab of the University of Texas at Arlington, Adrijana Torboverska of Farmahem DOOEL, Mengling Wong of Genentech, He Meng of Sanofi, and Michael Heidorn, Joel Stradtner, and Susanne Fabel of Thermo Fisher Scientific for providing useful editorial suggestions and comments. The content of this article stemmed from information and opinions gathered from the open literature, websites, and personal networking and observations at Pittcon 2020 and the year prior, and does not necessarily represent the views of those of LCGC, Pittcon, or any other organizations.

References

About the Author
Michael W. Dong is a principal of MWD Consulting, which provides training and consulting services in HPLC and UHPLC, method improvements, pharmaceutical analysis, and drug quality. He was formerly a Senior Scientist at Genentech, Research Fellow at Purdue Pharma, and 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.
Advancing Nontargeted Analysis of Water

Environmental analysis of water presents many challenges, one of which is the potential presence of unknown contaminants. Nontargeted analysis seeks to address that challenge, but has its own complications. Imma Ferrer, an associate research scientist at the University of Colorado, has spent the last 22 years developing methods for emerging contaminants using liquid chromatography–mass spectrometry (LC–MS) techniques, including nontargeted approaches. Here, she discusses some of her recent research on nontargeted analysis of water, including water from wastewater treatment plants, hydraulic fracturing wastewater, and environmental water samples.

Laura Bush

In a recent study, you used nontargeted analysis of water samples from wastewater treatment plants (WWTPs) to assess whether the treatment methods were effectively removing not only the toxic substance N-Nitrosodimethylamine (NDMA), but also its precursors (1). Why did you undertake this study and why is it important?

NDMA is known to be a carcinogen, and many states have regulations in place for drinking water. Over the course of many years, efforts have been made to understand what causes the formation of NDMA in water treatment plants, and one of the established theories is that some precursor compounds (such as pharmaceuticals and pesticides) have the ability to form NDMA, after source water is treated with chloramines. Thus, to be able to screen for these potential precursors, mainly by high-resolution mass spectrometry (HRMS) techniques, is of essential importance.

What approach did you use to qualify significant environmental analytes of importance?

The approach we used is based on nontargeted screening for a whole variety of compounds. Currently, we have a database of more than 200 compounds, including pharmaceuticals, pesticides, and surfactants. The data gathered by the high-resolution instrument (in this case an LC–quadrupole time-of-flight MS [LC–QTOF-MS]) system is then compared to the database, and possible identification hits are then scrutinized in terms of accurate mass and fragmentation patterns. In my opinion, there is no gold standard for this type of approach. Many papers in the literature have tried to come up with a universal and single way to carry out this step, but there are still a lot of variables that make each approach unique depending on the focus of the study. For example, experience analyzing hundreds of compounds and looking meticulously at their fragmentation data is no substitute for any large database or mass bank information on the web. I can look at mass spectra and quickly identify an analyte without the need for these tools; that saves a lot of time, and people usually do not account for this type of skill.

You also looked at neutral losses of dimethylamine using auto MS-MS. Can you briefly explain this approach?

The idea behind this process was that a precursor compound able to form NDMA will also have a common moiety related to chemical structure. In this case, the dimethyl-amine group, very common in pharmaceuticals and pesticides, would be the one susceptible to be fragmented from a precursor ion. So, by performing MS-MS fragmentation and identifying compounds that have this same group in the chemical structure, we would find out a list of potential NDMA precursors.

At the time of the study, commercial software for this type of screening...
was not available in the instrument. So, we imported the data to MatLab, and looked for the neutral losses that would indicate the presence of precursor compounds with that exact common moiety. This feature is now included in many software packages from instrument manufacturers, so it makes the process easier. That is why it is important that bench scientists like myself collaborate and talk to the big instrument companies to ask them to include those essential tools that make research much easier.

In what databases did you search for matches with your results? How important is the development and maintenance of such databases for nontargeted screening in environmental analysis? Are you involved in any database development yourself? Where might one find information about the available databases?

The development of databases for nontargeted screening is extremely important, in my opinion. I have used a relatively small database based on standards that we have analyzed throughout the years. But there are also commercial ones, such as Chemspider, Comptox-EPA, and PubChem. The main problem is to sort through these huge and extensive databases. Having a link (pathway) between instrument data and databases is the key. These two interfaces have to talk to each other. I just submitted a paper where we use a novel approach that combines MS-MS fragmentation data with a large database. The software we used is called MathSpec (www.mathspec.com), and takes into account not only accurate mass and formula generation, but also fragmentation data and fragmentation pathways. The idea behind it is that each fragment mass has to be interpreted in the context of other masses. What we learned is that good data will lead to successful identifications, even when no standards are available.

What results did you find in terms of the presence of NDMA precursors in treated wastewater treated with reverse osmosis or more advanced treatments? Can those results help us understand how to better manage treatment?

We found out that reverse osmosis and advance treatment processes, such as UV, are very effective to remove NDMA precursors. If no NDMA precursors are present, the formation of NDMA itself will be diminished. So, in general, these are good treatment techniques that can be used to minimize the presence of NDMA in our drinking water, and these results will help to determine what courses of action to take and better manage treatment.

What did you find in terms of the ability to detect NDMA precursors, either using auto MS-MS analysis or through nontargeted analysis using databases?

The approaches we used were successful in identifying a few NDMA precursors. But many of these precursors do not have the ability to form NDMA, or the yield is very small. However, in a previous study, we found the presence of methadone as an important precursor for NDMA. Prior to that date, the focus had been on ranitidine (a common pharmaceutical), so that finding broadened the focus to include other compounds.

How important is the role of nontargeted analysis in water quality investigations? How widely is it used?

Nontargeted analysis is widely used by everyone using HRMS techniques. I would say that is the main reason behind using this type of instrumentation, to be able to see beyond a targeted list of contaminants. As I mentioned above, there are lots of papers dealing with workflows, or universal ways to carry out this approach. Each one is unique, and focused on the type of application the researcher is conducting, and sometimes even on the instrument used. We still have a long way to go to make this approach universal and successful for everybody.

I personally do not like other terms, such as suspect analysis. Your analysis is either targeted (you know what you are looking for), or nontargeted (you are not looking for it, but the instrument can see it). With newer and more accurate instruments, the databases, including accurate mass and fragment ions, will definitely continue to grow and expand.

In another paper, you studied the identification of proprietary compounds, including poly(ethylene glycol)s, amino-poly(ethylene glycol) carboxylates, and amino-poly(ethylene glycol) amines, in hydraulic fracturing wastewater using LC–QTOF-MS with solid-phase extraction (SPE) (2). Why did you choose these analytical techniques?

SPE is one of the most important steps in sample preparation, and yet sometimes gets forgotten. We have been studying hydraulic fracturing waters for over eight years now, and all we had to do is directly inject the sample, because usually the main additives were in very high concentrations. However, there are always those compounds present at very trace levels that are invisible even to the most sensitive instrument, and those compounds can be enhanced by SPE techniques. In this case, we were able to identify new proprietary compounds that we had not seen before. Another issue is the high salt content usually present in this type of samples. This salt usually masks the detection of compounds by mass spectrometry. By using SPE, salt can be removed and detection can be enhanced, and instrument life will also be lengthened.

The reason for using LC-QTOF-MS is to be able to see beyond the list of targeted contaminants, as explained above. Moreover, these compounds were not in any database, so we used
our knowledge in accurate mass and fragmentation to be able to identify them. The instrument sees everything that is ionizable; it is the human eye that has to do the ultimate work of identification.

How is this study different from others that you or others have done on the analysis of hydraulic fracturing compounds in environmental samples?

This study combined the use of SPE and TOF for the analysis of hydraulic fracturing waters, which to date, was one of the first to combine these two techniques for this type of water samples.

What compounds were you able to identify, and with what level of confidence, in terms of their identification or link to hydraulic fracturing? Had these compounds been detected in other studies? Overall, what does this study tell us? What was your most surprising discovery of the compounds you detected, with masses in the range of \( m/z \) 120–986?

The level of confidence in the identification of these compounds was 100%, as we also purchased pure standards and confirmed retention time, accurate mass, and fragment ions (which is consider the gold standard in identification). These compounds had never been detected in other studies related to hydraulic fracturing. In fact, there are no known reports of the addition of these compounds to hydraulic fracturing fluids. The terms the oil and gas industry uses to refer to these compounds are general and do not include specific chemical names for additives. Therefore, their identification is an important step in understanding the chemistry, treatment, and possible toxicity of hydraulic fracturing wastewater.

You recently published a review paper on the occurrence of opioids in environmental water samples (3). For how many years have researchers been conducting such analyses? How many different opioids and their metabolites have been studied? Are there any important gaps in the data so far?

The reason behind publishing a review paper on the occurrence of opioids in environmental waters is the importance of the topic: Each year, thousands of deaths occur as a result of the consumption of these drugs. I also realized that not all opioids were accounted for, and, in fact, only a small group of them had been monitored over the years. One exchange student from abroad came along to spend a few months in our laboratory, and I thought that would be the perfect topic to work on. From what we found in the literature, it seemed that only 20% of the total number of opioids (>200 total) and their metabolites had been studied. One of the reasons for this is the difficulty to find pure standards, so scientists usually choose only a few targeted analytes to study. Our approach was to take advantage of the screening capability of accurate mass analysis to be able to see beyond that reduced group of analytes. In a second study we carried out, we detected the presence of some opioid metabolites that had not been reported to date.

What methods—including both sample preparation and analytical techniques—are most researchers using for these studies, and why? Has the thinking about which approaches are best changed over time?

The methods most researchers are using for these studies involve SPE and LC–MS techniques (both targeted and nontargeted approaches). Depending on what you are looking for, you might choose one approach or another. For example, if looking only at a relatively reduced number of
compounds (say, 20–30 compounds), then a targeted approach with tandem mass spectrometry techniques would be the best in terms of sensitivity (one can see down to the low ng/L most of the time). If a researcher is looking to expand the scope and look also for metabolites or degradation products, then a nontargeted approach using accurate mass techniques would be the ideal method. Lately, in the literature, there is a slight preference for the latter method, because instruments that do accurate mass have become more and more sensitive, so you see your chosen analytes of interest but also other contaminants that might be present in the sample, so that is an added bonus. Also, do not forget, there is always the possibility to perform retrospective data analysis with accurate mass techniques; the data gathered with the instrument will always be there.

What do the data so far show about the occurrence of opioids or transformation products of concern in different classes of water—wastewater, surface water, and drinking water?
The data showed that wastewater is the source of these contaminants in water, as we already knew from past studies. It also revealed that the concentrations in surface water were much lower, but it also showed that some compounds and their metabolites persist in water, and have the potential to end up in drinking water if not treated properly.

What is known so far about the presence of opioid metabolites and transformation products in environmental waters? You have mentioned that a few nontargeted studies have used high resolution accurate mass to determine the transformation products or metabolites. Why is this an important topic for future research?
Pharmaceutical compounds such as opioids are metabolized in the human body and excreted as metabolites. Once they reach surface waters, other processes, such as degradation, might occur and transformation products can be formed as well. These metabolites and transformation products are often omitted from targeted studies, and here is when high resolution mass spectrometry can aid to the detection of these compounds, by doing a full-spectrum scan of the whole sample. Not much is known about the toxicity of metabolites or degradation products, so that is an important topic for future research.

References

ABOUT THE INTERVIEWEE
Imma Ferrer is an Associate Research Scientist at the University of Colorado, in Boulder, Colorado. Her PhD at the University of Barcelona (Catalonia, Spain) focused on liquid chromatography–mass spectrometry (LC–MS) techniques to detect pesticides in environmental samples. She then conducted post-doctoral research with the U.S. Geological Survey at the National Water Quality Laboratory in Denver, Colorado, where she worked on tandem MS techniques to analyze pharmaceuticals in the environment. She then spent five years as an Assistant Professor at the University of Almeria (Almería, Spain), where her main focus was developing advanced LC–MS methodologies to analyze pesticides in food. In 2008, she moved back to the United States, and is currently an Associate Research Scientist and a co-founder of the Center for Environmental Mass Spectrometry at the University of Colorado in Boulder.
All analytical procedures should be fit for their intended use with appropriate measurement uncertainty (precision and accuracy), selectivity, and sensitivity. In this installment of “Data Integrity Focus,” we look at the impact of an analytical procedure on the integrity of data produced in regulated good manufacturing practices (GMP) and good laboratory practices (GLP) laboratories. Within the framework of the Data Integrity Model (1, 2), there is the right analytical procedure for the job at Level 2. The use of an accurate procedure is built on the foundation layer of data governance with management leadership, quality culture, procedures for data integrity, and training. This is applied to getting the right analytical instrument and application software that are qualified and validated respectively. Both levels now need to be applied to the development, validation, and use of any analytical procedure.

Analytical Procedure or Method?
You will notice that the title of this article uses the term analytical procedure, and not analytical method. The reason is that an analytical procedure covers all stages from sampling, transport, storage, preparation, analysis, interpretation of data, calculation of the reportable result, and reporting. An analytical method is a subset of this, and is typically interpreted as the instrumental analysis phase. After a discussion of the applicable regulations and guidance, I will focus on the analytical method portion of a procedure. After all, this is LCGC!

GMP Regulatory Requirements for Analytical Procedures
In 21 CFR 211.194(a), there is the following requirement for analytical methods used in pharmaceutical analysis:

(a) Laboratory records shall include complete data derived from all tests necessary to assure compliance with established specifications and standards, including examinations and assays, as follows: …
(b) A statement of each method used in the testing of the sample. The statement shall indicate the location of data that establish that the methods used in the testing of the sample meet proper standards of accuracy and reliability as applied to the product tested. …

The suitability of all testing methods used shall be verified under actual conditions of use (3).

What does this mean in practice? Any laboratory must know where the validation was carried out so that an inspector can access the data plus any method transfer protocol that was performed with the associated report to show that the procedure works in a specific laboratory. This interpretation is mirrored in EU GMP Chapter 6 on Quality Control, where clause 6.15 states:

Testing methods should be validated. A laboratory that is using a testing method and which did not perform the original validation, should verify the appropriateness of the testing method.

All testing operations described in the marketing authorization or technical dossier should be carried out according to the approved methods (4).

Reinforcing the European requirement, there is also EU GMP Annex 15 on Qualification and Validation, a very generic set of requirements covering all possible processes and equipment, where Section 9.1 notes for test methods:

All analytical test methods used in qualification, validation or cleaning exercises should be validated with an appropriate detection and quantification limit, where necessary, as defined in Chapter 6 of the EudraLex, Volume 4, Part I (5).

However, these regulations give broad direction, but not much detail. What do we need to do to validate an analytical procedure or test method?

GMP Regulatory Guidance for Validation
Currently in GMP, there is ICH Q2(R1) for method validation (6) that outlines the requirements for method validation for quality control (QC) testing. The emphasis in the document is mainly on chromatographic methods of analysis with parameters such as repeatability, intermediate precision, limits of quantification (LOQ), and limits of detection (LOD). There is no mention of method development in the guidance. However, there is an almost ritualistic approach to interpreting ICH Q2(R1): “If it says it, do it.” Therefore, we can find the stupid situation when validating a method for an assay of active component between say 90 and 110% of label claim, that the method also includes determination of LOQ and LOD. Why determine such parameters when the method will never be used near them? It is in ICH Q2(R1)™ always the answer. This is...
mirrored in EU GMP Annex 15, where at first reading all analytical procedures appear to require LOQ and LOD determination. However, the requirement does say “as appropriate.” Does anyone ever engage the brain and think in these situations?

In 2000, the FDA issued a draft guidance for industry on Analytical Procedures and Methods Validation (7) that outlined the FDA expectations for validation. The main problem is that this guidance did not address one of the most critical stages of the whole process: method development. In 2015, the FDA replaced the 2000 draft guidance with yet another draft guidance entitled “Analytical Procedures and Method Validation for Drugs and Biologics” (8), where there is a little, but insufficient, section on method development.

Bioanalytical Method Validation Guidelines

In the bioanalysis field, there are guidances issued by the EMA and FDA. The European Medicines Agency Guideline on Bioanalytical Methods Validation from 2011 states in Section 4.1 (9):

A full method validation should be performed for each analytical method whether new or based upon literature.

The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in a specific biological matrix, such as blood, serum, plasma, urine, or saliva. Moreover, if an anticoagulant is used, validation should be performed using the same anticoagulant as for the study samples. Generally, a full validation should be performed for each species and matrix concerned.

The final version of the FDA Bioanalytical Methods Validation guidance for industry in 2018 contains in the section on Guiding Principles the following selected statements (10):

The purpose of bioanalytical method development is to define the design, operating conditions, limitations, and suitability of the method for its intended purpose and to ensure that the method is optimized for validation.

Before the development of a bioanalytical method, the sponsor should understand the analyte of interest (determine the physicochemical properties of the drug, in vitro and in vivo metabolism, and protein binding) and consider aspects of any prior analytical methods that may be applicable.

Method development involves optimizing the procedures and conditions involved with extracting and detecting the analyte. Bioanalytical method development does not require extensive record keeping or notation…..

While this FDA guidance has started to include method development, it notes that documentation of this work does not need to be extensive. As we shall see later, this is the wrong approach to take, as method development is the single most important phase of an analytical procedure life cycle. Get this right, and the validation and operation of the method are easier to handle than a rushed development and validation. If a rushed approach is taken, then the analysts using the method pick up the tab with variable results and out-of-specification investigations.

In 2019, ICH M10 on Bioanalytical Method Validation reached step 2b and was issued for public consultation. Out of 60 pages, method development receives a scant half a page mention along with the need to include method development in the validation process.

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**TABLE I: C, N, X classification of method variables**

<table>
<thead>
<tr>
<th>Method Variable</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlled (C)</td>
<td>Variable controlled by:</td>
</tr>
<tr>
<td></td>
<td>• Explicit instructions in the analytical procedure</td>
</tr>
<tr>
<td></td>
<td>• Setting the value of an instrument parameter, for example, detector wavelength</td>
</tr>
<tr>
<td>Noise (N)</td>
<td>Variable difficult to control or predict and may vary randomly:</td>
</tr>
<tr>
<td></td>
<td>• Common variations are investigated experimentally, for example, precision</td>
</tr>
<tr>
<td>Experimental (X)</td>
<td>Variable that can varied deliberately</td>
</tr>
<tr>
<td></td>
<td>• Impact is investigated by experiment, for example, stability and robustness</td>
</tr>
<tr>
<td></td>
<td>• Establish acceptable ranges of performance</td>
</tr>
</tbody>
</table>

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**FIGURE 1:** A traditional view of analytical method development, validation, and use.
the lines of FDA and EMA above and noting that:

Bioanalytical method development does not require extensive record keeping or notation (11).

All the emphasis is on the validation, rather than the development of the assay. As we shall see, this is not the smartest approach, especially the majority of bioanalytical methods can be measuring analytes in biological matrices at the LOQ of the method. You really need to know what factors you need to control in the method, rather than hoping for the best.

Traditional View of Development, Validation and Use

Continuing this theme, the traditional view of analytical method development,
validation, and use is shown in Figure 1. The main emphasis is on a rapid method development phase and validation by an analytical development group. This is followed by a formal transfer to a quality control group to demonstrate that the method (possibly) works in their laboratory and then operational use by the QC staff. If changes are required, these need to be validated, and a method may need to be redeveloped in light of experience with use. As we shall discuss at the end of this article, most methods are regulated for the pharmaceutical laboratory, and need regulatory approval for any major change. How can this be simplified?

USP is Changing to a Lifecycle Approach

For 10 years, USP expert panels and committees have been publishing stimuli articles on analytical procedure lifecycle management (APLM). This new approach comes from the FDA’s updated guidance on process validation that took a lifecycle approach to the topic, rather than “three validation batches and all is good.” In addition to the “Stimuli to the Revision Process” articles published in Pharmacopoeial Forum, there is also a draft USP <1220> on Analytical Procedure Lifecycle Management issued for public comment in 2017 (12). At the end of this year, a revised draft of USP <1220> is expected to be published for comment.

The principles outlined in the current draft USP <1220> are a Quality by Design (QbD) approach to method development and validation (12) that is intended to deliver more robust analytical procedures. There is greater emphasis on the earlier phases of the lifecycle of an analytical procedure, such as defining the procedure specification in an Analytical Target Profile or ATP.

The overall process is shown in Figure 2. Although the USP is focused primarily on compendial analytical procedures, the sound scientific principles outlined in the draft USP <1220> are, in my view, applicable to bioanalytical methods as well. Shown also in the figure are the feedback loops from stage 3 to stage 2 and from stage 2 to stage 1, as well as to the ATP, representing continual improvement of the procedure. The key is continual improvement, as the pharmaceutical industry is regulated and some procedures that are part of a registration dossier might need to be modified under change control.

Stages of the Analytical Procedure Lifecycle

The lifecycle of analytical procedure advocated by USP <1220> in Figure 2 consists of three stages:

1. Procedure Design and Development (method development) derived from the ATP
2. Procedure Performance Qualification (method validation)

We are not very good at method development or monitoring performance of an analytical procedure in use. This new approach aims to provide a sound scientific basis throughout the whole analytical procedure lifecycle. We will discuss each stage of the lifecycle in overview; for a more detailed understanding of the USP <1220> process and best practices in analytical procedure validation, the reader is referred to the book by Ermer and Nethercote (13).

FIGURE 3: Method development workflow for an HPLC procedure (13).

Define the Analytical Target Profile

First, we need to define what are the objectives of the procedure and this is achieved by writing an Analytical Target Profile (ATP), as shown in Figure 2. An ATP should be considered the specification or intended use for any procedure. This term was developed by a US and EU pharmaceutical industry working group on Analytical Design Space and Quality by Design of Analytical Procedures, and has been incorporated by the USP into two Stimuli to the Revision Process articles on the ATP, as well as the draft USP general chapter <1220> (12–15).

The Analytical Target Profile (ATP) for an analytical procedure is a predefined objective of a method that encapsulates the overall quality attributes required of the method, including:

- sample to be tested
- matrix that the analyte will be measured in
- analyte(s) to be measured
- range over which the analyte(s) are to be measured for the reportable result
- quality attributes such as selectivity and precision and accuracy of the whole procedure or total measurement uncertainty (TMU).

This is the core of the lifecycle approach, as it defines the high-level objectives with no mention of any analytical technique used to meet the ATP as this could bias the analytical approach.
An example ATP could be:

To quantify analyte X over a range between a% and b% (or whatever units are appropriate) with X% RSD precision and Y% bias in a matrix of Z (or in the presence of Z).

This means that the requirements for an analytical procedure are defined before any practical work begins, or even an appropriate analytical technique has been selected. It provides the method developer with an explicit statement of what the procedure should achieve. This is a documented definition, and can be referred to during development of the procedure or revised as knowledge is gained.

Stage 1: Procedure Design and Development

This is most important part of an analytical procedure lifecycle, but it is missing from or minimal in the current regulatory guidance documents described above.

Knowing how sampling, transport, storage, instrumental analysis parameters, and interpretation of data impact the reportable value is vitally important to reducing analysis variability, and hence out-of-specification (OOS) results. The aim of a Quality by Design (QbD) approach is a well understood, controlled, and characterized analytical procedure, and this begins with the design and development of the procedure.

Knowledge Gathering

From the ATP we need to gather information and knowledge to begin the initial procedure design, such as:

• chemical information about the analytes of interest, such as structure, solubility, and stability (if known)
• literature search (if a known analyte) or discussions with medicinal chemists (if a new molecular entity, or NME).

From this knowledge, coupled with the ATP, the most appropriate procedure including the measurement technology can be derived, such as:

• type of procedure (for example, assay or impurity) in an active pharmaceutical product or determination of an NME in animal or human plasma.
• sampling strategy, such as the sample amount or volume required, how the sample will be taken, any precautions required to stabilize the analyte in the sample, and other factors
• design of the sample preparation process to present the sample to the instrument
• whether there is any need to derivatize the analyte to enhance detection characteristics
• appropriate analytical technique to use based upon the ATP and the chemical structure of the analyte (including, but not limited to, LC-MS, LC-UV, and GC-FID)
• an outline of separation needs based on previous analytical methods with
analytes of similar chemical structure, if appropriate.

In addition, business factors such as time for the analysis and cost should be considered when developing a method. Quicker is better, provided that the ATP is met, and UHPLC may be a better alternative to conventional HPLC, as an example.

Initial Design of the Analytical Procedure
Assuming that we are dealing with a liquid chromatographic analysis, solid samples need to be prepared so that a liquid extract can be introduced into the chromatograph for analysis. Development of the sampling, sample preparation, and separation should proceed in tandem and iteratively as shown in Figure 3. Some considerations for this phase of the development, covering all sample types and concentration or amount ranges defined in the ATP are:

- How much sample is required to achieve the ATP?
- Does the sample have to be dissolved, homogenized, sonicated, or crushed before sample preparation can begin?
- Does the analyte require derivatization either to stabilize the compound, or to enhance limits of detection or quantification?
- Screening experiments are run to see how the analytes run on a variety of columns and mobile phases of varying composition of organic modified and pH value of the aqueous buffers. It is important to note here that the KISS (Keep It Simple, Stupid) principle applies here. Don’t overcomplicate a method, as it will usually need to be established in one or more other laboratories, and unnecessary complexity makes method transfer more difficult.

A better approach for method screening is to automate it, using method development software to design and execute experiments using a statistical design (for example, factorial design such as Plackett-Burman). This is a more expensive option, but it will produce design space maps for optimum separation much faster than a manual approach. These design space maps provide the basis for a robust separation as the factors controlling the separation can be more easily identified and the optimum separation to meet the ATP can be predicted and then confirmed by experiment.

The overarching principle in method development and optimization is to keep the method as simple as possible to achieve the ATP requirements. For example, a commonly available column and simple mobile phase preparation should be the starting point for most separations, depending, of course, on the type of analyte involved. Use isocratic elution to achieve the ATP rather than a gradient, as the latter will increase the overall analysis time.

Risk Assessment and Management
Management of risk is a key element in the analytical procedure lifecycle approach. This involves identifying and then controlling factors that can have a significant impact on the performance of the separation. Such factors may be:

- pH value of the aqueous buffer or proportion of organic modifier used in an LC mobile phase
- type or dimensions of the column used
- autosampler and column temperature
- impact of light during sampling or sample preparation.

A formal risk assessment can be undertaken, such as Failure Mode Effects Analysis (FMEA), to identify the risk with the highest impact (13). The aim of risk assessment is to either mitigate or eliminate the risk posed by variables in the sample preparation, instrumental analysis, or operating practices. Method variables can be classified as controlled, noise, or experimental (C, N, or X), as shown in Table 1. A discussion of how these variables are investigated is outside the scope of this article, and the reader is referred to Ermer and Nethercote’s book for more details (13).

When key variables have been identified, then robustness studies can be started to understand the impact of each one on the overall analytical procedure. There will be a study design for robustness experiments, and the results will be examined statistically. The aim is to identify the acceptable range of each key variable; the greater the range means that the method is more flexible. Again, please see Ermer and Nethercote for more information about this approach (13).

Analytical Control Strategy: Identifying and Controlling Risk Parameters
The analytical control strategy for each procedure is based on the outcome of the risk assessment and, where appropriate, in combination with the robustness studies. This should provide a list of method parameters and variables that have significant impact on the method, and its performance as well as what to avoid when executing the analytical procedure. The outcome is the establishment of controls for critical parameters, such as how to perform a specific task with sufficient detail to ensure consistent performance, the type of integration, conditions in the procedure that have significant effects, or steps to avoid certain situations where outside variables (light, for example) can affect the stability of the analyte.

The outcome of the analytical control strategy is to have a set of instructions that are explicit and unambiguous when executing the procedure, such as:

- how to sample and the required sample size
- specification of sample containers, transport conditions to the laboratory, and storage conditions
- preparation of the sample for analysis
- preparation of reference standard solutions and mobile phases
- performance of the analysis, as well as integration and interpretation of data
- calibration method used
- identification of the system suitability test (SST) parameters to be used, and determination of the acceptance criteria for each one.

Procedure Development Report
The outcome of Stage 1 should be a comprehensive method development report describing the optimized procedure. It should also contain practical details for the procedure, including the robustness of the
analytical procedure, the analytical control strategy and the SST parameters to be used, and their acceptance criteria.

This is in stark contrast with the FDA bioanalytical method and draft ICH M10 guidance documents that suggest that bioanalytical method development does not require extensive recordkeeping or notation (10,11). If you don’t have any understanding of how critical parameters impact the performance of an analytical procedure, then how can you control them? In my opinion, method development needs a report that highlights those key parameters, and how they impact performance of the procedure. It is good analytical science, and an essential reference for all further work.

**Stage 2: Procedure Performance Qualification**

**Planning the Validation**

Procedure Performance Qualification (PPQ) or method validation should be simply confirmation of good method development and demonstrate that the analytical procedure is fit for purpose. PPQ demonstrates that the developed analytical procedure meets the ATP quality attributes, and that the performance is appropriate for the intended use. To control the work, there will be a validation plan or protocol describing the experiments to be performed, with predefined acceptance criteria to demonstrate that the ATP has been met. This will depend on the type of procedure, such as active pharmaceutical ingredient (API), impurities, or bioanalytical. The various experiments will depend on the criteria described in the ATP, and on the intended use of the procedure. For example:

- **Linearity experiments should be used to support the use of the specific calibration model used in the procedure (the calculations for which have been verified in Level 1 in the computerized system validation of the data system used for this work).**
- **Specificity or selectivity (depending on whether the instrumental technique is absolute or comparative) must be determined, including resolution for impurities and peak purity assessment for stability-indicating methods.**
- **Precision (injection precision, repeatability, and intermediate precision) should be set. The minimum number of runs could be two, but four or more provides better understanding of the intermediate precision for routine use.**
- **Accuracy can be run in the same experiments as precision.**
- **Analyte stability under storage, laboratory, and instrument conditions must be determined.**
- **System suitability test parameters, and their acceptance criteria, will be verified during this work.**

It is important that the acceptance criteria be defined in the validation plan, and are based on the information gathered from Stage 1, the procedure design and development. The plan will also define how the data from the various experiments will be evaluated statistically against the acceptance criteria.

**Validation Report**

Once the work is completed, a report is written that describes the outcome of the validation experiments and how the procedure meets the requirements of the ATP. As the draft USP <1220> (12) notes:

> The analytical control strategy may be refined and updated as a consequence of any learning from the qualification study. For example, further controls may be added to reduce sources of variability that are identified in the routine operating environment in an analytical laboratory, or replication levels (multiple preparations, multiple injections, etc.) may be modified based on the uncertainty in the reportable value.

The scope and the various parameters with the acceptance criteria for a bioanalytical method validation report are defined extensively in the updated FDA Guidance for Industry on Bioanalytical Method Validation and the draft ICH M10 guidance documents (10,11).

**Analytical Procedure Transfer**

Analytical method transfer is not always easy or straightforward, because there are always items that are not well described in, or even omitted altogether from, an analytical procedure. Well-documented method development (if existing) and validation reports will aid the transfer process immeasurably. The transfer must be planned, and a protocol developed, between the originating and receiving laboratories that includes predefined ways that the data will be interpreted with acceptance criteria. A report should be produced summarizing the transfer results against the data generated by the receiving laboratory.

To reduce the effort required when transferring an analytical procedure to another laboratory, a subject-matter expert could travel to the receiving laboratory to provide help and advice. Alternatively, an analyst from the receiving laboratory could go to the originating laboratory to learn the procedure. Management often looks at the up-front cost of this, but dismisses the hidden cost of time wasted in transferring the method without help from the originating laboratory.

When considering method transfer, one of the issues when using a contract research organization (CRO) laboratory is the quality of the written procedure used for method transfer. Often the originating laboratory (sponsor) may make a minimal effort at validation before passing the procedure to a CRO to complete the development and validation. This is not the best approach, and is planning for failure.

**Stage 3: Procedure Performance Verification**

Routine monitoring of an analytical procedure’s ongoing performance is an important element in maintaining control over the analytical procedure in operational use. It provides assurance that the analytical procedure remains in a state of control throughout its lifecycle, and provides a proactive assessment of a procedure’s performance. The aim of verification is that the reportable result is fit for purpose and can be used to make a decision.

Part of this verification can be trending of SST and sample replicates results...
over time. However, there is a note of caution that SST results can also be used to measure instrument performance directly (Group B and some Group C instruments) or indirectly (some Group C instruments) as part of an ongoing performance qualification. Data that could be collected and tracked are:

• SST test results including failures
• Trending of individual results and the reportable result including OOS and outliers from investigations.

These data should be monitored against limits, so that when there is a trend indicating a parameter is out of control, an investigation can be started early, before the situation gets out of hand (a proactive, rather than reactive, approach). When a root cause is identified in an investigation, it may be appropriate to update the analytical control strategy or to update the analytical procedure.

Pharma Is Going Lifecycle
Why have I discussed this new approach? The pharmaceutical industry is going lifecycle! ICH published in November 2019 a new guidance, ICH Q12, entitled “Technical and Regulatory Considerations for Pharmaceutical Product Lifecycle Management” (16). This document provides a framework to facilitate the management of post-approval Chemistry Manufacturing Controls (CMC) changes in a more predictable and efficient manner and the key concept is Established Conditions (ECs) for analytical procedures:

ECs related to analytical procedures should include elements which assure performance of the procedure. The extent of ECs and their reporting categories could vary based on the degree of the understanding of the relationship between method parameters and method performance, the method complexity, and control strategy (16).

Note that ECs are legally binding, as they will be part of a drug license application and changes will need regulatory approval. ECs are built up during the development and validation process of the lifecycle.

In addition, to further support the analytical procedure lifecycle management (APLM) approach, ICH is undertaking two projects:

• An update and expansion of ICH Q2(R1) to include other analytical techniques with a possible release for public comment of Q2(R2) as early as the end of this year (17)
• ICH Q14 on Analytical Procedure Development, which is beginning to be developed (18).

Common sense would suggest that combining the two into a single document would be the best approach. However, putting common sense and regulatory compliance in the same breath would be a novel idea.

What this means in practice is that the more you know about your analytical procedure, the more predictable the analysis becomes, thanks to the lower variation. There should be a lower regulatory burden to change a registered method. Most importantly, with robust analytical procedures there should be a lower incidence of OOS results attributed to analytical variation and subsequent investigations. OOS will be the subject of an article later in our series.

Summary
A key component for data integrity is accurate and precise analytical procedures that are validated for intended use. Changes in the way procedures are specified, developed, validated, and operated are coming. This “Data Integrity Focus” article should help prepare you for the changes.

Acknowledgments
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Overview of Methods and Considerations for Handling Complex Samples

Complex samples can be challenging, and there are many caveats that need to be considered to mitigate matrix effects. Matrix interferences can be detrimental to analysis, and the contents of a sample need to be evaluated to determine what could affect analysis. Approaches need to be tailored to remove or work around matrix interferences, to produce reproducible, reliable, and accurate methods of analysis. Here, methods of sample preparation, on-line sample treatment, and instrumental tools are outlined, and several examples of applications are discussed. Some general guidance is also outlined on how to evaluate the best option for your complex sample, and how to move forward.

Jamie L. York and Kevin A. Schug

There are many challenges related to complex sample matrices that analytical chemists have to overcome. These challenges can be met by skillful sample preparation, on-line sample clean-up, sample pre-treatment, or the use of instrumental tools. Sample preparation is an evident place to start, and includes methods such as solid-phase extraction (SPE), liquid-liquid extraction (LLE), salting-out, derivatization, filtration, and centrifugation, among many others. Multiple approaches can also be combined, but doing so can quickly become cumbersome for large sample sets. Online sample cleanup can be a welcomed alternative to relieve some of the manual steps and allow automation from the instrument of choice, but is not always a viable option. Lastly, the instrument’s abilities should not be undervalued. Multiple reaction monitoring (MRM) transitions can be very useful to gain specificity when using a triple quadrupole mass spectrometer, but can sometimes fall short when analyzing similar compounds that do not produce unique MRM transitions. Instrumental tools that allow deconvolutions are also possible when chromatography falls short, as in the case of vacuum ultraviolet spectroscopic detection for gas chromatography. In addition to this, capabilities like post-run spectral filters are a valuable tool to highlight certain classes of compounds in a convoluted complex matrix when analyzing by gas chromatography–vacuum ultraviolet spectroscopy (GC-VUV). All of these options for sample preparation, clean-up, and analysis should be taken into consideration when dealing with complex sample matrices, in order to retrieve meaningful, reliable, and reproducible data for the determination of target analytes.

Consider Your Sample

Before deciding on a technique to move forward with method development, it is important to consider what is in your sample, and what could be a possible interference. Consideration needs to be given to whether the analyte can be analyzed by GC, and, if not in native form, if it can be derivatized to be made more amenable to GC analysis. Derivatization can be a useful technique, but unless it can be automated, it is best to be avoided if the sample set is large, to save time and sanity. GC should not be written off too quickly, though, especially when dealing with complex samples. Headspace sampling can be a terrific technique paired with GC to save time on the front end during sample preparation, and, in many cases, no other sample clean-up is necessary. The measurement of ethanol content in blood samples is a great example of this technique and requires no clean-up of the matrix prior to injection (1). There are very few volatile substances that can be in a blood sample that will interfere with the measurement of ethanol, and if an appropriate column chemistry is used in conjunction with headspace sampling, no matrix clean-up is required.

Liquid chromatography (LC) is imperative for samples with higher molecular weight analytes, those that require extensive derivatization, or those that are otherwise non-volatile or thermally labile; these properties make them less amenable to GC analysis. Special attention needs to be given to cleanup of the sample matrix to ensure that the LC column is not compromised, that the lines do not get clogged, or that the system is not dirtied by the complex sample. Precipitation is also a concern for LC, so compatibility between the mobile-phase solvents, mobile-phase additives, and samples should be taken into consideration. Supercritical fluid chromatography (SFC) continues to re-emerge for use over a wide range of sample and analyte types, as many instrument manufacturers continue to improve their offerings; SFC is not covered in significant detail in this work, but it is acknowledged to be an interesting technique to bridge the gap between GC- and LC-amenable analytes. In recent work, online supercritical fluid extraction (SFE) was paired with supercritical fluid chromatography-mass spectrometry (SFE-SFC-MS), to analyze polycyclic aro-
matic hydrocarbons (PAHs) in soil (2). The coupling of these two techniques into one instrument and method allows for minimization of sample preparation, and reduction in loss of sample and sample contamination (3).

Food, environmental, and biological samples are some examples of matrices that can be especially tricky. In the case of food analysis, the USDA Food Composition Databases are a great tool to use when evaluating possible interferences (4). They can provide a general idea of what is expected to be in a sample, as far as components with different physicochemical properties, such as fats, carbohydrates, and proteins. Environmental samples can be challenging because of their non-uniformity, and will need tailored methods to mitigate any interferences, while still achieving as much consistency between sample sets as possible. Biological samples, and in some cases food samples, are plagued with large biomolecules and proteins that can greatly hinder analysis and make instrumentation dirty. In order to move forward, it is best to get a handle on what analytes to target and how to exclude or work around interferences.

Matrix Interferences and the Effect They Can Have on Analysis
Interferences can occur within the sample matrix, and affect the sample analysis in a number of different ways. Matrix effects can mask, suppress, augment, or make imprecise sample signal measurements. This can occur chromatographically, as in the case of coelution, or during ionization, in the case of mass spectrometric detection, and result in highly variable or unreliable data. To correct for matrix effects encountered during electrospray ionization, the use of stable isotopically labeled internal standards is recommended. This is so that the internal standard is nearly perfectly coeluted with the analyte of interest, experiences the same ionization suppression or enhancement as the analyte, and, thus, can be more effectively used to correct analyte response.

An example of this can be seen in previous research where estrogens were detected in phosphate-buffered saline-bovine serum albumin, gelded horse serum, and mouse serum (5). Internal standards were used to compensate for any fluctuation during the sample preparation procedure and ionization, but because deuterated internal standards were used, a deuterium isotope effect was observed, resulting in slightly different retention times between the internal standard...
and target analytes (5). The drawbacks of using an isotopically labeled internal standard can be availability and cost. Often, isotopically labeled internal standards are not readily available or can put a dent in the budget. If your laboratory is equipped, it might be advantageous to synthesize the internal standards. In previous research, a laboratory targeting the quantitation of lysosphingolipid bases was able to synthesize their own carbon-13 (13C) sphingoid bases to use as internal standards for their analysis by ultrahigh performance liquid chromatography electrospray ionization tandem mass spectrometry (UHPLC-ESI-MS/MS) (6).

It is worth noting that, when choosing an internal standard, it is important to find an internal standard that is physicochemically similar to the target analyte, but structurally unique, not present in the samples, and coeluted with your analyte but has unique MS transitions. Nitrogen-15 (15N) and 13C labeled internal standards are often preferred over deuterated standards, to eliminate deuterium isotope effects (7). Deuterium isotope effects, in terms of altered chromatographic retention, will be exacerbated the longer the analyte, and its deuterated internal standard are retained in the column, especially in reversed-phase LC mode.

One concern of sample interferences can also be reactivity, especially in the case of reactive analytes. This can happen when the contents of the sample react with target analytes, and is often not reproducible, and can hurt precision. The best way to alleviate this would be to remove the interference that is reacting, but this can be problematic, because it is not always clear what is reacting, especially in extremely complex or unknown samples. A specific case of this can be seen in the detection of formaldehyde, an extremely reactive analyte, in a sample of shale core and produced water. The formaldehyde originated from a resin-coating applied to a proppant, a hydraulic fracturing additive used in unconventional oil and gas extraction (8). The leaching of formaldehyde from the resin coating on the proppants was tested at laboratory simulated subsurface conditions by

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**FIGURE 3:** Online restricted access media (RAM)-LC settings for mobile phase concentration, flow rate, valve positions, and valve diagrams. From 0 to 6 min the sample is being loaded by pumps C + D onto the RAM column, while the analytical column is being equilibrated with pumps A + B. The analytes are back eluted from the RAM column by pumps A + B from 6 to 9 min and sent to the analytical column. The valves switch and pumps A + B perform the analytical separation from 9-14 min, while pumps C + D wash the RAM column. Finally, at 20.5 min the valves are switched to their starting position and the RAM and analytical columns are equilibrated for the next injection (29).

**FIGURE 4:** Example of isomers of dimethylnapthalene (DMN) coeluted and measured in the 200–220 nm range (blue). Deconvolutions were performed into the respective concentrations for each isomer (green and orange) using manufacturer software. The deconvolved individual contributions in this case are somewhat noisy because of the inherent similarity of DMN spectra. Even so, these isomers can be accurately deconvolved up to a 100:1 relative abundance (34).
heating to subsurface temperatures and with the addition of the shale core and produced water, two components that these additives are likely to contact during hydraulic fracturing (8). When the shale core and produced water were added to the sample matrix, the concentration and sometimes precision in measuring the formaldehyde was diminished, likely due to competing reactions taking place from the matrix (8). An example of the results for these experiments can be seen in Figure 1, where the proppants were left to soak in different matrices for 20 h, and then analyzed for their formaldehyde content (8). These data were obtained with the combined use of derivatization, to "trap" the reactive formaldehyde, in combination with headspace-gas chromatography-mass spectrometry (HS-GC-MS) analysis. In this case, better precision of the HS-GC-MS method was observed compared to a traditional LC-based approach, because all of the reaction chemistry, derivatization, and sampling could be performed in a sealed vial; this limited loss of the volatile analyte (8).

**Sample Preparation**

Ideally, sample preparation should be kept to a minimum to streamline sample throughput, but that is not always a viable option when handling complex samples. There are numerous techniques to choose from that can be implemented; the choices depend on the nature of your sample matrix and analyte.

Solid-phase extraction (SPE) is a sample preparation technique that can be of use in preconcentrating samples, removing interferences, or desalinating samples. This can be especially useful in aqueous environmental matrices, as in the case of detecting nonsteroidal anti-inflammatory drugs (NSAIDs) in drinking water, surface water, and wastewater, where the analytes are present in low concentrations (9). The setup usually consists of a manifold and cartridges that are used to trap and elute analytes. Large volumes of an aqueous sample can be loaded onto a cartridge, and eluted in a smaller volume, to pre-concentrate the analyte. The system can use positive or negative pressure, and a variety of sorbents are available from which to choose.

Solid-phase microextraction (SPME) can be used to extract volatiles and non-volatiles from a liquid or gas matrix. SPME consists of a fiber coated with a stationary phase, liquid polymer, or both, on the end of a plunger of a syringe or needle (10). SPME can be used to sample headspace or direct immersion by headspace sampling. This method of sampling is ideal for offsite sample collection, because it is easily transported to and from the site and back to the laboratory for analysis. An example of the portable capabilities of SPME comes from a recent research article describing a method that was developed to sample a plant’s volatile organic compound profiles. This analysis was performed using SPME on-site and subsequent GC–MS analysis in the laboratory (11). SPME can also be used as a sample cleanup technique. An example of this technique can be seen in a method developed for detecting short chain fatty acids, which are the end products of intestinal bacterial fermentation, from an in vitro gastrointestinal model (12). The model is made up of a very complex chemical composition, but by sampling using SPME, no additional extraction was needed, and only a simple sample treatment was used (12). Both SPE and SPME techniques require special apparatuses including cartridges, manifolds, and fibers that are available from manufacturers and can be somewhat costly. However, the selective extraction attainable through the use of these techniques can be very effective for eliminating unwanted matrix interferences prior to analysis.

Salting out can also be a useful technique for sample preparation. The addition of salts can help reduce the hydration of target analytes and make them more amenable to extraction. Salting out can be used to remove solid particles, fats, waxes, and even DNA from a sample. It can be used in combination with headspace or liquid-phase extraction techniques. Salting out is an integral part of the popular method referred to as QuEChERS (quick, easy, cheap, effective, rugged, safe), which also often includes dispersive solid-phase extraction (dSPE) (13). QuEChERS has been
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implemented in many different applications that would otherwise require more extensive cleanup of the matrix. One such application is the detection of pesticides in food samples, often a very complex sample matrix. In previous work, pesticides were detected in different fruit and vegetable samples using QuEChERS sample preparation followed by analysis with liquid chromatography–tandem mass spectrometry (LC–MS/MS) (14).

Salting out can also be advantageous during sampling when using the headspace technique coupled to gas chromatography, to facilitate partitioning of analytes into the gas phase. This can be performed with the addition of traditional salts (NaCl) or even ionic liquids (15). Liquid-liquid extraction (LLE) can also use salting out, called salting out-assisted LLE (SALLE), and is similar to QuEChERS. In one example from previous research, SALLE was implemented to extract oxytocin in plasma samples (16). These analytes are often difficult, due to their extremely low concentrations and the interferences present in the plasma. By using SALLE, the methods were able to overcome these challenges and obtain lower limits of detection.

Dispersive solid-phase extraction (dSPE) is another sample preparation clean-up technique if water, polar, non-polar, or pigmentation need to be removed from the matrix. As the name implies, in dSPE, solid-phase extraction particles are dispersed in the sample (rather than being used in a column format) and removed by centrifugation. In previous work using QuEChERS and dSPE as a sample cleanup technique, nicotine and its metabolites were detected in catfish, tuna, salmon, and tilapia (17). It was found that the different types of fish required different dSPE components to optimize the methods, but with the optimized methods minimal or no matrix effects were present. The judicious use of salts to aid phase partitioning of desired analytes can be a simple and economical alternative to purchasing additional sample preparation materials.

Various other simple and cost-effective sample preparation techniques can also be performed in the laboratory. Liquid-liquid extraction (LLE) is typically performed with two immiscible solvents, and can be used to extract certain analytes based on their relative solubilities in the two solvents. In one study, LLE was used to target chlorophenols from wastewater (18). In this example, hydrophobic ionic liquids were tested for their extraction capabilities of the targeted class compounds from the water layer. One aspect of using LLE as a sample preparation technique that needs to be considered is the difficulty of performing a multiclass compound extraction. This can be challenging, due to the differing degrees of solubility from compound class to compound class.

Filtering and centrifugation are very important steps in the sample preparation process, especially for LC analysis. Centrifugation can help remove solids or small particulates from a sample, and ensure that an autosampler and system do not become compromised. According to an old adage, “if you can dissolve it, you can HPLC it,” but, if you can’t dissolve it, it is going to clog your system. When trying to remove proteins, precipitation is a quick and easy tool to use. Typically, a chilled organic solvent, such as acetonitrile or acetone, can facilitate the precipitation of proteins, due to their limited solubility in these solvents. Finally, when targeting trace analytes from a complex matrix, concentration by dehydration can be used by drying down the sample with a stream of N₂ and regenerating with a solvent. This process also has the ability to be automated. Overall, many of these methods require materials that most laboratories are likely to already have (centrifuge, filters, glassware, gases, solvents), so they can be quite cost-effective.

Derivatizing the sample is sometimes a necessity. Derivatization is not just a technique to facilitate GC analysis. It can also be performed for LC to make a small molecule (>100 m/z) larger and more amenable for MS, or to give a molecule a UV- or fluorescence-active moiety. (19,20) In previous research, dansyl chloride was used to derivatize the active ingredient of over-the-counter medicines in the United States (21). A method was developed for methamphetamine and amphetamine chiral quantitation in blood plasma. Chiral differentiation of these compounds was achieved by the addition of Marfey’s reagent to derivatize these compounds into distinguishable analytes (diastereomers) by LC–MS/MS (21). If chiral columns are not available to differentiate chiral compounds from each other, chiral derivatization is a straightforward way to be able to differentiate compounds when necessary.

Three examples of common methods of derivatization techniques can be seen in Figure 2. When choosing which sample preparation technique is best for your complex matrix, it is important to consider your specific target analytes and the interferences you want to rid your sample of, in addition to how much time you want to spend handling each sample. Although derivatization is often referred to in a negative context, as a time-consuming step to justify an alternate approach, it is sometimes
actually quite straightforward. Many derivatization methods have been developed to be fast and essentially quantitative for the conversion of target analytes to facilitate either GC or LC analysis.

**Online Sample Treatment**

There are various other techniques that can be implemented online. Moving sample preparation and sample treatment online can be advantageous, because online sample treatment requires less manual sample handling, and provides increased recovery, improved limits of detection, less human error, and reduced exposure of compounds to the environment, which can be useful for analytes that are photosensitive or reactive to oxygen. One common method of online sample treatment is online SPE, which can be implemented to clean and preconcentrate target analytes in an automated fashion (9). Other forms of sample preparation that can be automated include the use of a continuous stirred tank reactor (CSTR) for online sample dilution. A CSTR contains a reservoir that allows fluid to travel through the apparatus. This device can be used to continuously dilute a sample injected into it. In previous research, a CSTR was used in the investigation of native carbohydrates to study the electrospray response factors by LC–MS/MS (23). By using the CSTR apparatus, analyte response data for a large range of analyte concentrations were able to be obtained with each single injection.

Restricted access media (RAM) can be particularly useful when dealing with complex matrices, especially when targeting small molecules and trying to rid the sample of large biomolecule interferences, prior to LC analysis (24–30). RAM columns work on a similar principle to size exclusion chromatography. The outer surface of the stationary phase has a non-retentive and size-restrictive layer, while the inner pores of the support material have a bonded group, like C4, C8, C18, and so on. Only the small molecules can access and interact with the inner-pore phase to be retained on the column, while the large molecules are largely unretained and washed to waste. This can be especially useful when looking at small molecules in complex matrices, such as whole blood or plasma, where there is an abundance of protein interferences. This is a technique that can be put in-line, in the flow path before the analytical column, so that the extraction process is completely automated. This can provide a welcomed alternative to extensive sample preparation and sample handling.

An example of this methodology was used in the quantification of lipid mediators in skeletal muscles using the RAM column coupled to LC–MS/MS (28). This technique requires the use of one or two high-pressure valves in the column oven, and some extensive LC programming for loading, eluting, and washing parameters. An example of LC settings and the valve setup can be seen in Figure 3. (29).

When dealing with trace analysis from complex samples, the RAM column can be loaded with large sample injections without affecting the peak shape. In addition to large sample volumes, the RAM can also be loaded with multiple injections to allow for ultratrace analysis to be performed. In previous research, bisphenol A was able to be detected in human saliva samples by using a RAM column in combination with LC–MS/MS (30). Parts per trillion levels of bisphenol A were detectable by performing multiple injections on the RAM column to concentrate the analyte and remove unwanted large biomolecules (30).

**Instrumental Tools**

Many instrumental tools can be very valuable and can save time and effort in sample preparation and method development. Instrumental tools can include things like multiple reaction monitoring (MRM), spectral filters, and programs that allow for deconvolution of coeluted signals. In the case of mass spectrometry, precursor-to-product ion MRM transitions allow for high sensitivity and specificity from complex matrices, as long as unique transitions can be acquired. Although useful for the differentiation of most compounds, this approach is not always an option when analyzing isomeric compounds. In previous work on cannabinoids, a number of isomers were analyzed and it was found that some of the compounds had common fragmentation pathways when using MRMs in GC–MS/MS (22). In order to differentiate analytes with common fragmentation pathways, the compounds had to be chromatographically separated. This was partially achieved by silylating the cannabinoids; however, some potential interferences still existed. It was still necessary to monitor less sensitive secondary and tertiary precursor-to-product ion transitions to provide adequate specificity.

In complex matrices, there is likely to be a case where the target analytes are isomeric, are not resolved chromatographically, and do not produce unique MRM transitions when analyzed by the mass spectrometer. These can still be accurately quantified using instrumental tools with the right detector. The vacuum ultraviolet (VUV) detector measures in the 120 to 240 nm range, where virtually all compounds have unique, compound-specific absorbance spectra (31,32). The VUV can be used to differentiate isomeric forms of compounds, such as in the case of illicit drugs. In previous research four classes of compounds were chosen that are listed in the Dutch drug legislation, as well as some of their uncontrolled isomers (33). These compounds were then analyzed and were able to be differentiated by GC-VUV analysis. Coeluted compounds, including isomers, can be distinguished and quantitatively deconvoluted using a VUV detector. Since Beer’s
law is additive, the overlapping VUV absorption signals arising from coelution can be easily deconvoluted (31,34). A straightforward least squares approach can be used to discern individual component contributions to the overlapping signals (34). An example of deconvolution can be seen in Figure 4, where coeluted isomers of dimethylnaphthalenes have been separated into their respective contributions to the coeluted peak.

In order to use this tool, the analytes have to first be amenable to GC, which can complicate things if the analytes are not volatile or thermally stable. The similarity of spectra between the coeluted compounds is a governing factor of how accurately the compounds can be deconvoluted.

The more distinct the absorbance spectra, the easier the compounds are to deconvolute over a wider range of relative abundance; the more similar the spectra are, the more difficult they are to differentiate.

Spectral filters can also be a great tool in conjunction with VUV for complex samples when certain classes of compounds are of interest. Post-run digital spectral filters can be applied to samples that are too complex to perform deconvolutions on because what compounds are coeluted with target analytes may not be known. In the VUV range, certain classes of compounds absorb more intensely in different wavelength ranges. Saturated compounds absorb in the 125–160 nm range, whereas unsaturated compounds absorb in the 170–240 nm range (34). This information can be used to build and apply a spectral filter to apply to a chromatogram post-run. In the same set of experiments performed for coeluted isomers of dimethylnaphthalenes, spectral filters were used on samples of diesel fuel and jet fuel, which are extremely complex, to show where naphthalene, methyl-naphthalenes, dimethyl-naphthalenes, and trimethyl-naphthalenes elute (34). This was accomplished by experimentally determining where naphthalene class compounds absorb strongly (210–220 nm) and then applying a digital filter in that range to selectively identify where the naphthalenes are eluted without performing deconvolutions (34).

How to Evaluate Which Is the Best Option to Use for Your Analysis

A good place to start when deciding what is best to try for method development in complex samples is to first take into account what you are targeting and what you want to remove. Knowing your sample and the possible interferences can facilitate at least an educated guess as to what types of sample handling should be implemented first and can save a lot of guess work, time, and money. Excessive fats, waxes, and proteins can dirty instruments, so special attention needs to be given to remove these from the matrix. Next, decide how removal of problematic compounds can be performed. Does the sample need extensive clean-up on the front-end to remove matrix interferences or can the use of an instrumental tool or on-line sample treatment mitigate unsavory effects on sample analysis, while maintaining reproducible results?

A flow-chart of the topics covered in this article for sample preparation, on-line sample treatment, and instrumental tools discussed in this article can be seen summarized in Figure 5. When starting with a complex sample, the first consideration is which method to choose, LC or GC. Once LC or GC is chosen, there are a number of different sample preparation and automated sample handling choices that are amenable for each technique. Finally, depending on the detector chosen, available tools may aid in the analysis of the complex sample and offer additional tools that can help with complex sample analysis. This flow chart is by no means exhaustive in its content, but can be a good starting point when considering the available options.

Conclusions

When starting a new project, it can be overwhelming to try to anticipate the problems your newly assigned complex matrix may give you, but there are many solutions that can be implemented to save time and money. After the sample matrix is evaluated and a method of analysis is chosen (GC or LC), the type of sample clean-up appropriate for the matrix can be determined. If the analytes are going to be analyzed via GC, headspace is a great option due to the limited need for sample clean-up; SPME sampling in solution or in the headspace might be the next best choice to consider. If using LC and excessive proteins are not a concern, a simple dilute, filter or centrifuge, and shoot with the right solvent might just do the trick. Ultra-trace analysis in a complex matrix can be accomplished with the use of a RAM column and multiple injections. The most important thing when trying to develop a method for a complex matrix is to start with literature and make the best educated guess you can, and to not be afraid to try something new. In the end, the proof of performance through method validation is key to acquiring reliable, reproducible, and accurate data.

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Preparing for GC Analysis—A Definitive Checklist

Tony Taylor

My fellow LCGC author John Hinshaw recently published an excellent article on the items in the gas chromatography (GC) toolkit and the uses for those tools (1). Inspired by John’s article, I wanted to produce a checklist for preparation of a GC or gas chromatography–mass spectrometry (GC–MS) system prior to analysis, referencing the actions, checks, tools, and consumable items that might be required. I write this having recently been involved in several troubleshooting exercises where more in-depth knowledge and a pragmatic approach to GC setup would have been a significant advantage for the user.

Check that gases are of the correct purity (99.999%+)
Turn on the cylinders. If cylinder bottle pressure is below 30 psi, replace it. If the line pressure (second regulator stage or gauge at the bench) is below 80 psi, increase the line pressure with cylinder or bench regulator.

Verify gas traps
Verify that the correct gas traps are installed, and not exhausted, and check when they were last replaced.

Is the GC column fit for purpose?
Has it been properly stored (out of sunlight with column ends capped)? How did it perform last time it was used? Check records.

Remove the inlet septum cap
Ensure you have the correct septum in terms of bleed profile and temperature. Use pinch-nose plastic tweezers to avoid scratching the metal surfaces of the inlet. Avoid applying excess torque pressure on the septum by following manufacturer’s guidelines for the inlet closure and septum nut tightening. Failure to do this will lead to septum coring and splitting.

Open the inlet
Inspect the inlet liner and check for cleanliness, including solid debris, discoloration, and dark patches of burned-on matrix contaminants. Ensure that inlet surfaces and seals are included in medium-term maintenance and replacement schedules.

Replace nuts and ferrules on each end of the column
Quarter a used inlet septum and slide one quarter onto both detector and inlet ends of the column, carefully removing any septum shards retained in the head of the column. These will be used to maintain the correct distance between the ferrule and the tip of the column on installation.

Trim and inspect column ends
Trim the ends of the column and inspect with a magnifier or low-power microscope to ensure the cut is straight and there are no rough edges. The quality of the column cut is directly related to peak shape and quantitative reproducibility, therefore good column cutting technique is essential.

Note your manufacturer’s recommended column insertion distance (inlet and detector)
Wipe the outer column surface of the column ends with a solvent suitable for removing contamination from fingerprints (isopropyl alcohol is a popular choice). Adjust the nut and ferrule position and hold in place with the quarter septum from two steps earlier.

Insert the column into the inlet
Tighten the ferrule and gently pull the column to check if the fitting is tight enough. Grip the column and continue to tighten about an eighth of a turn until the column can’t be moved. Repeat with the detector connection.

Input key information
Input into the instrument: column dimensions, carrier gas type, required flow rate, and whether constant flow or constant pressure is to be used. Verify column flow at the detector using an electronic flow meter.

Switch on the inlet and detector heaters, and the detector gas flows
Ignite the detector if it is flame-based.

Purge dissolved oxygen
Purge dissolved oxygen by allowing carrier flow through the column at ambient temperature for 10–30 min depending upon column dimensions. This step is important and should not be omitted. Failure to purge the phase may lead to unnecessary column damage and increased thermal equilibration times.

Set the oven settings
Set the oven to the conditioning temperature –10 °C above the maximum oven temperature of your method or at the gradient upper temperature limit of the column, whichever is lower. Allow to thermally condition for the period recommended in the guide below.

Cool the oven
Cool the oven to starting temperature of your analysis.

Load the test method into the GC and data system
Check the split flow at the split outlet port using an electronic flow meter. Check the septum purge flow using an electronic flow meter. Perform at least one full temperature program according to your analytical method.

Perform a test
Perform a test injection or system suitability test to check method performance.

Reference

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