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Dyes have been used over the centuries to give food more of an appeal to those eating it. Old foods were sometimes dyed to provide a more palatable appearance. Currently regulations have allowed the following dyes: Brilliant Blue FCF (FD&C Blue 1), Indigotin (FD&C Blue 2), Sunset Yellow FCF (FD&C Yellow 6), Tartrazine (FD&C Yellow 5), Allura Red AC (FD&C Red 40), Fast Green FCF (FD&C Green 3), and Erythrosine (FD&C Red 3). Though there are dyes from natural products used in the food and beverage industry, they have not garnered the ubiquity of the petroleum-based dyes. As such current studies have shown synthetic dyes to cause allergies, attention deficits, and in some cases lymphoma and other cancers.\(^1\)\(^3\)

The common approved and some unapproved dyes are identified with the help of reversed-phase HPLC. Synthetic dyes derived from petroleum products are analyzed in either their neutral state or anionic state. However, the anionic forms are generally not used due to the abundance of free silanols generated from alkaline mobile phases, which leads to poor analyte retention, peak tailing, and low efficiency. Though dyes in their anionic form tend to exhibit poor retention with silica-based columns, the opposite is valid with the Hamilton PRP-C18 column (5 µm).

With no silanol groups to convolute analyte retention, column longevity, and peak efficiency remain. The separation is completed in under 9 minutes and shows all 11 components fully resolved, including all colors of the rainbow; red, blue, yellow, green, and indigo. The polymeric PS-DVB backbone adds needed stability under the alkaline pH of 9.2, which is required to resolve two integral components, brilliant blue, and fast green. Additionally, the use of ammonium acetate as a mobile phase buffer allows the method to utilize a Mass Spectroscopy detector for increased analyte sensitivity.

**Column Information**

- **Packing Material:** PRP-C18, 5 µm
- **P/N:** 79676

**Chromatographic Conditions**

- **Gradient:**
  - 0.00 – 1.00 min, 9% B
  - 1.00 – 3.50 min, 9 – 25% B
  - 3.50 – 6.00 min, 25 – 33% B
  - 6.00 – 8.50 min, 33 – 70% B
- **Temperature:** 35 °C
- **Injection Volume:** 5 µL
- **Detection:** UV at 260 nm
- **Dimensions:** 150 x 4.6 mm
- **Eluent A:** 32 mM CH₃ COONH₄
- **Eluent B:** CH₃ CN : CH₃ OH 4:1
- **Flow Rate:** 2.0 mL/min

**References:**

1) A. Vojdani, C. Vojdani Altern Ther Health Med. 2015, 21, 1:52.

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Surfing on Mobile Phase, Part II: Impact of Mobile-Phase Composition Waves on Retention in LC

The most commonly used designs for modern liquid chromatography (LC) pumps produce mobile-phase streams with small short-term variations in mobile-phase composition. Understanding the origin of these variations, and their effects on chromatographic performance, can help us develop more robust methods, and monitoring for this aspect of pump performance is an important step in using the best troubleshooting tool—preventative maintenance.

Dwight R. Stoll

In the previous installment of “LC Troubleshooting,” I briefly reviewed the operating principles of liquid chromatography (LC) pumps that rely on low- or high-pressure mixing approaches, described how waves of solvent composition can develop in the mobile phase, and explained how these waves can affect detector noise and drift when using UV detection. In this installment, I continue this story by discussing the impact of waves in mobile-phase composition on retention time in reversed-phase LC. As I indicated last month, readers interested in a deeper dive into pump performance are encouraged to consider the two books by Kromidas (1) and Snyder and Dolan (2). Both books include chapters dedicated to discussing modern LC pump technology, and they also include details about performance specifications and descriptions of tests that can be used to evaluate pump performance. I also encourage readers to consider perusing through two “LC Troubleshooting” articles by John Dolan in 2006 (3) and 2014 (4), which describe case studies that illustrate what can happen when things go wrong in the pump. These are excellent resources for those looking to add to their LC troubleshooting knowledge.

Impact of Solvent Waves and Pump Parameters on Retention for Isocratic Separations

In support of the following discussion, I’ve used relatively simple simulations of analyte transport through a column to demonstrate that these solvent waves can also affect analyte retention in isocratic separations, and that parameters that can be controlled by the instrument user can strongly affect how serious, or not, the effect of the composition waves on retention is. We start with a very simple model for the waves themselves, which is a sinusoid. This is actually a reasonable representation of what happens with a low-pressure mixing pump. Simulating the wave patterns for high-pressure mixing systems require more sophisticated models. In our sinusoid, the frequency of the wave is the flow rate divided by the pump stroke volume (flow/stroke), and the amplitude of the wave is the maximum deviation of the mobile-phase composition from the set point. The family of waves shown in Figure 1 were constructed using an amplitude of 1% acetonitrile and a frequency of 200 µL/min / 100 µL (2 min⁻¹). The difference between the five sinusoids is that they are phase shifted relative to each other. These parameters (that is, the combination of flow rate and stroke volume) would not be optimal for this type of separation, but they are not completely unrealistic—an amplitude of 1% acetonitrile could be observed in a case where the proportioning valve in a low-pressure mixing system is not working well, or when a too-small mixer is used. One could certainly choose the combination of flow rate and stroke volume to give two strokes per minute, even though that would be a bad idea, for reasons I will explain below.

To simulate the impact of these waves on retention, we need to choose the column dimensions, and know the dependence of the retention of an analyte of interest on mobile-phase composition. For these simulations, I’ve assumed we’re working with a 50 mm x 2.1 mm i.d. reversed-phase LC column with a dead volume of 100 µL. For the retention dependence, I’ve used the linear solvent strength theory (LSST) model for reversed-phase LC separations (2), which relates the retention factor of the analyte (k) to the volume fraction of organic solvent in the mobile phase (φ, 0–1 scale):

\[ \ln k = \ln k_w - S \phi \]

where the parameters S and k_w are the slope of a plot of ln k vs. φ, and the extrapolated retention factor in completely aqueous mobile phase, respectively. Next, we assume that the mobile-phase composition wave travels through the column at the same velocity as the mobile phase (u_m), and that...
the velocity of the analyte through the column (\( u_x \)) is \( u_m/(1+k) \). Given these two velocities, we can calculate where the analyte is inside the column at any given time, and the local mobile-phase composition in the immediate vicinity of the analyte. Finally, we can calculate how far the analyte will move in the next, small interval of time as a result of its velocity that is determined by its local retention factor. We advance the solute by this distance along the axis of the column, recalculate the local retention factor, and repeat the process until the total distance travelled by the analyte is equal to the column length. The time elapsed to get to this point is the retention time.

Figure 1 shows a first set of chromatograms resulting from these simulations. The phase shift of each of the sinusoids relative to the first one at the top is indicated in each row. In chromatographic terms, the significance of the phase shift is that we are simulating the case where an injection of sample is made at a different point on the wave. This will be the case in real experiments unless the LC system is designed to synchronize injections with a certain point on these waves (some systems, but not all, do this). The analyte I chose in this case is acetophenone (LSST parameters are \( S = 6.3 \) and \( k_w = 45 \) for a C18 stationary phase). We see that the chromatograms obtained from the cases where the sinusoid is phase shifted yield a peak with a slightly higher retention, until we get to the point where the wave is shifted by \( 2\pi \). At this point, the sample is injected at the same point on the wave as in the very first case (at the top), and the resulting retention time is exactly the same. The mean retention time for the first four chromatograms is 2.309 min, the standard deviation is 0.007 min, and the percent relative standard deviation (RSD) is 0.3%. If the amplitude of the waves is larger than 1% acetonitrile because of a poorly functioning proportioning valve (low-pressure mixing) or poorly functioning check valves (high-pressure mixing), then the retention precision could be much worse (that is, higher RSD) because of these waves. If the amplitude is smaller than 1% because the pump has a low composition ripple or because a large mixer is used, then the retention precision could be much better (lower RSD).

In the next set of simulations, I used the same parameters as in Figure 1, but changed the wave amplitude to 2% acetonitrile so that we can easily see the dependence of retention precision on stroke volume (more specifically, stroke frequency).
Figure 2 shows overlays obtained for simulations with waves phase shifted by 0, π/2, π, or 3π/2, and stroke volumes of 100, 66, or 33 µL. One of the main conclusions of the recent paper by Gritt (5) describing the effects of solvent waves on baseline quality is that the effects of these solvent waves can be reduced dramatically by increasing the stroke frequency (by increasing flow rate, decreasing stroke volume, or both). We see the same effect here—that decreasing the stroke frequency dramatically improves the retention precision. As the stroke frequency increases, more and more of the variation in retention because of high and low acetonitrile percentage is cancelled out, which leads to the improvement in precision.

Impact of Solvent Waves and Pump Parameters on Retention for Gradient Separations

Everything discussed above concerns the effect of solvent waves on retention under isocratic conditions, but we can also examine these effects under gradient elution conditions, by superimposing the solvent wave on a linear increase of the percentage of acetonitrile over time, as in a typical gradient elution experiment. I emphasize again here that this is a pretty simplistic view of what happens inside the column, compared to more rigorous treatments of the situation (5), but this view is nevertheless useful for understanding how these waves can affect chromatographic performance. Figure 3 shows the results of simulations of gradient elution separations with waves phase shifted by 0, π/2, π, or 3π/2. In this case, I fixed the stroke volume at 50 µL, and the wave amplitude at 1% acetonitrile to start. Other conditions are given in the figure caption. The set of chromatograms in Figure 3a is for acetophenone, where we see that the retention precision is sevenfold worse for myoglobin (0.41 %RSD) compared to acetophenone (0.06 %RSD). However, if we decrease the wave amplitude to 0.2% (see Figure 3c), the retention precision improves dramatically to 0.08 %RSD. This result illustrates the value of a pump design characterized by low mobile-phase composition ripple in demanding applications involving analytes that are very sensitive to mobile-phase composition.

How to Avoid Solvent Waves

Avoiding mobile-phase composition waves starts with the decision about which pumping hardware to buy with your LC system. One of the advantages of a low-pressure mixing system is that it offers the flexibility of accessing four different solvents within a single LC method; these can be used as different pairs of solvents, or for making ternary (three component) or quaternary (four component) mobile phases. One of the prices paid for this capability, though, is that this design will produce composition waves, and relatively large mixers are needed to smooth out these waves, particularly for applications that require high quality detector baselines and good retention precision. Specifications for mobile-phase composition for modern pumps based on this design are typically on the order of 0.2 %RSD. On the other hand, pumps based on a high pressure mixing design tend to have tighter specifications for mobile-phase composition, even without the use of large volume mixers. These pumps also tend to be more expensive. So, one should carefully consider these tradeoffs at the point of purchasing an instrument.

Once the instrument is on the bench, one should periodically check the pump performance to see how it is doing in terms of the composition ripple. For modern pumps, there is usually a diagnostic test accessible through the instrument control software that enables automated execution of a method to measure the composition ripple, and assessment of the results (something like a “gradient composition test”). This test usually involves replacing the LC column with a restriction capillary and spiking one of the mobile-phase components with a tracer that can be detected by UV absorbance (something like acetone in water).
Next-Generation Sorbent-Based Extractions with Metal-Organic Frameworks

Metal-organic frameworks (MOFs) are coordination networks consisting of a metal ion linked with organic ligands. The resulting three-dimensional structures create pores that can be exploited for a number of chemical processes, including analytical extractions. The resulting sorbent-based extraction systems have several advantages, notably selectivity. The use of MOF in extraction has exploded in the last two or three years. In this article, we take a look at the current state of the art regarding analytical extractions utilizing MOF, including a description of what MOF are, their preparation, principles of use, advantages, and application areas.

Douglas Raynie

The field of sample preparation and analytical extractions faces a somewhat unique contradiction. On one hand, analysts are seeking high levels of selectivity during these preliminary stages. That is, there is a desire to isolate our analytes of interest to the exclusion of everything else prior to the actual analysis. On the other hand, advances in chromatography, mass spectrometry, and spectroscopy over the past couple of decades have allowed us to characterize samples of increasing impurity. Significant increases in gaining selectivity during the sample preparation steps of an analysis are gained with the use of selective adsorbents. These extractions include solid-phase extraction (SPE), solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), dispersive extractions (including QuEChERS, matrix-solid phase dispersion [MSPD], and dispersive SPE [dSPE]), and a host of similar techniques related to sorbent-based methods. Our last sample preparation trends survey, conducted five years ago (1), demonstrated steadily increasing use of these techniques. Historically, these sorbent-based extractions featured use of either chromatography stationary phases or general sorbents, like silica, carbon, or alumina. More recently, specialized sorbents are being used for sorbent-based extractions, such as molecularly imprinted polymers, restricted access media, crown ethers, and others.

Last year at this time (2), we reported on the major sample preparation advances in the previous year. One major advance, still in an emerging state, was the use of metal organic frameworks (MOFs) for extraction. This was highlighted by one of our thought leaders and featured prominently in an Analytical Chemistry fundamental review (3). During this past year, the use of MOFs in analytical extractions has exploded from the emerging state to the breakthrough phase. We can see in Figure 1 an almost exponential growth in the annual number of publications, as reported with the Scopus database using the search terms “metal organic frameworks” and “extraction,” over the past decade. In 2020, nearly 250 articles were reported, compared with just 8 in 2011! With this explosive growth, there must be some distinguishing features surrounding the use of metal organic frameworks (MOFs) for extraction. In this “Sample Prep Perspectives” installment, we explore this phenomenon, with a focus on the past year or two.

Metal Organic Frameworks

MOFs are coordination polymers or highly ordered crystalline structures, typically two- or three-dimensional, composed of metal cations or clusters connected with coordinating organic ligands (4,5). They are mesoporous with pores in the 2–50 micron diameter range. MOFs can have a surface area in the thousands of m²/g with a high number of pores and functional groups. Self-assembly of the metallic moieties with multifunctional organic ligands containing nitrogen and/or oxygen comprise the MOF. The first permanently porous MOF were reported in 1995 (6). The coordination between the metallic component and the organic ligand is described by the hard/soft acid/base (HSAB) theory. Because of the chemical nature of the MOFs, intermolecular forces, including electrostatic interactions, Van der Waals forces, hydrophobic interactions,
π–π interactions, ion exchange, Lewis acid-base, chelation, hydrogen bonding, and coordination can adsorb analytes from various mixtures during an extractive procedure (7–9).

Synthesis of MOF can be by a variety of routes, including slow evaporation, covalent assembly, chemical co-precipitation, hydrothermal, solvothermal, microwave-assisted method, mechanochemical, and electrochemical techniques (10,11). Because of these synthetic procedures, there is wide latitude in creating MOFs with crystalline properties, tunable porosity, and surface areas in the range of 2000–7000 m²/g. Additionally, variable pore volumes can be created, and uniform porous structures achieved, with high thermal and mechanical stability (8). MOFs functionalized with ionic liquids are even being produced (12). Given the number of available functional groups and metal ions or clusters available, it is conceivable that the potential number of available MOFs to be created is infinite.

**Extraction Modes**

Essentially all modes of sorbent-based extractions have been performed with MOFs, including the in-tube and in-syringe approaches to SPE. This is because of their key features. MOF sorbents have high tunable porosity and surface area, designable structures, internal functionalities, and outer surfaces available for molecular interactions. MOFs also have thermal and mechanical stability, structural cavities, and uniform active sites (13,14). The MOFs can be included as part of polymer matrices, or in the pores of organic monoliths. As a result of these properties, MOFs have been used in conventional and dispersive SPE, though compaction and flow irregularities with cartridge SPE seem to lead to the dispersive approach being favored. With dispersive SPE, the MOFs are rather easily dispersed with the sample matrix and recovery of the MOFs via phase separation is often straightforward. SPME and SBSE are also popular approaches for using MOFs during analytical extractions. One unique opportunity of MOFs is via magnetization of the metal component. After mixing the MOFs, frequently as nanoparticles, with the sample, recovery can be quite simple. Analyte enhancement factors using MOFs is large, often in the thousands. Extraction efficiencies with MOFs are similar to other sorbents and the extraction configuration; that is, the ratio of MOF to sample amount and analyte concentration, sample volumes, surface area, identify and volume of sample and eluent solvents, flow rates, extraction times, ionic strength, and sample phase can all play important roles in extraction efficacy, as they would in conventional and dispersive SPE, SPME, or SBSE. Thus, the selectivity, solvent use, recovery, and other advantages of these techniques still hold.

**Applications**

Given all of the stated advantages of MOFs and their explosive growth in the literature, one can expect that application of MOFs in sorbent-based extractions are manifold. These applications are found with both liquid and
### TABLE I: Representative application areas of extractions performed using MOFs

<table>
<thead>
<tr>
<th>Mode</th>
<th>Analyte</th>
<th>Matrix</th>
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</thead>
<tbody>
<tr>
<td>SPME, SPE, dSPE</td>
<td>Antibiotics</td>
<td>Water, milk, soil, sediments</td>
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<td>dSPE, SPE</td>
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<td>dSPE</td>
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<tr>
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<tr>
<td>SBSE</td>
<td>Benzylpenicillin</td>
<td>Biological samples, milk</td>
</tr>
<tr>
<td>SBSE</td>
<td>Phytohormones</td>
<td>Fruits</td>
</tr>
<tr>
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<td>Organophosphorus pesticides</td>
<td>Water, fruits</td>
</tr>
<tr>
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<td>Water</td>
</tr>
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<td>Beverages, urine</td>
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<tr>
<td>SBSE</td>
<td>Azo dyes</td>
<td>Water</td>
</tr>
<tr>
<td>SBSE</td>
<td>Carvedil, haloperidol</td>
<td>Water, plasma</td>
</tr>
<tr>
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</tr>
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<tr>
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<td>Chlorophenols</td>
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<td>Estrogens</td>
<td>Water, urine, milk</td>
</tr>
<tr>
<td>SPME</td>
<td>BTEX</td>
<td>Water, urine, smoke</td>
</tr>
<tr>
<td>SPME, magnetic, dSPE, SPE</td>
<td>Phenols</td>
<td>Water, beverages, vegetables, plants, honey</td>
</tr>
<tr>
<td>SPME</td>
<td>Odorants</td>
<td>Water</td>
</tr>
<tr>
<td>SPE</td>
<td>Nucleoside diols</td>
<td>Cells</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Bisphenols</td>
<td>Serum</td>
</tr>
<tr>
<td>Magnetic, dSPE</td>
<td>Triazole fungicides</td>
<td>Vegetables, plasma</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Bactericides</td>
<td>Fruits, vegetables</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Pacitaxel</td>
<td>Plasma</td>
</tr>
<tr>
<td>Magnetic, dSPE, SPE</td>
<td>Sulfonamides</td>
<td>Meat, water, milk, honey, meats</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Tetracycline</td>
<td>Water</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Nitroaromatics</td>
<td>Water, smoke</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Haloacetics</td>
<td>Urine, meats</td>
</tr>
</tbody>
</table>

Continued on Page 124
Multiply Your Sample Throughput
New Ultrafast Multiplex LC-MS System Maximizes Productivity

Shimadzu’s new **Nexera QX Multiplex LC-MS/MS System** incorporates innovative LC-MS multiplexing technology using dedicated sample introduction streams for continuous operation of the mass spectrometer. Combined with newly designed software control, Nexera QX significantly increases laboratory throughput and profitability.

- **Maximize Mass Spectrometer Productivity**
  Multiple Stream technology eliminates waiting for column equilibration or system flushing

- **Hardware Engineered for Multiplexing**
  Nexera SIL-40 UHPLC autosamplers, stream-dedicated injection valves and washing pumps deliver ultra-fast performance with ultra-low carryover

- **Software Designed for Multiplexing**
  Offers an intelligent, automated single point of control for multiple UHPLC streams and the LC-MS/MS

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solid samples and, while not exclusive, are found primarily in the biological, environmental, and food areas. Table I summarizes applications found in recent reviews (5,8,10,11,13,15–17).

A few key observations are gleaned from this table. Conventional SPE, SPME, and SBSE techniques using MOFs are somewhat evenly distributed, yet are the more minor approaches to MOF extractions. It is the approaches that take special advantage of the unique properties of MOFs that make up the majority of the applications. These approaches are dSPE and use of magnetic MOF. In the biological field, drugs of interest included antibiotics, nonsteroidal anti-inflammatories, penicillins, and methamphetamine in urine, milk, and tissues. Additional biomolecules isolated from these matrices include estrogens and hormones, carbohydrates, peptides, proteins, and aflatoxins. Each of the major environmental contaminants of recent interest are extracted with MOF, namely endocrine disruptors, most types of herbicides and pesticides, phthalate esters, parabens, polychlorinated biphenyls and polybrominated diphenyl ethers, polycyclic aromatic hydrocarbons, polyfluorinated compounds in environmental waters and wastewater, soils and sediments, body fluids, fruits and vegetables, and related sample types. Fruits, vegetables, beverages, edible

---

**TABLE I (CONTINUED):** Representative application areas of extractions performed using MOFs.

<table>
<thead>
<tr>
<th>Mode</th>
<th>Analyte</th>
<th>Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic</td>
<td>Perfluoro compounds</td>
<td>Water, milk, fruits, vegetables</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Alkaloids</td>
<td>Urine, plants</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Anthraquinones</td>
<td>Beverages</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Glycoproteins</td>
<td>Serum</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Trypsins</td>
<td>Tissues</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Phosphopeptides</td>
<td>Milk, serum</td>
</tr>
<tr>
<td>dSPE</td>
<td>Neurotransmitters</td>
<td>Biologicals</td>
</tr>
<tr>
<td>dSPE</td>
<td>Gallic acid</td>
<td>Urine, plasma, water</td>
</tr>
<tr>
<td>dSPE</td>
<td>5-Nitroimidazoles</td>
<td>Water</td>
</tr>
<tr>
<td>dSPE</td>
<td>Amphenicols</td>
<td>Water</td>
</tr>
<tr>
<td>dSPE</td>
<td>Glucocorticoids</td>
<td>Water, urine</td>
</tr>
<tr>
<td>SPE</td>
<td>Phenoxyacetic acid herbicides</td>
<td>Vegetables</td>
</tr>
<tr>
<td>SPE</td>
<td>Nitrobenzene</td>
<td>Water</td>
</tr>
<tr>
<td>dSPE</td>
<td>Sialic acids</td>
<td>Serum</td>
</tr>
<tr>
<td>dSPE</td>
<td>Domoic acid</td>
<td>Shellfish</td>
</tr>
<tr>
<td>dSPE</td>
<td>Aflatoxins</td>
<td>Food</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Penicillins</td>
<td>Milk</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Polybrominated diphenyl ethers</td>
<td>Milk, plasma, serum, water</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Polysaccharides</td>
<td>Algae</td>
</tr>
<tr>
<td>Magnetic</td>
<td>DNA</td>
<td>Blood, bacterial cells</td>
</tr>
<tr>
<td>SPE</td>
<td>Sulfamono-methoxine</td>
<td>Soil, sediment</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Aristolochic acid</td>
<td>Vegetation</td>
</tr>
<tr>
<td>dSPE</td>
<td>Methamphetamine</td>
<td>Urine</td>
</tr>
</tbody>
</table>
oils, and meats were investigated for herbicides and pesticides, hormones, and aflatoxins.

Conclusions and Future Prospects
An emerging type of sorbent material, MOFs, is presented for use in conventional and dispersive SPE, SPME, SBSE, and, especially, magnetic extractions. These MOFs are characterized by high surface area, controlled and tunable porosity, high stability, and significant functionalization. Applications to biological, environmental, and food samples abound. Analytical extraction with MOFs as a field is only about a decade old and growing rapidly. Consequently, continued use of MOFs for new application areas, including industry standard and regulatory methods, are the obvious growth area. Currently, there are no commercial MOF extraction materials and consumables available; such commercialization will help drive these additional applications.

References
Instrument Utilization: Context is Required

There is an increasing focus on capturing utilization data from instruments to improve overall lab operations, but without context, utilization data—such as service history, instrument information, and maintenance strategy—has limited value. Contextualizing improves the quality of the business decisions relating to the asset lifecycle.

**LCGC:** What are the use cases for capturing utilization? Why are labs capturing this data?

**SWEENEY:** There are five primary use cases for capturing utilization—three are related to the capital equipment planning process and two are for maintenance optimization. For the capital equipment process, utilization data is being captured to plan for future purchases, identify candidates for decommissioning, and repositioning underutilized assets. For maintenance optimization, this data is being used to help select the best preventative maintenance frequency and select the most cost-effective maintenance contract.

These decisions, historically, were made with little to no data. The data on utilization was derived from ad hoc discussions with equipment owners.

**LCGC:** What types or classes of instruments is utilization data being captured for today?

**SWEENEY:** There is interest in capturing utilization data for all instruments within the lab. Most often, the higher value instruments, such as chromatography, are the first types of instruments utilization data is captured for. But with the rapid advancements in technology, specifically internet of things (IoT), most instruments in the lab can have utilization data captured.

**LCGC:** Are there different approaches for capturing utilization data, and are there benefits associated with each approach?

**SWEENEY:** Yes, there are multiple approaches or technologies for capturing utilization data. For instruments that have a PC connected, embedded, or connected to a data system, utilization data through APIs or log files can be captured directly. For other instruments that may not have the data directly available, monitoring the power usage is a very good proxy for utilization data. Each of these approaches have benefits and challenges. For direct connection, the utilization can have additional information such as the description of the utilization events; for example, the method used. This data often
is historical, so utilization for the past year may be available. The challenge with this approach is getting to the data within a company’s rules for access. The benefit of the monitoring-power approach is you do not have the challenge of getting access to the data, as data is collected overtime through a sensor that is not part of the instrument. The challenge, however, is there is no context for the power consumption. A machine-learning model can be applied to determine stand-by power and other usage, but observation may be required to determine what was the other usage. For simple instruments, like a centrifuge, this may be enough.

**LCGC:** Can you expand on how context is required for utilization?

**Sweeney:** Utilization data on its own has some value but may not be enough to make decisions in the use cases we discussed at the beginning. When making capital planning or maintenance optimization decisions, data such as the age of the instrument, the type of lab, the instrument make and model, service history, instrument downtime, and the current maintenance contract can change the perspective on what the utilization data means.

**LCGC:** Do you have an example of how contextualization changes the perspective of utilization data?

**Sweeney:** One lab has been collecting utilization data for a thousand systems. During a review to identify candidates to remove from services, some LCs had relatively high usage and others with much lower usage. With utilization data only, the group with lower usage was the target. But once the instrument’s age and service history were reviewed, surprisingly, the group with the lower usage had newer instruments with no service issues.

**LCGC:** How do you envision the discussion/use of instrument utilization going forward?

**Sweeney:** I see tremendous interest in capturing utilization data throughout the labs and expect this to grow exponentially. As more utilization data is captured, the discussion will evolve from just talking about utilization and move into a discussion on overall asset performance. Utilization capture is a great start, but it is just the start of a journey toward asset performance management. As discussed, context is so important. Having all that information in one view or one dashboard will be the default for labs to improve the use cases.
Let’s Get Small: Powerful Gas Chromatography in Small Packages

The small size and low thermal mass of capillary columns offer many opportunities for miniaturizing gas chromatographs. Small instruments offer performance that until recently was only available in full-size laboratory systems. With full laboratory capability now available in systems with smaller footprint, lighter weight, lower power consumption, fewer consumables, and simpler maintenance, the possibilities for rethinking our use of gas chromatography (GC) both inside and outside the laboratory are almost endless. In this installment, we discuss how capillary GC has been miniaturized, how these small systems can potentially perform many analyses that were reserved for full laboratory systems until very recently, and the benefits and challenges involved with considering these newer, smaller gas chromatographs for typical analytical problems.

Nicholas H. Snow

When I began working with gas chromatography (GC) about 35 years ago, full-featured gas chromatographs were bulky and heavy, weighing 25–50 kg, and requiring two people to move them safely. The basic needs for the instrument generated large weight and size. Most of the bulk was taken by the column oven, which contained multiple packed or capillary columns. An oven that could accommodate two to four packed columns necessitated large size. The inlet, detector, and accompanying pneumatics and electronics were also quite bulky, necessitating the large size and weight. Instruments for capillary columns, which fundamentally should require less bulk than packed columns, were mainly redesigned packed column systems. In two recent installments of “GC Connections,” we discussed how the many analog operations performed by GCs, including gas flow controls, inlet and detector heating, column oven heating, and cooling and data processing, required bulky and often heavy analog equipment and how this has been improved somewhat using digital electronics (1,2).

Small and portable GC and gas chromatography–mass spectrometry (GC–MS) instruments have been available for decades, and have even appeared in popular culture. A portable gas chromatograph was featured in the 1992 movie “Medicine Man” with Sean Connery and Lorraine Bracco (3). Several field portable GC–MS systems were recently reviewed by the U.S. Department of Homeland Security, with the comparisons made by active field security professionals (4). Over the last two decades, typically for specialized applications, GC has been implemented on chips; however, even the smallest inlet, column, and detector configurations must still be handled by humans, placing limitations on the small size of the instrument (5–7). Searching the internet using search terms such as “portable GC” or “miniature GC” will provide an extensive range of products and possibilities. In this article, we do not discuss any individual small or portable gas chromatograph. Rather, the focus of this column is describing some of the basic principles and design concepts that are common to all of them and allow them to often demonstrate performance equal to or better than their larger cousins, with generally easier usage. Ease-of-use and smaller footprint are probably the most important characteristics and reasons for using smaller instruments.

The development of full microprocessor control and the miniaturization of many electronic devices over the past two decades has enabled gas chromatographs with more and more features to be designed into increasingly small packages. These smaller systems, however, have the same challenges as their larger brethren. They must still be capable of performing all the functions of a traditional benchtop system.

The column oven must accommodate a capillary column. Ideally, this should be a traditional column, so that the widest variety of stationary phases and column dimensions is available to the user. However, traditional capillary columns are wound onto cages that are 5–10 cm in diameter, necessitating a larger oven. Because capillary columns can
be wound into much tighter coils or etched into silicon wafers, most small gas chromatographs use specialized columns that may limit the column dimensions and stationary phases that are readily available.

One area where most small gas chromatographs have excelled is in simplifying the column connections to the inlet and detector. Connections and connectors have also advanced in traditional systems, with nearly all vendors having connectors that no longer require wrenches. Traditional systems still require blind connections and measurements as the column ends must still be inserted the correct distance into the inlet and detector. Miniaturized systems have generally done away with this, using columns installed in “snap-in” cartridges, as seen in Figure 1. This is not the only possible configuration; it is just an example that readily illustrates the concepts involved in making the column and the column oven smaller than with traditional instruments. The metal-clad column is tightly coiled and mounted into a frame, including a column holder and inlet and detector connections, at the factory. As in a traditional column, leak-free connections must be made at the inlet and detector ends. This assembly includes built-in end fittings that include replaceable O-rings to ensure a tight seal. The assembly is inserted into the column oven through a drawer on the front of the instrument. The column oven is only slightly bigger than the column, allowing rapid heating and
cooling and low power consumption. Since all handling of the column or fittings occurs outside of the column oven, there is no need for the oven to be large enough to accommodate the user’s hands.

The tradeoff with snap-in or slide-in columns like those shown in Figure 1 is that the column assembly must be purchased pre-assembled and may only be available from limited vendors. In this case, a metal clad column is permanently installed in the column holder. Because the column is custom installed into the holder, the stationary phases and column dimensions that are readily available and the choices of vendor for purchasing columns may be limited. Also, in this example, the metal clad column cannot be trimmed at the inlet end to remove contamination that builds up at the column head over time, potentially reducing column lifetime. However, the column ends are also not inserted directly into the continuously heated inlet and detector, which may increase the column lifetime.

Figure 2 shows a photograph of the interior of the cabinet of a small GC, looking down from above. The column assembly, as shown in Figure 1, is inserted into the column oven through a drawer on the front of the instrument, at the bottom of the photo. All the usual components are present, but they appear quite differently than in a traditional benchtop instrument. The figure is labeled showing the inlet, column oven, detector (flame ionization), connecting tubing, and electronics.

This instrument uses a traditional split/splitless inlet. One of the challenges in miniaturizing the overall instrument is that it must still have full functionality and the parts that the analyst must interact with, change, or adjust, still need to be easily handled. Therefore, the inlet and components such as O-rings and glass sleeves on this instrument are the same as those used on a popular line of traditional instruments. Unlike traditional systems, the column end is not inserted directly into the inlet. The column end is contained in an end-fitting on the column holder, as seen in Figure 1. This is inserted into the inlet and there is a short length of tubing between the inlet liner and column. Since the column cannot be trimmed, as is typical on many smaller systems, this provides some protection to the column head from nonvolatile contaminants that may be transferred.

### TABLE I: Specifications of some typical miniaturized gas chromatographs with comparison to a traditional instrument

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mini System 1</th>
<th>Mini System 2</th>
<th>Mini System 3</th>
<th>Mini System 4</th>
<th>Traditional Benchtop System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>16.5</td>
<td>15</td>
<td>1.3</td>
<td>32</td>
<td>43</td>
</tr>
<tr>
<td>Depth x width x height (cm)</td>
<td>46 x 27 x 29</td>
<td>45 x 57 x 22</td>
<td>13 x 19 x 11</td>
<td>69 x 27 x 51</td>
<td>54 x 51 x 44</td>
</tr>
<tr>
<td>Power (V)/(A)</td>
<td>120</td>
<td>120 / 10</td>
<td>120</td>
<td>120</td>
<td>115 / 15-20</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>He, N₂, H₂</td>
<td>HE, N₂, H₂</td>
<td>Air</td>
<td>He, N₂, H₂</td>
<td>He, N₂, H₂</td>
</tr>
<tr>
<td>Inlet</td>
<td>Split/Splitless</td>
<td>PTV</td>
<td>Direct</td>
<td>Multiple</td>
<td>Multiple</td>
</tr>
<tr>
<td>Maximum oven temperature (°C)</td>
<td>400</td>
<td>380</td>
<td>160</td>
<td>450</td>
<td>450</td>
</tr>
<tr>
<td>Maximum temperature program rate (°C/min)</td>
<td>Up to 60</td>
<td>UP TO 40</td>
<td>10</td>
<td>Up to 250</td>
<td>40</td>
</tr>
<tr>
<td>Column</td>
<td>Removable custom cartridges</td>
<td>Traditional</td>
<td>5 m megabore chip—not changeable</td>
<td>Removable custom cartridges</td>
<td>Traditional</td>
</tr>
<tr>
<td>Detectors</td>
<td>FID</td>
<td>FID/TCD/ECD/FPD</td>
<td>Capacitive</td>
<td>Multiple</td>
<td>Multiple</td>
</tr>
<tr>
<td>Cost</td>
<td>$$</td>
<td>$$</td>
<td>$</td>
<td>$$$</td>
<td>$$$</td>
</tr>
</tbody>
</table>
to the column within the inlet. Some systems protect the analytical column with a short guard column between the inlet and analytical column.

As discussed above, the column oven is much smaller than on a traditional instrument and represents much of the footprint and power savings. The total volume of this oven is about 0.6 L, compared to about 18 L in a traditional oven. Most of the volume of the traditional oven is air that must be heated (and cooled) to heat and cool the column. More direct heating of the column itself results in rapid heating for temperature programming with lower power consumption. These instruments can often heat the small column oven at rates usually reserved for 220–240 V power supplies using a 120 V power supply.

This system uses a flame ionization detector (FID), which is an excellent choice for utility, sensitivity, and for a range of analytes, but may be problematic if portability is desired because of the use of hydrogen for the fuel gas. However, this system, like other small systems, is still easily installed and plumbed into traditional laboratory environments. As seen in the references cited in this article, advances in miniaturized instruments led to smaller versions of most of the common detectors plus a range of specialized detectors.

Smaller footprint, lower weight, and lower power consumption are among the biggest advantages of miniature gas chromatographs. Table I shows specifications of some typical smaller gas chromatographs compared to a full-size system. Often, two or more smaller systems can fit in the same footprint as one full-sized system. Their lighter weight makes them easier to relocate within the laboratory, and opens interesting possibilities for field and outside the lab applications. Power consumption is also reduced, again allowing multiple systems to be operated on a single 120 V, 20 A electrical circuit, or opening the possibility of operating in the field from a generator or automobile engine. The main disadvantage of the smaller footprint is that these systems often can accommodate only one inlet-column-detector combination at a time.

These instruments come in a range of sizes, costs and capabilities. Not surprisingly, a wider range of capabilities and flexibility comes with a higher price and greater bulk. The smallest, simplest, and least expensive of these systems, Mini System 3 in Table I, is designed for education only and employs a nonremovable column on a chip, a specialized detector, and uses air for carrier gas. Mini systems 1 and 2 are typical of moderately priced instruments, with capabilities that cover most traditional routine laboratory operations using a single column. Mini System 4 and the traditional system allow multiple columns in the oven at once. Mini System 4 is almost as fully capable as a traditional benchtop laboratory system.

All these instruments are easily connected to laboratory data systems using USB ports and they employ digital control of the pneumatic and thermal zones. Like their traditional cousins, they can operate as stand-alone systems or be integrated into laboratory-wide information systems. Since they are simpler to operate and require fewer consumables and techniques to maintain, training on smaller systems can also be much faster than on traditional systems.

Gas chromatographs that perform most of the basic functions needed for routine analysis are becoming smaller. Today’s smaller systems offer simplified column installation and inlet maintenance combined with smaller laboratory footprint, weight, and power consumption. Many of them can lend themselves well to portability. They provide excellent, lower-cost platforms for routine applications and high sample throughput, often allowing two or more instruments to operate in the same space as one traditional system. For low cost and convenience, the tradeoff is flexibility: inlet, column, and detector choices may be limited, so I see these instruments as excellent solutions for educational institutions, laboratories that receive and use standard methods, that have especially low or high throughput or multiple users that require training. When considering your next purchase of a gas chromatograph, I highly recommend considering smaller systems.

References

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

INTRODUCTION

When we seek help to address challenges in our work, or improve our knowledge and skills, we want to turn to sources and people with solid experience and a strong track record. When it comes to solving problems in separation science, or expanding your knowledge, we hope you will agree that an invaluable and trusted source of information and support is LCGC, including everything in our print publications—such as advice from our expert columnists, peer-reviewed research on work to advance methods, and updates on the latest approaches and technologies—as well as all we offer digitally, through webcasts, virtual symposia, e-books, newsletters, and beyond. For training, we also hope that you think of our e-learning partner, CHROMacademy.

Another important source of helpful information should not be overlooked, however: the scientists who work for instrument and consumables manufacturers. To be successful, these companies must become acutely aware of the real challenges of chromatographers—whether those customers are carrying out advanced research or routine analyses. They then must seek to address those needs by providing new and improved products, by developing applications of their tools in a wide variety of fields, by working closely with customers to answer questions and solve specific problems, and in many cases, by providing training.

For that reason, from time to time, we like to create an opportunity for people from these companies to share helpful advice and best practices with our readers. An example is found here, in this short “Vendor Tips & Tricks” feature.

I hope you find the ideas and suggestions presented here useful in your work.

Laura Bush, Editorial Director, LCGC

VENDOR TIPS & TRICKS

Five Tricks for Troubleshooting Your LC Methods

James Hogbin, Application Scientist, ACD/Labs

1. Start by determining your key predictive sample set (KPSS).
   A good start can circumvent future troubleshooting. Understand the purpose of your method development first, and determine the fewest number of samples you will need to run, while including all necessary chemical components: PRIs, intermediates, real-time stability degradants, and so on.

2. Check solubility for all analytes, and consider pH and pKa for ionizable analytes.
   If you know your analyte structures, it’s a good idea to check their ionization states across a reasonable pH range. Ionization affects logD values, and a logD predictor will show you what pH works best. For a sample that contains multiple ionizable compounds, choose a pH where the logD curve for all compounds is relatively flat.

3. Screen a selection of orthogonal columns.
   Check that you’re screening a comprehensive selection of columns by calculating the difference between available columns based on their Tanaka parameters and choosing the most orthogonal ones. This can be done with online or software tools. The latter will give you radar graphs to help you visualize the differences between columns.

4. Check your LC set-up for sources of system dispersion.
   Large-volume tubing, long lengths of tubing, flow-cell volume—all these factors can broaden peaks. These extra-column volumes will differ between systems. Heating systems will also differ: active and passive systems have different efficiencies. Check tubing and heating when transferring a method as variations in these parameters can cause the same method to perform differently.

5. Check your system’s dwell volume or gradient delay volume.
   Changes in dwell volume can also cause method failures or shifting results when transferring a method, especially when changing column dimensions. The gradient-rate change may not be allowing enough time for the new mobile-phase composition to flow through the entire column.
The Benefits of the timsTOF Platform in Four Key Points

Bruker Daltonics

Introduction
Fundamental challenges in MS-based proteomics include the high sample complexity, the large dynamic range in protein concentration, and the resulting big-data computational analysis. Development in mass spectrometers towards higher sensitivity, faster sequencing speed and larger peak capacity address many of these challenges. With the introduction of the timsTOF platform the next evolution in technology is achieved by adding an additional dimension of gas-phase ion separation via the use of TIMS coupled to UHR-QTOF technology. This results in the transition from 3D-Proteomics (retention time, m/z and MS/MS fingerprint) into 4D-Proteomics™ (addition of intrinsic CCS values). 4D-Proteomics™ enabled by PASEF® allows scientists to dig deeper into their sample, provides more confidence in data, and increases data completeness by reducing the number of missing values. It is now possible to measure hundreds and thousands of omics samples due to the extremely high sequencing speed and proven robustness.

Benefits of the timsTOF platform
1. Increased Sensitivity and High Acquisition Speed at Ultra-High Resolution: PASEF enables MS/MS acquisition at >100 Hz, making high-throughput measurements using short gradients possible while maintaining deep proteome coverage or high protein depth measurements in less time. The time-focusing benefit of dual-TIMS funnel technology increases sensitivity thereby allowing for loading of lower sample amounts and simpler sample preparation techniques.

2. Mobility Offset Mass Aligned (MOMA): The MOMA benefit provided by TIMS technology identifies more near-isobaric peptides and post-translationally modified (PTMs) peptides per unit time. PTM-positional isomers, a common occurrence, are not only detected but can be quantified.

3. CCS-Aware Workflows and Open Data Format: CCS-Aware analysis software increases the confidence of identifications and quantitation, while increasing data completeness. Open data format easily allows timsTOF data to be used in custom developed bioinformatics solutions.

4. Increased Robustness: The orthogonal geometry of the inlet glass capillary to the axis of the mass analyzer combined with dual-TIMS funnel technology, greatly increases robustness, allowing for sustained MS performance over large sample cohorts, maximizing instrument uptime.

For complete details please go to: https://www.bruker.com/en/landingpages/bdal/grants.html
Five Tips & Tricks

A main goal of any U/HPLC analysis is to achieve the optimum sensitivity with the given instrumentation. Here are a few simple measures that can be easily implemented.

1. Mobile-phase considerations
   - **Choose the right solvent grade.** Solvents that are not “clean enough” will give high background signal. Several solvent grades are available for HPLC, for example “HPLC grade,” “LC–MS grade” or “UHPLC–MS grade”. Choose the solvent grade that matches your method requirements. Examining the specification sheet or Certificate of Analysis helps to decide which one is best suited. These should state whether, for example, an LC–MS-grade solvent has been tested for LC–MS suitability.
   - **To avoid ion suppression in LC–MS,** only highly pure volatile mobile-phase buffers and additives such as ammonium formate, acetate or triethylamine should be utilized, and at moderate concentrations. Make sure these reagents are MS grade quality. Also, since polyethylene glycol (PEG) is a common contaminant detected in LC-MS, find a solvent that has a specification for PEG, such as the LiChrosolv® UHPLC-MS grade solvents, for which levels of PEG are monitored in every batch produced.
   - **To avoid ion suppression in LC–MS,** only highly pure volatile mobile-phase buffers and additives such as ammonium formate, acetate or triethylamine should be utilized, and at moderate concentrations. Make sure these reagents are MS grade quality. Also, since polyethylene glycol (PEG) is a common contaminant detected in LC-MS, find a solvent that has a specification for PEG, such as the LiChrosolv® UHPLC-MS grade solvents, for which levels of PEG are monitored in every batch produced.

2. Equilibration time considerations
   - **In reversed-phase methods, avoid running long equilibration times under highly aqueous conditions,** because this leads to contaminant buildup in the column head. In gradient runs, these contaminants elute at specific retention times and compromise sensitivity or even generate ghost peaks. Figure 1 shows that both baseline noise and contaminant peak intensities increase with extended equilibration time.
   - **In reversed phase,** it is a good practice to periodically flush the column with 10 column volumes of 100% organic solvent, such as acetonitrile, to remove any contaminants that have built up.

3. HPLC column considerations
   - **Go small on the column i.d..** As column i.d. decreases (while geometrically scaling injection volume and flow rate accordingly), signal-to-noise ratio increases, which improves sensitivity.
   - **Go small on particle size.** If the LC system’s pressure limits allow, use smaller particle columns. Smaller particles give better separation efficiency.
   - **Go with fused-core or superficially porous particles (SPP).** SPPs provide high resolution and efficiency without the need to handle high backpressures.
   - **Go with monolithic columns.** These provide high matrix tolerance and enable rapid separations at low backpressure.

4. Instrument consideration: Avoid dead volumes
   - A proper setup of the HPLC system itself can contribute to increased sensitivity. It is important to minimize the dead volume, for example, the volume of all system parts from the injector to the detector cell but without the HPLC column volume. Large dead volumes can cause peak broadening, tailing, or splitting, leading to poor resolution and decreased performance—and hence lower sensitivity and the failure to detect low abundant analytes. Consequently, all system parts (tubing, connectors, fittings, and so on) must contribute to minimizing dead volumes.

5. Sample considerations: Clean up
   - When working with difficult matrices, using solid phase extraction (SPE) helps to remove major contaminant groups such as proteins and salts. Take advantage of selective sorbents for specific matrices such as zirconia-based hybridSPE® for removal of phospholipids and proteins in plasma or serum.
Things to Consider for Your Next GC–MS Instrument

Farai Rukunda, Customer Relations and Service Manager, Separation Science, LECO Corporation

It’s easy to line up a list of GC–MS instruments and rank them by price, sensitivity, or if they have a specific feature you’re looking for, but there are some factors that go into a purchase of this size you would do well to consider.

1) How much downtime is too much?
Every instrument is going to have some inevitable downtime, whether it’s from standard maintenance, hardware changes, system tunings, or unfortunate breakdowns. Look for instruments and support packages that actively work to minimize downtime, such as easily accessible parts, ion sources that don’t get dirty, automatic system tuning, or quality control checks you can pre-schedule to be finished before you come into the lab, and timely service responses.

2) How much do you want to spend on consumables?
Whether it’s helium gas or delicate columns, every GC–MS instrument has a secondary cost of consumables. Instruments that have automatic standby methods or are engineered for complete synergy with their consumables will ultimately cost you less in the long run.

3) How much manual management of your data is acceptable?
Imagine how much real work you could get done if you didn’t have to manage the various files of your project yourself, or if you could click a button and have the software handle the simple work of checking library values and reporting back identifications with accurate levels of confidence. When researching GC–MS instruments, look for software that can take the busywork out of analyses. Database-driven software that can handle the metadata and link files together saves you from opening multiple files to check on the method used, while a system that can do a full-spectrum scan means you don’t need to rerun your samples if the scope of your analysis changes.

4) How can the system grow?
There are always new advancements in science, and cutting-edge technology today can be obsolete tomorrow. Look for instruments with upgrades and options that will allow you to expand your abilities, such as upgrades from GC to GCxGC, without needing to expand your vendor list.

Pall. For Whatever’s on Your Plate

Pall’s AcroPrep™ 96-well filter plates with water-wettable PTFE membranes offer fast and easy sample prep for HPLC and LC–MS analytical workflows. For HPLC analysis of plants or cells, AcroPrep 96-well and 24-well 30–40 μm filter plates with PP/PE membranes rapidly and efficiently remove large particulates, helping protect the column. Complimentary, Pall’s AcroPrep Advance NAB 96-well Long Tip Filter Plate is designed for the specific purpose of extracting, isolating, and purifying genomic DNA (gDNA), RNA, and plasmid DNA (pDNA) from various starting materials such as bacteria, yeast, mammalian cultured cells, and plants, for analytical research applications. Pall’s filter plate portfolio unlocks the power of high-throughput processing for analytical QC workflows.
Rapid Simultaneous Determination of Five Major Alkaloids from Menispermi Rhizoma in Rat Urine by Ultrahigh-Pressure Liquid Chromatography–Tandem Mass Spectrometry (UHPLC–MS/MS) and its Application to a Urinary Excretion Study

Alkaloids are the main bioactive ingredients derived from menispermi rhizoma (MR), which is often used in traditional Chinese medicine. However, simultaneous determination of different types of alkaloids in urine has not been reported. A sensitive, rapid, and efficient ultrahigh-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method for rapid quantification of five major bioactive alkaloids (covering three different types) in rat urine has been established. Extraction of five alkaloids from rat urine was performed with acetonitrile precipitation. The separation was carried out on a C18 column, and the detection was performed with positive electrospray ionization (ESI) MS in multiple reaction monitoring (MRM) mode. The method was fully validated and successfully applied to a urinary excretion study of five alkaloids in rats after they were given an oral dose of MR extract. It is the first time that the urinary excretion profiles of dauricoside, acutumine, and acutumidine in rats has been reported. The results obtained lay the foundation for the clinical application and safety evaluation of MR.

Jinxia Wei, Yingying Yu, Yanan Li, Xingjie Guo, and Yubo Li

Menispermi rhizoma (MR) is the dried rhizome of Menispernum dauricum DC, which is a well-known Chinese herb and widely used in traditional Chinese medicine. It is reported in the Chinese Pharmacopoeia that the herb can treat sore throats, enteritis dysentery, and rheumatism arthralgia (1). In the last few decades, much attention has been focused on the chemical composition and pharmacological studies of MR. According to the literature, the ethanol extract of MR exhibits a variety of pharmacological effects, such as anti-inflammatory (2,3), anti-bacterial (4), antioxidant (5), anti-hypoxia (6), anti-tumor (7), anti-arrhythmia (8) effects, and the protection of injury induced by myocardial-cerebral ischemia (9,10). These pharmacological activities are presumed to result from the presence of various bioactive ingredients in MR.

Some phytochemical research on MR showed that it contains a large number of alkaloids, which include bisbenzylisoquinoline, protoberberine, and morphinane (11). Among these alkaloids, dauricine, dauricicoline, dauricoside, acutumine, and acutumidine (shown in Figure 1) are not only the most abundant alkaloids in MR, but also the most physiologically active ones. The biological activities of the five alkaloids have been widely reported. Dauricine has shown obvious effects on inhibiting blood platelet aggregation (12) and neuroprotection (13). Dauricine has also shown effects on anti-arrhythmia and anti-tumors (14). Moreover, according to the Chinese Pharmacopoeia, dauricine is used as the only component to assess the quality of MR. The antiarrhythmic effect of dauricine has been verified in several animal models, and it is widely used in cardiac arrhythmia patients (15). Meanwhile, dauricine exhibited a significant reverse use-dependent effect by blocking the Na+, K+, and Ca2+ ion currents of cardiac transmembranes (16). It is reported that dauricine can suppress hypoxia-inducible factor 1-alpha (HIF-1a) protein accumulation and vascular endothelial growth factor (VEGF) expression to inhibit cancer angiogenesis. Our previous study demonstrated that dauricicoline had a protective effect on hypoxia injury of EAhy 926 cells (6). Dauricoside has been proven to possess significant selective affinity to dopamine receptor D1 (17) and an inhibitory effect on blood platelet aggregation caused by adenosine 5’-diphosphate (12). Both acutumine and acutumidine belong to morphinanes, which have multiple biological activities. For example, they have significant anti-hypoxia effects (6). Acutumine has been reported to possess selective cell cytotoxicity (18). Acutumidine can also inhibit the production of hepatitis B surface antigen (HBsAg), and the IC50 value is 2.023 mM (19). To date, a large number of research studies on the pharmacology of MR have been carried out.

Generally, synergy among multiple constituents in vivo plays an important role in the pharmacological action of traditional Chinese medicines (20). Thus, simultaneous determination of multiple bioactive ingredients in vivo is crucial to illustrate the pharmacological and clinical effects of MR. Studies on MR in vivo still remain scarce; studies have mainly focused on the determination of these alkaloids in plasma (21–23).
Urine excretion of drugs is essential to investigation of interpreting in vivo dispersion, which is also closely related to the efficacy and safety of drugs (24). To date, only two articles have reported on the excretion of dauricine (25, 26). As a result, there is no successful approach for simultaneous determination of the five alkaloids in urine because of the lack of reference standards. Meanwhile, a urinary excretion study of the five alkaloids after oral administration of MR extract has not been reported. This is a great obstacle to further investigation of safe administration of MR in clinical applications. Thus, it is urgent to establish an available bioanalytical method for simultaneous determination of the five alkaloids in urine samples.

Currently, several analytical approaches, such as capillary electrophoresis (CE) (27), high-performance liquid chromatography (HPLC) with UV detection (28), diode array detection (DAD) (29), and tandem mass spectrometry (MS/MS) (30) have been employed to quantify alkaloids in MR. Among these methods, HPLC-UV, HPLC-MS/MS, and UHPLC-MS/MS have been used to quantify alkaloids from MR in rat plasma (21–23, 25). UHPLC–MS/MS combined with multiple reaction monitoring (MRM) mode has attracted much attention and become increasingly popular in bioanalysis because of its unique advantages, such as high speed, high selectivity, and strong specificity. Thus, the purpose of this study was to develop a rapid and sensitive UHPLC–MS/MS approach in MRM mode for simultaneous determination of five alkaloids (covering three different types) in rat urine and to apply it in a urinary excretion study. It is the first report on a UHPLC–MS/MS assay of five alkaloids in urine after dosing of MR extract. The results could be useful for the further studies on the usage, dosage, mechanisms of action, and pathology and toxicology of MR.

Materials and Methods

Reagents and Materials

The reference standard of dauricine was obtained from Shenzhen Meihe Biolog-
Mean cumulative urinary excretion-time profile of 5 analytes in rats after oral administration of menispermi rhizoma extract (5.77 g/kg). Axes labels for all figures are time (min) for x-axis and cumulative excretion (ng) for the y-axis.

**FIGURE 3:** Typical MRM chromatograms of the 5 active components and IS in rat urine: (a) blank rat urine sample; (b) blank rat urine spiked with the 5 analytes at LLOQ and IS; (c) rat urine 0-4 h samples collected after oral administration of menispermi rhizoma extract (5.77 g/kg). Axes labels for all figures are time (min) for x-axis and percent (%) for y-axis.

**FIGURE 4:** Mean cumulative urinary excretion-time profile of 5 analytes in rats after oral administration of menispermi rhizoma extract (n = 6). Axes labels for all figures are time (min) for x-axis and cumulative excretion (ng) for the y-axis.

Materia Medica, Shenyang Pharmaceutical University). A voucher specimen (Number 20141001) has been deposited in the Department of Pharmaceutical Analysis at Shenyang Pharmaceutical University. The internal standard (IS), verapamil hydrochloride with a purity of 99.00%, was obtained from the National Institutes for Drug and Food Control (batch number: 100223-200102, Beijing, China). HPLC grade acetonitrile and methanol were supplied by Fisher Scientific. Formic acid of HPLC grade was also obtained from Sigma-Aldrich. Tetrahydrofuran was purchased from Concord Technologies. Ultrapure water was purchased from Wahaha Corporation Ltd.

**UHPLC–MS/MS Conditions**

The liquid chromatography–triple quadrupole mass spectrometer consisted of an Acquity UPLC system and a Xevo TQ-S API triple quadrupole mass spectrometer system (Waters Corporation, Milford, Massachusetts, USA). The chromatographic separation of analytes and IS was conducted on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm). A Vanguard BEH C18 column (5 mm × 2.1 mm, 1.7 μm) acted as the guard column. The column temperature was kept at 35 °C. Gradient elution employed a mobile phase of 0.1% formic acid in water (A) and acetonitrile (B) with a flow rate at 0.3 mL/min. The gradient elution was programmed as follows: 0–2.0 min, 8% B; 2.0–4.0 min, 8–20% B; 4.0–8.0 min, 20–60% B; and 8.0–10.0 min, 60–8% B. The injection volume was 5 μL.

Analyste detection was conducted using positive ion electrospray ionization (ESI) in the MRM mode. Nitrogen was used as both nebulizing and drying gas. The optimized instrument parameters were as follows: The capillary voltage was set at 3.0 kV; the source temperature was 150 °C; the desolvation temperature was 350 °C; and the desolvation and cone gas flow were set at 700 and 150 L/h, respectively. The cone voltage, collision energy, and MRM transitions for each analyte and IS varied; their specific parameters were optimized and are displayed in Table I. Chromatograms and data acquisition were performed using a data qualitative and quantitative analysis system.

**Preparation of MR Extract**

MR powder (500 g) was mixed with 5.0 L ethanol:water (95:5, v/v) and refluxed twice (1.0 h each time). Then, extraction with ethanol:water (75:25, v/v) was performed twice using the same method. The extraction solutions were filtered and concentrated under vacuum. To calculate the quantity of drug administered, the amounts of five alkaloids in MR extract were measured by the external standard method. The contents of dauricine, dauricoline, dauricoside, acutumine, and acutumidine were 23.19, 3.63, 0.711, 0.167, and 0.762 mg/g, respectively.

**Preparation of Standards and Control Solutions**

Stock solutions of dauricine, dauricoline, dauricoside, acutumine, and acutumidine were separately prepared by dissolving the compounds in tetrahydrofuran and then precisely diluted with methanol to achieve final concen-
trations of 0.1 mg/mL. Then, an appropriate amount of the five stock solutions was mixed and gradiently diluted with methanol to obtain a linear concentration of standard working solutions. The working solution of the IS at 10.0 ng/mL was obtained from the stock solution.

Calibration standards and quality control samples were prepared in a glass tube with tapered bottom by evaporating 100 μL working solutions to dryness with nitrogen (N₂) and then mixing with a 100 μL of blank urine. The ranges of urine concentration were 5 to 2500 ng/mL for dauricine; 2.4 to 1200 ng/mL for dauricoline; 2 to 1000 ng/mL for dauricoside; 6 to 3000 ng/mL for acutumine; and 10 to 5000 ng/mL for acutumidine, respectively. Quality control (QC) samples were prepared at three concentration levels of 10, 141, and 2000 ng/mL for dauricine; 4.8, 68, and 960 ng/mL for dauricoline; 4, 57, and 800 ng/mL for dauricoside; 12, 170, and 2400 ng/mL for acutumine; and 20, 283, and 4000 ng/mL for acutumidine.

**Urine Sample Preparation**

A simple protein precipitation (PPT) method was used for the extraction of five alkaloids from rat urine. First, 20 μL of IS solution was put into glass tubes and evaporated to dryness with N₂. Next, 100 μL of the urine sample was transferred to the glass tube and vortexed for 1 min. Then, 500 μL of acetonitrile was added. After vortexing for 2 min, the mixture was centrifuged for 10 min at 15,000 rpm. The supernatant was removed to another polyethylene tube and evaporated to dryness with N₂. The residues were reconstituted with 100 μL of 50% aqueous acetonitrile. After vortexing

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Calibration Curves</th>
<th>Linear Range (ng/mL)</th>
<th>r</th>
<th>LLOQ</th>
<th>Nominal Concentration (ng/mL)</th>
<th>RSD (%)</th>
<th>RE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dauricine</td>
<td>( y = 6.19e^{-3} x - 2.26e^{-4} )</td>
<td>5.0–2500</td>
<td>0.9910</td>
<td>5.0</td>
<td>7.7</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Dauricoline</td>
<td>( y = 2.76e^{-3} x - 2.17e^{-4} )</td>
<td>2.4–1200</td>
<td>0.9902</td>
<td>2.4</td>
<td>8.0</td>
<td>-5.4</td>
<td></td>
</tr>
<tr>
<td>Dauricoside</td>
<td>( y = 6.01e^{-3} x - 2.47e^{-4} )</td>
<td>2.0–1000</td>
<td>0.9980</td>
<td>2.0</td>
<td>12.4</td>
<td>-3.0</td>
<td></td>
</tr>
<tr>
<td>Acutumine</td>
<td>( y = 9.63e^{-3} x - 1.35e^{-3} )</td>
<td>6.0–3000</td>
<td>0.9954</td>
<td>6.0</td>
<td>3.6</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Acutumidine</td>
<td>( y = 2.03e^{-3} x - 3.87e^{-4} )</td>
<td>10.0–5000</td>
<td>0.9972</td>
<td>10.0</td>
<td>5.1</td>
<td>-9.0</td>
<td></td>
</tr>
</tbody>
</table>
for 2 min and centrifuged for 10 min at 15,000 rpm, a 5 μL aliquot of supernatant was injected for UHPLC–MS/MS analysis.

Method Validation

The method was validated according to the guidelines set by the United States Food and Drug Administration (FDA) for bioanalytical method validation (31). Selectivity was evaluated by analyzing the chromatograms of six individual blank rat urine samples, blank urine samples spiked with the five analytes and IS, and the actual urine samples obtained from the excretion study after administration of MR extract.

Calibration samples were prepared and assayed in duplicate over three consecutive days. The calibration curve of seven concentration levels was constructed by plotting the peak area ratios (y) of each analyte to IS versus urine sample concentrations (x) of the analytes, using a weighted 1/x² linear regression. The limit of detection (LOD), depicted as the concentration of the analyte giving a signal-to-noise (S/N) ratio above 10, was determined at three concentration levels. The matrix effects of rat urine for five alkaloids and IS were evaluated by comparing the peak response of post-extracted blank urine samples spiked in post-extracted blank urine with those from the extracted QC samples with those of freshly prepared solutions, respectively. The recovery of five alkaloids and IS was measured by comparing the peak responses of five alkaloids and IS from extracted QC samples with those of samples spiked in post-extracted blank urine at an equivalent concentration. The matrix effects of rat urine for five alkaloids and IS were evaluated by comparing the peak response of post-extraction spiked samples with those from the non-extracted neat standard solutions at equivalent concentration. Both recovery and matrix effects of the five analytes were determined at three concentration levels, whereas for the internal standard, the recovery and matrix effect were determined at a single concentration of 2.0 ng/mL by determining six replicates of QC samples.

The stability of the five alkaloids in rat urine was determined by analyzing QC samples in six replicates at three concentration levels. The short-term stability of the analytes in rat urine was determined after keeping the spiked samples at room temperature for 8 h. Long-term stability was assessed after keeping the QC samples at −80 °C for two weeks. Freeze–thaw stability in rat urine was investigated after three freeze–thaw cycles. The post-preparation stability was evaluated after keeping the extracted QC samples in an auto-sampler at 10 °C for 12 h. The stability of the standard solutions (analytes and IS) was assessed by comparing the determined concentration of the solutions kept at 25 °C for 4 h and 12 h, and 4 °C for one month with that of the freshly prepared solutions, respectively.

When the concentrations of urine samples are higher than the upper limit of quantitation (ULOQ) of each analyte, it is required to dilute them with blank urine. To validate the dilution process, a 10x dilution of urine samples were assessed

### Table III: Precision, accuracy, recovery and matrix effect of 5 analytes in rat urine samples (n = 6)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (ng/mL)</th>
<th>Precision (RSD, %)</th>
<th>Accuracy (RE, %)</th>
<th>Recovery (%)</th>
<th>Matrix Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday</td>
<td>Interday</td>
<td>Intraday</td>
<td>Interday</td>
<td>Intraday</td>
</tr>
<tr>
<td>Dauricine</td>
<td>10.0</td>
<td>3.2</td>
<td>12.0</td>
<td>-10.2</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>141.0</td>
<td>6.8</td>
<td>7.7</td>
<td>-7.5</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>2.8</td>
<td>6.3</td>
<td>3.2</td>
<td>-6.3</td>
</tr>
<tr>
<td>Dauricoline</td>
<td>4.8</td>
<td>3.4</td>
<td>6.5</td>
<td>-5.4</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>68.0</td>
<td>5.3</td>
<td>7.4</td>
<td>-6.8</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>960.0</td>
<td>2.6</td>
<td>4.1</td>
<td>7.3</td>
<td>5.1</td>
</tr>
<tr>
<td>Dauricoside</td>
<td>4.0</td>
<td>10.4</td>
<td>11.5</td>
<td>2.1</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>57.0</td>
<td>11.2</td>
<td>10.5</td>
<td>11.2</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>800.0</td>
<td>6.7</td>
<td>7.2</td>
<td>7.5</td>
<td>-3.1</td>
</tr>
<tr>
<td>Acutumine</td>
<td>12.0</td>
<td>8.9</td>
<td>6.1</td>
<td>-6.6</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>170.0</td>
<td>12.1</td>
<td>7.8</td>
<td>-4.8</td>
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</tr>
<tr>
<td></td>
<td>2400</td>
<td>7.3</td>
<td>6.6</td>
<td>-3.1</td>
<td>-6.7</td>
</tr>
<tr>
<td>Acutumidine</td>
<td>20.0</td>
<td>10.6</td>
<td>7.4</td>
<td>-0.7</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>283.0</td>
<td>7.0</td>
<td>3.5</td>
<td>-3.0</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>6.9</td>
<td>2.1</td>
<td>-5.6</td>
<td>8.3</td>
</tr>
</tbody>
</table>
in six duplicates. The diluted QC samples were prepared at a concentration above the ULOQ sample level and diluted 10-fold with blank rat urine.

To confirm that carryover had no effect on the accuracy and precision of the assay, carryover effect was evaluated by six consecutive injections of a blank sample after the injection of an ULOQ sample.

**Excretion Study**

The described UHPLC–MS/MS method was applied to determine the urine concentrations of the 5 alkaloids after dosing of MR extract. Six male Wistar rats (220–260 g) were supplied by the Animal Center of Shenyang Pharmaceutical University. The rats had free access to food and water and were acclimatized in an environmentally controlled breeding room before experimentation. Under the above conditions, all the rats were acclimated for at least one week and then fasted for 12 h prior to experimentation. All experiments were done in strict accordance with the Regulations of Experimental Animal Administration. The MR extract was dissolved in 0.5% carboxymethyl cellulose sodium (CMC-Na) solution and was orally administered at a dose of 5.77 g/kg (equivalent to 133.78 mg/kg of dauricine, 20.95 mg/kg of dauricicoline, 4.10 mg/kg of dauricoside, 0.96 mg/kg of acutumine, and 4.40 mg/kg of acutumidine). Afterward, the rats were individually put in metabolic cages to collect rat urine. Urine samples were collected before administration and at the time intervals of 0–4, 4–8, 8–12, 12–24, 24–48, 48–72, 72–96, 96–120, and 120–144 h post dosing. The urine volume collected in each interval was recorded. All sample superna-

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**TABLE IV: Stability of analytes in rat urine samples under various storage conditions (n = 6)**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal concentration (ng/mL)</th>
<th>25 °C for 8 h</th>
<th>Frozen for 14 days</th>
<th>Three Freeze-Thaw Cycles</th>
<th>Post-Preparation Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precision (RSD, %)</td>
<td>Accuracy (RE, %)</td>
<td>Precision (RSD, %)</td>
<td>Accuracy (RE, %)</td>
<td>Precision (RSD, %)</td>
</tr>
<tr>
<td>Dauricine</td>
<td>10.0</td>
<td>11.2</td>
<td>-2.5</td>
<td>4.2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>141.0</td>
<td>7.5</td>
<td>-1.9</td>
<td>3.7</td>
<td>-7.9</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>6.9</td>
<td>3.2</td>
<td>5.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Dauricoline</td>
<td>4.8</td>
<td>5.6</td>
<td>-5.1</td>
<td>6.9</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>68.0</td>
<td>4.4</td>
<td>7.9</td>
<td>5.3</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>960.0</td>
<td>3.1</td>
<td>0.4</td>
<td>2.1</td>
<td>-3.7</td>
</tr>
<tr>
<td>Dauricoside</td>
<td>4.0</td>
<td>7.7</td>
<td>-11.0</td>
<td>10.5</td>
<td>-7.9</td>
</tr>
<tr>
<td></td>
<td>57.0</td>
<td>4.8</td>
<td>1.6</td>
<td>5.6</td>
<td>-9.0</td>
</tr>
<tr>
<td></td>
<td>800.0</td>
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tants collected were stored at −80 °C until further analysis.

Results and Discussion
Optimization of the UHPLC–MS/MS Conditions
In this study, a BEH C18 column (50 mm × 2.1 mm, 1.7 μm, Waters) compatible with low liquid flow rate was selected, which offered several attractive benefits, including higher peak intensity, lower solvent consumption, and a shorter runtime. Using this column, methanol and acetonitrile organic phases were investigated. A higher mass spectrometry (MS) response was obtained when rat urine was processed by LLE. Thus, the PPT method was adopted.

Sample Preparation Optimization
Sample processing plays an important role in bioanalytical assays. To improve recovery and minimize endogenous interference, both PPT and liquid–liquid extraction (LLE) were investigated in this study. The results indicated that low extraction recovery (especially for dauricoside) was obtained when rat urine was processed by LLE. Thus, the PPT method was adopted. Methanol and acetonitrile were tested as precipitation reagents. Eventually, acetonitrile proved to be a better extraction solvent for the five analytes.

Method Validation
Representative MRM chromatograms of blank urine, blank urine spiked with the target compounds (at LLOQs) and IS, and a urine sample after dosing are shown in Figure 3. No interfering peaks were detected at the retention times of five analytes and IS.

The standard calibration curves, linear ranges, linearity (r) and LLOQs of the 5 alkaloids are listed in Table II. The calibration curves showed good linearity with a correlation coefficient r ≥ 0.9902. The LODs for dauricine, dauricicoline, dauricoside, acutumine, and acutumidine were 1.5, 0.6, 0.6, 1.8, and 3.0 ng/mL, respectively. The LLOQs for dauricine, dauricicoline, dauricoside, acutumine, and acutumidine were 5.0, 2.4, 2.0, 6.0, and 10.0 ng/mL, respectively, which is sensitive enough to explore the excretion behaviors of the analytes.

The summary results for precision and accuracy of five alkaloids are listed in Table III. The intraday precision varied from 2.6% to 12.4%, while the interday precision varied from 2.1% to 12.0%. The intra- and inter-day accuracy ranged between -10.2% and 11.2%. It was found that both precision and accuracy values were all within the acceptable criteria (±15%, except ±20% at LLOQ).

The extraction recoveries and matrix effects of the five analytes and IS in rat urine are listed in Table III. The mean recoveries of dauricine, dauricicoline, dauricoside, acutumine, and acutumidine from rat urine were 91.4–96.0%, 89.4–96.9%, 89.3–98.6%, 76.4–85.6%, and 85.6–94.9% at the three QC levels. The mean recovery of IS normalized verapamil was 87.4% at a concentration of 2.0 ng/mL. The mean matrix effects of rat urine for analytes and IS ranged from 85.1–109.0%. Both the extraction recoveries and matrix effects met the acceptance criteria of the FDA.

The results of the standard solutions stability study indicated that the standard solutions of the analytes were stable for at least one month at 4 °C (RE: −4.3% to 3.5%, RSD <4.5%). The stability results of the analytes in rat urine are summarized in Table IV. It was found that the analytes were stable under various processing conditions including 10 °C for 12 h, three freeze-thaw cycles, 8 h at room temperature, and −80 °C for two weeks.

The precision and accuracy of the dilution integrity were validated. The results showed that precision and accuracy at each concentration were within the acceptable criteria (RSD <15%; RE ±15%). The samples with concentrations greater than the ULOQ could be met by appropriate dilution with blank matrix.

When injecting blank sample after ULOQ samples, no signal at the retention time of the analytes and IS was detected. The results indicated that the carryover from residues was negligible.

Urinary Excretion Study
The newly established UHPLC–MS/MS approach was applied to the rat urinary excretion study of the five alkaloids after dosing of MR extract (5.77 g/kg). The mean cumulative excretion-time profiles of the five alkaloids in rat urine are displayed in Figure 4. Results showed that five alkaloids were mainly excreted within 144 h after oral dosage. The cumulative excretion amount of the five alkaloids in urine over the 144 h period were 335118 ± 63965 ng for dauricine, 54564 ± 13915 ng for dauricicoline, 8860 ± 2019 ng for dauricoside, 16371 ± 5155 ng for acutumine, and 83130 ± 24784 ng for acutumidine.

Dauricine and dauricicoline exhibited a similar urinary excretion characteristic because they have the same structural skeleton. There was significant increase for them in their urine excretion after 12 h, and the rate of excretion continued to increase from 12 h to 48 h. The cumulative excretion of the two analytes reached to a plateau at 72 h after oral administration. Compared with the dosage, the recoveries for dauricine and dauricicoline within 144 h were about 1.255% and 1.302%, respectively.
respectively. The results suggest that dauricine and dauricicoline were mainly eliminated as metabolites in urine. In addition, the urine excretion characteristic of dauricine has been reported previously (25); the results of the report in the literature were higher than the results of this study. It is speculated that the other components in MR may promote the metabolism of dauricine in vivo. In terms of dauricoside, there was a significant increase in urine excretion from 0 h to 4 h, and the cumulative excretion of dauricoside reached a plateau at 72 h after oral administration. The cumulative urine excretion amount accounted for 1.080% of the total dose. Perhaps it was extensively metabolized under the action of intestinal enzymes and the excretion forms of dauricoside were its metabolites (including its aglycone). Thus, further experiments on its metabolism will need to be conducted. Both acutumine and acutumidine are morphine alkaloids, and showed a similar urinary excretion process. The excretion rate increased significantly from 12 to 24 h after dosing, and reached a plateau at 48 h. Total recoveries of acutumine and acutumidine over the 144 h period were about 8.50% and 9.45% of the total dose, respectively. Compared with the above three alkaloids, the morphine alkaloids have a higher excretion ratio in the prototype form, which may be attributed to their higher polarity. In this study, the urine excretion characteristics of dauricoside, acutumine, and acutumidine were reported for the first time.

**Conclusion**

This newly established UHPLC–MS/MS bioanalytical method was rapid, efficient, and highly sensitive, with a short run time of 10.0 min, and met all the requirements in bioanalysis of the FDA bioanalytical guidelines. The analytical method was successfully applied to excretion studies of the five alkaloids following administration of MR extract. This is the first time to report of excretion studies of dauricine, dauricicoline, dauricoside, acutumine, and acutumidine together in vivo after administration of MR extract. We believe that the described results will be of value in accelerating further research on the mechanism of action of MR and will provide useful information for the clinical application and development of MR.

**Acknowledgments**

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**References**


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Free Excel Software for Performing Virtual Liquid Chromatography

The “Practical HPLC calculator” is a new free simulator using Microsoft Excel® for virtually learning the concepts of high performance liquid chromatography (HPLC) and ultrahigh-pressure liquid chromatography (UHPLC). It has been developed by researchers from the University of Geneva in Switzerland to teach LC concepts using a virtual model calculator. This software is useful for students, professional educators, and trainers to teach and perform virtual HPLC experiments and to accomplish a limited number of real experiments using an HPLC instrument.

Davy Guillarme, Balazs Bobably, and Jean-Luc Veuthey

Following a very informative recent article from Charles A. Lucy published in LC GC North America (1) about simulation software available for chromatography, we wanted to highlight the existence of a new simulator for virtual liquid chromatography (LC) developed by researchers from the University of Geneva in Switzerland. With the current COVID-19 pandemic resulting in limited access to the laboratory, the existence and availability of such a tool can be extremely beneficial for teaching activities.

"Practical HPLC calculator" is a free Excel tool that can be downloaded directly from this website: https://ispso.unige.ch/labs/fanal/practical_hplc_simulator:en. There are two versions of this Excel spreadsheet available. The first version does not contain any macro, which is designed for those who are not allowed to use macro in Excel, for safety reasons. The second one includes several macros that can reset all the values to default by clicking on one button, configure the calculator to mimic a standard high performance liquid chromatography (HPLC) instrument, and configure the calculator to mimic a standard ultrahigh-pressure liquid chromatography (UHPLC) system. The second version with macro should be preferentially used whenever possible.

The main purpose of this calculator is to teach LC in a virtual way. Therefore, this calculator could be at the center of "virtual practical works" for students that are in college or currently undertaking internships. It can also be used by professional trainers during education courses to highlight some particular concepts related to HPLC, using simulated chromatograms.

In our School of Pharmaceutical Sciences at the University of Geneva, we use this tool to perform hybrid practical works. Students spend part of their time doing HPLC experiments virtually on the calculator (without the need to perform repetitive experiments), and once they master the basics of HPLC, they can do a very limited number of real experiments on a HPLC instrument to see how it works in practice. This is a valuable solution to reduce the number of instruments necessary in the practical work laboratory and the cost of operation.

This software allows an easy understanding of the impact of all chromatographic parameters on the final separation. For this purpose, the user has a choice between seven different mixtures of representative compounds composed of a number of molecules comprised between four and seven. Among the available mixtures, we can find parabens, nonsteroidal anti-inflammatory drugs, lidocaine formulation, cannabinoids, liposoluble vitamins, salbutamol, and related impurities, along with doping agents.

The software can also simulate the chromatogram obtained in any condition. The user will be able to tune the following parameters: mobile phase (pH and type of organic modifier), pump (elution mode, composition, flow rate, dwell volume), injector (volume injected, concentration, sample type, sample diluent), column (length, diameter, particle size, chemistry, porosity), oven (temperature), ultraviolet (UV) detector (cell volume, wavelength, time constant, data acquisition rate), and tubing used in the system (length and diameter).

Then, the software will show the corresponding simulated chromatogram, and the user will also have access to a variety of additional parameters, including retention times, retention factors (k’), peak widths at half height, efficiencies (N), peak capacities, asymmetries, resolutions, peak heights, peak areas, and background noise for all compounds under the simulated conditions. The software will also evaluate the efficiency loss because of the instrument (extra-column effects), the overall quality of the separation and will provide the UV spectrum and ionization profile of the analyzed compounds.

It is important to mention that more than 3500 practical experiments were performed (which cumulatively totaled to 37 molecules, four columns, two organic modifiers, four mobile phase pH, two mobile phase temperatures, and two gradient times) to develop this simulation tool and mimic in the best possible way the chromatographic behavior of all the compounds that can be analyzed with this tool. All these experiments were performed using an ultraviolet diode array detector (UV-DAD) from 200 to 350 nm to have reliable sensitivity at various wavelengths, as well as realistic background noise and baseline drift. Based on the practical experiments, the retention models (log k’ and S) were
calculated to simulate any isocratic or gradient conditions. Last, but not least, van’t Hoff parameters were also obtained from the experiments performed at two temperatures, which allowed the simulation of chromatograms at any other temperature suggested by the user.

For those involved in teaching, a document can be downloaded from the same address: https://ispso.unige.ch/labs/fanal/practical_hplc_simulator:en. It shows an example of a virtual practical work in HPLC that we have developed and can be used by anyone interested. It requires approximately one full day for a student to do this virtual practical work including the writing of the corresponding report summarizing the findings.

To develop some new HPLC exercises with this software, below is a nonexhaustive list of topics that can be explored with this calculator depending on the level of the users.

For a beginner, they can explore the following topics: a) the impact of modification of percent organic modifier on overall performance; b) the impact of the organic modifier nature on retention and selectivity; c) the impact of column chemistry on resolution; d) playing with gradient conditions to tune separation; and e) the impact of analytical conditions on back pressure.

For intermediate users, they can explore the following topics: a) the quantitative performance evaluation for an analytical method; b) impact of mobile pH on the final separation and relationship with ionization profile of the compounds; c) comparing HPLC and UHPLC conditions; d) impact of UV time constant and wavelength on sensitivity; and e) impact of mobile phase flow rate on efficiency (van Deemter curves).

Finally, advanced users can explore the following topics: a) the impact of instrumentation (extra-column band broadening) on the final separation; b) the evaluation of peak capacity in gradient mode based on the chromatographic conditions; c) understanding the impact of sample diluent nature on peak broadening; and d) assessing the impact of dwell volume in gradient mode.

We hope you will find this Excel tool useful, and if you have any comments to improve it, drop an e-mail to davy.guillarme@unige.ch. Since it is freely available, please do not hesitate to spread this tool to make it even more popular.

References
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Davy Guillarme, Balazs Bobaly, and Jean-luc Veuthey are with the Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO) and the School of Pharmaceutical Sciences at the University of Geneva in Geneva, Switzerland. Direct correspondence to: davy.guillarme@unige.ch
A Lifetime of Contributions for Advancing Research in Separation Science: Paul Haddad, the Winner of the 2021 Lifetime Achievement in Chromatography Award

Paul Haddad of the University of Tasmania in Australia is distinguished as the foundation director of the Australian Centre for Research on Separation Science (ACROSS). He is well known for his research in many analytical techniques, including high performance liquid chromatography (HPLC), ion chromatography (IC), capillary electrophoresis (CE), and capillary electrochromatography (CEC). He is the winner of the 2021 LCGC Lifetime Achievement in Chromatography Award, which honors an outstanding and seasoned professional for a lifetime of contributions to the advancement of chromatographic techniques and applications. He recently spoke to us about his research work and career.

Jerome Workman, Jr.

You are considered by many to be a leader in the study of separation mechanisms and methods of detection, with a view to developing mathematical models to improve fundamental understanding of these aspects of chromatography. What would you say are your most important contributions to the development of new chromatographic and electrophoretic methods of analysis?

Trying to identify my most important contributions is exceedingly difficult, because of the wide range of chromatographic fields in which I have worked, and the very large number of students and collaborators who have contributed. My publications list is in excess of 150 co-authors, and, for this reason, I feel that our contributions should be viewed as a whole, rather than trying to single out specific achievements. However, I will mention below just a couple of broad areas where I feel that our contributions have been significant.

I have always been interested in chromatographic retention mechanisms and electrophoretic migration mechanisms, especially in the derivation of mathematical models that describe these mechanisms. Once a suitable mathematical model is available, method optimization can be accelerated greatly because retention time can be predicted for a wide variety of conditions, and then used to identify the optimal conditions. My group has published widely on such retention models for ion chromatography (IC) and electrophoresis, and we have tried to focus on models that can be applied with minimal input data. We have derived IC retention models for isocratic elution, gradient elution, and complex eluent profiles involving multiple sequential isocratic and gradient steps, and these models have underpinned commercial IC simulation and optimization software sold by Thermo Fisher Scientific, which has had wide uptake by users.

You and your research group have recently developed a miniaturized deep-ultraviolet light emitting diode (LED)-based detector for use in a portable capillary-scale liquid chromatography (LC) system (1). This detector was incorporated into a briefcase-sized portable capillary HPLC system. In your estimation, how will this device improve field analysis using HPLC methods?

The universality of UV detection increases sensitivity, and also widens the range of compounds which can be detected. Thus, deep UV detection increases sensitivity, and also widens the range of compounds which can be detected. In the case of a miniaturized, portable instrument, there is a strong necessity to minimize weight and also heat production. The deep-UV LED is a perfect solution to these challenges, and the particular LED that we evaluated is a prototype, pre-production version that was supplied to us for evaluation.

You have reviewed the literature from 2015–2020 on the topic of predictive models for retention times in LC (2). What can you tell us about the state of the art in retention-time modeling? What additional future work would be helpful to the field of retention modeling?

Method development in liquid chromatography is generally a slow process, because of the need to equilibrate the system with each new mobile phase that is to be evaluated. For this reason, accurate prediction of retention time is a very desirable goal. Retention time can be calculated from mathematical models that are derived from a sound understanding of the chromatographic retention mechanism, but such models usually require some experimental input, and these models are applicable.
only when the actual retention mechanism is well understood. For these reasons, the focus in the field of retention prediction has been to use statistical approaches to model large databases of retention data based on the chemical structure of the analytes for which retention times are sought. This process, known as quantitative structure-retention relationships (QSRR), enables retention times to be predicted with sufficient accuracy (1–5%) to choose the best chromatographic system for a desired separation, based only on the structures of the analytes to be separation. However, once the optimal system has been selected, there is usually a subsequent step in which the fine details of the mobile phase composition are optimized, and this generally involves some experimental input.

We have recently completed a major QSRR project in which we worked with industrial partners (Pfizer, Thermo Fisher, and LC Resources) to devise some new approaches to the QSRR process. We have found that the most significant step in the development of a statistical QSRR model is the choice of the retention database compounds that will be used to train the model. It is tempting to think that using a large number of training compounds might give the best results, but in fact, the reverse is true. In particular, if one selects only those training compounds that have chemical similarity to the target compounds, good prediction accuracy can be achieved using fewer than 10 training compounds. It also turns out that the best results are achieved when a new QSRR model is made for each individual compound (that is, local models are derived rather than global models). In my view, the future directions of QSRR research will involve applications in nontargeted metabolomics, use of more comprehensive retention databases involving a wider variety of analytes and stationary phases (most probably accessed by crowd sourcing), application to the separation of enantiomers, and the use of the QSRR methodology to gain better insight into chromatographic retention mechanisms. Better access to very fast computers will also speed up the QSRR process.

**What do you consider the most important research publications you have produced science over your career in this field?**

As stated above, I am reluctant to choose particular publications, because to do so would require me to highlight some collaborators rather than others. However, the one publication that I am happy to mention is a book on IC, which I published in 1990 (Paul R. Haddad and Peter E. Jackson, *Ion Chromatography: Principles and Applications*, Elsevier, Journal of Chromatography Library, vol. 46). Peter and I are very proud of this book—it is nearly 800 pages long, it required

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**You have continued research and development of a miniature UV absorbance detector for capillary LC based on LED technology (3). This work is important for miniaturized analysis systems. How is your specific research approach different from your contemporaries for LC detection?**

There are several groups working currently and in the recent past on the development of a miniaturized, portable LC instrument, and some of these groups have produced very nice instruments. Our focus differs somewhat in two aspects. First, our research is strongly directed toward the needs of industry, and in the pharmaceutical industry in particular. We are actively collaborating with a local instrument manufacturer (Trajan Scientific and Medical) and a major pharmaceutical company (Pfizer) to design our portable instrument as a flexible platform that can be changed easily and sample preparation steps, such as dilution, can be incorporated. The second point of difference is cost. Most importantly, we are not trying to make a miniature ultra-high performance liquid chromatography (UHPLC) instrument capable of high-speed runs using high pressures. Rather, we are using low-cost syringe pumps capable of medium pressure, coupled with low-cost switching valves and LED-based detectors to come up with a purpose-built, inexpensive instrument that can be used in a wide variety of applications. We are not trying to replace benchtop LC systems in the pharmaceutical industry, but rather to complement them with a new instrument that is inexpensive and can therefore be applied in numerous at-line and on-line situations.
will contribute to higher peak capacity is 3-D printing, with the ultimate goal being to print a multidimensional separation system.

What words of advice would you share with young researchers just getting started, or even undergraduates considering a future career in science?

Do you have any specific advice for those interested in separation science as a career?

Separation science is a great career, with endless possibilities and directions. The advice that I always give to my students is to see their skills as general rather than specific, and to recognize that all forms of chromatography are connected. After doing an undergraduate degree and then a PhD, many students see their skills as being confined to the specific problem on which they have worked during their PhD. They can then be unwilling to look for a job in other areas of chromatography. I try to emphasize that what they have learned in one area of chromatography can be easily translated into another. I often relate my own experience as an early career researcher. I undertook a PhD in the field of molecular fluorescence spectroscopy applied to inorganic ternary complexes. When I returned to the same university as a junior staff member some five years later, my department head (who had also been my PhD advisor) instructed me to develop an interest in HPLC. At that time, I did not even know what the acronym meant, but I recognized that my PhD had provided me with generic skills in problem solving, experimental hypothesis and design, literature searching, and report writing, and that these skills would be sufficient to provide a strong base for moving into a new field.

References


details on this topic are referred to previous “LC Troubleshooting” columns (3,4), as well as the books referred to earlier in this column (1,2).

Summary
In this column and last month’s “LC Troubleshooting” installment, I have reviewed the operating principles of modern LC pumps based on low- and high-pressure mixing designs, and explained how these pumps produce mobile phase streams with small short-term variations in mobile phase composition. These composition “waves” can negatively affect detector baseline quality and retention time variability. If the characteristics of the mobile phase waves and retention properties of an analyte of interest are known, it is straightforward to estimate the impact of the waves (and associated instrument parameters, such as flow rate and pump stroke volume) on retention precision. We see that choices for some parameters, such as the pump stroke frequency, can have a dramatic impact on retention precision, and thus it is important to pay attention to these details, particularly for demanding applications involving compounds that are sensitive to small changes in mobile phase composition. Finally, the composition ripple is a metric that is pretty easy for any user to evaluate; this should be done regularly so that indications of degrading pump performance can be caught early and addressed before they lead to a serious dropoff in method performance (for example, measured by retention variability that is too high).

References

About the Column Editor
Dwight R. Stoll is the editor of “LC Troubleshooting.” Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 75 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC’s editorial advisory board. Direct correspondence to: LCGCed@mmhgroup.com

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n 1980, Atwood and Goldstein of Perki
Elmer published one of the earliest stud
ies on lot-to-lot selectivity variability across 24
lots of a commercial high-performance liquid
chromatography (HPLC) packing material (1).
In mid-August of 2020, I was asked by a major
column manufacturer to participate in a focus
group on a proposed new quality control (QC)
procedure for their LC columns. I guess, even
after 40 years, we have yet to find a perfect
solution to the problem of demonstrating and
achieving acceptable column precision.

During the conversation, I made an off-
hand comment that most end users probably
can’t remember the last time they looked at
the paperwork inside a column box. Indeed,
many vendors are moving toward minimal
packaging and inserts, whether motivated
by cost-saving measures or environmental
concerns. As long as the information is easily
retrievable, either from the internet or embed-
ded in the electronic data chips leashed to
some vendors’ columns, this seems like a
positive change. I still highly recommend that
ev\ry user read the column care guides on a
new column model. Knowing the difference
between a pH 7 and pH 8 upper limit (or 45
vs. 60 °C) can save you headaches, and the
guides usually provide the best storage and
rescue procedures.

Bad Column or Bad Method?
Aside from column care, most vendors pro-
\vide a Certificate of Analysis (CoA) with some
tests. Was the root cause a poor method (for
example, robustness issues, leveraging minor
column interactions, and so on), poor execu-
tion of the method (those pesky humans), or
insufficient vendor tests? Honestly, each of
these has been a factor at least once in my
history, but I believe improved column testing
could help prevent many of these instances.

Column Characterization
Column tests can span from the very simple
(plates or tailing factor on a single compo-
\nent), to what is most commonly seen (a few
components with a plate, selectivity, and tail-
ing factor assessment), to much more com-
prehensive probing of the actual chromato-
graphic interactions, such as those described
by the National Institute of Standards and
Technology (NIST) and Waters (2,3). The more
thoroug\h testing is usually performed at the
batch level, and not as an individual column
release test, because the latter are more
resource-intensive and move from a QC test
to true column characterization. A recent
review of the many possibilities of executing
this type of evaluation demonstrates there is
no “one size fits all” test, even within the rela-
tively constrained realm of reversed-phase
stationary phases (4).

Double Standards
In my industry (pharmaceuticals), there is the
expectation that our drug manufacture pro-
cess is fully characterized and controlled; yet
the tools we use to assess our processes, such
as chromatography columns, are not held to
the same standards. The nightmare scenario
for me is not being able to release life-saving
medicines because a new column failed to
perform consistently with previous lots. If I had
a magic wand, every column I receive would
have every chemical interaction identified
and tightly controlled by the manufacturer. I
had even hoped to use characterization tests,
such as the hydrophobic subtraction model
(5), to assess column manufacture precision (6).
The reality is that the execution of this type of
rigorous testing on every column, at present,
would be cost-prohibitive to the industry.

The Answer Is...
Kudos to those manufacturers that perform
more than the easy, simple column tests.
Laurels to those who listened to Atwood
and Goldstein’s advice “…to deliberately
choose test compounds that expose varia-
tions” (7). Accolades to those who put realisti-
cally appropriate specifications on those tests.
Please keep working toward “the perfect
test.” In pharmaceuticals, we’re counting on
you, the column makers, to deliver the same
product, day-in and day-out, for the life cycle
of our products.

To give an answer to the question I posed
as the title of this blog: No, the paper in the
box is irrelevant. Having the best tests pos-
sible on those CoAs, however, is absolutely
critical to us, the end users.

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