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Nicholas H. Snow
In this instalment we take a close look at Golay’s famous equation that most people see as relating HETP (height equivalent to a theoretical plate) to the carrier gas flow rate or average linear gas velocity in a capillary column.

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February/March Update

Welcome to the February/March issue of LCGC Asia Pacific. In this month’s cover story, a novel ion-exchange chromatography (IEX) method that uses non-aqueous mobile phases to purify a non-crystalline intermediate after a reductive amination with D-xylose is described. Often an overlooked technique, IEX is successfully used to deliver a step change in the quality of the non-crystalline intermediate and offers an alternative to traditional isolation methods.

This month’s LC Troubleshooting is focused on those topics that are the “bread and butter” of liquid chromatography (LC) troubleshooting—those elements that come up time after time. This instalment focuses on problems related to pressure—too low, too high, and fluctuating. Developing a list of the possible causes can help with troubleshooting.

GC Connections takes a look at Golay’s famous equation that most people see as relating HETP (height equivalent to a theoretical plate) to the carrier gas flow rate or average linear gas velocity in a capillary column. Developed in the 1950s, what is its relevance today?

In our Pharmaceutical Analysis Focus interview, Filip Cuyckens from Janssen R&D discusses his work developing innovative approaches to support drug metabolism and pharmacokinetic studies, and the role that two-dimensional (2D)-LC plays in his laboratory.

LCGC is a multimedia platform that helps chromatographers keep up to date with the latest trends and developments in separation science and supports them to perform more effectively in the workplace. Keep updated with our print and digital content by visiting the global website (www.chromatographyonline.com), subscribing to our newsletters, and attending our wide range of educational virtual symposiums.

Mike Hennessy Jr,
President and CEO, MJH Life Sciences®
High-quality active pharmaceutical ingredients (APIs) and intermediates are typically delivered using crystallization, distillation, precipitation, and chromatography. However, when organic synthesis delivers a material that will not crystallize, other approaches need to be considered. This article describes a frequently overlooked technique, ion-exchange chromatography (IEX), using non-aqueous mobile phases to purify a non-crystalline intermediate after a reductive amination with D-xylose. IEX delivers a step change in strength and purity of the intermediate allowing successful downstream processing.

- In this project, the intermediate product could not initially be isolated using crystallization, telescoping into the next stage was also unsuccessful, and in post-reaction work-up the intermediate product was of low quality.
- A solution involving IEX with a strong cation exchanger offered a means to increase the concentration and purity of the intermediate product, which could then be directly used in the next stage of the synthesis.
- IEX offers an alternative to traditional isolation methods.

The methods of choice to isolate compounds post-reaction work-up are typically crystallization or precipitation for solids, and distillation for liquids. If these techniques fail to isolate the desired compound at the correct quality, chromatography can be used to improve the purity (1). However, not all compounds are suitable for purification using traditional chromatography. The intermediate AZ13757020 (Figure 1), which is two steps removed from the active pharmaceutical ingredient (API), was synthesized as part of a planned manufacture to deliver 10 kg of the API to support clinical trials within the therapeutic area of cystic fibrosis. However, this intermediate could not be crystallized using classical techniques, leading to delivery of the material with low purity and low quality (% w/w assay). The product is also highly soluble in water, making extraction into an organic solvent difficult. The continuous process development of the intermediate AZ13757020 resulted in each feed (sample) being supplied with varying compositions of the product and impurities. The challenges with a traditional chromatographic purification of this compound were the separation at multi-kilogram scale of a weakly UV-active product, impurities, and starting materials. We chose to investigate the use of an ion-exchange chromatography (IEX) method using modified silica and high acid-base concentrations in a non-aqueous environment, which also posed a risk of stationary phase instability under such conditions.

**Purification Strategy**

As the product is highly soluble in water, extraction into an organic solvent was difficult; therefore, reversed-phase chromatography was not a viable option because lyophilization of multigram fractions was neither practical nor efficient. However, there is
TABLE 1: Selected by-product impurities from unwanted side reactions, the product, and calculated $pK_a$ values of the tertiary amine (where applicable)

<table>
<thead>
<tr>
<th>Product</th>
<th>Impurity 1</th>
<th>Impurity 2</th>
<th>Impurity 3</th>
<th>Impurity 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Chemical structure" /></td>
<td>$pK_a$ 8.55</td>
<td>$pK_a$ 9.01</td>
<td>$pK_a$ 7.9</td>
<td>$pK_a$ 6.86</td>
</tr>
</tbody>
</table>

Impurity 1 was generated via a double addition of the hexanal during reductive amination. Impurity 2 was generated via a double addition of the D-xylose during reductive amination. Impurity 3 was generated by an unwanted side reaction during the reductive amination. Impurity 4 was generated via reaction of the hexanal with sodium cyanoborohydride.

* Although the impurity 4 (2-hydroxyheptanenitrile) does not include an amine function, it is included in Table 1 as it was in the feed in an appreciable quantity (11%). An extremely small quantity of impurity 4 was detected in the product after release, showing that impurity 4 was not effectively captured by the resin.

FIGURE 1: Steps to intermediate AZ13757020.

![Chemical structure](image2)

- N-Boc-ethylenediamine
- Used directly without work-up/isolation

FIGURE 2: Protocol used for adsorption and desorption of the product.

![Protocol diagram](image3)

The following standard operations were used for the chromatography including fractionation:

A. **Conditioning:** Equilibration of the cation exchange resin with methanol 2 column volumes (CVs) prior to first injection only, ensuring that the resin was totally wetted allowing any analytes access to the whole surface area of the resin, fraction not collected.

B. **Load:** Injection of the feed on to the head of the column, fraction not collected.

C. **Retain and Wash:** Adsorption of the product and basic analytes throughout the resin bed using 2 CVs methanol, fraction collected for analysis. Fraction 1 (F1).

D. **Release:** Desorption (elution) of the product and basic analytes throughout the resin bed using 2 CVs of 2 M methanolic ammonia fraction collected for neutralisation no analysis carried out. Fraction 2 (F2).

E. **Wash:** Washing of the cation exchange resin with 2 CVs methanol to remove excess methanolic ammonia, fraction collected for analysis. Fraction 3 (F3).

F. **Regeneration:** The cation exchange resin was treated with 3 CVs of acidic methanol fraction collected for neutralisation no analysis carried out.

G. **Conditioning:** Washing of the cation exchange resin with 4 CVs of methanol to remove excess acid (ready for step B), fraction collected for neutralisation no analysis carried out.

When a feed solution containing an amine is passed through a column containing propylsulfonic acid-silica, the amine is retained or “caught” by this column. Non-cationic (non-basic) impurities are not retained and are further removed by washing the column with an organic solvent, such as methanol, acetonitrile, or ethers. The product is subsequently purified to intermediate AZ13757020:
“released” from the column by elution with a solution of ammonia in methanol (methanolic ammonia). Amine salts of weak conjugate acids (such as acetate and trifluoroacetate) are also exchanged onto the silica-based resin and are released as the free amine during the ammonia–methanol wash. The amine product is then typically isolated by removal of the volatile methanolic ammonia solution by evaporation.

**Experimental**

**Materials:**

*Cation Exchange Resins:* A strong cation exchange consisting of propylsulfonic acid-silica resin was selected for the experiments (Isolute SCX-2, Biotage), in pre-packed cartridges for milligram scale and in bulk for the multigram and multi-kilogram scale. The resin was supplied in H\(^+\) form, the physical and chemical properties are listed in Table A in Supplementary Information at https://bit.ly/34s6zpS.

*Chemicals:* See Table B in Supplementary Information at https://bit.ly/34s6zpS.

**Instrumentation**

*Milligram Scale:* A cartridge containing the chosen strong cation exchange was used. *Multigram Scale:* A 25-mm diameter glass column with a maximum bed length of 220 mm (YMC Europe Gmbh, Germany) containing the strong cation exchange resin was connected to a preparative chromatography system (Kronlab GmbH). The system comprised of an AP-250-250-3 quaternary pump, FC-250/250 fraction collector (Armen) with a UV diode array KN-A2700 detection flow cell (Kronlab GmbH). The data capture software, the valve, and flow control operating system was PrepCon 5.0 (SCPA GmbH).

*Multikilogram Scale:* A 300-mm diameter glass column with a maximum bed length of 490 mm (YMC Europe), containing the strong cation exchange resin, was connected to a WZ-76200-00 pump (Cole-Parmer). This pump was connected to a custom-built 316 stainless steel multi-port manual solvent manifold. The mobile phase reservoirs were under manual control; when the desired volume of a given mobile phase had been delivered to the column, the next mobile phase was selected as required.

*Inductively Coupled Plasma–Mass Spectrometry (ICP-MS):* An X-series II ICP-MS system (Thermo Fisher Scientific) was used for ICP-MS analysis. Standards were TraceCert (Sigma-Aldrich). Analysis was run using an external standard method: standards were prepared at 0 (blank), 0.01, 0.1, 1 mg/L (equivalent to 0, 1, 10, 100 ppm in the sample). Samples were prepared at ~10 mg/mL. All standards

---

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TABLE 2: Showing the change in purity profile pre- and post-IEX

<table>
<thead>
<tr>
<th>Component</th>
<th>Prior to ion-exchange</th>
<th>Post ion-exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>49% w/w*</td>
<td>87% w/w</td>
</tr>
<tr>
<td>HO</td>
<td>16% w/w</td>
<td>Not more than 0.5%</td>
</tr>
<tr>
<td>HO</td>
<td>1.00%</td>
<td>4 ppm</td>
</tr>
<tr>
<td>Boron</td>
<td>0.63%</td>
<td>0.07%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.82%</td>
<td>0.01%</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.00%</td>
<td></td>
</tr>
</tbody>
</table>

All assay values (%w/w) determined on a solvent-free basis by 1H NMR or ICP-MS. *49% w/w on solvent-free basis is determined based on 25.6% w/w product in solution containing 48.0% w/w of methanol and CPME combined (25.6 / (100–48.0)) = 49% w/w.

and samples were prepared in 20% nitric acid with 1 mg/L Yttrium internal standard. Nuclear Magnetic Resonance (NMR): An A500mHz NMR system (Bruker) was used for NMR analysis. Solvent D-methanol with maleic acid internal standard (20 mg of each in 1 mL).

High Performance Liquid Chromatography (HPLC): The 1100 HPLC system used with associated degasser, quaternary pump, autosampler, column compartment, and variable wavelength detector was from Agilent.

Data Handling: Data was processed using Atlas 8.2.3 software (Thermo Fisher Scientific).

Column Packing Method:

Milligram scale: The strong cation exchange resin was supplied as 10 g/15 mL pre-packed cartridges. The cartridge was flushed with three column volumes (CVs) of methanol to equilibrate the column. Backflush mode was used to avoid leaving non-wetted aggregated areas that could encourage the egress of air bubbles. Following the equilibration, the piston head was then lowered until it was just touching the resin bed. This enabled the resin bed length to be measured (190 mm) so a solvated bed density could be calculated. The piston head was then returned to its original height (1 cm above the resin). The solvated bed density was approximately 0.64 g/mL. No swelling of the resin was observed. Multikilogram Scale: Approximately 21 kg of the strong cation exchange resin was dry packed into a 300-mm glass column. The piston head was then inserted and lowered to 1 cm above the top of the packing. The column was then connected to the pump and manifold, as described in Figure 2. The column was then flushed with three CVs of methanol in backflush mode to equilibrate the column. Backflush mode was used to avoid leaving non-wetted aggregated areas that could encourage the egress of air bubbles. Upon completion of the equilibration, the piston head was lowered until the resin bed length was 490 mm. Solvated bed density was approximately 0.64 g/mL. No swelling of the resin was observed.

Chromatography (Including Fractionation): The protocol for adsorption and desorption are shown in Figure 2. Milligram Scale: Operations A–D were carried out at the milligram scale (no regeneration, the cartridges were discarded after use). The feed solution (2 mL, approximately 25% w/w solution in cyclopentyl methyl ether [CPME]) was charged to the cartridge in the manner described in Figure 2. The individual fractions were analyzed for the presence or absence of the product on a reversed-phase HPLC (see Supplementary Information at https://bit.ly/34s6zpS). The fractionation volumes were in the range of 40–60 mL. The product fractions were isolated by reduced vacuum distillation for downstream processing, the quality was confirmed by 1H NMR (see Supplementary Information at https://bit.ly/34s6zpS).

Multikilogram Scale: Operations A–G, as described in Figure 2, were performed at the multigram scale. These experiments were performed in a continuous manner by monitoring the UV signal and then manually collecting each fraction at the chromatographer’s discretion. Figure 3 shows a typical optimized run, with the unit operations A–G and the conditioning (A/G) steps, load (B), retain and wash (C), release (D), wash (E), regeneration (F) steps. The peak in C contains acidic and neutral non-retained impurities. The square wave peak in F and G is due to trifluoroacetic acid elution. The individual fractions were analyzed to confirm the presence or absence of the product on a reversed-phase HPLC system (see Supplementary Information at https://bit.ly/34s6zpS). The fractionation volumes were in the range of 70–80 mL. The product fractions were isolated by reduced vacuum distillation for downstream processing, and the quality was confirmed by 1H NMR (see Supplementary Information at https://bit.ly/34s6zpS).

Multikilogram Scale: Operations A–G, as
described in Figure 2, were performed at the multikilogram scale. The feed was provided in two separate batches. As a result of slight variations in the batches, a user trial was completed for each batch at the multigram scale to determine the optimum loading. Batch 1 was sub-divided into six aliquots and batch 2 into nine aliquots. The separation was performed in a continuous manner with fraction collection at the chromatographer’s discretion. Both batches were successfully processed. The individual fractions were analyzed for the presence or absence of the product using reversed-phase HPLC (see Supplementary Information at https://bit.ly/34s6zpS).

The fractionation volumes were in the range of 22–35 L. The product fractions were isolated by reduced vacuum distillation for downstream processing, and the quality was confirmed by 1H NMR (see Supplementary Information at https://bit.ly/34s6zpS).

Results and Discussion

Milligram Scale: Initial method development work was completed on a milligram scale, trying to understand and define the load, retain, wash, and release parameters. The desorption (elution) of the product from the resin at this scale initially used 7 M methanolic ammonia. The ammonia concentration was investigated and successfully reduced to 2 M with no impact on the release of the product from the resin or the quality of the material. This change was introduced to reduce the environmental impact of the emissions of ammonia during the vacuum distillation at the multikilogram scale.

Concerns were raised whether the silica-based ion-exchange resin would be stable with prolonged exposure to high pH at the proposed larger multikilogram scale. Exposure of silica to high pH is known to degrade (hydrolyze) silica-based stationary phases (3). Hence, after successfully demonstrating the process at the milligram scale using the cation exchange resin as described in Figure 2, a potentially more robust polymeric resin was investigated. It was proposed that a propylsulfonic acid modified styrene-divinylbenzene support would be chemically inert to the prolonged exposure to high and low pH at the larger multikilogram scale (4). However, this resin failed to retain the product. It was decided to return to the original cation exchange resin chosen, with the same feed source and volume as loaded on to the styrene-divinylbenzene resin, as this was successful.

At this time, it was thought that the resin would be single use, that is, at the multikilogram scale, a whole batch (4–5 kg) could be processed in a single run. Therefore, it was decided to continue to the multigram scale with the original strong cation exchange resin only on the basis that stability of the resin was not critical.

Feed loads greater than 0.05 g/g of resin (volumes above 2 mL load of the initial sample provided) exceeded the binding capacity of the resin and resulted in breakthrough...
UV output of the optimized parameters at the multi-gram scale. The peak in C contains acidic and neutral non-retained impurities. The square wave peak in F and G is due to trifluoroacetic acid elution.

![Figure 3](image)

The results clearly showed that the feed contained large quantities of cations (pre-IEX), and these were significantly reduced by the resin in the successful run (post-IEX). These results indicated that the failure of many of the experiments was a result of the presence of competing, smaller cationic impurities (non-amines). These stronger binding, small positively charged cations would preferentially bind with sulphonic acid ion-exchange sites, and when all the small cationic impurities were bound to the ion-exchange stationary phase, the amine-product would then bind to the remaining sites.

When these ICP results became available, the multikilogram manufacture of the product was already in progress and there was insufficient time or resource to develop a process to remove or reduce the cations present. Therefore, it was decided to perform a user trial of each batch at the multigram scale, to find a loading that would successfully scale-up the multikilogram scale. The user trial of each batch was based on trialling a volume at the multigram scale to find the optimum loading for the multikilogram scale. Using this data, the number of aliquots required to process a batch was calculated (see Supplementary Information at https://bit.ly/34s6zpS).

The results from the user trials demonstrated that the CV approach...
applied at multigram scale could be used to define the optimum load volume at multikilogram scale, making scale-up straightforward. The CV at the multi-kilogram scale (300 mm i.d. × 490 mm bed length) was calculated at 35 L (πr²h) for the first aliquot of the first batch. This was known to be an overestimation to take into account the dead volume (interstitial volume and pore volume with respect to the resin) of the system, ensuring that no product would be lost and that the regeneration and washing steps were not underestimated. After the first aliquot, it became clear that this calculated volume was excessive, and the actual CV was determined to be 22 L.

The 2 M methanolic ammonia base strength proved strong enough to remove the product and associated organic basic impurities (see Table 1). Serendipitously, the resin bound potassium, boron, and sodium were not displaced, otherwise these cations would have posed an issue for the downstream processing.

The most useful UV wavelength to monitor the chromatography was determined to be 235 nm. This ensured that the elution of the product could be observed without interference from the methanolic ammonia mobile phase–wash solvent (ammonia has a UV cut-off at 217 nm).

**Multikilogram Scale:** During steps A–G (Figure 2) of the first run at this scale, the column temperature was determined to be 235 nm, which ensured a powerful, productive, and effective technique and should be considered as part of the toolbox in process development and manufacturing for purification of pharmaceutical materials.

**Conclusion**

The use of IEX in 100% organic mode for scale-up to multigram has been successful to deliver a step change in the quality of a non-crystalline intermediate that cannot be purified by standard methods. This has been successfully applied to AZ13757020, converting material of 49% w/w to 87% w/w (Table 2). This allowed the high-quality material to be successfully used in subsequent manufacturing stages. As demonstrated in this article, IEX is a powerful, productive, and effective technique and should be considered for scale-up from multigram to multikilogram scale. The flow rates were governed by the maximum back pressure that the glass column could withstand (the maximum safe working pressure supplied by the manufacture was 5 Barg). For the aliquot subdivision of each batch, see Supplementary Information at https://bit.ly/34s6ZpS.

**Acknowledgements**

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**References**


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Some “LC Troubleshooting” topics never get old because there are some problems that persist in the practice of liquid chromatography (LC), even as instrument technology improves over time. There are many ways for things to go wrong in an LC system that ultimately manifest as deviations from the expected pressure. Developing a short list of the likely causes of these deviations can help streamline our troubleshooting experience when pressure-related problems occur.

Writing this “LC Troubleshooting” column and thinking about topics each month is interesting in the sense that there are some topics that just never get old. Whereas, in the chromatography research world, certain topics or ideas become obsolete as they are displaced by newer and better ideas, in the troubleshooting world there are certain topics that have remained relevant since the very first troubleshooting article appeared in this magazine (LC Magazine at that time) in 1983 (1). Over the last few years, I’ve focused several “LC Troubleshooting” instalments on contemporary trends (such as the relatively recent advances in our understanding of the effects of pressure on retention [2]) in liquid chromatography (LC) that are affecting the way we approach our interpretation of LC results, and approach troubleshooting with modern LC instruments. With this month’s instalment, I am starting a series focused on some of the “bread and butter” topics of LC troubleshooting—those elements that are essential for any troubleshooter, no matter the vintage of the system we are working with. The topics at the heart of this series will be highly related to the well-known “LC Troubleshooting” wallchart (3) that hangs in many laboratories. For the first instalment in this series, I’ve chosen to focus on problems related to pressure (too low, too high, or fluctuating). I hope LC users young and old will find some useful tips and reminders related to this important topic.

Everything is Possible
In the area of pressure problems, everything is possible. Sometimes pressure is unexpectedly low but stable. Other times the pressure is too low, and appears to steadily decrease. The same is true for pressures that are higher than expected. In other cases, the observed pressure may seem to be about right, but it is fluctuating more than usual. Figure 1 illustrates the idea that pressure problems appear in all kinds of different ways, and lists the specific situations that are discussed in this article. The list of pressure-related problems shown in Figure 1 is not exhaustive; in this instalment, I focus on those problems that I see most frequently in practice.

What Is To Be Expected?
A critical step in any troubleshooting exercise—but one that I think is underappreciated—is recognizing that there is a problem to be solved. Recognizing that there is a problem usually amounts to recognizing that what is happening with the instrument is different from what is expected to happen, and our expectations are formed from theories, empirical knowledge, and experience (4).

Before getting into details about what we can expect about pressure, a few words to clarify what it is and how it is measured in LC instruments are warranted. In LC, when we say “pressure”, we are really talking about a “pressure drop” or a “pressure difference”. These more precise terms are indicated in various equations that relate pressure drop to other variables, such as flow using the symbol ΔP. Most commercially available LC systems have a single pressure readout associated with the pump that reports the pressure drop between the pump and the outlet of the system (the outlet side of a detector flow cell) that, for all practical purposes, is zero, because the atmospheric pressure of about 1 bar is usually small compared to LC operating pressures. This single pressure readout quantifies the total pressure drop across the entire flow path, but does not tell
us anything about the pressure drops across individual elements of the flow path (for example, filters, different pieces of connecting tubing, and the column).

**Pressure Drop Across Connecting Tubing:** Most practical high performance liquid chromatography (HPLC) is done under conditions where flow through connecting tubing in the system is laminar. Under these conditions, the pressure drops across the different pieces of tubing can be calculated with the accuracy needed for troubleshooting purposes using Poiseuille’s Law:

\[
\Delta P_{\text{tub}} = 128 \cdot \frac{\eta \cdot L_{\text{tub}} \cdot F}{\pi \cdot d_{\text{tub}}^4} \quad [1]
\]

where \( \eta \) is the dynamic viscosity of the mobile phase, \( F \) is the flow rate, and \( L_{\text{tub}} \) and \( d_{\text{tub}} \) are the length and diameter of the tubing, respectively. Calculating the pressure drop using equation 1 is straightforward when all of the values needed are available; however, the dependence of viscosity on mobile-phase composition and temperature is a bit complex. Fortunately, there are some freely available web-based tools that take these factors into account (for example, see reference 5, and https://www.multidlc.org/dispersion_calculator), and provide users with quick estimates of the expected pressure drops for the tubes in their systems.

**Pressure Drop Across Columns:**

The pressure drop across the LC column itself (assuming the column is packed with particles) can be calculated using equation 2 (or similar). Like equation 1 for open tubes, the pressure drop depends on the column length, mobile-phase viscosity, and the flow rate (through the interstitial mobile phase velocity, \( u_i \)). Different in equation 2 are the \( \Phi \) term that quantifies the permeability of the packed particle bed and the particle size term, \( d_p^2 \).

\[
\Delta P_{\text{col}} = \frac{\Phi \eta u_i L_{\text{col}}}{d_p^2} \quad [2]
\]

As with equation 1, calculating the pressure drop across the column is straightforward once all of the values for length, viscosity, and other variables are in hand, but they are not all easy to come by. Once again, there are freely available simulators that can calculate the pressure drop for conditions of interest. Two such simulators that I am familiar with are the web-based LC simulator maintained by my group (https://www.multidlc.org/hplcsim), and the spreadsheet-based simulator developed more recently by Professor Davy Guillarme’s group (see [6]; https://ispso.unige.ch/labs/fanal/practical_hplc_simulator:en).

**Other Elements of the Flowpath:**

The other elements of an LC flow path that can contribute substantially to the pressure drop measured at the pump are inline filters (and guard columns, though these can be treated like columns as above). Most inline filters sold for use in analytical LC systems are designed in a way that they will not contribute more than a few bar to the total pressure drop under typical conditions (that is, less than 5 mL/min). When debris begins to accumulate on the filter, the pressure drop across the filter will increase, and become highly, and sometimes nonlinearly, dependent on operating conditions (for example, flow rate and mobile-phase composition). Because it is difficult to cope with this hard-to-predict behaviour, in my laboratory we simply change the filter if the pressure drop across it exceeds about 10 bar.

**Flow rate, 1 mL/min.; Temperature, ambient; mobile phase, 100% aqueous.**

**Situations Involving Pressure That Is Lower Than Expected**

There are two main problems that lead to pressures that are lower than expected.

- **Leaks:** There are many different ways that a leak can occur in a LC system. The most common ones I see are related to connections (for example, tubing to valve, or tubing to column). Usually, these leaks are relatively small (that is, a few microlitres per minute), and can be corrected by tightening the fitting slightly. However, be careful—forcing a metal ferrule into a connection too much can deform the port (such as a column endfitting or valve port). If you feel like the connection is already very tight, then it is better to throw the capillary away. I always tell my students that “too loose is much better than too tight”. Another way of saying this is that it is much cheaper.
LC TROUBLESHOOTING

Specific situations that are discussed in this article.

### Pressure

<table>
<thead>
<tr>
<th>Too Low (Decreasing)</th>
<th>Fluctuating</th>
<th>Too High (Increasing)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leak somewhere downstream from pump</td>
<td>Malfunctioning check valve (inlet or outlet)</td>
<td>Partially blocked element somewhere downstream from pump</td>
</tr>
<tr>
<td>Partially plugged solvent inlet filter</td>
<td>Air bubble in pump head</td>
<td></td>
</tr>
</tbody>
</table>

**FIGURE 1:** Illustration of the different ways pressure problems can appear, and the specific situations that are discussed in this article.

To replace several capillaries than it is to replace a valve stator because a capillary was overtightened and deformed or stripped the threads on a valve port. Significant leaks can also occur between the pump pistons and seals. We should never be able to see liquid emerging from the pump head in the area of the piston/seal. If liquid is observed, the seals are probably leaking and should be replaced. In my experience this problem does not occur nearly as frequently as it did with pumps 20 years ago. Finally, it is possible for polyetheretherketone (PEEK) tubing to burst, even when working below the advertised pressure limit of the tubing. When this happens it usually leads to a major, obvious leak, whereas leaks with connections and pump seals tend to be more subtle.

- **A partially obstructed solvent inlet filter.** LC pumps rely on a free, steady flow of solvent from the solvent bottle to the inlet check valve to work properly. If the inlet filter on the end of the line in the solvent bottle becomes partially obstructed by particulates or bacterial growth, this can slow the flow to the point where the pump is “starved” of solvent, and unable to deliver flow to the column at the specified flow rate, leading to lower than expected pressure. If one suspects that this might be the problem, a quick check involves simply removing the inlet filter from the solvent line. If the pressure returns to normal after the filter is removed, then the filter needs to be cleaned at a minimum, and it is usually best to just replace it altogether.

**Situations Involving Pressure That Is Higher Than Expected**

Most problems involving higher than expected pressure are somehow related to accumulation of debris somewhere in the system. The origins of this debris vary; it can come from particulate matter in the injected sample, molecules that are soluble in the sample solvent but precipitate in the mobile-phase stream, polymeric material shed by pump and injector seals, and so on. The specific nature of the problem that results from this debris depends strongly on how the system is configured. Determining where the obstruction is in the system can be tricky. A systematic approach to finding out where the problem lies involves removing components from the flow path one at a time, starting from the downstream end. For example, suppose we are running at 1 mL/min, and we observe a pressure at the pump of 600 bar, which is high compared to a normal operating pressure of 250 bar. With the flow off, remove the detector from the flow path. Turn the pump back on and record the pressure. If it has only decreased by 5 bar to 595 bar, then we know that the obstruction does not lie in the detector flow path. Again, with the flow off, remove the tubing between the detector and the column outlet. Turn the flow back on and record the pressure. If it has decreased another 10 bar to 585 bar, then we know that the tubing between the column outlet and the detector is not the source of the problem. Next, remove the column, turn the flow back on, and record the pressure. There should be a significant difference between the pressure recorded with and without the column connected. Suppose in this case that the pressure is still 365 bar even without the column connected, which would be abnormal in any typical analytical LC system. Next, suppose that upon removing the inline filter installed immediately upstream from the column the pressure drops to 20 bar. This would tell us that the pressure drop over the filter itself was 345 bar (far higher than expected), indicating that the filter should be thrown away and replaced. This “one-piece-at-a-time” approach can feel tedious when trying to get an instrument back on track, but it is the most reliable way to isolate the problem. The three most commonly encountered scenarios are:

- **An obstruction in an inline filter.** In my laboratory, partially plugged filters account for 95% of our high pressure problems. Once it is clear that a filter is obstructed, one could try backflushing them, but this solution is usually short-lived. It is far better in the long run to just replace the filter.
- **An obstruction in a piece of capillary tubing.** This does not happen very often if inline filters (0.2–0.5 µm porosity) are used immediately after the sample injector. If inline filters are not used, then the capillaries of the smallest diameters and the ones furthest upstream (that is, closest to the injector) are the most likely to become blocked. Reversing the flow through an obstructed capillary will occasionally be sufficient to remove...
the debris, but the most reliable solution is to just replace the capillary.

- An obstruction at the inlet of a column: This can also be largely prevented through routine use of an inline filter upstream from the column. If an increase in pressure drop across the column is observed over time, reversing the flow through the column can flush debris out of the inlet frit, but, in my experience, the reduction in pressure is usually short-lived. It is also important to note that some column manufacturers use frits with larger porosities at the column inlet; reversing the flow is then a bit risky, because some particles could go through the frit and be lost from the column (thank you to Professor Chuck Lucy for this reminder). The bottom line is that it is best to avoid problems like this with the column by protecting it, through routine use of inline filters and/or guard columns.

Situations Involving Pressure That Appears To Be Fluctuating

Most modern LC pumps are based on some variation of a reciprocating dual piston design, where the pressure variation that occurs at the end of each piston stroke can be minimized, but is difficult to eliminate entirely. The specification for modern pumps is that the pressure variation should not exceed about 1%. If the observed variation is much larger than 1%, then it is most likely because of one of two reasons.

- Malfunctioning check valves: A typical reciprocating dual piston pump design relies on two check valves to keep the mobile-phase flow moving in the direction of the column. When solvent is being pushed out of the pump head toward the column, the inlet check valve closes so that solvent cannot travel back towards the solvent bottle. When solvent is being drawn from the solvent bottle into the pump, an outlet check valve closes to prevent solvent from flowing backwards into the pump head. When working properly, these check valves open and close multiple times per minute. If one or both of them does not open or close properly, there will be a significant interruption in the flow to the column, which in turn manifests as a change in pressure. Determining which of the two check valves is faulty can be tricky. One approach is to first replace the inlet check valve with one that is known to be functional. If this does not solve the problem, then re-install the original inlet valve, and change out the outlet check valve with one that is known to be functional. If this does not reduce the pressure fluctuation, then the problem does not lie with the check valves.

- Gas bubbles in the pump head: Even a pump with properly functioning check valves can get tripped up by an air bubble. If air becomes trapped in a pump head (for example, after the pump has accidentally run dry, because an inline degasser is not working properly, or after a prolonged period without use), it can lead to severe flow or pressure fluctuations. Purging the pump at high flow rate (with the column disconnected) is often effective for dislodging the bubble and returning to normal operation. If this does not resolve the problem, purging the pump head with isopropanol is another approach that often works well.

Summary

In this first instalment on essential topics in LC troubleshooting, I have discussed situations where the observed system pressure is somehow different from what is expected or normal. Effective troubleshooting for this type of problem begins with a sense for what the expected system behaviour is, so that a deviation from those expectations is noticeable. While there are many different potential causes of pressure-related problems (too low, too high, or fluctuation), most problems can be connected to five or six specific causes. Understanding this short list of likely causes provides a good place to start troubleshooting, but it does not capture all possibilities. Readers interested in learning about a deeper list of causes and solutions are referred to the “LCGC Troubleshooting” wallchart (3).

References

3) https://www.chromatographyonline.com/view/troubleshooting-wallchart

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In this instalment of “GC Connections”, we take a close look at Golay’s famous equation that most people view as relating height equivalent to a theoretical plate (HETP) to the carrier gas flow rate or average linear gas velocity in a capillary column. We explore the original works of Golay and Keulemans. We also look closely at the original equation, the assumptions involved with its development, and the assumptions (both good and bad) that have been applied to HETP over the years. In part two, we will explore the consequences and relevance of this theory today, with temperature programming, very long (and very short) columns, high pressure drops, and vacuum outlet detectors.

The height equivalent to a theoretical plate (HETP) is one of the most important parameters used to measure column performance. HETP is the length of column required to generate one theoretical plate, with a theoretical plate being one transfer process of an analyte molecule between the mobile phase and the stationary phase. We are all familiar with considering the number of theoretical plates in a column or the number of theoretical plates in a length of column, as these are often stated by column vendors as a measure of column quality. More theoretical plates and more theoretical plates per metre are often considered good, leading to better resolution. A 30 m column with 100,000 theoretical plates is said to have 3333 theoretical plates per metre.

We use a chromatogram to measure theoretical plates (as shown in Figure 1), using equation 1, where \( N \) is the number of theoretical plates, \( t_r \) is the retention time of the peak and \( W_b \) is the peak width at the baseline. \( N \) and HETP (as discussed later) are dependent on many parameters, including temperature, carrier gas flow rate, inlet and outlet pressures, column dimensions, and the choice of carrier gas. One challenge in comparing plate numbers between different columns is that the experiments will almost always have been performed under different conditions, making true comparisons difficult.

\[
N = 16(\frac{t_r}{W_b})^3
\]  

[1]

HETP is then calculated using equation 2, where \( L \) is the column length and \( N \) is the number of theoretical plates calculated using equation 1:

\[
H = \frac{L}{N}
\]  

[2]

As we will discuss in detail shortly, \( H \), or HETP, is also defined as the rate of band broadening. As we know, the peaks or bands get wider in all chromatographic techniques as the peaks and bands traverse the length of the column. HETP provides a measure of the rate of the broadening, with a smaller HETP indicating slower band broadening, leading to sharper peaks.

Origins of Golay’s Famous Equation

How was HETP as a measure of the rate of band broadening originally defined? This definition goes back to the earliest days of gas chromatography (GC) in the 1950s and the work of A.J.P. Martin and A.T. James, the inventors of GC; J. Van Deemter, who developed the original theory of band broadening for packed columns; Golay, who developed the theory for capillary columns; and Keulemans, who provided a fundamental conceptual definition for a theoretical plate.
Keulemans presented a picture of the separation process in chromatography in a classic text, *Gas Chromatography*, published in 1959 (1). In the foreword to this short text, A.J.P. Martin described it as “an admirably clear account of all the practical and theoretical aspects of this rapidly growing method”. The clear influence of the text is seen in the highly recognized 1965 text, *Basic Gas Chromatography*, by McNair and Bonelli (2).

As illustrated in Table 1, Keulemans described each theoretical plate as a liquid–liquid extraction between immiscible solvents. Starting with a flask containing a solution of an analyte dissolved in a solvent, say, water. The solution is mixed with an equal volume of hexane. If the partition coefficient is 1, then equal masses of solute are present in each phase. The hexane layer is then removed and mixed with an equal volume of water in a second flask and fresh hexane is added to the first flask. Each flask now contains half of the original analyte, equally distributed between the two phases. This process can be repeated with a third, fourth, fifth, and up to any number of flasks, ultimately resulting in a distribution of analyte among all the flasks (seen in Table 1), calculated for 10 flask theoretical plates, 10,000 molecules, and a distribution constant between the two phases of one. The numbers in Table 1 represent the number of molecules out of the 10,000 in each flask or theoretical plate. We can note immediately that as the number of flasks increases, the number of molecules in each flask decreases, analogous to the chromatographic peak becoming broader and shorter. Also note that there are still some molecules in the initial flask, even after 10 extractions, an often-unappreciated consequence of chemical equilibrium: There are always some molecules left behind.

The description provided above makes several assumptions that, although illustrative of the chromatographic process, do not account for all processes that occur in real columns. First, it assumes that each theoretical plate occurs independently and has no direct contact with the other theoretical plates. Obviously in a column with a mobile phase, this assumption cannot be true as the mobile phase is continuously transporting solutes along the column. Second, it assumes that there are no

### TABLE 1: Illustration of a 10 theoretical plate distribution of 10,000 molecules with a partition coefficient of 1

<table>
<thead>
<tr>
<th>Step</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5000</td>
<td>5000</td>
<td></td>
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<td>10</td>
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<td>98</td>
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<td>2051</td>
<td>1172</td>
<td>439</td>
<td>98</td>
<td>10</td>
</tr>
</tbody>
</table>
barriers to mass transfer at the phase interfaces. In a real system, of course, the kinetics of mass transfer across the phase interfaces must be considered. Finally, there is no consideration of diffusion both within and between the two phases. In a column, the solute molecules distribute throughout the mobile and stationary phases. Golay’s equation, described below, uses a description of the chromatographic process like Keulemans, and accounts for these assumptions, plus the dimensions and physical conditions observed within columns.

The “Golay Equation”
Golay provided a thorough description of an equation describing the rate of band broadening in the proceedings of the “Gas Chromatography 1958” symposium organized by the Hydrocarbon Research Group of the Institute of Petroleum and the Koninklijke Nederlandse Chemische Vreninging in Amsterdam, The Netherlands, in May 1958. The symposium was published in 1959, edited by Desty (3). The equation for circular capillary columns is presented as equation 3:

$$\frac{du}{dx} = \frac{2D}{v_0} \left(1 + \frac{11k_v}{24(1+3k_v^2)} \frac{v_0r_0^2}{6(1+3k_v^2)} \right) dx \quad [3]$$

Note that Golay also described a similar equation for rectangular capillaries. At the time, there was considerable discussion about which would be better, and clearly circular capillaries have become far more popular. Interestingly, with recent interest in columns etched into silicon wafers for “laboratory on a chip” applications, rectangular capillaries are increasing in usage and importance. This equation is popularly called the “Golay Equation”, but it combines the work of many early researchers in chromatography.

Definitions of the many variables are discussed in the “deeper dive” below. Interestingly, the proceedings volume includes commentary following the formal text of the talk, in which substantive discussion among the symposium participants was captured. The names of those participating in that discussion read like an all-star lineup of modern chromatography inventors, including Golay, A.J.P. Martin, R.P.W. Scott, I.G. McWilliam, and others. It was traditional in the past to assign scribes to record the discussions. In one especially important exchange between Golay and Martin, they appear to agree, possibly begrudgingly, to use the definition of HETP presented by Keulemans and illustrated above (4).

In the original work, several related forms of the equation are presented, relating to specific situations that may occur in the making of capillary columns; the form used here is equation 31a from that work, chosen for simplicity in discussing the basic principles and consequences. The first thing to note is that this is a differential equation relating to $du$, a differential of peak width to $dx$, a differential of length or distance. The terms enclosed in the parentheses provide what we term as HETP or $H$, the height equivalent to a theoretical plate, so the equation could be conceptually simplified to equation 4. Interestingly, similar to the Keulemans description, the independent variable is the length of the column ($dx$), as the differential is distance and the dependent variable is the peak width, represented by the differential of the second statistical moment ($du$).

$$du = (HETP)dx \text{ or } \frac{du}{dx} = HETP \quad [4]$$

Equation 4 shows us that the rate of band broadening as the band traverses the column is equal to HETP.

As we have learned in short courses and textbooks, smaller HETP means a slower rate of band broadening and sharper peaks. In many textbooks and courses, the terms within the parentheses in equation 3 are simplified to the form shown in equation 5. As we will see in Part 2 next month, equation 5 is an oversimplification that can generate confusion and possibly lead to false directions in method development and optimization.

$$HETP = \frac{B}{v_0} + C_m v_0 + C_v v_0^2 \quad [5]$$

Note the average velocity of carrier gas ($v_0$) is usually chosen as the independent variable in equations 4 and 5, leading to the “van Deemter” or “Golay” plots often seen in discussions of column performance in the scientific literature. The three terms are often described as representing analyte diffusion in the mobile phase (the “$B$” term), analyte diffusion in the stationary phase (the “$C_m$” term), and analyte diffusion in the mobile phase (the “$C_v$” term). In developing equation 3, Golay noted the similarity in form to the equation of van Deemter, developed a few years earlier.

A Deeper Dive into Golay’s Equation
Whether we perform detailed calculations based on it or not, equation 3 provides a very useful description of the many variables and processes relating to band broadening and, ultimately, to peak widths and resolution. Some important general principles are discussed here; for a detailed description of all variables and how they are used to evaluate column performance, see these book chapters or the discussion in ChromAcademy, LCGC’s online chromatography learning platform (5–7). In short, to minimize HETP, the quantities in parentheses in equation 3 should be minimized.
In the first term within the parentheses, often called the “B” term using the terminology in equation 5, diffusion in the mobile phase is considered. The numerator, \( D \), is the diffusion coefficient of an analyte molecule in the gas phase, which for simplicity can be considered a constant for that analyte at a given temperature in a given carrier gas. Diffusion coefficients in the gas phase are mainly dependent on the identity of the analyte, the solvent gas, and the temperature. The denominator is the average carrier gas velocity in the column. Not surprisingly, this suggests an inverse relationship between HETP and average carrier gas velocity.

The second term, often abbreviated as \( C_m \), relates to mass transfer, also in the mobile phase, and is the major difference between Golay’s equation for capillary columns and the original van Deemter equation for packed columns. Capillary columns usually have much larger phase ratios (volume of mobile phase divided by volume of stationary phase) than packed columns, so mass transfer in the mobile phase becomes important. Note that in this term, the same diffusion coefficient described above is now in the denominator and the average carrier gas velocity \( (v_c) \) is in the numerator, suggesting a proportional (linear) relationship between HETP and carrier gas velocity.

Additional variables in the \( C_m \) term include the square of the column radius, \( r_c^2 \), and a complex polynomial of the retention factor \( (k) \). Note that all of the variables, except for the column radius are temperature dependent and that the retention factor is also related to the column dimensions and average linear gas velocity.

The third term, \( C_s \), relates to mass transfer in the stationary phase. Similar to the \( C_m \) term, the \( C_s \) term is linear with the average carrier gas velocity and the square of the column radius \( (v_c \text{ and } r_c^2) \). Also, similar to the \( C_m \) term, the denominator includes a diffusion coefficient, this time in the liquid phase, which depends on the specific analyte, liquid phase, and temperature. Additionally, the analyte partition coefficient \( (c) \) into the liquid phase is part of the denominator. The term also includes a polynomial function of the retention factor \( (k) \).

Taken together, the three terms of the expression describing HETP, as presented by Golay, have numerous dependencies, and many of the variables that make up the equation have their own dependencies. The column radius is fixed and appears in two of the three terms. All the other variables are dependent on temperature. The average carrier gas velocity is dependent on the choice of carrier gas, the column dimensions (length and inside diameter), and temperature. The partition coefficient depends on the chemical nature of the analyte and stationary phase, and the retention factor depends on the partition coefficient, column phase ratio, and is further related to the average carrier gas velocity.

In our next instalment, we will examine these dependencies and the assumptions upon which our traditional view of HETP, based on the “Golay Equation”, are founded. We will also discuss their relevance in today’s capillary GC. The major changes between thinking about GC from the 1950s, when these ideas were first developed, and today include: the nearly universal use of temperature programming, smaller diameter capillary columns, thinner stationary phase films, and high vacuum column outlets, as seen in GC–mass spectrometry (MS). We will see that many of the traditional views that we were all taught (and still teach) about HETP, peak broadening, and peak widths in gas chromatography deserve some new thinking.

References
2) H.M. McNair and E.J Bonelli, Basic Gas Chromatography (Varian, Palo Alto, USA, 1965).

ABOUT THE AUTHOR
Nicholas H. Snow is the Founding Endowed Professor in the Department of Chemistry and Biochemistry at Seton Hall University, and an Adjunct Professor of Medical Analysis. His research group is very active, with ongoing projects using GC, GC–MS, two-dimensional GC, and extraction methods including headspace, liquid–liquid extraction, and solid-phase microextraction. Direct correspondence to: amatheson@mjhlifesciences.com
Q. When did your laboratory start working with two-dimensional liquid chromatography (2D-LC) and when did you start using this technique routinely in your laboratory? What applications is it being used for?

A: We started with the first implementation around 2012, primarily focusing on improving sensitivity in radioactive drug metabolism studies by concentrating the samples online (1). Often we only consume a small portion of the samples we have at our disposal or that can be easily harvested. Injection of a much larger volume of sample can be a way to improve the sensitivity of your analysis.

We gradually expanded the approach with additional pumps, valves, and columns allowing online preconcentration in combination with heart-cut 2D-LC (2), providing additional selectivity in cases where we encounter coeluting radioactive metabolite peaks or to speed up the tedious purification process when structure elucidation with nuclear magnetic resonance (NMR) is required.

A downscaled micro-ultrahigh-pressure liquid chromatography–mass spectrometry (micro-UHPLC–MS) version was built based on the experience we obtained with the systems used for radioactive and UV analyses. This system is primarily applied for very sensitive LC–MS bioanalysis (3).

Q. You developed an online preconcentration method using high-volume injections of biological samples for sensitive metabolite profiling and quantification (2). What benefits does this approach offer and could it be useful in other applications?

A: Radiolabelled mass balance studies are a critical component of drug development to provide insight in the absorption, distribution, metabolism, and excretion (ADME) of a new drug. As radioactivity can be counted in exhaled air and excreta, and “what goes in, should come out”, it allows us to make up a mass balance and know when the majority of drug-related material has left the body, and from which route. In most studies, especially for the in vivo human study, the amount of radioactivity dosed is kept to a minimum, while sample volumes are rather large. Therefore, it is common practice to concentrate the samples prior to injection. We used to perform this step in an offline fashion but more frequently encountered recovery issues potentially related with advances in formulation science, which allow advance drug molecules that are “sticky” and/or have low solubility. Using an online preconcentration approach we could easily avoid losses of radioactivity, that is, compound-related material, and save a lot on otherwise time-consuming...
method development. Since the purpose of the drug metabolism studies is the identification and quantification of drug metabolites, the compounds we are analyzing are still unknown prior to analysis. Therefore, we cannot check their stability and recovery, and should avoid any related risk, such as drying steps, during offline sample concentration. Another approach we routinely apply is partial drying of a sample, for example, after protein precipitation with acetonitrile or methanol extraction of faeces. A fixed volume of dimethyl sulfoxide (DMSO) is added to the sample and dried to the DMSO content. This way, complete drying of the sample is avoided, which is when the majority of degradation usually occurs and also makes reconstitution of the compounds in solution much more difficult. The DMSO solution can be injected afterwards without affecting chromatographic performance thanks to an online dilution during sample loading with a high content of water.

The approach we use for online preconcentration was, of course, already widely used in many applications. The main difference with “trap-elute” approaches routinely applied is that we use a much larger 4.6 × 50 mm high performance liquid chromatography (HPLC) column for sample loading. This makes it possible to inject up to 100 mL of urine and tens of millilitres of protein-precipitated plasma or blood, faeces extracts, and so on, without deterioration of the chromatographic separation. Moreover, LC resolution often improves as a result of the back-flushing mode we use to elute the trapped sample compounds to the analytical column. We also notice a longer lifetime of our analytical columns when this approach is used. Often a replacement of the trapping HPLC column is sufficient to restore deteriorating LC performance after multiple large volume injections.

Q. You combined the method using very high-volume injections with heart-cut 2D-UHPLC for selective drug metabolite trace analysis (2). What is novel about this approach?

A: This is an extension of the online preconcentration approach discussed previously, which allows large volume injections (1–100 mL) in combination with heart-cut 2D-LC. This setup combines ultimate sensitivity with improved selectivity. The setup uses four columns: two 3-mm internal diameter (i.d.) analytical columns for the first and second dimension separation, each preceded by a 4.6-mm i.d. column to concentrate the sample or trap the compounds of interest. This way we were able to reach a quantification limit of 770 attogram/mL of a tritiated imipramine.
metabolite by injection of 100 mL of urine (2). For context, 770 attogram/mL is the same concentration as one 4 g sugar cube dissolved in more than 2 million Olympic swimming pools of 2.5 million litres each.

Q. What were the main challenges you encountered when developing this method?
A: These setups are not readily available, “off-the-shelf” systems. Most UHPLC users and vendors consider a few tens of µL already a large injection volume. Therefore, 10 years ago there were not many UHPLC systems available providing the flexibility to go beyond 50–200 µL injection volume. Since the ability to inject larger volumes is the only way to make a combination of UHPLC with online radioactive detection work for metabolism studies (4), we had already tackled this problem in previous years. Using a modular approach with separate autosamplers, pumps, valves, and so on—a lot of powerful combinations can be built. The most challenging part, however, is not the hardware configuration but finding the right software solution to control all the different modules in an easy and robust way. For this we heavily rely on the vendors, particularly their expertise in supporting less routine applications.

Q. What applications is this technique being used for in your laboratory and what benefits does it offer the analyst? Could this technique be useful in other applications?
A: Since the constituents of a metabolism sample are still unknown prior to analysis and often differ from sample to sample, we regularly encounter situations where new metabolites pop up that were not found earlier in other in vitro or in vivo samples. If these metabolites coelute with other metabolites, we can use the heart-cut 2D-LC approach to separate these, which is the only way for accurate quantification in radiochemical detection for which baseline separation is required.

Another application is selectivity enhancement—separating analytes of interest from interfering matrix components. Despite the fact that we are dealing with relatively dirty in vitro and in vivo samples (such as urine, blood, faeces, bile, and tissue), we usually keep sample preparation to a minimum since we don't know the metabolites we are analyzing yet and any sample preparation might introduce loss or degradation of some or all of the metabolites. The online preconcentration approach previously discussed is very good if a selective detection method such as radioactive detection or qualitative MS is used. If, however, the detection suffers from selectivity (such as UV) or matrix effects (for example, quantitative MS), heart-cut 2D-LC can be a solution. We use this, for example, to get baseline separated UV peaks of metabolites for which we don't have authentic standards. UV detection is more reliable to estimate the relative abundance of metabolites (if the chromophore is not dramatically changing relative to the parent drug) compared to MS, for which the response is more structure-dependent. The combination with an HPLC trapping column prior to the first dimension allows us to further boost the sensitivity of our analyses. Another application is the purification of metabolites for NMR structure identification, requiring relatively large amounts of compound in high purity. The combination of online preconcentration with heart-cut 2D-LC offers a way to purify large volumes of sample with high chromatographic performance on 3-mm i.d. columns packed with sub-2.5-µm particles. The purification could also be done with offline 2D-LC. The main benefits of the online approach, however, is that it reduces manipulation of the sample and, thus, reduces the time and potential errors and artefacts during sample preparation. A larger volume injection and trapping between the two dimensions is preferred over drying and reconstitution in a small volume, with the risk of degradation or incomplete recovery of the analyte of interest.

Q. The 4-column multidimensional micro-UHPLC–MS method you developed offers high sensitivity and selectivity for the quantitative analysis of drugs in biological samples (3). What is novel about this method and what benefits does it offer the analyst?
A: The micro-UHPLC–MS method was built based on the setup and experience obtained with the system using two 4.6-mm i.d. trapping columns and two 3-mm i.d. analytical columns. While the previous setup was aiming for sensitive and selective LC–radioactive and LC–UV detection, this setup was targeting ultrasensitive LC–MS quantification. Since sampling efficiency in electrospray ionization (ESI) largely improves with decreasing flow rates to the ESI emitter (6,7), a gain in MS sensitivity can be obtained in going to columns with a smaller internal diameter. Although in this perspective, nano-LC should provide the biggest gain in sensitivity, we are making use of micro-LC in this setup because it is considered the best compromise between sensitivity and throughput, which is also very important in the bioanalytical environment in which we are working. The sensitivity gain, often advocated for nano- and micro-LC–MS, should also be put in perspective of the sample volume available. If the sample volume is not the limiting factor, the sensitivity gain in going to nano- or micro-LC will usually be negligible compared to an injection of a larger volume of the same sample on a larger bore column that has a higher sample loadability. Trap-elute is a potential solution to increase loading on smaller bore columns, but we
often observe a less than proportional gain in sensitivity, for example, a 10-fold higher sample loading often results in only threefold higher sensitivity. This can be explained by the equally concentrated sample matrix constituents negatively affecting sensitivity by matrix effects (ion suppression). By using heart-cut 2D-LC, the analyte of interest can not only be concentrated but also separated from the sample matrix. Therefore, we usually see a proportional gain in sensitivity when using the 4-column micro-UHPLC–MS setup. By gradually decreasing column internal diameters from 2.1 mm (first trapping column) to 1 mm (first dimension analytical column) to 0.5 mm (second trapping column) and finally to 0.15 mm (second dimension analytical column), we are able to inject much larger sample volumes onto micro-LC with a reasonable throughput (±15 min total run times). As illustrated in reference 3, the lower limit of quantification (LLOQ) we can obtain with this setup are about 250- to 500-fold better than with direct injection on micro-LC and 10–50 times better compared to trap-elute. For midazolam and its 1'-OH-metabolite, we were able to reach an LLOQ of 100 fg/mL. This is comparable to a concentration of one 4 g sugar cube in 16,000 Olympic swimming pools of 2.5 million litres. In the midazolam example, we were limiting ourselves to the maximum injection volume feasible on the nano-LC system due to its maximum loop size and syringe volume. We are confident that, based on the experience we have with the larger-bore 4-column system, we can inject even higher volumes using multiple injection cycles without affecting the chromatographic performance.

Q. 2D-LC is often regarded mainly as a research tool and not suitable for routine analysis. Is this view changing in practice? Is there more scope to use 2D-LC routinely in pharmaceutical and biopharmaceutical analysis?

A: There is clearly a trend towards increased use of 2D-LC in pharmaceutical analyses, especially heart-cut 2D-LC (rather than comprehensive 2D-LC). While currently 2D-LC is primarily exerted in less routine, non-GXP environments. I am confident that the use in more routine analyses will grow further, especially in areas such as large molecule analyses (such as proteins and oligonucleotides), where sample preparation can be challenging and one-dimensional LC does not offer the required separation power.

More and more LC vendors are now offering 2D-LC instrumentation as a full package or as an optional expansion of regular LC systems, including the right software to warrant better robustness (for example, communication between the different modules) as well as improved user-friendliness. This proves the increasing interest of the industry in 2D-LC.

In order to be successful in the implementation of technology such as 2D-LC in the (bio)pharmaceutical industry, it is important to make its use as simple and straightforward as possible. There is no time to tweak the system for every new study, nor is there time to allow elaborate method development for every sample. This is also clear in the choices we made for the setups and methods we are using. The valve setup is chosen in such a way that it allows switching between the larger volume injections (large loop size) using online preconcentration and smaller direct injections (small loop size) without any replumbing. We use trap (5–10 μm particles) and analytical columns (2.2–2.5 μm particles) packed with the same stationary phase, avoiding that any analyte of interest would be more retained on the trap than on the analytical column. We use stationary phases in the first and second dimension that might not be extremely orthogonal but work for most drug-like molecules, such as C18 and phenyl-hexyl, and always combine this with an as orthogonal as possible pH (for example, acidic and basic) between both dimensions. The use of a trapping column in combination with solvent dilution between both dimensions might look more complex than a simple loop approach but allows the use of orthogonal conditions in the second dimension without having to worry about elution strength (8,9,10). The only thing that needs some optimization is the solvent strength for loading the sample and the dilution ratio with water to avoid breakthrough of analytes. A more apolar analyte will generally require a higher solvent strength to avoid loss by adsorption to the loop wall. Since metabolites are usually more polar than the candidate drug, we use the candidate drug for optimization and to test potential losses or carryover. Then, we adjust the loading pump/dilution pump flow-rate ratio to get the right dilution factor depending on the expected polarity of the metabolites.

References

This interview has been edited for length. Scan the QR code to read the full interview.
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For calibration of aqueous GPC/SEC/GFC systems, synthetic polymers, polysaccharides, and proteins are used. PSS ReadyCals are cocktails of molar mass reference materials pre-weighted into autosampler vials. New to the PSS ReadyCal family is the ReadyCal-Kit Protein comprising 10 proteins in three colour-coded 1.5 mL vials. A kit contains 15 vials for five calibrations.

[www.pss-polymer.com](http://www.pss-polymer.com)
PSS Polymer Standards Service GmbH, Mainz, Germany.

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[www.phenomenex.com](http://www.phenomenex.com)
Phenomenex, Torrance, California, USA.

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[www.gilson.com/cpc](http://www.gilson.com/cpc)
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[www.vici.com](http://www.vici.com)
VICI AG International, Schenkon, Switzerland.

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[https://markes.com/thermal-desorption-instrumentation/sorbent-tube/td100-xr](https://markes.com/thermal-desorption-instrumentation/sorbent-tube/td100-xr)
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