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News
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The Column spoke to Martin Giera from Leiden University Medical Center in the Netherlands, about the importance of gas chromatography-mass spectrometry (GC–MS) in bioanalysis applications and the latest advances he has made with this technique.

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Non-aqueous Ion-Exchange Chromatography Using High Acid-base Concentration: A Strategy for Purifying Non-crystalline Pharmaceutical Intermediates

Andrew S. Ikin, Stephen Norris, Andrew Stark, Craig Stewart, and Lee Timms, Early Chemical Development, Pharmaceutical Sciences, R&D, AstraZeneca, Macclesfield, UK

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 (RP) chromatography was not a viable option, because lyophilization of multigram fractions was neither practical nor efficient. However, there
is a tertiary amine (basic) for IEX and a secondary amide (non-basic) which is not suitable for IEX in the product (Figure 1). This suggested the opportunity to use an acidic ion-exchange resin (strong cation exchanger) to purify the product, exploiting the charge differences between the product and impurities (2). It was anticipated that this could be achieved using a classical load, retain, wash, release process. From the calculated pK\textsubscript{a} values for the tertiary amines in Table 1, it can be clearly seen that the impurities 1 and 2, and the product would preferentially be bound to a strongly acidic cation exchange resin here because impurities 3 and 4 should be less retentive. Propylsulfonic acid-silica is a strong cation exchanger with minimal non-polar character, used for the retention of basic compounds. It has been successfully used for the “catch and release” purification of amines. 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 “released” from the column by elution with a solution of ammonia in methanol (methanolic ammonia). Amines 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 was Isolute SCX-2 (Biotage) in pre-packed cartridges for milligram scale and in bulk for the multi-gram and multi-kilogram scale. The resin was supplied in H\textsuperscript{+} form, the physical and chemical properties are listed in Table A in Supplementary Information at https://bit.ly/3sKUJh5.


**Instrumentation**

*Milligram scale:* A cartridge containing the chosen strong cation exchange was used.

*Multi-gram 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
TABLE 1: Selected by-product impurities from unwanted side reactions, the product and calculated pKa 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></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.

Multi-kilogram 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 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 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).
associated degasser, quaternary pump, autosampler, column compartment, 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 cation exchange resin. The cartridge was then inserted and lowered to 1 cm above the top of the packing. The column was then connected to the UV diode-array detection flow cell. 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. 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.

**Multi-gram scale:** Approximately 60 g of the strong cation exchange resin was dry packed into a 25-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 UV diode-array detection flow cell. The column was then flushed with three CVs of methanol in backflush mode to equilibrate the column. Backflush mode was used to help avoid leaving non-wetted aggregated areas and help 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.

**Multi-kilogram 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 help avoid leaving non-wetted aggregated areas and help the egress of air bubbles. Upon completion of the equilibration, the piston head was lowered until the resin bed length was 940 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 analysed for the...
presence or absence of the product on a reversed-phase HPLC (see Supplementary Information at https://bit.ly/3sKlJh5). 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/3sKlJh5). Multi-gram scale: Operations A–G, as described in Figure 2, were performed at the multi-gram 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 (AVG) steps, load (B), retain and wash (C), release (D), wash (E), regeneration (F) steps. 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. The individual fractions were analyzed to confirm the presence or absence of the product on a reversed-phase HPLC (see Supplementary Information at https://bit.ly/3sKlJh5). 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/3sKlJh5). Multi-gram scale: Operations A–G, as described in Figure 2, were performed at the multi-gram 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 multi-gram 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/3sKlJh5). 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/3sKlJh5).

**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 multi-kilogram scale.

Concerns were raised whether the silica-based ion-exchange resin would be stable with prolonged exposure to high pH at the proposed larger multi-kilogram scale. Exposure of silica to high pH is known to degrade (hydrolyse) 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 multi-kilogram scale (4). However, this resin failed to retain the product. Accordingly 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 was successful.

At this time, it was thought that the resin would be single use, that is, at the multi-kilogram scale, a whole batch (4–5 kg) could be processed in a single run. Therefore, it was decided to continue to the multi-gram 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 of

### 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 H N O H</td>
<td>49% w/w*</td>
<td>87% w/w</td>
</tr>
<tr>
<td>H O</td>
<td>16% w/w</td>
<td>Not more than 0.5%</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>4 ppm</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.

**Multi-gram scale:** Following identification of an outline process at the milligram scale, the focus of the work moved to identification of suitable conditions on a multi-gram scale.

Initial experiments had varying degrees of success because the product was not consistently retained. It was observed that as an injection “plug” passed through the column, the feed in CPME diluent, remained in a tight band throughout elution, indicating that CPME had minimal miscibility with the eluent, methanol (MeOH). This raised concerns that the large difference in polarity between CPME and methanol may have affected the efficiency of the ion-exchange process. As part of the investigative work it was found that when the CPME feed solution was left standing for several hours, the feed was clearly biphasic. A non-homogenous feed would have different concentrations of the product in either layer, potentially leading to varying quantities being loaded onto the column for each injection. This could have been one of the contributing factors to the inconsistent retention of the product. Moving from CPME to MeOH (as feed diluent) produced a homogenous feed, eradicating the biphasic feed issue, and it was thought that this would lead to more consistent loadings and chromatography. This conjecture was later proved to be unfounded; the reason why many of the experiments failed was not due to the biphasic feed.

It was unknown if the resin would be stable to the high pH (2 M methanolic ammonia, product release (step D in Figure 2) or if the resin could be acid regenerated. Basic or acidic modifiers are usually present in the order of 0.1/0.5% v/v in chromatography eluents (5), but in this case the ammonia was present at 3.5% (2 M methanolic ammonia pH= 10.5). For resin regeneration, an acid soluble in methanol was required; water was not an option as the product is highly soluble in water. Hydrochloric acid could not be used as it was not compatible with the equipment at this scale and at the multi-kilogram scale (6). Sulphuric acid was not explored as it is a diprotic acid. Methanesulphonic acid resulted in the precipitation of ammonium methanesulphonate, which is insoluble in methanol and hence would have blocked the preparative system tubing. It was proposed that 2 M acetic acid in methanol would be sufficiently acidic for regeneration without corroding the 316-stainless steel tubing, steps (E–G) were carried out. Experimental work showed that the resin could not be regenerated using 2 M acetic acid. The resin was then subjected to 2 M TFA (23% TFA in MeOH) regeneration, steps (E–G). This successfully regenerated the ion-exchange stationary phase, however, the chromatography remained inconsistent.

As this is a cationic exchange process it was postulated that the inconsistent chromatography could be attributed to the presence of other cationic species present in the feed, for example, sodium and potassium, as overloading had been ruled out. This led to a sample of the feed and a sample from a successful run being analysed by ICP (see Supplementary Information at https://bit.ly/3sKJuH5). 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 multi-kilogram 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 multi-gram scale, to find a loading that would successfully scale up to the multi-kilogram scale. The user trial of each batch was based on trialling a volume at the multi-gram scale to find the optimum loading for the multi-kilogram scale. Using this data, the number of aliquots required to process a batch was calculated (see Supplementary Information at https://bit.ly/3sKJuH5).

The results from the user trials demonstrated that the CV approach applied at multi-gram scale could...

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**FIGURE 3:** 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.
be used to define the optimum load volume at multi-kilogram 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 (πr^2h) 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).

**Multi-kilogram scale:** During steps A-G (Figure 2) of the first run at this scale, the column temperature was monitored for excess heat because it was believed that the regeneration step would have the largest heat generation (acid-base reaction). However, this was found not to be the case, with the release step D, generating so much heat that the metal fittings on the exit line became quite warm. A reduction in flow rate controlled this heat generation.

An aliquot of feed was processed in 4 h. Hence, the processing of 17 aliquots took 68 h. The throughput was 284 mL of feed/kg resin/day (60 g of product/kg resin/day).

The pump flow rates were set between 1.0–1.5 L/min, which was not a linear scale up of the flow rates from multi-gram to multi-kilogram 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 sub-division of each batch, see Supplementary Information at https://bit.ly/3sKlJh5.

**Conclusion**

The use of IEX in 100% organic mode for scale-up to multi-gram 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. 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 as part of the toolbox in process development and manufacturing for purification of pharmaceutical materials.

**Acknowledgements**

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**References**


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Surfing on Mobile Phase, Part 2: Impact of Mobile Phase Composition Waves on Retention in LC

Dwight R. Stoll, LC Troubleshooting Editor

In the previous instalment 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 instalment, I continue this story by discussing the impact of waves in mobile phase composition on retention time in reversed-phase liquid chromatography (RPLC). As I indicated last month, readers interested in a deeper dive into these topics 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 effect 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 × 2.1 mm i.d. RPLC column with a dead volume of 100 µL. For the retention dependence, I’ve used the linear solvent strength theory (LSST) model for RPLC separations (2), which relates
the retention factor of the analyte ($k$) to the volume fraction of organic solvent in the mobile phase ($\phi$, 0–1 scale):

$$\ln k = \ln k_w - S \phi$$  \[1\]

where the parameters $S$ and $k_w$ are the slope of a plot of $\ln k$ vs. $\phi$, 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 sinuisoids 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, $\pi/2$, $\pi$, or $3\pi/2$, and stroke volumes of 100, 66, or 33 µL. One of the main conclusions of the recent paper by Gritti (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, $\pi/2$, $\pi$, or $3\pi/2$. 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 the retention precision is quite good at 0.06 %RSD. However, it is well known that the retention of larger molecules is much more sensitive to small changes in mobile phase composition, compared to smaller molecules. Figures 3b and 3c show simulation results for myoglobin, which is a 17 kDa protein with LSST parameters of 53 and 5.5 x 10^7 for $S$ and $k_w$, respectively (6). If the wave amplitude is fixed at 1% acetonitrile as in Panel A, 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). The tracer is used to determine the variation in composition of the mobile phase at the pump outlet, and this can be compared to expected values based on the instrument specifications. Readers interested in more 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” instalment, 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
Hydrophobic Interaction Chromatography (HIC) for the Characterization of Therapeutic Monoclonal Antibodies and Related Products, Part 1: Theoretical Aspects

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Hydrophobic interaction chromatography (HIC) is one of the historical analytical methods applied for the separation and purification of proteins. The main advantage of HIC is that separations are performed under mild—in most cases non-denaturing—conditions (ambient mobile phase temperature, aqueous mobile phase, and physiological pH). Thanks to the intense developments in the field of protein-based therapeutics, HIC has recently gained a huge interest and is currently considered as a reference method for the characterization of cysteine-linked antibody drug conjugates (ADCs) and widely used for the analysis of therapeutic monoclonal antibodies (mAbs) and bispecific antibodies (bsAbs), at both intact and sub-unit levels. In the first part of this two-part series, the current state of HIC is reviewed with a focus on retention and separation mechanism.
protein analysis including uni- and multi-dimensional separations. In this first part the focus is on theoretical aspects.

**Retention in HIC**

To retain proteins in HIC, kosmotropic salts are typically added to the mobile phase. These salts generally reduce protein solubility, while stabilizing protein structure and facilitate reversible interactions between the protein’s nonpolar (more hydrophobic) surfaces and the stationary phase’s hydrophobic functional ligands. As a result, protein binding occurs at high salt concentration, while elution takes place at low salt concentration (3,4,7,8,9). Then, the separation is based on reversible partitioning of proteins between a mobile phase containing the salt (such as ammonium sulfate) and a mildly hydrophobic stationary phase (for example, a butyl phase with low ligand density). Normally, increasing the salt concentration increases partitioning toward the stationary phase, while reducing the salt concentration increases partitioning toward the mobile phase. Because of the strong dependence of protein retention on salt concentration (“on-off”, or “bind and elute” like mechanism), HIC is often operated in gradient mode, starting at a high salt concentration and ending with a low salt concentration (often called inverse salt gradient) (10).

**FIGURE 1:** Impact of ammonium-sulfate concentration (molarity) on the retention of ribonuclease-A (left panel) and logarithmic retention factor vs. ammonium-sulfate concentration for myoglobin, ribonuclease A, BSA and chymotrypsinogen A (right panel). Adapted with permission from reference 11.

**FIGURE 2:** Schematic view of protein adsorption (a), unfolding (b), positive cooperative adsorption - preferred adsorption (c) and multilayer adsorption (d). Symbols: \( P \) is the protein in released state, \( P_n \) and \( P_u \) is the protein in the adsorbed phase in the native and unfolded form, respectively, while \( P_{nc} \) is the native protein adsorbed in positive cooperative adsorption. Adapted with permission from reference 29.

**FIGURE 3:** Effect of temperature on the elution behaviour of bsAb with a 1000–0 mM ammonium sulfate gradient in 20 column volumes (CV) at a flow rate of 0.5 mL/min. Adapted with permission from reference 33.

Chromatographic separation based on hydrophobic interactions was first reported by Tiselius (12). He used the term “salting-out chromatography”. The salting-out effect is based on electrolyte–nonelectrolyte interactions (corresponding to mobile phase-protein interaction in HIC), in which the nonelectrolyte becomes less soluble at high salt concentrations. The retention of proteins is mainly explained by the formation and collapsing of water layers surrounding the proteins and stabilized through H-bonding. When adding salts into the mobile phase, water molecules solvate the salt ions
predominantly. Therefore, the number of water molecules available to interact with the hydrophilic part of the protein will decrease. Under these conditions, the protein–protein and protein-ligand intermolecular interactions become stronger due to the decreased amount of surrounding water molecules and proteins will be retained (3). Later on, several terms were used to describe such types of separations, including “hydrophobic chromatography”, “hydrophobic affinity chromatography (HAC)”, “hydrophobic interaction chromatography (HIC)”, “salt mediated separation of proteins” or “salt-promoted adsorption chromatography (SPAC)”. The solvophobic theory is also often used to explain HIC retention. It describes the interactions between a polar solvent (aqueous mobile phase) and less polar solute (protein). As a result of H-bonding and other polar interactions, strong cohesive forces exist between the solvent molecules and provide a strongly structured order for the solvent (13). Therefore, less polar solutes tend to be insoluble due to the strong solvent–solvent binding interactions. According to this, the solute molecules bind to the surface of the stationary phase due to their rejection from the solvent and their affinity for the hydrophobic stationary phase. So, the retention is partly explained by the interactions between the solute and stationary phase and partly by the rejection of solute from the mobile phase solvent.

An entropy-driven model can also describe the retention in HIC. The structure of water is highly ordered thanks to dipole–dipole interactions in a three-dimensional structure, determined by the high surface tension of water. If hydrophobic moieties are dissolved in an aqueous system, the neighbouring water molecules have to be separated from each other thus forming a “cavity” for the less polar solute (14). This requires the investment of energy, depending on the surface area of the cavity and the surface tension. If two or more solutes (or solute and ligand) are associated, their hydrophobic contact surface area is reduced, and therefore energy is released. In other words, the interaction between hydrophobic molecules in aqueous solutions occurs spontaneously and is driven by the system’s entropy change (15,16,17). However, it has been shown that hydrophobic interactions are entropy driven at low temperatures, but enthalpy driven at elevated temperatures (18). Now it seems, that under practical conditions, both enthalpy and entropy control take place. It explains that in some conditions, retention increases with temperature while for other cases retention may decrease with temperature.

Is HIC Indeed Non-denaturing and Non-destructive?
HIC is often referred to as a non-denaturing and non-destructive chromatographic mode. Over the years, several studies have nevertheless proven that conformational changes may occur during the adsorption of proteins onto hydrophobic surfaces (19,20,21,22). Now, it seems that a certain fraction of the injected protein unfolds upon adsorption onto HIC stationary phases resulting in stronger binding and delayed elution, which can explain the loss of protein recovery observed in some cases (23,24,25). The partial unfolding of the proteins has been experimentally proven and a reversible unfolding mechanism was recently proposed (26). The rate of unfolding upon adsorption depends both on the type of stationary phase and on the protein properties. It was observed that “softer” proteins (which possess a more compressible structure) are more prone towards unfolding and showed higher retention (27). Based on melting point measurements (using differential scanning calorimetry), it could be shown that the conformational changes occurring in an antibody or antibody fragments upon adsorption onto HIC surface are directly proportional to the hydrophobicity of the stationary phase (28). Then upon elution (when protein is released from the HIC stationary phase), the protein takes back its original (native) conformation. It was also observed that antibody fragments which are less stable, are more likely to undergo conformational changes upon adsorption.

A model was recently developed, accounting for protein unfolding and intermolecular interactions in the adsorbed state (29). For the intermolecular interactions, we can distinguish so-called preferred and multilayer adsorption on the adsorbent surface, as well as exclusion effects due to size exclusion and electrostatic repulsion. The mechanism of protein binding and spreading on a HIC surface is shown in Figure 2. First, the protein adsorbs on a free active site on the adsorbent surface in its native form and then, the protein-binding surface spreads by attaching other active sites which results in unfolding. Additional protein molecules try to bind through positive cooperative adsorption, preferred adsorption, and multilayer adsorption. For proteins possessing unstable structures, the overall adsorption is dominated by the binding of unfolded species at low surface coverage and by positive cooperative adsorption at high surface coverage. Furthermore, exclusion effects strongly influence adsorption equilibrium, particularly at low surface coverages.
So in the end, simultaneously, several different equilibrium processes (that is, adsorption, desorption, unfolding, folding, positive cooperative adsorption, and positive cooperative desorption) determine the retention in HIC.

The strength of protein binding depends on the “footprint” of the protein, this is why aggregates are more retained than monomeric forms. Besides the size of the binding footprint, interactions between the protein surface and the stationary phase are likely to play an important role because they determine the preferred binding orientation. It has been demonstrated that even a small protein (for example, lysozyme) can interact with the chromatographic surface in orientation-specific ways, depending on various factors such as injected amount/volume, mobile phase pH, and resin structure (30,31). It has also been shown recently that for antibodies, the various mAb domains interact differently with the stationary phase, each contributing to the overall binding strength and selectivity. In practice, it can be assumed that a distribution of different binding states and binding orientations exists for most proteins with the most favourable binding configuration being the most probable determining the overall retention (32).

Since proteins can be oriented in different ways on the HIC stationary phase and can unfold upon binding in different extents (depending on their orientation, stationary phase hydrophobicity, temperature and pH), they can lose their secondary structure. The change in secondary structure can result in multiple peak elution. Some examples have been reported earlier, such as two-peak elution of α-lactalbumin, bovine serum albumin, and of β-lactoglobulin on various HIC resins (20,22,23). Very recently, Carta et al. showed a multiple (three) peak elution of bivalent bispecific antibodies from HIC phases (33). While three peaks were obtained at short residence times (binding time) and room temperature, residence times longer than 27 min or operating at 45 °C resulted in a single merged peak, indicating that the underlying mechanism occurs on time scales comparable to that of a chromatographic separation.

Holding the protein on the resins prior to elution enriched the late-eluting peak, indicating that multiple binding states formed on the chromatographic surface are responsible for this behaviour. The multiple and merged peak elution of the bsAb is shown at various temperatures on Figure 3.

It has also been shown that the rate of conformational changes upon adsorption in HIC depends on sample load (injection, feed) too (26). The unfolding phenomenon seems to enhance when increasing the salt content, and to decrease when increasing column loading. The latter phenomenon was explained by the reduction of available adsorption sites which aroused from steric hindrance to mass transport at the solid interface.

As discussed, protein retention is normally reduced by decreasing the salt concentration. However, in some cases, protein retention may increase at low salt concentrations resulting in a U-shaped retention factor curve (retention versus. salt concentration) (34). It has been shown that some proteins may elute partially in gradient mode and partially trapped in the column. Solvophobic effects and other adsorptive interactions can lead to such U-shaped retention factor curves in HIC. As an example, hydrophobic binding and protein unfolding on the stationary phase surface can lead to strong retention at low salt concentrations, weaker interactions at intermediate salt concentrations and strong binding again at high salt concentration. Moreover, U-shaped retention factor curves can be the result of interplay between hydrophobic and electrostatic interactions (35,36,37). MAbs can also exhibit U-shaped retention factor curves and a critical gradient slope can be determined beyond which 100% recovery is no longer possible (34).

Figure 4 shows retention factor curves, obtained for two mAbs and lysozyme. Elution with a reverse gradient is also demonstrated at low salt concentrations for these proteins. Understanding this
behaviour is important to design gradient HIC separations appropriately, since gradient slope may impact the recovery.

Conclusion

This article summarizes the most recent information that can be found in literature about hydrophobic interaction chromatography (HIC), which is widely applied for the analysis and purification of proteins. The first part of the two-series articles focuses on theoretical aspects, particularly on retention mechanisms observed in HIC, with the goal to develop more robust methods. Various contributions to retention have been discussed including hydrophobic interaction, hydrophobic effects, "long-range non-hydrophobic" interactions, solvophobic effects, salting-out, dehydration of proteins or structural rearrangement like reversible and irreversible conformational changes of proteins.

HIC is often considered as a non-denaturing chromatographic technique, which is advantageous for protein analysis. This article is also intended to decipher whether HIC can indeed be considered as a non-denaturing and non-destructive technique.

References


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Next Generation Sorbent-Based Extractions with Metal-Organic Frameworks

Douglas E. Raynie, Sample Preparation Perspectives Editor

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.

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” instalment, 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 favoured. 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 solid samples and, while not exclusive, are found primarily in the biological, environmental, and food areas. Table 1 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-inflammatory drugs, 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

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<td>SBSE</td>
<td>Carvediol, haloperidol</td>
<td>Water, plasma</td>
</tr>
<tr>
<td>Magnetic, SPME, dSPE</td>
<td>Polycyclic aromatic hydrocarbons</td>
<td>Water, soil, plasma, urine, meats, smoke, beverages, blood</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Neonicotinoid insecticides</td>
<td>Water, melons, vegetables</td>
</tr>
<tr>
<td>Magnetic, dSPE</td>
<td>Phenylurea herbicides</td>
<td>Fruits, soil, water, beverages, vegetable oils</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Flunitrazepam</td>
<td>Beverages</td>
</tr>
<tr>
<td>Magnetic</td>
<td>N-linked glycan</td>
<td>Egg proteins, serum</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Peptides</td>
<td>Serum</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Chlorophenols</td>
<td>Water, beverages, vegetables</td>
</tr>
<tr>
<td>Magnetic, SPME, dSPE</td>
<td>Organochlorine pesticides</td>
<td>Water, vegetables</td>
</tr>
<tr>
<td>Magnetic, SPE</td>
<td>Carbamates</td>
<td>Water, fruits, vegetables, beverages</td>
</tr>
<tr>
<td>Magnetic, SPE</td>
<td>Endocrine disrupting compounds</td>
<td>Water, juice, tissue</td>
</tr>
<tr>
<td>Magnetic, SPME</td>
<td>Pyrethroids</td>
<td>Vegetables, water</td>
</tr>
<tr>
<td>Magnetic, dSPE, SBSE</td>
<td>Hormones</td>
<td>Water, urine, cosmetics, vegetables, sewage</td>
</tr>
<tr>
<td>Magnetic, dSPE</td>
<td>Benzoylurea insecticides</td>
<td>Beverages, grains, water, honey, fruits</td>
</tr>
<tr>
<td>Magnetic</td>
<td>Anionic surfactants</td>
<td>Water</td>
</tr>
<tr>
<td>Magnetic, dSPE</td>
<td>Estrogens</td>
<td>Water, urine, milk</td>
</tr>
<tr>
<td>SPE</td>
<td>BTEX</td>
<td>Water, urine, smoke</td>
</tr>
<tr>
<td>SPME, magnetic, dSPE, SPE</td>
<td>Phenols</td>
<td>Water, beverages, 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>
</tbody>
</table>

Table 1: Representative application areas of extractions performed using MOFs
TABLE 1 (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>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>Sulfonylamides</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>Haloacetic acids</td>
<td>Urine, meats</td>
</tr>
<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>Domic acid</td>
<td>Shellfish</td>
</tr>
<tr>
<td>dSPE</td>
<td>Allatoxins</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>

related sample types. Fruits, vegetables, beverages, edible oils, and meats were investigated for herbicides and pesticides, hormones, and aflatoxins.

Conclusions and Future Prospects

An emerging type of sorbent material, MOFs, are 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


COLUMN EDITOR
Douglas E. Raynie “Sample Prep Perspectives” editor Douglas E. Raynie is a Department Head and Associate Professor at South Dakota State University, USA. His research interests include green chemistry, alternative solvents, sample preparation, high-resolution chromatography, and bioprocessing in supercritical fluids. He earned his PhD in 1990 at Brigham Young University, Utah, USA, under the direction of Milton L. Lee. Raynie is a member of LCGC’s editorial advisory board. Direct correspondence to: amatheson@mjhlifesciences.com
Let’s Get Small: Powerful Gas Chromatography in Small Packages

Nicholas H. Snow, Department of Chemistry and Biochemistry, Seton Hall University, New Jersey, USA

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 instalment, 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.

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 2–4 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 instalments 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 1990s movie “Medicine Man” with Sean Connery and Lorraine Bracco (3). Several field portable GC–MS systems were recently reviewed by the United States 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
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detector. Miniaturized systems have advanced in simplifying the column connections and connectors have also advanced 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, 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 labelled 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 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

**TABLE 1: 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>

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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, which 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 220–240V power supplies using a 120V 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 1 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 120V, 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 1, 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 standalone 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 throughout or multiple users that require training.

When considering your next purchase of a gas chromatograph, I highly recommend considering smaller systems.

**References**

1. N.H. Snow, LCGC Europe, **33**(11), 574–578 (2020).

**COLUMNS EDITOR**

Nicholas H. Snow is the Founding Endowed Professor in the Department of Chemistry and Biochemistry at Seton Hall University, USA, 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.

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www.biopharma.co.uk
Biopharma Group, Winchester, UK.

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YMC, Dinslaken, Germany.

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120 Starch Amylose and Amylopectin Molar Mass and Size Distributions by FFF-MALS
Rick White and Eija Chiaramonte, The Procter & Gamble Company
This practical application describes field-flow fractionation coupled to multi-angle light scattering (FFF-MALS) to separate AMY and AMP in order to calculate their mass ratio, determine molar mass distributions, the average molecular weights of AMY and AMP, and the z-average root-mean-square radius $R_{g,z}$ and polydispersity $M_w/M_n$ of the AMP component.

MEDICAL/BIOLOGICAL

121 SEC Separation of the ADC Brentuximab Vedotin from its Aggregates and Fragments
Daniel Eßer, YMC Europe GmbH
Drug-to-antibody ratios (DARs) of antibody-drug-conjugates (ADCs) are important for their therapeutic efficacy and pharmacokinetics. In this application note the ADC peak is separated from an aggregate and fragment peak using size-exclusion chromatography (SEC) in just one run.
FOOD & BEVERAGE

LC–MS/MS Analysis of Mycotoxins in Peanut Powder in 5.5 Min
Restek Corporation

• Fast analysis for higher sample throughput.
• Excellent separation improves accuracy for 12 mycotoxins.
• Quick and easy sample preparation (dilute-filter-shoot).

Certain fungi that can grow on agricultural products produce toxic metabolites known as mycotoxins. Modern food processing procedures cannot completely remove these compounds if they are present, so strict monitoring protocols have been established. Although a universal method for the analysis of mycotoxins would allow highly efficient screening, it is very challenging to develop such a method due to differences in physiochemical properties of mycotoxins, extraction efficiencies, and matrix effects. Zhang et al. published a multi-lab study (1) aimed at providing labs with an analytical procedure that could be broadly applied to the analysis of a variety of mycotoxins in many different matrices. Using that work as inspiration, we developed the following LC–MS/MS method that resolves Commission Regulation EC 1881/2006 regulated mycotoxins within the pressure limits of traditional HPLC instruments.

In this example, mycotoxins were analyzed in a peanut powder matrix. The use of a relatively short column format, the selectivity of the Biphenyl stationary phase, and the efficiency of 2.7 µm Raptor superficially porous particles provided excellent separations in a fast 5.5-min analysis (total cycle time of 7 min). A coeluting matrix compound that shared the most abundant MRM transition for mycotoxin HT-2 (447.3–285.3) was observed, so a less abundant transition (447.3–345.3) was selected for quantitation. To increase sensitivity, an ammonium buffer was used to promote better ionization of mycotoxins. The Raptor Biphenyl column worked very well for the 12 mycotoxins studied in the cited work, but for longer compound lists containing isobaric mycotoxins.

Table 1: Peak Identifications and Ion Transitions

<table>
<thead>
<tr>
<th>Peaks</th>
<th>tR (min)</th>
<th>Conc. (ng/g)</th>
<th>Precursor Ion</th>
<th>Product Ion 1</th>
<th>Product Ion 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Deoxynivalenol</td>
<td>0.62</td>
<td>50</td>
<td>297.3</td>
<td>249.3</td>
<td>231.2</td>
</tr>
<tr>
<td>2. Fumonisin B1</td>
<td>2.45</td>
<td>50</td>
<td>722.5</td>
<td>352.4</td>
<td>334.5</td>
</tr>
<tr>
<td>3. HT-2</td>
<td>2.60</td>
<td>50</td>
<td>447.3</td>
<td>345.3</td>
<td>285.3</td>
</tr>
<tr>
<td>4. Fumonisin B3</td>
<td>2.85</td>
<td>50</td>
<td>706.5</td>
<td>336.4</td>
<td>318.4</td>
</tr>
<tr>
<td>5. Fumonisin B2</td>
<td>3.23</td>
<td>50</td>
<td>706.5</td>
<td>336.3</td>
<td>141.2</td>
</tr>
<tr>
<td>6. T2</td>
<td>3.31</td>
<td>50</td>
<td>489.3</td>
<td>245.2</td>
<td>387.4</td>
</tr>
<tr>
<td>7. Aflatoxin G2</td>
<td>3.74</td>
<td>5</td>
<td>331.2</td>
<td>313.3</td>
<td>189.3</td>
</tr>
<tr>
<td>8. Zearalenone</td>
<td>3.96</td>
<td>50</td>
<td>319.3</td>
<td>283.3</td>
<td>187.2</td>
</tr>
<tr>
<td>9. Aflatoxin G1</td>
<td>4.22</td>
<td>5</td>
<td>329.2</td>
<td>243.2</td>
<td>200.2</td>
</tr>
<tr>
<td>10. Aflatoxin B2</td>
<td>4.43</td>
<td>5</td>
<td>315.3</td>
<td>287.3</td>
<td>259.2</td>
</tr>
<tr>
<td>11. Aflatoxin B1</td>
<td>4.99</td>
<td>5</td>
<td>313.3</td>
<td>285.2</td>
<td>241.2</td>
</tr>
<tr>
<td>12. Ochratoxin A</td>
<td>5.19</td>
<td>5</td>
<td>404.2</td>
<td>239.3</td>
<td>358.3</td>
</tr>
</tbody>
</table>
with similar structures, the Raptor FluoroPhenyl phase may be necessary to provide adequate chromatographic resolution. The selectivity of the Raptor Fluorophenyl column is demonstrated in an analysis of 20 mycotoxins that can be found by visiting www.restek.com and entering LC_FS0511 in the search.

This method showed excellent precision and accuracy for the 12 mycotoxins that were evaluated during a validation study that covered a variety of matrices (including multiple sources of cornmeal and brown rice flour, in addition to the peanut powder example shown here).

Restek would like to thank Dr. Zhang for his technical support during this project.

**Experimental**

**Column:** Raptor Biphenyl (cat.# 9309A52); Dimensions: 50 mm × 2.1 mm i.d. Particle Size: 2.7 µm; Pore Size: 90 Å; Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm i.d., 2.7 µm (cat.# 9309A0252); Temp.: 40 °C; Inj. Vol.: 5 µL.

**Mobile Phase:** A: Water, 2 mM ammonium formate, 0.1% formic acid; B: Methanol, 2 mM ammonium formate, 0.1% formic acid.

**Gradient (%B):** 0.00 min (30%), 0.6 min (30%); 0.7 min (50%); 3.00 min (70%); 4.5 min (75%); 5.0 min (90%); 5.2 min (90%); 5.21 min (75%); 6.00 min (75%); 6.01 min (30%); 7.00 min (30%)

**Flow:** 0.5 mL/min;

**Detector:** MS/MS; Ion Mode: ESI+; Mode: MRM.

**Instrument:** UHPLC

**Notes:** Weighed 1.00 gram of peanut powder in a 50 mL centrifuge tube and added 2.00 mL of water. Vortexed at 3000 rpm for 5 min followed by the addition of 4.0 mL of extraction solvent (50:50 water:acetonitrile, v/v). The tube was then vortexed at 3000 rpm for 5 min followed by centrifugation for 15 min at 4200 rpm. 475 µL of the supernatant was filtered through a Thomson SINGLE StEP Nano filter vial (0.2 µm, cat.# 25882). The sample was then fortified with 25 µL of a standard solution prepared in water at 1000 ng/mL (100 ng/mL for aflatoxins and ochratoxin A) as part of the matrix-matched calibration curve. Vortexed at 3000 rpm for 1 min prior to analysis.

**References**


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**Table 2: Raptor Biphenyl LC Columns (USP L11)**

<table>
<thead>
<tr>
<th>Length</th>
<th>2.1 mm cat.#</th>
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<th>4.6 mm cat.#</th>
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<tr>
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<tr>
<td>100 mm</td>
<td>9309212</td>
<td>930921E</td>
<td>—</td>
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<td>9309262</td>
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<td>2.7 µm Columns</td>
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<td>9309A3E</td>
<td>9309A35</td>
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**Table 3: Raptor FluoroPhenyl LC Columns (USP L43)**

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<tr>
<td>100 mm</td>
<td>9319212</td>
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<td>9319A5E</td>
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<td>250 mm</td>
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Starch Amylose and Amylopectin Molar Mass and Size Distributions by FFF-MALS

Rick White and Eija Chiaramonte, The Procter & Gamble Company

Starch contains large homopolymers of linear amylose (AMY) and branched amylopectin (AMP). With average radii in the hundreds of nm, and molecular weights ranging into the hundreds of millions, starch polymeric components cannot be separated by GPC. However, field-flow fractionation coupled to multi-angle light scattering (FFF-MALS) is well suited to starch analysis. FFF performs non-shearing separation by hydrodynamic size up to 1000 nm, and MALS analyzes absolute molar mass and size regardless of conformation.

This note demonstrated the application of FFF-MALS to separating AMY and AMP in order to calculate their mass ratio, determine molar mass distributions, the average molecular weights of AMY and AMP, and the z-average root-mean-square radius $R_{g,z}$ and polydispersity $M_w/M_n$ of the AMP component.

Materials and Methods
Separation was performed by an Eclipse® FFF system with short channel, a 350 µm spacer, and a 10 kDa cutoff regenerated cellulose membrane. Detection was accomplished with DAWN® 18-angle MALS and Optilab® RI detectors.

Results and Discussion
Figure 1 shows that FFF-RI fractograms separate into distinct peaks for amylose and amylopectin. The molar mass distributions determined for these samples ranged from 10 kDa to 1 GDa, though there is evidence for smaller starch components as well.

Table 1 summarizes the results of the analyses. Integration of the respective peak areas enabled calculation of the AMY:AMP ratios, in excellent agreement with the nominal values. The values for $M_w$ and $R_{g,z}$ fall within the generally accepted limits found in the literature.

Polymer conformation is assessed by comparing $R_g$ with molar mass. Conformational plots for the AMP component (not shown) indicate a log-log slope of 0.39–0.41 for all starches measured, verifying their branched nature.

Conclusions
Starch characterization by GPC can be limited due to shear degradation, filtration of high-molar-mass fractions, and the size range of GPC columns. The open-channel separation of FFF eliminates these limitations. The molar masses and mass ratios of AMY:AMP were accurately determined over five orders of magnitude. FFF-MALS fully addresses the multiple analytical challenges presented by starch.

Wyatt Technology Corp.
6330 Hollister Ave, Santa Barbara, California 93117, USA
Tel.: 1 (805) 608 9009
Website: www.wyatt.com

![Figure 1: FFF-MALS-RI fractograms for five native starches of varying AMY:AMP ratio with molar mass values overlaid.](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>AMY:AMP (nominal)</th>
<th>AMY:AMP (measured)</th>
<th>Amylose $M_w$ (g/mol)</th>
<th>Amylopectin $M_w$ (g/mol)</th>
<th>$R_{g,z}$ (nm)</th>
<th>$M_w/M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi-Maize</td>
<td>75:25</td>
<td>74:26</td>
<td>227,900</td>
<td>109,200,000</td>
<td>145</td>
<td>2.4</td>
</tr>
<tr>
<td>Hylon VII</td>
<td>75:25</td>
<td>77:23</td>
<td>204,000</td>
<td>94,340,000</td>
<td>158</td>
<td>1.9</td>
</tr>
<tr>
<td>Hylon V</td>
<td>55:45</td>
<td>56:44</td>
<td>367,000</td>
<td>251,100,000</td>
<td>245</td>
<td>2.5</td>
</tr>
<tr>
<td>Melo jel</td>
<td>25:75</td>
<td>26:74</td>
<td>419,000</td>
<td>418,500,500</td>
<td>316</td>
<td>2.9</td>
</tr>
<tr>
<td>Amioca</td>
<td>1:99</td>
<td>1:99</td>
<td>—</td>
<td>321,000,000</td>
<td>261</td>
<td>2.1</td>
</tr>
</tbody>
</table>
SEC Separation of the ADC Brentuximab Vedotin from its Aggregates and Fragments

Daniel Eßer, YMC Europe GmbH

Drug-to-antibody ratios (DAR) of antibody-drug-conjugates (ADCs) are important for their therapeutic efficacy and pharmacokinetics. Typically, the DAR of second generation ADC such as Brentuximab vedotin (Adcetris®) is determined by hydrophobic interaction chromatography (HIC).

Comparably crucial, fragmentation and aggregation of ADCs have to be monitored. Given that, it is important for quality control (QC) purposes to obtain a satisfying resolution of the high and low molecular weight range. If fragmentation and/or aggregation is of interest, size-exclusion chromatography (SEC) is the mode of choice.

In this application note the ADC peak is separated from an aggregate and fragment peak using a YMC-SEC MAB column. YMC-SEC MAB is designed to separate an antibody or, as in this case, an ADC from its aggregates and fragments in just one run. The column provides high resolutions for the high and low molecular weight range.

Two different mobile phase options are used, while the other parameters

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{skeletal_formula.png}
\caption{Skeletal formula of brentuximab vedotin (Adcetris®).}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{Table 1: Chromatographic conditions} & \\
\hline
\textbf{Column:} & YMC-SEC MAB (3 μm, 25 nm) 300 x 4.6 mm i.d. \\
\hline
\textbf{Part No.:} & DLM25S03-3046WT \\
\hline
\textbf{Eluent:} & 0.1 M phosphate buffer (pH = 7) cont. 0.2 M NaClO₄ \\
& 0.1 M phosphate buffer (pH = 7) cont. 0.2 M NaCl/2-propanol (85/15) \\
\hline
\textbf{Flow rate:} & 0.165 mL/min \\
\hline
\textbf{Temperature:} & 25 °C \\
\hline
\textbf{Detection:} & UV at 280 nm \\
\hline
\textbf{Injection:} & 4 μL (2.5 mg/mL) \\
\hline
\textbf{Sample:} & Brentuximab vedotin (Adcetris®) for injection \\
\hline
\end{tabular}
\end{table}
The first mobile phase is fully aqueous and consists of 0.1 M phosphate buffer at pH 7 containing 0.2 M sodium perchlorate referring to Wang et al. as recommended by the FDA. They stated that the use of perchlorate works better than other chaotropic salts because they found it more effective in overcoming protein-column interactions (1).

The other mobile phase consists of an aqueous mobile phase A, a neutral 0.1 M phosphate buffer containing 0.2 M sodium chloride, and an organic mobile phase B, 2-propanol. The ratio is 85/15. Both mobile phases were applied here and gave similar results with no significant differences in Rs between main ADC peak and aggregate as well as fragment peak.

Figure 2: SEC analysis of Brentuximab vedotin using different mobile phases containing different salts with or without organic solvent.

Reference

Acknowledgement
The author would like to acknowledge the work of Prof. S. Manabe, from Hoshi University, Tokyo/Tohoku University, Sendai Japan.
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- Sue Sisley, MD

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