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The Utility of a Cation Exchange Column for Successful Charge-Variant Analysis of PEGylated Proteins

Polyethylene glycol (PEG) is a biocompatible compound with extensive use in a wide variety of medical, biopharmaceutical, and industrial applications. It is a popular compound for modifying peptides, proteins, and other biologics through covalent-bond attachment for therapeutic drug applications. PEGylation is the process of attaching PEG chains to a protein drug to shield it from proteolytic enzymes and improve pharmacokinetic properties. However, analytically characterizing PEGylated proteins can be a complex process, and many different methodologies are used.

Protein drugs are PEGylated for many reasons. “PEG has a number of unique properties: It’s a water-soluble polymer, bioinert, and non-immunogenic,” said Athena Papasodero, associate scientist, analytical development at the Pelican Expression Technology™ business unit of Ligand® Pharmaceuticals Inc. “When PEG is conjugated to a protein, it can provide many positive advantages that the native molecule lacks.”

Papasodero explained it increases half-life, reducing dosage frequency and extending the circulation lifetime in the human body. And because PEG is hydrophilic, when it attaches to proteins and other biomolecules, it decreases aggregation and increases solubility. PEG can lower toxicity, immunogenicity, and antigenicity and can also improve stability and decrease degradation.15

PEG conjugation masks the protein surface and increases the molecular size of the protein. It is transparent and nonfluorescent and therefore non-detectable by itself.

Current Methodologies

Currently, there are three major techniques for determining charge-variant analysis of proteins: ion exchange (IEX) chromatography, imaged capillary isoelectric focusing (iCIEF), and capillary zone electrophoresis (CZE). All three high-resolution techniques separate biomolecules based on differences in the net surface charge and enable for charge-variant characterization of both PEGylated and non-PEGylated proteins.

According to a 2020 study, “iCIEF has become the current industry standard for isoelectric-point (pI) determination and charged-variant quantification of proteins and antibodies.”16
“Variants that can be separated and analyzed using IEX chromatography, iCIEF, and CZE include post-translational modifications such as glycosylation, as well as other modifications including deamidation and fragmentation of the protein,” Papasodero said. “For IEX chromatography, these types of separations are carried out using one of two gradient techniques: salt gradients or pH gradients.”

- Salt gradients have the advantage of historical use; therefore, they are more common and more familiar to scientists in the bioanalytical lab.

- pH gradients are a newer development and offer certain advantages, including the ability to be mass-spectrometry friendly when compared to salt gradients.

However, there are challenges with charge-variant analysis for PEGylated proteins. “The main challenge in characterizing charge variants of PEGylated proteins is the charge-shielding effect‘ in which the steric-bulk of the PEG residue hinders the electrostatic interactions between the amino acid residues of the protein and the charges surface of the stationary phase of the IEX column,” Papasodero explained. “For lysine conjugated PEGylated proteins, the net cationic charge of the protein also decreases as the number of PEGylated lysine residues increase, which can alter selectivity and hinder resolution.”

As researchers from the department of biologics process development at Bristol-Myers Squibb discovered, iCIEF separation of PEGylated proteins creates broad peaks. “Charged variants of PEGylated proteins merge into one broad peak during iCIEF, most likely due to the masking of proteins by the surrounding PEG chain and the increased hydrodynamic volume cause by PEGylation.”

Papasodero explained it also produces distorted peak shapes and modification of pharmalyte contents, methylcellulose concentration, protein concentration, focusing time, and other additives do not yield reasonable peak shapes for iCIEF methods.

The Experiment

To address these challenges, the Pelican Expression Technology team assessed the ability of the YMC’s BioPro IEX SF column to analyze PEGylated protein charge variants reproducibly.

“We developed a characterization method for PEGylated biotherapeutic proteins that can provide increased information without compromising peak shape and resolution,” Papasodero said, which, as seen above, are two factors that at times are sacrificed when characterizing PEGylated proteins.

The stability indicating method developed by the Pelican Expression Technology team created exceptional separation of charge-variant constituents using BioPro IEX SF. The method can enable others who have similar separation requirements to develop methodology from a proven and effective protocol.
Materials and Reagents

<table>
<thead>
<tr>
<th>Name</th>
<th>Brand</th>
<th>Catalog #</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride</td>
<td>JT Baker</td>
<td>3628</td>
<td>(≥99.0%), mw 58.44</td>
</tr>
<tr>
<td>MES Hydrate</td>
<td>Sigma Aldrich</td>
<td>PHG0003</td>
<td>195.24 (anhydrous basis)</td>
</tr>
<tr>
<td>Ammonium Hydroxide</td>
<td>Sigma Aldrich</td>
<td>338818</td>
<td>28% NH₃ in H₂O, ≥99.99% trace metals basis</td>
</tr>
</tbody>
</table>

Experimental Conditions

Sample Preparation: A sample of PEGylated protein was diluted to 10 mg/mL using Milli-Q water.

Degraded Sample Preparation: A sample of PEGylated protein was diluted to 10 mg/mL in formulation buffer. The sample was held at 40°C for 30 days.

Column Specifications: BioPro IEX SF; particle size 5 μm; column size length X.I.D. (mm) 100 x 4.6; flow rate 0.2 - 0.8 mL/min; pH range: 2 - 12; temp. range 4 - 60°C; column material PEEK.

Chromatographic Conditions

<table>
<thead>
<tr>
<th>LC System</th>
<th>Thermo Scientific Vanquish UPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>Vanquish VF-P20-A</td>
</tr>
<tr>
<td>Autosampler</td>
<td>Vanquish VF-A10-A</td>
</tr>
<tr>
<td>Column Compartement</td>
<td>Vanquish VH-C10-A</td>
</tr>
<tr>
<td>Pump</td>
<td>Vanquish VF-P20-A</td>
</tr>
<tr>
<td>Viper Fitting</td>
<td>MP35N 018 x 350 mm</td>
</tr>
<tr>
<td>Column</td>
<td>BioPro IEX SF 100 x 4.6 mm I.D. 5 μm P/N: SF00S05-1046WP</td>
</tr>
<tr>
<td>Mobile phase A*</td>
<td>20 mM MES, pH 5.9</td>
</tr>
<tr>
<td>Mobile Phase B*</td>
<td>25 mM MES, 0.5 M NaCl, pH 5.9</td>
</tr>
<tr>
<td>Column temp.</td>
<td>35 °C</td>
</tr>
<tr>
<td>UV Wavelength</td>
<td>280 nm (reference OFF/16nm-bandwidths)</td>
</tr>
</tbody>
</table>

* Use ammonium hydroxide to adjust pH

Gradient Table

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow [mL/min]</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.750</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
The Results

The effect of pH on charge variant analysis of a PEGylated protein is shown in Figure 1. The panels show UV traces at 280 nm for (A) full-sized chromatograms of PEGylated protein during the mobile phase pH screen and (B) expanded chromatograms of PEGylated protein during the mobile phase pH screen.

**Figure 1**

<table>
<thead>
<tr>
<th>pH</th>
<th>Retention Time</th>
<th>Peak area acidic variants (%)</th>
<th>Peak area main peak (%)</th>
<th>Peak area basic variants (%)</th>
<th>USP Resolution (Acidic-MP)</th>
<th>Peak-to-Valley Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>10.910</td>
<td>6.8</td>
<td>81.1</td>
<td>12.1</td>
<td>1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>5.9</td>
<td>10.387</td>
<td>7.2</td>
<td>85.7</td>
<td>7.1</td>
<td>1.4</td>
<td>3.7</td>
</tr>
<tr>
<td>6.0</td>
<td>10.253</td>
<td>7.0</td>
<td>86.1</td>
<td>6.9</td>
<td>1.6</td>
<td>5.1</td>
</tr>
<tr>
<td>6.1</td>
<td>10.160</td>
<td>7.1</td>
<td>86.8</td>
<td>6.1</td>
<td>1.8</td>
<td>5.7</td>
</tr>
</tbody>
</table>

Table 1 reports the sum of the acidic variants, the peak area percentage for the main peak (MP), and the sum of the basic variants. Resolution (R) between the acidic residues and main peak and the ratio of peak height to valley height (End p/v) for the main peak are also reported.

**Figure 2** displays the effect of pH on IEX of a PEGylated protein. **Chart A** shows the pH effects on the main peak area percentage. **Chart B** shows a heat map visualization of the method attributes: area percent of the acidic and basic charge variants, retention time, USP resolution between the major acidic peak and the main peak, and the peak-to-valley ratio between the major acidic peak and the main peak as a function of mobile phase pH.
In Figure 3, the IEX of a PEGylated protein characterized with the BioPro IEX SF column is shown. The panels show UV traces at 280 nm for (A) full-sized and expanded trace of mobile phase A blank, (B) full-sized and expanded trace PEGylated protein, and (C) full-sized and expanded trace degraded PEGylated protein. The optimized cation-exchange (CEX) high-performance liquid chromatography (HPLC) chromatogram for the PEGylated protein is shown in Figure 3B. One major acidic peak with a slight shoulder is observed in the pre-peak region of the chromatogram. Two small peaks are observed in the post main peak region. Six small basic peaks are eluting between 11-14 minutes, and one large basic peak eluting at 13.3 minutes. Over 100 injections were performed in a single run with no loss of resolution or peak shape of the PEGylated protein and charge variants. Figure 3C also shows the chromatogram following heat degradation of the PEGylated protein. The effects of heat degradation on the sample are clear: The dominant major acidic peak, the two smaller post main peaks, and the major basic peak increase following heat degradation.
Figure 4 shows peak-to-valley ratios used to judge peak separation for CEX-HPLC chromatograms of a PEGylated protein. The panels show UV traces at 280 nm for (A) an example showing a peak that is integrated as a main peak without baseline contact. It is separated by two neighboring peaks with the peak delimiters being on a valley, (B) expanded trace PEGylated protein, and (C) expanded trace degraded PEGylated protein.

Carryover from injection to injection can be a significant problem in PEGylated-protein analysis. As shown in Figure 5, the BioPro IEX SF column minimizes carryover: In the 118th injection using this column, there is minimal carryover. The percentage of carryover was calculated using this equation:

\[
\frac{\text{PEGylated protein area in blank injection for carryover}}{\text{PEGylated protein area in sample injection}} \times 100
\]
Conclusion

YMC’s BioPro IEX SF column provides a tool for the analysis and confirmation of PEGylated protein and PEGylated protein charge variants, increasing mobile phase pH results in earlier retention times. From visual inspection of the data, similar chromatographic profiles are visible; however, a buffer pH 5.9 results in the elution of two small acidic variant species not present when differing pH mobile phases were screened. The column also exhibited good retention time, area, and relative-area reproducibility.

“Conjugation of the PEG group to a protein increases the shielding effect, causing the protein to elute early before the salt or pH gradient begins,” Papasodero said. “Our results suggest the combination of BioPro IEX SF column and optimized salt and pH buffer compositions can enhance charge-variant characterization of PEGylated molecules. The difference in profiles between non-PEGylated and PEGylated variants of the protein can offer rapid insight into the degree of PEGylation during PEGylation optimization and development.”

Overall, this method is superior to current charge-variant analyses of PEGylated proteins because it can be easily replicated and yield repeatable results; it also has little carryover and the potential to be used as a platform method for charge-variant characterization of PEGylated proteins, lowering both cost and the development time required.

The BioPro IEX SF column provides an illuminating analytical approach for charge-variant drug characterization.

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Read more: https://bit.ly/3H6Hek0

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Development of New Centroiding Algorithms for HRMS
Researchers have developed two new algorithms capable of converting centroided data—generated during HRMS analysis—to mass peak profile data and vice versa.
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February Update
Our February cover story is focused on a novel mode of bioanalytical solid-phase extraction (SPE) that has been developed and used for a quantitative application involving an RNA oligonucleotide therapeutic candidate. Previously established protocols and media had shown little recovery, and so this new uncharted approach, based on hydrophilic interaction liquid chromatography (HILIC) with aminopropyl bonded phase on a silica support, was explored.

This month’s LC Troubleshooting looks at kinetic plots: what are they, how they can be constructed, and how they can be used in practice?

GC Connections continues to explore Golay’s equation for HETP (height equivalent to a theoretical plate). This instalment discusses the consequences, both good and challenging, of the Golay theory, its continuing relevance, and some alternatives.

In Column Watch we return to a very popular topic last featured in 2018: metal organic frameworks (MOFs). The large amount of research that has continued to be observed in the field is reviewed by Dave Bell, and he examines their use in liquid chromatography (LC).

Data integrity continues to be a major topic for regulated pharma laboratories. In Questions of Quality, we ask the question how static are static data? And have some regulatory data integrity guidance documents failed to comply with their own regulations?

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Mike Hennessy Jr,
President and CEO, MJH Life Sciences®
Biologics, such as peptides, proteins, and oligonucleotides, are more prevalent as therapeutic candidates than ever. In the exciting arena of oligonucleotide bioanalysis, there are well-known challenges when it comes to any quantitative platform including liquid chromatography–mass spectrometry (LC–MS). The author’s group has recently been focusing on creating quantitative bioanalytical approaches without the use of ion-pairing reagents, and has previously worked with hydrophilic interaction liquid chromatography (HILIC) preceded by polymeric-based weak cation exchange (WAX) solid-phase extraction (SPE) (1). This article focuses on the sample preparation and extraction aspect of a recently developed and unique bioanalytical SPE method that also does not require ion-pairing reagents and could possibly be useful for general applicability in analogous applications in the future. Despite the success of the polymeric WAX in the one referenced application (1), and another with a more laborious protocol (2), we found that for one particular application, for an RNA oligonucleotide known as GNV705 AS, we had to find and explore a new realm of selectivity for decent recovery (3). GNV705 AS is the 22-mer antisense strand of a duplex-based RNA therapeutic candidate. Along the GNV705 AS strand, there are numerous methylations and fluorinations, and towards each terminus there are phosphorothioate linkages, typical features for stabilization, and the central region is populated by phosphodiester linkages.

With protocols that were based on references (1) and (2), it was found that recoveries (even after altering the set of key variables in the SPE) were never in excess of 20–30% and, more importantly, were non-reproducible. In the context of oligonucleotide SPE, this ballpark of recovery is not unusually low (4,5), but we wanted as much as possible to achieve concrete method reproducibility and, ultimately, sensitivity. At this point, thoughts turned to producing something...
distinct on a physicochemical basis but still poised to exploit the innate excellent degree of polarity and ionization of the oligonucleotide analyte. This is even more prominent for RNA compared to DNA because the extra hydroxylation in the ribose moiety offers slightly more polarity.

Considering the pronounced polarity of the silica base, the clear established contribution that it has to HILIC in the LC context (6), and specifically how the highly polar silica chemistry aligns with nucleic acids’ intrinsic deep-rooted polarity, the fundamental affinity for the analyte on such a support should be prominent, in contrast to a polymeric support, which has much more of a hydrophobic constitution. This should be particularly valid if the silica support has a polar bonded phase with an appropriate electrostatic functionality.

In principle, such an idea can certainly work. This group’s own previous research from 2016 demonstrated an application where HILIC has been utilized within the SPE for a bioanalytical application for the therapeutic peptide bivalirudin in human plasma. (7) This produced a high-recovery, validated method.

There was also work reported recently (8) that employed SPE using unmodified silica and HILIC operation, but it was a dual extraction with several liquid–liquid treatment steps in precedence to SPE loading and the remainder of the extraction. This presents an undesirable laborious element and more sources of variability, even though the ultimate extract cleanliness could be excellent.

For the work described here, we aspired to a more high-throughput protocol where plasma is simply diluted in the appropriate aqueous diluent prior to loading, more aligned with classical SPE in the regulated pharma industry context.

The key feature was that this SPE would follow a HILIC scheme, with rudimentary polar and electrostatic interactions being harnessed.

**Experimental**

**Chemicals and Materials:** The reference material for the analyte GNV705 AS, as well as the N-1 and N-2 metabolite reference materials, were kindly gifted by Genevant Sciences.

Acetonitrile, concentrated phosphoric acid (85%), concentrated ammonium hydroxide (25%), concentrated formic acid, and ammonium formate were all used.
obtained from Sigma Aldrich and were LC–MS-grade, with the exception of phosphoric acid, which was ACS-grade.

Water was purified in-house with a Thermo Scientific Barnstead Nanopure purification system by reverse-osmosis filtration and subsequent deionization to a resistivity of 18.2 MΩ cm. Control cynomolgus plasma with K₂EDTA anticoagulant was obtained from BioIVT, including from six individual donors for differential tests and selectivity.

Calibration Standards and Quality Control Samples: The GNV705 AS primary solution and a candidate internal standard (IS) were prepared at 100 µM in 9:1 (v/v) water–acetonitrile. Calibration quality control (QC) sample spiking solutions and the IS spiking solution were prepared in 9:1 (v/v) water–acetonitrile. Calibration standards were prepared in 9:1 (v/v) water–acetonitrile in polypropylene tubes. Plasma was prepared in analogous polypropylene tubes at concentrations of 0.5, 2, 5, 10, 20, 50, 100, 200, 500, 1000, and 2000 nM for calibrant samples and at 0.5, 2, 5, 10, 20, 100, and 2000 nM for QC samples. Volumes of spiking solution were no more than 2% of the volume of plasma spiked.

Optimized Sample Preparation:

As a final optimized procedure, the solid-phase extraction of GNV705 AS from cynomolgus plasma was performed as follows. The SPE sorbent was United Chemical Technology NAX, 100 mg, an aminopropyl phase on a silica base, in 96-well format. The 96-well 1-mL round-bottom collection plates used to receive the final eluent were regular DNA LoBind polypropylene from Eppendorf. Each step where liquid was applied was allowed to pass through gravity alone. The time taken was three to four minutes for this to occur, corresponding to an adequately slow linear flow velocity conducive to best performance under the circumstances of high-energy ionic interactions being formed and broken.

The candidate IS, in 1:9 (v/v) acetonitrile–water at 2500 nM, was added in 20 µL aliquots to 100 µL plasma within 1.5 mL regular polypropylene tubes. This resulted in an internal standard concentration of 500 nM in matrix. Then, a 2-s vortex of each tube took place. This was followed by the addition of 700 µL 6% H₃PO₄ (aq.) to each sample and another vortex step. Sorbent conditioning involved the application of 1 mL 9:1 (v/v) acetonitrile–water at 2500 nM, was added in 20 µL aliquots to 100 µL plasma within 1.5 mL regular polypropylene tubes. This resulted in an internal standard concentration of 500 nM in matrix. Then, a 2-s vortex of each tube took place. This was followed by the addition of 700 µL 6% H₃PO₄ (aq.) to each sample and another vortex step. Sorbent conditioning involved the application of 1 mL 9:1 (v/v) acetonitrile–(2% formic acid [aq.]), and there was no subsequent equilibration step prior to loading the diluted plasma sample. The prepared samples (800 µL) were loaded onto the conditioned sorbent beds. This was followed by the application of a 1 mL wash with 9:1 (v/v) acetonitrile–(2% formic acid [aq.]). The next wash was with 1 mL 8:2 (v/v) acetonitrile–(2% ammonium hydroxide [aq.]). Analyte elution was affected by the application of 2 × 400 µL 2% ammonium hydroxide in (3:7 acetonitrile–water) into the 1-mL round-well 96-well collection plate.

Optimised loadings were evaporated under oxygen-free nitrogen at 40 °C and then reconstituted in 200 µL 4:6 (v/v) acetonitrile–0.05% ammonium hydroxide in 10 mM ammonium formate [aq.]. The block was then sealed, put on a plate shaker at 500 rpm for 10 min,
before being placed in the autosampler compartment at 10 °C awaiting injection.

**Liquid Chromatography:** The analytical column for the GNV705 AS quantitative method was a 2.1 × 50 mm, 1.7-μm Waters Acquity Premier UPLC BEH Amide. The LC front-end system was a classic Waters Acquity UPLC unit that included pump, degasser, autosampler, column heater, and mobile phase pre-heater. The autosampler compartment was maintained at 10 °C. Gradient elution was employed, with mobile phase components of 0.05% ammonium hydroxide in 10 mM ammonium formate (aq.), pH measured at 9.1, and acetonitrile, delivered at 0.45 mL/min and with mobile and stationary phases at 40 °C. For each gradient cycle, initially the mobile phase composition began at 20% aqueous, and the mobile phase composition underwent a linear excursion over the next 5.0 min to 50% aqueous. This composition was held for 0.5 min and then re-equilibration took place over the remaining 1.0 min of the 6.5 min overall runtime. The injection volume was 6.0 μL and partial loop with needle overfill (PLNO) mode was used, along with a strong wash composition of 0.05% ammonium hydroxide in 10 mM ammonium formate (aq.) and acetonitrile, delivered at 4.5 mL/min and with mobile and stationary phases at 40 °C. The peak area-based integration for the quantitative endpoint involved the internal standard, which elutes last.

**Mass Spectral Detection:** The high resolution mass spectrometer was a Sciex ZenoTOF 7600 System equipped with an Optiflow ion source and auxiliary gas heated to 550 °C, with no split of the LC flow into the source. A negative MRM acquisition was applied for quantification, where the precursor ion m/z target was set as 1832.3 and a MS/MS scan range was 100–2000 m/z. The extracted ion chromatograms (XICs) of transitions 1832 → 586.1134, 1832 → 664.0810, and 1832 → 604.1199, where all values denote m/z, were generated using Sciex OS software with the extraction window width set at 0.02 Da. The precursor ion involved was the same as for nominal mass, corresponding to the charge state of [M-4H]+. The selected product ions were analogous to the nominal mass scheme. Again, these peaks were selected on the basis of most compelling intensity and verifiability. The peak area-based integration for the quantitative endpoint involved the summation of the peaks in each transition.

**Results and Discussion**

**HOLIC:** To dwell briefly on the chromatography, the HILIC nature of the dynamics was a critical component of the methodology in terms of the requirement for no ion-pairing reagents to be present. The setup just described represents the bones of what we have assigned the moniker “HOLIC” (3), a play on the HILIC abbreviation interfaced with the term “oligo”. We felt it was merited particularly on the basis of observed applicability to a wide range of different oligonucleotide methods as performed in our laboratory (data not shown). A representative chromatogram is shown in Figure 1. The baseline resolution of the analyte GNV705 AS from the earlier-eluting N-1 and N-2 metabolite pair is evident, and the similar resolution from the internal standard, which elutes last.

**Hydrophilic-Phase Extraction:** The extraction is clearly the centrepiece of this article. In the previous SPE work of fundamentally similar nature reported for the therapeutic peptide bivalirudin (7), HILIC was proven to be the critical functional mode throughout the bulk of the SPE process, and for this new oligonucleotide application, the anticipation and outcome were no different. This time, instead of the cation exchanger used for the peptide capture and subsequent release, an aminopropyl function was used, appropriate for the negative charge strongly and abundantly present with oligonucleotides. All things considered, at the pivotal load step it was ideal for the most efficient capture. In the absence of the classical partitioning HILIC model because of the high aqueous content—in terms of the fundamental physicochemical affinity as alluded to earlier—the profound polarity of the silica support in conjunction with the short-chain charged bonded phase matched the acute polarity of the RNA analyte.
The initial attempts using a 100 mg bed weight of the aminopropyl phase on a silica base brought improved recoveries from what we had seen at any point prior in this project. From then, it was a straightforward series of experiments to fully optimize the procedure.

For the 100 mg packed sorbent, a procedure prior to elution, and the sample load notwithstanding, based on 1 mL solvent application at each step was appropriate. In all steps, a minimum 10% aqueous was maintained to preserve HILIC conditions and avoid oligonucleotide precipitation via lack of organic solubility that may lead to loss of oligonucleotide precipitation via lack of electrostatic bonds. In the absence of enough neutralization, low energy electrostatic bonds are forming, albeit involving a little more neutralization of the phosphate backbone and accordingly more nonpolar interactions, low energy thus rapidly manifesting, a phenomenon that we had used in previous work (1) to better align with the low plateau of pH attained by phosphoric acid with increasing concentration. This did appear to make a difference for the first few moments where the high-energy electrostatic bonds are forming, albeit involving a little more neutralization of the phosphate backbone and accordingly more nonpolar interactions, low energy electrostatic bonds are forming, albeit involving a little more neutralization of the phosphate backbone and accordingly more nonpolar interactions.

The single condition was therefore identical to the condition—again the high “oligo-phobic” organic helping to prevent analyte breakthrough.

For the plasma sample dilution and loading, initially it was decided to use 6% phosphoric acid (aq.) as the diluent, a little more concentrated than what we had used in previous work (1) to better align with the low plateau of pH attained by phosphoric acid with increasing concentration. This did appear to make a difference for the first few moments where the high-energy electrostatic bonds are forming, albeit involving a little more neutralization of the phosphate backbone and accordingly more nonpolar interactions, low energy electrostatic bonds are forming, albeit involving a little more neutralization of the phosphate backbone and accordingly more nonpolar interactions.

The wash after the sample load was identical to the condition—again the high “oligo-phobic” organic helping to prevent analyte breakthrough.

TABLE 1: Inter-assay data. Adapted and reproduced from reference 3.

<table>
<thead>
<tr>
<th>Nominal concentration (nM)</th>
<th>0.5</th>
<th>2</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>100</th>
<th>2000</th>
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</thead>
<tbody>
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<td>4.81</td>
<td>10.4</td>
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<tr>
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<td>10.2</td>
<td>15.7</td>
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<td>10.4</td>
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<tr>
<td>RE (%)</td>
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<td>-10.0</td>
<td>-3.90</td>
<td>3.56</td>
<td>6.35</td>
<td>12.7</td>
<td>9.03</td>
</tr>
</tbody>
</table>

The wash after the sample load was identical to the condition—again the high “oligo-phobic” organic helping to prevent analyte breakthrough. The wash after the sample load was identical to the condition—again the high “oligo-phobic” organic helping to prevent analyte breakthrough.

As mentioned, the first wash was identical to the single-step condition, 1 mL of 9:1 (v/v) acetonitrile–[2% formic acid (aq.)]. The following wash immediately prior to elution was with 1 mL 8:2 (v/v) acetonitrile–[2% ammonium hydroxide (aq.)]. This introduced a little eluotropicity by increasing in aqueous content and featuring a slightly alkaline pH; without enough alkalinity to neutralize the key ionic moiety in the bonded phase, particularly in the high-acetonitrile conditions, retention persisted. Elution was applied, with 2% ammonium hydroxide in [3:7 acetonitrile–water], highly aqueous to fully solubilize the analyte, and sufficiently alkaline, with a pH of 11.5 as measured in the aqueous component, to neutralize the sorbent. For the 100 mg packed bed, we anticipated a void volume of 150 μL and thus we included a test with 200 μL total volume. This was not enough to effect full elution. Only once 2–3 void volumes had passed—a total of 400 μL—did we see the full response corresponding to the elution of what remains on-cartridge. To err on the side of caution, and with knowledge of how incurred samples can differ quite profoundly in their natural matrix.

The single condition was therefore 1 mL of 9:1 (v/v) acetonitrile–[2% formic acid (aq.)], with the acidity maintaining the ionized state of the key ionic moiety of the bonded phase.
The performance data are impressive, compositions, we decided to establish the final elution volume as 800 µL total to afford sufficient eluting power in unusual conditions that may demand it. Decisively, in all the available numerical detail over the low QC (LQC) and high QC (HQC) nominal levels, the recovery for GNV705 AS was above 60% and the candidate internal standard was in the same region as calculated over the replicate set at the associated one nominal level. Specifically, the analyte recovery was 64.1% and 62.0% at the LQC and HQC levels, respectively, and 57.4% for the internal standard.

It may be speculated that a marriage between a HILIC-based sample extraction and a HILIC LC–MS analytical endpoint may not result in clear orthogonality of selectivity between these two methodological components. However, we believe that this ever-desirable feature is actually largely preserved because of the details in each case. Primarily, the chemistry of the stationary support was different between the analytical column and the solid-phase packed cartridges—amide on hybrid polymer-silica compared to aminopropyl on silica. The extraction used a comprehensive wash scheme involving both acidic and alkaline applications prior to elution, which is almost unique apart from the portfolio of our own work (1,3,7), and will have a large bearing on selectivity and the elimination or resolution of interferences. The performance data are impressive, underlining the adequate selectivity.

The final HPE schematic is shown in Figure 3. It is not difficult to see that we have a liking for monikers in this group, and for this we have used “hydrophilic phase extraction” (HPE), since it can be none other than hydrophilic interactions manifesting at the key stages of the process, for the reasons outlined. Inter-Day Analysis: To demonstrate quantitative method ruggedness, three inter-day analytical batches were performed. From these, the bias and precision ranged from -12.0% to 12.7%, and 6.54% to 25.0%, respectively, over the calibration range of 500 pM to 2000 nM. The inter-day performance data are shown in Table 1.

Outlook: Over the decades, silica-based SPE has become less popular in regulated bioanalysis, while the popularity of polymeric products has risen accordingly. However, particularly with the importance of SPE for reliable quantification of biologics, this work may help reopen the door for the silica base in such applications, with sound underlying chemistry-based reasons. Silica may find a niche to be used—in conjunction with polar and ionizable bonded phases—in HILIC mode, distanced from reversed-phase operation, which has dominated the history of silica in bioanalytical application. This could apply to all biologic-based analytes with an LC–MS bioanalytical endpoint, in light of the accompanying polarity at hand.

Conclusion Within the development of a quantitative bioanalytical LC–MS method for an antisense RNA oligonucleotide, and within the valuable theme of maintaining a complete absence of ion-pairing reagents, a novel mode of SPE has been developed and used for this, and the basic framework likely has a great deal of general utility. It is distinct from regular means of bioanalytical SPE in that HILIC is principally manifest and governs the band migration along with ion-exchange via the aminopropyl functionality. Only one condition step was used and, after sample load, a double-wash regime was applied at both low and high pH, thereby stepping up the aqueous and the basicity to finally effect elution. We have given the extraction the moniker “HPE”, hydrophilic-phase extraction. The method was proven to be rugged and reliable over a sequence of analyses using an analytical endpoint featuring our default HILIC setup, “HOLIC”, interfaced with the latest in accurate mass detection.

References
2) “Oligonucleotide purification products: OTX phase detail”: https://www.phenomenex.com/Products/Detail/Clarity%20(SPE)/OTX

Robert MacNeill is Head of Method Development at LabCorp Drug Development. He received his bachelor's degree with honours in chemistry from Heriot-Watt University, and his M.Sc. in analytical chemistry from the University of Huddersfield, UK. Robert is also a Chartered Chemist and Fellow of the Royal Society of Chemistry, and has 24 years of experience in all aspects of quantitative bioanalytical LC–MS method development—with 15 of these spent heading method development activities within LabCorp Drug Development at the Somerset (New Jersey, USA) site. Taking a prominent role in innovation projects with related R&D activities, Robert has been an active contributor to the scientific literature for many years.
Choosing a liquid chromatography (LC) column for a particular application can be a surprisingly challenging task. On the one hand, column manufacturers give us many options to choose from, including particle types, pore sizes, particle sizes, and different lengths and diameters. On the other hand, we usually do not have time to experimentally evaluate many combinations of these parameters, and sometimes we end up picking something similar to the columns that are already in the drawer. The “kinetic plot” is a powerful graphical tool that can help leverage the best available theory to help us understand how different combinations of parameters (such as particle size and length) will perform in relation to the time needed to get to a particular column efficiency (and thus resolution), and therefore make well-informed decisions when choosing columns.

Fifty years into the evolution of modern liquid chromatography (LC) we are still observing remarkable growth in the commercial offerings of LC columns. Each year, we see introductions of columns prepared with superficially porous particles (SPPs) from more manufacturers, new column formats (such as pillar array columns), more particle types suitable for bioanalysis (such as wide pore particles), and so on. From an analyst’s perspective, these developments are wonderful because they give us more choices, and increasingly they give us options that are designed with specific applications in mind (such as columns sold specifically for the purpose of characterizing monoclonal antibodies [mAbs]). However, having all these options to choose from can also make purchasing decisions complicated. How do we know which column is the “right” one? Of course, with purchase prices in the $500–$1000 range, trying a large number of them is simply not realistic. In the worst-case scenario, making the wrong choice can lead to real disappointment and sunk costs. If we choose the wrong combination of particle size and column length and diameter and pore size for a particular application, we may find that the performance we observe (as measured by analysis time, resolution, and sensitivity) is not as good as we expected, or perhaps even worse than what we were already using.

The analysis time–resolution compromise is one of the most studied topics in the history of LC research. Although the issue may have been considered settled in the late 1990s, new developments over the last 20 years, including the use of pressures greater than 400 bar, particles smaller than three micrometres, new stationary phase supports, and the surge in interest in the analysis of biopharmaceuticals, are keeping things interesting (1,2). One very powerful framework that is used to understand how all these factors influence what columns we should use for which applications is the “kinetic plot”. For this first installment in a multipart series on the construction and use of these plots in practice, I’ve asked kinetic plot expert Professor Ken Broeckhoven to join me in describing what a kinetic plot is and how it relates to the practical
factors we care about most in LC—analysis time and resolution. In subsequent instalments, we will go on to show how these plots can be constructed from experimental data of your own, or data accessible to you, and finally how the resulting plots can be used to guide decision making when choosing columns, and help troubleshoot situations where a particular column does not deliver the performance expected based on intuition and community conversations.

Dwight Stoll

Analysis Time and Resolution Are Connected

A primary goal of most analytical separations is to achieve a certain resolution between one or more pairs of compounds in the shortest possible analysis time. More-efficient columns enable realization of this resolution in shorter times. Column efficiency (plate number, $N$) as defined in equation 1 makes the connection between plate number and peak width ($w_{1/2}$) clear:

$$N = 5.54 \left( \frac{t_R}{w_{1/2}} \right)^2 \quad [1]$$

If we compare two separations where an analyte of interest is eluted at the same retention time ($t_R$), the separation with the higher efficiency will have narrower peaks because of the inverse relationship between $N$ and $w_{1/2}$. Because resolution is also inversely related to peak width, higher plate numbers lead to higher resolution. As soon as we start discussing analysis time, the mobile phase flow rate matters: Higher flow rates lead to shorter analysis times, and vice versa. However, the plate number also depends on the flow rate, so optimizing a separation is not as simple as using the highest possible flow rate. The conventional framework used to relate the plate height and flow rate involves the concept of the plate height ($H$), or height equivalent to a theoretical plate (HETP), which is related to the column length ($L$) and plate number through equation 2:

$$H = \frac{L}{N} \quad [2]$$

In rough terms, the plate height is a property of the material inside the column, but is also affected by the mobile-phase flow rate and the properties of the analyte. Increasing the diameter of the particles inside the column generally leads to larger plate heights and lower efficiencies, for a given column length. The relationship between the plate height and the mobile-phase flow rate has the characteristic shape shown in Figure 1(a), and is most commonly referred to as a van Deemter curve (3,4). Although for real separations we are mostly interested in the mobile-phase flow rate, van Deemter curves are usually plotted with mobile-phase velocity on the x-axis because the velocity is independent of the column diameter, and thus allows a comparison of the curves constructed from data obtained using different columns. The most valuable takeaway from the van Deemter curve is the realization that the smallest plate heights—and thus highest plate number and resolution—are obtained at intermediate velocities (not too low and not too high). However, the mobile-phase flow rate that produces the optimal (minimum) plate height—and thus the maximum plate number and resolution—is not necessarily the best flow rate in practice because analysis time is so heavily dependent on the flow rate.

It is instructive to examine the chromatograms that we would observe in experiments conducted with flow rates corresponding to three characteristic points on
These three points are highlighted in Figure 1(a), and the corresponding chromatograms are shown in panels (c–e) (full timescale) and (f–h) (zoomed view). These chromatograms make clear the practicality relevant trade-offs that are encoded in the van Deemter plot, but can be difficult to appreciate without a lot of experience working with these plots. We call your attention to three main points:

1. Working at flow rates below the optimum flow rate (where $H$ is at its minimum) is almost never a good idea. Under these conditions the analysis will take longer than necessary—compare panel (c) to (d) and (e)—and the resolution will be poorer than what can be obtained at the optimum flow rate.

2. The highest resolution is obtained by working at the optimum flow rate, which is clear in panels (f–h) where we see that the valley between the peaks is greatest in panel (g).

3. Much shorter analysis times can be realized by working at flow rates above the optimum, so long as some resolution can be sacrificed in the interest of a faster analysis. This trade-off, and the extent to which this game can be played with real separations, strongly depends on how steep the $H$ vs. $u$ curve is in the C-term regime. This slope strongly depends on particle size (slope generally increases with particle size, see Figure 1[a]), and the properties of the analyte (slope generally increases with molecular weight).

At this point, we encounter a significant limitation of the use of van Deemter curves alone for optimizing a separation—the plot does not explicitly communicate anything about the pressures required to realize chromatograms like those shown in Figure 1(c–h).

The significance of this limitation is made dramatically clear in Figure 1(b), which shows the pressures required to produce the data points plotted in Figure 1(a). For example, it is true that a 100-mm column packed with 1.7-µm particles will yield a plate number at the optimum flow rate that is roughly three times higher (29,400 compared to 10,000) than that for a column of the same length packed with 5-µm particles, and it will do so in one-third the time (optimum
velocity of 6 mm/s compared to 2 mm/s). However, this improvement in plate number and speed comes at the cost of a significantly higher pressure requirement for the smaller particles. The pressure at the optimum for the 1.7-µm particles is 800 bar, whereas it is only 33 bar for the 5-µm particles. We also see here that one can also achieve a plate number of about 29,000 using a 20-cm long column packed with 3.5-µm particles at the optimum velocity, which would require a longer analysis time (roughly four times longer) compared to the column with 1.7-µm particles, but only one-fourth the pressure (200 compared to 800 bar). The van Deemter plot alone cannot help us manage the compromise between resolution (plate number), analysis time, and pressure requirements. However, the kinetic plot can help with this, and this is why it is so useful as a conceptual framework for optimizing separations and choosing columns.

**Introduction to the Kinetic Plot Concept**

Although many variants of the kinetic plot have been introduced and discussed over the years, a kinetic plot, in principle, is any method of presenting data that relates plate number to analysis time (5,6). This type of representation is not new and dates back to some of the early publications of Giddings (7,8). Figure 2 shows the relationship between a van Deemter curve and a kinetic plot. To go from the van Deemter curve in panel (a) to the first type of kinetic plot in panel (b), the y-axis is transformed from $H$ to $N$ using equation 2. The x-axis is transformed from mobile phase velocity ($u_0$) to column dead time ($t_0$) using equation 3:

$$t_0 = \frac{L}{u_0} \quad [3]$$

The kinetic plot in panel B makes the implications of the relationship between $H$ and $u_0$ for analysis time clear. Moving to the right on the plot—which corresponds to the $B$-term regime discussed above—leads to longer analysis times and poor efficiencies, which is not good. On the other hand, moving to the left of the maximum in the curve leads to much shorter analysis times, albeit at a slight cost of reduced plate number. The kinetic plot in Figure 2(c) is a simple transformation of the plot in panel B. The axes are reversed, and each axis is presented on a logarithmic scale, which helps visualize the relationship between $t_0$ and $N$ over a time range that spans from a few seconds to tens of minutes.

**Adding the Dimension of Pressure to the Kinetic Plot**

The kinetic plot in Figure 2(c) is sometimes referred to as a “fixed length kinetic plot”. Although it does make explicit the connection between analysis time and plate number, it does not make clear how the pressure limitations of a particular LC system (or column type) influence column selection. The fixed length plot can be extended as shown in Figure 3 by adding multiple curves, each representing a different column length. On each curve, moving from the top of the plot towards the bottom corresponds to an increase in flow rate until the maximum pressure of the system is reached, where the curve terminates in “X”, and intersects the grey line. This grey line represents the “kinetic performance limit”, which defines the maximum achievable plate number for a given analysis time, or the shortest time needed to obtain a given plate number, depending on your perspective. Such families of curves can yield very instructive insights related to column selection. Consider the vertical dashed black line that intersects the curves for the 10-, 15-, and 25-cm columns. This view makes it immediately clear that there are multiple ways to obtain a plate number of 10,000 using...
columns of different lengths, but all packed with 3.5-µm particles.

The most time-inefficient way is to use a 25-cm column and operate it at low flow rate. In this example, the column dead time would be approximately 15 min (intersection of the dashed vertical line and the green curve), which is in spite of the fact that we generally think of long columns as being more efficient. Although it is true that longer columns have the potential to produce larger plate numbers, this potential can only be realized when the column is operated the “right” way. The next best option out of those shown here is to use the 15-cm column, which would produce 10,000 plates with a dead time of about 4 min. By far, the best way is to use the 10-cm column, operated at a velocity above the optimum, where the dead time would be about 10 s, a 90-fold improvement over the use of the 25-cm column at a low flow rate. Another way to look at this is to fix the analysis time at 0.9 min (horizontal black dashed line). Using a 10-cm column, this corresponds to only a little more than 10,000 plates are obtained (Note that by operating the same column length in the C-term, the same efficiency is reached in only 10 s, as shown for the vertical line). Operating a 15-cm column at the optimum velocity, a much higher efficiency of 21,600 plates is obtained, clearly making it a better choice. However, when operating a 25-cm long column slightly above its optimum flow rate, even 33,000 plates can be obtained in 0.9 min.

Summary

This installment of “LC Troubleshooting” is the first in a multipart series aimed at providing a framework for troubleshooting separations that don’t perform as expected based on perceptions about the influence of variables including column length, particle size, and flow rate on column efficiency and resolution. The van Deemter curve—in spite of its frequent use for relating plate height and mobile-phase velocity—does not explicitly indicate the trade-off between analysis time and plate number (and thus resolution).

The kinetic plot is a graphical tool that does make this relationship clear, and can also incorporate practical limits on the use of certain columns based in the pressure limitations of a LC system. In subsequent instalments in this series, we will explain how you can create your own kinetic plots using your data, or data from the literature, and then use these plots to guide decision making when choosing columns, and troubleshoot situations where the performance of a column in use does not seem to be as high as expected. Readers interested in a more comprehensive discussion of kinetic plots and related optimization tools are referred to several recent papers in this area (9–12).

References


ABOUT THE CO-AUTHOR

Ken Broeckhoven received his PhD in 2010 from the Vrije Universiteit Brussel (VUB), in Brussels, Belgium. Following postdoctoral research at VUB and work as a visiting researcher in the separation processes laboratory at ETH Zurich in Switzerland, he became a research professor at VUB in 2012. He was subsequently promoted to Assistant Professor and then to his current position as Associate Professor in 2017.

ABOUT THE COLUMN EDITOR

Dwight R. Stoll is the editor of “LC Troubleshooting.” Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota, USA. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 75 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC’s editorial advisory board. Direct correspondence to: amatheson@mjhlifesciences.com
Is Golay’s Famous Equation for HETP Still Relevant in Capillary Gas Chromatography? Part 2: Assumptions and Consequences

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

This instalment is the second part of a discussion on the origins and uses for determining the height equivalent of a theoretical plate (HETP) calculations in gas chromatography (GC). This month, we discuss the good and challenging consequences of the Golay theory, its relevance, and some alternatives. Many of the variables that chromatographers view as independent have some interdependence, and we see several limitations of the theory. The complex relationships between HETP and temperature present a particular challenge when thinking about temperature programmed GC compared to isothermal GC. This column concludes with comments regarding the continued relevance of this classical theory.

In the January 2022 instalment, we examined Golay’s famous equation describing the height equivalent to a theoretical plate (H or HETP), one of the most common measures of capillary column performance (1). The equation originally derived by Golay is shown below in equation 1. A commonly used simplified form, which was developed sometime later, is likely a misinterpretation of Golay (2). As in last month’s column, equation 31a from Golay’s paper is used here.

\[ du = \left( \frac{2D}{v_0} + \frac{(1+6k+11k^2)v_0^2}{24(1+x)cD_i} + \frac{k^2v_0^2}{6(1+x)c^2D_i} \right) dx \] \[ \text{HETP} = \frac{B}{v_0} + C_n v_0 + C_i v_0 \] \[ \text{[1]} \]

Definitions of the variables are discussed in detail in last month’s instalment. As we concluded last month, the many variables in equation 1 are themselves interdependent in ways that are not always obvious and can cause confusion for chromatographers in understanding and using the equation to assist in column and method optimization. Next, we ask three key questions about equations 1 and 2 that lead to comments about the use and relevance of the two equations.

1. Is the average carrier gas velocity independent of the other variables?
2. What is the effect of the polynomial expressions of the retention factor \( k \) on the mass transfer terms?
3. What about temperature and the relevance to temperature programming?

Figure 1 shows a simplified “Golay plot” of the form seen in countless textbooks on analytical chemistry, chromatography, and in nearly every course on gas chromatography (GC) that discusses theories. In experimental work, such plots are often used to evaluate new columns and methods, to compare experimental conditions, or to make a claim that a column is performing adequately. In this column, we use the three above-mentioned questions to critique this plot to better understand why the literature includes many experiments that do not appear to follow this behaviour, and to answer our larger question of the relevance of Golay’s equation and these plots in today’s capillary GC. Interestingly, the version of equation 2 presented in Figure 1, which seems to be the most common presentation, has the two mass transfer terms reversed in order from Golay’s original equation.

Is the Average Carrier Gas Velocity Really Independent of the Other Variables?

In making plots as seen in Figure 1, \( H \) is plotted against the average carrier...
gas velocity ($u$ or $v_0$) as seen in equation 2, and the expected shape of the plot as the carrier gas velocity increases is seen in Figure 1. Although this behaviour is seen in many cases, it is not universal. The first example of this is seen in the same initial symposium where Golay presented and discussed equation 1, following a paper by Dujkstra and de Goey. Sjenitzer commented on this paper and showed a plot of the value of the polynomial term in the mobile phase mass transfer term of Golay’s equation versus retention factor.

An example challenge with using the simplified form shown in equation 2 is that the average carrier gas velocity influences the other variables, most notably the retention factor, $k$, which appears in both mass transfer terms. We are reminded of the definitions of $k$ and its relationship to the gas hold-up time, $t_u$, and the average linear gas velocity, $v_0$, in equation 3:

$$k = \frac{t_M - t_u}{t_u} = \frac{K}{B} \text{ and } v_0 = \frac{L}{t_u} \quad [3]$$

Both the average carrier gas velocity and $k$ are calculated in practice from $t_u$, which itself is a complex function of carrier gas properties, column dimensions, and the inlet and outlet pressures (4,5). We see that $k$ and $t_u$ are closely related; therefore, $k$ and $v_0$ are also closely related. As a result, the use of $v_0$ in equation 2, as a variable independent of the other terms in the more detailed equation 1, is clearly an oversimplification that may cause confusion.

Since the average carrier gas velocity itself is a derived quantity, a complex function of column dimensions, identity of the carrier gas, and the inlet and outlet pressures, and those same factors also influence the retention factor and the gas phase diffusion coefficient ($D$), we see that the relationship between $H$ and $v_0$ is much more complex than implied by equation 2.

### What is the Effect of the Polynomial Expressions of the Retention Factor $k$ on the Mass Transfer Term?

The interesting polynomial functions of $k$, seen on the two mass transfer terms, are often overlooked as impacting the determination of $H$ and the resulting determination of $N$, the number of theoretical plates. We know that $k$ is analyte-, temperature-, and stationary-phase dependent, yet plates and plates per metre are often reported with little specifics. As an example, Figure 2 shows the dependence of the polynomial function of $k$, with $k$ ranging from 1 to 20, as seen in the mobile phase mass transfer term in equation 1.

Note in Figure 2 that the result varies considerably with retention factor with a low of approximately 3 for $k = 0.5$ to approximately 10 for $k = 10–20$. A larger $k$, which means longer retention times, results in the function roughly approaching a constant, indicating little influence of the retention factor on $H$.

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**FIGURE 1:** Simplified form of Golay’s equation with the expected form of a plot of $H$ versus carrier gas velocity. Reprinted with permission from ChromAcademy, LCGC’s online learning platform (accessed January 2022).

**FIGURE 2:** Plot of the value of the polynomial term in the mobile phase mass transfer term of Golay’s equation versus retention factor.

<table>
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<tr>
<th>Linear velocity (cm/s)</th>
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<table>
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<th>Retention factor ($k$)</th>
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</table>

**Golay Equation**

$$HETP = \frac{B}{u} + (C_s + C_m)u$$

- $C_s$: mass transfer in the stationary phase
- $C_m$: mass transfer in the mobile phase
- $HETP$: HETP
- $u$: linear gas velocity
- $B$: term of Golay’s equation versus retention factor.
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and by extension \( N \). As \( k \) gets smaller, the polynomial term also gets smaller with greater influence seen at smaller \( k \).

There are several practical consequences of this result. First, because \( k \) is analyte dependent, each analyte sees a different result for the polynomial and, therefore, a different \( H \) and \( N \). Often, \( N \) is quoted for a particular column, but this must be stated with the caveat that it was determined for a specific analyte under a specific set of instrumental conditions. In Figure 1, we see that mobile phase mass transfer is often the dominant term in determining \( H \) and \( N \). In Figure 2, we see that \( k \), on which the mobile phase mass transfer term depends, may vary by as much as a factor of three, depending on experimental conditions. Therefore, the actual number of theoretical plates experienced by an analyte in practice may vary significantly from the number reported for the column, and \( N \) can be expected to vary significantly from analyte to analyte and condition to condition.

A similar impact is seen in the \( k \)-related terms in the stationary phase mass transfer term. This term also has a polynomial function of \( k \) and, largely because of the presence of the partition coefficient in the denominator, generally has a smaller impact on \( H \) and \( N \), which is seen in Figure 1. When the retention factor term and the partition coefficient are combined using equation 3, the result is a polynomial term that ranges from 0.2 at \( k = 0.5 \) to approximately 0.04 at \( k = 20 \).

**What About Temperature and the Relevance to Temperature Programming?**

When van Deemter and Golay developed the theory of band broadening in the 1950s, nearly all practical GC was done using packed columns and isothermal analysis. Both equations 1 and 2 assume constant temperature, so this is another variable that must be controlled if experiments are to be comparable. Prior to the development of fused-silica capillary columns in the late 1970s, capillary GC was seen as more of a novelty than a technique for routine analysis. Today, almost all laboratory gas chromatographs are capable of temperature programming and most capillary column-based methods are temperature programmed. As discussed in an earlier “GC Connections” instalment, the peaks in a temperature-programmed separation do not broaden as rapidly as they do in an isothermal separation. In that article, I raised the question, “Why are all those peaks so sharp?” (6).

Equation 1, as it assumes constant temperature, does not fully explain this, but the concepts seen in the three terms and the variables can provide a qualitative discussion.

Equation 1 provides some qualitative insight about why the peaks in temperature programming appear sharper than in an isothermal analysis with similar retention times. The sharp peaks indicate that the rate of band broadening in temperature programming must be much smaller (slower) than in isothermal analysis. As seen in Figure 1, the mobile phase diffusion term is relatively low and nearly constant at most practical carrier gas velocities and temperatures, so the main impact on \( H \) would be seen in the two mass transfer terms. We also have seen that the mobile phase mass transfer term has the greater impact on \( H \). Higher temperatures reduce \( k \), which diminishes the value of the polynomial and increases \( D \), reducing the overall value of the mobile phase term. Therefore, the mobile phase mass transfer term lowers \( H \) and leads to a reduction in the rate of band broadening and sharper peaks.

**Is Golay’s Equation Still Relevant?**

Golay’s equation, shown in equation 1, that was developed in the 1950s is still relevant today, although the context in which we use and think about it has changed. With most methods employing temperature programming, the practical utility of Golay plots and plate height calculations today is limited. The need for the exact matching of conditions, such as temperature, carrier gas, flow rate, and analyte, make column-to-column comparisons difficult, and the requirement for constant temperature makes conclusions about column performance based on these plots not readily translatable to temperature-programmed methods. The simplification of \( H \) plotted as a function of \( \nu_c \), as seen in equation 2, is an oversimplification that does not do proper justice to the many interrelationships between the many variables in the original equation and should be used with caution. Equation 2 would be better expressed as:

\[
HETP = B + C_o + C_i \quad \text{or} \quad \frac{du}{dx} \text{HETP} = B + C_o + C_i
\]

with the carrier gas velocity included in each term, not expressed as an independent variable, which now expresses HETP as a rate and then requires discussion of each term tailored to the experiment being performed.

Golay’s original intent is why his equation is still relevant today. It describes the separation process...
in measurable terms based on physical properties of the column, carrier gas, and analyte. Unless experimental conditions are carefully controlled to ensure an “apples to apples” comparison, we should be cautious when using Golay’s equation to attempt direct comparisons of column performance from one column or one set of instrumental conditions to another. We should avoid extending conclusions about column performance derived from Golay’s equation under isothermal conditions to temperature-programmed conditions. Finally, as an extension, determinations of the number of theoretical plates in a column, or the number of plates per metre, are only valid for the exact analyte and isothermal experimental conditions under which the determination was made. We should be careful using such data to compare the performance of one column to another, especially if we intend to conduct temperature-programmed analysis. In spite of these limitations, Golay’s equation remains relevant in helping us understand the many complex processes that relate to the rate of band broadening in chromatography.

References
Early investigations utilizing metal-organic frameworks (MOFs) for liquid chromatography (LC) were discussed in a 2018 issue of this column series (1). As one of my most widely read contributions and given the large amount of research that continues to be observed in the field, it seems prudent to provide a current review and comment on advancements in the field since 2018.

Research on the utilization of MOFs for chromatography includes applications in sample preparation, gas chromatography (GC), and LC, among others. In monitoring activity over the past several years, the majority of publications within the chromatography realm continue to focus on materials for sample preparation, with fewer focusing on dynamic chromatography. With that being said, interest in MOF-enabled LC and GC are clearly on the rise. This paper focuses on the newest developments in LC.

One of the major issues noted regarding the use of MOFs for LC in the 2018 “Column Watch” paper was that the generated particles are often irregularly shaped and possess large particle size distributions. These qualities result in poor chromatographic efficiency when packed in LC columns. At that time efforts to build MOFs on silica particles in an attempt to utilize the former’s controlled particle size, shape, and uniformity were just starting. Much of the research conducted since then has built upon such initial efforts, including the control and distribution of the MOF material. These efforts include improved synthesis and control of the MOF distribution on the silica surface, improved understanding of the role of the silica substrate, as well as the addition of other materials, such as polymers, to enhance the utility, robustness, and reproducibility of resulting composites.

This column describes some of the more pertinent research conducted over the past few years. The contents are by no means meant to be exhaustive, but represent just a few of the interesting trends towards the development of MOFs for LC.

**Discussion**

**Recent Efforts to Improve MOF@Silica Composites:** Several early attempts at developing MOF@silica composites (note that in this work the convention of “shell material” @ “core material” is used to describe composite construction) resulted in improved efficiency over the native MOF particles but still fell short of modern standards. It is expected that this is largely because of uncontrolled growth of the MOF layer on the silica substrate. An attempt to improve the efficiency of MOF@silica composites was reported by Si and co-workers (2). By controlling the distribution of MOF growth on the surface of silica through first modifying the surface with 2-methylimidazole to seed the reaction prior to the
addition of the metal ions, the authors reported moderate but significant improvements in performance as compared to previous synthetic methods.

Another attempt at improving MOF@silica composites was published by Qu and others (3). In this method, the formation of zeolite imidazolate framework-8 (ZIF-8) on the surface of the silica was kinetically controlled through modifying the dimethylformamide (DMF) to methanol solvent ratio employed in the synthesis. The authors used an efficient separation of xylene isomers to demonstrate the applicability of their synthetic approach. Another “kinetically controlled” MOF@silica composite was separately reported by the Qu team where HKUST-1 nanocrystals (1,3,5-benzenetricarboxylate and copper[II]) were confined in the pores of the silica. This reaction was also controlled by manipulating the solvent system utilized in the reaction (4).

UiO-66 is a popular MOF that has been shown to be effective in various separation device formats. UiO-66 is based on a ZrO$_6$(OH)$_2$ octahedron and 1,4-benzene-dicarboxylate (BDC) linkers. Its cubic three-dimensional (3D)-pore structure consists of an array of octahedral cavities that have a diameter of 1.1 nm, and tetrahedral cavities with a diameter of 0.8 nm (5). In another example of “seeding”, to better control the growth of the MOF, Gao and co-workers carried out the synthesis of UiO-66@silica composites by combining a carboxylate-terminated silica, ZrCl$_4$, and terephthalic acid. The authors compared a heated stirring procedure to the more common solvothermal process. Here, the use of the composite MOF materials under more controlled synthetic conditions were found to provide superior chromatographic efficacy compared to columns packed with UiO-66 crystals alone (5).

A layer-by-layer synthetic approach was reported by Ehrling and others (6). In their work, both a relatively hydrophilic MOF (MIL-101[Fe]-NH$_2$[FeCl$_3$/2-aminoterephthalic acid]) and a relatively hydrophobic MOF (UiO-67 [Zr$_6$O$_4$(OH)$_4$/4,4’-biphenyldicarboxylate]) were employed to produce core–shell silica composite particles for
application as stationary phases for LC. The layer-by-layer method consisted of immobilizing the metal ions onto the surface of the silica particles followed by exposure to the acid linkers, with washings taking place after each step. The cycles can be repeated until the desired materials are obtained. The authors reported excellent separations of various classes of compounds under normal phase conditions employing columns using MOF@silica composites prepared in this manner. In another layer-by-layer approach, Liu and co-workers synthesized MOF-74@silica composites and subsequently packed efficient LC columns with these materials. MOF-74, in this case, consisted of 2,5-dihydroxyterephthalic acid as the organic ligand and nickel as the metal centre. The authors provide several chromatographic applications demonstrating hydrophilic and hydrophobic character as well as alternative selectivity from other MOF composites.

Building three-dimensional MOFs on the surface of silica may result in a certain amount of disorder. For example, the MOF crystals may be randomly placed and various sized crystal structures may result. The net impact is the potential for poor efficiency in dynamic chromatography. One report intended to solve this issue proposed using two-dimensional (2D) or nanosheets of MOFs when building the MOF@silica composite materials. According to the authors, a 3D MOF (MOF-FDM-23) was first prepared then treated via ultrasound to produce 2D structures that were then assembled onto a silica surface. Subsequent analysis of the 2D MOF-FDM-23@silica column demonstrated superior efficiency in comparison to the 3D MOF-based stationary phases tested. The synthetic control of the MOF formation, as noted above, impacts the overall efficiency of the composite material. However, the silica substrate can also have bearing on the final results. Wei found that the pore size of the silica substrate can have a significant impact on the uniformity of ZIF-8 MOFs that are grown on them. The authors reported that wide-pore silicas provided improved uniformity of the ZIF-8 and that the uniformity could also be enhanced further by modifying reaction solvent systems (here, DMF–methanol ratios). The improved efficiencies were demonstrated through several chromatographic applications.

Studies Incorporating Additional Polymer Materials: The incorporation of additional components to MOF@silica composites, such as polymers, has also been a subject of study. Si and co-workers reported on an efficient synthesis of core–shell composite material built upon silica microspheres that were modified with both the metal organic framework (MOF-235) and polyethylene glycol polymer (PEG) (10). MOF-235 is constructed from iron (III) chloride and terephthalic acid. The MOF-235@PEG@silica stationary phase was shown to provide both hydrophilic and hydrophobic character, with separation properties superior to the MOF-235@silica composite also studied in this paper. The authors investigated several parameters, such as pH, buffer concentration, and the ratio of organic modifier in the mobile phase, to support the retention mechanisms claims. Another publication supporting the addition of polymers to MOF@silica composites was also provided by Si and others. In that study, polyvinylpyrrolidone (PVP) was synergistically immobilized with a nickel organic framework onto a silica surface. The resulting Ni-MOF-PVP@silica composite was shown to exhibit both hydrophilic and hydrophobic character as well as some enhanced performance owing to the PVP modification. In a similar publication, the same group discussed the modification of a MOF-235@silica composite with PVP via a modified process documented in the literature, stating that the PVP helps to suppress silanol activity, enhances composite material robustness, and improves the water compatibility of the original MOF-based stationary phases.

Other Interesting Finds: An article focused on the use of MOFs for preparative work was interesting. The authors utilized MIL-53 (aluminium terephthalate) as a substrate to understand the adsorption isotherms of m-cresol and p-cresol. The studies on the adsorption process for the analytes indicated a complex, multistage process. Although the process was complicated to model, purification was effective. It is highly possible that MOF-based materials will find their way into preparative processes in the future. MIL-53(AI) is typically prepared from trivalent aluminium (aluminium nitrate) and terephthalic acid via a solvothermal process. In an interesting report from Aqel and co-workers, the authors used polyethylene terephthalate (PET) bottles as their “green” terephthalic acid source for the MOF-53(AI) synthesis and demonstrated...
equivalent separation performance to the traditional approach using standard chemical sources of terephthalic acid (14). Perhaps this will inspire other innovative “green” approaches for MOF synthesis.

Because of the sheer number of developments in the field of MOFs, experimental exploration to find the right MOF for an application, or design the right material for a given purpose, is becoming more difficult. As with other “big data” issues, computational methods are likely to assist greatly. Yan and others recently published an interesting review intended to highlight current research in this field as related to MOFs. High-throughput screening and machine-learning assisted high-throughput screening methods are discussed (15).

Chiral: As mentioned in the 2018 “Column Watch” article, the use of MOFs for chiral separations is of great interest. There have been a considerable amount of articles published over the past few years, focusing on chiral MOFs and their MOF@silica composites for use in LC. There has been enough activity in this realm that the subject likely warrants its own independent treatment, which is planned for a future installment. For those interested in delving into this subject sooner, a recent review by Taima-Mancera and Pino provides a good start (16).

Conclusions
Interest in MOFs for application in LC continues to rise. Early issues packing efficient LC columns arising from the irregular shape and large particle size distribution of native MOFs were initially improved through the development of MOF@silica composites. These composites take advantage of the controlled particle size distribution of silica and the improvement in efficiency this characteristic brings. However, simply building the crystalline MOF structure on the surface of the silica still limits the efficiency of the resultant materials. Over the past several years, most of the effort has been focused on improving the synthetic approaches used to prepare MOF@silica composites. The careful seeding of the silica surface with either the organic ligand or the metal centre has been used to better distribute the growth of the MOF evenly across the surface of the substrate. Several researchers have demonstrated improvements in efficiency through a “layer-by-layer” approach towards building the MOF structures. In almost all cases, careful control of reaction solvent conditions were reported to impact the efficiency of the final materials. Studies intended to better understand the impact the properties of the silica substrate have on the eventual MOF@silica composite are also expected to result in improved materials.

Although MOF-based materials for dynamic LC separations are not commercially available at this point, the rapid development and the large number of research efforts observed in this field indicate that this may not be far off from being realized.

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How Static Are Static Data?

R.D. McDowall, RD McDowall Ltd, Bromley, Kent, UK

A balance printout is a fixed record, and is also called static data. But how static are static data when the weight is used in a chromatographic analysis? Also, have some regulatory data integrity guidance documents failed to comply with their own regulations?

Data integrity continues to be a major topic for regulated pharmaceutical laboratories, which has resulted in health authorities issuing guidance documents to ensure the reliability and integrity of good manufacturing practice (GMP) records (1–5). Some of the guidance refers to GMP records and data as being either static or dynamic (2–5). Static and dynamic data are generally accepted terms, as seen in other data integrity publications (6–8).

Defining Static and Dynamic Data

Table 1 presents the definitions of static and dynamic data, with examples quoted from question 1(d) from the FDA Data Integrity Guidance (4). The definition of static data gives the impression that nothing happens after the record is created and they remain immutable for the record retention period. In contrast, dynamic data require interpretation by an analyst to obtain the reportable result. As you can see from Table 1, chromatography data and spreadsheets are examples of dynamic data; static data involve a balance printout.

What I want to discuss in this “Questions of Quality” instalment is that some static data are inputs into dynamic data processing. In these circumstances, static data are converted into dynamic data. However, creating a static record where the weight is manually entered into a chromatography data system (CDS) is an inefficient and error-prone process. Instead, laboratories should automate the complete process to eliminate the static data and enable sharing of all analytical data for review and to comply with the complete criterion of ALCOA+.

Furthermore, the focus on printouts from analytical instruments (5) is inconsistent with US and EU regulations for keeping current with technology (9,10), in addition to some recent FDA warning letters (11–13) that will be discussed later.

Static and Dynamic Data in Practice

CDS dynamic data as presented in Table 1 require a user to interpret peak integration for each data file in the sequence. One more requirement for dynamic data is that they must remain dynamic throughout the record retention period (2), therefore:

- Electronic records are primary and printouts are secondary in the data set.
- Paper alone can never be the GMP record from an electronic record generated by a computerized system.
- Hybrid records are tolerated currently but two data integrity regulatory guidance documents encourage their replacement (3,5).
- Dynamic data cannot be printed to PDF and stored in an informatics application.

The PDF file is a static electronic record as a user cannot interact with it.

In contrast, once static data have been created, the user cannot interact with them. Right?

But are static data really static with no further user interaction?

How Static are Static Data?

Back to the laboratory bench and imagine you are weighing either sample aliquots or a reference standard on an analytical balance and the weighing sequence (for example, vessel weight, tare, material weight) is printed out. It is generally accepted that this is a static record where the balance printout is attached to a laboratory notebook or analytical batch record. Indeed, the 2021 PIC/S guidance in section 8.9.1 (5) states:

Some very simple electronic systems, e.g. balances …which do not store data, generate directly-printed paper records. These types of systems and records provide limited opportunity to influence the presentation of data by (re-) processing, changing of electronic date/time stamps. In these circumstances, the original record should be signed and dated by the person generating the record and

...
information to ensure traceability, such as sample ID, batch number, etc. should be recorded on the record. These original records should be attached to batch processing or testing records.

This approach is repeated in the FDA data integrity guidance in question 10 (4):

10. Is it acceptable to retain paper printouts or static records instead of original electronic records from stand-alone computerized laboratory instruments, such as an FT-IR instrument?

A paper printout or static record may satisfy retention requirements if it is the original record or a true copy of the original record (see §§ 211.68(b), 211.188, 211.194, and 212.60). During data acquisition, for example, pH meters and balances may create a paper printout or static record as the original record. In this case, the paper printout or static record, or a true copy, must be retained (§ 211.180).

The stated regulatory expectation of both the PIC/S and FDA guidance documents for simple analytical instruments is for paper printouts that are attached to an analytical batch record or a laboratory notebook. Whilst there is limited ability to interfere with the printout, without an audit trail and e-records, weighing can be repeated until the “right” result is obtained. Whether a static record is the right approach shall be discussed later in this column.

**Static Data are an Integral Part of an Analytical Procedure**

Simple instruments such as balances generate data that are critical to the whole of an analytical procedure because the values are inputs to any quantitative chromatographic analysis, for example:

- Determination of purity
- Stability testing
- Impurity determination
- Residual solvent measurement

Here static data become an integral component of a dynamic data process. However, many laboratories still have inappropriately designed data processes for the small instruments that generate critical data on paper printouts. This is compounded by manually entering the weight into a computerized system or spreadsheet, resulting in transcription error checking by the reviewer.

We can look at this situation further in Figure 1; on the left of the diagram is the generation of a static data record: the balance printout. On the right, weights of samples and standards are manually entered into a CDS that generates dynamic data used to generate the reportable result. Here we can see the conversion of static

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**TABLE 1: Static and dynamic data definitions and examples from the 2018 FDA Data Integrity Guidance (4)**

<table>
<thead>
<tr>
<th>Static Data</th>
<th>Dynamic Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static is used to indicate a fixed-data record.</td>
<td>Dynamic means that the record format allows interaction between the user and the record content.</td>
</tr>
<tr>
<td>Paper record or an electronic image</td>
<td>A dynamic chromatographic record may allow the user to change the baseline and reprocess chromatographic data so that the resulting peaks may appear smaller or larger. It also may allow the user to modify formulas or entries in a spreadsheet used to compute test results…</td>
</tr>
</tbody>
</table>

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**FIGURE 1: Converting static data into dynamic data.**

**FIGURE 2: An analytical procedure with hybrid records.**
data into dynamic data. If the analyst entering weights into the CDS mistypes a value that must be corrected later with a corresponding audit trail entry, what was static data on the printout is now dynamic data in the CDS.

This poses a key question: Why make data static first, only to make it dynamic later?

**Is Regulatory Guidance Correct?**
The heading poses a fundamental question: Where is the event horizon when considering if data are static or dynamic? Consider the situation on the left side of Figure 1:

- **Static Data:** Analytical balance and printout in isolation: Weigh sample + print result = static data
- **Dynamic Data:** The balance and sample weight are integral parts of an overall CDS analytical procedure: Weigh sample + print result + type weight into CDS + acquire data + generate result = dynamic data

The dynamic equation only takes a high-level view of an analytical procedure, what also must be considered are all the contextual metadata generated throughout the process.

The records generated are a mixture of paper printouts (in yellow) and electronic records (in green) and yet more paper, as shown in Figure 2. Whilst there may be some static data records generated at the start with sample preparation and the balance printout, the overall process generates dynamic data. We now have an analytical procedure that generates hybrid records.

Even if the instrument data system in Figure 2 were to use electronic signatures, only one item of paper would be eliminated (the paper printout on the right of the figure). A hybrid analytical procedure still remains.

Figure 2 shows the complete record set that needs to be reviewed and archived, but the problem is that there are several static paper records plus electronic records.

Are hybrid analytical procedure records an adequate situation to be in?

The one regulatory guidance document that advocates process efficiency is the WHO Good Record and Data Management Practices Guidance from 2016 (3), which states in section 5.6:

> A data management programme developed and implemented on the basis of sound QRM principles is expected to leverage existing technologies to their full potential. This, in turn, will streamline data processes in a manner that not only improves data management but also the business process efficiency and effectiveness, thereby reducing costs and facilitating continual improvement.

It is unusual to find a regulatory guidance document recommending business process efficiencies and effectiveness. However, there are two points to be made here:

1. Use informatics solutions to streamline processes.
2. Eliminate paper and have only a single medium to review and retain.

This is coupled with the statements that “hybrid systems are not encouraged” and “hybrid systems should be replaced at the earliest opportunity” (3). The same approach should be applied to hybrid analytical procedure records because it is not just computerized systems but also processes that require assessments of data vulnerabilities (2,3,5).

However, are static records from simple instruments and the use of hybrid systems acceptable in the third decade of the 21st century? To find the answer we need to travel back to 1978.

**Understanding the c in cGMP**

Most analytical scientists working in a quality control or analytical development laboratory will refer to GMP regulations; however, the formal title of 21 CFR 211 is *Current Good Manufacturing Practice for Finished Pharmaceutical Products* or cGMP regulations.

Is current important? What does current mean?

A question was posed in the introduction of this white paper: Are the regulators failing to follow their regulations and guidance when it comes to the regulated analytical laboratory? To answer this question, we must go back to 29 September 1978 where the preamble to FDA regulation 21 CFR 211 on *Current Good Manufacturing Practice for Finished Pharmaceutical Goods* can be found. Preamble comment 17 has the following statements that have been edited for brevity (9):

> Several comments objected to the word “current” in the title and text of the regulations…
> Several of these comments reflect, the Commissioner believes, a misunderstanding regarding the use of the word “current”….
> Although the practices must be “current” in the industry, they need not be widely prevalent.
> Congress did not require that a majority or any other percentage of manufacturers already be following the proposed mandated practices as long as it was a current good manufacturing practice in the industry e.g. it has been shown to be both feasible and valuable in assuring drug quality.
The takeaway message from this is that laboratories must keep current with technologies and implement new approaches and systems to keep “current”. This is also mirrored in a page on the FDA website (14) that says:

Accordingly, the “C” in CGMP stands for “current,” requiring companies to use technologies and systems that are up to date in order to comply with the regulations. Systems and equipment that may have been “top-of-the-line” to prevent contamination, mix-ups, and errors 10 or 20 years ago may be less than adequate by today’s standards.

Is this approach to keeping current unique and only applicable to the FDA? No, it is mirrored in Article 23, §1 of European Directive 2001/83/EC (10) that requires a marketing authorization holder to:

After a marketing authorization has been granted, the marketing authorization holder shall, in respect of the methods of manufacture and control provided for in Article 8(3)(d) and (h), take account of scientific and technical progress and introduce any changes that may be required to enable the medicinal product to be manufactured and checked by means of generally accepted scientific methods.

Therefore, both FDA and EU regulations expect laboratories to keep up with technologies and be subject to continual improvement as required by ICH 10 on Pharmaceutical Quality Systems (15) and EU GMP Chapter 1 (16).

However, the situation in many laboratories does not match the requirements either to keep current or to take account of scientific and technical progress. In general, a lazy approach is prevalent: if it was good enough last inspection, it will be good enough for the next one. Further arguments may involve citing a lack of money for investment. These cease immediately after receiving regulatory citations when money for remediation flows like water over Niagara Falls.

Apart from the WHO guidance presented above, none of the regulatory guidance documents really consider the requirement for keeping current. This is particularly the situation with PIC/S PI-041 and the FDA quotations cited earlier for simple instruments to print outputs (4,5). Do these guides leave the pharmaceutical industry with a complacent attitude that paper records are OK?

Digitization of the Laboratory
Keeping current with respect to GMP and digitization of a regulated laboratory are two sides of the same coin. Both require automation, computerization, and elimination of paper records that result in complete electronic records in the same informatics system, thus making records and data easy to search and share.

One approach to keeping current with existing technology is shown in Figure 3. The informatics applications used are:

- Laboratory information management system (LIMS), shown in yellow, for managing sample information and collating test results;
- Instrument data system, typically developed by the instrument manufacturer, is interfaced with an analytical balance (interfacing other instrument types is also possible);
- CDS in green;
- Laboratory execution system (LES), in blue, is an option for documenting sample preparation and transferring data to the CDS.

Sample and batch information are transferred to the LIMS and then downloaded to the LES and CDS applications. Sample preparation is automated using the LES and the CDS. In Figure 2, the weight from the analytical balance is manually entered into the chromatography data system, which is slow and error-prone. In Figure 3, we have an instrument data system with an electronic workflow that acquires the balance sample weight and transfers it to the CDS. Further technical controls
ensure that the right type of balance is used or even a specific instrument identified by its serial number. There is an audit trail to ensure that samples are not weighed into compliance.

The CDS now has all the data required. The sequence is run. Chromatographic data files are interpreted and a reportable result generated. Second person review is accomplished electronically in the various informatics systems. The scope of review is limited by effective use of validated technical controls within the application, for example:

- No user has deletion privileges, so a reviewer does not need to look for deleted data.
- Data can only be stored in a single location; therefore, no searches need to be conducted for orphan data.
- Audit trails in the applications highlight modified data, allowing a reviewer to review by exception (5).

The informatics applications ensure that all data are complete, consistent, and accurate. This approach also leverages existing technologies as advocated by the WHO data integrity guidance discussed earlier (3), in addition to meeting the expectation of current in cGMP (9, 17).

If required, the values from many results sets can be monitored for trends as required by EU GMP Chapter 6.9 (18) or used to identify errors. This work would not be possible with static data without extensive manual data entry into a spreadsheet.

The Regulatory Driver for Laboratory Digitization

The FDA are keeping current in cGMP by interpreting the same regulation differently over time. For example, there is no explicit mention of audit trail review in 21 CFR 211 regulations, the bulk of which was issued in 1978 (9). However, since 2005 and the Able Laboratories fraud case (19), the regulatory expectation of the FDA is that audit trail entries must be reviewed. EU GMP has taken a different and better approach by updating Annex 11 for Computerized Systems where there is an explicit requirement for audit trail review of GMP-relevant changes and deletions (20). Audit trail review is now a feature of all regulatory authority data integrity guidance documents (1–5).

Another, less subtle, driver for automation is evidenced by some recent FDA warning letters. In July 2020, FDA issued warning letters to Stason Pharmaceutical (11) and Tender Corporation (12). Although they were cited under different clauses of 21 CFR 211, the extensive remediation required by the FDA was virtually identical for the two companies. A detailed review of these two warning letters and a discussion on understanding the cost of non-compliance is available (21). One specific remediation requirement for both organizations was:

- Technological improvements to increase the integration of data generated through electronic systems from standalone equipment (e.g., balances, pH meters, water content testing) into the LIMS network.

This is a clear message that paper records from standalone systems are not acceptable. The citation also mentions a LIMS network, which can include different informatics applications such as LIMS, LES, ELN, and instrument data systems, as shown in Figure 3.

It is better to acknowledge the problems in your laboratory and have a plan to remediate them at your pace rather than have a tight deadline imposed to appease a regulatory authority. It is much cheaper as well.

However, is interfacing a balance to a LIMS the only way forwards?

Connecting the balance to an instrument data system may be a better option as additional metadata can be acquired during the weighing process to ensure the integrity of data. As shown in Figure 3, the instrument data system has the advantage of using the weights from a balance in another instrument workflow. Also, the use of an instrument data system also provides the laboratory with additional resilience if the LIMS is unavailable due to loss of connectivity to the central system or the Cloud.

If an analytical instrument is purchased, how a laboratory uses it can also result in a regulatory citation as BBC Group found out in a warning letter in August 2021 (13):

Your viscometer and UV–vis spectrophotometer had the capability to save data from product/material testing. Despite having this capability, your analysts failed to save the complete, dynamic testing data, and therefore the data was not available for review by the FDA investigator.

If an instrument has the capability to store electronic records and this feature is not used, it can result in a regulatory citation. Equally so, an old instrument operating in a laboratory without the capability to either connect to a printer or store data is a problem. It falls into the FDA argument that it is not top of the line (14), as discussed above.

If a laboratory does not digitize there can be expensive remediation costs, with a timescale determined by the need to convince a regulator that the company is serious about compliance.

Business Driver for Laboratory Digitization

Regardless of the regulations, organizations should consider digitization
from a business efficiency perspective alone. Quality control (QC) testing is often at the end of manufacturing and can be seen as slowing the release of a batch of product. If a laboratory was more efficient with analysis and release of the certificate of analysis, what would be the impact on company cash flow if each and every batch were released to the market one day earlier? The way to do this is to work smarter and work electronically through digitization of the laboratory and the removal of paper.

The Covid-19 pandemic has forced laboratories to view a new way of working—some analysts working in the laboratory and others remotely. This can only work if data are electronic and not on paper. You cannot share paper remotely. Imagine doing laboratory work such as second person review remotely in an electronic environment. Review of data and audit trail entries as well as signing results and reports can be performed remotely. Trending can also be performed electronically to meet the requirements of EU GMP Chapter 6.9 (18). You cannot do these tasks with working practices that involve static data and hybrid records.

**Conclusion**

Some static data are less static than others because the values are manual inputs into a dynamic analytical procedure. Original records from simple instruments must be complete and include all metadata and must be subject to review. However, the review includes transcription error checking, which is a slow and error-prone process. Regulatory guidance documents on data integrity—with the exception of that from the WHO—do not discuss the need for regulated laboratories to keep current but should do so to be compliant with the regulations that they are supposed to enforce. However, the benefits of managing data in a fully electronic process provides significant business benefits and regulatory compliance, with data stored in one medium. This approach facilitates efficiency and effectiveness including ease of performing data analytics.

This is the way to keep current with cGMP.

**Acknowledgement**

I would like to thank Gunnar Danielson for helpful comments during the preparation of this column.

**References**

4) US Food and Drug Administration, Guidance for Industry Data Integrity and Compliance With Drug CGMP Questions and Answers (FDA, Silver Spring, Maryland, USA, 2018).

**ABOUT THE COLUMN EDITOR**

Bob McDowall is Director of R.D. McDowall Limited, Bromley, UK. He is also a member of LCGC Europe’s editorial advisory board. Direct correspondence to: amatheson@mjlifesciences.com
UHPLC–MS Columns
1 mm YMC-Triart UHPLC high-performance columns for LC–MS use are now available. The company reports that excellent sensitivities can be achieved with very low sample amounts/concentrations. These columns are dedicated for labs that routinely use LC–MS and need to deal with very small concentrations where high sensitivities are important.

https://ymc.eu/d/brDmC
YMC Co., LTD., Kyoto, Japan.

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

www.pss-polymer.com
PSS Polymer Standards Service GmbH, Mainz, Germany.

GC Detector
VICI's Model D-3-1-8890 is a plug-and-play pulsed discharge detector for easy installation and configuration on the Agilent 8890 GC. This detector is optimized for trace-level work in helium photoionization mode, and is a non-radioactive, low maintenance universal detector with a wide linear range, according to the company. The system also uses the electronics and power supply of the host GC.

www.vici.com
VICI AG International, Schenkon, Switzerland.

SEC Columns
Phenomenex Biozen DSEC-2 LC columns are packed with low-pore volume silica coupled with proprietary hydrophilic diol-type bonded surface chemistry that prevents unwanted interactions with protein samples. According to the company, the proprietary DSEC-2 particle technology results in consistent reproducible results and an increase in column lifetime.

www.phenomenex.com
Phenomenex, Torrance, California, USA.

Sample Filtration
Teknokroma’s range of Olimpeak filter vials provide efficient, safe, fast, and sustainable sample filtration prior to HPLC analysis, according to the company. Manufactured from highly inert, high purity materials and designed for easy use, they fit directly into most autosamplers and are reportedly the ideal solution for laboratories of all sizes. They are available with a wide variety of membranes.

Teknokroma Analítica S.A., Barcelona, Spain.

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The Sciencix CTS-21591 PM Kit is designed for G4226A models of the Agilent 1290 autosampler and includes a metering device seal, peristaltic pump, rotor seal, needle assembly, and needle seat to maintain consistent peak performance.

www.sciencix.com
Sciencix, Inc., Burnsville, Minnesota, USA.
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https://chiraltech.com
Daicel Chiral Technologies Europe, Illkirch, France.

**Field-Flow Fractionation**
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www.wyatt.com/eclipse
Wyatt Technologies, Santa Barbara, California, USA.

**Method Translator**
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www.restek.com/Pages/Pro-EZLC-Method-Translator
Restek Corporation, Bellefonte, Pennsylvania, USA.

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www.go-jsb.co.uk/assortiment/chromatografie_oplossingen/valving_JSsolutions/eclipse__process_gc_wasson

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www.shimadzu.eu
Shimadzu Europa GmbH, Duisburg, Germany.

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www.microplates.com/draft/kingfisher-compatible-96-well-microplate/
Porvair Sciences Ltd, Wrexham, UK.

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https://www.separations.eu.tosohbioscience.com
Tosoh Bioscience GmbH, Darmstadt, Germany.

**EAF4 System**
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www.postnova.com
Postnova Analytics GmbH, Landberg, Germany.

**High Performance Pulse Dampers**
Baseline fluctuation is a thing of the past, according to the company. Knauer’s Pulse Dampers reportedly provide excellent damping performance while remaining simple to integrate into HPLC systems due to their membrane-free assembly.

https://www.knauer.net/
Knauer Wissenschaftliche Geräte, Berlin, Germany.

**MS Bench**
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www.ionbench.com
ionBench, Sens, France.
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