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Organophosphorus pesticides (OPPs) have been widely used for preventing or decreasing damage caused by pests, weeds, and plant disease, and are very important for high-yield production in agriculture (1,2). However, as a result of extensive use, these OPPs enter the environmental water system by various sources, and as a result, both human and animal populations are exposed to OPPs through drinking water. The most harmful outcome, however, may be the formation of mutagenic compounds during conventional oxidation processes (3). Therefore, it is necessary to develop an effective method for monitoring OPPs in real environmental water, which is useful for ensuring environmental and health safety.

The determination of OPPs has been carried out with a variety of techniques, such as gas chromatography with a flame photometric detector (GC-FPD) (4,5), liquid chromatography–mass spectrometry (LC-MS) (6), gas chromatography–mass spectrometry (GC-MS) (7), and gas chromatography with nitrogen–phosphorus detector (GC-NPD) (8). Even though most OPPs are analyzed by GC, high performance liquid chromatography (HPLC) is preferred for its higher accuracy and precision.

Analysis of Organophosphorus Pesticides by HPLC Using Magnetic SPE with Nitrogen-Doped Reduced Graphene Oxide/Fe₃O₄ Nanocomposite as the Adsorbent

Yi Gao¹ and Qingqing Pan², ¹Department of Analytical Chemistry, Southwest University, Chongqing, The People's Republic of China, ²Department of Chemistry, Southwest University, Chongqing, The People's Republic of China

In this study, a method was developed for extraction and preconcentration of trace amounts of organophosphorus pesticides (OPPs), including fenitrothion, chlorpyrifos-methyl, and chlorpyrifos in environmental water. The method uses a new magnetic solid-phase extraction (SPE) technique, followed by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. Nitrogen-doped reduced graphene oxide-iron oxide (Fe₃O₄) nanocomposite, used as the adsorbent of organophosphorus pesticides, was successfully prepared using an easy hydrolysis process. The nanocomposite was synthesized and characterized by X-ray photoelectron spectroscopy (XPS), Fourier-transform infrared (FT-IR) spectroscopy, and X-ray diffraction (XRD). The effects of type and ratio of eluents, amount of the graphene oxide (GO)/Fe₃O₄ nanocomposite, pH, ionic strength, equilibrium time, and ultrasound time on the quantitative recoveries of fenitrothion, chlorpyrifos-methyl, and chlorpyrifos were investigated. On the basis of the best condition, the recoveries of the target analytes in real water samples were between 84.40% and 105.05%. The relative standard deviations varied from 1.21% to 4.22%. Finally, the method was successfully applied to the determination of three organophosphorus pesticides in real environmental water samples.

KEY POINTS

- The monitoring of organophosphorus pesticides (OPPs) in the environment is important because of their mutagenic properties.
- A successful method to analyse trace amounts of OPPs in environmental waters has been developed using solid phase extraction with magnetic adsorbent and UV detection.
- The method has been successfully used to analyse three OPPs in real environmental water samples.
preferred to GC for analysis of OPPs because of the thermal lability of these compounds. In addition, HPLC is one of the most sensitive and selective analytical methods for the determination of organic pollutants in environmental water.

However, there are challenges in using HPLC for such analyses, because OPPs in environmental water samples, such as tap water, underground water, farmland water, lake water, and river water, are often present at low concentrations, and these matrices are considered to be very complex. Therefore, for HPLC analysis of OPPs in environmental water samples, an appropriate sample preparation technique is required to isolate and enrich the target analytes (9–11). To address this challenge, various sample preparation methods for analysis of OPPs have been researched, such as liquid–liquid extraction (LLE) (12), ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) (13), liquid–liquid microextraction based on solidification of floating organic droplets (DLLME-SFO) (14), and other traditional sample pretreatment methods.

In recent years, there has been much interest in the study of the magnetic materials, such as iron oxide (Fe₃O₄) nanoparticles, which have been widely used for enrichment of organic pollutants in water, owing to various advantages, including their stability, good separation characteristics, availability, and avoidance of secondary contamination of samples (15–19). However, Fe₃O₄ nanoparticles, have a tendency to agglomerate into larger particles because of the magnetic tendencies of this compound (20–24). The most effective technique is to load Fe₃O₄ onto supporting materials, such as activated carbon or graphene. There are many researchers who load Fe₃O₄ onto graphene to analyze organic contaminants in water (21,25,26). However, in recent studies, the structure of the activated carbon was changed after treatment by strong oxidation (18,27). In another study, therefore, a new magnetic material, a nitrogen-doped reduced graphene oxide-Fe₃O₄ nanocomposite (N-RGO/Fe₃O₄), which was based on Fe₃O₄, was synthesized using an easy method. N-RGO/Fe₃O₄ has high adsorption capacity and strong catalytic ability for removing OPPs at low concentrations from environmental water, and the N-RGO incorporated with magnetic Fe₃O₄ nanoparticles is able to effectively overcome the challenge of the separation of adsorbent from aqueous solution (28).

However, N-RGO/Fe₃O₄ has long been used for electrochemical super capacitors and electrochemical catalysis (29–32). The aim of the present study, therefore, is to establish a new method of magnetic solid-phase extraction (MSPE) for enriching and separating OPPs in water samples. This is the first time that the novel N-RGO/Fe₃O₄ has been used as a solid-phase extraction agent for the enrichment of OPPs including fenitrothion, chlorpyrifos methyl, and chlorpyrifos in real water samples. This separation process can be performed directly in crude samples containing suspended solid materials without the need for filtration or additional centrifugation, which makes separation faster and easier. Furthermore, the recoveries of OPPs and reusability of the N-RGO/Fe₃O₄ were also evaluated. This method using sample pretreatment with MSPE followed by HPLC analysis offers several advantages, including good precision and recoveries, low cost, and good reproducibility. Given these advantages, this new method may be broadly useful in many other fields.

**Materials and Methods**

**Chemicals**

Fenitrothion (purity >98%), chlorpyrifos methyl (purity >98%), chlorpyrifos (purity >98%), GO (purity >98%, layers <3), FeSO₄·7H₂O, NaCl, acetic acid, and NH₄H₂O were used.
obtained from Aladdin Ltd. The reagents used for the elution, including methanol, ethanol, acetone, acetonitrile, and ethyl acetate, were purchased from Taixin Ltd. Deionized water was provided using a laboratory system. All chemicals were used as received, without further purification. A stock solution containing fenitrothion, chlorpyrifos methyl, and chlorpyrifos at 100, 430, and 500 mg/L concentration, respectively, was prepared in methanol, and stored at 4 °C.

**Instruments**

An LC-20AT series HPLC system equipped with a solvent delivery pump, an SPD-20A UV-vis detector (Shimadzu), and an LC solution workstation were used for the analyses. An HY-5 mechanical shaker (Jintan Eton Electric Corp.) was used in this work. An SZ-2 system (Shanghai Lu West Analytical Instruments) was used to prepare double deionized water. A high-speed centrifuge was employed to centrifuge the sample solutions (Model 800).

**Preparation of N-RGO/Fe₃O₄**

The N-RGO was prepared by adding 260 mg graphene oxide (GO), 100 mL water, and 4 mL aqueous ammonia into a 250 mL round-bottom flask, and then heating at 180 °C for 24 h, followed by adding 5.2 mL of NH₃·H₂O with sonication for 7 min to thoroughly mix the solution. Finally, 8 mL of 0.35 g/mL freshly prepared FeSO₄·7H₂O was added to the solution, and heated in a water bath at 85 °C for 6 h to form N-RGO/Fe₃O₄. After being cooled to room temperature, negative-pressure filtration was used, and then N-RGO/Fe₃O₄ solids were washed with deionized water to neutralize them. Finally, N-RGO/Fe₃O₄ solids were placed in an oven to dry, and the dry solids were placed in storage for the next phase of the experiment.

**HPLC Conditions**

The HPLC separation was performed on a C18 column (150 mm × 4.6 mm, 5-µm, Beijing Jingkerida Technology Co., Ltd.) with equivalent elution using methanol and water at a rate of 1 mL/min and detection at 285 nm. The composition of equivalent elution was 85% methanol and water (0.1% acetic acid). The injection volume was 20 µL, and the column temperature was 35 °C.

**Sample Preparation**

We added 30 mg of N-RGO/Fe₃O₄ into 150 mL water to which was added 0.075 mL fenitrothion, 0.015 mL chlorpyrifos methyl, and 0.015ml chlorpyrifos, with a concentration of 5.0 mg/L for each analyte. The mixture was sonicated for 1 min to disperse the graphene, and then the conical flask was shaken on the platform of an orbital incubator for 30 min for adsorption equilibrium. Subsequently, an external magnet was placed on the bottom of the tube. After the solid phase was aggregated,
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the water phase was discarded. Simultaneously, the collected sorbents adsorbing the target analytes were eluted with 0.5 mL methanol and 0.5 mL ethyl acetate to desorb the analytes, which was sonicated for 5 min. Separation of solid and liquid phases was the same as above. The eluted solution was collected, then dried under a stream of nitrogen at 55 °C, and dissolved with 1 mL methanol. Finally, after filtration through 0.22 µm membrane, 20 µL of the solution was analyzed by HPLC.

Results and Discussion

Characterization of N-RGO/Fe₃O₄

In this study, the N-RGO/Fe₃O₄ was synthesized and characterized by Fourier-transform infrared (FT-IR) spectroscopy, X-ray diffraction (XRD), and X-ray photoelectron spectroscopy (XPS).

FT-IR was used to identify the presence of functional groups and chemical bonds in material. Figure 1 shows the FT-IR spectra of N-RGO/Fe₃O₄ powders. The intense band at 592 cm⁻¹ is assigned to the Fe-O bonds stretching of the magnetite, and the intense band at 3440 cm⁻¹ is attributed to stretching of the amino group, which can provide the evidence to demonstrate that amino group were successfully grafted onto the surface of the RGO/Fe₃O₄ during preparation. The band at 1635 and 1128 cm⁻¹ could be assigned to the aromatic skeleton C=C and C-C stretching vibration of the autoxidized graphitic domains and stretching vibrations of the epoxy, further confirming the above results.

XRD is a common technology to determine the crystal structure of the superparamagnetic nanoparticles. The XRD pattern of the N-RGO/Fe₃O₄ is depicted in Figure 2. It shows diffraction peaks at 2θ = 18.10°, 30.13°, 35.69°, 36.36°, 43.04°, 53.51°, 57.07°, and 62.64°, which correspond to crystal indexes of (220), (311), (400), (222), (422), (511), and (440), respectively (JCPDS no. 19–0629) (30,33). For N-RGO/Fe₃O₄, the disappearance of the diffraction peaks corresponding to GO (24.6°) and N-RGO (43.1°) is attributed to inhibition of the restacking of graphene layers by the crystal growth of Fe₃O₄ during preparation. The band at 1635 and 1128 cm⁻¹ could be assigned to the aromatic skeleton C=C and C-C stretching vibration of the autoxidized graphitic domains and stretching vibrations of the epoxy, further confirming the above results.

XPS was employed to further investigate the chemical composition of N-RGO/Fe₃O₄. As shown in Figure 3a, the XPS wide scan spectra of N-RGO/Fe₃O₄ clearly exhibited that the magnetic material mainly consisted of Fe, C, N, and O. Figure 3a also shows that the observation of Fe 2p3/2 and Fe 2p1/2 signals at 711 and 725 eV arose from the magnetic Fe₃O₄ core of prepared material, and
from Figure 3a, and binding energy of N 1s was about 400 eV, which was attributed to the amino group. Thus, the presence of the peak for N 1s suggested the formation of amino group on surface of Fe$_3$O$_4$. Figures 3b and 3c show the high-resolution XPS of C 1s and O 1s. As can be seen from C 1s, the appearance of peaks at the binding energies of 284.7, 286.4, and 288.8 eV were corresponding to C-C in aromatic rings, C-OH, and C=O, respectively, which confirmed that RGO was successfully immobilized on N-Fe$_3$O$_4$ by acylation reaction. For O 1s, the typical peak at 530.3 and 531.8 eV were corresponding to O-H and C=O, respectively, which suggest there were different oxygen-containing groups. Thus, these indicated the successful preparation of N-RGO/Fe$_3$O$_4$.

**Optimized MSPE Procedures**

In the present study, a 150 mL sample to which was added 0.075 mL fenitrothion, 0.015 mL chlorpyrifos methyl, and 0.015 mL chlorpyrifos for each analyte, was used to study the extraction performance of OPPs under different experimental conditions. During optimization, the experiments were analyzed them at least in duplicate.

**FIGURE 5:** (a), (b) Replicate chromatograms of tap water and lake water.

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Selection of Elution Solvents and Ratios

In this study, four elution solvents, including methanol, acetone, acetonitrile, and ethyl acetate, were used. Methanol has a better elution effect on chlorpyrifos and chlorpyrifos methyl than acetone, acetonitrile, or ethyl acetate. However, in addition to ethyl acetate, other elution solvents have little elution capacity for fenitrothion. This may be accounted for by their better solvation capabilities for the target analytes. Based on the above considerations, a mixed solvent of methanol and ethyl acetate was selected as the elution solvent.

The rate of the elution solvent was also an important factor affecting the extraction and recoveries efficiency. To achieve good extraction and recoveries for the target analytes, different ratios of methanol and ethyl acetate (for example, V_{methanol}/V_{ethyl acetate}=5:1; 3:1; 1:1; 1:3; and 1:5, respectively) were compared at these studies. As shown in Figure 4a, the results showed that the extraction recovery of fenitrothion was affected by the ratio of methanol to ethyl acetate, and when the ratio of methanol and ethyl acetate was 1:1, the recoveries of fenitrothion and chlorpyrifos methyl were highest. However, the greatest recovery of chlorpyrifos appeared at 1:3. This may be owing to the strong polarity of ethyl acetate for chlorpyrifos. Considering that the change of extraction recovery of chlorpyrifos and chlorpyrifos methyl with the ratios of methanol and ethyl acetate is less obvious than fenitrothion, V_{methanol}/V_{ethyl acetate}=1:1 was selected as the optimum rate for the following experiments, at a total volume equal to 1 mL.

Selection of the Amount of N-RGO/Fe_3O_4

To improve the enrichment efficiency of the extraction method, the amount of N-RGO/Fe_3O_4 was investigated, from 10 to 50 mg. As shown in Figure 4b, when the amount of N-RGO/Fe_3O_4 added was 30 mg, the recovery reached peak; thus, the optimal salt addition was determined to be 5%.

Selection of Sample Solution pH

To examine the effect of the sample solution pH, the pH was studied ranging from 3.0 to 11.0, which was adjusted by using CH_3COOH and NH_4H_2O solution. From the results, as shown in Figure 4c, it was found that the recoveries of OPPs increased when increasing the pH of the sample solution from 3.0 to 6.0. This is due to the electrostatic interactions that the three tested OPPs exhibit on the surface of the N-RGO/Fe_3O_4. At pH < 6.0, both the analyte and the adsorbent surfaces are positively charged, and the electrostatic repulsion between two positive charges is a robust force. Further increases in solution pH were also examined, and it was found that the peak areas of the OPPs decreased when the pH was above 6.0. This result was attributed to the fact that more oxygen-containing groups on the N-RGO/Fe_3O_4 surfaces are ionized when the pH is above 6.0, which causes lower extraction of analyte materials. To sum up, a pH of 6.0 was selected for follow-up experiments.

Selection of Ionic Strength

To investigate the effect of ionic strength on the adsorption percentage of OPPs on N-RGO/Fe_3O_4, the ionic strength was set over the range of 0–11% (adding NaCl of 0, 4.5, 7.5, 11.5, and 14.5 g), and the results are shown in Figure 4d. As can be seen, the recoveries rose rapidly with the increasing NaCl concentration at the beginning, due to the salting out effect and the higher viscosity, which may make the process of extraction between the target analytes and adsorbents more effective. Meanwhile, previous studies suggested that an excessive amount of NaCl may reduce the extraction kinetics. Thus, the optimal salt addition was determined to be 5%.

Selection of Equilibrium Time

Adsorption equilibrium time is an important factor for evaluating the adsorption capacity of the materials, because MSPE is a partition equilibrium process of analytes between

<p>| TABLE 1: Regression equations, linearity, the detection limits, and repeatability of the developed HPLC method for the analysis of water |
|---------------------------------|----------------|----------------|---------------|----------------|</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Intercepts</th>
<th>Linear Range (μg/mL)</th>
<th>R²</th>
<th>LOD (μg/mL)</th>
<th>RSD (n = 5, %)</th>
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<tbody>
<tr>
<td>Fenitrothion</td>
<td>Y = 4015.98+9.80*10^6X</td>
<td>0.6–600</td>
<td>0.9993</td>
<td>0.04</td>
<td>2.21</td>
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<tr>
<td>Chlorpyrifos</td>
<td>Y = 10708.56+1.71*10^6X</td>
<td>0.6–600</td>
<td>0.9995</td>
<td>0.05</td>
<td>1.53</td>
</tr>
<tr>
<td>Chlorpyrifos methyl</td>
<td>Y = 17878.43+3.2*10^6X</td>
<td>0.8–800</td>
<td>0.9999</td>
<td>0.06</td>
<td>2.90</td>
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**TABLE 2: Results of determination and recoveries of real water spiked with three OPPs (n = 5)**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Spiked (mg/L)</th>
<th>Laboratory Water</th>
<th>Lake Water</th>
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<tr>
<td></td>
<td>Found (mg/L)</td>
<td>RECOVERY (%)</td>
<td>RSD (%)</td>
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<tr>
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<td>5</td>
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<td>20</td>
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ND: Not detected.
adsorbents materials and sample solution. To screen out the optimum conditions, the effect of extraction time from 10 to 50 min were explored. As shown in Figure 4e, the maximum recoveries were obtained when the extraction time was 30 min for the three analytes. Thus, 30 min was selected as optimum adsorption equilibrium time for the further experiments.

**Selection of Ultrasound Time**

Ultrasound time is the minimum time needed for quantitative desorption of the analytes, which is another essential factor that affects the recoveries of the analytes. As shown in Figure 4f, for screening out optimum ultrasound time, 3–11 min was set for experiments. For fenitrothion, 3 min was enough to get desorption, while 5 min was needed for chlorpyrifos and chlorpyrifos methyl. In terms of total recovery for the three OPPs, 5 min was selected as the optimal ultrasound time.

**Validation of the HPLC Method**

To validate the developed HPLC method for the analysis of OPPs, the linearity was evaluated using a series of standard mixture solutions of the analytes. Concentrations from 0.020 to 800 μg/mL were obtained for the establishment of the calibration curve. For each level, five repetitive measurements were carried out. Relative standard deviations (RSDs), enrichment factors (EFs), and limits of detection (LODs) were examined to evaluate the HPLC method.

The recovery percentage (RSD) was calculated by following equation 1 (34):

\[
\text{RSD} = \left( \frac{C_1 - C_2}{C_1} \right) \times 100\% \tag{[1]}
\]

where \(C_1\) and \(C_2\) are the final concentration of the analyte in the spiked sample and in the unspiked sample, respectively.

The enrichment factor was calculated with equation 2 (34):

\[
\text{EF} = \frac{C_2}{C_1} \tag{[2]}
\]

where \(C_2\) and \(C_1\) are the final concentration of the analyte in the organic phase (extraction solvent) and the initial concentration of the analyte in the aqueous phase, respectively.

The results are shown in Table 1. Three OPPs exhibited good linearity with correlation coefficients (\(R^2\)) between 0.9993 and 0.9997, and the RSDs varied from 1.58% to 2.90%. The LODs of 0.04–0.06 μg/mL for the analytes were obtained based on the ratio of signal-to-noise \((S/N = 3)\), with the RSDs \((n = 5)\) lower than 3.58%. Finally, EFs were in the range of 133 to 166, which demonstrates that the HPLC method has high values and good extraction performance. Above all, it can be concluded that good sensitivity and reproducibility could be achieved with the developed HPLC method.

**Application to Real Samples**

The proposed N-RGO/Fe\(_3\)O\(_4\) was applied to determine three OPPs, including fenitrothion, chlorpyrifos, and chlorpyrifos methyl residues in environmental samples under the above optimum conditions established. No OPPs were found in the real samples; the recovery was carried out by spiking known concentrations of mixed standard OPPs into the samples before adding the N-RGO/Fe\(_3\)O\(_4\). For each concentration level, three replicate experiments with the whole analysis process were performed. The results are listed in Table 2, and show that this method has good extraction efficiency for analyzing of three OPPs in real water samples. The recoveries of the studied OPPs were between 84.40% and 105.05%. Figure 5 shows the typical chromatograms of tap water and lake water.

**Conclusions**

In this study, a method based on MSPE-HPLC with N-RGO/Fe\(_3\)O\(_4\) as the adsorbent was proposed for analyzing three organophosphorus pesticides—fenitrothion, chlorpyrifos, and chlorpyrifos methyl—in environmental water. A rapid separation of organophosphorus pesticides was achieved. The developed method provided fast analysis process, high EFs, low LODs, wide linear range, and good anti-interference ability. This method fully embodies the high speed and efficiency of HPLC. It is suitable for the speciation of OPPs in environmental studies.

**Acknowledgments**

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**References**


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Rules of Thumb for Reversed-Phase LC: What’s In Your Chromatographic Mind?

Dwight R. Stoll, LC Troubleshooting Editor

A handful of approximate rules about the behaviour of reversed-phase liquid chromatography (LC) can facilitate more efficient work, both during method development and in troubleshooting problems that arise with LC systems.

As with other fields of analytical science, such as spectroscopy and mass spectrometry, professionals practicing chromatography carry a lot of information and knowledge in their minds that facilitates problem solving in their daily work. I think this is most evident in method development activities for chromatography. For example, knowing the general retention behaviour of a carboxylic acid-containing analyte in reversed-phase liquid chromatography (LC) enables the method developer to make the quick decision to decrease the mobile phase pH if they observe that the retention of this analyte is too low. To me this kind of knowledge is akin to knowing a set of “math facts” so that one does not have to always reach for a calculator when making estimates for simple calculations.

Here, carrying in our minds knowledge of the general behaviour of a carboxylic acid-containing analyte in reversed-phase LC allows us to avoid looking in books or journal articles for commentary on the behaviour before taking the next step in method development. In this case, the rule is that generally retention of a carboxylic acid-containing analyte will increase as the mobile phase pH is decreased from above the pK_a of the carboxylic acid functional group to below the pK_a.

In my work with students and practicing chromatographers around various aspects of LC, I find that they generally are not aware of as many of these rules as they could be. Increasing the amount of chromatographic knowledge we carry around in our minds will not only be helpful during method development, but also in troubleshooting problems with LC systems. Very often a critical part of the troubleshooting process is understanding how the system should behave so that irregular behaviour can be spotted, and troubleshooting efforts can be focused in this area. The more rules we have in mind, the more readily we can spot irregular behaviour. This instalment of “LC Troubleshooting” articulates several rules in one place. They are not new ideas, but information about them tends to be spread out across many resources. In this article, I have consolidated some of them as a starting point for increasing the knowledge in our chromatographic minds.

Rules #1-4: Mobile Phase Effects on Retention of Small Molecules in Reversed-Phase LC

#1: Effect of the Level of Organic Solvent in the Mobile Phase

In many cases, the most powerful determinant of retention in reversed-phase LC is the volume fraction of organic solvent (commonly referred to as “%B”) in the mobile phase, and thus it is very helpful to have a sense for how much the retention of an analyte of interest should change in response to a change in %B. As part of my ongoing work in my laboratory, we have collected retention data for a diverse set of small molecules that includes both non-ionogenic (that is, no net charge in aqueous solutions) and ionogenic (both acids and bases) compounds. Since we have retention factors for these compounds in several different mobile phases varying in %B, we can calculate the degree of change in retention factor for a certain degree of change in %B.

Figure 1 shows a histogram of these results. Here, a 100% increase in retention means that the retention factor...
for a given compound doubles when the %B is decreased from 40% to 30%, a 200% increase means that the retention triples, and so on. From these data we make two practically significant observations: 1) the degree to which retention increases for these compounds in response to the same change in mobile phase composition varies by about a factor of four; and 2) the degree of change for the majority of the compounds falls in the range of 90% to 150%. Based on this, we can adopt the rule that the retention of an average small molecule will roughly double in response to a 10% decrease in the %B in the mobile phase; these numbers are easy to remember. For a more precise estimate of the expected change, more information about the analyte and separation conditions are needed. For example, it is known that the degree of change in retention in reversed-phase LC is highly correlated with the molecular weight of the analyte (1), which we also see in the data.

#2: Effect of the Type of Organic Solvent
Acetonitrile (ACN) and methanol (MeOH) are the organic solvents most commonly used in reversed-phase LC mobile phases. However, they are not interchangeable in terms of their effects on retention and selectivity in separations of small molecules. The effects of these solvents on selectivity are compound- and stationary phase-specific, and thus we should be careful not to assign too much weight to general statements about their effects on retention. However, a generalization is still useful for method development and troubleshooting purposes. The diagram in Figure 2 is known as a nomogram, which is useful for estimating the composition of one mobile phase that will lead to roughly the same retention as another mobile phase containing a different organic solvent. The dashed blue line helps visualize how this tool can be used in practice.

Suppose we have an isocratic separation where a particular analyte has a retention factor of five in a mobile phase that is 20% (v/v) acetonitrile. The nomogram shows us that we could expect to observe similar retention for the same compound on the same column if we use about 8% more methanol (28%) or 5% less tetrahydrofuran (15%) in the mobile phase. Inspection of the nomogram shows that these percentages that will give similar retention vary as one moves across the scale from 100% aqueous to a 100% organic solvent mobile phase. However, a rule to remember here is that when changing from a mobile phase of acetonitrile water to methanol water, use about 10% more methanol than acetonitrile to get comparable retention; when using tetrahydrofuran water, use about 5% less tetrahydrofuran than acetonitrile to get comparable retention.

#3: Effect of Mobile-Phase pH on Retention of Carboxylic Acids
The fact that some small molecules change their ionization state over the pH range of aqueous solutions can present challenges in method implementation, but also opportunities. With non-ionogenic small molecules, we are limited to variables, including mobile-phase composition, temperature, and stationary phase chemistry to adjust retention and selectivity. However, with ionogenic analytes (such as carboxylic acids) and amines, the pH of the mobile phase can also be a powerful determinant of retention and selectivity.

Figure 3 illustrates the general retention behavior of these types of molecules under reversed-phase LC conditions. The carboxylic acid functional group (Figure 3a) is protonated and neutral (that is, not charged) in a mobile phase with a pH that lies below the $pK_a$ of the acid (in this case, $pK_a = 5$). When protonated, the acid is less water soluble and retention will be higher compared to the case where the acid is deprotonated and negatively charged (that is, when pH > $pK_a$). The limiting retention of the two forms when the mobile phase is buffered well below or above the $pK_a$ depend on the nature of the rest
of the analyte structure. A useful rule here, then, is that the reverse-phase LC retention of a carboxylic acid-containing analyte will be significantly higher in a mobile phase buffered at a pH much lower than the pKₐ compared to when the mobile phase is buffered well above the pKₐ.

### #4: Effect of Mobile Phase pH on Retention of Amines

As shown in Figure 3b, the dependence of retention on mobile phase pH is quite different for amine-containing analytes compared to carboxylic acids. In this case, the protonated and positively charged form of the amine is favoured in mobile phases buffered well below the pKₐ of this acid, while the deprotonated and neutral form of the amine is favoured when the pH is above the pKₐ. It is this difference in the ionization behaviour of the two functional groups that leads to the very different retention behaviours depicted in Figure 3. In the case of amine-containing analytes, a useful rule is that the retention will be significantly lower in a mobile phase buffered at a pH much lower than the pKₐ of the acidic form of the amine compared to when the mobile phase is buffered well above the pKₐ.

### Relevance to Troubleshooting

On occasions where peaks do not appear where they are expected to appear in a chromatogram (whether in absolute terms, or relative to each other), the list of possible reasons is pretty long. Is the flow rate correct? Was the mobile phase made properly? Is the solvent proportioning valve working properly in the pump? This short version of the list has several elements related to potential problems with the mobile phase, and so it is very helpful to have in mind the expected effect on retention of a change in either the type or amount of organic solvent in the mobile phase and in the mobile phase pH. Knowing whether or not the magnitude of the observed change in retention could possibly be explained by a problem with the mobile phase composition can help reduce the number of likely causes of the problem to pursue, and ultimately help the analyst arrive at a solution more quickly.

### Rules #5 and 6: Mobile Phase Viscosity and Pressure Drop in Reversed-Phase LC

Pressure is an important topic in modern LC. Contemporary methods are often run at pump pressures on the order of several hundred bar, and problems with partially blocked connecting tubing, filters, or columns can result in an over-pressure situation that must be resolved before analysis can continue. We can also use measured pump pressures to our advantage during troubleshooting, however. Under most reversed-phase LC operating conditions in common use the flow in the LC system is laminar, which...
means that the pressure drop between the pressure measurement point in the pump and the detector outlet is highly predictable. For example, under these conditions, the pressure drop between the inlet and outlet of a piece of connecting capillary is related to the mobile phase viscosity through Poiseuille’s law, shown in equation 1:

$$\Delta P = \frac{128 \eta L F}{\pi d^4} \quad [1]$$

where $\Delta P$ is the pressure drop across the capillary, $F$ is the flow rate of mobile phase, $\eta$ is the mobile phase viscosity, $L$ is the capillary length, and $d$ is the capillary diameter. In the case where all of the variables except viscosity are fixed (as in a typical LC analysis), we can predict the change in pressure drop if the change in mobile phase viscosity is known or predictable, as in gradient elution, for example. Figure 4 shows the dependence of the viscosity of acetonitrile:water or methanol: water mixtures on the fraction of organic solvent in water.

**FIGURE 3:** Illustration of the effect of mobile phase pH on retention of (a) carboxylic acids and (b) amines in RPLC. The $pK_a$ of the generic carboxylic acid in this example is assumed to be 5, and the $pK_a$ of the protonated, acidic form of the amine is assumed to be 10.

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Since the pressure drop is directly proportional to the first power of the viscosity, we can expect the pressure measured at the pump to change in a way that looks very similar to these curves when we execute a solvent gradient from 0% to 100% organic solvent (or a piece of these curves if a narrower gradient is used). Indeed, the observed pressure profiles look very much like this in actual experiments, and these pressure measurements can be useful when troubleshooting LC problems. A useful rule for acetonitrile: water mixtures is that the maximum viscosity and pressure occurs around 20% acetonitrile, and that 100% acetonitrile has a viscosity that is about one-third of that of water. For methanol: water mixtures, a useful rule is that the maximum viscosity occurs around 50% methanol, and the viscosity of 100% methanol is slightly lower than that for water.

Relevance to Troubleshooting
The direct relationship between pressure drop and mobile phase viscosity means that we can use the pressure measured at the pump as an indirect (relative) measure of the viscosity of the mobile phase flowing through the system. This can be quite valuable for troubleshooting a number of problems, including leaky pump parts, malfunctioning gradient proportioning valves, errors in mobile phase preparation, and errors in method setup and implementation. Again, knowing the pressure changes we should expect to see during gradient elution, or when switching from 100% acetonitrile to 100% water better prepares us to determine when something does not look quite right; these are often the first clues needed in a successful troubleshooting adventure.

Summary
In this installment of “LC Troubleshooting”, we have discussed several chromatographic rules that approximately describe some of the behaviors we observe in reversed-phase LC systems, which are useful in both method development and troubleshooting. While there are certainly exceptions to these guidelines, and they only describe average behavior, carrying these general ideas in our chromatographic minds can make our method development and troubleshooting work more efficient by facilitating quick decisions about next steps, and identification of potential problems when the observed behavior of the system does not look quite right.

Acknowledgement
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References

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Gas chromatographs today are easy to use. With modern web-based controls and data analysis, you don’t even have to be in the laboratory to run the instrument and collect the data. In this first instalment on how this magic happens, we discuss signal generation and processing from a classical flame ionization detector (FID), so that you can use the data to make decisions. The fundamental operation and chemistry of signal generation in an FID is unchanged since the 1960s, yet the data are accessed, processed, and stored much more easily today. We will discuss analogue signal generation in the FID using historical references, analogue-to-digital conversion, and the storage and processing of digital data that happens with today’s instruments. In future instalments, we discuss how the magic of controlling today’s “smart” and remote controlled GC’s works, more detail on how the analogue signal is converted to digital data for the computer, and more on best practices and tools that our data systems can do with chromatographic data.

I write this column in my socially-distanced home office on a laptop computer, wirelessly connected to the internet, along with my cell phone streaming music in the background and playing it on a Bluetooth-connected wireless speaker. I cannot help but marvel at how easy these tasks have become since my first desktop computer in 1983, a TRS-80 Model III from now-defunct Radio Shack. I also cannot help but think about how far gas chromatographs and their data and control systems have come since I performed my first manual injection in 1985 with the chromatogram recorded on a strip-chart recorder. Some knowledge of electronics and circuits was necessary just to assemble and operate most instruments. In a recent blog post, Jim Grinias discusses the “lost art of electronics” in chromatography and analytical chemistry (1). He is correct in that today’s “plug and play” systems have moved much of this into the background. As a direct result of instruments becoming more versatile and easy to use, the need to modify them to suit a specific analysis is greatly reduced. We now think much more about modifying the chemistry (changing the stationary phase, sample preparation, or detector) than about modifying the instrument itself. However, the same electronic principles, and sometimes the same electronics as in the distant past, still form the heart of modern instrument control and data analysis systems.

Inside a gas chromatograph (GC), however, the chemistry and the fundamental electronics needed to produce an electrical signal at a detector when an analyte passes through it are not much different today than when most of our detectors were invented in the 1950s and 1960s. GC is unique among instrumental methods in that most of the classical detectors were invented or adapted to the specific needs of detection in GC, which, in this case, is high sensitivity and selectivity in a rapidly moving, vapour phase eluent stream. Using the flame ionization detector (FID) as an example, we will explore how the detector generates a signal, what that signal is, and how it is processed into the chromatograms and other information that is stored and provided by a modern data system. We will do this by walking through the evolution of data processing in GC from the early days to today, examining how the various components work and how they were ultimately integrated into the instrument.

A classical schematic of a flame ionization detector is shown in Figure 1, adapted from early works (2–4). There were several early designs, including single and multi-jet. Today’s FIDs use a single jet design, as seen in Figure 1. In short, the column effluent is mixed with hydrogen and air (or, in the case of some of the early work, hydrogen and nitrogen, the most common carrier gas back then), and ignited, generating a flame between two electrodes. The flame temperature of about 2000 °C is not sufficient to ionize water vapour, but is sufficient to ionize a small portion of the carbon dioxide produced by the combustion of organic compounds. The ionized CO$_2$ present in the flame then allows the circuit to be completed and electrical current to flow. The amount of current (amperes) is proportional to the mass of CO$_2$ generated in the flame.
Variations in the chemistry of combustion reactions in the flame lead to the need to determine response factors, and provides the selectivity of the FID for organic (carbon-containing) analytes (5). A complete description of how to operate an FID can be found on LCGC’s learning platform, CHROMacademy (6). While the techniques and electronics we use to measure and analyze the signal have changed over the decades, the fundamental combustion chemistry that generates it has not changed.

The electrical current produced by the FID is usually measured in picoamperes (pA) by an electrometer that may also convert the current into a voltage for output to a data system. The output of an FID is an analogue signal, in which the output, an electrical current, varies continuously with the input, the mass of carbon entering the detector. This signal must be further processed in order to produce a chromatogram, perform calculations and store the data.

Figure 2 shows a simplified block diagram of the data processing steps in GC over the years. First, the current is amplified (think about an old stereo with an amplifier) and then may be converted to a voltage. The electrometer output (volts or amperes) is represented by the third block in the middle of Figure 2. In predigital-age GC, shown by the green box in Figure 2, the voltage was plotted against time on a continuous roll of paper using a strip-chart recorder that was connected to the electrometer by a cable. The voltage signal (y-axis) and time (x-axis) scales could be adjusted to obtain a proper appearance for the chromatogram, but there was no data storage capability. If you wanted to make the peaks appear larger or smaller to fit on scale, you usually had to rerun the sample. Quantitation was most often done using peak height (again, the scale was limited to what would fit on the paper), which was much simpler than peak area. To measure the peak area, one could count the little square blocks on the paper under the peak, carefully cut the peak out with scissors and weigh it, or use a challenging device called a planimeter (7).

The analogue output and strip chart recorder combination was the most common means of data collection in GC until the 1980s, when microprocessors became available in desktop or bench top computers. In the 1980s and 1990s, digital electronic integrators, specialized small computers, were commonly used for data collection and analysis. Like a strip chart recorder, these devices printed chromatograms on rolls or sheets of paper. In addition to printing out the chromatogram, the raw data could be digitized and stored for later processing or analysis in computer memory within the integrator.

To understand how analogue signals are transferred to a digital electronic computer or integrator, we need some definitions of the language and standards for digital data storage and transmission. Digital signals use binary (or base-2) numbers and logic. A binary number is code for a simple switch that may be either ON (1 or one) or OFF (0 or zero). A single binary data point is called a bit. For example, when we discuss internet service provider speeds at 100 Mbps, they mean that you can transmit up to 100 megabits (100 million bits) per second. A string of eight bits is termed a byte. A byte of data can be thought of as the equivalent of a single alphanumeric character (a letter or a number). A memory card with one GB of storage space can

**FIGURE 1:** Diagram of a classical FID showing the electrical circuit and how the flame completes the circuit and generates the current that is measured.

**FIGURE 2:** Block diagram showing the components of a modern GC and data system. Coloured boxes show the components internal to the GC as the generations of instruments have progressed. Green = 1950s to 1980s. Blue = 1980s to Present.
hold one gigabyte or approximately one billion characters of information.

To standardize the use of alphanumeric characters in digital storage, nearly all computers use ASCII or the American Standard Code for Information Interchange, which provides a seven-bit representation of all of the letters, numbers, and characters on a standard United States keyboard plus representations for various control functions such as carriage returns and line feeds. When eight-bit microprocessors, such as the Z80 and 8088, the precursors to the microprocessors in today’s personal computers, were developed in the 1980s, ASCII was extended to eight bits, allowing for additional special characters. ASCII is still in use today, included as the first 128 characters in the Unicode standard that now includes over 140,000 different characters and symbols (8). The Unicode standard is how your cell phone knows which emoji is which, so your smiley face emoji does not turn into a frown (or worse) on someone else’s phone.

The second aspect of communicating between a data system and an instrument, besides having a standard code or rubric for converting letters numbers and symbols into bits and bytes, is a common standard for transferring the actual electronic signals. This is usually accomplished using a serial port on the computer. There have been many standards used over the decades, but the most common for GCs are RS-232, GPIB, USB, and Ethernet. RS-232 uses a 9- or 32-wire connector, and a ribbon cable to transfer the signals with one of the wires actually carrying the signal and the others related to a “handshake” between the devices in that both would have to trade the correct separate signals to demonstrate that they were ready to send or receive data. RS-232 is the classical serial port used in personal computers, but that port is slow by today’s standards as the single signal wire means that one bit is transferred at a time, hence the term “serial”. With the other control lines available, instrument manufacturers often modified the standard RS-232 connections to make their instrument and data system connections proprietary. GPIB, or General Purpose Interface Bus (also called HPIB, Hewlett Packard Interface Bus and IEEE-488) took serial communications one step further, with eight signal lines, allowing the transfer of a byte of data instead of a bit at one time. In the 1980s and 1990s, GCs with less requirements for fast data transfer often used RS-232. Meanwhile, many GC–mass spectrometry (MS) systems with need for higher throughout used GPIB. Today, most instrument communications are based on USB and Ethernet, which provide much greater speed and more strict standardization, so there is much better connectivity of instruments and data systems between vendors. While Ethernet and USB are much faster RS-232 and GPIB, the same basic principles apply. Both instrument and data system must be ready to send and receive data, the connection must be working, and the data must be transferred and
stored according to industry standards.

The third necessary component is an analogue to digital converter that converts the analogue signal into the binary digital numbers for the computer to store and process. An analogue to digital converter may be included in the GC itself, or it may be added as a separate converter box. External converters were common in the 1990s and 2000s, with an example shown in Figure 3, showing a common data system interface box of 1990s vintage. The right-side image is the rear of the box where all the connections are shown. The left-side image shows a side view of the box with the cover removed to show the electronic circuitry. The back shows several types of connectors that made this interface mostly universal in that it worked with almost any GC on the market. The inputs on the top left are analogue detector inputs. These could be connected directly to the analogue detector outputs on the GC. Below these are connectors for remote starting and stopping the instrument, controlling an autosampler, and several connectors for activating valves or switches on the GC. To the right are both types of serial connectors, RS-232 and GPIB, that connect to a personal computer data system.

Looking at the left side of Figure 3, we see an electronic circuit board showing the various components allowing this box to function. Some key components include the analogue-to-digital converter circuitry in the top right, within the silver rectangle. The large square microprocessor in the bottom middle is a Zilog Z80 microprocessor, already mentioned in this column. This was the microprocessor used in my first computer, revealed at the beginning of the article. There were no graphics, the display was monochrome, and a separate modem allowed me to communicate with other computers over the phone lines at a whopping 300 bits per second. In the 1990s and 2000s, while no longer used in personal computers, the Z80 was commonly used in digital electronic integrators and today it is still used in many devices in the “internet of things”, such as appliances. The white chip to the left of the Z80 contains the box manufacturer’s own firmware. The large chips between the Z80 and the cable connectors on the right provide the interface between the microprocessor and the communication cables to the PC. Finally, the rest of the chips provide memory. In short, these control boxes were computers in their own right that provided an interface between the GC and the data system.

Figure 4 shows the back panel of a new GC purchased in 2019, showing the connections and capabilities that are now inside the cabinet of a modern GC. In this case, all of the functions of the control box shown in Figure 3 are now internal to the GC. Several control ports, along with input and output ports, are shown at the top of the panel. These allow the GC to send and receive signals from other devices such as a headspace sampler. This GC has a specialized port for an auto-sampler and seen with the cable attached. This port uses Ethernet to communicate with the computer, which is also seen with the cable attached. The analogue-to-digital converter is now contained within the GC. If needed, a classical analogue output is still available. Note that these functions are all very similar to those shown on the control box in Figure 3.

Looking back at Figures 3 and 4, we saw several additional connectors for control lines used to send and receive various commands to or from the instrument. Most commonly, these are based on transistor to transistor logic (TTL) that allows each to act as a switch that is either “on” or “off” or “1” or “0”, respectively. Each of these lines represents an opportunity for the user to activate or deactivate an electronically actuated switch or valve in the instrument or to start or stop an external device. This logic is also used to send the “start” signal between the data system and the instrument to signal the start of a run or a “stop” signal in either direction to indicate the end of a run. These lines are connected to the GC through a remote control port, such as the one shown in Figure 5, from a 1990s-era GC. Each pin on the control port activates or deactivates a certain function on the GC, such as “start” and “stop”. There are multiple lines for each command to allow
multiple devices to communicate. This port has lines for ready; they indicate that the instrument or device is ready to run, that it can both send and receive start signals, that it can send out information on its configuration, and ground.

In this installment, we have discussed the basics of how a GC generates signals and transfers them to a data system to generate your data. By looking “inside the box” of a data transfer device, we have seen how the analogue signal converts to a digital signal and transfers to the data system using standardized digital communications protocols. In future installments, we will look more closely at the processes for controlling the GC from the data system or from anywhere in the world and how “smart GCs” work, the process of analogue to digital conversion and at best practices and tips for data systems and analysis in GC.

Remember that even with all the new technology in the foreground, a GC is still performing the same basic functions that it has done for decades: injection, separation, and detection. Fundamentally, an inlet, column oven (with a column in it), and detector have not changed. The basic digital and analogue electronics that provide our ability to collect data accurately, precisely and conveniently are still there under the covers and require our understanding.

References

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Field-flow fractionation (FFF) offers a solution for the characterization of submicron particles and macromolecules. To discuss flow FFF, multi-angle light scattering (MALS), and more, LCGC sat down with Christoph Johann, global product manager at Wyatt Technology. In this thought-leader series, Johann talks about Wyatt’s history with FFF products and methods, what the technology means for the company, and his expectations for FFF. He also discusses the benefits of coupling light scattering online, FFF’s role in the pharmaceutical industry, its acceptance by regulatory agencies, the company’s new model Eclipse NEON, and more.

LCGC: You are the product manager for FFF products at Wyatt Technology—why is Wyatt investing in FFF?

Christoph: Wyatt’s MALS detectors are widely utilized in conjunction with size-exclusion chromatography (SEC) to analyze the molar mass, size, and conformation of macromolecules. However, SEC has a limited upper size range, and it prevents using SEC-MALS to characterize larger species such as very high-molecular-weight polymers, liposomes, viruses, large protein aggregates, and similarly sized analytes.

On the other hand, FFF is a separation technique that covers a range of 1 to 1000 nanometers, and it can be coupled to Wyatt’s online instruments. FFF-MALS determines with high-resolution the absolute and fully quantitative size distribution for various nanoparticles and macromolecules, which is not possible with SEC. Yet, it’s not subject to the limitations of small particle numbers that affect other methods such as nanoparticle tracking techniques.

So, FFF greatly expands the range of applications for our light-scattering detectors, and we see some really important markets opening up for these products.

LCGC: Although FFF has been around for more than 50 years, it’s still not a mainstream method. What are your expectations for the future development of FFF?

Christoph: Flow FFF is used in food, pharmaceutical, environmental, and polymer applications, but it has been perceived as a complex method and adoption has not been comparable to GPC. I believe it’s ready to break into a wider market as a result of two key factors: the advent of nanoparticle drug and gene delivery technologies that require the capabilities of FFF coupled to MALS and dynamic light scattering (DLS) to bring the promise of these medical advances to patients; and upcoming instrument and software improvements that will eliminate the issues of complexity and user intimidation, so the method can be transferred easily from R&D scientists to quality-control labs.

LCGC: Where do you see the increasing need for FFF in the pharmaceutical industry?

Christoph: There are two parallel and similar paradigm shifts going on at full speed. In traditional small-molecule pharmaceuticals, more drugs are formulated as nanoparticles, whether as emulsions, nanosols, or...
Encapsulation in liposomes or other nanocarriers, including lipid nanoparticles, polymer micelles, polymersomes, albumin particles, polyplexes, etc.

In the biopharmaceutical world, commercialization of gene therapies—the delivery of DNA or RNA by viral or non-viral gene vectors—is in high gear. SEC-MALS is suitable for small vectors like adeno-associated virus, but larger vectors such as lentivirus or adenovirus require separation by FFF. Non-viral vectors are very similar to small-molecules nanodrug-delivery systems, e.g., lipid nanoparticles or polymersomes. In both cases, the trend is to deliver therapeutic payloads in carrier vehicles that are in the size range of 30 to 300 nanometers, which is very different from the size of current drugs based on small molecules, peptides, proteins, or microparticles.

The standard tools in place for characterizing these new modalities—whether batch DLS or nanoparticle tracking analysis for nano drug delivery systems (DDS) or qPCR and ELISA for gene vectors—are simply insufficient to meet the challenges and analytical needs presented by these complex therapeutics. FFF with MALS, DLS, and spectroscopic detectors provide a powerful and versatile characterization platform that is perfectly matched to these products.

**LCGC:** What is new with Wyatt’s Eclipse flow FFF products?

**Christoph:** I am really excited about the next-generation Eclipse NEON. It was redesigned to incorporate Wyatt’s advanced NEON platform in order to meet critical requirements for usability, robustness, and performance. All of Wyatt’s online detectors, including the DAWN, Optilab, and ViscoStar, have already been released in this platform and the benefits have been enormous in the marketplace.

The most prominent new feature is a front-panel interface with built-in intelligence implemented on a 10-inch multitouch display. The system health indicators eliminate the guesswork—they let the user know whether the instrument is ready for an optimal run, or it provides guidance on what to do to achieve ready status. What’s more, the new interface enables manual control of the Eclipse FFF controller directly from the instrument display, eliminating the need to use the software for basic setup, cleaning, and maintenance. Our aim is to ensure a perfect analysis each time, with a streamlined workflow and maximum reliability.

On top of that, we have two new hardware features, the dilution control module (DCM) and the Eclipse Mobility. DCM reduces the sample dilution that normally occurs at the outlet of the channel. The dilution can be reduced by the DSM module with a corresponding increase of the detector signal by a factor of up to 10X for higher sensitivity. Fractions that are collected downstream of the last detector, using a standard fraction collector, come out with the same increase in concentration, so the process is much more effective.

Eclipse Mobility combines flow FFF with an electrical field by adding two electrodes to the channel. Applying an electric field changes the retention time of sample components according to their charge, in addition to size-based retention. From the resulting shift in retention time, the electrophoretic mobility and zeta potential can be calculated. We have shown in a peer-reviewed publication that the values are consistent to those obtained by electrophoretic light scattering (ELS).

The advantage of the Eclipse Mobility over standard ELS is that the charge of several populations within a mix can be determined. In a semiquantitative way, it can be established whether the charge is uniform within a broad distributed sample or whether it isn’t homogeneous. This helps to understand interactions between nanoparticles that have different or opposing charges. One example is a conjugation of RNA or DNA with a positively charged lipid nanoparticle.

**LCGC:** How does the new model relate to your previous offering?

**Christoph:** The Eclipse NEON replaces the Eclipse Dualtec and Eclipse AF4; they supported different types of separation: center-downstream injection and tip injection. With the Eclipse NEON, both center and tip injection are supported for analytical, semi-prep, and frit-inlet AF4 channels, and tip injection for hollow fiber (HF5) channels or SEC. An automated dual-channel switching configuration is still available.

**LCGC:** Where is FFF-DLS-MALS in terms of acceptance by chemistry, manufacturing, and controls (CMC) departments and regulatory agencies?

**Christoph:** The need for FFF-MALS-DLS in characterization for regulatory filings of drugs, and eventually quality control of nanomedicines, is fully recognized by regulatory agencies and the institutions and organizations developing standards for the pharmaceutical industry. These organizations have been developing protocols and technical documents, as well as publishing reviews and introspection papers, to support the adoption of this method across the pharmaceutical industry. I can say with confidence that it will become an essential characterization tool for nano DDS and gene vectors. The improvements we are making to performance, simplification, robustness, and GMP compliance should meet the needs and expectations of CMC departments, regulatory agencies, as well as quality-control departments.
What is on Your HPLC Particle? A Look at Stationary Phase Chemistry Synthesis

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In 1973, Waters Corporation launched the first commercial 10-μm particle C18 column using a bonded monofunctional silane (μBondapak C18). Almost 50 years later, the C18 phase is still the reigning champion in the reversed-phase liquid chromatography (RPLC) arena, and many chromatography companies are still bonding using the same or a very similar synthetic approach. Several innovative bonding chemistries have been developed over the years to mitigate some of the common problems associated with the traditional C18 bonding approaches. These may include low and high pH stability, undesirable silanol activity, and a lack of polar retention. Nevertheless, particle technologies based on silica have received the most attention because of their undisputed chromatographic benefits. Silica supports offer high mechanical strength, allowing the formation of packed beds that are stable for long periods under high operating pressures. Controllable surface area, diversity in particle morphology, and higher efficiency values when compared to other support materials are just some of the advantages of silica-based columns. Advances in platforms that drift away from legacy products in terms of both the solid supports and in device architecture are trending in the literature. Some of these areas of interest include monoliths, open tubular columns (OTCs), microchip based columns, metal-organic frameworks (MOFs), and overall column and instrument miniaturization. Such developments will challenge traditional approaches toward surface chemistry modification. This article will review historical bonding techniques still in use for manufacturing high performance liquid chromatography (HPLC) stationary phases today, and also examine some emerging technologies that may be able to tackle unmet needs in novel platforms and phase construction.

Various advances in silica manufacturing have paved the way for modern chromatography. Since their inception in the 1970s, superficially porous particles (SPPs) have offered good performance and are indispensable in high-speed high performance liquid chromatography (HPLC) (1). Recent trends show the preference towards using SPP, especially in emerging markets such as cannabis (2); nevertheless, conventional particle sizes are still beneficial due to their higher surface area and their high loadability required for preparative scale applications.

These advancements on solid supports have contributed great benefits to every industry where chromatography takes place, but novel approaches to functionalization of such platforms has remained stagnant for decades. According to the United States Pharmacopeia (USP), there are 858 C18 liquid chromatography phases registered under code L1 (octadecysilane chemically bonded to porous silica or ceramic microplates, 1.5 to 10 μm in diameter, or a monolithic rod) (3). The vast amount of commercially available C18 columns are constructed on a wide range of solid supports, and may exhibit ancillary options such as endcapping and aqueous compatibility (AQ). They can also be mixed with other reversed-phase liquid chromatography (RPLC) phases. Although silica particle manufacturing has become more normalized in the last few decades, these subtle differences in commercial C18 make it extremely difficult for the novice, and even the seasoned chromatographer, to "grab a column and go" for a given application.

Silica is an amorphous polymer of silicon and oxygen. This polymer's
surface contains reactive silanols (Si-OH) that offer a number of possibilities for the synthesis of chemically-bonded phases. Organosilanes have been used as early as the 1950s to functionalize filter paper for the separation of steroids (4). In 1973, Locke correctly predicted that organosilanes would transform HPLC columns via chemically-bonded phases, mentioning that polymerization of silanes would be the synthetic route taken in general with chemical reactions being carried out to produce a primary organosilane layer (5). At the same time, he hoped for new developments in bonding stationary phases through the introduction of specific groups onto the organosilane bonding reagents; however, almost half a century later, organosilanes are still being grafted onto silica particles via the same chemistry described back then. Although new synthetic approaches have been applied to reversed-phase ligands, the bulk of the commercially available phases are still manufactured via conventional methods (6).

**C18 Ligand Chemistries**

Using reactions developed by John Speier at Dow Corning, USA, Waters' scientists successfully synthesized octadecyldimethylchlorosilane (ODS), leading to the first commercial monomeric-bonded C18 column in 1973 (7). Since then, traditional bondings of reversed phases use a monofunctional silane in order to maximize ligand coverage and avoid unwanted polymerization that could affect batch-to-batch reproducibility. A typical monofunctional silane will yield a ligand density of around 3-4 µmol/m² under optimal bonding conditions, leaving behind up to 50% of the original amount of silanols based on an average of 8 µmol/m² on a typical silica surface. HPLC phases labelled as “end-capped”, “maximum coverage”, or “high density” still abide by this maxima, and any residual silanol would still be able to interact with analytes, contributing to the overall adsorptive properties of the bonded phase.

Over the years, traditionally-bonded phases have suffered from disadvantages tied to the use of silica as a solid platform. The tethering of the ligand to the silica surface is subject to hydrolytic cleavage at pH < 2, leading to loss of bonded ligand while silica particles are prone to dissolution at pH > 8. However, several bonding chemistries have been developed to circumvent such disadvantages, and numerous commercially-available phases are able to mitigate these problem areas.

Figure 1 shows some of the innovative bonding constructs for several C18 phases. Element (a) shows a conventional monofunctional octadecylsilane and the most common phase available on the market. Element (b) shows a difunctional silane that is formed when a dichloro- or a dialkoxysilane is chemically bonded to silica; this double attachment is thought to increase the stability of the ligand at lower pH values and decrease phase bleed. Some manufacturers claim to use trifunctional silanes (not shown), but the exact control of tridentate reactions with the silica surface is still open to debate, and may lead to lower reproducibility of manufacturing. Element (c) shows a “bulky” silane where an isopropyl or isobutyl group hinders the siloxane linkage from hydrolytic cleavage at
low pH values (8). Element (d) shows a “bridged” phase, where a bidentate organosilane is grafted on a silica particle, better shielding the surface from dissolution at a high pH. This latter bridging (crosslinking) technology can extend from one to several protective organic and inorganic layers, rendering high stability to the phase overall (9). This is not to be confused with hybrid silica particles where the organic moiety is a main component of the particle construction, and not just surface functionalization (10).

Element (e) in Figure 1 shows an alkyl chain much like its C18 counterparts, but contains a polar group intrinsic to the chain (amide, urea, and carbamate). These polar-embedded groups (PEG) have led to a new class of phases that offer some surface silanol shielding and additional polar retention (11). The polar-embedded moiety yields good peak shape towards basic analytes while making the phase compatible in 100% aqueous mobile phases without the “dewetting” effect. These polar functionalities can be obtained via a single step, using a pre-formed PEG containing silylating ligand directly onto silica, or a multi-step process, as outlined in Figure 2 (12). Amides and hydroxyl groups react with acid chlorides to yield amides and esters respectively; however, since ester groups are more unstable at low pH, amide linkages have been the preferred one among HPLC phases, along with sulfonamides (13). However, due to their toxicities, high reactivities, and non-selective behaviours, acid chlorides are rarely used in amide coupling reactions currently. Instead, several peptide-coupling reagents have been developed over the years to fulfill the safe and efficient processes required in drug development, and eventually applied towards manufacturing HPLC phases. Some of these popular reagents are highlighted in the following manuscripts for the construction of chiral and achiral ligands: 1,1´-carbonyldiimidazole (CDI) (14), hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) (15), 1-hydroxybenzotriazole (HOBT) (16), and N-Ethyl-N´-(3-dimethylaminopropyl) carbodiimide (EDC) (17).

The other major categories of polar-embedded groups are ureas and carbamates. They are obtained by reacting isocyanates with primary amines and alcohols respectively. These functional groups provide additional polar interactions as hydrogen bond acceptors and less ionic interactions when compared to alkyl phases, leading to selectivity differences for polarizable compounds while improving peak shape of basic analytes (18). Additionally, carbamate linkages have been used specifically in the derivatization of chiral selectors such as Pirkle type (19) and polysaccharides due to the abundance of hydroxyl groups while enhancing their chiral recognition (20).

**Silanization Chemistries**

While the diversity of column chemistries for all types of separation modes is never ending, the chemistry of ligand attachment or grafting is not nearly as varied. Chlorosilanes, alkoxysilanes, and silazanes have been the workhorses of silica functionalization. They react through hydrolysis, condensation, and polymerization reactions, where a new siloxane bond (Si-O-Si) forms while yielding a small molecule, typically hydrochloride gas, diethylamine, methanol, ethanol, or water. The result of reacting an organosilane with silica’s surface is not only the bridging between organic and inorganic materials, but also is what imparts the main mode of separation to each stationary phase.

The advance of silicon-related technologies in material science has driven silane ligand synthesis. However, recent acquisitions of two major silane manufacturing companies leads to uncertainty of the current silane portfolio (21,22). Silane synthesis, although straightforward, suffers from one caveat: purification. Flash column chromatography is the method of choice when purifying a newly-synthesized compound from a mixture. However, a desired chlorosilane or alkoxysilane may permanently bind to the silica gel, leading to poor recoveries. Although some protocols exist to passivate silica gel from interacting with the silane ligand (23), the bulk of the reactive organosilane purification have relied on
simple distillation. Such an approach leads to the confinement of commercially available ligands to a specific molecular weight range and containing functional groups that are thermally stable enough to endure purification by this technique. An evolutionary bonding technology uses hydrosilanes, which Pesek developed through the development and the application of hydrosilylation chemistry in the production of HPLC stationary phases (24). The “Type C” silica possesses silica hydride (Si-H) at the surface of the particle and lacks the negative effects of silanols found on Type A and Type B silica. In order to functionalize this surface, a terminal alkyne or alkene will undergo hydrosilylation with Si-H in the presence of a platinum metal catalyst, leading to a phase with improved resistance to conditions that may cause hydrolysis in Type B silica columns. This is partially due to the much more hydrophobic surface offered by the silicon hydride moieties versus the usual hydrophilic silanol (25).

Figure 3 displays the reaction outcomes of alkene and alkyne hydrosilylation of a hydride-terminated particle. Alkenes may undergo a double hydrosilylation resulting in a bidentate attachment onto the surface, although most of the ligand will be singly attached via silicon-carbon double bond (Si=C) due to steric hindrance (26). The presence of hydrides, or the lack of silanols (<5%), and the strong Si-C or Si=C resulting from hydrosilylation is what gives these phases their chemically stability and interesting selectivity for chromatographic applications. Several metal catalysts have been used throughout the years but platinum remains the gold standard. Hexachloroplatinic acid (H2PtCl6), also known as Speier’s catalyst, and later the silicone-soluble Karstedt’s catalyst was adopted for this type of chemistry (27).

A look into the hydrosilylation platform synthesis leads to some unanswered questions and challenges. The first challenge is regarding the complete removal of the platinum reagent after the bonding reaction. The catalyst may form colloidal platinum that could deposit deep inside the particle making it almost impossible to remove; therefore, defeating the purpose of using Type B silica as the very first solid support (28). The second challenge is the catalyst’s selective functional group tolerance, leaving the weak anion-exchange and many mixed-mode phases out of the hydrosilylation platform (29). The third challenge,
which it is still subject to debate, is whether the coverage of Si-H is enough (~95%), hindering as many surface silanol and the inertness of such moiety during the lifetime of the column (30,31). The last challenge is phase availability, and the difficulty of finding equivalent columns by other manufacturers (32).

**Functionalization of Novel Solid Supports**

The most common formats for analytical columns remain 4.6 mm and 2.1 mm i.d. dimensions, although smaller, capillary-size columns have found a place within the HPLC arena, especially for complex biological applications. Capillary (0.3–0.5 mm i.d.) and nano formats (0.075–0.1 mm i.d.) have become more popular in the last few years, and many vendors offer a decent variety of stationary phases in these dimensions. The sub-millimeter internal diameter columns are packed with functionalized silica particles via slurry methods similar to their larger dimension counterparts; nevertheless, this process has shown to be challenging, and much research has been devoted into this field (33,34). As an alternative, other phase supports, including monoliths and pillar arrays, have gained traction in recent years, and although both of them promise either lower backpressure or higher efficiencies than the particle packed beds (35), very little has been mentioned in regards to the pragmatic functionalization of such formats.

Monolithic beds are usually created in situ by free radical polymerization of monomers in the presence of porogens. Although thermal polymerization is a viable option, temperature fluctuations can occur in the confined spaces of the fluidic path, altering bed homogeneity along the column (36). Monomers can be organic (styrene, or acrylate-based) or siliceous in nature and offer the right amount of synthetic handles to permit surface functionalization. However, due to the nature of the inorganic monolith requiring a high temperature calcination step during its manufacturing, the appropriate functionalization must take place in situ as well. Hilder and co-workers developed a flow method for the ODS grafting on a 100 x 4.6 mm i.d. silica monolith, and its performance benchmarked against commercial C18 monolithic and a particle packed column (37). Since then, similar flow-like protocols have been applied to other column dimensions; however, slow reagent flow rates (mL/min) and high temperatures are still needed to graft a common C18 ligand, leading to bonding times of up to 24 h for a single analytical size column (38).

Organic-based monoliths offer a wider variety of synthetic functionalization that are out of the scope of this article; however, their pervious behaviour towards organic solvents and their hindered mass transfer kinetics for small molecules result in the chromatographic preference towards silica-based monoliths. For more information, El Rassi and co-workers recently published a review discussing several post-polymerization functionalization strategies (39).

Another column format involves microfabricated devices with microfluidic channels. Sepaniak and co-workers describe the functionalization of a pillar array architecture, which includes submerging the silicon oxide layers of the pillars in pure octadecyltrichlorosilane (OTS) and heating it to 170 °C for 2 h (40). On the other hand, De Malsche describes a flow method to functionalize the silica porous layer of radially elongated pillars (REP) where a solution of ODS is infused under 40 bar of pressure overnight. Although this method successfully grafted a C18 phase, the process only allows for a single “column” functionalization in about a 24-h window (41).

As novel formats show promising chromatographic benefits, whether they are open tubular columns, chip-based platforms, or overall miniaturization of the column compartment, the grafting protocols must deviate from legacy silanization chemistry to facilitate their large scale manufacturing while securing low cost and high batch-to-batch reproducibility. Vendors are currently able to manufacture functionalized silica in kilogram scale within 24 h, which can be used to pack hundreds or maybe even thousands of conventional analytical or capillary size columns. New solid supports and platforms such as metal-organic frameworks will obligate scientists to figure out interesting ways to functionalize them, given their lack of attachment points while at the same time widening their applicability in separation science (42).

**Conclusion**

Neue wrote that, when compared to gas chromatography (GC), variety in HPLC stationary phases is not necessary since scientists have control over the mobile phase composition, which is a powerful tool over the selectivity of the separation (43). However, we are seeing an influx of novel stationary phases in the literature and the market, especially in the mixed-mode arena, to fulfill wider customer demand for solutions. Many innovative chemistries have been designed to tackle the common problems of reversed-phase associated with silica as solid support from bulky silanes to polar embedded ones; nevertheless, with the exception of hydrosilylation platform, the actual silanization technique has remained
largely the same for almost half a century. Miniaturized chromatography systems are already on the market, but the bulk of surface modification processes cannot be adapted into these new formats, especially at the rate of customer needs. Flow functionalization has shown to be an alternative to conventional particle bonding methods but reactions times per column manufacturing remain excessively high. Historically, silanization techniques have been developed by both academia and the silicon industry; furthermore, these new methods are readily available in the literature and require metal-free conditions, room temperature, and faster kinetics. It is time for chromatography firms to implement such innovative grafting protocols.

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The Development of Biopharmaceuticals and the Impact on Separation Scientists

Separation science is key to the successful development of biopharmaceutical medicines. Understanding the challenges of biopharmaceutical analysis, optimizing column design, and improving experimental conditions help minimize production costs while satisfying regulatory requirements. Here, LCGC speaks with Paul Ferguson, New Modalities Product Development, AstraZeneca, about the shift toward alternative chromatography approaches, tips for analyzing oligonucleotides, and regulatory implications on biopharmaceutical analysis.

**LCGC:** The growth of biopharmaceuticals has had a significant effect on the therapies available for patients. What impacts are you seeing within the pharmaceutical industry across various departments?

**Ferguson:** Traditional small molecules will always have a place in the development of new medicines, but there is an increasing focus on biomolecule development including oligonucleotides, synthetic peptides, proteins, and monoclonal antibodies, as well as even more complex combinations like antibody–drug conjugates. As the COVID-19 pandemic has shown, vaccines also play an important role in that portfolio, too.

Increasingly, complex drug formulations are also becoming more prevalent. Such formulations are designed to better reach and act at the required target in vivo and include drug-polymer conjugates, lipidic formulations, and nanoparticles. These new modalities have diverse requirements and pose various chromatographic and sample preparation challenges. As a result, there is a move away from the traditional reversed-phase liquid chromatography used for small-molecule development towards alternative chromatographic approaches such as ion exchange chromatography (IEX), size exclusion chromatography (SEC), supercritical fluid chromatography (SFC), and electrophoretic characterization techniques to ensure medicine quality and patient safety.

**LCGC:** What chromatographic challenges do oligonucleotides pose, and what approaches will be important to address these challenges?

**Ferguson:** The first point to consider for manufacturing QC analysis is determining the best time to use manual versus automated sample preparation solutions. In my environment, we always have a manual sample prep approach for QC methods. For high-volume products, an automated method is often also required. With automation, it is important to consider many aspects including required throughput, mode of sample preparation, consumable costs, and vendor support infrastructure.

One interesting development in automation is the use of “cobots,” or collaborative robots, to perform unattended, repetitive tasks. Cobots are able to perform tasks alongside an analyst and may be mobile—having vision systems to help accurately locate themselves within a lab. Unlike robots that perform one task repeatedly, cobots can be programmed to do multiple tasks and act upon experimental outcomes. A good example of this may be found at Liverpool University where researchers are using cobots in the lab to identify and design new polymeric and inorganic materials.

**LCGC:** What aspects of sample preparation are being introduced into QC analysis, and is there an impact on other facets of analytical procedures?

**Ferguson:** Approximately 800 oligonucleotides are currently in development as drug therapies from pre-clinical to Phase 3 studies. These molecules include antisense oligonucleotides (ASOs), siRNA, and mRNA, and are exciting because they can provide a highly targeted approach to treating particular diseases.
Oligonucleotides possess a phosphate backbone connected to sugars and nucleotides, which makes them highly polar. The sugar ring is often modified to decrease non-specific protein binding and increase their accuracy in targeting specific proteins. The phosphate backbone can be modified to prevent unwanted degradation by enzymes.

There are some significant chromatographic challenges associated with oligonucleotides. They are manufactured through solid-phase synthesis using phosphoramidites. Initial purity of these is critical to the final purity of the oligo. The longer the oligo, and the more impure the phosphoramidite—the higher the impurity levels in the final product.

There are also process-related impurities generated through modifications. One key modification is the derivatization of the phosphate backbone with a sulfur atom to increase chemical stability. This induces chirality in the phosphate group, which can lead to thousands of diastereomeric impurities that are impossible to resolve chromatographically.

The analysis of oligos is typically done using a liquid chromatography/mass spectrometry (LC/MS) approach based on reversed-phase chromatography with ion-pairing reagents such as trimethylammonium acetate or tetrabutylammonium acetate. Occasionally, the acidic modifier hexfluorooracopropanol (HFIP) is used as an additive to enhance ion pairing. If you’re using these methods, the cleanliness of the LC/MS instrumentation is critical. It often takes up to a month to clean and remove potential metal contamination in the system, which can potentially complicate the identification or quantification of oligonucleotide impurities through adduct formation. Standard C18 columns are often used as the stationary phase, but it would be interesting to see the development of columns that could provide increased retention with lower amounts of ion-pairing reagents because higher ion-pairing reagent concentration can compromise MS sensitivity.

**LCGC: Are you seeing any impacts from recent regulatory introductions in terms of analytical procedures or in terms of the approach to developing assays?**

**Ferguson:** Quality by Design (QbD), introduced by the US Food & Drug Administration (FDA) initially focused on process chemistry and the resultant medicine quality, but was later extended into analytical chemistry processes such as method development. QbD nowadays is rarely commented on by the FDA with regard to method development because these processes are so well embedded within pharmaceutical companies. Regulatory agencies expect that companies will use a rational method development process including aspects such as forced degradation of molecules, screening of columns and mobile phases, in-silico retention modeling and in-silico robustness assessment.

Additionally, the ICH Q12 guideline focuses on the technical and regulatory considerations for pharmaceutical product life cycle management. This is for post-approval method changes and is meant to guarantee that medicine quality isn’t impacted by method parameter changes. In-silico method development tools aid compliance with ICH Q12 and help analysts understand how methods might vary if parameters are changed within a chromatographic procedure.

Currently under review is the ICH Q14 Analytical Procedure Development, which focuses on enhanced method flexibility.

It is hoped that, for example, based on established scientific theory, UHPLC methods may replace older HPLC approaches without the need for complete revalidation and full re-registration of methods with regulatory bodies.

**LCGC: What’s the future of reversed-phase columns for small and large molecule analysis?**

**Ferguson:** For large molecules, only a small fraction of the analytes structure can interact with the stationary-phase ligands at any moment in time within the chromatographic column while the rest of the molecule remains in the bulk mobile phase. Therefore, it can be difficult to modify selectivity for large molecules by varying the stationary phase alone under reversed-phase conditions. Other separation approaches become increasingly important such as SEC which discriminates based on molecular size or shape. For polymer-drug conjugates, SFC is becoming increasingly popular for characterizing the polymeric parts of the molecule and more focus is required by vendors on designing stationary phases to expand capability. Another exciting area is microfabricated pillar arrays. There are a number of articles describing biomolecule separation using pillar arrays, which give incredible chromatographic efficiencies to aid biomolecule characterization.

The smallest particle size routinely used in UHPLC is 1.5 µm, which places significant strain on instruments because the pressure generated from those particle sizes is very large. Moving to smaller particles to generate higher chromatographic efficiencies is not beneficial because it will simply make the instrumentation more prone to breakdown. However, I expect to see more developments with superficially porous particles (which can provide higher chromatographic efficiencies compared to fully porous particles of the same diameter and back pressure) such as increased high pH stability and alternative selectivities.

**LCGC: What are some bottlenecks in chromatographic method development and how can technology or a better understanding of science address them?**

**Ferguson:** Method development relies on selecting the right starting conditions from the outset, namely the stationary and mobile phases. An appropriate mobile phase pH can be guided by knowledge of analyte functionality and in-silico tools are available to aid with this. However, it can be much more difficult to choose the “best” stationary phase as there are literally hundreds to choose from. Therefore, many companies use a fixed set of mobile phases and column types as “generic” starting points. These companies use a screening process to observe analyte separation on various combinations of generic columns and mobile phases and, largely through visual inspection, choose what appear to be good starting conditions. Some companies and academics are investigating machine learning approaches to help with identification of these initial starting conditions, such as quantitative structural retention relationship approaches, in order to determine the best starting point based on analyte structure. A lot more work is needed in this area.
Are Spreadsheets a Fast Track to Regulatory Non-Compliance?

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This article uses case studies to explore the use and misuse of spreadsheet calculations in conjunction with a chromatography datasystem (CDS) in regulated GXP laboratories. What wonders of non-compliance will we find? How and when should spreadsheets be used in chromatographic analysis?

“It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness...”. These are the opening lines of a book by that great chromatographer, Charles Dickens, and they serve as an excellent introduction to the subject of this column: spreadsheets. Is it the best application in the world? Are spreadsheets a fast track to regulatory non-compliance? The answer to both questions is YES! This column is focused on the use of spreadsheets for the post-run calculation of reportable results in a regulated chromatography laboratory.

The title, abstract and introduction pose a number of questions that I will discuss and answer in this column. The examples quoted here are based on real examples and have been anonymised (mostly) to protect the guilty. If any examples come close to what you do in your laboratory, what are you going to do about it?

Enter the Dragon

Reader discretion is advised at this point as this is where the laboratory finds out the regulatory mess it is in. An inspector has arrived. They want to see some high performance liquid chromatography (HPLC) analysis records. As an auditor, I am a great believer in auditor’s (and inspector’s) luck: whatever records I ask for turn to dust in front of the audited (or inspected). Let us look at a recent FDA warning letter—I don’t have to write fiction, as real life is posted on the Agency’s warning letters “wall of shame” on their website.

Tismor Health and Wellness, an Australian company, received an FDA warning letter in December 2019 that did not do much for the company’s health or wellness. Apart from minor transgressions with the Chromatography Data System (CDS) such as analysts with administrator privileges, performing trial injections, aborting runs, repeating work to obtain passing results plus data deletion, there was also a citation for failing to control spreadsheets:

Your firm failed to validate the spreadsheet used to perform the assay calculation for your <redacted>. Your procedures lacked guidance on how to check and manually verify the calculation sheets. During the inspection, our investigator identified a calculation error within the spreadsheet. The incorrect formula for averaging the Internal Standard peak area was used... (1)

Let begin the non-compliance saga: no checks to verify the calculations and failure to validate the spreadsheet is bad. But then we plumb the depths: a calculation error was found by the inspector!

It goes from bad to worse as during the remediation:

During the review, you identified another error within your spreadsheets.

Please read the warning letter to see the list of remedial actions required by the FDA (1). This is not the best situation to be in and it could so easily be avoided if the CDS was used to perform the calculations.

Main Chromatography Data System Functions

Let’s set the scene for the rest of this column by listing the main functions of a CDS which are:

- Input of the sample injection sequence (standards, blanks and samples), sample identities, sample information (batch number), as well as factors, weights, and dilutions as necessary. Injection sequence in the CDS should match the order of vials in the autosampler.
- Control of chromatographs including auto injection from the autosampler according to the sequence file.
- Acquire and store data from the detector for each injection.
- Integrate each chromatogram to calculate peak areas of the analytes.
- If permitted, allow the analyst to intervene to change processing parameters.
Adeno-associated viruses (AAVs) are increasingly used for gene therapy due to their versatility and safety. One of the biggest concerns for manufacturing a uniform AAV suspension is the presence of viral aggregates, which can create problems with transduction efficiency, biodistribution, and immunogenicity. These large AAV aggregates are challenging to separate and characterize by traditional column-based chromatography techniques such as size exclusion chromatography (SEC).

Asymmetrical Flow Field-Flow Fractionation with Multi Angle Light Scattering (AF4-MALS) can separate and size large AAV aggregates, and discern a difference in aggregate concentration due to the stressing protocol. Some or all of the large aggregates would be filtered out by SEC, resulting in incorrect determination of the aggregate content or the false conclusion that no aggregates are present.

For more details visit www.postnova.com and search for ‘virus’.

Contact us for more information: www.postnova.com
or manually integrate peaks (2–4)
• Calculate parameters from the system suitability test (SST) injections to determine whether the run is acceptable
• Select a calibration model and use it to calculate the amount or concentration of the analyte in the samples
• Automatically adjust the calculated amounts to account for weight, purity, dilution, etc.
• Apply any other post-run calculations to calculate the reportable result of the sample
• The performer of the analysis checks their work, makes any applicable corrections and then electronically signs the report
• A reviewer checks all data entered manually is correct and work was performed as required by the applicable procedure, reviews the audit trail and checks their prohibited actions, such as unofficial testing, aborted runs, etc., and electronically signs the report to approve the result.

Looks simple and straightforward doesn’t it? All work can be performed in a single application. All chromatography files and associated contextual metadata are found in a single location. The only transcription checks that need to be conducted are when data are manually input from the sample preparation stage. Validated calculations can be performed automatically with the results signed and reviewed electronically.

Chromatographic and regulatory perfection! Why do anything else? Why even think about using a spreadsheet? Especially as many of the functions listed above cannot be performed by a spreadsheet:
• Control a chromatograph? Not a chance.
• Inject a sample? What a joke.
• Integrate a peak? No way.
• Reposition a baseline? Pull the other leg!

However, when we come to post-integration calculations, spreadsheets sometimes manage to get a foot in the door, a leg or even the whole body. CDS suppliers have gone to great trouble to include functions that enable a laboratory to work electronically, automatically calculate SST values, calculate results, adjust results with weights, dilutions and other factors, and perform custom calculations. This poses the key question: why on earth do laboratories use spreadsheets?

Seductive and Siren Spreadsheets
Enter stage left, the spreadsheet. Turn on any workstation in your laboratory or office, what is the one icon that you see consistently on each screen? The short-cut to a spreadsheet. Spreadsheets are everywhere and they can be used and abused for a multitude of uses. We will focus on the parallel universe found in chromatographic analysis. The known universe is that of the CDS which can perform the majority of calculations as well as sign reports electronically, as described earlier. The parallel and dark universe is inhabited by the spreadsheet – a pervasive force with a seductive voice whispering in your ear saying, “I’m easy to use and you don’t need to read the CDS manual”. If listened to, the laboratory is now heading down the very slippery slope to transcription error checking hell.

The Great and Not So Good
Who is to blame for this? There are two key individuals who contribute to this debacle:
• Dopey, the laboratory manager, for allowing this to happen and also failing to invest in the proper automation of the chromatographic process that is essential to perform any laboratory’s business function
• Stupid, from Quality Assurance (QA), for simply checking that the regulations are apparently being followed but failing to point out the compliance holes and regulatory risks.

To this list of the great and the good, three more players are added:
• Nerdy, the laboratory’s spreadsheet guru. The spreadsheet champion—any calculation or any task that could be performed by a spreadsheet is. A spreadsheet developed by Nerdy in the morning is the laboratory standard by the afternoon break. Does Stupid in QA know about this new spreadsheet? Probably not. Is it validated? Please don’t ask such questions.
• Sloppy, the chromatographer who knows chromatography inside out but is a little light on the documentation aspect of analysis and has a short attention span. Sloppy’s data entry lapses can have some interesting impacts, such as releasing out-of-specification products if not caught in the second person review.
• Picky, the second person reviewer who must be on the top of their game to pick up Sloppy’s mistakes. The role requires great attention to detail and concentration to review the multiple data transcription checks resulting from numerous manual data entries from a CDS printout into the spreadsheets used in the analysis.

The World’s Most Expensive Electronic Ruler
Enter stage right is our CDS in the hands of Dopey the lab manager. Seductive voices from Nerdy, the laboratory’s spreadsheet gnu—sorry guru—suggest that a quicker way of getting results would be to use spreadsheets to do all the post-integration calculations. If Dopey agrees, the laboratory can apply to the Guinness Book of World Records to see if they have created the world’s most expensive electronic ruler.

Dopey, aided and abetted by Nerdy, is now opening the door to a potentially uncontrolled and hidden software factory that appears at first glance to be a cheap solution. However, there are big hidden costs and great regulatory risks...
that can become unmanageable as we shall see later. Of course, a laboratory goes down the spreadsheet route in the full knowledge that a spreadsheet has a full audit trail (no it does not) and is technically Part 11 compliant (no it’s not) and is also a hybrid system that are not recommended by WHO (5).

Ski at the ready? This will be a rapid descent down the slippery slope to spreadsheet hell.

A Case Study of Analytical Incompetence

Let’s see how this works in practice in a QC laboratory working to Good Manufacturing Practice (GMP). Sloppy, the analyst analyses the samples as normal but the only data entered into the CDS is the sequence of vials and their corresponding identities, for example, standard, sample, blank, etc. There is no input from the sample preparation phase of weights, dilutions, etc. as these are saved for the spreadsheet extravaganza later in the process. Post-analysis, Sloppy checks the peak integration and when acceptable, the chromatograms and peak areas are printed out.

Dumb Move Number 1:
- The laboratory has now created a hybrid system with electronic records and signed paper printouts.
- The analysis records are now in two locations rather than one: one in the CDS and one in the signed printout, these require additional review checks by Picky to ensure that the data correlate.
- Stupid (our QA hero) has mandated that each page of the printout must be dated and initialled by the analyst. Of course, Sloppy with his legendary attention to detail does his best to do this but thinks that Picky will let him know of any omissions.

Next, Sloppy transcribes the peak areas of the SST injections from the CDS printout into a spreadsheet to calculate the SST parameters to see if the run passes or fails the predefined acceptance criteria. As mentioned earlier, Sloppy has acquired a reputation for a lack of attention to detail.

Dumb Move Number 2:
- All CDS applications calculate SST parameters automatically. Why wait until the whole run is finished, check the integration, print and then enter the data into a spreadsheet only to find you have wasted your time as the SSTs have failed and the run is invalidated? Only masochists should apply to this laboratory.
- SST acceptance criteria can be set.

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Workflow advantages and future developments.

Who can benefit from automated method-modeling software? Probably more laboratories and research teams than you think—perhaps even your own. That’s because all the method-development experience in the world is no match for an automated modeling software package that can eliminate human error, provide a 360-degree view into the design space, and accelerate time to development of a truly robust method. As DryLab Specialist at Molnár-Institute for Applied Chromatography, Arnold Zöldhegyi understands this better than anyone. So, LCGC Europe sat down with him to discuss the benefits of the new DryLab/Empower connection in pharmaceutical analysis and to get a peek at future goals and advances for the automation module.

**LCGC Europe: What are the benefits of using high performance liquid chromatography (HPLC) modeling software such as DryLab for method development?**

**Zöldhegyi:** The main benefit of modeling software is that it literally illuminates the whole possible design space—a design space that easily consists of more than a million work points or method-parameter combinations. For example, in the case of DryLab, all it takes is 12 distinct input runs to visualize all the chromatographic interactions inside the design space.

Some packages are statistically based and will run numerous experiments, keeping your instrumentation busy day-in and day-out in an automated way. DryLab takes a different approach, probably because it was programmed by two scientists—Lloyd Snyder and John Dolan—who greatly contributed to the understanding of HPLC. A typical statistical package will tell you if your method works or fails, but it won’t demonstrate what’s going on in your design space the same way DryLab does. DryLab will visualize the interactions of your separation under all possible method conditions so you see precisely which areas your method will work in, which parameters can be varied, and which need strict control.

Some scientists believe following one’s intuition is better than using modeling software. Having a hunch and working by trial-and-error may be helpful in some areas, but it is the wrong approach when you’re trying to receive marketing authorization for a new drug product. In fact, CROs that do contract method-development work daily—clearly very experienced chromatographers—use DryLab to avoid heading in the wrong direction and to substantiate their decision-making for filings. Modeling software also saves a lot of time wasted in other approaches.

**LCGC Europe: How does automated method development relate to Analytical Quality by Design (AQbD), and what advantages does it bring to pharmaceutical analysis?**

**Zöldhegyi:** The term “AQbD” implies a new method development that’s based on scientific understanding—on HPLC’s underlying theories. Because DryLab takes advantage of the absolute accuracy of the laws describing the retention mechanisms in HPLC, you’ll only need 12 input runs. Now, adding automation to DryLab further lowers the threshold of using this AQbD software to the level that you’ll be using it on an everyday basis.
What then really determines the quality of your DryLab model is the pristineness and reproducibility of your input data. In other words, you can expect a close-to-perfect model if you rule out transcription errors and other slips—which is exactly what automation does.

**LCGC Europe: How do the new automation module and its Empower connection facilitate the DryLab workflow, and in what regard does this make the user’s life easier?**

**Zöldhegyi:** The automation module connects directly to the Waters Empower CDS, writes the sample sets—making sure that the proper re-equilibrations occur between runs—and then acquires the integrated data from Empower back to DryLab. It does this across the DryLab workflow, which first involves designing and running the 12 input experiments on Empower, then executing and acquiring confirmation runs, and finally running and acquiring robustness verification runs to confirm the robustness assessment.

If you’re a subject-matter expert in charge of a separation center that delivers to analytical operations, you’ve probably set up an SOP that implements the daily use of DryLab. But, not everyone is experienced in method modeling. Here, having the Empower automation really makes life easier, with all data transferring seamlessly across the DryLab workflow.

Not only does the new automation module and its Empower connection facilitate DryLab modeling for less-experienced users, but it also prevents all users from making mistakes when writing method sets, exporting from the CDS, importing to DryLab and copy-pasting additional peak data from spreadsheets. It really brings DryLab’s AQbD modeling to your daily routine on all those levels, plus it vastly saves time—your sample set is written with one click.

**LCGC Europe: Now, assessing the robustness of an analytical method is one central point of Q12 (LCM). How does DryLab assess method robustness, and how is this process facilitated by DryLab’s new automation module?**

**Zöldhegyi:** Robustness is a key performance criterion of analytical methods. The way DryLab has been assessing robustness since we first made the module in 2011 turns out to be in-line with what ICH Q12 recommends.

But the reason for industry to turn to the robustness module is that it gives you a very good understanding of how well your method will perform in routine use, and which areas of your design space it can be run across its lifecycle without facing any out-of-spects. This is highly relevant to many of our customers because their business models depend on their ability to yield profits in a limited window of opportunity. When it comes to analytical development, this means that methods must perform flawlessly in subcontractor labs in regulatory systems across six continents.

So DryLab’s robustness assessment works as follows: First, the design space and its chromatographic interactions are modeled based on scientific theory. Then, based on that knowledge, the method-operable design region (MODR) is identified. Instrumentation precision is taken into account including the range of gradient sensitivity, temperature accuracy, pH accuracy, and other specs that could vary such as flow rate. This information is added to the DryLab model to evaluate a work point or workspace’s robustness. Once your MODR has been scrutinized, the chromatographer then validates the robustness assessment.

From the systematic way your model has progressed so far, you can see the points in your MODR at which the API will elute earliest and latest, which gives you a range that you can expect in routine use. This will be highly relevant for your system sustainability test. Also, you’ll see where in your MODR peaks of interest, for instance, the critical peak pair, will have their lowest critical resolution.

You then take these strategically relevant points from in-silico and run them for confirmation with fully automated sample sets written and executed through DryLab’s Empower connection, and then acquired from Empower and compared to confirm the model in DryLab.

The whole point of doing this with regard to Q12 is to use the software capabilities of visualizing the interactions going on, and to determine which parameters affect your separation in routine use. This information is gathered and structured in DryLab’s knowledge-management document as the scientific basis for your post-approval lifecycle management. Flexible regulatory approaches regarding later changes would derive from, for instance, a downgrading of certain parameters from prior approval to notification.

**LCGC Europe: Are you seeing new areas of application in which industry is using DryLab?**

**Zöldhegyi:** We have some amazing customers using DryLab extensively across techniques: ion-exchange, ion-pairing, hydrophobic- and hydrophilic-interaction chromatography and, of course, normal and reversed-phase. We also see a lot of protein analysis with large teams of analytical scientists in the industry using DryLab for separation work on polypeptides and oligonucleotides, and the DryLab workflow being laid down in their SOPs. We see very impressive applications in monoclonal antibody work.

Something new we’re seeing is industry companies adding DryLab knowledge-management document to the pharmaceutical-development section of their CTDs. It facilitates approval because it provides all the relevant information that’s missing if you only file validation results. Validation results alone do not justify post-approval leeway, which regulators may have granted if you’d turned in documentation of your analytical procedure development.

In this regard, customers filing DryLab’s knowledge-management documentation may have the potential to advance the field in the coming years.
in a CDS to determine automatically if the run passes or fails
- Some CDS systems can stop a run if the SST injections fail one of the parameters. This avoids the situation where the whole run is injected and next day peaks are integrated, peak areas typed into the spreadsheet and SST results calculated to find that the SST results have failed. Instead, if the run is stopped automatically after the SST injections fail acceptance criteria. This allows a chromatographer to find the problem, resolve it, and restart the injection sequence for an overnight run, making the laboratory more efficient.
- Is the spreadsheet validated?
  Of course not!
- Is the completed spreadsheet file saved as part of the complete data (6) or raw data (7) of the analysis? Probably not!
- Sloppy also must remember to enter the sample weights and any dilutions, etc. into the spreadsheet against the correct sample identities
- More typing generates a greater probability of typographical errors
- This process is totally unnecessary
- This process is very slow.

Who is Paying the Bill?
Here’s where Dopey’s laboratory picks up the bill for an amazingly abysmal and inefficient process. Enter Picky, the poor unsuspecting individual who is going to review all the records and data to see that work has been performed correctly under EU GMP Chapter 6 (9) and 21 CFR 211.194(a)(8) (6). Picky must check all records to ensure that no mistakes have been made, the data are complete and accurate and signed off. What does Picky have to review? Instead of the sample preparation records and all data in the CDS if you had designed the process well, Picky has a mess of records spread across two media: paper and electronic.

Figure 1 shows the process of chromatographic analysis and spreadsheet calculations in green. Below each task in the process are the records that are created: paper records are in yellow and electronic ones are in blue. Some observations:
- Consider the complexity of the records that must be reviewed: three separate sets of electronic records and five sets of paper records. This is a compliance nightmare and a mess.
- Transparency and consistency of the data: results in the CDS must be consistent with the data entered in the spreadsheets and accurately entered. The files used must be the right file used?
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and printouts. These are shown by the dashed lined connecting the e-records and printouts.

- Data calculated in one spreadsheet may need to be transferred and manually input into the next spreadsheet bringing joy to the reviewer of yet another task of transcription error checking.
- Picky’s review process is shown at the bottom of Figure 1 in orange. The linkages between the various paper and electronic records that must be reviewed have been omitted as the number of arrows required would make the diagram look like a still photograph of Custer’s Last Stand.

This mess ensures that any properly conducted second person review takes longer than the actual analysis to perform. Now you are probably thinking that I’m making all of this up and no laboratory works this way. I wish I was but this description is based on a real laboratory. I have only changed the names of the people involved; the names used here are much better and far more appropriate.

What is missing from Figures 1 and 2 is the instrument log book. The entries in the log must also correlate with analysis record sets generated either from the spreadsheet or electronic processes.

**Do It Right: A Spreadsheet Free Zone**

What should have happened when an inspector dropped in for a cozy fireside chat is that they should have been presented with the records generated from a streamlined electronic chromatography process, as shown in Figure 2 and based on the process described at the start of this column. The advantage for the inspector and the laboratory is that all the records are in the CDS database and the inspector can review them on-screen by directing a skilled CDS user where they want to look. As all appropriate technical controls are in place and the process is validated, there should be no issues and the inspector can go and terrorise—I mean inspect—somebody else.

**Key Review Areas of the Electronic Process**

Consider the situation where we have an electronic process with validated calculations and electronic signatures in the CDS, as described at the start of this column. Where are the main problem areas? Manual data input of weights, factors, and dilutions as well as the entry of sample identities in the sequence file and placement of the vials in the corresponding order in the autosampler. Picky’s job as a reviewer is focused on these areas as these are critical data.
entered manually that require a check as required by EU GMP Annex 11 (10), plus checking that peaks have been integrated correctly. The remaining part of the process is fixed and validated and should not require much review scrutiny. Comparing the spreadsheet-driven process in Figure 1 with the streamlined electronic process in Figure 2, shows the simplicity of an electronic process. But the process in Figure 2 is not just to keep an inspector happy. They may only see the process every two years or so with regular facility audits. What about the staff in Doppy’s laboratory who will operate the process daily?

• The whole process is validated with calculations performed automatically
• Sloppy, the analyst, does not need to print the chromatograms, enter peaks areas into an army of spreadsheets then save, print, and initial the paper records.
• Picky’s work as a reviewer is much reduced. They need to check that data entered manually from sample preparation records are correct and all chromatographic data, especially the peak integration, has been performed appropriately within the CDS
• Both Sloppy and Picky have simpler and faster processes to perform and training will be much easier for new staff
• Inspectors, QA staff or auditors have easier tasks as many records are in a single location
• Business benefits accrue to the laboratory each and every time an analysis is performed far outweighing any costs to implement and validate a process. OK, job done? Not always.

**Interface the CDS!**

An earlier Questions of Quality column discussed a laboratory information management system (LIMS) interfaced with a CDS (11). Here sample identities and weights for an analysis are transferred from the LIMS to the CDS reducing the manual data input to the sequence file of factors and dilutions. This limits Sloppy’s data input activities and consequently reduces Picky’s workload as the integrated process is validated and under control.

**Alright, There Are Exceptions**

Having spent the whole of this column until now saying that spreadsheets should be exterminated from the face of all chromatography laboratories, there are inevitable exceptions to this rule. In some situations where an experiment requires data from more than one chromatographic run, then a spreadsheet is one way that can be used to collate the data and perform the calculations with current CDS applications. However, the spreadsheet does not have an audit trail and is a hybrid system which are not great attributes to boast about in the current data integrity environment.

One way to overcome this is to use an electronic laboratory notebook (ELN) or similar application where data from the CDS can be imported and used to automatically populate the spreadsheet for preparing the required calculations; in this way manual input is avoided and the associated transcription error checking. The ELN has the security, audit trail, and the ability to work electronically, enhancing the compliance of using a spreadsheet. The use of spreadsheets in this way should be a minority of cases in a chromatography laboratory.

**Summary**

Let’s revisit the words I quoted from Dicken’s *Encyclopedia of Chromatography* at the start of this column:

• It was the best of times: we don’t use spreadsheets for post-run calculations
• It was the worst of times: we use uncontrolled and unvalidated spreadsheets
• It was the age of wisdom: we spent time designing and validating a paperless process and eliminating spreadsheets
• It was the age of foolishness: we got caught and have lots of expensive remedial work to fix the problems.

Need I say more?

**Acknowledgements**

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YMC Europe GmbH, Dinslaken, Germany.

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Postnova’s simultaneous electrical and asymmetrical flow field-flow fractionation (EAF4) system is designed to enhance the separation and characterization of biopharmaceutical, environmental, and nanomaterials. In an EAF2000 system electrical and cross flow fields are applied simultaneously to enable separations by particle size and particle charge based on electrophoretic mobility to characterize complex proteins, antibodies, and viruses, as well as environmental and charged nanoparticles or polymers.

www.postnova.com
Postnova Analytics GmbH, Landberg, Germany.

**Method Translator**
Pro EZLC method translation software makes it possible to scale down an existing LC method to a smaller column format so that users can speed up run time, increase sample throughput, and reduce solvent use, according to the company. The user can input current column dimensions and method conditions, then specify the dimensions of the new column that they want to try.

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

**Column Selection App**
Columns are the heart of any GPC/SEC system. However, it can be a challenge to find the best matching column from the wide range of options. PSS has therefore developed the Column Selection App to support users. The app offers the following choices: How to Replace Existing Columns, Column Recommendations, USP methods/EP methods, and Application Searches.

www.psscolumnselector.com
PSS GmbH, Mainz, Germany.
Determination of Psilocin and Psilocybin in Magic Mushrooms Using iHILIC®-Fusion and MS
Tibor Veress¹, Norbert Rácz², Júlia Nagy¹, and Wen Jiang³
¹Department of Drug Investigation, Hungarian Institute for Forensic Sciences, Budapest, Hungary; ²Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary; ³HILICON AB

Determination of Pesticide Residues in Cannabis Sativa Using an Optimized QuEChERS Method
Hans Rainer Wollseifen, Torsten Kretschmer, Johannes Brand, and Detlef Lambrecht, Macherey-Nagel GmbH & Co. KG

Analysis of Chloroquine, Hydroxychloroquine, and Desethylchloroquine in Urine Using SPE and LC-MS/MS
Abder Abdelkaoui, UCT, LLC

RT-MALS End-Point Determination of a Polysaccharide Depolymerization Process
Wyatt Technology Corp.

Separation and Sizing of a Virus Mixture Using Asymmetrical Flow Field-Flow Fractionation Coupled to Multi-Angle Light Scattering
George Bou-Assaf¹, Andy Blum¹, Omar Matalka¹, Ruth Frenkel¹, Robert Reed², and Soheyl Tadjiki², ¹Analytical Development, Biogen, Cambridge, Massachusetts, USA, ²Postnova Analytics Inc, Salt Lake City, Utah, USA

SEC-MALS Analysis of Biotherapeutics
Tosoh Bioscience

High Throughput DAR Determination of Brentuximab Vedotin (Adcetris®) by Reducing the Analysis Time
Daniel Eßer, YMC Europe GmbH
Determination of Psilocin and Psilocybin in Magic Mushrooms Using iHILIC®-Fusion and MS

Tibor Veress¹, Norbert Rácz², Júlia Nagy¹, and Wen Jiang³, ¹Department of Drug Investigation, Hungarian Institute for Forensic Sciences, Budapest, Hungary, ²Department of Inorganic and Analytical Chemistry, Budapest University of Technology and Economics, Budapest, Hungary, ³HILICON AB

Hallucinogenic mushrooms, known as magic mushrooms, contain psychoactive compounds such as psilocin and psilocybin (Figure 1). This hallucinogenic effect means they are constantly offered on the black market. Therefore, the reliable quantification of these compounds is a particularly important task for forensic analysis because their results have a significant impact on the judgement passed by the courts.

Although there are many analysis methods available in forensic laboratories and in the scientific literature, the majority of them are based on reversed-phase liquid chromatography (LC) separation (1–6). Due to the highly hydrophilic nature of psilocybin and psilocin, reversed-phase LC is not able to provide sufficient retention for them. Moreover, it is crucial to develop new methods and techniques that can improve the analysis detectability, selectivity, and productivity. To fulfill these goals, the application of hydrophilic interaction liquid chromatography (HILIC) and mass spectrometry (MS) is investigated.

In this study, we aimed to use a charge modulated iHILIC®-Fusion HILIC column for the analysis of extracts from hallucinogenic mushrooms and evaluate its potential for forensic application.

**Experimental**

**LC–MS System:** Agilent 1100 LC system and Bruker Esquire 6000 ion trap mass spectrometer, operated in positive ionization mode (ESI+). Chromatographic data were acquired and evaluated with ChemStation Rev. A. 10.02.

**Column:** 150 × 4.6 mm, 3.5-µm iHILIC®-Fusion (P/N 110.154.0310, HILICON AB, Sweden)

**Mobile Phase:** 80:20 (v/v) acetonitrile–ammonium formate (10 mM, pH 3.5)

**Flow Rate:** 0.5 mL/min

**Column Temperature:** 12 °C

**Sample Preparation:** Quasi-counter current extraction with methanol at 60 °C in a Shimadzu 10/A HPLC system. A 50-mg measure of air-dried and homogenized hallucinogenic mushroom was filled in the extractor chamber (an empty 250 × 4.6 mm HPLC column). The standard solutions were 5 µg/mL and 500 µg/mL for psilocin and psilocybin, respectively. Methanol was used as the solvent.

**Injection Volume:** 1 µL

**Results and Discussion**

In our previous study (2), the methanolic mushroom extract was first separated under the conditions within a designed

**Figure 1:** Chemical structures of (a) psilocin and (b) psilocybin.

**Figure 2:** Total ion chromatogram (m/z 40–400) of the methanolic mushroom extract and extracted ion chromatograms of m/z 205 (psilocin) and m/z 285 (psilocybin).
experimental space with a total of 18 model establishment points and two approval points, considering the mobile phase composition, pH, and temperature. The factors that affect the separation selectivity and resolution on three iHILIC® columns were studied using DryLab® and STATISTICA®. It was found that iHILIC®-Fusion provides best separation regarding separation selectivity and efficiency. Figure 2 illustrates the separation of mushroom extract and also the extract ion chromatograms at $m/z$ 205 (psilocin) and $m/z$ 285 (psilocybin), respectively. It is clear that iHILIC®-Fusion was able to separate psilocin and psilocybin from each other and also from the major matrix compounds within 15 min. An unique feature is that psilocybin elutes with a retention factor two times greater than that of psilocin. In addition, the sample preparation consists of few steps to minimize error sources and assure reliable results.

In the second step of this work, we separated the methanolic solution of psilocin and psilocybin standards to confirm the detection of these two alkaloids in the mushroom extract. As shown in Figure 3, both psilocin and psilocybin have identical retention times to the standards compared to those peaks from the mushroom extracts. Therefore, the developed method is selective for the two target compounds and can be used for the quantification as described in our early work (1).

**Conclusion**

This work illustrates how to use an iHILIC®-Fusion column and MS detection to separate and identify psilocin and psilocybin in hallucinogenic mushrooms or “magic mushroom” extracts. This developed HILIC–MS method can be utilized in forensic and clinical applications.

**References**


**Figure 3:** Total ion chromatogram ($m/z$ 40–400) of psilocin and psilocybin standards and extracted ion chromatograms of $m/z$ 205 (psilocin) and $m/z$ 285 (psilocybin).
This application note describes the determination of pesticide residues in Cannabis sativa using a QuEChERS method for most effective sample clean-up. Interfering substances (for example, lipids and pigments), which were also extracted with the organic layer, are almost completely removed with clean-up salt mixes with high amounts of GCB (graphitized carbon black) and CHROMABOND® C18 ec adsorbents. The organic extracts are finally analyzed by HPLC-MS/MS.

While medical and recreational use of cannabis are legalized in more and more countries, the markets for cannabis and cannabis-based products (such as concentrated oils, soda, candy, and other edible forms) have grown year by year in North America and other countries (1). A huge demand for marijuana has lead to professional cultivation forms of hemp to improve growth yields. The use of pesticides is a common tool into a 50 mL centrifuge tube
- Add 100 μL of standard solution ($\beta = 1 \mu$g/mL for each analyte in acetonitrile) for determining recovery rates
- Add 10 mL water and shake
- Add 10 mL 1% acetic acid in acetonitrile and shake for 30 min
- Add the CHROMABOND® QuEChERS extraction mix I (Macherey-Nagel REF 730970) or the competitor mixes (3)
- Shake vigorously for 2 min and cool down the mixture in an ice bath
- Centrifuge the mixture at 4500 rpm, for 5 min at 4 °C

**Dispersive Solid-Phase Extraction (dSPE)**
- Weigh out 1 g of homogenized sample (milled in a grinder) into a 50 mL centrifuge tube
- Add 10 mL 1% acetic acid in acetonitrile and shake for 30 min
- Add the CHROMABOND® QuEChERS extraction mix I (Macherey-Nagel REF 730970) or the competitor mixes (3)
- Shake vigorously for 2 min and cool down the mixture in an ice bath
- Centrifuge the mixture at 4500 rpm, for 5 min at 4 °C

**Figure 1:** Separation of pesticides on Bluebird RP 18 column (QuEChERS extract of Cannabis sativa spiked with $\beta = 100 \mu$g/g).
The presented QuEChERS method leads to an average recovery rate for pesticides of 92.3%, for 162 pesticides. Most of the pesticides (138 analytes) show recovery rates between 70–120%. On the other side, high matrix reduction yields were also possible by using the presented clean-up mix. The amount of dry substance after clean-up is reduced to less than 70% of raw acetonitrile extracts. With this clean-up approach interfering substances (such as lipids and pigments) are successfully removed due to high amounts of GCB and CHROMABOND® C18 ec adsorbents.

The chromatographic separation of pesticides was performed by using core-shell particles that are well known for fast and high-efficient separations combined with a reasonably low back pressure. In this work, a subsequent analytics was developed on a NUCLEOSHELL® Bluebird RP 18 column.

Conclusion

This application note shows the reliable and successful determination of pesticide residues from marijuana samples with an optimized QuEChERS method. The optimized composition of QuEChERS salt clean-up mixes leads to high reduction of matrix components and to high recovery rates for pesticides.

References

(3) “Determination of Pesticide Residues in Cannabis Sativa Using an Optimized QuEChERS Method with High Matrix Reduction”, Macherey-Nagel, tech-chroma@mn-net.com, (Düren, Germany, 2020).

Figure 2: Comparison of distribution of recovery rates between Macherey-Nagel and different competitors.
Analysis of Chloroquine, Hydroxychloroquine and Desethylchloroquine in Urine Using SPE and LC-MS/MS

Abder Abdelkaoui, UCT, LLC

Since the outbreak of the Novel Coronavirus (COVID-19) triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), controversy over the use of the antimalarial drugs Hydroxychloroquine and Chloroquine to treat the virus has surfaced as the side effects and multiple risks associated with these medications have not been fully evaluated. This application note describes a simple and robust solid-phase extraction (SPE) procedure for Chloroquine, Hydroxychloroquine, and the primary metabolite, Desethylchloroquine, in urine.

Table 1: Extraction/Analytical Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSDBX063</td>
<td>Styre Screen® DBX 60 mg, 3mL Column</td>
</tr>
<tr>
<td>SLDA100ID21-3UM</td>
<td>Selectra® DA HPLC Column 100 X 2.1 mm, 3 µm</td>
</tr>
</tbody>
</table>

Procedure

1. **Sample Prep:** To 1 mL of urine add 1 mL of pH 6 phosphate buffer (0.1M) and internal standard(s). Mix/vortex briefly.

2. **Condition Cartridge:**
   - 1 × 1 mL Methanol
   - 1 × 1 mL DI H₂O

3. **Apply Sample:** Load sample at 1–2 mL/minute

4. **Wash Cartridge:**
   - 1 × 1 mL PH 6 phosphate buffer (0.1M)
   - 1 × 1 mL Methanol

   Dry cartridges under full vacuum or pressure for 2 minutes

5. **Elute Analytes:**
   - 1 × 2 mL MeOH:NH₄OH (98:2)
   - Collect at 1–2 mL/minute.

6. **Dry Eluate:** Evaporate to dryness at < 40°C.

7. **Reconstitute:** Reconstitute sample in 100 µL of mobile phase or other appropriate organic solvent.

**Instrumental**

LC-MS/MS: Shimadzu LCMS-8050

Column: UCT Selectra® DA HPLC Column 100 × 2.1 mm, 3-µm

Guard Column: UCT Selectra® DA Guard Column 10 × 2.1 mm, 3-µm

Injection Volume: 5-µL

Mobile Phase A: D.I. H₂O + 0.1% Formic Acid

Mobile Phase B: Methanol + 0.1% Formic Acid

Column Flow rate: 0.40 mL/min

Results

**Table 2: Absolute Method Recovery Values**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Recovery (n=5)</th>
<th>RSD (%)</th>
<th>25 ng/mL</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>95%</td>
<td>5.6</td>
<td>100%</td>
<td>3.3</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>94%</td>
<td>3.0</td>
<td>99%</td>
<td>4.2</td>
</tr>
<tr>
<td>Desethylchloroquine</td>
<td>80%</td>
<td>5.8</td>
<td>88%</td>
<td>5.9</td>
</tr>
</tbody>
</table>

**Conclusion**

This application note outlines a simple SPE procedure for the analysis of Chloroquine, Hydroxychloroquine, and the primary metabolite Desethylchloroquine in urine using UCT’s Styre Screen® DBX polymeric SPE cartridge. Excellent recoveries for all three compounds were obtained using the outlined procedure, namely ≥80% at the 2.5 ng/mL level and ≥95% at the 25ng/mL level. RSD values at both concentration levels were ≤6%. In addition, the chromatographic separation of these analytes was challenging due to the extreme polarity of all analytes. However, the use of a Selectra® DA polyaromatic HPLC column resulted in excellent retention and baseline separation for all the compounds included in the method.
RT-MALS End-Point Determination of a Polysaccharide Depolymerization Process

Wyatt Technology Corp.

Polysaccharide depolymerization is typically monitored offline, using SEC-MALS to determine molar mass. However, this cannot provide timely feedback for process control. This application note describes the use of real-time MALS for online monitoring and timely endpoint determination.

Molar mass plays an important role in the solubility, potency, and stability of polysaccharide-based vaccines. The production process includes a critical depolymerization step to reduce the polysaccharide’s initial weight-average molar mass, $M_w$, from over 1800 kDa down to less than 350 kDa.

Current methods to monitor molar mass during polysaccharide production employ off-line SEC-MALS analysis, during which a single run can take up to 30 minutes. With a typical depolymerization time of about 90 minutes, off-line analytics cannot provide timely feedback on reaching the endpoint. Real-time multi-angle light scattering (RT-MALS) fills this need and ensures that the process ends as soon as the endpoint criterion is achieved.

Materials and Methods

A polysaccharide solution was depolymerized by ultrasonication at 20 kHz. Molar mass was monitored online, in near-real-time, by continuously pumping a small fraction of solution from the reactor through an ultraDAWN RT-MALS instrument (Wyatt Technology) by means of a quaternary HPLC pump. The solution was diluted continuously by a factor of 10 in order to reduce viscosity for flow through capillary tubing. The flow rate was 5 mL/min, resulting in a lag time (RTD, residence time delay) of just 3 minutes between the time product was pulled from the reactor and time of measurement. OBSERVER software was configured to control the HPLC pump, acquire data from the ultraDAWN, calculate $M_w$ 30 times per minute, and send a trigger to stop the sonication when $M_w < 350$ kDa, as determined by the release specifications.

Results and Discussion

RT-MALS demonstrably tracked reduction of the polysaccharide’s $M_w$ and triggered reaction shutdown once it fell below 350 kDa. SEC-MALS analysis of the final product confirmed the desired critical quality attribute (CQA) value. Multiple off-line analyses were eliminated, the time spent by the drug substance in this portion of the process was cut by 25%, and total person-hours spent in this part of the process was reduced by at least 50%.

Figure 1: Trace in OBSERVER software of weight-average molar mass measured by ultraDAWN, indicating the achievement of the desired process endpoint.

Conclusions

Polysaccharide antigens are high-value-add products with substantial cost benefits to be gained by in-process determination of the primary attribute impacted by the specific process. Here, the goal of the depolymerization process is to modify the polymer’s molecular weight, so the ability to determine molecular weight in near-real-time via RT-MALS reduces uncertainties due to process drift or variations in raw material properties, resulting in perfect depolymerization with every run and appreciable cost savings.
Separation and Sizing of a Virus Mixture Using Asymmetrical Flow Field-Flow Fractionation Coupled to Multi-Angle Light Scattering

George Bou-Assaf¹, Andy Blum¹, Omar Matalka¹, Ruth Frenkel¹, Robert Reed², Soheyl Tadjiki², ¹Analytical Development, Biogen, Cambridge, Massachusetts, USA, ²Postnova Analytics Inc, Salt Lake City, Utah, USA

Biogen and Postnova present data on the analysis of adeno-associated viruses (AAV) using Asymmetrical Flow Field-Flow Fractionation coupled to Multi-Angle Light Scattering detection (AF4-MALS). AAV are promising gene therapy delivery vehicles, whose efficacy may be negatively affected by the presence of viral aggregates. Due to its gentle separation and broad applicable size range, AF4-MALS is able to characterize AAV and their aggregates with high resolution and precision thereby overcoming the drawbacks that column-based chromatography techniques often face when dealing with samples larger than 50 nm in size.

Viruses are increasingly used as gene therapy delivery vehicles due to their versatility and safety. They can be loaded with DNA or RNA and delivered to a specific location in the body to treat or cure a disease (1). One of the biggest challenges for manufacturing a homogeneous virus sample is the presence of viral aggregates, which negatively affect transduction efficiency, biodistribution, and immunogenicity (2). Due to their relatively large size, often over 50 nm in diameter, virus aggregates are challenging to separate and characterize by column-based chromatography techniques such as size-exclusion chromatography (SEC). In this Application Note, we present data on separation of a virus mixture using Asymmetrical Flow Field-Flow Fractionation (AF4) and measurement of their radius of gyration ($R_g$).

A schematic for the AF4 channel is shown in Figure 1. The combination of cross flow and channel flow enable size separation over the course of the analysis: small particles elute and reach the connected detectors before larger particles, including aggregates.

**Experimental Details and Results**

A virus mixture sample was created by combining smaller adeno-associated viruses (AAV) with larger adenovirus type 5 (Ad5) in solution to simulate a sample with virus monomer and aggregates. To separate the viruses by size, an AF4 (Postnova AF2000) was used, coupled to a Postnova 21-angle multi-angle light scattering (MALS, PN3621) detector for measuring the $R_g$. Both the AAV-only and virus mixture samples were analyzed by AF4-MALS to highlight the differences between the samples. The carrier solution was phosphate buffered saline (PBS). The AF4 membrane used was 10 kDa regenerated cellulose.

The MALS response for the AAV-only sample is shown in Figure 2. The main peak corresponds to the AAV monomer eluting between 17 and 20 min. The $R_g$ measured for the

---

**Figure 1:** Schematic of the AF4 separation principle.
monomer is ~ 12.5 nm, very consistent with an expected diameter of 25 nm. There is a small shoulder to the right of the monomer peak, and the increasing $R_g$ for this shoulder peak indicates the presence of a small amount of dimer/trimer/small aggregates in the sample. Fully separating the monomer from any aggregate species was beyond the scope of this work. Further method optimization is required to achieve this goal.

In Figure 3, the virus mixture is separated into multiple peaks, with the $R_g$ plotted as black and red dots. The monomers have measured $R_g$ values consistent with AAVs, at about 12.5 nm, with the slight shoulder (aggregates) still observed as in the AAV-only sample. A second peak between 33 and 40 min corresponds to the Ad5 virus, whereas a third peak which elutes between 40 and 50 minutes are aggregates of the Ad5 virus. The Ad5 virus and its aggregates have radii in the range of 30–55 nm, most likely too large to be successfully separated by SEC.

**Conclusion**

The data presented here demonstrates that AF4-MALS is a powerful tool in the separation of virus particles. It can separate viruses and aggregates from a few nm up to >100 nm. This means that AF4-MALS can easily separate and size multimodal virus samples, including the larger Ad5 virus and its aggregates with high resolution and precision.

**References**


The analysis toolbox for biotherapeutics contains many chromatographic techniques. When it comes to aggregation and fragmentation of a therapeutic protein, size exclusion chromatography (SEC) is the method of choice. Here we report the detection of absolute molecular weight of a recombinant protein sample using a UHPLC compatible Multi-Angle Light Scattering (MALS) detector for UHPLC-SEC-MALS analysis.

Therapeutic proteins must remain free from impurities such as fragment, dimer, and other higher order aggregates as they may cause immunogenic response. Historically, size-exclusion is the preferred mode of chromatography used for separation and characterization of such applications. Here we report the online detection of absolute molecular weight of a recombinant protein sample using a size exclusion UHPLC column directly connected to the LenS Multi-Angle Light Scattering (MALS) detector.

**UHPLC Conditions**

- **Column:** TSKgel UP-SW2000, 300 mm × 4.6 mm, 2-μm
- **Mobile phase:** BupH modified Dulbecco’s phosphate buffer
- **Flow rate:** 0.20 mL/min
- **Detection:** UV @ 280 nm, Tosoh LenS MALS
- **Temperature:** 25 °C
- **Injection vol.:** 10 µl
- **Sample:** Recombinant Protein (~90 kDa) at 3.64 mg/mL

**Results**

The multi-detector setup was calibrated using a bovine serum albumin (BSA) solution. The one-step calibration procedure in the SECview™ software adjusted the dead volume between the detectors and corrected for the band-broadening effect while determining the detectors’ calibration constants and offsets. Figures 1 illustrates the UV detector overlay for two consecutive injections of the 90 kDa protein. The zoomed-in figure (inset) shows the excellent separation between the monomer and the aggregates.

Figure 2a depicts the molecular weight (green) profile for the sample. Looking closer at the UV trace, the monomer peak seems to have a very slight shoulder on the higher MW region, suggesting a bimodal shape. The molecular weight (MW) trace of the MALS detector reveals two separate MW populations. Figure 2b zooms in on the monomer peak with the two molecular weight plateaus, 1a (MW 93106 Da) and 1b (MW 93602 Da).

**Conclusion**

These data demonstrate that the molecular weight species including monomer and multiple aggregate levels present in the sample could be determined and quantified using a UHPLC-SEC-MALS configuration. TSKgel UP-SW2000 delivers excellent separation of the higher order aggregates from the monomer, as well as the slightly higher molecular weight temperature-induced impurity that almost co-elutes with the monomer. The MALS detection using LenS produces reproducible, accurate results in terms of MW determination for all peaks, in addition to area calculations, even at extremely low concentrations/presence of the aggregates.

TSKgel and Tosoh Bioscience are registered trademarks of Tosoh Corporation

LenS is a trademark of Tosoh Bioscience LLC.

SECview is a registered trademark of Tosoh Bioscience LLC in the USA and EU and of Tosoh Corporation in Japan.
High Throughput DAR Determination of Brentuximab Vedotin (Adcetris®) by Reducing the Analysis Time

Daniel Eßer, YMC Europe GmbH

Drug-to-antibody ratios (DAR) of antibody-drug-conjugates (ADCs), such as brentuximab vedotin, are important for their therapeutic efficacy and pharmacokinetics. Therefore, control of DAR is a key factor for ADC quality control. Given that, it is important for QC purposes to obtain sufficient resolution of all DARs and the resulting average ratio calculation, whilst performing the separation in the shortest time.

Analysis of Brentuximab Vedotin

Brentuximab vedotin (Adcetris®) is an ADC targeting different types of lymphoma with an average DAR of four (1). The average number of drugs attached to the monoclonal antibody (MAb) (DAR) is one of the most important quality attributes of an ADC because it can directly affect safety and efficacy of ADCs. The DAR determines the amount of drug that can be delivered to tumour tissue (2). The analysis used to determine DAR depends on the chemistry used for the linkage of the drug to the antibody. Second generation cysteine-linked ADCs, such as brentuximab vedotin, are generated after the reduction of the interchain disulfide residues, resulting in free sulfhydryl groups that can be conjugated to specific linker groups. The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero, two, four, six, or eight drugs per antibody molecule. Therefore, the determination ADC molecules loaded with different numbers of drugs per antibody is a key factor for ADC quality control.

The relative hydrophobicity increases with the drug load of the ADC because the small molecules attached to the MAb are often relatively hydrophobic. Therefore, the accepted technique to monitor the DAR is hydrophobic interaction chromatography (HIC).

In this application note YMC’s latest hydrophobic interaction chromatography column, BioPro HIC HT, was used. A mobile phase of sodium phosphate buffer at neutral pH with decreasing gradients of the lyotropic salt, ammonium sulphate, was used.

Rigid particle allows higher flow rates

The rigid 2.3 µm non-porous polymer particles with butyl modification are pressure tolerant up to 400 bar and allow rapid analyses through increased flow rates without loss of resolution.

<table>
<thead>
<tr>
<th><strong>Table 1: Chromatographic conditions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column:</strong></td>
</tr>
<tr>
<td><strong>Part No.:</strong></td>
</tr>
<tr>
<td><strong>Eluent:</strong></td>
</tr>
<tr>
<td><strong>Gradient:</strong></td>
</tr>
<tr>
<td><strong>Temperature:</strong></td>
</tr>
<tr>
<td><strong>Detection:</strong></td>
</tr>
<tr>
<td><strong>Injection:</strong></td>
</tr>
<tr>
<td><strong>Sample:</strong></td>
</tr>
</tbody>
</table>
This allows flow rates to be increased by 2.5 times with savings of about 60% in time. Such fast runs are not possible with other HIC columns due to their much lower pressure limit, which is usually only the half that of BioPro HIC HT.

**Conclusion**

This application describes a fast, high-resolution method for the drug-to-antibody ratio determination of antibody-drug-conjugates using YMC’s most recent HIC column, BioPro HIC HT. Due to its novel surface chemistry and high-pressure tolerant beads, it allows very rapid analyses with high resolution. BioPro HIC is also the ideal choice for high throughput analyses. These properties, together with the excellent batch-to-batch reproducibility of YMC columns, make BioPro HIC HT the perfect solution for quality control applications. The reproducibility, together with the high column stability, ensures the highest reliability for every separation, time and time again.

**References**

(2) A. Wakankar et al., *mAbs.* 3(2), 161–172 (2011).

**Figure 1:** Skeletal formula of brentuximab vedotin.

**Figure 2:** The separation of Brentuximab vedotin using YMC’s BioPro HIC HT at flow rates of 0.5 mL/min and 1.2 mL/min.
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**Excellent analysis features** such as long-term stability, reliability and reduced need for calibration

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[Sulfur Chemiluminescence Detection Gas Chromatograph System](http://www.shimadzu.eu/nexis-scd-2030)