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Crawford Scientific Acquires Anatune Ltd.
By Lewis Botcherby
In December, 2018, Crawford Scientific announced the acquisition of Anatune Ltd., which will continue to operate as a separate legal and commercial entity, while allowing Crawford Scientific customers access to Anatune’s expertise in automation and trace analytical measurement.

“Over the many years we have known Ray Perkins and his team at Anatune, we have been impressed by their expertise in applications development for the most challenging analytical separations, and their ability to automate these solutions,” said Tony Taylor, group technical director at Crawford Scientific Holdings Ltd. “We welcome Ray and his team to the Crawford Scientific family, and look forward to many successful collaborations with our clients.”

“Anatune becoming part of the Crawford Scientific Group means we can match the growing demand for automated sample preparation solutions. Both organizations are oriented around first-class technical support and have similar cultures and missions. We are excited by the new opportunities before us,” said Ray Perkins, CEO of Anatune.

The acquisition of Anatune Ltd. follows Crawford Scientific’s acquisition of VR Analytical late last year. Based in Oregon, VR Analytical partnered with Hall Analytical Laboratories in Manchester, UK as part of the deal, and offered clients extractables and leachables analysis, as well as electronic nicotine delivery systems (ENDS).

“We are delighted to be joined by Ray Colton and his excellent team at VR Analytical, which presents exciting opportunities for our customers in this rapidly expanding and technologically challenging testing market,” commented Sam Crawford, managing director of Crawford Scientific Holdings Ltd.

Tosoh Bioscience LLC Moves to Acquire Semba Biosciences
Tosoh Bioscience LLC (King of Prussia, Pennsylvania) has invested in Semba Biosciences (Madison, Wisconsin) with the intention of acquiring full ownership. The transaction was approved by the Board of Directors of both companies, and an equity participation agreement was executed in November.

Tosoh and Semba have been collaborating on various downstream biomanufacturing projects since 2007. Semba Biosciences President Robert Mierendorf said in a statement that his company is pleased to team up with Tosoh Bioscience to offer customers an advanced platform for multicolumn continuous biopharmaceutical chromatography. The technologies of both companies fit perfectly together, he said, to provide an integrated solution for bioprocess research, development, and continuous manufacturing.

Ali Soleymanneshad, the director of sales and marketing at Tosoh, also expressed optimism about the deal. “By combining Semba Biosciences’ advanced Simulated Moving Bed technology with Tosoh Bioscience’s best-in-class resins, we will be able to offer the most productive and the most efficient solution in bioprocess chromatography while maintaining the highest quality,” he said.

YMC Acquires Pharmaceutical Systems Business of Lewa-Nikkiso America
YMC Co., Ltd. (Kyoto, Japan), a supplier of separations resins, services, and systems for the pharmaceutical market, has acquired the pharmaceutical systems business of Lewa-Nikkiso America, Inc. (Devens, Massachusetts). The business, which was part of the Lewa division of Nikkiso Corporation, provides production-scale chromatography systems for the biopharmaceutical market. This includes the manufacturing site located in Devens, Massachusetts.
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Reversed-Phase Liquid Chromatography and Water, Part I: How Much is Too Much?

When can we use completely aqueous eluents with reversed-phase stationary phases, and what happens if we make a mistake?

Dwight R. Stoll

Reversed-phase liquid chromatography (LC) is an incredibly powerful mode of separation that is applicable to a wide variety of applications, ranging from the separation of small organic acids to 150 kDa proteins. Reversed-phase separations have limitations, however, with one of the most practically significant ones being low retention for compounds that are highly water soluble (that is, hydrophilic). Understanding the general trend for reversed-phase separations that retention increases as the fraction of water in the eluent increases, and encountering situations where retention is too low for an analyte of interest, pushes us to use eluents with higher and higher levels of water. This, then, leads to the question, “How much water is too much?” Jumping to the end of this article, the short answer will be “It depends.”

In some cases, using completely aqueous eluents (that is, containing no organic solvent such as methanol or acetonitrile) may be completely acceptable and, in fact, can provide very useful separations of highly hydrophilic molecules. In other cases, completely aqueous eluents can cause some reversed-phase stationary phases to behave in undesirable ways, and should be avoided. This is not a new topic by any means (1,2), but the options we have for handling such situations are constantly changing. It is worth taking the time here to refresh our perspective on the topic as column manufacturers introduce new stationary phase chemistries and particle morphologies, and our knowledge of what goes on inside the column improves through fundamental research. In this first segment on this topic, I will review the basic concepts that are important for reversed-phase separations in highly aqueous eluents, summarize recent advances in our understanding of what goes on inside the column, and provide examples of bad column behavior and potential remedies.

Basic Concepts for Reversed-Phase Separations with Aqueous Eluents

It has been observed since the early days of LC that operating a typical C18-type reversed-phase column in a completely aqueous eluent can lead to gradual or sudden decreases in retention time (3). For at least a couple of decades, the prevailing idea seems to have been that the observed decrease in retention time was due to a change in the conformation of C18 chains bonded to the particle substrate in aqueous

FIGURE 1: Retention factor for uracil measured before and after stopping the flow for 10 minutes. Chromatographic conditions: Columns, Halo AQ-C18 (black points) or Halo C18 (red points), 50 mm x 2.1 mm i.d., 2.7 μm superficially porous particles; eluent, 10 mM phosphoric acid in water; flow rate, 0.40 mL/min; temperature, 40 °C. Retention factors were calculated using thiourea as a dead time marker, and injections of the thiourea and uracil analyte mixture were made once per minute. Halo is a trademark of Advanced Materials Technology, Inc.
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eluents from highly extended chains (that is, perpendicular to the substrate surface), to ones that laid down on themselves (that is, parallel to the surface). The latter state was commonly referred to as phase collapse (1–3). These observations also led to the notion that operating reversed-phase columns in completely aqueous eluents was generally a really bad idea, so much so that this idea made it into the Top 10 Myths of LC addressed by Ron Majors just five years ago (4). Around the late 1990s, however, experimental evidence led a number of groups to adopt the idea that the decrease in retention time was due instead to “dewetting” of the stationary phase (5). Specifically, the idea is that the high surface tension of an aqueous eluent in contact with a hydrophobic surface causes the eluent to extrude from the pores of the stationary phase particle. If there is no liquid in the pores of the particle, this effectively reduces both the column dead volume (that is, the volume of mobile phase inside the column, $V_m$) and the volume of stationary phase that is accessible to the analyte. Indeed, significant decreases in $V_m$ have been measured for C18-type phases when switching from organic-rich to completely aqueous eluents, and these decreases are correlated with decreases in retention observed for analytes of interest (3,6). The extent to which this extrusion of the eluent occurs under actual separation conditions depends on a number of factors, including the particle pore size, stationary phase chemistry, column temperature, and operating pressure (through this last factor, we could say dewetting depends on particle size and flow rate, as well).

For over a decade, Siepmann, Schure, and coworkers have been using Monte-Carlo molecular simulations to study the microscopic details of mobile and stationary phases in an effort to better understand how reversed-phase separations work (7). The results of these studies complement experimental work, and provide insights that cannot be obtained easily by experiment. Among a number of topics, they have addressed the question of what happens to reversed-phase stationary phases in aqueous eluents. They have found that the results of simulation support the idea that the observed decreases in retention must be due to loss of eluent from the particle pores, rather than physical collapse of the stationary phase chains onto themselves (8).

The equation of Young and Laplace is most commonly used to rationalize the effects of different chromatographic variables on the dewetting phenomenon (1,5,6,9). This equation, shown in equation 1 (10), provides a relationship between the pressure ($P$) required to force a liquid into a capillary, the contact angle of the liquid on the interior surface of the capillary ($\theta$), the surface tension of the liquid ($\gamma$), and the radius of the capillary ($r$):

$$ P = \frac{-2\gamma \cos \theta}{r} \tag{1} $$

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ified silica, is greater than 90°, a positive pressure is required to force water into the pore of a particle. Under conditions typical of modern LC separations, the pressure required to push the eluent through the particle bed will exceed the pressure required to push the eluent into the pores of the particles at most points along the column length. However, when the flow is stopped (for example, if the LC is not operated overnight), an aqueous eluent can be spontaneously extruded from the pores of a hydrophobic stationary phase particle, and the retention behavior will look very different the next time the column is used. With this framework in mind, we can think about how different chromatographic variables will affect this behavior. As the stationary phase becomes less hydrophobic, the contact angle for water will decrease. When the angle is less than 90°, the pressure indicated by equation 1 becomes negative, meaning that the eluent will spontaneously be drawn into the pores of the particles. Among chro-

**FIGURE 2:** Representative chromatograms for the thiourea and uracil analyte mixture obtained before and after stopping the flow. Conditions are as described in Figure 1. A) AQ-C18 before flow stop, B) AQ-C18 after flow stop, C) C18 before flow stop, D) C18 after flow stop.
matographers, we refer to this as wetting of the pores. Equation 1 also suggests that the particle pore size ought to play a role (that is, through $r$), with the pressure required to force eluent into the pore increasing as the pore size decreases.

The Washburn equation provides a helpful framework for thinking about the effects of these parameters, but, of course, the pores of chromatographic particles are not ideal by any means. The pore structure itself is heterogenous, with a distribution of diameters and shapes, and the chemistry of the pore surface is locally heterogeneous with some unbonded silanol sites, stationary-phase ligands (for example, C18), and endcapping functional groups (for example, trimethylsilyl groups). And so, we look to experimental results for the definitive answer to the question, “How much water is too much?” The chromatographic literature provides useful data that at least establish trends, even if the results are not exactly transferrable to a particular set of conditions of interest. For example, Bidlingmeyer and Broske showed results that speak to the effect of particle pore size, stationary-phase chemistry, and column temperature on the extent to which dewetting occurs in aqueous eluents (11). They found that for one type of C18 stationary phase there was a retention loss of 80% for particles with a pore diameter of 80 Å, but, with a diameter of 150 Å and the same stationary phase, there was no measurable retention loss. On the other hand, they found that very little retention loss was measured for a phenyl-type stationary phase, even for particles with 80 Å pores. Walter and coworkers observed similar trends, and also described results for the dependence of retention loss on stationary phase bonding density, the concentration of methanol in the eluent, and use of a post-column restrictor to increase pressure inside the column (5).

Testing for Dewetting, and Some Remedies

One good way to assess whether or not a reversed-phase stationary phase is susceptible to dewetting under a particular set of conditions is by first equilibrating the column under conditions where the stationary phase is highly solvated, or fully wetted. For most reversed-phase stationary phases, this could be an eluent high in methanol or acetonitrile content. Flushing the column at a modest flow rate for a time equivalent to about 20 column volumes should be more than enough. Then, we switch to the aqueous eluent (or whatever eluent is useful for the application at hand), begin injecting a mixture of two or three probe compounds that have reasonable retention under these conditions, and monitor the change in retention factor ($k$) as the column equilibrates. One could simply wait for 20 column volumes to pass first before injecting the test sample, but, if we start injecting right away, we also learn something about how quickly the column equilibrates when switching from the organic-rich to the aque-
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ous eluent. Then, once the retention has stabilized after this initial equilibration step, turn the flow off, wait 10 min, then turn the flow back on, and start injecting the test mixture again. If there is a significant difference between the retention before and after stopping the flow, dewetting is likely to be a serious problem under these conditions. Retention will vary from day to day, depending on the post-column flow restriction in the system, and peak shapes may deteriorate and become variable.

Figures 1 and 2 show representative results from such a test, conducted with two C18-type stationary phases that are otherwise very similar (that is, they use the same base silica), but one (AQ-C18) is designed for use in highly aqueous eluents. In other words, the AQ-C18 is engineered to avoid the dewetting phenomenon in completely aqueous eluents. Figure 1 shows the retention factor for uracil measured on these two columns in a completely aqueous eluent, injecting sample once per minute, before and after stopping the flow for 10 min. For the very first injection, the retention is slightly lower than the rest of the points, because the column has not equilibrated from the 50:50 organic:water flushing solvent to the completely aqueous eluent. After equilibration, the retention of uracil on the two phases is remarkably similar. After turning the flow off for 10 min, we observe that the two phases behave very differently. For the AQ-C18, there is no statistically significant change in the retention of uracil. On the other hand, the retention of uracil on the C18 column decreases by about 75%.

Representative chromatograms for the two columns before and after stopping the flow are shown in Figure 2. The chromatograms for the AQ-C18 column are indistinguishable, as expected. In the chromatograms for the C18 column, though, we see that there is not only a change in the retention factor of uracil, but also a 23% decrease in the measured dead time. This is consistent with the idea discussed above, that eluent is extruded from the pores of the stationary phase particles when dewetting happens, effectively decreasing the dead volume of the column.

The good news is that dewetting does not have to be a death sentence for reversed-phase columns. As is the case when recovering a column that has dried out during storage (12), columns that have dewetted can be recovered by flushing with several column volumes of organic-rich eluent. Figure 3 shows a comparison of chromatograms obtained for the analyte butyrophenone in 50:50 acetonitrile:10 mM phosphoric acid in water using the C18 A) before, and B) after the dewetting experiment. All other conditions are the same as in Figure 1.

FIGURE 3: Comparison of chromatograms obtained for the analyte butyrophenone in 50:50 acetonitrile:10 mM phosphoric acid in water using the C18 A) before, and B) after the dewetting experiment.
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phase chromatography. I have discussed the basic principles that explain why and when this occurs, so they can be used as a guide during method development. Since the extent of dewetting depends on a number of factors, including particle pore size, stationary phase chemistry, and operating conditions, it is a good idea to test for dewetting using the stop-flow during method development of applications involving an alkyl bonded phase (for example, C8 or C18), if they will be used with eluents containing less than 5% organic solvent. This piece has been restricted to isocratic conditions (that is, the use of completely aqueous eluents with reversed-phase stationary phases). In a future installment, I will discuss the implications of using solvent gradient elution involving these highly aqueous eluents.

Acknowledgements

I’d like to thank Dr. Stephanie Schuster of Advanced Materials Technology for providing the columns used in this work, and Dr. Mark Schure and Dr. Richard Henry for helpful discussions on the topic of dewetting.

References


ABOUT THE COLUMN EDITOR

Dwight R. Stoll is the editor of “LC Troubleshooting.” Stoll is a professor and co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 60 peer-reviewed publications and three book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC’s editorial advisory board. Direct correspondence to: LCGCedit@ubm.com
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GCxGC: From Research to Routine

Comprehensive two-dimensional gas chromatography (GCxGC) is becoming increasingly popular. Since its inception in the 1990s, and its original commercial availability about 15 years ago, GCxGC has moved from a strict research realm into more routine applications and use. Although not yet a standard capability on all gas chromatographs, GCxGC is rapidly becoming the technique of choice for analysis of highly complex samples as diverse as petroleum, pharmaceuticals, biological materials, food, flavors, and fragrances. This installment of “GC Connections” begins with a brief introduction to GCxGC, follows with examples of how GCxGC opens additional avenues of analysis, and concludes with information about how to learn more.

Nicholas H. Snow

GCxGC is continuing to see increased attention throughout the scientific community. Until fairly recently, most GCxGC work was performed and published by chromatography experts. Much like traditional gas chromatography (GC), which is now routinely used by analysts with a wide variety of scientific backgrounds, GCxGC is finding its way out of the chromatographer’s laboratory. “Should I try GCxGC?” is one of the most common questions I receive when discussing GC or GCxGC. Following a short introduction, this article discusses scenarios in which GCxGC has proven useful and applicable to routine analyses, and some avenues for obtaining additional help and information.

A simple schematic of the basic instrumentation and data processing in GCxGC is shown in Figure 1. The instrumentation is based on a traditional GC system, with the same inlet, main column oven, and detector. A second column oven, usually smaller for ease of heating and cooling, is added to house the second-dimension column within a separate heated zone. Also, a modulator is added to provide the sample transfer between the two columns. These are all controlled by a specialized data system. A traditional column is used in the first dimension, and the second column is very short (often 1–2 m), to perform a fast separation. As analytes elute from the first column, they are refocused at the head of the second column, and injected very rapidly, with second dimension separations lasting for only a few seconds. This transfer is performed by the modulator, which is programmed to perform this step at regular intervals, essentially cutting the chromatogram into short slices. As seen in Figure 1, GCxGC has one major instrumental advantage over other multicolumn techniques in that it employs only one detector. LCGC’s training partner ChromAcademy provides a summary of the various configurations of multidimensional GC that are available (1).

The right side of Figure 1 shows the data analysis process, performed by the data system. As there is one detector, a single chromatogram is generated, shown at the top. The solid vertical lines represent the beginning and end of each slice. The chromatogram is separated into these slices, which are then organized side-by-side, to generate the three-dimensional plot shown below.

Figure 2 shows a photograph of the column oven in a GCxGC instrument. The most important difference versus

FIGURE 1: Simplified schematic of a GCxGC instrument and the data analysis process.
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GC is the addition of the second column oven and the modulator hardware. In this configuration, the second column oven is installed within the main column oven; usually, the second column is maintained at the same or higher temperature as the main oven. The two columns are connected with a simple capillary column butt-connector. The modulator sits at the head of the second column. In this system, a two-stage thermal modulator is used with alternating cold liquid nitrogen and heated gaseous nitrogen gas jets that focus and inject the analytes into the second column, without allowing unwanted carryover from the first.

There are numerous reviews and vendor websites that provide more detail about instrumentation, modulators and data analysis in GCxGC (2). A simple online search using “GCxGC” as keyword will point you to major vendors.

One major advantage of a GCxGC instrument is that the additional components (modulator and second column oven) are installed between the traditional inlet and detector in a gas chromatograph, allowing them to be used without modification. Given that GCxGC is most commonly used for analysis of extremely complex mixtures, most applications have been developed using split injection, which ensures a rapid transfer of the analyte mixture to the first-dimension column. Other injection techniques, and nearly all on-
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off-line sample preparation techniques, have been attempted with GCxGC. In this article, examples using split, splitless, and solid-phase microextraction (SPME) injections are shown. In detection, there is one important caveat in GCxGC: The detector must be fast. The short second-dimension column generates peaks that are eluted with widths of 100–200 ms or less. If 10 data points are required to generate a properly symmetrical peak for good quantitative analysis, the detector must be able to respond by providing a signal within 10–20 ms. The data collection rate must therefore be set at a minimum of 50–100 Hz for proper peaks to be generated. A flame ionization detector is easily capable of this, but most benchtop mass selective detectors in full-scan mode are not, without careful optimization. Most GCxGC–MS applications are therefore performed using time-of-flight mass spectrometry (TOF-MS), which allows extremely fast full-scan rates.

The decision to consider GCxGC should be based on the overall needs and goals of the project, and on the other capabilities that can be built into the method. There is also a cost consideration, as the hardware for GCxGC and for GCxGC–TOF-MS requires larger capital and ongoing expenses than for traditional GC and GC–MS, and additional training and skill development for system operators are needed. Less expensive alternatives, such as heart-cutting or additional sample preparation, should also be considered. GCxGC has been described as a “super-resolution” technique, so the need for high resolution or peak capacity (the number of peaks that can theoretically be fit into the chromatogram) is the main driver for choosing it (3).

This main consideration leads to three situations where a multidimensional approach to the column in GC, leading to GCxGC, mainly apply.

- First, there is a complex sample matrix with many peaks and a smaller sub-set of them are of interest
- Second, there are many peaks of interest that must all be separated.

---

**FIGURE 4:** GCxGC–TOF-MS chromatogram of a “brown mousse” sample from the surface of the Gulf of Mexico following the Deepwater Horizon oil spill. Columns: 1D: DB-5MS, 30 m x 0.25 mm x 0.25 μm; 2D: Rtx-200 1.5 m x 0.25 mm 0.25 μm. Temperature program: 1D: 40 to 300 °C at 10 °C/min; 2D: 45 to 305 °C at 10 °C/min. Modulator: 0.90 s hot pulses; 1.60 s cold pulse. MS: EI, full scan 40–600 amu.
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Third, there are a few very closely spaced or overlapping peaks, and they are not separated following optimization of the method.

The first major application for GCxGC was in petroleum analysis, perhaps the most extreme example of the first situation. Petroleum-related samples often contain thousands of compounds, yet often only a few are of interest. They must still be separated from the rest of the extremely complex sample matrix. Figure 3 shows a traditional GC–MS chromatogram of a sample of “brown mousse” obtained from the surface of the Gulf of Mexico following the explosion of the Deepwater Horizon oil platform in 2010. A photograph of a brown mousse sample in a jar is included. The sample has the physical consistency of chocolate mousse, and the odor of another well-known brown substance. Extraction and chromatographic conditions are included with the figure. This chromatogram is typical of petroleum analysis in one dimension. The spikes represent signals from straight chain alkanes, and the “hump” results from a huge number, perhaps thousands, of overlapping hydrocarbon isomers. It is very difficult to detect individual compounds or compound classes of interest from such a complex one-dimensional chromatogram.

Figure 4 shows a GCxGC–TOF-MS separation of the same extract. The chromatogram is viewed as a contour plot, with the bright spots representing peaks against the blue baseline. The horizontal axis represents the traditional retention time on the first-dimension column; the total run time is 40 min, similar to the one-dimensional example. The vertical axis represents the short second-dimension retention time. As described above, the full chromatogram was separated into 8-s slices, and reassembled into the three-dimensional plot. The brighter and more red the spot, the taller the peak, while the blue color represents the baseline. The black dots represent the apexes of all the individual peaks identified by the data system. In this case, it is possible to generate a mass spectrum of each of these peaks, if desired.

We were interested in polycyclic aromatic hydrocarbons (PAHs). The PAHs were easily identified using extracted ion chromatograms; some are circled on the plot. This is the first and most important benefit of GCxGC: Individual compounds or compound classes of interest are much more easily separated from the remaining matrix components. With judicious choices of column sets, the chromatography can be made very highly selective for specific compounds of interest. In this case, the PAHs are found together near the “top” of the chromatogram in the circled region. In our application, GCxGC was used to separate and identify a small number of components of interest that share a common chemistry, from a large and complex sample matrix. Figure 4 also shows a few peaks near the bottom of the chromatogram. This is an example of “wraparound,” an effect caused by second dimension peaks being retained longer on the second-dimension column than the modulation period (in this case...
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Given that the analytes of interest were PAHs and related compounds, we chose to extract \( m/z \) 128, which is representative of naphthalene and related compounds. Figure 5 shows the extracted ion chromatogram (EIC) of an oil spill sample. This chromatogram demonstrates the very high selectivity that can be obtained when combining the separation power of a traditional first-dimension column with the added selectivity of the second-dimension column followed by a selective detector. The bright spots represent the peaks that included signals for \( m/z \) 128. Note that the large signals for the alkanes no longer appear. The black dots, which represent the maxima of all the peaks, are left in the figure to demonstrate the unique selectivity obtainable in GCxGC, especially when using MS detection, that enables the isolation of a few peaks from hundreds or thousands.

In the second situation, there is a large number of analytes, which must be fully separated, either for good quantitative analysis (perhaps detection is performed with a flame ionization detector or other traditional detector, not a mass selective detector [MS]) or for resolution that must meet regulatory requirements. One example of this type of problem is the analysis of residual solvents in pharmaceuticals, which is governed in the United States by the United States Pharmacopeia, General Chapter <467> (USP <467>) (4). In 2008, Crimi and Snow published an article in LCGC North America describing the use of GCxGC–FID for this analysis (5). This article is available freely online. A two-dimensional separation of the complete set of class I (high toxicity), class II (moderate toxicity), and class III (low toxicity) residual solvents is described, along with discussion of the method development process.

Figure 6 shows the two-dimensional separation of the class II, moderately toxic solvents. These include common moderately toxic solvents, such as methanol, ethanol, acetonitrile, dichloromethane, and others. A full list of the analytes can be found in the article. In this example, the analytes were dissolved in methanol, which is not a traditional diluent for residual solvents analysis. As seen in the figure, the components are all well-separated from each other, from matrix components and from interferences inherent in GC analysis. The inset shows the separation of acetonitrile and dichloromethane, as an important system suitability test. Note the large solvent peak, methanol. It is also fully separated from these two analytes, including the long tail. In GC, most solvent peaks can exhibit a detectable tail long after the solvent has eluted. In traditional one-dimensional GC, the solvent peak tail would lie underneath all analyte peaks, possibly affecting quantitation. In GCxGC, the analytes are fully separated from the solvent peak tail. Also note the row of small, sharp peaks.

**FIGURE 7:** Selected-ion monitoring (SIM) chromatogram for SPME-GC–MS analysis of nine drugs. The drugs are identified in the article. Reprinted with permission from Reference 6.

**FIGURE 8:** SPME-GCxGC–TOF-MS contour plot chromatogram of 14 drugs extracted from tap water. Drugs are identified in the article. Reprinted with permission from Reference 6.
These are indications of a small amount of septum bleed. In a traditional separation, they would appear as “extra” peaks, possibly interfering with the analysis. In GCxGC, like the solvent peak, they are fully separated from the analytes. A complete separation of all the International Council for Harmonization (ICH) Class I, II, and III residual solvents (57 compounds) and full description of the separation conditions is provided in the article. In this case, GCxGC allowed separation of all 57 analytes, with separation from the solvent peak tail and other interferences.

In the third situation, GCxGC can be used to separate several closely eluted or overlapping peaks that are not separated in more traditional method development. This situation may arise, especially in cases where the first-dimension separation is optimized for speed. A faster separation generally means less separating power and lower resolution. Figure 7 shows a one-dimensional separation, really a non-separation, of several steroids on a traditional 5% phenyl polydimethylsiloxane column, 15 m × 0.25 mm × 0.25 μm (6). I use this stationary phase and column dimensions for many separations in my own laboratory, because it represents an excellent compromise between separating power and analysis time. It is a good starting point for method development. There are about 10 overlapping peaks eluted in a very short time window at about 12 min. This situation is common with close structural isomers and closely related structures. There are several possible approaches for optimizing this separation, including further optimizing the temperature program (see Hinshaw’s recent “GC Connections” column using a computer simulation (7)), changing the column to one with greater selectivity for steroids, or heart-cutting, a classical form of multidimensional GC in which the small portion of the chromatogram with the overlapping peaks is transferred to a second, more selective column (8). Heart-cutting is still often the method of choice in this situation, as the instrumentation required is simpler than for GCxGC.

In this case, we used GCxGC to separate the steroids. The resulting chromatogram is shown in Figure 8. The first-dimension column was the same as in the single dimension separation, so the retention times of the overlapping peaks are about the same and the total run time is about the same. The second-dimension column was 1.5 m × 0.25 mm × 0.25 μm trifluoropropyl methyl polysiloxane, which is more polar, and has better selectivity for steroids and other drugs due to greater opportunity for dispersive and dipole–dipole interactions between the trifluoropropyl methyl moieties on the stationary phase and the various functional groups on the drugs. This separation demonstrates increased resolving power of GCxGC in a similar run time to GC, with the formerly overlapping analytes now fully separated. Also note that SPME was used for the sample preparation and injection.

There are now myriad applications of GCxGC in the chemical literature, and several instrumental configurations. Most major instrument manufacturers offer multidimensional GC in some form, ranging from simplified heart-cutting to fully implemented GCxGC. A simple search in Google Scholar using “GCxGC” as the keyword yielded about 5870 results (9). To quickly learn more about GCxGC, consider attending the International Symposium on Capillary Chromatography and GCxGC Symposium in Fort Worth, Texas, May 12–17, 2019 (10,11). This is the continuation of an annual series of small meetings of the best minds in capillary chromatography that began in Hindenlang, Germany, in the 1970s. The meeting is now held in alternating years in Ft. Worth (USA) and Riva del Garda (Italy). One of this year’s plenary lectures, titled “Don’t Bring a Football to a Baseball Game: Get GCxGC to Have its Own Rules, Field, and Fan Base,” is being given by Chris Reddy of Woods Hole Oceanographic Institute. Chris Reddy is one of the leaders in bringing GCxGC from the realm of chromatography research laboratories into the “real world.” Unlike most conferences, this (Continued on page 130)
Milos V. Novotny and Ken Broekhoven are the winners of the 12th annual LCGC Lifetime Achievement and Emerging Leader in Chromatography Awards, respectively. The LCGC Awards honor the work of leading separation scientists for lifetime achievement and emerging potential (Table I). For the fifth year, the award winners will be honored at an oral symposium as part of the Pittcon 2019 conference, held this year in Philadelphia on March 19. The presentations will feature talks by each award winner, as well as by Gert Desmet, Robert T. Kennedy, and Milton L. Lee. The Awards Session will be held on Tuesday, March 19, from 1:30 pm to 4:55 pm.

The Lifetime Achievement Award

The Lifetime Achievement in Chromatography Award honors an outstanding and seasoned professional for a lifetime of contributions to the advancement of chromatographic techniques and applications.

Milos V. Novotny, the 2019 winner, received his undergraduate and graduate degrees in biochemistry from the University of Brno (now Masaryk University), in what is now the Czech Republic. In 1968, he emigrated to Sweden, and held a position of Research Assistant Professor at the Royal Karolinska Institute in Stockholm in mass spectrometry. He then moved to the United States as a postdoctoral fellow at the University of Houston, in Texas. In 1971, he was appointed an assistant professor of chemistry at Indiana University; he was promoted to associate professor in 1974, and full professor in 1978. In 1988, he was named the James H. Rudy Professor of Chemistry, in 1999 a Distinguished Professor, and in 2000 became additionally the Lilly Chemistry Alumni Chair. He was also the Director of the National Center for Glycomics and Glycoproteomics there from 2004 to 2009. He is currently the Director of the Institute for Pheromone Research and the Director of the Novotny Glycoscience Laboratory.

Novotny’s contributions to the field of chromatography span a wide range of activities. He was a pioneer in virtually all capillary separation techniques, starting with significant surface treatment technologies in the preparation of glass capillary columns for gas chromatography (GC). He developed the first credible combination of capillary GC with mass spectrometry (MS) in the late 1960s, and continued with his conceptually important work in capillary liquid chromatography (LC) and supercritical fluid chromatography (SFC) in the late 1970s. This was followed by seminal contributions to capillary electrophoresis (CE) and capillary electrochromatography (CEC) of peptides and carbohydrates about 10 years later. He is known as a major contributor to important methodologies, based on LC–MS and CE, in the areas of glycomics and glycoproteomics. He also made a unique contribution to separation science and society at large by designing two chromatographic columns that were an important component of miniaturized GC–MS equipment that was landed on the surface of Mars in July 1976 by the U.S. National Aeronautics and Space Administration (NASA) in the Viking Mission.

Novotny received an Honoris causa doctorate from Uppsala University (Sweden) in 1991, a Doctor of Science degree from his alma mater Masaryk University in 1992, and an honorary doctorate from Charles University, in Prague, in 2007. Novotny was named a foreign member of two scientific academies: the Royal Society for Sciences, Sweden (1999) and the Learned Society/Academy of Czech Republic (2005). He has received more than 25 national and international awards for his work.

A Love Affair with Chromatography

Novotny says that his “love affair” with chromatography started during his undergraduate research in 1961. “While the methodology I was using (mostly paper chromatography) was clearly primitive by today’s standards, I could somehow relate to the impact that A.J.P. Martin and...
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- Extraction of PCBs From Dry Soy Meal & Fish Meal
- Extraction of PCB’s From PUF Filters
- Extraction of PCB’s From Soil, Loam or Clay (EPA 3545A)
- Extraction of Pesticides From Cannabis Plant
- Extraction of Pesticides From Dry Spice Samples
- Extraction of Pesticides From Rice
- Extraction of Pesticides From Hops
- Extraction of Pesticides From Strawberries
- Extraction of Pesticides From Avocados
- Extraction of Pesticides From Wet Food Sample
- Extraction of Phthalates From Polyvinyl Chloride
- Extraction of Pigments From Plastics
- Extraction of Polyphenols From Cacao
- Extraction of Polyphenols From Beans
- Extraction of SVOC’s From Soil, Loam or Clay (EPA 3545A)
- Hydrolysis & Extraction of Total Fat From Cookies
- Hydrolysis & Extraction of Total Fat From Chocolate
his colleagues made in separating and detecting the mixtures of amino acids at the microscale,” he said. But it wasn’t love at first sight. He was trying to separate components of an alkaloid mixture and the paper chromatography was slow, and the solvent systems were inefficient. His frustrations quickly ended, however, when he heard about a new technique, thin-layer chromatography (TLC). “TLC was, to me, chemistry at its best: I was able to play with different adsorbents, quickly optimize the solvent systems, and even come up with my own ways to visualize and detect the separated TLC spots,” he says. “I became thus a sort of local TLC expert as a second-year university student who was ready to solve everyone’s problems through chromatography.” After choosing biochemistry for his graduate studies, Novotny used more-established chromatography techniques to purify proteins and began appreciating the real structural complexity of biological systems, a perspective that he feels serves him well throughout his research career. “I was even then an analytical chemist by heart, but my biochemistry background was important as a long-term incentive to develop better ways to separate and identify important biological compounds,” he says.

It was at this point that Novotny’s “American journey in chromatography” began. After completing graduate school, he went to the Karolinska Institute in Stockholm to work as a research assistant professor in mass spectrometry. But the political situation in Czechoslovakia was worsening, and he had to decide where to live permanently. “I thought an English-speaking country would be professionally preferable at the time,” he says. “Why not America?” This took him from a cool summer in Sweden to the sweltering heat of Houston in August of 1969, for a two-year post-doctoral position with Albert Zlatkis, a move he describes as an extremely important transition in his entire career. “I owe much gratitude to Al Zlatkis for many things, but perhaps most for his insistence that I apply for an academic job,” says Novotny. He had thought he wanted a job with an instrument company, but at Zlatkis’ insistence, applied to five academic institutions and received invitations to visit three. “The interviews opened my eyes to academia,” Novotny says. “I never regretted my decision to accept.” Thus began Novotny’s career at Indiana University, where he developed a highly respected research program and mentored future leaders of the field.

**Groundbreaking Work Across Capillary Techniques**

Novotny’s groundbreaking work began with GC in his early research in surface wettability problems in preparing highly efficient glass GC capillaries (1,2). The surface treatment techniques enabled high-performance separations for biological mixtures, which was revolutionary. This work also led to pioneering research in the early demonstration of capillary GC–MS and its applications to complex mixtures. Novotny applied glass capillary columns to the separation of complex steroid mixtures, which became one of the milestones in the development of chromatography (3,4). This work became internationally known very quickly and stimulated a great deal of interest in high-resolution separations.

Novotny’s group at Indiana was one of the first to apply serious chemometrics to chromatographic problems (5,6). This work was spearheaded by graduate students Mike McConnell and Jerry Rhodes, who applied non-parametric pattern recognition in the analysis of urinary volatile profiles from diabetic patients. “The metabolic patterns at 95% correct classification were subjected to a feature-extraction algorithm for selection of metabolically significant components for the subsequent GC–MS identification,” Novotny explained. “While these procedures may seem trivial through the eyes of today’s computational technologies in the ‘omics era,” ours were developed during the 1970s!”

Novotny then moved into new areas: capillary LC and SFC. In 1978, two key papers were published (7,8), which are considered by many to be the beginning of the field of capillary LC. The intellectual foundation for this idea was inspired by the theoretical insights of J. Calvin Giddings of the University of Utah. “As a young scientist in Czechoslovakia, I had almost religiously studied his classy 1965 book Dynamics of Chromatography and related papers,” he says. “There it was: To counter the much slower solute diffusion in the liquids, you have to resort to very small, preferably spherical, particles, with small diameter columns, which eventually necessitate the use of unusually high column inlet pressures.”

The development of capillary SFC is quite a different story, Novotny says, but also has connections to the observations made by Giddings and coworkers in the 1960s, in this case on the solvating properties of dense gasses near the critical point. “My former graduate student, and later a faculty member at Brigham Young University, Milton Lee, spent two highly productive summers at Indiana, and I introduced him to my thoughts on ‘rejuvenating’ Giddings’ dense-gas ideas in terms of much improved capillary column technologies,” Novotny recalls. “Milton and my students collaborated and it worked; the rest is history.” Capillary SFC enriched both his and Lee’s research programs, attracted other groups into the field, and was successfully commercialized, Novotny says, while noting that further developments in SFC later took “some unexpected turns.”

**A Broad Impact**

What are Novotny’s biggest contributions to chromatography? We asked leading researchers for their perspectives.

Milton Lee, the Emeritus H. Tracy Hall Professor of Chemistry at Brigham Young University, emphasized the broad nature of Novotny’s work. “Milos has unique insight into the whole field of separation science. He has had first-hand experience in all forms of chromatography and electrically driven separations, and their coupling with mass spectrometry,” he said. “I always come away with new ideas, understanding and enthusiasm from discussions with him, as I am certain is the case for others.”

Jim Jorgenson, the Kenan Professor of Chemistry at the University of North Carolina at Chapel Hill, also cites Novto-
ny’s prolific contributions: “His extremely early work in capillary GC and capillary GC–MS. His pioneering work in profiling metabolites (1970s) that is the forerunner of metabolomics. His extremely early work (around 1978) in capillary LC. His 30 years of devotion to developing analytical methodologies for complex carbohydrates. His 45 years of highly successful studies of mammalian pheromones.” This is a short list of Novotny’s accomplishments.

Bob Kennedy, a chemistry professor at the University of Michigan, also cites Novotny’s diverse contributions, including his development of capillary gas chromatography columns, capillary liquid chromatography columns, and methods for glycobiology.

“I think that the capillary LC is especially interesting because many people didn’t believe that it would ever have an impact in analytical chemistry, but now techniques based on work he did are routinely used,” Kennedy says. “His early work in this area made possible my PhD work on single cell analysis.” Kennedy appreciates Novotny’s work in glycobiology because it addresses such a hard problem; the structures are complex and analysis and interpretation are challenging. “By just working on it, [Milos] inspired many others to get involved and help move the field forward,” Kennedy says. “Now we know how important these glycosylations and related modifications are.”

Pat Sandra, an Emeritus Professor at Ghent University in Belgium and the Director of the Research Institute for Chromatography (RIC), also praised Novotny’s fundamental contributions to the present-state-of-the-art of all pressure- and electro-driven separation techniques: capillary GC, microcolumn LC, SFC, CE, CEC. He also applied these techniques to study small molecules (identifying the first mammalian pheromones, and glycans) and large molecules (proteomics). “Needless to say, he also developed unique sample preparation methods for biological samples,” Sandra adds. “Very few colleagues were covering such a broad research field, giving him a helicopter view over separation methods and their applicability.”

Susan V. Olesik, a professor and chair at Ohio State University, has known Novotny since she was a postdoctoral researcher in his laboratory in 1982. They collaborated on the first Fourier-transform–infrared

![FIGURE 3: Novotny in 1977 in Amsterdam, The Netherlands, on the occasion of the 12th International Symposium on Advances in Chromatography organized by Al Zlatkis. Left to right: Leslie Ettre, Al Zlatkis, Joseph Huber, and Milos Novotny (Photo courtesy of Pat Sandra).](image-url)
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(FT-IR) interface for SFC. Olesik feels that Novotny’s most significant contributions to the field include capillary GC systems to collect and describe pheromone analysis long before others even considered such work. “He and Milton Lee started the entire field of analytical separation science,” she says. “He led analytical separation science into microscale packed capillary column LC with very efficient and relevant separations.” Later in his career, Olesik says, “[Novotny] revolutionized the study of glycopeptides based on high-efficiency separations combined with mass spectrometry.”

A Unique “School of Chromatography”

Even given the groundbreaking nature of so many of Novotny’s advances in capillary techniques, many cited his work as an educator among his greatest accomplishments. For example, Milton Lee says that even though Novotny is recognized as one of the earliest pioneers in both capillary GC and LC, and especially their application to biochemical problems, his greatest contribution may be his influence on countless separation scientists worldwide through training students, postdocs, and visiting scientists. “Milos is passionate about the field of separation science and is uniquely gifted in his ability to motivate and inspire others, especially young people and those new to the field,” he said. “He will always be an active voice in promoting excellence.”

Kennedy agreed, pointing out that Novotny mentored so many students who became exceptional analytical chemists. “Certainly, the quality of his research instruction has contributed to the outstanding accomplishments of his many former students and post-doctoral associates including Jim Jorgenson, Milton Lee, Victoria McGuffin, Takao Tsuda, and Susan Olesik,” He said. “This is a remarkable group of people, all of whom received their education from Milos.”

“Milos created a unique ‘chromatographic school’!” said Sandra. “For example, several of his students are recipients of the ACS Chromatography award, such as Milton Lee, Jim Jorgenson, and Susan Olesik.”

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Have you ever noticed that you can test the same analytes on two different C18s and you will get two very different chromatograms? We hear this a lot from our customers, so we thought it might be useful to show some of the reasons why this might happen. All four of the columns tested above are C18s, and as you can see, each one produces a different chromatogram. So, why are they so different? We are used to thinking about C18s in terms of carbon load and hydrophobicity, but that is only part of the story. Here we show, not only the hydrophobic nature of these columns, but also their electrostatic interaction and steric selectivity, each of which contribute to a column's total separation ability. Electrostatic interactions can be caused by exposed unreacted surface silanols, or in the case of Cadenza CX-C18, polar end-capping. This can be desirable if you want to take advantage of a polar difference between two molecules, or in the case of unreacted surface silanols, it could contribute to poor peak shape, especially for basic compounds. In which case, a reduction in electrostatic interaction might be preferred, as with Dacapo DX-C18, the world's first 100% silanol-free C18. Steric selectivity can provide faster baseline resolution when you have two similar compounds that do not appreciably differ hydrophobically. We first discovered how to improve steric selectivity with Cadenza CD-C18, by increasing ODS ligand density. We have since improved on this technique with Cadenza CX-C18 and Dacapo DX-C18. Please see our LCGC application note for more details.
As one of Milos’ first graduate students, Milton Lee had firsthand experience of Novotny’s mentorship. Lee started his graduate studies in 1971, the same year Novotny arrived at Indiana University. “As a graduate student, I interacted with Milos almost every day that he was at the university,” Lee recounts. “He was keenly interested in our daily progress and visited the laboratory often, sometimes several times a day; we didn’t need to make appointments – there was plenty of opportunity to talk with him rather informally.” And Novotny’s influence on Lee and on his career did not end with his degree at Indiana University, but has continued ever since. “He is still my greatest professional colleague and advocate, and I know that this is also the case for his other students,” Lee says. “He is extremely proud of all of our accomplishments.” Over the years, he and Milos became the best of friends.

Jim Jorgenson also has fond memories of his graduate school experience with Novotny. In particular, Jorgenson appreciated the encouragement to be independent and curious. “He did not try to force me to just be focused on my thesis project (pheromones) but supported and encouraged me to pursue a whole range of ideas in separations,” Jorgenson recalls. “It was these additional projects that became the core of my entire career as an independent researcher. Had Milos not provided that freedom and encouragement my life would likely not have turned out as exciting and fulfilling for me.”

That encouragement has extended beyond Novotny’s immediate graduate students. As an academic “grandchild”—having earned his PhD from Jim Jorgenson, one of Novotny’s students—Kennedy felt that Novotny always took extra interest in him and his career. “For example, I can remember often seeing him in the audience when I gave a talk,” Kennedy says.

### Table I: Winners of the LCGC Awards

<table>
<thead>
<tr>
<th>Year</th>
<th>Lifetime Achievement</th>
<th>Emerging Leader</th>
</tr>
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<tbody>
<tr>
<td>2008</td>
<td>Walt Jennings</td>
<td>Gert Desmet</td>
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<tr>
<td>2009</td>
<td>Harold McNair</td>
<td>Kevin Schug</td>
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<tr>
<td>2010</td>
<td>Georges Guiochon</td>
<td>Jared Anderson</td>
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<tr>
<td>2011</td>
<td>James W. Jorgenson</td>
<td>Dwight Stoll</td>
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<tr>
<td>2012</td>
<td>Lloyd Snyder</td>
<td>Emily Hilder</td>
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<tr>
<td>2013</td>
<td>Peter W. Carr</td>
<td>Davy Guillarme</td>
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<tr>
<td>2014</td>
<td>Fred E. Regnier</td>
<td>Andre’ De Villiers</td>
</tr>
<tr>
<td>2015</td>
<td>Joseph Jack Kirkland</td>
<td>Caroline West</td>
</tr>
<tr>
<td>2016</td>
<td>Milton L. Lee</td>
<td>Debbby Mangelings</td>
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<tr>
<td>2017</td>
<td>Pat Sandra</td>
<td>Deirdre Cabooter</td>
</tr>
<tr>
<td>2018</td>
<td>Ronald E. Majors</td>
<td>Zachary S. Breitbach</td>
</tr>
<tr>
<td>2019</td>
<td>Milos V. Novotny</td>
<td>Ken Broeckhoven</td>
</tr>
</tbody>
</table>
The FFF - MALS Platform
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“He has always made a point afterwards to talk to me about it and provide insights.”

Kennedy adds that Novotny has been one of his most important mentors after his PhD and post-doctoral research advisors. “He has great insight into working at the interface of biology and chemistry, and I would consider him one of the founders of bioanalytical chemistry,” he says. “I learned from him, for example, that working on biological problems is a great way to be inspired for new analytical challenges.” Kennedy remembers seeing early talks of Novotny’s on diabetes complications where he used methods he developed to study the effects. “I knew that was what I wanted to do when I saw it,” recalls Kennedy.

Novotny’s personal impact went far beyond his students, affecting other researchers and collaborators. Pat Sandra relates his first encounter with Novotny’s work. “Through literature study, I came across the work of Milos Novotny and was immediately very impressed by his work,” he said. “For example, in 1969, he described not only the preparation and characterization of open tubular columns but also their hyphenation to mass spectrometry. This was definitely the approach I should apply to unravel the contribution of hops to beer (my very Belgian research).”

Sandra and Novotny first met almost a decade later, in 1977, and ultimately ended up collaborating. “We worked scientifically together in some projects related with the determination of chemical signal profiles from biological media,” Sandra says. “Milos was an authority in pheromone research.” And with time, the collaboration turned into friendship. “Over the years, we became very close friends sharing not only science but also topics such as culture, art, and European history,” Sandra says. “Milos indeed has a very broad knowledge and interest and our conversations and discussions, often with a glass of red wine, were very lively and stimulating.”

So as researcher, mentor, teacher, colleague, and friend, Milos Novotny has given much to the field of separation science. Olesik succinctly sums up his impact. “He has such a wealth of knowledge both in the analytical separation and biochemistry try that the number of fields that he might impact [now and in the future] are too numerous to list.”

**The Emerging Leader Award**

The Emerging Leader in Chromatography Award recognizes the achievements and aspirations of a talented young separation scientist who has made strides early in his or her career toward the advancement of chromatographic techniques and applications.

Ken Broeckhoven, the 2019 winner, received his PhD in 2010 from the Vrije Universiteit Brussel (VUB), in Belgium. Following post-doctoral research at VUB and work as a visiting researcher in the separation processes laboratory at the Swiss Federal Institute of Technology (ETH Zurich), in Switzerland, he became a research professor at VUB in 2012. He was subsequently promoted to assistant professor and then to his current position as an associate professor in 2017.

Important areas of Broeckhoven’s work include theoretical and experimental studies applying the kinetic plot method to gradient chromatography, laying the basis for the application of the method to other liquid chromatography (LC) modalities and toward its use in supercritical fluid chromatography (SFC) and gas chromatography (GC). He developed a novel solution for the effects of viscous heating in ultrahigh-pressure liquid chromatography (UHPLC); and the first demonstration of the use of very high operating pressure (up to 2600 bar) in typical column dimensions (2.1 mm inner diameter [i.d.] columns) used in UHPLC instrumentation, and later extended this approach to gradient chromatography. He has also shed light on the future direction and limits of separations power in liquid chromatography, including the importance of extracolumn band broadening as one of the limiting factors for further improvements in separation speed and efficiency. Broeckhoven’s work revealed the significant effect that turbulent flow in the connection tubing can have on the operating pressure and separation results in supercritical chromatography.

Broeckhoven has published 61 papers, with more than 850 citations. He has also given more than 40 oral and 52 poster presentations at scientific conferences, one of which received first place in the poster award competition at HPLC 2008 conference in Boston.

![FIGURE 5: Ken Broeckhoven.](image)

One of Broeckhoven’s most cited papers (80 citations) involves a description of the kinetic plot method. This method was originally developed for isocratic separations and was extended for use in gradient elution separations (9). For any data set containing the separation efficiency and the flow rate from a single column, the data may be converted into a kinetic performance limit curve of the tested separation medium. This paper presents experimental and numerical data corroborating the conclusion that the kinetic performance limit curves are independent of the column length for the collected data.

In another highly cited paper (10), Broeckhoven and colleagues described their work to double the operating pressure for UHPLC. In addition, the paper describes kinetic gain factor expressions, while constructing the corrected Knox and Saleem limits. The work even considers the effect of extracolumn band broadening. The paper reported, “The equations show that the possible gain by moving from 1200-bar to 2400-bar instruments would at most lead to a 40% increase in efficiency and only 20% in resolution or peak capacity, while analysis time would halve.” This method demonstrates a requirement for radical design changes for optimized chromatographic systems.
Advancing the Fundamentals of HPLC and UHPLC

Broeckhoven’s most cited papers to date (11–13) discuss advances related to questions of optimization and efficiency in designing and applying peak capacity in HPLC. This work represents novel, in-depth research into existing technology. He began his interest in this technology through initial studies as a chemical engineer in the field of LC. He began working with Pat Sandra and Frederic Lynen at Ghent University, where he began collaborating with Deirdre Cabooter. His first investigation was an inquiry into why very narrow bore (1 mm i.d.) columns, ideally suited for UHPLC, showed poor performance compared to 2.1- and 4.6-mm i.d. columns. “Unfortunately, the project failed due to the much too large system dispersion common for earlier generation LC systems, but I got a lot of hands-on experience in LC which landed me in the follow-up project on kinetic plots originally developed by my promotor Gert Desmet,” Broeckhoven says. “By developing a framework from gradient LC, we also paved the way the extend the kinetic plot methodology to other chromatographic techniques.”

Broeckhoven’s research has moved from whole system measurements to elucidating the different contributions of individual components such as the injector, the detector, and tubing. These new discoveries are yielding information for new design work for the development of the next generation of LC instruments. For example, Ken points out that recently Prof. Attila Felinger of the University of Pécs, in Hungary, showed that the column hardware (frits and inlet) has a significant effect on column efficiency. This is an area of research that had received little attention previously.

One of Broeckhoven’s papers describes the advances in UHPLC technology and system design over the past three years (12). “Just a few years ago,” he says, “many UHPLC systems were simply upgraded HPLC systems that were capable of operating at high pressures.” Today that has changed; manufacturers have redesigned and optimized UHPLC systems for ultra-high operating pressures and minimized system dispersion. “As a result, not only the pumps, but other parts, are fully optimized, including the injectors (for example, having the needle seat integrated on the injec-

![Figure 6: Ken Broeckhoven speaking at the 9th Balaton Symposium on High-Performance Separation Methods, in Hungary, in 2013 (Photo courtesy of Ken Broeckhoven).]
tion valve), column ovens (different thermal modes, active preheating) and detectors (frequency and response time),” he notes.

In his most recent publications (14,15), Broeckhoven addresses band broadening of contemporary injectors in UHPLC. In this work his team was able to show that peak dispersion (variance) from the injection is not simply related to the square of the injection volume as commonly assumed. “Reducing the injection volume still reduces the peak widths, but not as much as one expects,” he says. Although the team had some theories to explain this, it was not until they decided to go back to computational fluid dynamics simulations of the injection processes that they fully understood what was going on (15). “My colleague Sander Deridder, using a simplified model of injector geometry, showed that flow and diffusion occurring during sample load and injection inherently put a limit on how much you can reduce injection dispersion and that we would need to develop novel ways of introducing our sample if we want further improvements in injection band broadening,” he says.

Broeckhoven feels that his main (and perhaps most useful) research contribution for the broader field of LC is in the methodology of how to extend the kinetic plot method to gradient LC. This work has enabled gradient LC to be used for optimization of real-life applications. “This theoretical basis from the gradient kinetic plot also serves to extend it to other techniques such as SFC, GC, and so on,” he explains. In his future work, he plans to investigate a wide range of topics. “I want to look at topics ranging from the optimization of individual separation techniques (higher performance, faster separations, lower system dispersion), to the coupling of these techniques (hyphenation of LC, SFC, GC, and MS) and the necessary software to find the optimal separation conditions in a more automated way,” he said.

The Contributions of a Young Researcher in Chromatography

Researchers and collaborators share the view that Broeckhoven is truly an emerging leader in chromatography. “Ken is a worthy winner of the emerging leader in chromatography award,” says David McCalley, a professor of Bioanalytical Science at the University of the West of England, in Bristol, UK. He notes that Broeckhoven already has an impressive publication record, and a publication h index of 20, despite his young age. “This achievement is all the more remarkable because his first major publication was as recent as in 2007,” McCalley says. “He has also in this time had to contend with the great pleasures (and distractions!) of a young family.”

McCalley appreciates many specific aspects of the work Broeckhoven has done, including contributing greatly to the understanding of kinetics in LC. “He was part of the group under professor
Gert Desmet (his doctoral supervisor) in Brussels that developed the kinetic plot method for the rationalization of column performance, both in isocratic and in gradient elution chromatography,” he notes. “Using these techniques, he has (amongst other studies) compared the properties of totally and superficially porous particles and helped to explain the differences in their performance.” McCalley also values Broeckhoven’s work to investigate aspects of current HPLC instrumentation including the band broadening contribution produced by ultraviolet (UV) detectors of different design, his study of the effects of UHPLC systems in excess of 2600 bar, and his investigations of column particle sizes smaller than those used in current commercial ultrahigh pressure systems. “This work has enabled researchers to understand the limitations of current instrument design and performance,” McCalley concludes.

Desmet, a full professor at Vrije Universiteit Brussels, was Broeckhoven’s supervisor for his MSc and PhD studies. But he had already noticed Broeckhoven when he was an undergraduate. “He stood out as the smart kid, always asking the right questions during my course on reactor technology,” he said. And when he did his master’s research, his MSc thesis work was so good that the legendary Michel Martin was under the impression that it was a PhD thesis. This outstanding work was presented as an oral contribution at the 2007 HPLC conference in Ghent by Gerard Rozing.

David S. Bell, a director of research and development at Restek, and the editor of LCGC’s “Column Watch” column, agrees. “Ken has contributed much to the fundamental understanding of the physical contributions to separation technologies,” he says. “He brings an engineering perspective to a largely chemistry oriented group.”

Caroline West, an associate professor at the University of Orleans, in France, particularly appreciates Broeckhoven’s work in SFC. “I mostly know Ken’s fundamental work in SFC, where he has contributed to improving the understanding of band broadening effects, taking account of the specificities of the compressible CO₂-based mobile phase,” she says. “Because the conditions there are trickier than in other forms of chromatography, with many contradictory effects, this is a very challenging question.”

Davy Guillarme, a senior lecturer at the University of Geneva, and a jury member of Broeckhoven’s PhD defense in 2010, has known Broeckhoven for more than ten years and collaborated with him within the of Desmet’s group. “From my point of view, Ken’s most important contributions are around the fundamental understanding of chromatography, in particular kinetic performance (the Van Deemter equation, kinetic plots, and so on), frictional heating, band broadening,
and simulation and modelization of the chromatographic behavior," he says. Guillarme also notes that Broeckhoven has been recently involved in the development of a new UHPLC system capable of working at very high pressures and doing work in SFC studying the interactions of turbulent flow, temperature effects, and isopycnic plots, among other subjects.

Deirdre Cabooter, an associate professor at the University of Leuven, in Belgium, has known Broeckhoven since he began his master’s thesis in Desmet’s lab in Brussels in 2006. When discussing his most significant research, Cabooter also cites his work to advance fundamental understanding. “His work on mass transfer in liquid chromatography, the extension of the kinetic plot method to gradient separations, his work on extracolumn band broadening, and his work on UHPLC, at pressures of 2500 bar—these are his greatest contributions to date,” she says.

Monika Dittmann, Principal Scientist at Agilent Technologies explains that Ken is an excellent scientist with the ability to work across experimental as well as theoretical aspects of chromatography. “His contributions to the field of mass transfer and kinetic performance of modern stationary phases as well as his studies of instrument related aspects have helped the entire community,” she says.

The Future
Given Broeckhoven’s accomplishments to date, his collaborators expect more good things from him in the future. Guillarme is convinced that Broeckhoven will continue to work in the field of fundamental chromatography, adding that this knowledge is essential for the practitioners of chromatography. “Ken is already leading a sizeable research group at the University of Brussels, and his work has had strong impact on the community of separation scientists,” he concludes. “I’m sure his research group will continue to grow.”

Cabooter also sees great opportunity for her friend in his role in a tenured position at VUB. She believes he really likes the combination of teaching and research, in an environment where he is free to explore the fundamentals of chromatography. “Ken has already demonstrated that he can easily adapt to new concepts, for example by extending the kinetic plot method to supercritical fluid chromatography and gas chromatography,” she says. “I foresee much more interesting and high-quality fundamental work from him in the future.”

Desmet points out that Ken already has many important contributions to the field, with some of the highlights including the theory for gradient kinetic plots, intermediate cooling as a generic solution for viscous heating chromatography using 2.1 mm bore columns at 2600 bar and using SFC at 1200 bar.

“No doubt, Ken will become one of the leading experimental and theoretical specialists on the fundamentals of chromatography, working in close collaboration with industry wherever and whenever possible,” concludes Desmet.

Bell believes Broeckhoven will continue to probe the limits of chromatography—exploring extremes of pressure, temperature, and flow. “If there is a breakthrough, I suspect he will jump to the next edge that interests him,” Bell says.

References


Jerome Workman, Jr. is Senior Technical Editor for LCGC North America and Spectroscopy. Direct correspondence about this article to Direct correspondence to jerome.workman@ubm.com
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Analytical Solutions for Biosimilar Characterization

Analytical techniques for biosimilars are necessary in order to compare protein sequence, post-translational modifications, function, and quality to the reference product.

A biosimilar is a biologic drug that is “similar” to a reference product that has already been approved by the FDA. To meet the criteria for biosimilarity, analytical studies must demonstrate that the biological product is highly similar to the reference product, aside from minor changes in clinically inactive components. LCGC recently sat down with Cara Tomasek, Product Management Group Leader at Tosoh Bioscience, to discuss an ultrahigh performance liquid chromatography (UHPLC) workflow solution for biosimilar characterization.

**LCGC:** What are biosimilars and why are they important?
**Tomasek:** A biosimilar is a biological product that’s highly similar to a reference or innovator product. While it’s unlikely to be an exact copy of the reference product, it has undergone rigorous evaluation and testing to ensure that no differences in safety, purity, and potency exist when compared to the reference product. Biosimilars can be granted an interchangeability designation, which indicates that it meets additional criteria and can be substituted for the reference product by a pharmacist without involvement of the prescribing physician.

To address the second half of your question, biosimilars are important because they generate competition in the market, thus expanding patient access to critical medications. They seek to generate an environment similar to that which was generated after the introduction of generic small molecule medications.

**LCGC:** How are biosimilars different than the generic drugs?
**Tomasek:** Generic small-molecule drugs are chemically synthesized to be identical to the originally developed and patented material. They’re easy to characterize and have a small well-defined structure. Biosimilars, on the other hand, are very complex molecules with many post-translational modifications. They’re grown in complex living systems, which make it exceptionally challenging to produce exact replicates of the reference material. And with this increased complexity, biosimilars require extensive analytical characterization.
**LCGC:** Do you anticipate an increase in the prevalence of biosimilars over the next five years?  
**Tomasek:** Yes, I believe that we will see an increase in the number of approved biosimilars available to consumers in the next four to five years. While it can cost upward of $1.2 billion to develop a new biologic, a biosimilar molecule can be developed for roughly $375 million, which indicates a significant cost savings to pharmaceutical companies for a product that’s proven to work effectively. This cost savings coupled with the growing prevalence of chronic disorders globally contribute to the success of the biosimilar market and its anticipated continued growth.

**LCGC:** What analytical techniques are currently used to analyze the similarity between the reference product and the biosimilars?  
**Tomasek:** When developing a biosimilar, the manufacturer first must understand the protein sequence, post-translational modifications, biologic function, and variability in the quality attributes of the reference product. Then, as lots of biosimilars are produced, they can be compared against the reference product standard. There’s a vast array of analytical techniques used in the analytical characterization of biosimilars and reference products, so I’ll specifically focus on analysis using UHPLC. Commonly employed UHPLC techniques include peptide mapping using reversed phase LC–MS analysis to elucidate the primary structure as well as post-translational modifications; size exclusion chromatography (SEC) to detect protein aggregation or higher order species; ion exchange chromatography for charge distribution analysis; and hydrophilic interaction chromatography to unveil present glycosylation.

**LCGC:** What does Tosoh offer to assist the researchers examining biosimilarity?  
**Tomasek:** Tosoh Bioscience offers a workflow solution for biosimilar characterization using the renowned TSKgel® column portfolio. TSKgel ODS-100V is a traditionally fully porous C18 column, and it can be used for primary structure and post-translational modification identification. TSKgel CM-STAT is a cation exchange column that contains non-porous particles and provides fast and high-resolution separations of charged isoforms. TSKgel Amide-80 is a hydrophilic interaction (HILIC) column that utilizes a unique surface chemistry to selectively resolve released glycans. And, the most recent addition to the TSKgel portfolio is the TSKgel UP-SW3000 SEC column that provides fast and high-resolution separation of aggregates and fragments.

**LCGC:** You mentioned size exclusion chromatography is an analytical technique that can be used to compare protein aggregates and fragments in the reference product and biosimilar candidate. What are some considerations the researchers should note when selecting a SEC column?  
**Tomasek:** When selecting a SEC column, a few important aspects to consider are column length, column internal diameter (ID), particle size, pore size, and pore volume. Other somewhat immeasurable characteristics such as packing efficiency and presence or absence of secondary interactions with the analyte of interest also play a role in column selection. However, these characteristics will need to be examined after initial selection.

**LCGC:** What impact does each of these considerations have on chromatographic performance?  
**Tomasek:** Because SEC separations take place in one column volume, length and ID play a pivotal role in separation. Longer columns may be required to get the desired resolution. Typically, SEC columns are approximately 30 centimeters in length. Reduction in ID can decrease resolution, but can provide heightened sensitivity in situations with limited sample volume. With regard to particle size, the trend is maintained with SEC, and the smaller particles inherently provide better resolution and higher efficiency separations. A reduction in particle size may enable the researcher to reduce length and/or ID while maintaining the desired resolution between analytes of interest. In taking a look at pore size and pore volume, it is important to consider the overall accessibility of the pores. A calibration curve should be used in order to determine the appropriate pore size for the molecules you are trying to resolve. In general, a 250-angstrom pore works well for monoclonal antibody-based drugs and related biosimilars. Pore volume also must be examined because larger pore volumes can provide more space for separation and, in turn, higher resolution. TSKgel columns are known to provide a high pore volume for high-efficiency separations of aggregates, monomers, and fragments.
Data Integrity Focus, Part II: Using Data Process Mapping to Identify Integrity Gaps

Understanding and mitigating risks to regulatory records is an important part of a data integrity program. We discuss data process mapping as a technique to identify data gaps and record vulnerabilities in a chromatographic process and look at ways to mitigate or eliminate them.

R.D. McDowall

Welcome to the second installment of "Data Integrity Focus." In the last part, we looked at the overall scope of a data integrity and data governance program via a four-layer model (1–3). In this part, we look at a simple and practical methodology that can be applied to identify the risks with any process in a regulated "good practice" (GXP) laboratory. Once identified, the risks can be mitigated or eliminated to ensure the integrity of data and records. The methodology is called data process mapping, and it is a variant of process mapping, which some of you may be familiar with if you have been involved with implementation of a computerized system or six sigma improvement project. Once the process is mapped, the data and records created, modified, or calculated are identified and assessed to see if there are any data vulnerabilities in a paper process or computerized system.

Ignore Paper Processes at Your Peril!

It is very important to understand that data integrity is not just a computer or information technology (IT) equipment problem. There are many manual process generating paper records that occur in the laboratory, such as sampling, sample preparation, calculation, and review (3–5). Many observation tests such as appearance, color, and odor are typically recorded on paper. Even with a computerized system, there are additional and essential paper records, such as the instrument and column log books.

What Do the Regulators Want?

What do the regulatory guidance documents say about assessment of processes? There are three documents that I would like to focus on. The first is the World Health Organization (WHO) in their guidance document (6), that notes:

- 1.4. Mapping of data processes and application of modern quality risk management (QRM) and sound scientific principles throughout the data life cycle;
- 5.5. Record and data integrity risks should be assessed, mitigated, communicated, and reviewed throughout the data life cycle in accordance with the principles of QRM.

The second is the UK’s Medicines and Healthcare products Regulatory Agency (MHRA) in their 2018 GXP guidance, which makes the following statements about assessment of processes and systems (7):

- 2.6 Users of this guidance need to understand their data processes (as a life cycle) to identify data with the greatest GXP impact. From that, the identification of the most effective and efficient risk-based control and review of the data can be determined and implemented.
- 3.4 Organizations are expected to implement, design, and operate a documented system that provides an acceptable state of control based on the data integrity risk with supporting rationale. An example of a suitable approach is to perform a data integrity risk assessment (DIRA) where the processes that produce data or where data are obtained are mapped out and each of the formats and their controls are identified, and the data criticality and inherent risks documented.

The third and final guidance is from the Pharmaceutical Inspection Cooperation Scheme (PIC/S) (8):

- 5.2.2 Data governance system design, considering how data is generated, recorded, processed, retained and used, and risks or vulnerabilities are controlled effectively;
- 5.3.2 Manufacturers and analytical laboratories should design and operate a system which provides an acceptable state of control based on the data integrity risk, and which is fully documented with supporting rationale.
- 5.3.4 Not all data or processing steps have the same importance to product quality and patient safety. Risk management should be utilized to determine the importance of each data or processing step. An effective risk management approach to data governance will consider data criticality (impact to decision making and product qual-
ity) and data risk (opportunity for data alteration and deletion, and likelihood of detection or visibility of changes by the manufacturer’s routine review processes).
From this information, risk-proportionate control measures can be implemented.
Summarizing the guidance documents:
- Processes should be assessed to identify the data generated and the vulnerabilities of these records, and this assessment should be documented.
- Vulnerabilities and risks to records must be mitigated or eliminated, and the extent of the controls used depends on the data criticality and risk to the records.
- In some cases systems should be replaced, and there should be a plan for this over a reasonable timeframe,
- Management must accept the process risk and support the migration plan.

Enter the Checklist
Typically, assessment of computerized systems involves a checklist where questions are posed for a spectrometer and the associated computerized system, such as:
- Does each user have a unique user identity?
- Is the audit trail turned on?
- Is there segregation of duties for system administration?
The checklist questions can go on, and on, and on, and, if you are (un) lucky, it can go into such excruciating detail that it becomes much cheaper and safer than a sleeping pill. There are three main problems with a checklist approach to system assessment:
- The checklists are not applicable to all computerized systems, as the questions may not cover all functions of the application
- Checklists can mislead an assessor into focusing too much on the checklist at the risk of not seeing additional data risks posed by a specific system
- Typically, checklists don’t cover manual processes, of which there are many in a laboratory.
If a checklist is not the best tool, what tool should be used to identify data and records and then understand the risks posed?

Principles of Data Process Mapping
Instead of starting with a fixed checklist, start with a blank whiteboard or sheet of paper together with some Post-it notes, pencils, and an eraser. Why the eraser? You are not going to get this right the first time, and you’ll be rubbing out lines and entries on the notes until you do. You’ll need a facilitator who will run the meeting and two to three experts (perhaps laboratory administrators) who know the process,
and, if software is involved, how the application works at a technical level.

The first stage is to visualize the process. Define the start and end of an analytical process (for example, from sampling to reportable result). The process experts should write the stages of the process down on the notes, and place them on the whiteboard or paper in order. The first attempt will be rough and will need revising, as the experts can miss activities, or some activities will be in the wrong order, or the detail is uneven. The facilitator should encourage and challenge the experts to revise and refine the process flow, which may take two or three attempts. Although you can use a program like Visio to document the process, this slows the interaction between the participants during the initial mapping. I would suggest paper and pencil or whiteboard is an easier, and more flexible, option at this stage. When the process is agreed, then commit the final maps to software.

The second stage is to document data inputs, outputs, processing, verification steps, and storage for each of the process activities. This can involve manual data recording in log books, laboratory notebooks, and blank forms, as well as inputs to and outputs from any computerized systems involved in the process. Typically, such a process has not been designed, but has evolved over time, and can often look like a still from Custer’s Last Stand with the number of arrows involved. This is the data process map or what we can call the current way of working.

Once the process is complete and agreed, look at each step and document:

- How critical is each activity within the overall process (for example, product submission, release, stability, analytical development, and so on)?
- Where are the data and records stored in each activity?
- Are the data vulnerable at each stage?
- What is the reason for the vulnerability? (Reasons may include, but are not limited to, manual recording, or manual data transfer between a standalone instrument and another application)
- Are data entered into a computerized system manually, how is this checked, and how are corrections documented?
- Who can access the data? (Consider both paper and electronic records)
- Are the access controls for each application adequate, and are there any conflicts of interest?
- Are data corrections captured in an audit trail and, most importantly, are the entries understandable, transparent, and clear (9)?
- Are the responsibility for all steps and data clearly described, such as decisions or further actions taken and attributed to an individual?
- Is the data verification process clearly described, such as assurance of accuracy of measurement, double checks performed (if any), and the review of the whole data package?

Any vulnerabilities need to be risk assessed, and remediation plans need to be developed. These plans will fall into two areas: quick fix remediation and long-term solutions. We will look at these two areas now for an example involving a chromatography data system.

**Practical Example for Chromatography**

From the theory, we need to look at how data process mapping could work in practice with a chromatograph linked to a chromatography data system (CDS). Welcome to a chromatography laboratory near to you operating the world’s most expensive electronic ruler, as shown in Figure 1.

Let me describe the main features of the simplified process:

- The chromatograph and CDS are set up for the analysis (we’ll not consider the manual, paper-based sampling and sample preparation, because this was discussed recently by Newton and McDowall...
(4), together with the related data integrity problems).

- Although there are several instances of the same CDS, they are all standalone systems, and not networked together.
- There is a shared log on for all users, and this account has all privileges available, including the ability to configure the software.
- Paper printouts are considered the raw data from each instance of the CDS.
- Electronic records are backed up by the chromatographers when they have time, using a variety of media such as USB sticks and hard drives.
- Peaks are integrated, but there is no standard operating procedure (SOP) or control over the integration, such as when manual integration can or cannot be used (2,10).
- The integrated chromatograms are printed out.
- The calculations are printed and signed, but the spreadsheet file is not saved.
- The results are entered manually from the spreadsheet printout into a laboratory information management system (LIMS) for release.
- The second-person review is not shown in this figure for simplicity, but this is a crucial part for ensuring data integrity (11).

Some of you may be reading the process with abject horror, and may think that this would never occur in a 21st century chromatography laboratory. Based on my experience, and this is also seen in numerous U.S. Food and Drug Administration (FDA) warning letters, this process is more common than you may think. Remember that the pharmaceutical industry is ultraconservative, and if it worked for the previous inspection, all is well. However, to quote that world-famous chromatographer, Robert Zimmerman, the times, they are a-changin’. Hybrid systems (discussed in the next part of this series) are not encouraged by at least one regulator (6), and now some inspectors are unwilling to accept procedural controls to mitigate record vulnerabilities.

Identifying Record Vulnerabilities

Once the process is mapped, reviewed, and finalized, the data vulnerabilities can be identified for each process step. The main data vulnerabilities identified in the current chromatographic process steps are listed in Table I. To put it mildly, there are enough regulatory risks to generate a cohort of warning letters. There are many data integrity red flags in this table, including the fact that work cannot be attributed to an individual, defining raw data as paper, and failing to backup, or even save, electronic records. There is also the shambles of the business process, due to the use of the spreadsheet to calculate all the values from SST parameters and reportable results. Overall, the process is slow and inefficient. These risks need to be mitigated as an absolute minimum or, even better, eliminated entirely.

Fix and Forget or Long-Term Solution?

Enter stage left that intrepid group: senior management. These are the individuals who are responsible and accountable for the overall pharmaceutical quality system, including data integrity. The approaches that a laboratory will take are now dependent on them.

Figure 2 shows the overall approach that should happen to resolve data integrity issues. There are two outcomes:

1. Short-term remediation to resolve some issues quickly. Ideally, this should involve technical controls where available (for example, giving each user a unique user identity, or creating and allocating user roles for the system and segregation of duties). However, remediation often involves procedural controls, such as the use of SOPs or log books to document work. This slows the process down even further, and will result in longer second-person review times (11).

2. Long-term solutions to implement and validate technical controls, to ensure that work is performed correctly and consistently. This should involve replacement...
of hybrid systems with electronic working and ensuring business benefit from the investment in time and resources.

The problem is management. In many organizations, they want only to focus on the first option (fix and forget) and not consider the second, as it would detract from the work or cost money. While this may be thought to be an option in the very short term, it is not viable when regulatory authorities become more focused on hybrid systems with procedural controls.

In organizations that claim there is no money to provide long-term solutions, however, the financial taps are quickly turned on following an adverse regulatory inspection. However, it is better, more efficient, and cheaper to implement the long-term solution yourself, because then the company, not the regulator, is providing the solution.

Quick Fixes and Short-Term Remediation

From the data process map in Figure 1, some short-term solutions can be implemented as shown in Figure 3. Rather than attempt to fix a broken and inefficient process, use the CDS software that the laboratory has paid for to calculate the SST and final results. This would eliminate the spreadsheet, as well as manual entry into the spreadsheet and subsequent transcription checks.

Attention must also be focused on the CDS application, and some of the main changes for immediate implementation must be:

- Unique identities for all users
- Implement user types with access privileges, and allocate the most appropriate one to each user
- Segregation of application administration from normal laboratory users is more difficult, as the systems are currently standalone, and will probably require a two-phase approach: Short-term with laboratory administrators having two access types, one with administration functions (user account management and application configuration) but no access to CDS functions, and vice versa. This approach should only be used as a temporary fix, and not a permanent solution.
- Write an SOP for chromatographic integration, and specifically control when manual integration can be used (2,10,12), and train the staff.

Where feasible, restrict a user's abil-

---

**TABLE I: Main data vulnerabilities identified in a chromatography process**

<table>
<thead>
<tr>
<th>Process Step</th>
<th>Data – Record Vulnerability</th>
</tr>
</thead>
</table>
| 1. Acquire Data | • No attribution of action: all users share one account  
• Conflict of interest: all users are administrators  
• Standalone system  
• Backup is performed by the lab users and is ad hoc  
• Ability for users to configure the CDS: turn functions on and off  
• Manual input of sample and sample preparation data, such as identity, lot number, sample weights, dilutions |
| 2. Interpret Data | • No attribution of action: All users share one account  
• No standard operating procedure (SOP) for integration  
• No control of manual integration |
| 3. Print Peak Areas | • Paper is considered the raw data by the laboratory  
• Risk of paper and e-records being different |
| 4. Type into Spreadsheet | • Typographical errors when peak areas entered  
• No verification that calculations are correct as spreadsheet is unvalidated |
| 5. Print Spreadsheet | • Failure to save the electronic spreadsheet file  
• No record signature linking to comply with Part 11 between paper printout and e-record  
• Must re-enter all data in a new spreadsheet if there is a typographical error |
| 6. Enter Results into LIMS | • Typographical errors when results are entered  
• Second person review only looks at paper records |
ity to perform manual integration for some methods.

- Validate and use the CDS application ability to calculate SST parameters and eliminate the spreadsheet calculation (the former should be relatively easy to implement). Calculation of the reportable result may have to wait until the CDS is networked. In the latter case, the spreadsheet calculations will need to be validated and all files saved.

This should result in an improved business process, as shown in Figure 3. The CDS is still a hybrid system, but the spreadsheet has been eliminated, along with manual entry to a second system, but the process is under a degree of control. Left like this (the fix and forget option from Figure 2), there is substantial risk remaining in the process, such as backup of the standalone systems and the need for plans for a long-term solution.

Implementing Long-Term Solutions

Long-term solutions require planning, time, and money. However, with the potential business and regulatory benefits that can be obtained, management should be queuing up to hand over money. Let us look at some of the remaining issues to try and solve with this process:

- Standalone CDS systems need to be implemented into a networked solution including the migration of existing data to the central server. This has several advantages: IT backup of records, IT application administration, and time and date stamps from the network time server.

- Consistency of operation: The same methods can be applied across all chromatographs.

- Removal of a hybrid system: Design the networked CDS for electronic working and electronic data transfer to the LIMS, which results in minimal or zero paper to be printed out.

- Efficient, effective, and faster business process, as shown in Figure 4, and this should be compared with that in Figure 1.

The regulatory risks of the original process have been greatly reduced or eliminated at the end of the long-term solution. The laboratory can face regulatory inspections with confidence.

Acknowledgement

I would like to thank Christine Mladek for helpful review comments during preparation of this column.

References

(3) R.D McDowall, Data Integrity and Data Governance: Practical Implementation in Regulated Laboratories (Royal Society of Chemistry, Cambridge, UK, 2019).

R.D. McDowall
is the director of RD McDowall Limited in the UK. Direct correspondence to: rdmcdowall@btconnect.com
The inlet liner is designed to hold a certain volume of gas (typically 500 to 950 mL), and vents such as n-hexane with a non-polar to optimize GC–FID performance.

By choosing a sample solvent that matches the stationary phase polarity as closely as possible, peak shape will be optimized with no tailing or broadening, which might otherwise reduce the signal-to-noise ratio. Here, we think of choosing nonpolar solvents such as n-hexane with a non-polar stationary phase such as 1% polydimethyl siloxane (DB1, RTX-1) or methanol with polar stationary phases such as waxes.

High Head-Pressure (Pressure Pulsed) Injection

The inlet liner is designed to hold a certain volume of gas (typically 500 to 950 mL), and we need to avoid overfilling the liner with sample gas, or problems will occur such as poor quantitative reproducibility and carry over. By choosing a sample solvent with a lower expansion coefficient, and increasing the pressure within the liner during the injection, to constrain the volume of sample gas created, larger injection volumes can be made. Calculators, typically known as backflash or vapor volume calculators, can be used to determine the maximum sample volume that can be injected before liner overfilling occurs.

Optimize Splitless Time

In splitless injection mode, the split vent valve is closed, and all of the contents of the inlet are directed into the GC column. After a predetermined length of time (the splitless time), the split vent value is opened, and the remaining inlet contents are vented to the atmosphere. This helps to prevent wide and badly tailing solvent peaks, increased baseline noise, and a rising baseline during the analysis. If the split vent is opened too early, there is a risk of analyte loss, and, if opened too late, the broad and tailing solvent peak may reduce the sensitivity of early eluting analytes; and noisier baselines will reduce the signal-to-noise ratio for all analytes. One must experimentally determine the optimum splitless time to achieve the best reproducibility and sensitivity from the splitless injection. For proper solvent focussing to occur, the initial oven temperature must be maintained around 20 °C below the boiling point of the sample solvent during the injection phase of the analysis.

Initial Oven Temperature Hold

On-column sample focusing during splitless injection takes a finite amount of time, and, during this time, the oven temperature must be held constant. However, if this initial hold time is too long, some degree of analyte dispersion may occur, reducing the peak efficiency, and, hence, the signal-to-noise ratio. The initial hold time should be carefully evaluated if optimum sensitivity is to be achieved.

GC Column Choice

Shorter columns (10–15 m) with narrow i.d. (0.18–0.25 mm) and thin films (<0.3 mm) will give the best peak efficiencies, and therefore the optimum signal-to-noise ratio. Further, less-polar stationary phase chemistries will show less inherent bleed, again improving the signal-to-noise ratio, although the choice of stationary phase chemistry will be sample dependent. One should choose the least polar column with the thinnest film that results in a satisfactory separation, in order to reduce column bleed to the minimum possible.

Carrier Gas Operating Mode

Ensure that the carrier gas operating mode is set to constant flow, rather than constant pressure, to ensure that the carrier flows at the same linear velocity during the whole temperature program. This approach prevents the carrier from slowing as the temperature increases, which can lead to broadening of later-eluted peaks and sub-optimal detector response.

FID Optimization

Optimize the fuel (hydrogen) to oxidizer (air) ratio of the FID to ensure the best response for your analytes; typically, start with a 10:1 ratio and adjust the fuel gas in steps of +/- 5 mL/min. Also, ensure that the make-up gas flow rate (nitrogen is recommended by many manufacturers as the most effective make-up gas) is optimized, and note that the make-up gas flow rate can have a pronounced effect on analyte sensitivity. Start with a 1:1 ratio of make-up gas to fuel gas (typically hydrogen), and adjust in +/- 5 mL/min. steps to investigate the optimum range.

Thermal Gradients

Ballistic (short and fast) thermal gradients will produce the sharpest peaks and the best signal-to-noise ratios, but ensure the oven heater motor can follow the required temperature profile in a reproducible way. Of course, the use of ballistic thermal gradients will depend upon the separation requirements and the complexity of the sample.
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The Emerging Leader in Chromatography Award recognizes the achievements and aspirations of a talented young separation scientist who has made strides early in his or her career toward the advancement of chromatographic techniques and applications.

Winners will be honored in an oral symposium at Pittcon 2019.
Selecting the best stationary phase to purify or separate a compound can be a struggle. Typically, bench chemists simply use whatever stationary phase is handy in their laboratory, or work with whatever their predecessors used. It’s much wiser to think like a chef and consider the full “spice rack” of stationary phases now available. LCGC recently sat down with David Schurer, executive vice president/co-founder & partner at Sorbent Technologies, Inc., to explore why thinking beyond “salt and pepper” when choosing a stationary phase is the best recipe for achieving accurate results: resolution, purity, yield, kinetics, and cost.

Also, when we meet chemists at industry events, they want us to help them with their issues to get immediate solutions, but it takes more than just a two-minute discussion at a tradeshow. To really assist them, it requires a more detailed conversation with our in-house scientists. And we regularly have discussions to address their specific applications. We also address these issues on our website, and hope to in future webinars and podcasts.

Chemists really need to think beyond simply developing an analytical separation and only using what’s available at their bench. They must think bigger-picture and understand the downstream ramifications of what they’re doing.

LCGC: Can you share some insights to help researchers better understand the various stationary phases and how to select the best one for their work?

Schurer: Sure. Numerous stationary phases are available, but first we must ask specific questions to help them select the right one. Such as:

- Which stationary phase or phases have you tried, and why?
- What is the compound being separated or purified, including the molecular weight? A chemdraw is always helpful, if we can get it from them.
- What is the matrix, the functional groups, and impurity profile?
- What is the solvent system, pH, and the compound’s moisture, air, or light sensitivity?
• How much of the target compound is in the matrix?
• Is this a multipurification-step process?
• What scale is required (e.g., a gram or up to metric-ton quantities)?

These questions must be answered before an effective solution can be defined. Every compound is unique, and each laboratory and situation presents its own set of unique challenges.

LGCG: What are you doing to address “chroma-phobia” with your customers?

Schurer: We’re working with them to minimize the fears associated with chromatography—and chroma-phobia—and to help chemists work more effectively from bench to scale-up.

From the onset, the bench chemist is—or should be—determining which stationary phase is appropriate to use when the time comes for scale-up. If this is addressed from the get-go, then scale-up is much more effective. Typically, most chemists simply grab the stationary phase they see on the shelves nearest their bench, or they work with what’s been done by other chemists previously. Rarely do they think about the various stationary phases now available to them, similarly to what a chef would do when making a new recipe.

LGCG: What prohibits chemists from considering the range of stationary phases available to them?

Schurer: They are often under time constraints, unfamiliar with what’s available, or don’t have a relationship with a knowledgeable supplier to guide them. Additionally, they’re not thinking about the ramifications of the overall scale-up process. Chemical engineers who are working downstream to scale up the process must source the material that’s being used, and sometimes that stationary phase is either not available in a scale-up particle size, or it’s simply just too expensive.

It’s made more challenging because suppliers haven’t been as open to educating chemists regarding which stationary phases are best to use, and how to use them. Also, the study of stationary phases hasn’t been taught in universities.

Additionally, as technology continues to evolve, more “spices” are available to cook with. A chemist may realize they can slightly deviate a process using C18 to perhaps be more robust for their particular compound or application. And with C18, there are many phases to select from, such as an aqueous or pH-stable phase, not to mention the various particle sizes and pore sizes available.

Most researchers usually think of using silica gel and C18—“salt and pepper.” They aren’t aware of other spices, such as the benefits of spherical silica gel, or other phases such as C4, C8, amino, diol, phenyl and others. And depending on the application, there are very good reasons to use alumina, polymeric resins, or other stationary phases.

Selecting the stationary phase for your unique compounds or even standard compounds is very complicated. And that’s why today, it’s really more important than ever for chemists and labs to partner with a good supplier to help them select the right stationary phase to optimize their process so they aren’t wasting a lot of time and money.

An example that I’ve had recently is that a chemist may not take into consideration the molecular weight of the compound when selecting a stationary phase. And they need to select the correct pore size to fit that molecule. So, with a compound with a molecular weight greater than a 1,000 Daltons, they need a 100-angstrom pore or even larger, but most grab what’s on the bench, which is a 60-angstrom pore.

LGCG: When you start scaling up, what issues can occur that’ll become problematic moving forward?

Schurer: When I have discussions with chemists about scale-up, we have to address method development. And there are multiple variables to consider.

First, the technician: How good are they at what they do? Do they have the skillset and experience to prepare the material correctly prior to putting it through chromatography?

Then, you have the chromatography media to consider: How selective, robust and available is it?

The solvent system is a variable: How well has the purity been maintained?

Is there something happening on the pilot or process column that didn’t occur in method development, such as crashing of the compound, decomposition or unidentified impurities?

When scaling up, perhaps they didn’t use the same ultra-high-purity silica gel; maybe there are some metals causing a reaction or creating a sidechain reaction.

Also, there’s the sample feed and its quality and purity; maybe the initial sample they developed in their lab was done by their technician and was high purity and quality, but now they’re using a contractor to make it and that can create a problem.

LGCG: Why aren’t all these factors always considered in the lab?

Schurer: Often—and most of the time—labs cut corners to achieve the most bang for their buck. But even more importantly, they’re moving extremely fast because they’re under a time crunch. They need a large quantity of material, and they need it now.

It’s common for our customers to come to us somewhere in the middle of the process when they’re stumped. Very often, the problem lies in one of the numerous steps before column chromatography is performed. We’ve had experiences where we reduced steps in pharmaceutical processes by addressing the chromatography upfront and saving hundreds of thousands of dollars for our customers.

There are many considerations, but it’s always possible to keep their process on one path and reduce or eliminate the variables, particularly if we’re working with them from the start.

There’s so much more to discuss and consider with these challenges, and we intend to continue the dialogue with our customers and anyone else who has interest in the ongoing discussion.

SPICE-RACK CHROMATOGRAPHY: GOING BEYOND SALT AND PEPPER TO OPTIMIZE PURIFICATION AND SEPARATION
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**Silicone-free silica-based C18 column**
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www.imtaktusa.com

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**Merlin Instrument Company,**  
Centennial, CO.  
www.merlin.com/arrow

**Laboratory water system**
MilliporeSigma’s redesigned Milli-Q IQ 7003/7005 Ultra-pure and Pure laboratory water system is designed to provide Type 1 and Type 2 water directly from tap water. According to the company, the system’s design features include system auto-rinsing prior to production; an integrated vent filter; a built-in ech₂o bactericidal UVC LED lamp; and automatic recirculation of stored water through the purification loop.  
**MilliporeSigma,**  
Burlington, MA.  
www.emdmillipore.com

**Trapping column**
The PharmaFluidics trapping column is designed to perform large-volume (≥5 μL) peptide sample injections on μPAC analytical columns with minimal impact on the separation performance. According to the company, the micromachined column features an identical stationary phase support morphology as the 50-cm, cr and 200-cm long analytical μPAC columns.  
**PharmaFluidics,**  
Ghent, Belgium.  
www.pharmafluidics.com

**Hemp analyzer**
Shimadzu’s hemp analyzer is designed as a high-performance liquid chromatograph for quantitative determination of cannabidiol and cannabinoid content. According to the company, the analyzer includes all required hardware, software, consumables, and analysis workflow to enable the operator to run samples.  
**Shimadzu Scientific Instruments,**  
Columbia, MS.  
www.ssi.shimadzu.com

**Submicrometer coating**
SilicoTek’s Dursan submicrometer coating is designed to provide the inertness of PEEK and the robustness of stainless steel. According to the company, the coating can be applied directly to ready-made stainless steel components to render them inert.  
**SilicoTek Corporation,**  
Bellefonte, PA.  
www.SilcoTek.com

**Flash cartridge**
Sorbtech’s Purity flash cartridge is packed with Premium RF silica gel, and designed to withstand pressures up to 300 psi. According to the company, the cartridges are compatible with its EZ flash system, and are 100% compatible with a variety of other leading systems.  
**Sorbent Technologies, Inc.,**  
Norcross, GA.  
www.sorbtech.com
Automated solvent extraction system
The EDGE automated extraction system from CEM Corporation is designed to perform solvent extraction in less than 5 min. According to the company, the system combines traditional pressurized fluid extraction with dispersive solid-phase extraction, and can process 12 samples per hour.
**CEM Corporation,**
Matthews, NC.
www.cem.com/en/edge

Headspace syringe
Hamilton Company’s HDHT headspace syringe is designed for high-temperature applications up to 200 °C. According to the company, the syringe’s high-dynamic HD plunger uses a spring in the plunger tip that compensates for the materials’ different expansion coefficients.
**Hamilton Company,**
Reno, NV.
www.hamiltoncompany.com

GCxGC TOF-MS system
The Pegasus BT 4D GCxGC TOF-MS system from LECO is designed to interrogate challenging samples. According to the company, the system is now available with flow modulation, an option that replaces thermal modulation.
**LECO Corporation,**
St. Joseph, MI.
www.leco.com

Ion chromatograph
Metrohm’s Eco IC ion chromatograph is designed for the routine analysis of anions, cations, and polar substances in water. According to the company, the chromatograph includes a suppressor, a conductivity detector, and software, and allows for automatic analysis of up to 36 samples.
**Metrohm USA,**
Riverview, FL.
www.metrohmusa.com

Fittings
EXP2 All-One fittings from Optimize Technologies are designed with an integral ferrule. According to the company, the fittings provide liquid chromatography connections that can be hand tightened to over 18,000 psi (1250 bar), and are suitable for making HPLC and UHPLC connections that fit into tight spaces, such as injection valves, column couplers, sample loops, and column ovens.
**Optimize Technologies,**
Oregon City, OR.
www.optimizetech.com

Artificial body fluids
Pickering’s artificial body fluids are designed to meet official product testing specifications from AATCC, ISO, DIN, B5, EN, and other worldwide standards organizations. According to the company, the artificial body fluids are suitable for product development, quality testing, and research applications.
**Pickering Laboratories,**
Mountain View, CA.
www.pickeringlabs.com

Syringes
VICI Precision Sampling Pressure-Lok analytical syringes are made with polytetrafluoroethylene (PTFE) plunger tips. According to the company, the tips are designed to remain smooth, without the seizing or residue of conventional metal plunges, and have leak-proof seals.
**Valco Instruments Co., Inc.**, Houston, TX.
www.vici.com

Separation system
The Eclipse DualTech separation system from Wyatt Technology is designed for both hollow-fiber flow fieldflow fractionation (HFFS) and asymmetric-flow field-flow fractionation (AF4) techniques. According to the company, both techniques may be integrated into one instrument and coupled to the company’s DAWN HELEOS II detector.
**Wyatt Technology Corp.**, Santa Barbara, CA.
www.wyatt.com/separation
(Continued from page 101)
symposium (of about 500 attendees) is built around discussion, well-attended poster sessions, and a strong social program where all attendees have opportunities to rub elbows and discuss chromatography in an informal setting with the leaders in the field. The GCxGC meeting begins with a GCxGC short course on Sunday, May 12, followed by the opening of the GCxGC symposium on Monday and the rest of the capillary chromatography program on Tuesday. This is an unparalleled opportunity to learn about GC and GCxGC directly from leaders and pioneers.

GCxGC is fully quantitative and automated as easily as GC. If “super-resolution” is a priority, GCxGC is clearly ready to take its place alongside GC as a major technique for both research and routine analysis in its own right.

Acknowledgement
The author is grateful to Prof. John R. Sowa, Jr. (now at Governor’s State University) for providing the brown mouse sample and to Dr. Brian B. Barnes (now at ExxonMobil) for obtaining the chromatograms.

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SUPPLEMENT TO

THE APPLICATION NOTEBOOK

February 2019
www.chromatographyonline.com
Polymer HPLC columns have a lot of benefits. They don’t require any functionalization for reversed-phase separations, and rigid polymeric supports intrinsically resist chemical and pH degradation, a fundamental problem with silica columns. Plus, polymer’s inertness to most chemical environments makes it a robust and economical solution.

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Cover Photography: Getty Images
Utilizing Automated SPE to Improve the Recovery of Semivolatile Compounds in Compliance with EPA Method 8270

Michael Ebitson, Stephen Panos, and Andrew Taylor, Biotage

Groundwater is one of our most precious, yet easily contaminated resources. Contaminants can originate from a multitude of sources. Industrial sources include underground storage tanks, septic systems, farms that have been treated with pesticides, and hazardous waste sites. Smaller contributors, such as individual households, can pollute local groundwater with items such as household cleaners, disinfectants, laundry detergents, stain removers, motor oil, gasoline, paint, and paint removers. Even an inkjet printer can be a source of groundwater contamination.

Consuming contaminated drinking water is a well-known public health risk (1), and can produce effects ranging from mild illnesses to serious diseases, and even death. Effects can be challenging to predict with 100% accuracy as they depend on a number of factors, including the chemical properties of the contaminants, and the degree and length of exposure, on top of factors such as the person’s age and whether he or she has pre-existing health conditions. The key to ensuring our safety is to minimize our exposure to those contaminants with known health hazards.

The U.S. EPA monitors a variety of compounds that pose public health risks when they are present in our air, soil, or water. The 8000 Series EPA Methods outline the quantification of contaminants in groundwater, and Method 8270 specifically addresses semivolatile compounds (2). The method outlines the extraction of over 200 semivolatile compounds that fall into a range of compound classes, including: nitrosamines, pesticides, ethers, ketones, phenols, polyaromatic hydrocarbons (PAHs), anilines, pyridines, and aldehydes. While most laboratories do not quantify all 243 compounds outlined in the method, they often measure at least 100 compounds from several different compound classes and in a variety of challenging sample matrices.

This applicate note presents the use of an automated solid-phase extraction (SPE) system to improve the accuracy and precision of semivolatile extractions, in compliance with EPA Method 8270. In this work, a Biotage® Horizon 5000 automated SPE system was used to extract 114 semivolatile organic compounds from groundwater samples. The extraction system was fitted with an Atlantic® One-Pass SPE Disk, combined with a One-Pass Carbon Cartridge, to streamline the extraction and reduce losses due to precipitation or volatilization. The extracts were concentrated using a DryVap® Concentrator before being transferred to a GC–MS instrument for measurement. Results for a pair of matrix spike (MS) and matrix spike duplicate (MSD) samples are presented in Table I.

As indicated in Table I, the extraction and concentration workflow is ideally suited for extracting a wide range of semivolatile organic compounds with precision and accuracy that is compliant with EPA Method 8270E. With the exception of challenging compounds such as pyridine, NDMA, hexachlorocyclopentadiene, and 2-picoline, all matrix spikes were recovered between 50–150% of their spiked concentration and relative percent differences were below 20%, in compliance with Method 8270E requirements.

References

Table I: Spike recovery results for matrix spike (MS) and matrix spike duplicate (MSD) samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Results for Matrix Spike 1</th>
<th>Results for Matrix Spike 2</th>
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<tr>
<td></td>
<td>Avg. Spike Recovery (%)</td>
<td>RPD (%)</td>
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<tr>
<td>1,3,5-Trinitrobenzene</td>
<td>88.2</td>
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</tr>
<tr>
<td>2,3,4,6-Tetrachlorophenol</td>
<td>90.6</td>
<td>3.9</td>
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<td>2,4-Dinitrotoluene</td>
<td>92.1</td>
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</tr>
<tr>
<td>1-Naphthylamine</td>
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<tr>
<td>2-Methyl phenol</td>
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<td>3-Nitroaniline</td>
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<td>4-Bromophenyl phenyl ether</td>
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</table>

Biotage
16 Northwestern Drive, Salem, NH 03079
tel. (603) 893-3663, fax (603) 893-4994
Website: www.biotage.com
Analysis of Amino Acids in Oxidized and Unoxidized Feed Samples

Maria Ofitserova, PhD, Pickering Laboratories, Inc.

Commission Regulation (EC) No 152-2009, published in The Official Journal of European Union, laid down the methods of sampling and analysis for the official control of feed. The regulation describes methods of analysis to control the composition of feed materials and compound feed products. Establishing the amino acids profile is an important way to control quality and nutritional value of feeds. This regulation specifies HPLC with post-column derivatization with ninhydrin reagent as the method of analysis for total and free amino acids. Pickering Laboratories developed an analytical method to comply with all the chromatographic requirements of Commission Regulation (EC) No 152-2009. The same method is used to analyze oxidized and unoxidized feed samples.

**Method**

**Analytical Conditions**

| Column:     | High-efficiency sodium cation-exchange column, 4.0 × 150 mm, Catalog Number 1154150T |
| Guard:      | Cation-exchange GARD™, Catalog Number 1700-3102                                      |
| Flow rate:  | 0.4 mL/min                                                                          |
| Mobile Phases: | Na270, Na740, RG011.                                                                 |
| Injection Volume: | 10 uL                                                                               |

**Table I: HPLC Program**

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<th>RG011, %</th>
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**Table 2: Column oven program**

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<td>65</td>
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<tr>
<td>42</td>
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</table>

**Figure 1:** Chromatogram of a standard solution of amino acids
Post-Column Conditions

Post-Column System: Pinnacle PCX
Reactor volume: 0.5 mL
Reagent: Trione®
Column Temperature: See method in Table II
Reactor Temperature: 1 30 ºC
Flow rate: 0.25 mL/min
Detection: UV-VIS 570 nm for primary amino acids, 440 nm for secondary amino acids

Figure 2: Chromatogram of an oxidized feed sample

Figure 3: Chromatogram of a non-oxidized feed sample
**LC–MS/MS Analysis of Mycotoxins in Peanut Powder in 5.5 Min**

Restek Corporation

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

Certain fungi that can grow on agricultural products produce toxic metabolites known as mycotoxins. Modern food processing procedures cannot completely remove these compounds if they are present, so strict monitoring protocols have been established. Although a universal method for the analysis of mycotoxins would allow highly efficient screening, it is very challenging to develop such a method due to differences in physiochemical properties of mycotoxins, extraction efficiencies, and matrix effects. Zhang and associates published a multi-lab study (1) aimed at providing labs with an analytical procedure that could be broadly applied to the analysis of a variety of mycotoxins in many different matrices. Using that work as inspiration, we developed the following LC–MS/MS method that resolves 12 FDA regulated mycotoxins within the pressure limits of traditional HPLC instruments.

In this example, mycotoxins were analyzed in a peanut powder matrix. The use of a relatively short column format, the selectivity of the Biphenyl stationary phase, and the efficiency of 2.7-μm Raptor superfi cially porous particles provided excellent separations in a fast 5.5-min analysis (total cycle time of 7 min). A coeluting matrix compound that shared the most abundant MRM transition for mycotoxin HT-2 (447.3-285.3) was observed, so a less abundant transition (447.3-345.3) was selected for quantitation. To increase sensitivity, an ammonium buffer was used to promote better ionization of mycotoxins. The Raptor Biphenyl column worked very well for the 12 mycotoxins studied in the cited work, but for longer compound lists containing isobaric mycotoxins with similar structures, the Raptor FluoroPhenyl phase may be necessary to provide adequate chromatographic resolution. The selectivity of the Raptor Fluorophenyl column is demonstrated in an analysis of 20 mycotoxins that can be found by visiting www.restek.com and entering LC_FS0511 in the search.

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

Restek would like to thank Dr. Zhang for his technical support during this project.
Raptor Biphenyl LC Columns (USP L11)

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Reference

Why are C18s So Different? A Focus on Contributions of Steric Selectivity

Robert Puryear*, Piotr Macech*, Dustin Austin*, Hiroshi Tachikawa†, and Itaru Yazawa†,
*Imtakt USA, Portland, OR, Imtakt Corp., †Kyoto, Japan

One of the fundamental tenets of chromatography is the interaction between an analyte and stationary phase. We often assume that this should be consistent between different columns with the same stationary phase, but this is not always the case. With C18 columns, this highlights the contribution of factors other than hydrophobicity, such as electrostatic interaction, when a column has exposed unreacted surface silanols, or steric selectivity, which is related to surface ODS density (Figure 1).

Experimental Conditions are shown in each figure.

Result and Discussion

Figure 2 shows that the columns tested had significantly different separation profiles for o-terphenyl and triphenylene, despite having nearly identical hydrophobic resolution ($\alpha = 1.4–1.5$, data not shown). This comparison shows how steric selectivity can contribute to variation, with Brand XB ($\alpha = 1.4$) having the least resolution, followed by Cadenza CD-C18 ($\alpha = 1.6$), then Cadenza CX-C18 ($\alpha = 2.2$), with Dacapo DX-C18 ($\alpha = 2.5$) having the highest.

Conclusion

We first discovered how to improve steric selectivity with the development of our Cadenza CD-C18 column. In Figure 1 you can see that, by having a higher ligand density, this creates a narrower space between the C18 chains, which presents a different geometry of interaction for molecules that are more planar. We have developed this technology further with Cadenza CX-C18 and Dacapo DX-C18, improving steric selectivity to $\alpha$ values of 2.2 and 2.5, respectively. The advantage of high steric selectivity can be seen in Figure 3, where four steroids with only minor differences were baseline resolved. In this case, the differences were minute, as with hydrocortisone vs. prednisolone, where a single bond is replaced by a double bond, and in cortisone vs. hydrocortisone, where a ketone is replaced by an alcohol.

In summary, we have shown here that four ODS columns can have very different retention characteristics, despite all having the same ligand, with steric selectivity significantly contributing to this variation. We have also shown the importance of steric selectivity in providing separation of compounds that are very similar to each other. This suggests that the use of a high steric selectivity ODS column should be considered when separating compounds such as isomers or impurities, which may have very little difference between them.
Separation of Five Steroids on a Hamilton PRP™-C18 Reversed-Phase HPLC Column

Steroids represent a chemically distinct class of hormones with wide-ranging biological functions. Synthetic derivatives of endogenous steroid prototypes are used medically in birth control and in the treatment of asthma, arthritis, inflammation, and osteoporosis.

Steroids share a characteristic polycyclic structure, and have varying degrees of lipophilicity (log P). In this study, a reversed-phase HPLC method was developed for separation of five steroid hormones with partition coefficients ranging from 1.47 (cortisone) to 4.5 (pregnenolone) on a Hamilton PRP-C18 HPLC column.

For more information on Hamilton HPLC columns and accessories or to order a product, please visit www.hamiltoncompany.com or call (800) 648-5950 in the US or +41-81-660-60-60 in Europe.
Characterization of TSKgel® FcR-IIIA-NPR HPLC Column by Top Down Mass Spectrometry

Tosoh Bioscience

Monoclonal antibodies (mAbs) comprise the largest class of glycosylated protein therapeutics currently on the market, and glycosylation is known to be a major source of mAb heterogeneity. N-glycosylation of IgG-Fc of mAbs is known to impact drug therapeutic mechanism of action (MOA), thus monitoring glycan critical quality attributes (CQAs) is an essential part of biopharmaceutical development. Glycosylation is a critical factor in drug product solubility, kinetics, stability, efficacy, and immunogenicity. Analytical methods utilize a suite of chromatographic modes using high performance liquid chromatography (HPLC) to analyze glycosylation of both intact and digested protein molecules.

The TSKgel FcR-IIIA-NPR column is a high performance affinity chromatography column for the analysis of IgG glycoforms. The stationary phase utilizes a recombinant FcR-IIIA protein bound to a nonporous polymethacrylate polymer. The retention mechanism is based on the interaction between the FcR ligand and the sugar moieties attached to the ASN amino acid in the conserved region of the monoclonal antibody. The resulting elution profile of the glycoprotein mimics ADCC activity, which is correlated to the composition of the N-glycans.

The purpose of this study is to demonstrate the use of mass spectrometry to characterize the elution profile of a typical IgG1 molecule separation on a TSKgel FcR-IIIA-NPR column, and verify the observations that certain glycan structures impart higher activity to the monoclonal antibody, especially as it relates to the presence of terminal galactose sugars.

**Experimental HPLC Conditions**

**TSKgel FcR-IIIA-NPR Separation**

- **Column:** TSKgel FcR-IIIA-NPR, 5 μm, 4.6 mm ID × 7.5 cm
- **Instrument:** Agilent 1200
- **Mobile phase:** A: 50 mmol/L Na citrate, pH 6.5  
  B: 50 mmol/L Na citrate, pH 4.5
- **Gradient:** 0 min: 0% B, 20 min: 100% B, 30 min: 100% B
- **Flow rate:** 0.85 mL/min
- **Detection:** UV @ 280 nm, 25 Hz
- **Temperature:** 15 ºC
- **Injection vol.:** 5 μL
- **Sample:** NIST mAb fractions; 5 mg/mL in mobile phase A

**Top Down MS Characterization**

- **Column:** TSKgel Protein C4-300, 3 μm, 2.0 mm ID × 15 cm
- **Instrument:** Shimadzu Nexera® XR
- **Mobile phase:** A: 0.1% formic acid in water  
  B: 0.1% formic acid in acetonitrile
- **Gradient:** 0 min: 10% B, 40 min: 95% B, 50 min: 95% B
- **Flow rate:** 0.2 mL/min
- **Detection:** Sciex X500B Q-TOF, ESI positive, m/z 900-4000
- **Temperature:** 50 ºC
- **Injection vol.:** 5 μL
- **Sample:** NIST mAb fractions; 100 μg/mL in LC-MS water

**MS Conditions:**

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<thead>
<tr>
<th>Source gas 1</th>
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Figure 1: Zoomed view of the elution profile of NIST mAb on TSKgel FcR-IIIA-NPR. The boxes highlighting each peak represent fractions that were collected.
Figure 2: The TIC, extracted spectrum, and reconstructed spectrum for a NIST mAb control sample. The glycoforms observed for this sample are in agreement with accepted literature on characterization of this molecule.

### Results and Discussion

Figure 1 demonstrates the separation of NIST mAb on the TSKgel FcR-IIIA-NPR column. IgG₁ molecules yield this typical type of elution profile based on glycoform composition that is consistent with ADCC activity. This offers a fast orthogonal chromatographic method for determination of antibody activity and comparisons of antibody heterogeneity.

The three largest eluting peaks were collected and analyzed by offline mass spectrometry. Peak fractions were pooled from successive 25 μg on column injections, concentrated, and buffer exchanged to LC/MS grade water.

Figures 2 and 3 illustrate analysis of the NIST mAb standard compared against the collected peak fractions. It is observed that each peak has a unique composition of intact mAb glycoforms and that the selectivity of the stationary phase is based on the amount of terminal galactose units on the glycan moiety. This conclusion agrees with studies that show antibodies with higher amounts of G1- and G2-containing sugars show greater ADCC activity. Because of some peak overlap in the initial separation, there is some overlap of different galactose-containing species in the MS profile, though the general trend between galactose and activity has been confirmed.

### Conclusions

The separation of an IgG₁ molecule was demonstrated using the TSKgel FcR-III-A-NPR column and peaks from that separation were characterized by high resolution mass spectrometry. The results support that the stationary phase selectivity is based on the same Fc-glycan/Fc receptor interaction as ADCC activity. The glycoform composition of each peak is consistent with previous published observations on the activity of N-glycan sugars with higher amounts of terminal galactose.

This application demonstrates the efficacy of this approach and characterization data that demonstrate the proof of concept of this chromatographic technique for a fast orthogonal analysis to evaluate mAb ADCC activity, potentially for early cell line development, bioreactor modeling and lot-to-lot comparability of therapeutic antibodies.
**Figure 3:** Reconstructed spectra for each of the isolated peak fractions, indicating that later eluting fractions have a greater proportion of terminal galactose glycan sugars, consistent with observations of antibody activity and percentage of galactose.

<table>
<thead>
<tr>
<th>Peak</th>
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<th>Glycoform</th>
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<th>Mass</th>
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<td>147620</td>
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Extraction of Loperamide and N-Desmethyl Loperamide from Blood Followed by LC–MS/MS Analysis

Tina Fanning, UCT, LLC

Loperamide is an over-the-counter antidiarrheal drug that has been considered to be safe when used as directed. However, as the opioid epidemic continues to ravage our nation, there has been an increasing number of reported cases of loperamide overdose. This application note outlines a simple three-step method for the extraction of loperamide and its main metabolite, N-desmethyl loperamide, from blood. UCT’s Clean Screen® XCEL I column provides users with the same level of sample clean up as traditional SPE, while allowing the elimination of timely conditioning and wash steps.

Procedure:
Sample Pretreatment:
1. To 1 mL blood sample add 3 mL Acetate Buffer pH 5 and appropriate amount of internal standard
2. Vortex Samples for 30 s to mix

SPE Procedure:
1. Apply samples to SPE tubes without any preconditioning
2. Allow samples to flow through the column at a rate of 1–2 mL/min.
3. Wash samples with 2 mL of D.I. H₂O
4. Wash SPE column with 2 mL of 98:2 MeOH:Glacial Acetic Acid
5. Dry column for 5 min at full vacuum or pressure
6. Wash samples with 2 mL of Hexane
7. Dry column for 10 min at full vacuum or pressure
8. Elute compounds with 2 mL of 78:20:2 DCM:IPA:NH₄OH
9. Collect eluate at a rate of 1–2 mL/min
10. Evaporate to dryness at < 50 °C
11. Reconstitute sample in mobile phase

Instrumental:
LC–MS/MS: Thermo Scientific™ Dionex™ 3000 (LC) TSQ Vantage™ (MS/MS)
Column: UCT Selectra DA HPLC Column
100 × 2.1 mm, 1.8 μm

Results:

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<tr>
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<th>5 ng/mL</th>
<th>10 ng/mL</th>
<th>50 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-desmethyl-loperamide</td>
<td>106</td>
<td>97</td>
<td>96</td>
</tr>
<tr>
<td>loperamide</td>
<td>102</td>
<td>95</td>
<td>93</td>
</tr>
<tr>
<td>Average recovery</td>
<td>104</td>
<td>96</td>
<td>94.5</td>
</tr>
</tbody>
</table>

Conclusion:
This application note effectively outlines a simple three-step method for the extraction of loperamide and its main metabolite, N-desmethyl loperamide, from blood. UCT’s Clean Screen XCEL I column provides users with the same level of exceptional sample clean-up as traditional SPE while allowing the elimination of timely conditioning and wash steps in addition to a concentration step. Sample extracts are analyzed via UHPLC–MS/MS utilizing UCT’s Selectra C18 1.8 μm analytical column.
Absolute Molar Mass Analysis of Chitosans

Wyatt Technology

Chitosans are analyzed by SEC-MALS to determine molar mass moments and distributions.

Chitin is one of the most abundant biopolymers on earth (the other is cellulose). Chitin, or poly-N-acetylglucosamine, is the major polymer in the exoskeleton of marine arthropods and can also be found in fungi and yeasts.

Chitosan is deacetylated chitin. It can be obtained from shrimp or crab shells. Its applications vary from the therapeutic, such as wound healing, to cosmetics and dietary supplements.

For many of these applications, it is useful to fully characterize the molar mass moments and distributions of chitosan products. Size-exclusion chromatography, in combination with multi angle light scattering detection (SEC-MALS), provides an easy method to obtain these properties in an absolute manner, free of molecular references. In this note, we describe the results for two chitosan samples analyzed by SEC-MALS.

Experimental Conditions

A DAWN® MALS detector (Wyatt Technology, Santa Barbara) and an Optilab® differential refractive index (dRI) detector (Wyatt Technology) were plumbed downstream of the GPC column. Data collection and analysis were performed in the ASTRA® software (Wyatt Technology) using empirically determined differential refractive index increments (dn/dc). Polymer molar mass $M$ was calculated at each elution volume using signals from the two detectors.

The differential refractive index is a property of the polymer–solvent system. It is measured by injecting a series of known concentrations into the Optilab, using solutions that are often prepared by the dry weight method for accuracy, and the GPC mobile phase as solvent. ASTRA collects and analyzes the results to determine $dn/dc$.

Results

The molar masses of two chitosan samples are plotted as a function of elution volume in Figure 1. The molar mass decreases logarithmically, indicating that chromatographic conditions were optimal. The offset in elution volume for a given molar mass may be a result of conformation (short-chain branching, SCB) or non-ideal analyte–column interaction. SCB may be further investigated by making use of simultaneous size (radius of gyration, $R_g$) and molar mass analysis by MALS.

A cumulative molar mass distribution plot, depicted in Figure 2, clearly differentiates the two chitosan samples. ASTRA software can also report weight fractions above, below, or between the molar masses of interest. As an example, the weight fractions of molar masses below 50 kDa and above 500 kDa for these samples are given in the table in Figure 2. These calculations and the cumulative molar mass distribution plot are ideal for quality control applications.

Conclusions

The results described herein show that MALS detection combined with SEC provides a useful tool for biopolymer characterization. Absolute molar mass and molar mass distribution can be readily obtained without the use of any standards or empirical relations, simplifying QC and routine analyses while enhancing in-depth analysis with absolute measurements of size as well as molar mass.
Develosil UHPLC C18 and C30 for Oligonucleotide Analysis

Satoshi Horikiri, Develosil USA

Reversed-phase HPLC is common for analysis of small to midsize molecules, but careful experimental design and selection of columns are both required for successful separation.

Here we applied our newly developed UHPLC C18 and C30 columns to oligonucleotide analysis. This new type of column is designed and optimized for the rapid separation of small to midsize molecules, utilizing 1.6-μm diameter particles with 11-nm diameter pores to achieve higher resolution. Theoretically, this pore size should allow separation of molecules of up to 25,000 kDa with this column.

Experimental Condition

Table I shows the analytical conditions. For Develosil 1.6 μm UHPLC columns, C18 has a higher density of alkyl chains on the silica gel surface than C30. This results in the C18 columns having higher retention of hydrophobic compounds. The lower density of alkyl chains in the C30 column allows the mobile phase more access to the endcapped silica, which can influence the separation of hydrophilic compounds. Because of this, modulation of the pH and organic solvent content of the mobile phase can result in larger changes in retention of hydrophilic species such as oligonucleotides on C30 when compared to C18.

Results and Discussion

Figure 1 shows an oligodeoxythymidine ladder standard mixture (DNA ladder standard, oligos at 15, 20, 25, 30, 35, and 40 mer) separated using Develosil UHPLC C18 1.6 μm. The sample has higher concentrations at each 5-mer increment from 15-mer and up. This chromatogram shows each additional individual nucleotide length is a clearly separated peak.

We then applied Develosil UHPLC C30 1.6 μm to this analysis (Figure 2). Compared to C18, the C30 column retains nucleotides more strongly, leading to longer retention times under the same conditions. Increasing the final concentration of mobile phase B by 0.5% results in similar retention times to that of the C18 column. During the process of optimizing the mobile phase, we also found that small differences in mobile phase composition will affect retention time on C30 much more so than on C18.

Conclusion

Using the 1.6 μm particle of Develosil UHPLC C18 and C30 showed clear and sharp peaks of oligonucleotides up to 40 mer. The resolution was higher on C30. This indicates that choosing different column lengths or mobile phase conditions can enable analysis of oligonucleotides 40 nucleotides or even longer.
Add More Confidence to Your UHPLC–MS Analysis

Rudolf Köhling, PhD, MilliporeSigma

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  • ESI or APCI (-) < 10 ppb
• Lowest impurity profile: for interference-free baselines (Test 2)
• Microfiltration through 0.2 μm filter (Test 3)
  • Prolonged lifetime of filters and mechanical parts in HPLC systems
  • Reduced risk of column clogging
• Packaged in borosilicate glass bottles: minimized contamination with metal ions
• Lowest levels of trace metal impurities: for minimized metal ion adduct formation
  • <5 ppb
• Lowest level of polyethylene glycol (PEG) impurities in our entire UHPLC-MS solvent lineup to give you confidence in your results (PEG S/N signal-to-noise-ratio < 50)

LiChrosolv Methanol for UHPLC–MS shows a flat baseline and by far the lowest impurity profile compared to the competition. Both competitor’s high purity UHPLC-MS products A and B show a baseline drift and significant impurity peaks.

Test 1: UHPLC-MS gradient run with LiChrosolv acetonitrile for UHPLC–MS shows a clear detection and identification of 1 ppb reserpine, 500 ppt propazine, and 4 ppb prednisolone, with very low background interferences.

Test 2: Comparison of LiChrosolv methanol for UHPLC–MS (blue line) with two competitor UHPLC-MS products.

To read the rest of this application please visit SigmaAldrich.com/UHPLC–MS.
Simultaneous Analysis of Ten Water-Soluble Vitamins Using a Polymer-Based Reversed-Phase Column—Shodex™ RSpak DE-413L

Showa Denko America, Inc.

Vitamins are micronutrients essential for the metabolism of living organisms. Since humans cannot produce vitamins, the intake of vitamins must be part of their intake. There are many commercial foods and drinks supplemented with vitamins for nutrient enhancement purposes, including most processed foods.

Methods using microbiological assays, absorption spectrophotometry, and HPLC have been used to analyze vitamins, creating a long process. A typical HPLC method to separate and quantify vitamins can use an ODS column with an addition of ion-pair reagent. However, the ion-pair reagent tends to remain on the column and the flow-lines, resulting in increased background level and lowers the sensitivity.

Therefore, in this application, a simple method to simultaneously analyze various water-soluble vitamins was developed. Shodex DE-413L, a polymer-based reversed phase column, without the use of an ion-pair reagent. We further applied the developed method to quantify vitamins in a commercial multi-vitamin supplement.

Experimental

Ten vitamins (thiamin HCl, pyridoxine HCl, nicotinamide, ascorbic acid, nicotinic acid, calcium pantothenate, cyanocobalamin, folic acid, riboflavin, and biotin) were used as standards. A 4-mM standard solution was used for biotin and 2-mM standard solutions were used for other vitamins. More standards were dissolved in 250-mM phosphoric acid. Five levels of multi-vitamin calibration standards were prepared using standard solutions and 250-mM phosphoric acid. We used 250-mM of phosphoric acid to prevent the oxidation of ascorbic acid.

A Shodex RSpak DE-413L column (4.6 mm I.D. × 250 mm, 4 μm) was used with a PDA detector (190–400 nm). The eluent conditions were as follows: (A) 10 mM H3PO4 aq. / (B) CH3CN, linear gradient (high pressure); (B%) 0% (0 min) → 30% (5-10 min) → 0% (10.1–20 min). The column was kept at 50 ºC and the flow rate was 1.0 mL/min.

Results and Discussion

Figure 1 shows the UV chromatograms of the standards. Peaks of the ten vitamins were fully resolved using the developed method. The UV absorbance was measured at 254 nm. However, since the absorbance of pantothenic acid and biotin at 254 nm were low, 210 nm was used for the measurement.

This simple method using phosphoric acid and acetonitrile as the eluents demonstrated a successful simulated analysis of ten water-soluble vitamins in 20 min, including the column equilibration time.

We analyzed the extract of a commercial multi-vitamin supplement. We used a guard column (Shodex RSpak DE-G 4A) during the sample analysis (Figure 2).

A method for simultaneous analysis of ten water-soluble vitamins was developed using the Shodex RSpak DE-413L column. The eluents used consisted of a mixture of an acid and acetonitrile. One sample measurement completes in 20 min.

The Shodex RSpak DE series provide a stable analysis even under highly aqueous eluent conditions, without the concern of column deterioration due to the polymer-based packing materials compared to using silica-based material.

References


Shodex™/Showa Denko America, Inc.
420 Lexington Avenue Suite 2335A, New York, NY 10170
tel. (212) 370-0033, X109
Website: www.shodexHPLC.com
Thermal Extraction of Phthalates in Polymers Based on IEC Method 62321-8 Using a Pyroprobe

Karen Sam, CDS Analytical

This application note presents calibration plots, RSDs, and MDLs for IEC Method 62321-8 using a CDS Model 6150.

Certain phthalate additives are known to be harmful to humans, resulting in regulations regarding their use. With growing environmental awareness and perceptions, the use of phthalates has been restricted in many countries, including the European Union and the United States of America. As a result, a few international standards and conformity assessment bodies such as the International Electrotechnical Commission (IEC) and the American Society for Testing and Materials (ASTM) have published standards for determining certain phthalates in polymeric materials. The recent IEC Method 62321-8 defines approaches to determine di-isobutyl phthalate (DIBP), di-n-butyl phthalate (DBP), benzyl butyl phthalate (BBP), bis-2-ethyl hexyl phthalate (DEHP), di-n-octyl phthalate (DNOP), di-isononyl phthalate (DINP), and di-iso-decyl phthalate (DIDP) in electronics using GC–MS and TD–GC–MS.

Experimental Conditions

The samples were pyrolyzed in a CDS Pyroprobe 6150 with an autosampler, equipped with Drop-In-Sample-Chamber (DISC) technology. A DISC tube was used as the sample vessel.

Pyro Chamber

Ramp 1: 200 to 300 °C at 20 °C/min
Ramp 2: 300 to 340 °C at 5 °C/min
IsoZones: 300 °C

GC-MS

Column: 5% phenyl (30 m × 0.25 mm)
Carrier: Helium, 50:1 split
Injector: 320 °C
Oven: 80 °C for 13 min
20 °C/min to 300 °C
Mass Range: 50–1000 amu

Results

Thermal extraction of additives is a straightforward approach involving only a few steps, and therefore the possibility of greater recovery when compared to solvent extraction techniques exists. Sample is simply placed in a sample tube, and dropped into the DISC of a 6150 Pyroprobe. The Pyroprobe thermally extracts the sample using two sequential temperature ramps as defined in the experimental conditions, straight to a single quadrupole GC–MS instrument. Resulting chromatograms closely match the chromatograms in Annex C.2 of the IEC Method (Figure 1). Calibration curves based on a one-point calibration (as indicated in the method) are also shown in Figure 1.

The statistical measures related to reproducibility depend on temperature precision, along with sample related issues like homogeneity and sample preparation. Eight replicates a 500 ng phthalate standard provided area RSDs of around 3% for most of the phthalates. Furthermore, when method detection limits were studied in accordance with the IEC method, seven replicates produced calculated MDLs ranging from 9.4 to 21.7 mg/kg, 78–91% lower than the 100 mg/kg requirement (Table I).

Conclusion

The latest version of the Pyroprobe from CDS Analytical ensures repeatable, reliable results for thermal extraction of phthalates in accordance with standard methods, like IEC Method 6321-8 for determination of phthalates in electrotechnical products.

Table I: Area RSDs and calculated MDLs of regulated phthalates

<table>
<thead>
<tr>
<th>Phthalate</th>
<th>Quant Ion</th>
<th>RSD (%)</th>
<th>MDL (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIBP</td>
<td>223</td>
<td>3.2</td>
<td>21.7</td>
</tr>
<tr>
<td>DBP</td>
<td>223</td>
<td>2.3</td>
<td>21.0</td>
</tr>
<tr>
<td>BBP</td>
<td>206</td>
<td>4.3</td>
<td>21.0</td>
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<tr>
<td>DEHP</td>
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<td>2.9</td>
<td>14.7</td>
</tr>
<tr>
<td>DNOP</td>
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<td>3.2</td>
<td>9.4</td>
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<tr>
<td>DINP</td>
<td>293</td>
<td>3.0</td>
<td>17.9</td>
</tr>
<tr>
<td>DIDP</td>
<td>307</td>
<td>3.2</td>
<td>13.6</td>
</tr>
</tbody>
</table>
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