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

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

Dwight R. Stoll

As with other fields of analytical science, such as spectroscopy and mass spectrometry, professionals practicing chromatography carry a lot of information and knowledge in their minds that facilitates problem solving in their daily work. I think this is most evident in method development activities for chromatography. For example, knowing the general retention behavior of a carboxylic acid-containing analyte in reversed-phase liquid chromatography (LC) enables the method developer to make the quick decision to decrease the mobile phase pH if they observe that the retention of this analyte is too low. To me this kind of knowledge is akin to knowing a set of “math facts” so that one does not have to always reach for a calculator when making estimates for simple calculations. Here, carrying in our minds knowledge of the general behavior of a carboxylic acid-containing analyte in reversed-phase LC allows us to avoid looking in books or journal articles for commentary on the behavior before taking the next step in method development. In this case, the rule is that generally retention of a carboxylic acid-containing analyte will increase as the mobile phase pH is decreased from above the pKₐ of the carboxylic acid functional group to below the pKₐ.

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

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

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

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

Figure 1 shows a histogram of these results. Here, a 100% increase in retention means that the retention factor for a given compound doubles when the %B is decreased from 40% to 30%, a 200% increase means that the retention triples, and so on. From these data we make two practically significant observations: 1) the degree to which retention increases for these compounds in response to the same change in mobile phase composition varies by about a factor of four; and 2) the degree of change for the majority of the compounds falls in the range of 90% to 150%. Based on this, we can adopt the rule that the retention of an average small molecule will roughly double in response to a 10% decrease in the %B.
Most natural waters contain natural organic matter (NOM), which is primarily composed of humic and fulvic acids. Natural waters are used both as potable and non-potable sources and both need to be disinfected with an oxidant to deactivate pathogens from either use. The disinfection of NOM with an oxidant produces disinfection by-products (DBP). The disinfection of NOM can be achieved through the use of various oxidation sources, such as: UV, ozone, chlorine, or chloramination. Depending on the oxidant and the source water, various halo DBPs can be formed. Each source generates multiple DBP’s. One of the main components to the nearly 600 identified DBP’s are haloacetic acids (HAA) which have been detected in our ecosystem and affect overall human health. As utility companies utilize more influent waters containing higher salinity or desalinated sea/brackish groundwater, a growing concern has mounted for HAA’s. The higher concentrations of bromide and iodide converted in these waters change the speciation of DBP’s toward their brominated and iodinated analogues rather than their more recognized chlorinated species. These species have been documented as more toxic than the chlorinated analogs and are not routinely tested for by regulatory administrations.

NOM in general contains healing properties, however, oxidation of NOM generates toxicity when the HAA’s react with themselves to form halocitric acids. When ingested, halocitric acids bind calcium in the citric acid cycle. The sequestered calcium leads to hypocalcemia through the inhibition of the citric acid cycle. Accurate detection of HAA’s is paramount for the prevention of over oxidation, while still managing enough water sanitation to eliminate water borne pathogens. As such, a need for a fast and repeatable method for the quantification of HAA’s is of great demand for sanitation departments to accurately and quickly determine if their eluent is environmentally friendly. This method isolates fluoro, chloro, bromo, and iodoacetic acids from water samples which highlights the effectiveness and robustness of the Hamilton PRP-X300, 7 µm ion exclusion column (150 x 4.1 mm). The simple isocratic method utilizes a mobile phase of 3 mN H2SO4 and acetonitrile (95:5) to make reproducibility fast and easy.

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

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

### #3: Effect of Mobile-Phase pH on Retention of Carboxylic Acids

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

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

---

**Figure 1**: Increases in the retention of 49 small molecule analytes in response to a 10% decrease in the organic solvent level (%B) in the mobile phase of RPLC separations. Chromatographic conditions: column, Agilent SB-C18; temperature, 40 °C; mobile phase A solvent, 25 mm ammonium formate, pH 3.2.

**Figure 2**: Nomogram for the comparison of acetonitrile-, methanol-, and tetrahydrofuran-containing mobile phases used for RPLC. Adapted from reference (2).
#4: Effect of Mobile Phase pH on Retention of Amines

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

Relevance to Troubleshooting

On occasions where peaks do not appear where they are expected to appear in a chromatogram (whether in absolute terms, or relative to each other), the list of possible reasons is pretty long. Is the flow rate correct? Was the mobile phase made properly? Is the solvent proportioning valve working properly in the

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

![Figure 4](image-url): Dependence of mobile phase viscosity on volume fraction of organic solvent in water at 20 °C and ambient pressure. The values plotted are based on the data in reference (3), which were fitted as described in reference (4). The blue line is acetonitrile, and the red line is methanol.

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pump? This short version of the list has several elements related to potential problems with the mobile phase, and so it is very helpful to have in mind the expected effect on retention of a change in either the type or amount of organic solvent in the mobile phase and in the mobile phase pH. Knowing whether or not the magnitude of the observed change in retention could possibly be explained by a problem with the mobile phase composition can help reduce the number of likely causes of the problem to pursue, and ultimately help the analyst arrive at a solution more quickly.

Rules #5 and 6: Mobile Phase Viscosity and Pressure Drop in Reversed-Phase LC
Pressure is an important topic in modern LC. Contemporary methods are often run at pump pressures on the order of several hundred bar, and problems with partially blocked connecting tubing, filters, or columns can result in an over-pressure situation that must be resolved before analysis can continue. We can also use measured pump pressures to our advantage during troubleshooting, however. Under most reversed-phase LC operating conditions in common use the flow in the LC system is laminar, which means that the pressure drop between the pressure measurement point in the pump and the detector outlet is highly predictable. For example, under these conditions, the pressure drop between the inlet and outlet of a piece of connecting capillary is related to the mobile phase viscosity through Poiseuille’s law, shown in equation 1:

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

where \(\Delta P\) is the pressure drop across the capillary, \(F\) is the flow rate of mobile phase, \(\eta\) is the mobile phase viscosity, \(L\) is the capillary length, and \(d\) is the capillary diameter. In the case where all of the variables except viscosity are fixed (as in a typical LC analysis), we can predict the change in pressure drop if the change in mobile phase viscosity is known or predictable, as in gradient elution, for example.

Figure 4 shows the dependence of the viscosity of acetonitrile:water or methanol: water mixtures on the fraction of organic solvent in water. Since the pressure drop is directly proportional to the first power of the viscos-
ity, we can expect the pressure measured at the pump to change in a way that looks very similar to these curves when we execute a solvent gradient from 0% to 100% organic solvent (or a piece of these curves if a narrower gradient is used). Indeed, the observed pressure profiles look very much like this in actual experiments, and these pressure measurements can be useful when troubleshooting LC problems. A useful rule for acetonitrile water mixtures is that the maximum viscosity and pressure occurs around 20% acetonitrile, and that 100% acetonitrile has a viscosity that is about one-third of that of water. For methanol water mixtures, a useful rule is that the maximum viscosity occurs around 50% methanol, and the viscosity of 100% methanol is slightly lower than that of water.

Relevance to Troubleshooting

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

Summary

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

Acknowledgment

I would like to acknowledge Gustavus student Simerjit Kaur for compiling the retention information shown in Figure 1.

References


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Dwight R. Stoll is the editor of “LC Troubleshooting.” Stoll is a professor and the co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. 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 four book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC’s editorial advisory board. Direct correspondence to: LCGCedit@mmhgroup.com

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Diego A. Lopez, Ahren Green, and David S. Bell

Various advances in silica manufacturing have paved the way for modern chromatography. Since their inception in the 1970s, superficially porous particles (SPPs) have offered good performance and are indispensable in high-speed high performance liquid chromatography (HPLC) (1). Recent trends show the preference towards using SPP, especially in emerging markets such as cannabis (2); nevertheless, conventional particle sizes are still beneficial due to their higher surface area and their high loadability required for preparative scale applications. These advancements on solid supports have contributed great benefits to every industry where chromatography takes place, but novel approaches to functionalization of such platforms has remained stagnant for decades.

According to the United States Pharmacopeia (USP), there are 858 C18 liquid chromatography phases registered under code L1 (octadecylsilane chemically bonded to porous silica or ceramic microplates, 1.5 to 10 μm in diameter, or a monolithic rod) (3). The vast amount of commercially available C18 columns are constructed on a wide range of solid supports, and may exhibit ancillary options such as endcapping and aqueous compatibility (AQ). They can also be mixed with other RPLC phases. Although silica particle manufacturing has become more normalized in the last few decades, these subtle differences in commercial C18 make it extremely difficult for the novice, and even the seasoned chromatographer, to “grab a column and go” for a given application.

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

**C18 Ligand Chemistries**

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

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

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

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

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

**Silanization Chemistries**

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

The advance of silicon-related technologies in material science has driven silane ligand synthesis. However, recent acquisitions of two major silane manufacturing companies leads to uncertainty of the current silane portfolio (21,22). Silane synthesis, although straightforward, suffers from one caveat: purification. Flash column chromatography is the method of choice when purifying a newly-synthesized compound from a mixture. However, a desired chlorosilane or alkoxysilane may permanently bind to the silica gel, leading to poor recoveries. Although some protocols exist to passivate silica gel from interacting with the silane ligand (23), the bulk of the reactive organosilane purification have relied on simple distillation. Such an approach leads to the confinement of commercially available ligands to a specific molecular weight range and containing functional groups that are thermally stable enough to endure purification by this technique.

An evolutionary bonding technology uses hydrosilanes, which Pesek developed through the development and the application of hydrosilylation chemistry in the production of HPLC stationary phases (24). The “Type C” silica possesses silica hydride (Si-H) at the surface of the particle and lacks the negative effects of silanols found on Type A and Type B silica. In order to functionalize this surface, a terminal alkyne or alkene will undergo hydrosilylation with Si-H in the presence of a platinum metal catalyst, leading to a phase with improved resistance to conditions that may cause hydrolysis in Type B silica columns. This is partially due to the much more hydrophobic surface offered by the silicon hydride moieties versus the usual hydrophilic silanol (25).

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

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

**Functionalization of Novel Solid Supports**

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

Monolithic beds are usually created in situ by free radical polymerization of monomers in the presence of porogens. Although thermal polymerization is a viable option, temperature fluctuations can occur in the confined spaces of the fluidic path, altering bed homogeneity along the column (36). Monomers can...
be organic (styrene, or acrylate-based) or siliceous in nature and offer the right amount of synthetic handles to permit surface functionalization. However, due to the nature of the inorganic monolith requiring a high temperature calcination step during its manufacturing, the appropriate functionalization must take place in situ as well. Hilder and co-workers developed a flow method for the ODS grafting on a 100 x 4.6 mm i.d. silica monolith, and its performance benchmarked against commercial C18 monolithic and a particle packed column (37). Since then, similar flow-like protocols have been applied to other column dimensions; however, slow reagent flow rates (μL/min) and high temperatures are still needed to graft a common C18 ligand, leading to bonding times of up to 24 h for a single analytical size column (38). Organic-based monoliths offer a wider variety of synthetic functionalization that are out of the scope of this article; however, their pervasive behavior towards organic solvents and their hindered mass transfer kinetics for small molecules result in the chromatographic preference towards silica-based monoliths. For more information, El Rassi and co-workers recently published a review discussing several post-polymerization functionalization strategies (39).

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

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

Conclusion

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

References

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Workflow advantages and future developments.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In this regard, customers filing DryLab’s knowledge-management documentation may have the potential to advance the field in the coming years.
From Detector to Decision: How Does the GC Instrument Generate Your Data?

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

Nicholas H. Snow

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

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

A classical schematic of a flame ionization detector is shown in Figure 1, adapted from early works (2–4). There were several early designs, including single and multi-jet. Today’s FIDs use a single jet design, as seen in Figure 1. In short, the column effluent is mixed with hydrogen and air (or, in the case of some of the early work, hydrogen and nitrogen, the most common carrier gas back then), and ignited, generating a flame between two electrodes. The flame temperature of about 2000 °C is not sufficient to ionize water vapor, the product of hydrogen combustion, but is sufficient to ionize a small portion of the carbon dioxide produced by the combustion of organic compounds. The ionized CO₂ present in the flame
then allows the circuit to be completed and electrical current to flow. The amount of current (amperes) is proportional to the mass of CO₂ generated in the flame. Variations in the chemistry of combustion reactions in the flame lead to the need to determine response factors, and provides the selectivity of the FID for organic (carbon-containing) analytes (5). A complete description of how to operate an FID can be found on LCGC’s learning platform, CHROMacademy (6). While the techniques and electronics we use to measure and analyze the signal have changed over the decades, the fundamental combustion chemistry that generates it has not changed.

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

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

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

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

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

The second aspect of communicating between a data system and an instrument, besides having a standard code or rubric for converting letters numbers and symbols into bits and bytes, is a common standard for transferring the actual electronic signals. This is usually accomplished using a serial port on the computer. There have been many standards used over the decades, but the most common for GCs are RS-232, GPIB, USB, and Ethernet. RS-232 uses a 9- or 32-wire connector, and a ribbon cable to transfer the signals with one of the wires actually carrying the signal and the others related to a “handshake” between the devices in that both would have to trade the correct separate signals to demonstrate that they were ready to send or receive data. RS-232 is the classical serial port used in personal computers, but that port is slow by today’s standards as the single signal wire means that one bit is transferred at a time, hence the term “serial.” With the other control lines available, instrument manufacturers often modified the standard RS-232 connections to make their instrument and data system connections proprietary. GPIB, or General Purpose Interface Bus (also called HPIB, Hewlett Packard Interface Bus and IEEE-488) took serial communications one step further, with eight signal lines, allowing the transfer of a byte of data instead of a bit at one time. In the 1980s and 1990s, GCs with less requirements for fast data transfer often used RS-232. Meanwhile, many GC–mass spectrometry (MS) systems with need for higher throughput used GPIB. Today, most instrument communications are based on USB and Ethernet, which provide much greater speed and more strict standardization, so there is much better connectivity of instruments and data systems between vendors. While Ethernet and USB are much faster RS-232 and GPIB, the same basic principles apply. Both instrument and data system must be ready to send and receive data, the connection must be working, and the data must be transferred and stored according to industry standards.
The third necessary component is an analog to digital converter that converts the analog signal into the binary digital numbers for the computer to store and process. An analog to digital converter may be included in the GC itself, or it may be added as a separate converter box. External converters were common in the 1990s and 2000s, with an example shown in Figure 3, showing a common data system interface box of 1990s vintage. The right-side image is the rear of the box where all the connections are shown. The left-side image shows a side view of the box with the cover removed to show the electronic circuitry. The back shows several types of connectors that made this interface mostly universal in that it worked with almost any GC on the market. The inputs on the top left are analog detector inputs. These could be connected directly to the analog detector outputs on the GC. Below these are connectors for remote starting and stopping the instrument, controlling an autosampler and several connectors for activating valves or switches on the GC. To the right are both types of serial connectors, RS-232 and GPIB, that connect to a personal computer data system. Looking at the left side of Figure 3, we see an electronic circuit board showing the various components allowing this box to function. Some key components include the analog-to-digital converter circuitry in the top right, within the silver rectangle. The

![Figure 4: Modern USB and ethernet connectors.](image-url)

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

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

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

**Conclusion**

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

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

**References**


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Semi-Automated Cleanup of Persistent Organic Pollutants in Environmental Samples—Complete Separation of PCDD/Fs and PCBs for Extracts in Toluene

Analysis of polychlorinated dibenzo-p-dioxins (PCDDs), furans (PCDFs), and biphenyls (PCBs) by environmental laboratories in North America has continued for many years. Increasingly analytical needs have focused on complete separation of PCDD/Fs and PCBs during sample processing with all existing 209 PCBs being analyzed. A semi-automated method (EZPrep), which combines elements of fully manual and fully automated procedures, was developed and tested. It uses pre-packaged silica, alumina, and carbon column kits for sample cleanup. The system uses a vacuum pump to elute solvents through these chromatographic columns for sample purification and preparation. The method described here allows for the sample extracts to be in toluene. With this method all PCBs are collected in one fraction and all PCDD/Fs in another fraction. 13C labeled recoveries for 10 g soil extracts were 75 to 95% for PCDD/Fs and 75 to 90% for PCBs. Similar tests for 2 g salmon extracts yielded recoveries of 80 to 105% for PCDD/Fs and 75 to 95% for PCBs. The semi-automated method can process six samples in parallel within a 50 min period.

Ruud Addink and Tom Hall

Persistent organic pollutants (POPs), such as polychlorinated dibenzo-p-dioxins (PCDDs), furans (PCDFs) and biphenyls (PCBs), have been analyzed and studied in a variety of samples for decades (1). These compounds are regulated under the 2004 Stockholm Convention, which the majority of countries in the world have acceded (2). Human health risks include possible damage to the immune system, the nervous system, reproductive functions, increased risk of cancer, endocrine disruption, and chloracne (3). PCDD/Fs occur as byproducts of various processes such as combustion, incineration, metalurgical industry, and pulp and paper bleaching production (4). PCBs were produced from the 1920s to the 1970s (1.5 million tons globally) and used in capacitors and transformers as flame retardants, hydraulic fluids, sealants, and vacuum pump fluids (5).

PCDD/Fs and PCBs have been strictly regulated in North America, creating a steady flow of samples analyzed by both small and large environmental laboratories. PCDD/Fs and PCBs sample processing is labor-intensive and prone to error. Compliance with regulatory procedures and accreditation requirements can result in a lengthy method validation effort. Oftentimes, strict quality assurance (QA) requirements apply. The complexity of sample matrices and the possibility of native background interferences, which can be orders of magnitude higher than analytes, can be very challenging.

Since these compounds are often present at very low concentrations (nano-, pico-, or even femto- gram levels, \(10^{-9}-10^{-15}\) gram) of sample elaborate extraction and cleanup methods have been developed to remove interferences as much as possible. Environmental samples can be air, sediment, soil, or water, and food stuffs are often analyzed. Sample extraction for solid samples is usually done by techniques such as pressurized liquid extraction (PLE) and Soxhlet extraction; solid phase extraction (SPE) can be used for liquid samples.

Manual procedures for cleanup of sample extracts (typically in hexane) include use of open column chromatography with the various steps: 1) Use acidified silica (often mixed with sulfuric acid) to oxidize lipids or other components of the extract (such as chlorophenols). Sometimes, neutral-basic silica is used behind the acidic silica. Depending on the amount of oxidation desired, the amount of acidified silica used will vary. Silver nitrate can be added to the column downstream of the silica to remove any sulfur components present in
the extract. 2) Basic alumina is used after the silica steps to remove chlorobenzenes and other chlorinated components. 3) Carbon columns can be used to further purify the extracts and also to fractionate and separate the mono- and di-ortho chlorinated PCBs from the PCDD/Fs and so-called coplanar PCBs. This is because the carbon retains flat molecules such as PCDD/Fs and coplanar PCBs while letting the other PCBs through.

The solvents that are eluted from the columns in the cleanup steps include benzene, dichloromethane, hexane and toluene. Meanwhile, the column elution is gravity-driven (6).

Automation of sample cleanup has focused on the use of a closed loop system to reduce background contamination, use of certified pre-packaged columns kits to further reduce background, lower solvent use, and elution by using a positive pressure pump. An automated system may allow for running more sample extract cleanups in parallel and faster processing through use of preprogrammed methods via a built-in computer (7).

A combination of aspects of both manual and automated methods can lead to a semi-automated system which is closed and uses pre-packaged certified column kits (thus reducing chances of background contamination) but requires less capital investment than a fully automated system. Use of a vacuum pump for column loading and elution reduces processing times compared to fully manual procedures and has the advantage of limiting potential downtime since the vacuum pump is the only mechanical part of the set-up. It also uses less electricity than an automated system.

Commercial environmental laboratories in North America with high sample throughput are often interested in analyzing PCDD/Fs and PCBs in completely separate fractions. This reduces the need for analyzing each sample more than twice and makes the analysis of all 209 PCBs in a separate fraction easier. In addition, Soxhlet-based methods for sample extraction are often carried out in toluene. Cleanup of sample extracts in concentrated toluene without the need for further evaporation and solvent-exchange to hexane is considered highly desirable.

The work described here offers a simple method for cleanup of sample extracts in between 2–10 mLs of toluene using a semi-automated system. The method results in complete PCDD/Fs and PCBs separation.

Materials and Methods

A closed loop system (EZPrep; Fluid Managements Systems, Watertown, MA) was used that utilizes the certified pre-packaged column kits mentioned above. The semi-automated system uses a rotary workstation with a vacuum pump to perform the entire...
sample cleanup in two stages. Figure 1 shows the small and large solvent reservoirs at the top and on the sides of the system. Black clamps (column holders) are shown for large size acidic silica columns and small clamps for the other columns. The turntable that the system is mounted on allows to rotate it 180° so that either side can face forward. Note that no columns are in place in Figure 1. The Stage 1 manifold has six positions for installing columns when the solvent eluents are directed to waste. The Stage 2 glass manifold (“fish tank”) is meant to contain collection tubes for when the desired analytes are eluting from the columns. The three-way valve controls the vacuum by directing it to either Stage 1 or Stage 2 or putting in “neutral” (off).

Figure 2 shows the system with columns being conditioned (eluents to waste) on the Stage 1 side. The flow of solvents is shown with arrows going from the solvent reservoirs to the columns, then through the Stage 1 manifold to a wash bottle for waste collection and to the vacuum pump. The vacuum pump is the only mechanical part of the set-up and is used to

![FIGURE 3: 13C PCDD/F recoveries (%) for 10 g soil in toluene extracts (n = 6).](image)

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pull the samples and elution solvents through the columns either to waste or for collection.

In the work presented here, three columns in a pre-packaged kit were used: high capacity acidified silica, 6 g alumina, and carbon. Depending on the amount of lipid or other oxidizable material in the sample, an acidified silica column with lower or higher capacity can be used. Columns available have between 0.15 g and 5 g of lipid capacity. The high capacity columns used can oxidize up to five grams lipid. The alumina and carbon columns used always have the same size. Columns can be ordered as kits (Table 1, Fluid Management Systems, Watertown, MA, www.fms-inc.com).

Procedure
Stage 1: Columns were assembled in the order high capacity acidic silica–alumina (no carbon used here). The columns connect easily using male and female SNAP connections that are built in. The use of a 4 g neutral-basic silica column between the acidic silica and alumina columns are optional. The solvent reservoirs were filled with 60 mL hexane, the vacuum was turned on, and the columns were conditioned. The hexane was pulled through the columns by the vacuum pump to waste. Afterwards, the vacuum was turned to neutral.

Stage 2: The column assemblies were removed and put on the Stage 2 side of the turntable on top of the glass manifold. Glass sample collection tubes had previously been put inside the manifold. Samples were placed in syringe vials on top of the acidified silica columns and the vacuum was turned on. Samples were loaded across both the acidified silica and alumina columns in 2–10 mL toluene and collected as Fraction #1 (PCBs). The syringe vials were removed and the columns were subsequently eluted with 60 mLs hexane from the solvent reservoirs (Fraction #1, PCBs). The vacuum was turned to neutral, the high capacity acidic silica columns
were then removed and discarded (all remaining PCBs and PCDD/Fs had been transferred to the alumina), and the vacuum was turned back on. The alumina columns were now eluted with 30 mLs 10% dichloromethane in hexane from the solvent reservoirs (Fraction #1, PCBs). After this step all PCBs had been collected in Fraction #1 and the PCDD/Fs remained on the alumina. The vacuum was turned to neutral (off) and the collection tubes with Fraction #1 were removed.

Stage 3: The alumina columns were put back on the Stage 1 manifold and connected at the bottom to the top of the carbon columns (which had been connected to the manifold). Solvent reservoirs were filled with 50 mL dichloromethane, the vacuum turned on, and both columns were eluted with 50 mL dichloromethane (vacuum, waste). This transferred the PCDD/Fs onto the carbon columns. The vacuum was then turned to neutral.

Stage 4: Alumina columns were disconnected from the carbon and discarded. The carbon columns were turned upside down and put on the Stage 2 manifold while the sample collection tubes were put in place in the manifold for collection. Solvent reservoirs were filled with 60 mL toluene. The vacuum was turned on and the carbon was eluted in reverse with 60 mL toluene collecting all PCDD/Fs (Fraction #2). The vacuum was turned to neutral and the tubes with Fraction #2 were removed. Because the carbon has strong adsorptive properties, the PCDD/Fs eluting from the alumina onto the carbon in the previous step were mostly present in the upper half of the carbon column; hence, the reason to reverse them before elution

**TABLE I:** Column kits available (contains three columns: acidified silica, alumina and carbon)

<table>
<thead>
<tr>
<th>Part #</th>
<th>Lipid Capacity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZ-LCNA-KIT</td>
<td>0.15</td>
</tr>
<tr>
<td>EZ-CLNA-KIT</td>
<td>0.5</td>
</tr>
<tr>
<td>EZ-MIDNA-KIT</td>
<td>1.0</td>
</tr>
<tr>
<td>EZ-HCNA-KIT</td>
<td>2.5</td>
</tr>
<tr>
<td>EZ-XHCNA-KIT</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Two ions were measured for each analyte. Thermo high resolution DFS GC–MS analysis was done on a Thermo high resolution DFS GC–MS with a 60 m x 0.25 mm ID x 0.25-um film thickness DB-5 type column. Two ions were measured for each analyte (Multiple Ion Detection). GC–MS analysis was approximately 40 min for PCBs and 60 min for PCDD/Fs.

Discussion

Runs were carried out with 10 g soil and 2 g salmon extracts in toluene. Note that the presence of up to 10 mls of toluene in the extracts pushes the analytes faster down the acidic silica column than if the extracts had been in hexane. This results in less hexane needed after sample loading to elute PCDD/Fs and PCBs from the acidic silica onto the alumina and then into the collection vessel. Because of the toluene present, all effluent eluting from the columns is collected and nothing goes to waste. As soon as the samples in toluene are loaded, some of the PCBs are eluted, and then carbon with 60 mL toluene. The total run time was approximately 50 min.

13C PCDD/Fs and PCBs spiked soil and salmon extracts in toluene were run on the cleanup system. Afterwards, collection tubes were put in an automated concentrator (Fluid Management Systems, Watertown, MA), preheated for 10 minutes at 55 °C, followed by heating in sensor mode under a 8–10 psi nitrogen flow which assured automatic shut-off at 0.5–1.0 mL. Samples were reduced in volume further in a vial evaporator (Fluid Management Systems, Watertown, MA) to 10 µL and spiked with recovery standards. Analysis was done on a Thermo high resolution DFS GC–MS with a 60 m x 0.25 mm ID x 0.25-um film thickness) DB-5 type column. Two ions were measured for each analyte (Multiple Ion Detection). GC–MS analysis was approximately 40 min for PCBs and 60 min for PCDD/Fs.

Complex matrices such as fish and soil can be rapidly cleaned up with this method. Total solvent use per sample is reduced because of the presence of toluene in the sample extract. Depending on the capacity of the acidic silica column, 250–300 mL solvent is used per sample. Compared to manual sample cleanup the semi-automated method is faster and incorporates the use of pre-packaged columns reducing the risk of native background contributions. Compared to automated systems, the semi-automated method requires relatively little investment and has less chance of breakdown because the vacuum pump used is the only mechanical part.

Note that the semi-automated EZPrep method is compatible with US EPA methods 1613 for PCDD/Fs, (8), and 1668C for PCBs (9). This is important since these methods are often followed by environmental laboratories in North America and also for work that is done under individual state certification.

Conclusions

Very good recoveries, all well within windows required by EPA methods 1613 and 1668C, were seen with the semi-automated system (Figures 3–6). Because the system is closed and mostly composed of disposable parts, risk of cross-contamination is low. A complete separation of all PCBs and PCDD/Fs was achieved. No solvent exchange to hexane was needed and the extracts in toluene were loaded as such. The method presented here can be seen as an alternative to fully manual or fully automated cleanup. Processing times are about 50 min which is shorter than most manual procedures. Use of certified pre-packaged column kits reduces chance of native background contamination. Individual column kits with varying sizes of acidified silica make the method applicable to low lipid level matrices such as serum and to demanding samples such as edible oils that require a high oxidizing capacity.

References


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Not (Only) Reversed-Phase LC–MS: Alternative LC–MS Approaches

Electrospray ionization (ESI) and other ambient ionization techniques have allowed a successful interface between liquid chromatography (LC) and mass spectrometry (MS). The coupling of these two high-resolution techniques has fostered the use of analytical science in various fields, including, but not limited to, the clinical, pharmaceutical, and forensic fields, enabling the analysis, identification, and characterization of thousands of molecular components in a large diversity of complex mixtures. For many years, reversed-phase LC has remained the most commonly adopted chromatographic mode, due to its rather straightforward applicability to the analysis of a wide variety of compounds (from small to large molecules), as well as its direct compatibility with ESI-MS. However, reversed-phase LC–MS has shown relevant limitations in a number of analytical applications. This encouraged the development of alternative MS-compatible chromatographic techniques, including hydrophilic interaction chromatography (HILIC), supercritical fluid chromatography (SFC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), and hydrophobic interaction chromatography (HIC), which provide analyte separation in the liquid phase based on different retention mechanisms compared with reversed-phase LC. Here, we present these alternative chromatographic approaches, highlighting the recent relevant applications in various fields, and discussing their potential in future of analytical science investigations.

Isabelle Kohler, Mingzhe Sun, Gino Groeneveld, and Andrea F.G. Gargano
FIGURE 1: (a) Number of publications per year in LC (dark blue), MS (red) and %LC–MS (black) literature between 1970 and 2019. Results have been extracted from Scopus using the following search: “liquid chromatography”, “mass spectrometry” and “LC–MS” as keywords. The result on the right y-axis “%LC–MS (vs. LC publications)” are obtained by calculating the percentage of LC–MS publications with respect to the LC publications in the same year. (b) Overview of the chromatographic modes used in LC–MS analysis. Results for reversed-phase LC–MS have been retrieved from Scopus using the following search: RPLC–MS (TITLE-ABS-KEY (liquid AND chromatography AND mass AND spectrometry) AND TITLE-ABS-KEY (reversed-phase AND chromatography) AND LIMIT-TO (DOCTYPE, “ar”) AND LIMIT-TO (SUBJAREA, “CHEM”). Similarly, the same filters have been applied for the other chromatographic modes with the exception of the descriptor “TITLE-ABS-KEY” which has been replaced by “hydrophilic-interaction” for HILIC–MS, “ion-exchange” for IEC–MS, “size-exclusion” for SEC–MS, “normal-phase” for NPLC–MS, “supercritical-fluid” for SFC–MS, and “hydrophobic-interaction” for HIC–MS.

simplicity in the coupling with ESI and other ambient ionization interfaces. This is reflected in the much higher number of studies using reversed-phase LC–MS published since 1970, compared to all other chromatographic techniques, as shown in Figure 1b.

However, the concept of “one-size-fits-all” does not always apply in analytical science. Indeed, in many cases, reversed-phase LC–MS may not offer the selectivity or resolving power needed for a given analytical investigation. This review showcases a selection of relevant technological developments and applications coming from different areas, such as analysis of small molecules, proteins, and synthetic polymers. In these applications, the coupling of alternative chromatographic techniques—namely, hydrophilic-interaction chromatography (HILIC), supercritical fluid chromatography (SFC), size-exclusion chromatography (SEC), ion-exchange chromatography (IEC), and hydrophobic interaction chromatography (HIC)—to MS has proven to offer advantages over the conventional reversed-phase LC–MS-based approaches. These examples of alternative LC–MS developments and applications have been carefully selected to further inspire analytical scientists, and provide them with additional options to solve a large diversity of analytical questions.

Hydrophilic Interaction Chromatography (HILIC)

HILIC–MS is considered the second most common chromatographic mode used in LC–MS analysis (Figure 1b). The reasons for its widespread diffusion include an orthogonal selectivity to reversed-phase LC, allowing for the retention of polar-ionizable compounds often poorly retained in reversed-phase LC (11), a lower mobile phase viscosity (enabling the use of longer columns leading to higher efficiencies), and a high sensitivity when using ESI-MS due to the use of high organic percentages in the mobile phase (12,13).

A large number of papers in the literature have investigated HILIC retention mechanisms, describing the multimodal nature of this chromatographic mode (14). The retention is driven by the partition of analytes between a polar station-ary phase and a relatively hydrophobic mobile phase (for example, an aqueous–organic mixture containing a high proportion of acetonitrile). Under appropriate conditions, namely, a concentration of 5 to 40% water in the eluent, a water-enriched layer is formed at the surface of the stationary phase, allowing for the hydrophilic partitioning. HILIC retention also involves ionic interactions, dipole–dipole interaction, and hydrogen bonding (15,16). The stationary phases can be classified on the basis of their chemistry as neutral, such as diol and amide, and charged stationary phases, such as bare silica, zwitterionic, and amine (17).

The analyte retention strongly depends on the selection of the stationary phase chemistry, as well as the buffer composition used in the mobile phase. Moreover, in order to ensure reproducible experiments, careful attention has to be paid to essential operating parameters, such as the composition of the injection solvent and the use of a reproducible mobile phase composition, as well as adequate equilibration time between runs. We refer to published literature for more insights on method development in HILIC–MS (18,19).

HILIC–MS has significantly matured over the last years, along with the commercialization of columns ensuring an improved batch-to-batch reproducibility and the introduction of stationary phases equipped with sub-2-µm particles. HILIC–MS is currently used in a wide range of fields, including pharmaceutical analysis, metabolomics, lipidomics, and glycomics. Moreover, recent investigations have demonstrated the suitability of HILIC–MS to study large molecules, such as intact proteins and their subunits, making it an attractive orthogonal tool for the analysis of glycosylated biotechnological products.

From Metabolomics to Lipidomics

One of the major challenges in metabolomics is the wide diversity in physicochemical properties observed between metabolites, showing the need for complementary approaches enabling a larger coverage of the metabolome.
The human metabolome encompasses a large number of (highly) polar metabolite classes such as amino acids, small organic acids, nucleosides, nucleotides, and phosphate derivatives, as well as saccharides, which are playing key roles in multiple (patho)physiological processes. Such metabolites show poor retention, and suffer from severe matrix effects using reversed-phase LC, leading to poor quantitative accuracy and low sensitivity. However, they are typically well-retained using HILIC. HILIC–MS also typically leads to a broader metabolome coverage in untargeted metabolomics compared with reversed-phase LC–MS (14,20). For all these reasons, HILIC–MS is now considered by the metabolomics community as essential as reversed-phase LC–MS, being integrated in the state-of-the-art analytical toolbox.

One of the major challenges in HILIC-based metabolomics is to develop methods offering a good compromise between metabolome coverage and acceptable peak shapes for different metabolite classes. The presence of broad peaks results in additional challenges during data pre-processing that may lead to poor quantitative accuracy. Better peak shapes for all compounds can be obtained by using multiple HILIC methods in successive experiments. However, this strategy is often not possible, due to time constraints and sample or resource availability. Most reported applications have therefore been carried out using one single HILIC method; for example, using a zwitterionic or diol column in untargeted metabolomics approaches, sacrificing part of the coverage for a better analytical efficiency.

Peak broadening has been significantly reduced by adding micromolar concentrations of phosphate to the mobile phase buffer (5 μM phosphate, corresponding to an estimation of ca. 40 nmol introduced to the column during each run). In the presence of trace amounts of phosphate, a significantly better peak shape, signal intensity, and improved coverage have been observed for a set of 65 polar compounds, including neurotransmitters, small organic acids, nucleosides, nucleotides, biogenic amines and sugars (Figure 2) (21). Similar improvements have been observed when a comparable amount of phosphate was added to the sample injection solvent. Moreover, in addition to the chromatographic effects, a slight increase of ionization efficiency has been observed in presence of phosphate (5 μM in the mobile phase), that is, 6% for negatively ionizing compounds and 16% for positively ionizing compounds, respectively. The significant improvement in peak shapes led to a more accurate automatic peak detection, representing a key advantage in the data preprocessing pipeline for untargeted applications. Interestingly, no sign of source contamination or instrument failure was observed after a year of experiments.

With additional experiments, the authors showed that the presence of trace phosphate improved the chromatographic performance by shielding electrostatic interactions between the analytes and the polymeric-based zwitterionic stationary phase, similar to what is observed when increasing the concentration of salts in the mobile phase. Indeed, a similar behavior in performance enhancement was observed when increasing the concentration of ammonium acetate in the mobile phase (i.e., from 5 to 200 mM) compared with the addition of trace phosphate (21). The analytes being the most positively affected by the presence of phosphate were also the ones requiring a higher concentration of ammonium acetate to achieve optimal peak shape. Moreover, phosphate – which has a high charge density – showed to be highly beneficial to improve the peak shape of compounds whose elution profiles were negatively affected by strong electrostatic interactions.

The exact mechanisms underlying this shielding effect remain to be fully understood, as different effects were observed depending on the column batch used. Indeed, irregularities in column manufacturing – as well as column conditioning – may modify the accessibility of phosphate to the electrostatic sites of the stationary phase. Moreover, phosphate blocks trace metals within the stationary phase matrix, influencing the peak shape. Inter-
hestingly, this study not only highlighted the benefits of adding trace phosphate to the mobile phase, but also revealed that electrostatic interactions may be the predominant cause of poor chromatographic performance when using HILIC, notably in metabolomics.

Lipidomics is a subdiscipline of metabolomics focusing on the large-scale study of the structure and functions of lipids. Lipids are involved in a plethora of physiological functions, including energy storage, signaling, and regulation of protein function. More than 60% of all human metabolites have been annotated as lipids (22). Reversed-phase LC–MS is widely used in lipidomics, typically using a C₁₈-based column combined with a highly organic gradient, allowing for the separation of lipids based on their fatty acyl chain length and unsaturation.

HILIC–MS, on the other hand, offers an orthogonal chromatographic separation where lipids are separated based on the lipid headgroup polarity, however typically leading to the co-elution of all lipids of a specific lipid class (23). The latter is considered advantageous in quantitative analysis, as the target lipids of different classes are co-eluting with their labeled internal standard (ISTD), chosen for each class. In reversed-phase LC–MS, ISTDs are usually eluted at a different retention time, possibly showing different matrix effects than the target lipids. Lange and associates evaluated the quantitative performance of HILIC–MS and reversed-phase LC–MS for the analysis of five lipid classes in human plasma (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, and lysophosphatidylethanolamine) using the so-called “one ISTD-per-lipid class” approach (23). They demonstrated that, despite the obvious difference in matrix effects, both workflows can be equally used for quantitative analysis, as similar concentrations were measured for most of the lipid classes assessed, consistent with reported NIST consensus values (except for highly unsaturated phosphatidylcholines).

**Analysis of Intact or Subunits of (Glyco)Proteins**

HILIC–MS has recently emerged as an attractive analytical tool for proteins analysis, in particular for the characterization of glycoproteins. Glycosylation is a common post translational modification (PTM), where oligosaccharides (referred to as glycans) are covalently bonded to an amino acid residue of the protein. Glycoproteins have an heterogeneous glycan composition; differences in their distribution may have a major impact on the biological functions of the protein and its stability, solubility, antigenicity, folding, and half-life (24,25). The glycosylation pattern is therefore considered a Critical Quality Attribute of recombinant pharmaceutical proteins, and should be carefully monitored in drug development pipelines to ensure quality, safety, and efficacy of the bio-pharmaceutical product.

Similar to what is observed for small molecules, HILIC allows for the discrimination between protein sample components based on a mechanism orthogonal to reversed-phase LC (26). In particular, optimized HILIC–MS methods have led to the separation of glycoforms of glycoproteins, such as monoclonal antibodies (27,28) and their fragment crystallizable portion (29), biopharmaceuticals (30), and neo-glycoproteins (31).

In most cases, protein samples are solubilized in water–acetonitrile mixtures, typically high in water content and containing an acidic ion-pair (such as trifluoroacetic acid). Small volumes (<10% of column volume) of this solution are injected and analyzed using acetonitrile–water gradients on amide stationary phases. The mobile phase conditions allow for ion-pair formation, with the basic protein residues at low pH reducing ion-exchange interactions with the stationary phase. As a result, the (neutral) sugars of protein glycans substantially contribute to the retention, resulting in glycoform resolution based on the overall glycan size and composition.

Figure 3 shows an example of the resolving power that can be obtained with HILIC–MS for the characterization of a lipase enzyme used in the food industry (results obtained in our laboratory) (32). Whereas the reversed-phase LC–MS analysis resulted in a single peak, the HILIC–MS method revealed the different glycoforms of the glycoprotein, resolved into distinct peaks according to the num-
The number of glycose units and the glycosylation site occupied (Figure 3). The HILIC–MS method resulted in the detection of over 100 glyco-proteoforms, allowing for the detection of glycoforms with two and three glycosylation sites occupied that were not observed with reversed-phase LC–MS. Interestingly, the total ion current profile obtained was similar to the SDS-PAGE den- sitogram. Off-line fractionation of the HILIC-UV separation confirmed this observation.

An important aspect in HILIC–MS being currently actively investigated is the replacement of ion-pair additives, such as trifluoroacetic acid, with more MS-friendly acidic ion-pair, such as formic acid, to increase the sensitivity of the method while maintaining its selectivity. In this context, an interesting development has been presented by the group of Wirth and co-workers, who used stationary phases with a special polymer coating to reduce the ionic interactions between the stationary phase and the proteins, preserving the same HILIC selectivity while significantly reducing the concentration of trifluoroacetic acid needed (33–35).

**FIGURE 3:** HILIC–MS analysis of a lipase enzyme. (a) Total ion current (TIC) with annotated glycoform elution windows (xN-yM), where (x) indicates the number of N-glycosylation sites occupied (0–3) and (y) the number of mannose units present next to a single N-acetylglucosamine present in each glycan. (b) Deconvoluted mass spectra obtained for the corresponding elution windows (7.5–7.7 min, 8.7–8.8 min, 8.8–9.0 min and 9.0–9.3 min), showing signals for the mature form of the protein (intact sequence, Ma), a mature form without a C-terminal tryptophan (Ma-W) and a mature form without a C-terminal serine and tryptophan (Ma-SW). (c) SDS-PAGE of the lipase showing the derived densitogram (top trace) aligned with the TIC trace obtained with HILIC-MS. Reproduced from reference (32) with permission.

**Supercritical Fluid Chromatography (SFC)**

First introduced in the last century with minor success, supercritical fluid chromatography (SFC) has regained a substantial popularity in recent years due to the remarkable technology advances carried out in modern SFC instrumentation, which have greatly improved its reliability, reproducibility, and robustness (36). SFC is based on the use of supercritical fluids as...
Hyphenation of SFC with MS. (a) The two commercially available interfaces used to hyphenate SFC with MS (pre-BPR splitter with sheath pump interface and BPR and sheath pump with no splitter interface). (b) UHPSFC–MS interface design developed to improve the spray stability. Note: A is commercially available interface and B is the modified prototype interface. Adapted from references (42,45) with permission.

FIGURE 4: Hyphenation of SFC with MS. (a) The two commercially available interfaces used to hyphenate SFC with MS (pre-BPR splitter with sheath pump interface and BPR and sheath pump with no splitter interface). (b) UHPSFC–MS interface design developed to improve the spray stability. Note: A is commercially available interface and B is the modified prototype interface. Adapted from references (42,45) with permission.

FIGURE 5: SFC–MS for the simultaneous analysis of hydrophobic and hydrophilic metabolites. (a) SFC–MS chromatogram showing the simultaneous injection of tricosanoic acid and raffinose (see reference [47] for experimental conditions); (b) Scatter plot showing the molecular weights of the detected metabolites as a function of their retention times. Blue dots, metabolites detected from reference [47] for experimental conditions; (b) Scatter plot showing the molecular weights of the detected metabolites as a function of their retention times. Blue dots, metabolites detected from reference [47] for experimental conditions.

Additives in the mobile phase influence the critical parameters of the fluid, which is then closer to the so-called subcritical conditions (38).

Most of the reversed-phase, normal-phase, and HILIC stationary phase chemistries can be used in SFC, overall offering a wide selectivity range (39). Moreover, multiple SFC-specific stationary phases have been developed and commercialized over the last years. A large number of the commercially available columns are also available with fully porous sub-2-µm and superficially porous sub-3-µm particles, enabling ultrahigh-performance SFC (UHPSFC) analysis which leads to excellent kinetic performance with a low pressure drop compared with UHPLC (40,41).

Hyphenating SFC with MS remains more challenging than the rather straightforward LC–MS configuration, due to the depressurization of the mobile phase which may lead to compound precipitation and cooling effects (36). Many approaches have been recently proposed to interface SFC with MS, with two designs currently available on commercial instruments, namely, the pre-backpressure regulator (pre-BPR) splitter with sheath pump, and the backpressure regulator (BPR) and sheath pump with no splitter interfaces, both illustrated in Figure 4a (42). In the pre-BPR splitter with sheath pump interface, the effluent is mixed with an added make-up solvent flow, and split into two parts, with the smaller proportion directed to the MS, and the remaining larger proportion directed to BPR to maintain the adequate pressure in the system. In the BPR and sheath pump with no splitter interface, the effluent is mixed with the make-up solvent flow, and directed to the MS inlet placed after the BPR. Adding a make-up flow to the SFC–MS interface has two purposes: enhancing the ionization efficiency, and avoiding a possible precipitation along the tubing. With such interfaces, the conventional ESI and atmospheric-pressure chemical ionization (APCI) sources can be used. Compared to LC–MS, SFC–MS typically shows higher sensitivities, due
to an improved evaporation process, as a rapid evaporation of CO₂ occurs at the MS inlet, which leaves the analytes in a small amount of easily-evaporated organic modifier (42). Finally, most of the common mass analyzers used in LC–MS, such as single quadrupole, triple quadrupole, and time-of-flight, as well as hybrid instruments are also suited for SFC–MS analysis (41,43,44).

Due to the nonpolar nature of CO₂, SFC–MS has been mostly used for the analysis of relatively non-polar lipids, steroids, tocopherols, and vitamins. As an example, a validated workflow for the quantitative analysis of 14 fat-soluble vitamins and carotenoids in human plasma was developed using UHPSFC–MS (45). Using a C₁₈ stationary phase and adding a small proportion of water to the mobile phase, multiple isomers and tocopherols were successfully separated in a single 8-min run. The commercial SFC–MS interface hardware was improved to minimize the post-decompression volume and allow for a better control of the chromatographic effluent density before the ESI process (Figure 4b). Combined with specific make-up solvent conditions, this new prototype interface led to a more stable spray, reducing the occurrences of spiky peaks, and resulting in an improved repeatability and sensitivity.

SFC–MS is not only well-suited for the analysis of hydrophobic compounds, but also fully applicable to the analysis of (highly) hydrophilic compounds using optimal experimental conditions. Moreover, with one of the major advantages of SFC–MS being its great versatility, the simultaneous analysis of both hydrophobic and hydrophilic compounds has become achievable, as demonstrated in recent studies (46–50). As an example, a comprehensive study has demonstrated the applicability of UHPSFC–MS in metabolomics for the simultaneous analysis of both nonpolar and highly polar metabolites within one single run, which remains very challenging using UHPLC-based methods (47). The complete separation of the compounds set composed of 57 metabolites of different polarities (-6 < logP <11) was achieved in a single UHPSFC–MS run, using optimized conditions (a mobile phase gradient ranging from 2% to 100% of modifier, a column packed with sub-3 μm superficially porous particles and a mix of water/acetonitrile [50:50, v/v] as sample diluent) (47). Figure 5a shows the separation of two compounds with a large polarity difference (tricosanoic acid [logP = 9.3] and raffinose [logP = -6.3]), thus demonstrating the suitability of UHPSFC–MS for metabolomics.

Besides metabolome coverage, another important aspect in metabolomics is the presence of matrix effects, which may significantly affect the quality of the quantitative data and represent a crucial parameter during method optimization. In their study, Guillarme and coworkers also investigated the importance of matrix effects in plasma and urine for a set of representative metabolites (48). Overall, limited matrix effects were observed for the analysis of both biological fluids, with 30% of the metabolites suffering from matrix effects in plasma and 25% in urine, respectively. Moreover, the repeatability of the UHPSFC–MS coupling was remarkable, with average relative standard deviation (RSD) values for retention time repeatability between 0.3 and 0.5% over a period of three weeks. The authors also evaluated the performance of UHPSFC–MS in metabolomics using a commercial library containing 597 metabolites. With UHPSFC–MS, 66% of the compounds present in the library were detected, notably highly polar compounds such as amino acids, nucleosides, and carbohydrates, as well as hydrophobic analytes such as steroids and lipids (Figure 5b). Phosphorylated metabolites, however, led to poor performance.

SFC–MS does not only show interesting advantages for the analysis of low-molecular weight compounds, but has also demonstrated a great potential for the analysis of proteins. As an example, SFC (here referred to as enhanced fluidity liquid chromatography by the authors) was used to separate intact proteins using hydrophobic interaction chromatography stationary phase combined with a dual gradient elution that consisted of an LC solvent gradient with simultaneous addition of an increasing amount of CO₂ (51). Compared to conventional LC analysis, improved efficiency was observed using SFC, due to the faster mass transfer caused by the presence of CO₂ (Figure 6a). As the desolvation of the mobile phase in the MS was also enhanced by the addition of CO₂, a "supercharging" effect was also observed, resulting in higher ionization efficiencies and shifts of charge state (Figure 6b).

Overall, the major advantage of SFC–MS compared with LC–MS is its suitability for the simultaneous analysis of a wide
The development of high performance analytical applications.

Such chemical features include molecular weight, molecular structure organization.

Synthetic polymers are chemical products used in a large diversity of everyday life applications, such as plastics and coatings. Polymers are produced through polymerization of chemical units (referred to as monomers), resulting in a heterogeneous distribution of molecules.

The development of high performance polymers (used in applications such as drug delivery systems) relies on tailoring the distribution of chemical features to achieve the desired product properties. Such chemical features include molecular weight distribution, end-group functionality, topology (branching vs. cyclic), and polymer sequence distribution.

SEC-UV is typically used to determine the molecular-weight distribution of soluble polymeric products, but it does not provide any information on the molecular composition of the sample. On the other hand, MS can provide information on multiple chemical distributions (52). Therefore, the combination of SEC with MS is highly relevant in the field of polymer analysis. However, the common mobile phase systems used in SEC-UV (apolar solvents like THF) are not compatible with ESI-MS. An interesting approach is to couple SEC–MS using a post-column make-up flow (53). In this set-up, the LC eluent is mixed post-column with an organic solvent (such as methanol containing an ionization agent such as NaI) (54). Furthermore, supercharging agents can be used as additives to improve the ionization and detect higher molecular weight (MW) polymers showing a wide variety of chemistries (such as polystyrene, acry-
lates, and polyesters), also enabling the ionization of hydrophobic polymers (55).

The combination of the resolving power of SEC, partly discriminating the sample polydispersity, with ESI-MS with supercharging agents significantly extends the MW range that can be measured. As a result, SEC–MS with supercharging agents allow for the analysis complex synthetic polymers up to 10 kDa range. Figure 7 shows an example of SEC–MS for the analysis of a branched polyester resin sample (unpublished data from Groeneveld and associates [56]). Species up to \([\text{M + 6Na}]^{6+}\) were observed, allowing for the detection of species up to 6 kDa distributed in a highly complex MS spectrum.

Beside polymer characterization, SEC–MS is often used for the characterization of biotechnological products, notably native SEC–MS, which is gaining more attention. Size and conformation based separation are of interest as often the active form of a protein is determined by its higher order structure distribution (dimerization). The information on the protein structural organization is lost when using denaturing methods such as reversed-phase LC and HILIC for the analysis of biopharmaceuticals. Native (or non-denaturing) separation approaches are indeed only accessible using mild conditions, such as water-based solutions at neutral pH and separation at room temperature. Coupling native SEC to MS allows for the analysis of protein complexes and aggregates. Under native separation conditions, water-based mobile phases with relatively high concentrations (100 mM) of volatile salts are used. These conditions result in a mitigation of ionic interactions with the stationary phase and maintain non-covalent interactions during measurements, enabling to obtain protein structural information (57). An example is illustrated in Figure 8 with the analysis of monoclonal antibodies (mAbs), where dimer, monomers and mAbs fragments of are separated using SEC–MS (58). Similar workflows can also be used to desalt and buffer-exchange pro-

![Graph](image-url)

**FIGURE 8:** Online SEC-native MS analysis of a temperature-stressed NIST mAb sample. (a) Overlaid UV chromatogram of stressed (red trace) and unstressed (blue trace) NIST mAb. (b) Native mass spectra of peaks (1) and (2) observed for the SEC analysis of the temperature-stressed NIST mAb sample. Figure adapted from reference 58 with permission.
protein products, allowing for the analysis of very large molecular complexes (59).

The (Not Necessarily) Salty: Ion-Exchange Chromatography (IEC) and Hydrophobic Interaction Chromatography (HIC)–MS

The growing interest in characterizing protein-based biotechnological products in their intact forms under nondenaturing conditions has fostered further developments in alternative chromatographic approaches such as ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC) and capillary electrophoresis, focusing on the development of MS-compatible liquid-phase conditions.

IEC is commonly used to characterize charge variants of mAbs, such as those from amino acid variants, or PTMs such as deamidation. Typically, IEC methods are based on the use of cation-exchange stationary phases and mobile-phase gradients where either the concentration of salts (NaCl) is increased during analysis, or the pH is changed during analysis. Reference methods however make use of nonvolatile buffers or salt components; they are therefore not suitable for direct MS coupling and can only be used combined with desalting procedures.

Recent results, such as reported by Füssl and associates (60,61), have demonstrated the feasibility of performing pH gradient-based IEC separations using low concentrations of volatile salts such as ammonium acetate. These results underline the importance of a separation step to obtain a detailed characterization of molecules having similar masses (62,63). Figure 9 illustrates a striking example of the analytical power of such method, with the separation of the deamidated forms of the mAb trastuzumab, differing in only 1 Da on a molecule with a MW of ca. 150 kDa (64)! This detailed characterization is not possible with direct MS (65) or using denaturing reversed-phase LC–MS methods, as they lack the sufficient selectivity to differentiate the charge variants.

Trends in this field focus on the development of optimized methods to further enhance the overall sensitivity and widen the applicability of IEC-MS to other protein-based therapeutics, as well as other biotechnological products.

HIC separates proteins based on the hydrophobicity of the residues exposed in the protein tertiary structure under nondenaturing conditions, allowing for the preservation of noncovalent interactions. Similar to IEC, nonvolatile salts (ammonium sulphate, for example) are commonly used in HIC, but Bifan and colleagues have demonstrated that ammonium acetate in combination with small percentages of organic solvents is suited for HIC–MS analysis (66). However, with this volatile salt, most of the proteins are not retained using conventional HIC columns. Interestingly, a relatively wide spectrum of proteins can be retained using columns functionalized with selectors with higher hydrophobic character, for instance with selectors with a longer chain length. In their study, the authors clearly demonstrated the feasibility of HIC–MS, showing relevant examples in the context of top-down proteomics analysis (66) and analysis of mAb samples (67). These convincing proof-of-principle results require follow-up studies where further steps are taken to reduce the amount of salt used (here over 1M) and improve the separation efficiency.

Conclusions

Reversed-phase LC–MS has long remained the gold standard chromatographic technique for the analysis of a large diversity of compounds, from metabolites to biopharmaceuticals via lipids, glycans, and peptides. Over the last decade, remarkable technological developments have been carried out in the fields of HILIC, SFC, SEC, IEC, and HIC, fostering their use in many different applications. Those developments include the commercialization of novel stationary phase chemistries and new instruments, as well as experimental conditions enabling the direct coupling to MS. Overall, these innovations have raised the interest of the analytical science community, seeking alternative chromatographic options that would help solving the challenges encountered with reversed-phase LC–
MS. Moreover, alternative selectivities may be a valuable tool to address analytical questions when not having access to the latest MS technology platforms.

Most of the alternative chromatographic techniques are now reaching a level of maturity that allow them to be used outside academic research. HILIC, SFC, SEC, IEC, and HIC, together with important liquid-based separation approaches that were not covered here, such as capillary electrophoresis, multi-dimensional liquid chromatography, normal phase chromatography and chiral chromatography, are not expected to replace reversed-phase LC–MS, but will become increasingly important in analytical sciences, enabling the scientific community to tackle analytical challenges that cannot be entirely solved with reversed-phase LC alone. We, therefore, find it essential that the new generation of young scientists become familiar with those techniques as early as possible, together with reversed-phase LC–MS.

References


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(26) A.F.G. Gargano, L.S. Roca, R.T. Fellers,
The topic of chromatographic integration and data interpretation raises a lot of questions about data integrity. Is it acceptable to integrate data? What are the limits? We asked Mark E. Newton, of Heartland QA, in Lebanon, Indiana, to weigh in.

It’s interesting. I would say manual integration is acceptable. So long as you make a provision in your process that says when I do manual integration, I recognize there is risk here and therefore commensurate to that risk there is an additional set of review. So for example, in your laboratory you may say when somebody engages in manual integration, there should be somebody who is a data reviewer with a lot of experience who is perhaps a senior individual in that laboratory. They’re able to look at your chromatography and say it’s reasonable chromatography, it’s scientifically sound, and therefore it stands as it is.

I would encourage everybody as much as possible to work on automating their integration. If for no other reason, it’s far more efficient. I remember we ran some numbers back a few years ago and said that for every chromatogram that we could integrate automatically we would realize a savings; it was in the range of 15 to 20 minutes per injection so you can save some time simply by trying to move your methods to automated integration as opposed to manual integration. If for no other reason you just gain efficiency on it, so it’s a win in that regard as long as the risk is commensurate and known.

I think the one dangerous thing to me is if you’re not going to do manual integration, your other choice would be to actually take the chromatography integration parameters and adjust them as needed to fit the particular run that you’re dealing with. The issue is you’ve got to be careful about is that you can literally get to where you’re manipulating the system to do what you would have done manually, so you’re just using this system to do the same thing. So even though one of them is automated and one of them is manual, you need to look at the record of what’s being done and why is it being done and ask: is it reasonable to do that? This is because people can use the automated system to manipulate data as well.

So just because it’s automated versus manual doesn’t mean it’s safe versus dangerous. It means that the more data you have, the less robust the method. It also means that the more intervention that is required on the part of the user, either in parameters or in manual integration, the more review is necessary to determine that it’s scientifically reasonable and valid.

Are there best practices that companies should follow with respect to chromatographic integration?

The first rule would be to follow Norm Dyson’s and get a good method first. Unfortunately, people are too quick to rush in to try and put lipstick on the pig. And sometimes you have a method that is your problem, not your manner of the integration of it. And so I would tell people if you’re having a hard time getting chromatography to work for you, why don’t you take a step back and look at your chromatography first? Try a different method or try a newer technique for doing this rather than doing the old method that you ran the last 15 or 20 years. Try something new and see if you can’t get a better method out of the process because I’ve seen situations where you’d scratch your head and think why you are doing this. You know, the other metric I would encourage people to consider is what would be ‘right first time.’ How many times can you set up a run, do the run, process the run, and release the data the first time? By considering ‘right first time,’ I don’t have to go back and re-inject something, and I don’t have to go back and reprocess it all over again. How many times can I just workflow it all the way through to the end and do that time after time?

If you’d start keeping metrics on how often we get it right the first time versus how often we have to either reprocess or reinject something to get an acceptable set of results, I think you could find pretty quickly where your problems are in your lab and where it would be worth devoting your resources.

Mark E. Newton is the principal at Heartland QA in Lebanon, Indiana. Direct correspondence to: mark@heartlandQA.com.
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534 Separation and Sizing of a Virus Mixture Using Asymmetrical Flow Field-Flow Fractionation Coupled to Multi-Angle Light Scattering
George Bou-Assaf¹, Andy Blum¹, Omar Matalka¹, Ruth Frenkel¹, Robert Reed², and Soheyl Tadjiki²
¹Analytical Development, Biogen, Cambridge, Massachusetts
²Postnova Analytics Inc, Salt Lake City, Utah
Per- and polyfluoroalkyl substances (PFAS) are a group of manufactured chemicals that are used in a wide variety of industries because of their resistance to stains, grease, and high temperatures. They possess a chain of linked carbon atoms with fluorine atoms branching off the main chain. The presence of the strong carbon-fluorine bond contributes to the stability of these compounds, earning them the nickname “forever chemicals.” PFAS are used in products such as nonstick cookware, firefighting foam, and stain-resistant carpets.

Because of their persistent nature and their widespread use, this group of substances has leached into the environment with limited methods of remediation. Furthermore, these compounds have been found to bioaccumulate in animals and humans, and exposure in humans has been shown to cause adverse health outcomes, including cancer, infertility, and endocrine disruption. Thus, the assessment of the levels of PFAS in the environment is important to the health and safety of humans.

The EDGE, an automated solvent extraction system, was used to extract a subset of PFAS molecules from spiked soil samples. The EDGE was able to extract the soil samples in less than 10 min. The extraction yielded excellent recoveries and standard deviations. Furthermore, there was no carryover found between samples. The EDGE is an excellent choice for laboratories seeking to automate their PFAS extraction.

**Method**

**Reagents**
Clean sandy loam was purchased from MilliporeSigma. A PFAS standard containing 24 different compounds (Part number 99207) was purchased from Absolute Standards, Inc. HPLC-grade methanol, HPLC-grade water, HPLC-grade formic acid, and ammonium hydroxide were purchased from Fisher Scientific.

**Sample Preparation and EDGE Method**
Five grams of clean sandy loam was weighed directly into a Q-Cup® containing the S1 Q-Disc® stack (C9-G1-C9 sandwich). Each sample was spiked with 2 ng or 200 ng of standard in HPLC-grade methanol, resulting in low spike and high spike samples, respectively. Each set of spikes was done in triplicate. The sample was extracted with the EDGE using 80:20 methanol:water with 0.3% ammonium hydroxide using the method provided. Each set of spikes was extracted on the EDGE, and then a blank extraction of the system was done with a Q-Cup containing the S1 Q-Disc stack (C9-G1-C9 sandwich) to assess the level of carryover. Each extraction was collected in a polypropylene conical tube using an EDGE rack. The sample was brought up to a final volume of 20 mL using 80:20 methanol:water with 0.3% ammonium hydroxide, and 20 µL of formic acid was added to each sample to neutralize the sample. The samples were then analyzed by Pace Analytical.

**EDGE Method**
- **Q-Disc:** S1 stack (C9-G1-C9 sandwich)
- **Extraction Solvent:** 80:20 methanol:water with 0.3% ammonium hydroxide
- **Cycle 1**
  - **Top Add:** 10 mL
  - **Bottom Add:** 0 mL
  - **Rinse:** 0 mL
  - **Temperature:** 65 °C
  - **Hold Time:** 3 min
- **Cycle 2**
  - **Top Add:** 10 mL
  - **Bottom Add:** 0 mL
  - **Rinse:** 0 mL
Results
The recovery data in Table I, from the low and high spikes, indicated that the samples were extracted with high efficiency, with recoveries ranging from 63% to 101%. The resulting RSD values were also low, indicating the recovery data were reproducible. The extraction data from the empty Q-Cup indicated that there was no carryover of the spiked compounds within the system, indicating the wash was aggressive enough to remove any residual PFAS compounds.

Conclusion
The analytical assessment of PFAS compounds is critical because of their widespread nature, high stability, and adverse health effects. The EDGE was able to rapidly and efficiently extract spiked soil samples with excellent recoveries and RSD values. The EDGE also saw no carryover after extractions into the subsequent extraction. The EDGE is an excellent extraction tool for laboratories seeking to automate their PFAS extractions with great efficiency.

Table I: Average recovery results from the low spike and high spike soil samples

<table>
<thead>
<tr>
<th>Compound</th>
<th>Low Spike</th>
<th>RSD ($n = 3$)</th>
<th>High Spike</th>
<th>RSD ($n = 3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1H, 1H, 2H, 2H-perfluorodecane sulfonic acid (8:2 FTS)</td>
<td>83.00%</td>
<td>7.81%</td>
<td>87.00%</td>
<td>7.00%</td>
</tr>
<tr>
<td>1H, 1H, 2H, 2H-perfluoroctane sulfonic acid (6:2 FTS)</td>
<td>80.00%</td>
<td>3.00%</td>
<td>87.67%</td>
<td>7.09%</td>
</tr>
<tr>
<td>1H,1H,2H,2H-perfluorohexane sulfonic acid (4:2 FTS)</td>
<td>86.67%</td>
<td>10.02%</td>
<td>87.67%</td>
<td>5.13%</td>
</tr>
<tr>
<td>N-ethylperfluoro-1-octanesulfonamide (EtFOSAA)</td>
<td>85.67%</td>
<td>6.66%</td>
<td>86.00%</td>
<td>5.57%</td>
</tr>
<tr>
<td>N-methylperfluoro-1-octanesulfonamidocetic acid (MeFOSAA)</td>
<td>78.67%</td>
<td>1.15%</td>
<td>81.33%</td>
<td>4.16%</td>
</tr>
<tr>
<td>Perfluoro-1-butanesulfonic acid (PFBS)</td>
<td>78.00%</td>
<td>3.00%</td>
<td>86.00%</td>
<td>1.73%</td>
</tr>
<tr>
<td>Perfluoro-1-decanesulfonic acid (PFDS)</td>
<td>77.00%</td>
<td>6.56%</td>
<td>84.33%</td>
<td>0.58%</td>
</tr>
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<td>Perfluoro-1-heptanesulfonic acid (PFHpS)</td>
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Temperature: 65 °C
Hold Time: 4 min

Wash 1
Wash Solvent: Methanol
Wash Volume: 10 mL
Temperature: 50 °C
Hold: 3 s

Wash 2
Wash Solvent: 80:20 methanol:water with 0.3% ammonium hydroxide
Wash Volume: 10 mL
Temperature: None
Hold: None

CEM Corporation
3100 Smith Farm Road, Matthews, NC 28104
tel. (800) 726-3331
Website: www.cem.com
Cylinder Free Auto-GC–MS for Continuous SVOCs, VOCs, and PAHs Monitoring in Air

Franck Amiet, Chromatotec Group, France

Ambient air is polluted by many semi-volatile and volatile organic compounds (SVOCs and VOCs) originating from anthropogenic and natural sources. These compounds contribute significantly to the formation of tropospheric (ground-level) ozone (O\textsubscript{3}), resulting primarily from the reaction of VOCs with NO\textsubscript{x} (NO and NO\textsubscript{2}) in the presence of sunlight, and secondary organic aerosol (SOA) by oxidation.

Automatic, continuous identification and quantification of VOCs in ambient air is possible through artificial intelligence; software compares results from FID and MS systems for validation of results, and identifies molecules by their ion’s spectrum. The system does not need an expert to interpret and analyze data.

Due to the large number of molecules, the complete separation of all compounds is difficult to perform using gas chromatography (GC) columns. Mass spectrometry (MS) detection must be added to identify a list of VOCs with expertise, also allowing identification of unknown compounds with FID or MS.

To respond to those needs, a new Field Dual Thermal Desorption (TD)-GC-MS/Flame Ionization Detector (FID) solution has been developed. This standalone system provides automatically- and continuously-validated results for the identification and quantification of more than 120 different VOCs as standard, directly on the field without the need of any daily operator.

Introduction

The airmOzone expert has been selected by the United States Environmental Protection Agency (USEPA). The USEPA requires Photochemical Assessment Monitoring Stations (PAMS) to collect ambient air pollutant and meteorological data to attain the National Ambient Air Quality Standard (NAAQS) for ozone nationwide.

The VOCs measured are gaseous aliphatic and aromatic hydrocarbons in ambient air up to C\textsubscript{20}, including the components of interest at the PAMS program, as well as the extra chlorinated compounds covered by Compendium Methods TO (Toxic Organic)-14 (EPA 625/R-96/010b) and TO-15. The data collected at the PAMS sites include measurements of a target list of VOCs such as paraffins, olefins, aromatics, and alkenes (1).

Additionally, the system is used by various reference institutes and universities to monitor Polycyclic Aromatic Hydrocarbons (PAHs) in industrial sites, tropical forests, and on-site under decontamination. Volatile PAHs measured in standard are: naphthalene, 2-methyl-naphthalene, 1-methyl-naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, and anthracene. Benzo(a)pyrene can now also be analyzed.

Instrumentation

The system consists of two automatic gas chromatographs (airmoVOC C2-C6 and airmoVOC C6-C20) both equipped with a FID detector, one for light compounds (C\textsubscript{2}–C6) and the other for heavy compounds (C\textsubscript{6}–C20). Both TD-GCs are coupled to a single Quadrupole MS.

Air generators (airmoPure, Chromatotec\textsuperscript{®}) were used for the flames of FIDs, valve actuations of the auto-TD-GC-FIDs, and drying of samples for C\textsubscript{2} to C\textsubscript{6} analysis, and hydrogen generators 99.9999% with dew point below -15 °C (Hydroxychrom, Chromatotec\textsuperscript{®}) were used as carrier gas and to fuel the FID flames.

It can automatically identify and quantify VOCs down to ppt levels. Results are obtained every 30 min for molecules from 2 to 20 carbon atoms simultaneously. LOQ for Benzene is as low as 1 ppt in automatic.

The equipment is specially designed for field applications with the best performance and flexibility, optimizing the space. It can be placed in one cabinet or in two smaller ones, each one occupying less than one square meter of floor space. This miniaturized system is transportable, thanks to its wheels, and the inside is easily accessible thanks to the rack sliders, perfect for installation in mobile laboratories for on-site analysis.

Results

Characteristics of the Analytical Method

The stability and accuracy of the system is automatically checked every day by the internal calibration system, with benzene permeation tube for validation of the complete system and to provide automatic data validation. Both detectors gave an excellent correlation between their response and the concentration (>\textsuperscript{2}R=0.999).

The robustness and long-term stability of the complete system allow validating data without the need for frequent calibration. The results are extremely stable at 10 ppb level with, for example, on
benzene peak area RSD at 3.77% for MS detector and 2.51% for FID detector over more than 120 days without tuning or calibration during this period (Figure 1). The concentrations are automatically computed using VistaMS software which calculate the compounds concentration analyzed using high intensity on selected ion for each species.

The performance of the system was studied using permeation tubes, certified cylinders containing VOC mixtures (PAMS, TO14 and TO15), and a dilution system equipped with two mass flow controllers and a dilution chamber. With this system, concentrations ranging from 0 ppb up to 40 ppb were generated.

### Solving Coelutions in Ambient Air Samples

Compounds taking part in a coelution can be identified by FID coupled to MS detection. When a single FID detector is used, interferences may occur but the additional MS can identify individual components of a peak by choosing the compound with the best matching mass spectra pattern available in the NIST library. Thus, the peak at 680 s, identified as only N-Octane in Figure 2(a), could be deconvoluted into N-Octane and tetrachloroethene in Figure 2(b).

### Validation of the Results

The determination of validated results by the automatic intercomparison of FID and MS results was performed automatically by VistaMS software following an algorithm that privileges the result obtained by FID because it is a very accurate and sensitive detector. In case of coelution, validated results are considered those obtained using the MS detector, due to its ability to identify individual compounds. Thus, dual-detection improved the accuracy of the identification and quantification of the compounds in ambient air by 14%, with precision values expressed as RSD better than 5%.

### Identification of Unknown Compounds

Two unidentified peaks of less than 1 ppb were found in the GC–MS data. Compound identification was performed automatically by comparison of the mass spectrum (m/z values and the relative intensities) obtained with those from the NIST Library by VistaMS software. Figure 3 shows the excellent spectral match obtained for Hexanal and Furfural by C6–C20 analysis.

Thanks to NIST library, all molecules SVOCs, VOCs, and PAHs from 2 to 20 carbon molecules can be identified by MS and quantified by FID using theoretical response factors. Thus, the standard parameter list can be adapted according to the needs of the measurement campaign (for example, depending on the geographical area) or if the requirements of the PAMS, TO14, and TO15 methods change.

### Conclusion

In this study, 120 different VOCs from C2 to C20 range were quantified at ppb and ppt level. Potentially coeluted compounds were identified and validated by VistaMS software thanks to the combination of FID and MS detectors. Any unexpected or unknown compounds are automatically identified thanks to the internal NIST library linked to VistaMS in the internal computer of the GC–MS system.

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**Figure 2:** Chromatograms, intensity of the signal [A] vs. retention time (s), of the N-Octane and tetrachloroethene coelution observed by FID detection (a) solved with the MS (b) for C6–C20 analysis.

**Figure 3:** Automatic comparison between mass spectra obtained (top) and reference (bottom).

This fully automatic, intrinsically linear, precise, and very stable system with data validation allows non-specialist operators to access expertise-level results. No cylinders are required: the FID can be operated continuously using hydrogen and zero air generators. All data collected are stored to assure their full traceability and availability.

**Reference**

Promoter or Polluter? Separation of Triazines on HxSil C18 Column

Adam L. Moore, PhD, Hamilton Company

Large industrial scale farms use triazines to help increase the production of foods through the blocking of broad-leaf weeds. The inhibitors are primarily used on farms growing corn, sugar cane, palm oil, and citrus, and, to a lesser degree, weed control in roadway applications.

The primary mode of action for triazine herbicides in plants is through the binding of proteins involved in the electron transfer chain of the photosystem II pathway of photosynthesis (1). Triazine compounds block the water reduction pathway, which normally generates protons that are used in subsequent steps along the electronic cascade. With the plants’ proton pump deactivated, the gradual destruction of chloroplasts occurs, culminating in plant death (2, 3).

Triazines have been found as contaminants in waterways and drinking water supplies. Multiple studies have shown that triazines exhibit endocrine disruption in female and male reproduction in reptiles, rats, and humans (4).

As such, drinking and waste water regulations involving triazine compounds are now employed. Structural similarities between the seven federally approved triazines adds to the difficulty of identification, isolation, and quantification. Hamilton Company has achieved a separation using the 3 µm HxSil HPLC column. The column utilizes C18 moieties chemically bonded to silica as its primary mode of separation. The ample end-capping applied to the column helps to facilitate longer lifetimes while limiting free silanols. The Hamilton ODS column is a great choice when analyzing analytes that exhibit Lewis basicity and therefore contribute more extensively to acidic hydrogen bonding in the stationary phase. The separation of the seven triazines occurs within 15 min, with baseline resolution of all critical pairs.

References

(3) A. Cessna, Non-biological degradation of triazine herbicides photolysis and hydrolysis. (2008).
Shodex Rapid SUGAR Series: Fast Fermentation Monitoring and Sourdough Cultures

Alexander Schrum, Ronald Benson, and Marlon Rettis, Showa Denko America, Inc.

Living organisms (yeast and bacteria) were not identified as the agents that produced or spoiled common food items until the 19th century. Baking, especially of bread, was among the first industries to be transformed by the emerging scientific understanding of fermentation. The introduction of commercialized yeast in the early 20th century steadily reduced the time needed to bake bread both at home and on the production line, making way for modern packaged breads (1). However, traditional fermentation techniques, including sourdough breads, have resurfaced in popularity. Typical sourdough cultures are created from yeast and bacteria populations naturally present in the air and on many surfaces, providing sourdough loaves with their characteristic rise and “tangy” flavor by converting fermentable sugars into CO₂ and flavor compounds like lactic acid. In this application note, a “homemade” sourdough starter culture was analyzed to determine how key fermentation products changed over the course of 24 h with the new Rapid SUGAR SH1011 8C column.

The Shodex™ Rapid SUGAR SH1011 8C column contains a styrene divinylbenzene base material ligand exchange column, and is designed for rapid fermentation monitoring, allowing for the analysis of saccharides, organic acids, and ethanol in less than 5 min. The method uses simple aqueous conditions and RID detection, ideal for large sample workflows and QC environments.

Experimental

Seven common fermentation compounds (maltotriose, maltose, glucose, lactic acid, acetic acid, glycerol, and ethanol) were used as standards. The sourdough starter culture was adapted from King Arthur (2), with the procedure repeated daily for two weeks.

Sample preparation

Approximately 10 g of sample was homogenized with 90 mL of distilled water (for 30 s, with the blender on maximal speed). Five milliliters of 1 mol/L HClO₄ solution was added to a 10 mL aliquot of the homogenate. The mixture was centrifuged for 15 min at 4000 g at 15 °C, the supernatant was neutralized (pH 7.0) with 2 mol/L KOH, and the volume was adjusted to 25 mL with distilled water. After 30 min precipitation on ice, the solution was filtered on 0.45 mm cellulose filter, adapted from reference 3. Samples were taken starting on Day 15 at three time points:

- Sample 1: Immediately after mixing in flour and water, labelled time 0
- Sample 2: 12 h after time 0
- Sample 3: Day 16, approximately 24 after time 0

Shodex SUGAR SH1011 8C (8.0 mm I.D. × 100 mm, 6 μm) was used with a Shodex RI-501 detector. The eluent conditions were: 1 mM H₂SO₄ aq. The column was kept at 75 °C, and the flow rate was 1.0 mL/min. The chromatography was performed on a Shimadzu Prominence LC 2030 instrument.

References


Shodex™/Showa Denko America, Inc.
420 Lexington Avenue Suite 2335A, New York, NY 10170
tel. (212) 370-0033, X116, fax: (212) 370-4566
Website: www.shodexhplc.com
MALDI Guided SpatialOMx® Uncover Proteomic Profiles in Tumor Subpopulations of Breast Cancer

Janina Oetjen*, Romano Hebeler*, Frédéric Dewez†, Corinna Henkel*, Benjamin Balluff†, and Ron Heeren†

* Bruker Daltonik GmbH, Bremen, Germany
† Maastricht MultiModal Molecular Imaging Institute (M4I), University of Maastricht, Maastricht, The Netherlands

The timsTOF fleX system bridges a current gap by providing MALDI Imaging and in-depth proteomics analysis in just one instrument. The instrument offers all benefits of a timsTOF Pro for time-efficient and sensitive proteomics, combined with a high-resolution MALDI source and stage. Using PASEF technology, it is possible to retrieve high protein ID rates with small sample amounts. Here we present the new SpatialOMx® workflow to identify distinct proteomic profiles for different tumor subpopulations in breast cancer as an example for this powerful approach.

Since tissue and disease are correlated, SpatialOMx provides the unique opportunity to combine regiospecific information from MALDI Imaging with deep proteomic coverage for biomarker discovery and molecular characterization. MALDI imaging has the advantage of providing spatial and molecular information that can be used to detect Region of Interests (ROIs) in tissue sections. As a discovery tool, MALDI Imaging lacks the possibility of in-depth molecular characterization of classical LC-MS/MS-based OMICs methods. Classical OMICs strategies, however, do not retain the spatial information.

Here we present SpatialOMx for microproteomic characterization of tumor subpopulations in breast cancer. In this efficient workflow, unsupervised segmentation of MALDI imaging data with SCiLS Lab is used to define ROIs. Image processing strategies provide boundary information of segments of tumor subpopulations for laser capture microdissection (LMD). Protein extraction and tryptic digestion of small microdissected material is followed by proteomic analysis on the timsTOF fleX using PASEF (Parallel Accumulation Serial Fragmentation). Analysis of proteomics data provides a comprehensive mechanistic understanding of cell type specific biological processes in situ to complement the workflow.

Methods

MALDI Imaging Analysis, Staining, and Clustering
Fresh breast cancer tissue was collected by the Tissue Biobank of the University of Liège, frozen in liquid nitrogen, and stored at -80 °C. The standardized protocol was approved by the Ethics Committee of the University Hospital Center of Liège. Informed consent was obtained from the participant included in this study.

Fresh and frozen breast tumor sections were cut at 12 µm thickness and mounted on PEN (polyethylene naphthalate) membrane slides (Leica Microsystems). These slides are compatible with MALDI imaging and with LMD. After drying, the slides were coated with norharmane matrix (7 mg/mL in CHCl₃:MeOH, 2:1, v:v) using a TM-sprayer (HTX Technologies) for lipid analyses. 12 matrix layers were applied with a 30 s break between each layer at a flow rate of 0.120 mL/min, a velocity of 1200 mm/min and a nozzle temperature of 30 °C. The slides were scanned with a slide scanner (TissueScout, Bruker) to obtain a grayscale reference image with teach marks. Lipid MALDI Imaging analysis was performed on a timsTOF fleX instrument over the entire section with 50 µm spatial resolution in the mass range 300–1600 m/z.

After MALDI imaging analysis, the matrix was removed, and the section was stained with hematoxylin and eosin, scanned with a digital slide scanner for pathological evaluation, and coregistered to the MALDI imaging data using the previously applied teach marks. MALDI imaging data were imported into SCiLS Lab 2019c. Spectra from the pathological annotation of the tumor region were analyzed by unsupervised clustering using the k-means algorithm to define three tumor subpopulation regions of interest (ROIs).

Image Processing of Cluster Results and LMD
As segmentation by k-means provides fine structures that are not processable by LMD, the segmentation image data had to be smoothed. Therefore, segmentation data of the tumor subpopulation areas were imported to MatLab R2018a for

Figure 1: The left image shows the H&E stained section with the tumor area marked in blue by a pathologist. The right image is the tumor area zoomed in after segmentation analysis using k-means clustering, which produced three segments representing tumor subpopulations.
smoothing, removing small objects, filling holes, and detecting boundaries. Hereafter, the different segmentation images were up-scaled to the resolution of the optical image. This up-scaled segmentation information was then transferred to a consecutive un-stained and un-measured section located on a separate PEN slide. This was achieved through the coregistration of both sections using prominent morphological features of the sample. The untouched section was also surrounded by teach marks which are visible in the LMD and can therefore function as reference points for the recalculation of the boundary coordinates to the coordinate system of the LMD. Areas containing approximately 2000 cells were dissected from each segmented tumor subpopulation with a Leica LMD7000 and directly transferred to reaction tubes for microextraction and tryptic digestion.

Sample Preparation for Proteomics

Samples for protein identification by PASEF on the timsTOF fleX were processed as described (2). Briefly, extraction solution was added, and samples were heated at 99 °C for 1 h. The pH was adjusted to 7.4, samples were reduced, alkylated, and digested overnight at 37 °C with trypsin. After digestion, samples were dried and desalted using ZipTip purification.

Proteomics Data Acquisition Using PASEF Technology on the TimsTOF fleX

Microextracted peptide samples were run on a Bruker nanoElute system using a one column setup equipped with an Auro ra 25 cm × 75 µm 1.7 µm C18 column (IonOpticks, Parkville, Australia). 0.1% formic acid and 2% acetonitrile in water were used as eluent A while 0.1% formic acid in acetonitrile was used as eluent B with a 400 nL/min gradient at 50 °C oven temperature. The Bruker CaptiveSpray nano source was used as an inlet into the timsTOF fleX system using 3.0 L/min dry gas at 180 °C and 1.6 kV capillary voltage. Data were acquired in the mass range of 100–1700 m/z and the mobility range was set to 0.6–1.6 1/k0. For MS/MS fragment spectra acquisition, 10 PASEF scans at 100 ms were run, from which each contains around 12 MS/MS events resulting in a greater than 100 Hz acquisition rate.

Analysis of Proteomics Data

PEAKS X (Bioinformatics Solutions Inc, Waterloo, Canada) was used for protein identification. Database searches were carried out against a SwissProt human database using a parent mass error tolerance of 15 ppm and a fragment mass tolerance of 50 mDa. Trypsin was used as proteolytic enzyme and maximally two missed cleavages were allowed. Carbamidomethylation was set as fixed modification, acetylation at the protein N-terminus, deamination (NQ), and oxidation (M) were allowed as variable modifications. For biological process characterization of the different tumor subpopulations, the gene ontology tool PANTHER V.13.1 was used.

Results

MALDI Imaging Defines Tumor Subpopulation ROIs

Lipid imaging on the timsTOF fleX from tumor sections was used to define ROIs by segmenting intratumor heterogeneity for laser microdissection (LMD) based microproteomics. The workflow is supported by the compatibility of PEN (polyethylene naphthalate) membrane slides with MALDI Imaging on the timsTOF fleX. These slides also support histological staining and are compatible with LMD. Lipid MALDI Imaging has the advantage that the same tissue section can be used for imaging, staining and LMD based microproteomics. Moreover, discernible lipid profiles are the basis for segmenting intratumor heterogeneity.

The breast tumor section was stained with hematoxylin and eosin (H&E) after MALDI Imaging data acquisition and tumor areas were annotated by a pathologist. MALDI Imaging data with 50 µm spatial resolution were imported into SCiLS Lab 2019c and the annotated tumor areas analyzed by unsuper-

Figure 2: Image processing pipeline to produce ROI boundary information as x,y-coordinates for laser capture microdissection from segmentation raw data. The workflow includes smoothing, removing of small objects, filling holes, detecting boundaries, and upscaling to the optical image.
vised clustering using the SCiLS Lab segmentation algorithm. k-means segmentation highlighted three subpopulations in the annotated tumor region (Figure 1).

ROI Boundary Coordinates are the Basis for Precise LMD
Fine structure segmentation ROIs require image processing to allow viability of LMD. Currently, the segmentation image raw data contains small objects or edged segment boundaries that prevent direct usage of the coordinates for LMD. After image processing, boundaries of the tumor subpopulation areas were transferred to a coregistered optical image of a consecutive section and referenced to teach marks that were scanned together with the section (Figure 2). This allows coregistration of the MALDI Imaging segment boundaries to the LMD for precise excision of the defined areas.

Proteomics of Microextracted MALDI Imaging Segments by PASEF Technology Implemented in timsTOF fleX
The timsTOF fleX system supports PASEF (Parallel Accumulation Serial Fragmentation) technology for time efficient and sensitive TIMS device which separates incoming ion packages from one scan according to their ion mobility and elutes these ions in a time focused manner. While ions from the continuous ESI process are separated and eluted in the second TIMS device, ions are accumulated in parallel in the first part of the dual TIMS cartridge allowing for a 100% duty cycle. Alternating precursor and fragment ion scans complete assignment of fragment ion spectra to their respective precursor spectra. PASEF allows for scan rates of more than 100 Hz without losing sensitivity or resolution and is the optimal system for small sample amounts and therefore for SpatialOMx.

Tryptic digests of the extracted proteins from the excised MALDI Imaging tumor subpopulation areas were run on the timsTOF fleX using PASEF. Number of protein IDs per tumor subpopulation segment (a) and biological process characterization per segment as revealed by PANTHER (b).

Proteomics of Microextracted MALDI Imaging Segments by PASEF Technology Implemented in timsTOF fleX

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Figure 3: Proteomics of tumor subpopulations and biological process characterization. Proteins from microdissected tissue (approx. 160 ng) were extracted, digested with trypsin and peptide extracts were run on the timsTOF fleX using PASEF. Number of protein IDs per tumor subpopulation segment (a) and biological process characterization per segment as revealed by PANTHER (b).

In summary, SpatialOMx provides guidance to specific regions of interest for microextraction and in-depth proteomics analysis to characterize regiospecific molecular changes in detail.

References
Thyroid hormones play critical roles in the regulation of biological processes, including growth, metabolism, protein synthesis, and brain development. Specifically, both 3,3',5,5'-tetraiodo-L-thyronine (thyroxine or T4) and 3,3',5-triiodo-L-thyronine (T3) are essential for development and maintenance of normal physiological functions. For a clinical laboratory, measurements of total T4 and total T3, along with estimates of free T4 (FT4) and free T3 (FT3), are important for the diagnosis and monitoring of thyroid diseases. Most clinical laboratories measure thyroid hormones using immunoassays, which offer a relatively rapid, high patient sample throughput that lends itself to automation, but are compromised by problems with assay interference and by changes in protein levels that alter free hormone availability (1).

Liquid chromatography–mass spectrometry (LC–MS) can offer superior specificity and speed over immunoassays for determination of thyroid hormones in biological matrices such as serum and tissues. The present work demonstrates successful online solid-phase extraction (SPE) with LC–MS for rapid determination of T4, T3, and 3,3',5'-triiodo-L-thyronine (rT3) from biological matrices.

**Experimental**

**Materials:** Supel™ Genie RP-Amide (RPA) and C8 (results not shown) online SPE cartridges (2 cm × 4.0 mm i.d.); human serum; and protein precipitation solvent: methanol with 1% (w/v) ammonium formate.

**Sample Processing:** Human serum spiked with analytes was protein precipitated by vortex mixing with the precipitation solvent at a 1:3 ratio. The mixture was then centrifuged at 10,000 × g for 3 min, and the supernatant was collected and directly injected for online SPE and LC–MS analysis.

**Online SPE–LC–MS Setup:** Six-port switching valve and two pumps; one for sample loading–washing, the other for sample elution. To minimize potential peak broadening from the cartridges, the flow of sample loading–washing and the subsequent elution are in reversed directions.

**Results and Discussion**

Conventional SPE typically involves multiple labor-intensive and time-consuming steps: conditioning, sample loading, washing, elution, evaporation, and reconstitution. Supel Genie online cartridges were developed to automate the sample preparation process, minimize hands-on time and human error, and reduce overall sample processing time. We used RPA online cartridges with LC–MS analysis for the detection of thyroid hormones from human serum. The samples were protein precipitated with methanol containing ammonium formate and after centrifugation then directly injected for online SPE and LC–MS analysis. Sample loading–washing was performed entirely by the instrument, eliminating the time-consuming solvent evaporation and reconstitution steps.

The RPA cartridges captured a trace amount (100 ng/mL × 2 µL in this case) of thyroid hormones from human serum (Figure 1). All three analytes were well resolved with a peak width at half height <6 s and tailing factor from 1.5–1.8. The total run time was within 6 min.

An online SPE–LC–MS method can be used for rapid detection of thyroid hormones in human serum with minimal hands-on effort and time-consuming steps.

**Reference**

Analysis of Chloroquine, Hydroxychloroquine and Desethylchloroquine in Urine Using SPE and LC–MS/MS

Abder Abdelkaoui, UCT, LLC

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

Extraction/Analytical Materials

<table>
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<td>Selectra® DA HPLC Column 100 × 2.1 mm, 3 µm</td>
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**Procedure**

**Step #1: Sample Prep**
To 1 mL of urine, add 1 mL of pH 6 phosphate buffer (0.1M) and internal standards. Then, mix or vortex briefly. Keep in mind that a hydrolysis protocol may be required if conjugated compounds are to be included into the above drug panel.

**Step #2: Condition Cartridge**
For the condition cartridge, add 1 × 1 mL MeOH and 1 × 1 mL DI H₂O.

**Step #3: Apply Sample**
The next step is to load the sample at 1–2 mL/min.

**Step #4: Wash Cartridge**
For the wash cartridges, add 1 × 1 mL PH 6 phosphate buffer (0.1M) and 1 × 1 mL MeOH to each. After completing this step, dry the cartridges under full vacuum or pressure for two min.

**Step #5: Elute Analytes**
For the elute analytes, add 1 × 2 mL MeOH: NH₄OH at a ratio of 98:2. Then, collect at 1–2 mL/min.

**Step #6: Dry Eluate**
To dry the elute, set the temperature to less than 40 °C and evaporate to dryness.

**Step #7: Reconstitute**
Once the elute is dried, reconstitute sample in 100 µL of mobile phase or other appropriate organic solvent.

**Instrumental**
This procedure uses the following instruments:
1) LC–MS/MS: Shimadzu LCMS-8050
2) Column: UCT Selectra® DA HPLC Column 100 × 2.1 mm, 3 µm
3) Guard Column: UCT Selectra® DA Guard Column 10 × 2.1 mm, 3 µm
4) Injection Volume: 5 µL
5) Mobile Phase A: D.I. H₂O + 0.1% Formic Acid
6) Mobile Phase B: MeOH + 0.1% Formic Acid
7) Column Flow rate: 0.40 mL/min

**Results**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Recovery (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 ng/mL</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>95%</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>94%</td>
</tr>
<tr>
<td>Desethylchloroquine</td>
<td>80%</td>
</tr>
</tbody>
</table>

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

Wyatt Technology Corp.

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

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

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

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

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

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¹Analytical Development, Biogen, Cambridge, Massachusetts ²Postnova Analytics Inc, Salt Lake City, Utah

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

Viruses are increasingly used as gene therapy delivery vehicles due to their versatility and safety. They can be loaded with DNA or RNA and delivered to a specific location in the body to treat or cure a disease (1). One of the biggest challenges for manufacturing a homogeneous virus sample is the presence of viral aggregates, which negatively affect transduction efficiency, biodistribution, and immunogenicity (2). Due to their relatively large size, often over 50 nm in diameter, virus aggregates are challenging to separate and characterize by column-based chromatography techniques such as size-exclusion chromatography (SEC). In this application note, we present data on separation of a virus mixture using Asymmetrical Flow Field-Flow Fractionation (AF4) and measurement of their radius of gyration (R₉).

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

**Experimental Details and Results**

A virus mixture sample was created by combining smaller adeno-associated viruses (AAV) with larger adenovirus type 5 (Ad5) in solution to simulate a sample with virus monomer and aggregates. To separate the viruses by size, an AF4 (Postnova AF2000) was used, coupled to a Postnova 21-angle multi-angle light scattering (MALS, PN3621) detector for measuring the R₉. Both the AAV-only and virus mixture samples were analyzed by AF4-MALS to highlight the differences between the samples. The carrier solution was phosphate buffered saline (PBS). The AF4 membrane used was 10 kDa regenerated cellulose.

The MALS response for the AAV-only sample is shown in Figure 2. The main peak corresponds to the AAV monomer eluting between 17 and 20 min. The R₉ measured for the monomer is ~ 12.5 nm, very consistent with an expected diameter of 25 nm. There is a small shoulder to the right of...
the monomer peak, and the increasing $R_g$ for this shoulder peak indicates the presence of a small amount of dimer/trimer/small aggregates in the sample. Fully separating the monomer from any aggregate species was beyond the scope of this work. Further method optimization is required to achieve this goal.

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

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

References
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