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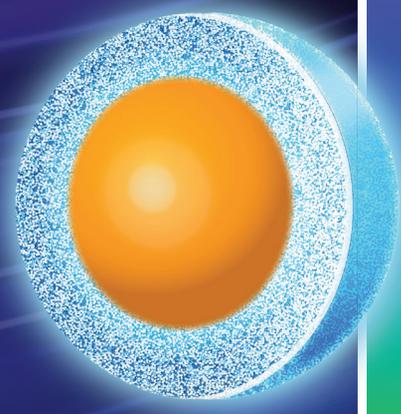
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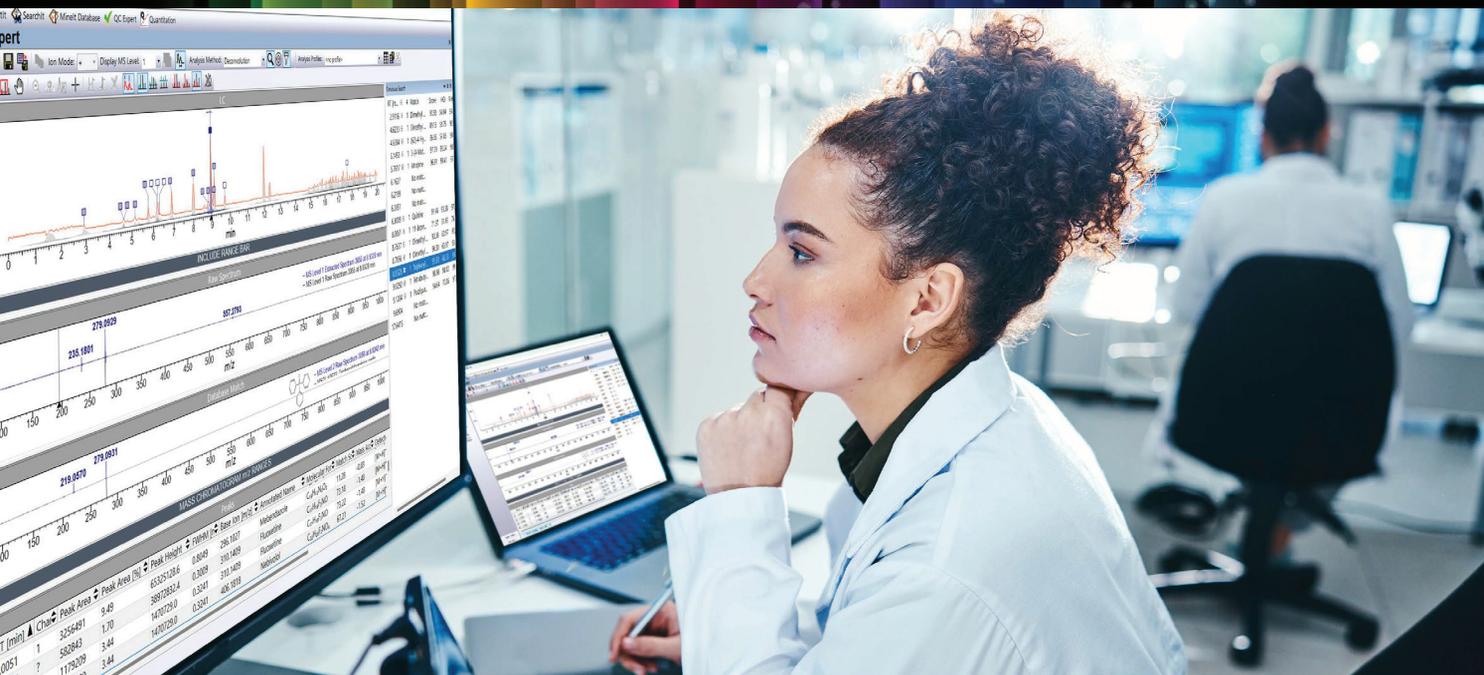
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NOTE FROM THE CEO

A **WE START TO SEE** the first signs of spring and the temperatures beginning to warm up, the conference season is starting to heat up as well. As this March issue of *LCGC International* goes to press, members of our editorial and sales teams kicked off the conference season at Pittcon 2025, which was held from March 1–5, 2025 in Boston, Massachusetts. Conference coverage is a core part of what we do, so keep up to date with the latest developments at conferences by social media platforms, signing up for our newsletters, and www.chromatographyonline.com

The “matrix effect” is a common challenge that analysts face when using liquid chromatography for quantitative analysis, even though the concept itself is relatively simple. In this month’s “LC Troubleshooting” column, Dwight Stoll breaks down what “matrix effects” actually mean and explores some of the possible origins of these effects in practice. He also outlines effective strategies for reducing these effects to improve accuracy and precision, especially when working with complex sample matrices. Understanding the impact of matrix effects and knowing how to mitigate them is crucial not only for developing new methods that prevent future issues, but also for troubleshooting quantitation challenges in existing methods.

In the third installment of the “GC Connections” series on optimizing splitless injections in gas chromatography, Nick Snow offers valuable tips for setting up the inlet and making the necessary connections for optimal injections. He covers checking key components such as the septum nut, glass liner, and column connections, as well as optimizing column installation and gas flow settings to achieve the best results. The article emphasizes that some general principles can help to obtain the best inlet setup for successful, reliable, and reproducible splitless injection methods.

This month’s “Biopharmaceutical Perspectives” column explores hydrophilic interaction liquid chromatography (HILIC) as a promising alternative to traditional ion-pair reversed-phase liquid chromatography (IP-RPLC) for separating oligonucleotides (ON). The article delves into the use of HILIC for separating ON sequence and length variants, duplexes, and single-stranded components. The authors investigate key method variables such as ionic strength, temperature, mobile phase composition, and gradient optimization. Additionally, a sequential injection procedure is proposed to help eliminate breakthrough effects and peak splitting.

The March edition of “The Application Notebook” showcases how leading technology companies can help chromatographers achieve the results they want using modern technologies in practice. Applications this month include “Identification and Quantification of Microplastic Polymers in Food Packaging Using Micro-Furnace Pyrolysis-GC-MS”; “Comparison of Organic Compounds in Natural Wine, Red Wine and Grape Juice by HPLC”; “Comprehensive Metabolite Profiling with HILIC-LC-MS”; and “Implementation of EPA 8270E Using Solid-Phase Extraction and Hydrogen as Carrier Gas for GC-MS Analysis.”

If you have any interesting suggestions for topics you would like us to cover in 2025 on our multimedia platform, please reach out to associate editorial director Caroline Hroncich (chroncich@mjlhifesciences.com). Happy reading! ■

Mike Hennessy, Jr.

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Matrix Effects on Quantitation in Liquid Chromatography: Sources and Solutions

Dwight R. Stoll

The “matrix effect” is one of the sticky details that can make the use of liquid chromatography (LC) for quantitative purposes challenging, even though it is conceptually quite straightforward. In this installment, I review what we are referring to when we talk about “matrix effects,” and discuss some of the potential origins of the phenomenon in practice. I then go on to describe some of the commonly-used approaches to mitigate these effects, providing a path to accurate and precise results, even when working with highly complex sample matrices. As with many troubleshooting topics, developing a solid understanding of matrix effects on quantitation and potential strategies for mitigation can be helpful, both during the development of new methods to prevent future problems, and troubleshooting problems with quantitation observed with existing methods.

A **S PART OF** my preparation for these installments, I generally like to review what has been written about a topic in the past. It is often interesting to see how our approaches to specific aspects of liquid chromatography (LC) troubleshooting evolve over time. In some cases, our approaches change dramatically, frequently in response to new understanding of phenomena or new technology being introduced into the marketplace. In other cases, our approaches don't change much, usually because there are long-standing causes of problems in the practice of LC for which there simply are not good, foolproof solutions yet. Part of my reading in preparation for this installment included one of John Dolan's early articles from 1984, which was simply titled “Autosamplers.” I found it strangely comforting to read John's description of problems related to the “electronics” and “sensors” found in autosamplers, realizing that I too spent a lot of time early in my career troubleshooting problems of this type. Fortunately, I would say that this category of problem has largely been addressed—at least with

modern samplers manufactured in the last decade. I simply do not experience many problems with the electronics or sensors in these modules like we used to. That doesn't mean they never fail—they are sophisticated instruments, after all—we simply spend less time troubleshooting this type of problem than we did in the past.

On the other hand, there are some other sample-related topics Dolan addressed in 1984 that are still generally important, but also relevant to the focus of this installment on matrix effects. Thus, before diving into the central topic, I think it is worth taking some time to review these long-standing ideas:

- **Labeling:** In 1984, Dolan wrote that one of the biggest sampler-related problems was mislabeled vials. This is not the sampler's fault, of course, but it is certainly true that properly labeling vials and telling the chromatography data system which vials are in which locations is a very important (and sometimes overlooked) part of the analytical process. It is striking that, 40 years later, we are still writing on vials by hand, but perhaps this is one area where artificial intelligence (AI) can help us soon.

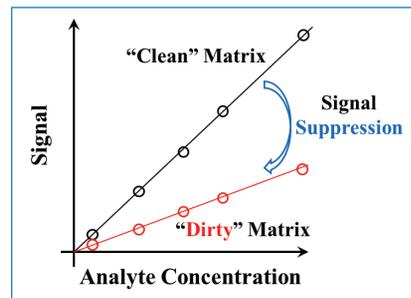


FIGURE 1: Comparison of calibration curves obtained using different sample matrices.

- **Freeze-thaw:** Subjecting samples to a freeze-thaw cycle process prior to analysis has the potential to cause a number of problems. For example, proteins may aggregate, leading to results that are very different from those that would be obtained if the sample were never frozen. Also, organic or aqueous mixtures may phase-separate, leading to very heterogeneous results depending on which part of the solution is sampled, if the sample is not mixed thoroughly after thawing.

- **Evaporation:** Analytes or sample solvent components may evaporate if vials are not capped tightly, or following an initial puncture of the septum if the septum is not

replaced. Obviously, direct evaporation of the analyte will affect quantitative results, but evaporation of the solvent can also cause problems, either directly through a reduction in the volume of sample that contains the analyte, or indirectly by affecting the solubility of the analyte in the sample solvent.

This list of reminders is good for preventing problems before they occur, but it also offers a good short list of possible explanations to consider when sample-related problems do occur.

Matrix Effects: A Definition for Our Purposes Here

The conventional definition of the sample matrix is that it is the portion of the sample that is not the analyte—that is, most of the sample. We talk about the matrix potentially affecting different parts of an LC method, including effects on extraction efficiency and recovery in cases where a sample preparation step precedes the LC separation, apparent retention time, peak shape, and apparent quantity of the analyte (typically measured by peak area). In this installment, I am focusing on potential effects of the matrix on quantitation, so it is helpful here to be precise about what “matrix” is relevant to the part of the LC method where the analyte is actually detected. This certainly includes components of the sample matrix that have retention properties similar to the analyte, and are thus coeluted from the LC column and enter the detector along with the analyte of interest. However, from the point of view of the detection step, the matrix that the analyte is detected in very importantly contains the mobile phase components, including any impurities that might be present in addition to the intended ones (for example, ammonium and acetate ions, water, and acetonitrile). So, for the purposes of discussion here, the “matrix” includes both components of the sample other than the analyte, and the mobile phase components.

The Fundamental Problem with the Matrix Effect on Quantitation

The fundamental problem we have to face is that the matrix the analyte is detected in can either enhance or suppress the detector response to the presence of the analyte. An ideal detection principle would be one in which the matrix components have no effect whatsoever on the detector response to the analyte. This ideal situation rarely occurs in

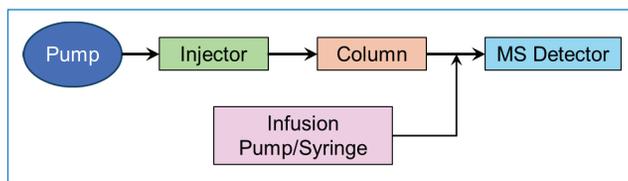
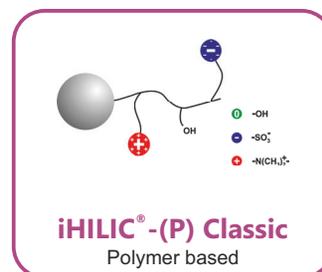
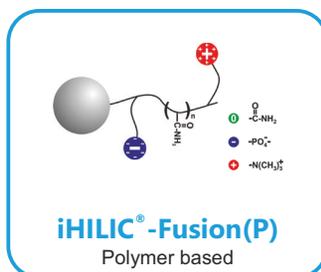


FIGURE 2: Post-column infusion setup. A dilute solution of the analyte of interest is continuously infused into the effluent stream between the column outlet and the detector inlet.



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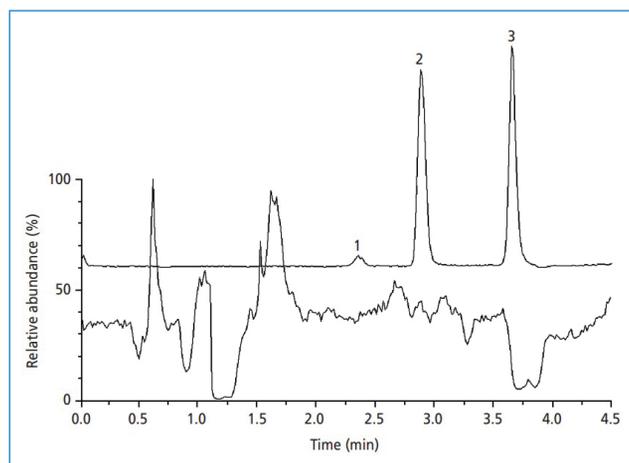


FIGURE 3: Results from infusion of target compound while sample matrix components are separated using the LC method conditions. Peaks 1–3 show the locations of peaks of interest injected from a clean sample. Peak #3 in particular elutes in a region where there is suppression of the signal for the target compound by sample matrix components. Reprinted with permission from reference (3).

practice; however, some detection principles are less prone to matrix effects than others, to the point where the effect may be too small to measure in the presence of typical measurement noise. In these cases, we could say that there is no measurable matrix effect. Some examples of phenomena that lead to detector response enhancement or suppression are:

- **Fluorescence Quenching (Fluorescence Detection):** Matrix components can affect the quantum yield of the fluorescence process for the analyte, leading to suppression of the signal observed with fluorescence detection. Readers interested in learning about fluorescence as a detection principle for LC in general, and the quenching phenomenon specifically, are referred to reference (1).
- **Solvatochromism (Ultraviolet/Visible [UV/Vis] Absorbance Detection):** The absorptivity of analytes can be affected by mobile phase solvents, leading to increases or decreases in the observed absorption of UV/vis light for a given analyte concentration. Readers interested in learning more about the phenomenon of solvatochromism are referred to a recent review article on the topic (2).
- **Ionization Suppression/Enhancement (Mass Spectrometric [MS] Detection):** Most well-known in the case of electrospray ionization, analytes compete with matrix components for available charge during the desolvation process, leading to enhanced or suppressed ionization of the analyte, depending on other constituents of the matrix present in electrospray droplets (3,4).
- **Effects on Aerosol Formation (Evaporative Light Scattering [ELSD] and Charged Aerosol Detection [CAD]):** Both ELSD and CAD involve evaporating volatile components of the mobile phase and the formation of aerosol particles that are detected either by the degree of light scattering or particle charging from a corona discharge. Mobile phase additives (for example, buffering agents) can influence the aerosol formation process, resulting in significant enhancement or suppression of the detector response to specific analytes (5).

These are only very brief descriptions of the most common phenomena. Readers interested in learning more about the details associated with these phenomena should refer to the references cited for more information.

The First Step Toward a Solution: Knowing You Have a Problem

Generally speaking, a simple comparison of detector responses under different conditions is an effective way to determine whether or not we have a matrix effect to deal with. For example, we might compare the detector response to our analyte when using different mobile phase additives, or when the sample is prepared using water as a diluent compared to when phosphate-buffered saline is used as the diluent. In this type of comparison, we are interested in knowing if the slope of the calibration curve we observe depends on these changes in conditions, as shown in Figure 1. If the slopes are different, this tells us the matrix is affecting the detector response, and we should be careful to either systematically study the matrix effect so that we can properly control it over time, or implement one of the solutions described below that are designed to mitigate matrix effects on quantitation.

A second approach, which is most commonly used to assess sample-dependent matrix effects when using mass spectrometry (MS) detection, is to add a dilute solution of the analyte of interest to the effluent stream by infusion between the column outlet and the MS inlet. A simple schematic of the setup for this is shown in Figure 2. Figure 3 shows an example of the type of information this approach yields, where we see regions of the chromatogram in which the signal for the analyte of interest is clearly suppressed. These regions correspond to zones of elution of sample matrix compounds. An ideal outcome from this experiment would be one where the analyte signal is constant across the entire chromatogram, indicating no significant suppression or enhancement due to matrix components. Here, we see examples where this is not the case, and we should be mindful of strategies that can be used to mitigate the matrix effect going forward with method development.

Commonly Used Approaches Designed to Mitigate Matrix Effects

There are several approaches that can be used to mitigate matrix effects on quantitation, each with their own advantages and disadvantages. In the following sections, I briefly describe the concepts behind the most commonly used approaches, and provide references for those interested in learning about any of them in more detail.

Internal Standard Method of Quantitation

The internal standard method of quantitation is a very potent way to mitigate matrix effects on quantitation. Unfortunately, it is not always practical, but when it can be used, it is one of the most effective tools we have for maintaining good accuracy of quantitation when working with complex sample matrices. The basic concept is as follows: a known amount of an internal standard compound is added to every sample. For example, if our target analyte is toluene, we might choose ¹³C-labelled toluene as our internal standard, because it will behave



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very similarly to toluene, yet it can be detected separately from toluene via MS (with a 6-Dalton mass shift if every carbon in the ring is labelled). Then, rather than making a calibration curve for quantitation by plotting the detector signal (for example, peak area) versus analyte concentration, two ratios are plotted. On the *y*-axis, we use the ratio of the signal for the target analyte to the signal for the internal standard, and on the *x*-axis, we use the ratio of the target analyte concentration to the internal standard concentration. An example that is often used to illustrate the utility of this approach is a situation where we encounter highly variable sample injection volume, such as with manual injections of small volumes by inexperienced users into a gas chromatograph. When we use a traditional calibration approach using external standards (that is, standards containing only the analyte of interest) an error of 10% in the volume of sample injected (for example, injecting 0.9 μL instead of 1.0 μL) leads to terrible precision and accuracy. In this case, we have no way of knowing that the injection volume was not correct, and thus, we have no way of recovering from the error. However, if we use an internal standard and spike every sample with the same amount of internal standard, this gives us an easy way to observe when something is not right—in this case, measured by highly variable, or outlying, values for the signal due to the internal standard. Moreover, because we ultimately base the quantitation of our target analyte on the ratios of signals and concentrations for the target analyte and internal standard, the approach is self-correcting. If we only inject 0.9 μL instead of 1.0 μL , the signals for both the target analyte and internal standard will be lower than expected, but the ratio will be unaffected. In other words, this type of error is completely cancelled out.

In the case of the incorrect injection volume, it is intuitively obvious that this error should affect both the target analyte and the internal standard in the same way. In the case of sample matrix effects on quantitation, the extent to which the internal standard approach will help depends largely on the extent to which the sample matrix affects the detector response to the target analyte and internal standard to the same degree. Consider Figure 4, which illustrates the hypothetical effects of the matrices of different samples (S1–S3) on the detector response for the analyte (X) and internal standard (IS). Here we assume that X and IS are spiked into the samples at the same levels in each case, and that the samples did not contain either compound before spiking. Here, we would expect the detector response to be the same for the analyte in each sample, but clearly it is not, because due to the analyte, the signal has been suppressed in Samples 1 and 3 relative to Sample 2. However, we see that the signal due to the internal standard has been affected in the same way, and thus, when we instead plot the ratio of signals for all three samples, they are the same, as expected.

The major potential advantage of the internal standard approach is that it can be highly effective, particularly when using stable isotope-labelled internal standards and MS detection, even when working at trace levels, and with complex sample matrices. Among the most significant potential disadvantages are the fact that isotope-labelled standards are not commercially available for many compounds, and when they are, they are often quite expensive.

Once one considers implementing the internal standard approach, we are immediately confronted with a number of practical questions.

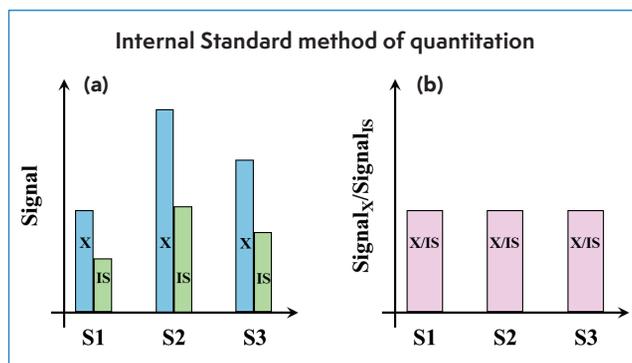


FIGURE 4: Illustration of the benefit of the internal standard method for mitigating matrix effects. Hypothetical results for three samples (S1–S3) all spiked with the same levels of analyte (X) and internal standard (IS). (a) In this example, sample S1 exhibits the strongest matrix effect, suppressing the signal relative to that observed for samples S2 and S3. (b) However, using the ratio of the signal for the analyte relative to the signal for the internal standard mitigates the variation in signal due to the matrix effect, improving both the precision and accuracy of quantitation.

Exactly what compound should I use for the internal standard? Is the best candidate molecule commercially available at a reasonable cost? Should the internal standard be separated chromatographically from the target analyte, or not? Are there other compounds in the sample matrix that might interfere with my ability to accurately determine the detector response to the internal standard? These questions, while very important, are too numerous to answer here in any detail. Readers interested in learning more about the internal standard approach are referred to a previous installment of “LC Troubleshooting” by Dolan focused on this topic (6).

Standard Addition Method of Quantitation

The standard addition method of quantitation can also be very effective for overcoming sample matrix effects on quantitation, but its advantages and disadvantages are quite different from those of the “internal standard” approach. The concept is a bit simpler in this case. Briefly, a sample of interest is divided into multiple portions, and varying but known amounts of the target analyte are deliberately added to each portion prior to analysis (one portion remains unspiked). Then, the detector signal due to the analyte (again, typically peak area) is plotted versus the mass of analyte added to the sample portions. The amount of analyte originally present in the unspiked sample is then estimated by simply dividing the *y*-intercept of this plot by the slope; the resulting value is the *x*-intercept of the calibration line. One way of thinking about the meaning of the *x*-intercept here is that it is the amount of analyte that would have to be removed from the unspiked sample to bring the detector response to zero. Figure 5 illustrates the powerful simplicity of this approach. If the sample matrix affects the detector response to the target analyte, the slopes of the calibration lines for samples with different degrees of matrix effects will be different, even if the unspiked samples contain exactly the same amount of target analyte. However, these lines will all intersect at the same *x*-intercept. In other words, we will always get the same analyte

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concentration estimate if the actual concentration is the same, no matter how large or small the sample matrix effect is. This is the good news about this approach. The major downside is that splitting a single sample into multiple portions and analyzing each of them increases the amount of instrument time dedicated to each sample by approximately a factor of five. Readers interested in learning more about the practical details associated with this approach are referred to reference (7).

Separating the Analyte of Interest from Interfering Matrix Components

As discussed above, many sample-related matrix effects on quantitation are fundamentally related to the influence of matrix molecules on the detector response of our target analytes. Therefore, anything we can do to reduce the occurrence of interfering molecules entering the detector at the same time as our analytes should reduce the matrix effect. There are a number of strategies to consider here as well. First, selective sample pretreatment prior to the actual LC analysis can be very effective in this regard. Methods such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE) are commonly used prior to LC analysis for this reason. Readers interested in the benefits and practical application of these and other related approaches are referred to Doug Raynie's excellent "Sample Prep Perspectives" articles in this magazine (for example, see the overview of different techniques in reference [8]).

Second, the selectivity and elution conditions of the LC separation can be adjusted to move peaks containing analytes of interest away from regions of the chromatogram that are known to contain interfering compounds. Figure 3 shows a good example of this; peak #3 is partially eluted in a region where sample matrix components severely suppress the detector response for the analytes of interest. In his article discussing this and related results, Dolan described the influence of several parameter changes (for example, changing gradient slope or column chemistry) on the location of peak #3 relative to the suppressing region. Ultimately, he concluded that simply changing the gradient delay volume provided the selectivity change needed for this particular application (3).

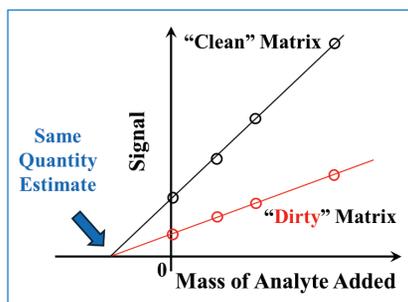


FIGURE 5: Illustration of the benefit of the standard addition method for mitigating matrix effects.

Finally, multidimensional LC is a powerful way to very selectively separate sample matrix components that might influence the detector response to analytes of interest. In this approach, the sample is injected into a first column where some separation occurs, but when dealing with very complex sample matrices, some degree of peak overlap between analytes of interest and matrix components is inevitable. To address this, a small portion of the effluent from the first column containing the analyte of interest is transferred to a second dimension column for further separation. The parameters of this second separation can then be tuned to achieve additional separation of the target analyte from matrix components. Readers interested in learning more about the use of multi-dimensional separations to reduce sample-related matrix effects are referred to references (9–11).

Matrix Compensation (ELSD and CAD)

As discussed above, the composition of the mobile phase used with ELSD and CAD detection can affect the detector response. This can be challenging in the context of gradient elution separations, where the mobile phase composition is changing throughout the analysis, and thus, the detector response may change, even for the same analyte, depending upon where the compound is eluted during the gradient elution program. One approach to mitigate this problem is to use a setup like that shown in Figure 2, but infuse a "counter-gradient" between the column and detector, such that the composition of the effluent arriving at the detector is nominally constant throughout the analysis. For example, if, in a reversed-phase separation,

the concentration of organic solvent in the mobile phase is increased as the analysis proceeds, we would program an infusion pump to deliver a solvent gradient that increases in the concentration of water as the analysis proceeds. Readers interested in learning more about this approach are referred to reference (5), which discusses the topic in more detail.

Summary

In this installment, I have discussed matrix effects that can influence the quantitative performance of LC methods. Although matrix effects are often associated with sample matrices, a broader definition of matrix is warranted here that considers mobile phase components that enter the detector at the same time as our analytes of interest. Once in the detector, matrix components can influence the detector response to an analyte, either enhancing or suppressing the response. This influence can in turn affect both the accuracy and precision of quantitation. Thus, it is important to: 1) recognize when matrix effects are affecting the detector response; and 2) employ strategies to mitigate their effects on quantitation. I have briefly discussed approaches to address both points, including the use of post-column infusion to assess the magnitude of matrix effects, and the internal standard and standard addition approaches to quantitation, which are designed to overcome matrix effects. Developing a solid understanding of matrix effects on quantitation and potential mitigation strategies can be helpful both during development of new methods, to prevent future problems, and troubleshooting problems with quantitation observed with existing methods. ■

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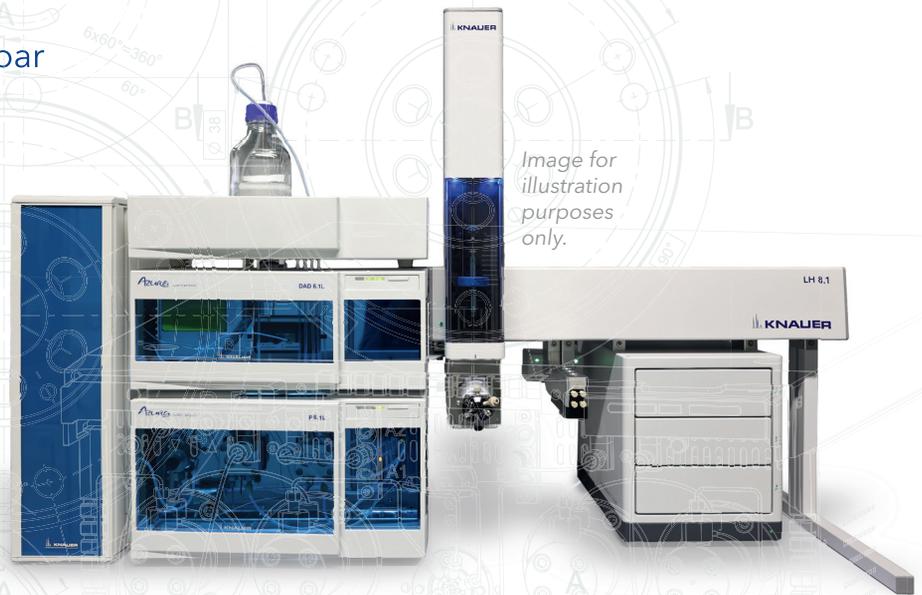


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Optimizing Splitless Injections in Gas Chromatography, Part III: Setting Up and Optimizing the Inlet

Nicholas H. Snow

In the first two parts of this series, we explored the rationales by which splitless injection is most commonly used for trace quantitative analysis, and the band broadening and focusing mechanisms that impact our ability to effectively perform splitless injections. In this installment, we will discuss techniques for setting up the inlet and connections for optimized injections. We will examine the connections, including septum nut, glass liner, and column connections, along with column installation and gas flow setting. While this review is not comprehensive, we see that some general principles can guide inlet setup efforts and ensure successful, rugged, and reproducible splitless injection-based methods.

MOST PROBLEMS IN gas chromatography (GC) are caused at or before the sample injection. When the inlet is set up correctly, GC is among the most rugged and reproducible instrumental techniques. When properly installed, set up, and maintained, columns and detectors generally perform properly for hundreds or thousands of injections. The column will separate, and the detector will detect whatever reaches them. The main challenge, as we have seen in parts I and II of this series on splitless injection, is getting the analytes to the column in sharp, reproducible, and quantitative bands (1,2).

Make Sure that Your Inlet is Clean

Cleanliness is paramount when performing capillary gas chromatography. With sensitive detectors that can easily detect nanograms or less material, even small amounts of contaminants can lead to ghost peaks or an elevated baseline. As you perform each of the setups and optimizations discussed below, keep cleanliness in mind and avoid

introducing contaminants, including fingerprints, into the inlet.

Make Sure Your Inlet is Properly Set Up and Maintained

There are several considerations for ensuring that the inlet and injection are properly set up. These include the syringe and autosampler, the septum, the glass liner, column installation, setting gas flows, inlet temperature and pressure initial column temperature, and split purge valve timing. Besides setting the various temperature and pressure set points, each of these requires continuous attention and maintenance.

Use a Properly Maintained Autosampler and Syringe

Nearly all gas chromatography today is performed using an autosampler. Prior to each sample batch, visually inspect the syringe for evidence of leaks or damage, such as a slightly bent needle, encrustation, or resistance in the travel of the plunger. If you have any questions about the syringe, replace it. It is a good idea to have spare syringes on hand. Today's autosamplers generally do not require maintenance, except for periodic

maintenance performed by the vendor and syringe replacement.

Choose the Correct Glass Liner

Glass liner selection is possibly the most confusing part of gas chromatographic method development because there is no single glass liner that will serve all analytes and samples. Testing several glass liners should be part of any method development process. When trying different glass liners, all other instrumental conditions should remain constant. Several manufacturers of glass liners have provided selection guidelines and they all recommend the following considerations; each makes a recommendation for a specific glass liner to begin method development. Remember that there is no "killer app" with glass liners; they must be tested experimentally with your own samples (3,4).

Sample concentration is usually the first consideration; for splitless injection, we can assume relatively low sample concentration in the low part per million or part per billion range. A single taper liner with (for dirty samples) or without (for cleaner samples) wool is a good place to start. For solvents with higher vapor volume, such as polar

solvents like ethanol, a double taper liner can help prevent backflash of excess solvent vapor into gas lines and other tubing. If the analytes are polar or reactive, a deactivated liner should be used. These quick recommendations provide a start; again, glass liners should be tested and tried.

For splitless injections, the glass liner should be inert, deactivated by the vendor. In the past, it was common for laboratories to purchase less expensive non-deactivated liners and deactivate them on their own. I recommend against using such “home brew” methods for liner deactivation; use the deactivated liners provided by the vendors. Vendors offer several levels of deactivation, ranging from none (least expensive) to highly inert materials (most expensive). The glass liner should be checked often for contamination. If obviously contaminated, it should be replaced. Figure 1 shows an image of a contaminated glass liner. Note the discoloration near the area where the

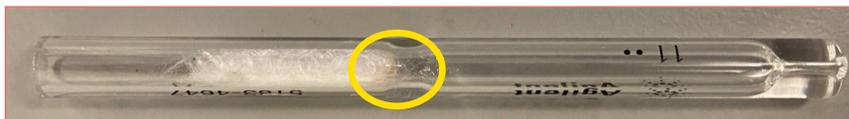


FIGURE 1: Photograph of a contaminated glass liner. Note the contamination on the glass wool (circled); this glass liner should be replaced.

syringe has penetrated and deposited sample material.

Properly Cut and Install the Column

An often overlooked, but critical, aspect of inlet performance is properly cutting and installing the column. The inlet-column connection is often blind, unwieldy, and requires practice to perform correctly. One of the most important practical characteristics of working with capillary columns is that the columns themselves are easily trimmed. Column vendors offer videos demonstrating inserting columns into ferrules and connectors and trimming them (5). Trimming columns requires training; a good way to do this is to use an

old column for practice, and to inspect your practice column cuts with a magnifying glass or your phone camera, zoomed.

Figure 2 shows the results of some column cuts and handling, both good and bad. The up-close images were obtained using a cell phone camera on maximum zoom, with the phone held steady using a ring stand and clamp. Figure 2a shows a properly cut column end. Note that it is square, and there is no evidence of cracked glass or degradation of the polyimide coating. As seen in the figure, care should be taken with the scoring tool to not cut all the way through the tubing; just make a score and then quickly snap off the end with a flick of a finger.



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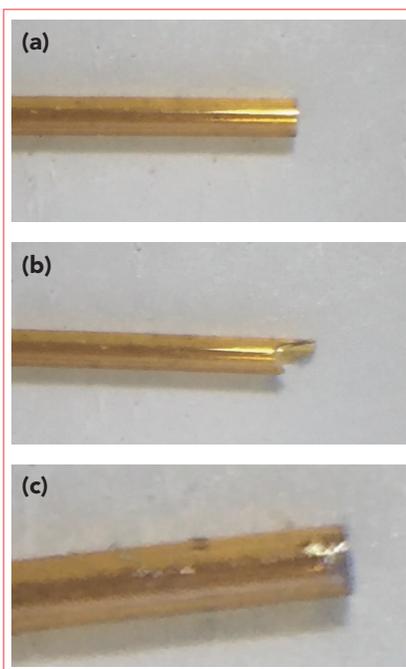


FIGURE 2: Photographs of cut capillary column ends. (a) Proper column cut; (b) Improper column cut; (c) Proper column cut with graphite ferrule contamination.

Figure 2b shows an improperly cut column end, cut by scoring too hard with a column scoring wafer. The uneven cut is easily seen, along with exposed glass surfaces and cracks in the polyimide coating. Unwanted adsorption of polar analytes can happen on these uneven surfaces, causing poor peak shapes and loss of reproducibility. This is an extreme example for illustration purposes. Typical poor column cuts may be more subtle and difficult to see with the naked eye. After every cut, use a magnifying glass or zoomed cell phone camera to closely inspect the cut. If you have any questions about the cut quality, make another cut. A meters-long column will not miss a few extra centimeters cut off.

Figure 2c shows a properly cut column end that was incorrectly threaded through a graphite ferrule following cutting. One subtle point of column trimming and installation is to thread the column through the ferrule prior to trimming. When the column passes through the hole in the soft ferrule, it collects graphite, which then will contaminate the inlet, causing possible sample adsorption or ghost peaks in the chromatogram. This

is avoided by threading the column onto the ferrule prior to cutting; the ferrule contamination is removed when the column is trimmed.

Install the Column Correctly into the Inlet

Proper column installation is critical to successful injections and chromatography. Each inlet from each vendor has a specific distance above the ferrule that the end of the column must protrude to be properly inserted into the inlet. If the column is inserted too far or too little into the inlet, analyte transfer into the column may not be complete. Be careful to not mix up the column and detector installation instructions; I have seen situations where the column was inserted all the way into the inlet until it stopped and then slightly pulled back, similar to many detectors, such as a common flame ionization detector. The user then wondered why they had no peaks. Most instrument vendors provide video instructions and devices that assist in properly setting the column length above the ferrule to ensure proper column insertion into the inlet (6).

Figure 3 shows a close-up of a column installed into an inlet the “old-fashioned” way, to illustrate the key point that the installation is blind, yet the length of column above the ferrule protruding into the inlet, is critical. In this figure, an old, used septum is threaded onto the column (prior to trimming!) through the hole made by the syringe, to hold the column nut and ferrule in the proper position; the column nut is then threaded into the inlet and tightened. An old septum can also be used to seal the column ends, if a column is removed from the instrument and stored.

Use Proper Technique When Tightening Column Nuts and Ferrules

Tightening the nut and ferrule, especially for the column connection with the inlet, is an art form. The nut should be tightened enough that the column is firmly held, and the connection is leak-free, but not over-tightened so that the soft ferrule material is crushed or cracked. A crushed or cracked ferrule, while it may not leak, can introduce contaminants. Generally, the nut should be



FIGURE 3: Close-up photograph of a capillary column installed through an inlet column nut the old-fashioned way. The column is threaded through the hole in an old septum to mark the correct insertion distance into the inlet.

tightened finger-tight, plus about one-quarter turn with a wrench. Once tightened, a gentle tug on the column can test whether the column is firmly seated. If the column moves with a gentle tug, re-set and tighten.

At the top of the inlet are two additional nuts that require care in tightening. The glass liner is held in place by a nut with an O-ring that maintains a seal. When changing the glass liner, also change the O-ring and ensure that it is not deformed or discolored. When tightening the nut, again, finger tighten, plus about one-quarter turn until moderate resistance is felt. Remember that the glass liner is rigid; over-tightening of the nut can actually crack or break the glass liner. Septa should be replaced on a regular schedule, typically about every 50 injections. The septum nut should be finger-tightened until moderate resistance is felt. Under-tightening results in leaks; over-tightening can deform the septum, also resulting in leaks.

Be Gentle

In general, when working with connections and tightening nuts, either finger tight or with wrenches, if you feel you are using a lot of force to tighten the connection, it is a good idea to stop and check that the connection is seating properly. As the connections are screw-threaded, the need for too much force can lead to stripping of the threads.

Ensure that the Carrier Gas Flows Properly, and Check for Leaks

Once the column is installed in the inlet, but prior to installing in the detector, establish carrier gas flow. Check for column flow by placing the column outlet into a vial of solvent and looking for bubbles emanating from the column end. The bubbles indicate gas is flowing; then connect the column to the detector. Use an electronic leak detector to check all of the connections: septum nut, glass liner, column nut, and detector nut. If any leak, another one-quarter turn to tighten will usually stop it.

Heat the inlet, column oven, and detector and allow them to equilibrate. Once the inlet is properly set up, the column is installed and not leaking, take your time with this heating step. Although the inlet, column oven, and detector will come to the temperature settings very quickly, they may not be fully equilibrated at the set temperature for an hour or two. Once heated and equilibrated, run the system through 2–3 temperature cycles of the method (blank runs are fine here) and recheck for leaks. Leaks may appear after a few heating and cooling cycles because of dissimilar materials used in the connections. Again, be gentle and judicious with connections and wrenches. If you feel the need to force the connection to achieve a leak-free seal, it is best to have the connection repaired.

There are many critical steps to follow in properly setting up an inlet. Our current series has focused on the splitless inlet, but the general principles discussed in this installment apply to all inlets. Keep the inlet clean. Use care in making connections. Ensure that glass liners and septa are replaced on a regular basis. Optimize the glass liner and, as discussed in Parts I and II, the temperature and gas flows. The column is often termed the “heart of the separation;” the inlet, then, is the lungs. The gases and samples that flow through the column must first pass through the inlet. If the inlet is not properly optimized, the column and detector will also not perform their best. ■

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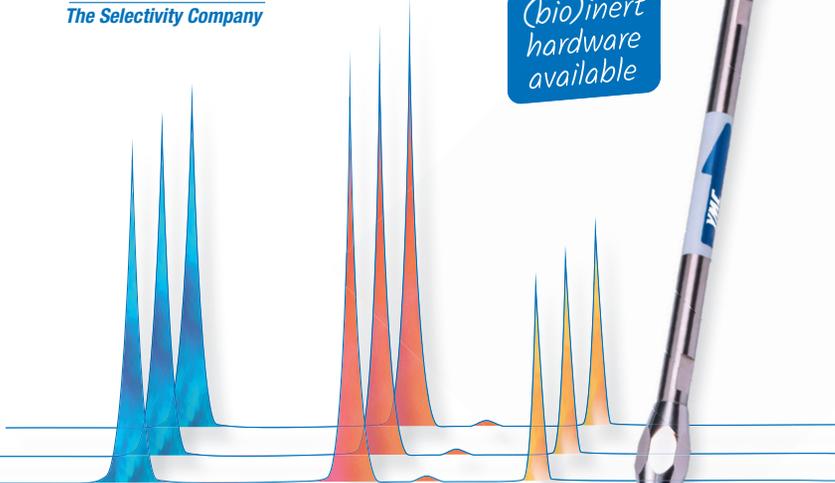
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Hydrophilic Interaction Liquid Chromatography for Oligonucleotide Therapeutics: Method Considerations

Szabolcs Fekete, Makda Araya, Balasubrahmanyam Addepalli, and Matthew Lauber

Hydrophilic interaction liquid chromatography (HILIC) has emerged as a promising alternative to traditional ion-pair reversed phase liquid chromatography (IP-RPLC) methods for separating oligonucleotides (ON). This work investigates the application of HILIC to the separation of ON sequence and length variants, duplexes, and single-stranded components. Method variables, including ionic strength, temperature, mobile phase composition, and gradient optimization, are investigated in detail. A sequential injection procedure is also proposed to eliminate breakthrough effects and peak splitting. Some considerations for generic (platform) methods are also suggested.

RECENTLY, **HYDROPHILIC** interaction liquid chromatography (HILIC) has gained increasing interest for oligonucleotide (ON) separations (1–3). HILIC-mode separations offer attractive, sensitive, and robust alternatives to the most commonly used ion-pairing reversed-phase liquid chromatography (IP-RPLC) methods. HILIC has shown particular promise for ON diastereomer separations. It has been shown that high ionic strength and low mobile phase temperature favor diastereomer selectivity (3). HILIC is also capable of separating duplex forms from their single-stranded components because it can be readily operated in either denaturing or non-denaturing (native) conditions depending on the mobile phase temperature (4). Another important advantage of HILIC is that it allows the use of ion-pair-free mobile phases and so is favored for more versatile mass spectrometry (MS) applications (5,6). HILIC has also been successfully applied for the bottom-up sequencing of single guide RNA (sgRNA) in liquid chromatography–mass spectrometry (LC–MS) and LC–tandem MS (LC–MS/MS) setups (7).

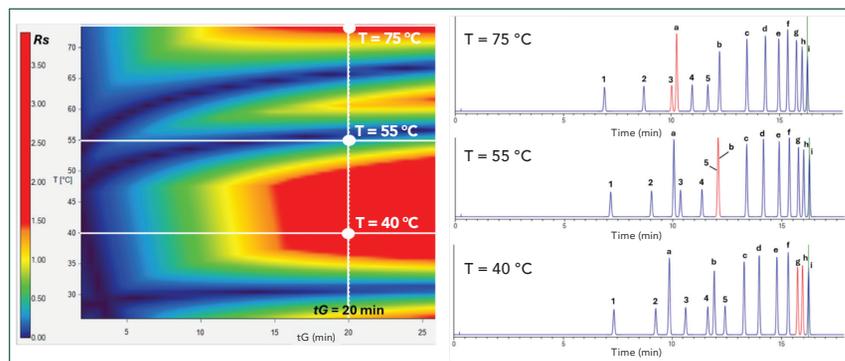


FIGURE 1: Resolution map (left) and chromatograms at three different temperatures (right). The resolution map shows the critical resolution (R_s) as a function of temperature (T) and gradient time (t_G). Red colors correspond to high resolution conditions ($R_s > 1.5$), while the blue areas indicate low resolution conditions. On the chromatograms, t_G is fixed at 20 min, while T is set at 40, 55, and 75 °C. Mobile phase buffer: 50 mM ammonium acetate, flow rate $F = 0.4$ mL/min. Peaks: 15, 20, 25, 30, and 35 nt o-dTs (1–5) and 20, 30, 40, 50, 60, 70, 80, 90, and 100-mer ssDNAs (a–i).

Although robust methods can be developed in HILIC for ONs, the retention mechanism is not fully understood. Some authors mention that ionic interactions may be involved in the retention mechanism (note that in this case it should be repulsive effect) (1), while others conclude that HILIC analysis of ONs is mainly driven by hydrogen bonding interactions, either by adsorption

or partitioning (3). It is thought that mobile phases with high ionic strength appear to stabilize the immobilized water layer on the surface of the polar (that is, amide) stationary phase and minimize ionic interactions (3). However, some chromatographic models suggest the opposite: when salts are added to aqueous mobile phases, water molecules will predominantly solvate salt ions

such that the highly ordered structure of water is disrupted. This would reduce the number of water molecules available to interact with a hydrophilic surface, and there would be a corresponding reduction in the thickness of the immobilized water layer. It is also acknowledged that HILIC columns provide good peak shape and similar selectivity when a protic co-solvent (that is, methanol) is used instead of acetonitrile. This fact further complicates the understanding of the retention mechanism. Finally, it should be noted that the amount of water required to elute ONs from HILIC columns (20–60% water) is quite high, much higher than the amount required to elute glycans or glycoproteins (15–35% water) (8). Bartlett concluded that the mechanism of retention of ONs in HILIC is a complex and unique multi-modal mechanism involving partitioning, hydrogen bonding, and electrostatic interactions (9). Because of the higher water content in the mobile phase, HILIC of ONs occurs with mobile phase compositions that are quite unique when compared to “traditional” HILIC. Non-specific binding appears to be a greater challenge for HILIC when compared to other types of chromatography; this is probably because of its optimal pH being between 5 and 8, which causes greater metal surface charging for non-metal-free column hardware (9).

The character and effect of the polar functional groups of HILIC phases on ON retention and selectivity has recently been investigated (10). A modified Tanaka test (adapted to HILIC of ONs with specific test compounds) was proposed to characterize the HILIC phases, but it was found that small molecule probes do not necessarily translate to the behavior of larger complex molecules (ONs). The amide phase was found to make the greatest contribution to hydrophilic partitioning and is likely to form the thickest adsorbed (immobilized) water layer surrounding the stationary phase (10).

There is little mention of systematic method development for ON separations in HILIC. Very often, the same or similar conditions are used. Ammonium acetate, formate, or a bicarbonate-modified aqueous solution is applied along with acetonitrile. The ionic strength of the mobile phase additive, temperature effects, and pH have been studied (1,3,11), but very often separately (one factor at a time [OFAT]) rather than in a combined design of experiments (DoE) or multidimensional retention model.

Here, a systematic approach was used to compare three ammonium salts. The effect of the mobile-phase temperature and gradient programming was evaluated by retention modeling. Breakthrough effects were studied and a sequential (bracketed) injection mode was proposed to avoid peak splitting. Some generic (platform) method conditions were also proposed.

Materials and Methods

Method Screening and Optimization

First, for screening purposes, a three-factor DoE was applied to study the effect of gradient steepness (gradient time, $[t_G]$), temperature (T), and ionic strength (C). The impact of t_G and T was studied at two levels while the effect of C was studied at three levels. Then, the gradient program and temperature were simultaneously further optimized (using chromatographic modeling software) to

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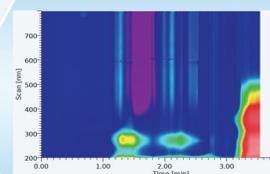
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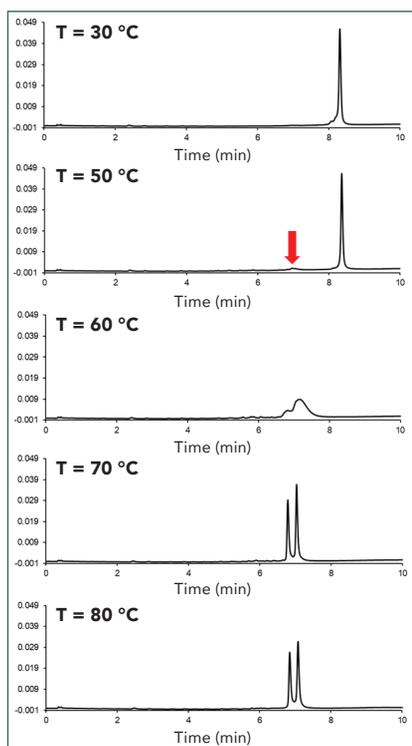


FIGURE 2: Illustration of temperature effects in HILIC on duplex stability. Column: 50×2.1 mm, $1.7\text{-}\mu\text{m}$ 300 \AA amide-bonded. Mobile phase buffer: 50 mM ammonium acetate, flow rate $F = 0.4$ mL/min, $t_C = 10$ min. Sample: siRNA standard (mixture of annealed 25mer and 27mer RNA strands).

obtain a 10-min separation that can be generally applied to different oligonucleotide ladder samples (for a broad range of ON length variants).

Studying Breakthrough Effects and Sequential Injections

Breakthrough effects (or partial peak splitting) often occur in HILIC because of an inherent mismatch between the sample diluent (biomolecules such as proteins or oligonucleotides must be solubilized in aqueous media) and the mobile phase, which is mostly comprised of aprotic solvent (acetonitrile) (12). Practicing chromatographers often make a compromise to avoid breakthrough effects by diluting the sample in an acetonitrile–water mixture that can lead to sample precipitation or denaturation. This study proposes a more elegant solution: the addition of pre- and post-injection plugs of

a weak solvent (acetonitrile) while the sample is injected in a pure aqueous solvent plug (“bracketed” injection). Direct injections of different sample volumes will be compared to the bracketed injections performed, considering different ratios of solvent to sample plug volume.

Mobile Phase Preparation

High performance liquid chromatography (HPLC)- and MS-grade solvents were used. For the LC study, pre-mixed mobile phases were considered to avoid precipitation and solubility problems. Mobile phase A was 30:70 water–acetonitrile, while mobile phase B was 90:10 water–acetonitrile (Honeywell). It was found that such a mobile phase composition could elute all oligonucleotide standards with adequate retention when running linear gradients of 0–100%B. To investigate the effect of additives in the mobile phase, three different ionic strengths ($C_1 = 10$ mM, $C_2 = 25$ mM, $C_3 = 50$ mM) were considered for the three ammonium salts (acetate/formate/bicarbonate [Sigma Aldrich]) in both A and B. For the final optimized LC–UV methods, 50 mM salts were added to both mobile phases A and B.

For the LC–MS application, neat acetonitrile (Honeywell) and water were used (not premixed) to decrease leachate effects from the reservoir bottle. Mobile phase A was 100 mM ammonium acetate (Waters Corporation) in water and kept in a low density polyethylene (LDPE) bottle while B was neat acetonitrile (Honeywell) kept in a glass bottle.

Samples

Various ON standards and ladders were used (all Waters Corporation): an oligo dT ladder, single-stranded DNA (ssDNA) ladder, a lipid-conjugated antisense oligonucleotide (ASO), a small interfering RNA (siRNA), and an sgRNA. Samples were reconstituted in HPLC-grade water, except for the LC–MS analysis; the lipid-conjugated ASO was reconstituted with 20% acetone and the sgRNA in nuclease-free water.

Column and Chromatographic System

Low adsorption columns in dimensions 50×2.1 mm were packed with a 300 \AA $1.7\text{-}\mu\text{m}$ amide-bonded stationary phase (Waters

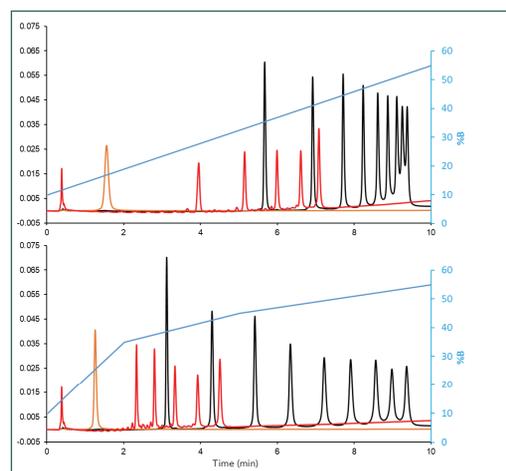


FIGURE 3: Comparison of linear (top) and concave multi-linear (bottom) gradients for ONs HILIC separation. Column: 50×2.1 mm, $1.7\text{-}\mu\text{m}$ 300 \AA amide-bonded. Mobile phase A: 50 mM ammonium acetate in 30:70 water–acetonitrile. Mobile phase B: 50 mM ammonium acetate in 90:10 water–acetonitrile, gradient: 10–60%B in 10 min, $F = 0.4$ mL/min, $T = 50$ °C. Samples: lipid conjugated ASO oligonucleotide (orange trace), oligo dT ladder (red trace), and ssDNA ladder (black trace).

Corporation). The experiments were performed on a low dispersion and low adsorption binary ultrahigh-pressure LC (UHPLC) system coupled to a UV and/or time-of-flight mass spectrometry (TOF–MS) detector (Waters Corporation).

Results and Discussion

The Effect of Additive: Comparison of Three Ammonium Salts

The effect of ionic strength was found to be important when $C \leq 40$ mM and retention decreased with decreasing ionic strength. Beyond 40 mM, retention was found to be robustly stable, suggesting minimal electrostatic effects (with all three ammonium salts). Note that this mobile phase ionic strength limit may depend on the column hardware material and the stationary phase. If the additive concentration was too low ($C = 10\text{--}20$ mM), then breakthrough and/or peak splitting might be significant even if the retention of the first eluted peaks is set to be apparently high (that is, the apparent retention factor is $k_{app} \sim 5$).

From a chromatographic separation point of view, ammonium acetate and

formate seem equivalent. The same gradient program can be used with both salts, resulting in almost identical retention, selectivity, and peak patterns. However, ammonium bicarbonate resulted in slightly lower retention than acetate or formate salts, so the gradient program must be adjusted (shifted by approximately -5% B) to achieve similar retention. Note that the selectivity was slightly different for bicarbonate as well. With bicarbonate, some early eluting peaks were more sensitive to peak splitting and breakthrough (particularly at elevated temperatures), which is a disadvantage compared to the other two salts. However, this effect can be eliminated by using a bracketed injection.

The Effect of Temperature

In general, temperature was found to be a very important variable in tuning the selectivity and even the elution order of different ON species; therefore, temperature is recommended to be optimized for a given sample. For all samples, retention decreased with temperature decrease, suggesting that ONs were present in extended form with multiple sites for adsorptive interaction and that retention was mostly controlled by enthalpy changes upon adsorption-desorption steps (transfer of the solutes between stationary and mobile phases) in HILIC. For all ON samples, van't Hoff plots (the logarithm of retention vs. $1/T$) followed a linear relationship. The positive slope of the van't Hoff plots suggests that ON solubility was favored toward the stationary phase (note that an immobilized water layer was adsorbed onto the surface of the stationary phase) rather than the mobile phase. Figure 1 illustrates the effect of temperature on selectivity and resolution for a complex ON mixture (including both length and sequence variants). Peaks 1–5 are 15, 20, 25, 30, and 35 nucleotide (nt) long oligodeoxythymidines (o-dTs), while peaks *a-i* are 20, 30, 40, 50, 60, 70, 80, 90, and 100-mer ssDNAs. Peak pairs "*a*" – 3, and "*b*" – 5 change their elution order with the changing temperature.

Another important observation was that peak splitting (for early eluting species)

and breakthrough became more pronounced at elevated temperatures ($T \geq 50^\circ\text{C}$).

As well as tuning the selectivity and elution order of different species, temperature is also critical in setting "less" denaturing or "more" denaturing separation conditions in HILIC. ON duplexes can be stabilized in non-denaturing conditions below the duplex

melting temperature (T_m). Melting temperature is the property used to measure duplex stability; however, it is not fully understood how the chromatographic conditions affect the duplex stability (4). Pressure, temperature, pH, and co-solvents can cause significant shifts in the apparent melting temperature.

An siRNA standard (a mixture of annealed 25mer and 27mer RNA strands)



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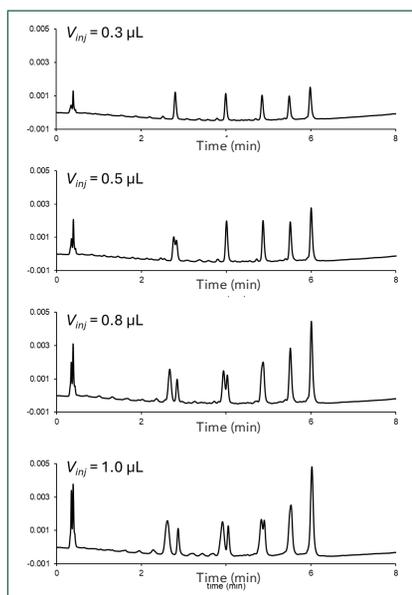


FIGURE 4: Illustration of injection breakthrough effect in HILIC. Column: 50×2.1 mm, $1.7\text{-}\mu\text{m}$ 300 \AA amide-bonded. Mobile phase A: 50 mM ammonium bicarbonate in 30:70 water–acetonitrile. Mobile phase B: 50 mM ammonium bicarbonate in 90:10 water–acetonitrile, gradient: 5–55%B in 10 min (linear gradient), $F = 0.4$ mL/min, $T = 50$ °C. Sample: oligo dT ladder.

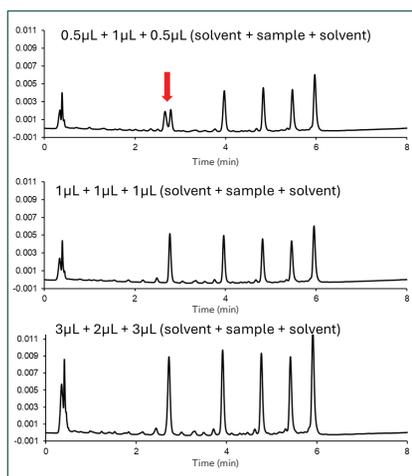


FIGURE 5: Bracketed injections (acetonitrile solvent pre-plug + sample plug + acetonitrile solvent post-plug) performed with different ratios of solvent-to-sample plugs. Conditions and sample: the same as described in Figure 4.

was used to study temperature effects on duplex stability. Below 55 °C, a single peak was observed. At 60 °C, a very broad zone appeared on the chromatogram

and its retention time decreased significantly compared to the sharp single peak, and when the temperature was increased above 65 °C, two sharp peaks were observed on the chromatogram. These results suggest that this particular siRNA begins dissociating into its single strands at 60 °C. Figure 2 shows the temperature effects on duplex stability of a siRNA.

The Effect of Gradient Steepness and Shape

When optimizing the separation of complex ON mixtures, the two most important method variables are probably gradient steepness (t_G) and mobile phase temperature (T). First, an appropriate mobile phase system (that is, 50 mM ammonium acetate and acetonitrile as co-solvent) must be selected to allow elution of all species in the sample mixture. This may require some screening experiments. Then, the best practice is to apply a retention model combining t_G and T to optimize the peak resolution (R_s). The entire optimization process requires only four experiments ($2 \times t_G + 2 \times T$). By adjusting t_G , the entire elution window of all peaks and the R_s between short and long ONs (size variants) can be adjusted. Meanwhile, by changing the temperature, the selectivity between sequence variants of similar ON lengths can be improved. The retention of length variants is primarily sensitive to gradient steepness, while the relative retention of sequence variants is mainly determined by temperature.

The empirical observation in LC that the retention of compounds of a homolog series (that is, the length variants of ONs) increases in logarithmic fashion with an increasing number of homolog units is called *the homolog rule* (13;14). As a result of the homolog rule, the selectivity between $n-1$ -mers decreases significantly for longer ONs when running linear mobile phase gradients. Very often, unnecessarily high selectivity is obtained for the short ONs and very low (limited) selectivity for the long ONs. Therefore, a more uniform selectivity (retention pattern) should be established for the separation of length variants to approach uniform selectivity across the entire elution window of ON homologs. An

elegant approach to set up a generic (platform) method is based on an “inverse function” or “inverse gradient” method (15;16). If a linear mobile phase gradient (linear input function) results in a logarithmic (“concave”) retention time distribution (logarithmic response function) for a set of compounds and the analytes elute by on-off mechanism (like ONs do), then programming a logarithmic concave gradient program is predicted to yield a linear retention time distribution across the entire elution window (inverting the input and response functions) (17). This inverse gradient approach has recently been applied for ONs in IP-RPLC (18). The same considerations apply in HILIC. Figure 3 compares peak patterns obtained by running a linear vs. a concave gradient program for different ONs.

It is worth noting that for ON separations, HILIC and IP-RPLC modes may be alternative separation techniques but they are not entirely orthogonal. The strength of the interaction increases with the length of the ON, whether it's a hydrophobic (RP) or polar (HILIC) retention mechanism. If the nucleotide sequence is uniform (homopolymer or homolog), the ONs will elute according to their length. If the sequences vary, retention and selectivity can change significantly.

Breakthrough Effects and Bracketed Injections

Sample injection in HILIC can cause problems because of the higher eluent strength of the injection solvent compared to the mobile phase. In this case, where there is a solvent mismatch between the mobile phase composition and the injection solvent, the sample is not sufficiently focused at the column inlet (12). Fine adjustment of the injection diluent could minimize this mismatch (that is, increasing the percentage of acetonitrile in the sample diluent). However, this adjustment is not always possible because of the nature of the sample, which may cause denaturation, sample artifacts, or even precipitation. This solvent mismatch usually results in undesirable effects on the chromatographic separation, ranging from slight broadening to severe peak deformation or even splitting and analyte breakthrough. Breakthrough is a phenomenon where part of the sample

migrates through the column with little interaction with the stationary phase and therefore elutes close to the column dead time, whereas the other part of the peak elutes at the normal retention time.

In our examples, the oligo dT ladder was the most sensitive to peak splitting. When the injection volume was set to 0.5 μL , the first compound (15 nt) eluted in a split peak. When the injected volume was increased, the later eluting ONs also showed significant splitting and some of the solutes eluted at the column void (Figure 4), which limits the column load and sensitivity.

A bracketed injection sequence can help eliminate breakthrough effects. In a bracketed injection, the sample is injected into the column head together with a pre- and post-plug of weak solvent (in this case acetonitrile). Using this bracketed injection, the sample injection volume could be increased up to 2 μL for the dT ladder without splitting. Figure 5 shows a comparison between bracketed injections performed with various solvent-to-sample plug volume ratios when

1 and 2 μL of sample plug is introduced into the flow path. Note that with a normal injection mode, only a 0.3 μL sample could be safely injected, while the bracketed injection allowed the sample load to be increased to 2 μL (a factor ~ 7 improvement).

HILIC-MS Application

With insights collected on the tunability of ON HILIC methods, the direct hyphenation of these separations with mass spectrometric detection was studied. Two unique methods were optimized to analyze a CRISPR sgRNA molecule as well as a lipid-conjugated ASO. For the sgRNA, an aqueous eluent modified with 100 mM ammonium acetate was applied. In the case of the lipid-conjugated ASO, a lower, 25 mM concentration of ammonium salt was employed. Both cases produced effective levels of ion signal that could be directly interpreted with an accurate mass measurement derived from a monoisotopic peak or from MaxEnt deconvolution of the raw mass spectra.

For the CRISPR sgRNA synthesized with a human HPRT sequence complementarity, an intact molecular weight of 32,276.97 Da was obtained, which is in close agreement to the sequence of this sgRNA—inclusive of its 5' and 3' modified termini. Results obtained for the lipid-conjugated ASO are shown in Figure 6. In contrast to the sgRNA, the lipid ASO was not strongly retained on the HILIC column. This can be attributed to it being a significantly smaller oligonucleotide that is conjugated to a C16 lipid. This lipid ASO contains a fully phosphorothioated backbone, along with several other modifications. A high temperature separation was found to reduce peak splitting (partial separation of diastereomers). With a final method using a 25 mM ammonium acetate eluent, two isobaric species were separated (Peaks 1 and 2 in Figure 6). In addition, a sample impurity lighter in mass by 419 Da was resolved.

Conclusion

It has been demonstrated that HILIC presents a viable and effective alternative to

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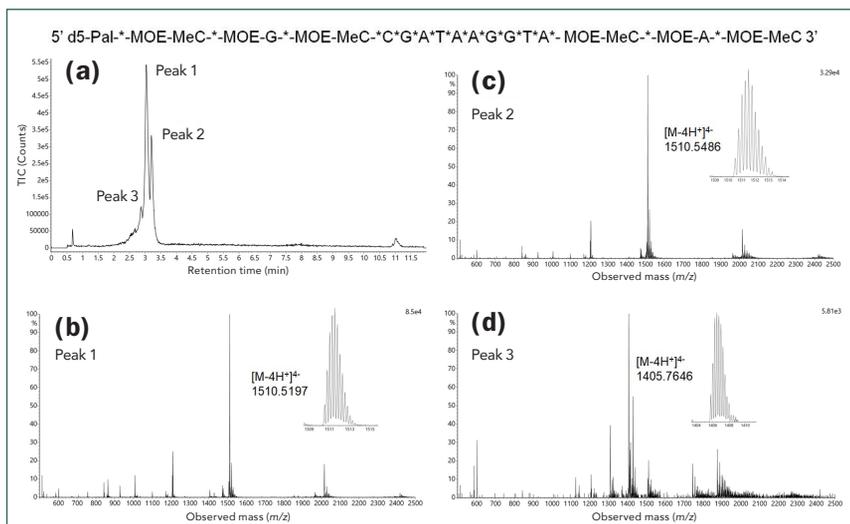


FIGURE 6: HILIC–MS analysis of a lipid conjugated ASO. Column: 50×2.1 mm, $1.7\text{-}\mu\text{m}$ 300 Å amide-bonded. (a) Total ion chromatogram from a 50 pmol injection. Mass spectra acquired for (b) Peak 1, (c) Peak 2, and (d) Peak 3. A monoisotopic mass of 6046.11 Da is determined for the main species. An isobaric species is chromatographically resolved as Peak 2, and Peak 3 is determined to be predominately comprised of a 419 Da lighter impurity. Peak 3 $M_i = 5,627.09$ Da.

traditional IP-RPLC methods for separating ONs. Through a systematic investigation of various method parameters such as ionic strength, temperature, and gradient steepness/program, optimized conditions have been established that improve selectivity and resolution of ON sequence and length variants. The importance of mobile phase temperature to help analysts prepare for both denaturing and non-denaturing conditions must be highlighted. The use of a bracketed injection effectively mitigates breakthrough effects, allowing for increased sample load without compromising separation quality.

Generic (platform) method conditions are also proposed that can be broadly applied to diverse ON samples. These results emphasize the potential of HILIC for applications in mass spectrometry, where the molecular weights of ONs such as CRISPR sgRNAs and lipid-conjugated ASOs can be quickly determined along with the masses of their impurities.

Continued exploration into the retention mechanisms of ONs in HILIC will further enhance the robustness and applicability of this technique in the rapidly evolving field of oligonucleotide therapeutics.

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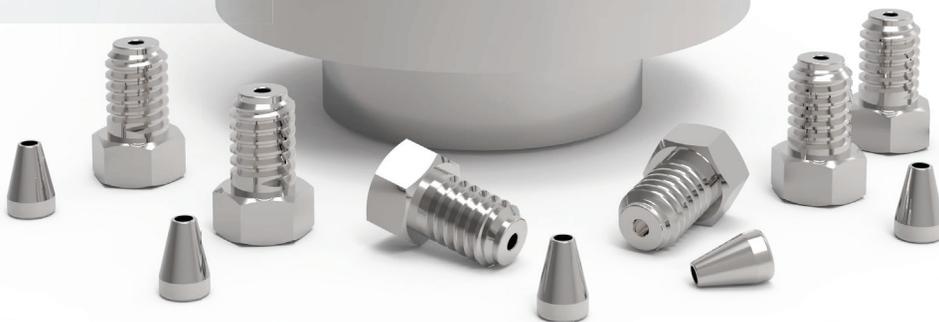
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Comparison of Organic Compounds in Natural Wine, Red Wine, and Grape Juice by HPLC

J. Wesolowski, Y. Krauke, G. Greco, KNAUER Wissenschaftliche Geräte GmbH

Natural wine is the latest trend among wine lovers. It is produced by natural fermentation of grape juice without external additives. But what is the difference from classical wine? Here, we investigated sugars, alcohols, organic acids and phenolic compounds in natural wine, red wine and grape juice using two simple and rapid chromatographic methods. The versatility of various column materials and detectors allows for the analysis of a wide range of compounds. This analysis is also crucial in daily beverage analysis (Fig. 1) and essential to ensure product quality and compliance with regulations, as well as food standards and labeling requirements.

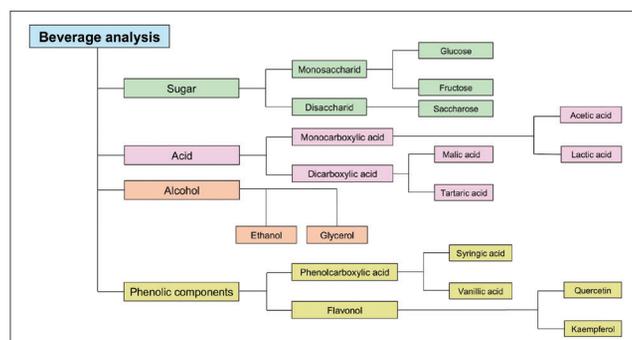


FIGURE 1: Overview of common analytes in the beverage analysis

Sample preparation

All standards (sucrose, glucose, fructose, glycerol, ethanol, sorbitol, tartaric acid, lactic acid, acetic acid, citric acid, succinic acid, malic acid, shikimic acid, syringic acid, vanillic acid, p-coumaric acid, ferulic acid, benzoic acid, quercetin, kaempferol) were dissolved in an appropriate solvent to a desired concentration. The standard mix was made from the individual standards and dissolved in water/acetonitrile 50:50 (v/v) to a concentration of 0.1 mg/ml. The samples (Tab. 1) were filtered with a CA (cellulose acetate) filter (0.45 µm) before injection. For a detailed description please refer to KNAUER application note VDF0194.

Results

For the determination of the most important organic acids, sugars and alcohols in wine and grape juice, a method using Eurokat columns, refractive index detector (RID) and diode array detector (DAD) was applied. Eurokat columns are not

Sample type			
Unadulterated wine	Red wine	Red Wine	Grape juice
Sample ID			
Natural wine	Wine 1	Wine 2	Grape juice



TABLE 1: Sample description

only environmentally friendly but also has the advantage that water soluble sugars can be easily injected. The Eurokat H column was specially developed for the analysis of organic acids, monosaccharides, alcohols. Even complex mixtures of these substances can be separated. In order to be able to clearly identify the substances in the samples, all standards were measured separately (Fig. 2).

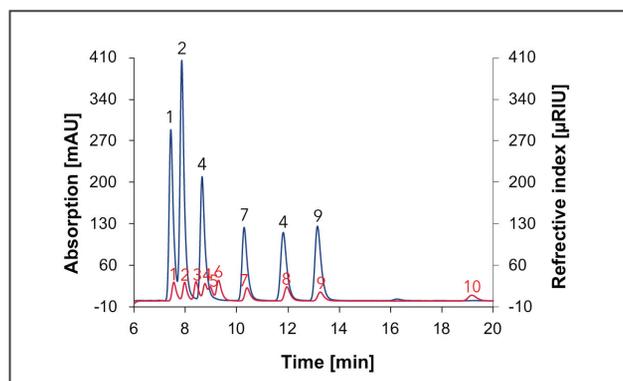


FIGURE 2: Chromatogram of standard mix measured with Eurokat H, DAD (blue) and RID (red), 1: citric acid, 2: tartaric acid, 3: glucose, 4: malic acid, 5: fructose, 6: sorbitol, 7: succinic acid, 8: glycerol, 9: acetic acid, 10: ethanol.

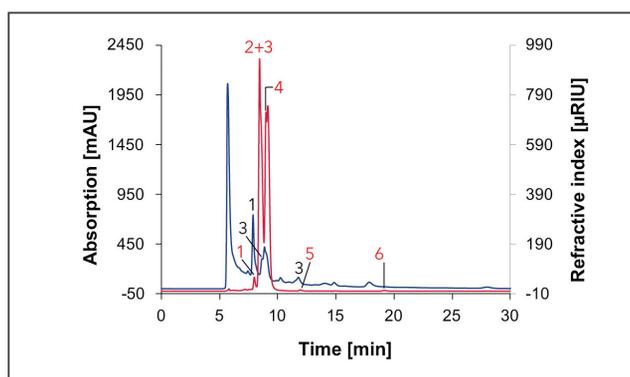


FIGURE 3: Chromatogram of grape juice measured with Eurokat H, DAD (blue) and RID (red), 1: tartaric acid, 2: glucose, 3: malic acid, 4: fructose, 5: glycerol, 6: ethanol

The detection of high levels of glucose and fructose in grape juice (Fig. 3) and low levels in red wine was as expected. Natural wine was the only sample that contained no sugar. Ethanol was also found in all beverage samples as this can be found not only in alcoholic beverages, but also in low concentrations in various foods such as different juices. The detection of organic acids did not differ between the wine samples, but there were clear differences compared to grape juice. For example, acetic acid and succinic acid were not found in grape juice, whereas malic acid was found in grape juice but not in wine samples. In addition, a Eurokat Ca column was tested for better sugar separation and gave results consistent with the Eurokat H column. In addition, the sugar alcohol sorbitol could be identified due to the better separation (Fig. 4).

Furthermore, a reversed-phase LC method was developed and optimized on a common C18 column with DAD and gradient elution for the determination of phenolic compounds.

The phenolic compounds were similar among the samples, with the exception of p-coumaric acid which was the only

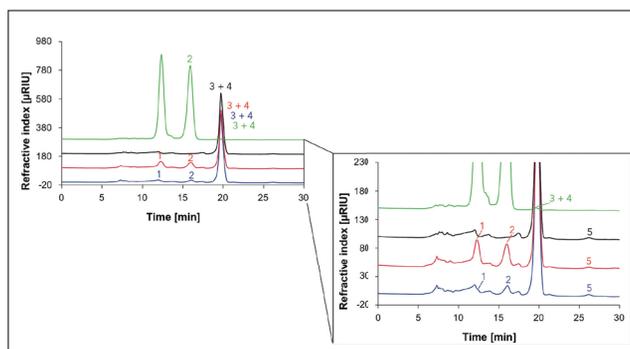


FIGURE 4: Overlay chromatograms with samples measured with Eurokat Ca and RID, blue: wine 1, red: wine 2, black: natural wine, green: grape juice, 1: glucose, 2: fructose, 3: glycerol, 4: ethanol, 5: sorbitol.

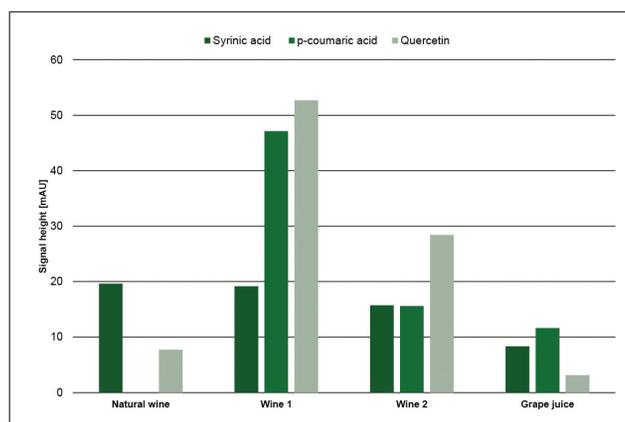


FIGURE 5: Signal heights of syringic acid, p-coumaric acid and quercetin in the four different samples.

substance not detectable in natural wine (Fig. 5). The absence of p-coumaric acid in natural wine is due to its conversion to 4-ethylphenol by the yeast *Brettanomyces*, which thrives in minimally intervened wines like natural wine⁸.

Conclusion

Wine is a complex mixture of hundreds of compounds, each present in various concentrations. High performance liquid chromatography (HPLC) is one method for this analysis. A dedicated system with two detectors (Fig. 6) and different columns enables the detection of the most commonly substances in beverage samples such as wine and juice. ■



FIGURE 6: System setup; from top to bottom: eluent tray with bottles, pump, DAD, RID, autosampler, right: oven

Reference

- (1) Salameh, D., Brandam, C., Medawar, W., Lteif, R. & Strehaiano, P. (2008). Highlight on the problems generated by p-coumaric acid analysis in wine fermentations. *Food Chemistry*, 107(4), 1661-1667.

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Comprehensive Polar Metabolite Profiling with HILIC-LC-MS

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METABOLIC PROFILING STUDIES using LC-MS technology have enabled the sensitive and reproducible detection of a wide range of metabolites in various biological samples, including biofluids, cells, tissues, and organisms. However, the analysis of polar/hydrophilic metabolites, such as small organic acids, amino acids, nucleotides, and sugars, meets challenges due to their poor retention in traditional reverse-phase LC-MS methods without using ion-pairing reagents in mobile phase or sample derivatization (1).

Hydrophilic interaction liquid chromatography (HILIC) offers a different retention mechanism that is advanced for straightforward separations of hydrophilic compounds (2). Although earlier works showed HILIC technology is effective for a small set of metabolites, it currently has achieved remarkable progress on improving chromatography separation and measurement repeatability, paving the way for comprehensive polar metabolite profiling.

In this application note, we describe a HILIC-LC-MS method using a polymeric iHILIC-(P) Classic column to accomplish a comprehensive polar metabolite profiling of several hundred metabolites in a single run. The results indicate remarkable chromatographic separation and repeatability of retention time. The importance of incorporating medronic acid in HILIC separations (3) and a straightforward sample extraction procedure are also touched.

Experimental

Sample preparation:

- 1) Metabolite standard samples were prepared in acetonitrile-methanol-water mixture (40:40:20, v/v%) and stored at -80 °C. Their final concentration was 10 μM.
- 2) 100 μL plasma or DMEM was mixed with 400 μL ice-cold methanol-acetonitrile solution (50:50, v/v%) by vortexing for 3-5s. The mixture was then incubated overnight at -80°C. Whereafter, samples were centrifuged at 15,000 rpm for 15 min at 4°C. Supernatant was transferred to a sample vial for LC-MS analysis.
- 3) The 293T cells after medium removal were extracted with 500 μL ice-cold acetonitrile-methanol-water solution (40:40:20, v/v %) for about 10 min. The cell extracts were transferred to 1.5 mL tubes and stored overnight at -80 °C. They were further cleaned by centrifuging to remove proteins at 15,000 rpm for 15 min at 4°C. The supernatant was used for LC-MS analysis.

LC-MS/MS system:

A Shimadzu ExionLC AC HPLC system was connected to a TripleTOF 6600+ mass spectrometer from AB SCIEX. Electrospray ionization (ESI) in both positive and negative mode were used for detection. The ESI source parameters: source temperature at 500 °C, ion source gas 1 and 2 at 60 psi, curtain gas (CUR) at 35 psi, ion spray voltage floating (ISVF) at 5.5 kV or -4.5 kV for positive or negative modes. Mass spectrometer was set at TOF masses of 70–1200 Da.

HILIC separation:

Columns:

150 × 2.1 mm, 5 μm/200Å, iHILIC®-(P) Classic (P/N 160.152.0520, Sorbent Lot: 160-0520-10117 HILICON); Flow rate: 0.2 mL/min
Column temperature: 30 °C

Eluents:

A) 95:5:20 mM ammonium acetate and 0.1% ammonium hydroxide (v/v%) in water/ACN with 2.5 μM medronic acid.
B) Acetonitrile

TABLE 1: Gradient programs for separation with iHILIC-(P) Classic

time [min]	% B
0	85
2	85
7	60
12	35
12.1	20
15.9	20
16	85
23	85

Results and Discussion

Polymeric iHILIC-(P) Classic columns signify an outstanding advancement in HILIC separation under basic pH conditions, which enables successful separation of several hundred hydrophilic metabolites in a single run. This makes them a valuable tool for untargeted metabolomics studies in biological samples, especially when combined with a simplified extraction technique and ESI-MS detection.

Figure 1 demonstrates the untargeted metabolomics measurements for real biological samples. After excluding metabolites belonging to “lipid or lipid-like” classes, a dataset containing 699 unique hydrophilic metabolites was obtained. Among them, 286 were found exclusively in the 293T cells, 300 in plasma, and 113 in both. We were able to use our dataset of 154 metabolite standards to identify 61 metabolites exclusively in the 293T cells, 34 in plasma, and 59 in both. Our method is highly effective for a broad spectrum of metabolites, particularly for those belonging to the class of organic acids and their derivatives and nucleotides.

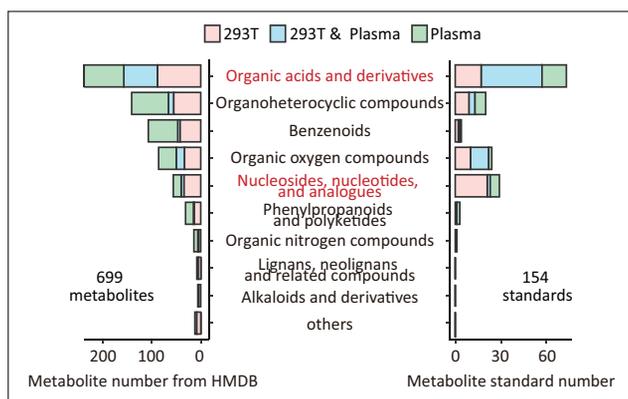


FIGURE 1 Untargeted profiling of hydrophilic metabolites in the 293T cells and plasma with HILIC-MS method in ESI- mode.

The described HILIC-LC-MS method is effective for several biologically important polar metabolites that were difficult to measure. As shown in Figure 2A, highly polar compounds with multiple phosphate groups, such as fructose 1, 6-bisphosphate and inositol hexaphosphate, are easily identified. Additionally, four biologically important metabolites and their isomeric forms were efficiently separated as well, shown in Figure 2B. Moreover, it's worth emphasizing that our sample extraction methods, which avoid drying and reconstitution, ensure the preservation of fragile metabolites, like nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADP), as illustrated in Figure 2C.

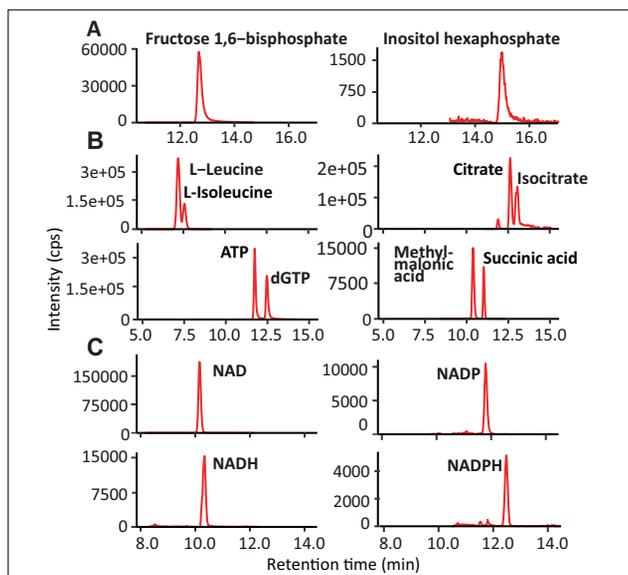


FIGURE 2 Extracted ion chromatograms of polar metabolites from standards or samples.

Figure 3 demonstrates the chromatographic repeatability of the polymeric iHILIC-(P) Classic columns. When analyzing identical amino acid mixture samples, we observed excellent peak shapes and retention time (RT) reproducibility across three different

columns (serial number: #20308, #20504, and #40174), marked in green, blue, and red, respectively. In addition, the column (#40174) showed consistent RTs with a median delta RT of less than 0.03 min for >1000 injections of real samples and standards over a four-month period between July and October 2024.

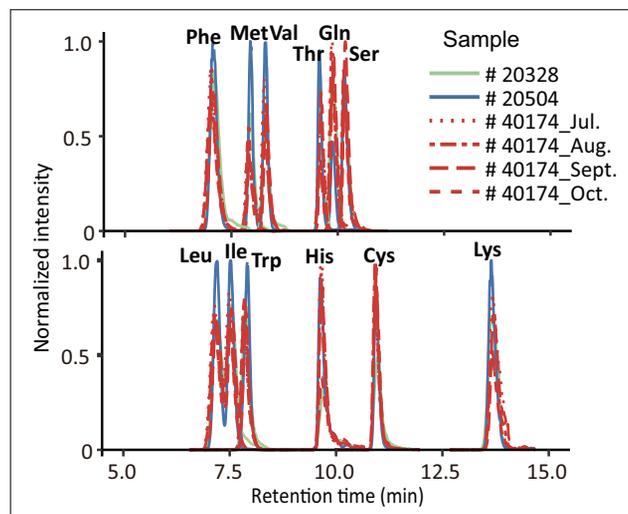


FIGURE 3 Extracted ion chromatograms of amino acids from Dulbecco's Modified Eagle Medium (DMEM) samples.

Conclusion

In summary, the current HILIC-LC-MS method profiles more than 500 polar metabolite standards across various categories in metabolomics studies. It revealed excellent chromatographic separation and retention time stability over several months. Along with straightforward sample preparation, the method enables comprehensive polar metabolite profiling of biological samples.

References

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Identification and Quantification of Microplastic Polymers in Food Packaging Using Micro-Furnace Pyrolysis-GC/MS

Food packaging materials consist of multilayer film polymers. These polymers provide heat resistance, sealability, and printability and act as gas barriers. Detailed identification and quantification of these multilayer polymers is critical in quality control and manufacturing. Many food safety and packaging industries must comply with specific regulations. They seek analytical techniques to accurately and rapidly identify and quantify different compositions while collecting reproducible data.

Introduction:

In this technical brief, as a proven technique, the micro-furnace pyrolysis-GC/MS is used to analyze mixed samples of 11 polymers to identify and quantify a multilayer polymer sample. The micro-furnace multi-mode pyrolyzer directly interfaces with a benchtop GC/MS to perform a continuous (single-step) pyrolysis to analyze the polymeric composition of the sample. In addition to continuous pyrolysis, the micro-furnace is used for other analytical techniques, such as Evolved Gas Analysis (EGA), Thermal Desorption (TD), and heart-cutting (fractionated pyrolysis).

Experimental Conditions:

A three-layer film for bread packaging bags of known composition (mass composition ratio; polypropylene (PP) 82.5 : polyethylene (PE) 12.5 : polybutene-1 (PB) 5.0) was used as the sample. 46 µg of the sample was placed inside an inert sample cup. The micro-furnace temperature was set at 600 °C.

A UAMP column kit consisting of a pre-column and a metal separation column was used. An MP calibration standard (MPCS-SiO₂)

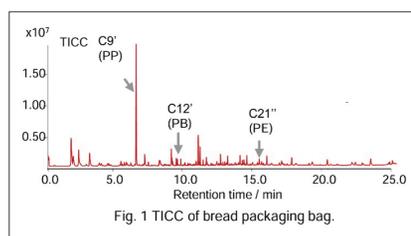


FIGURE 1: TICC of bread packaging bag.

TABLE 1: Match rates of bread packaging bag sample.

Polymer	PP	PE	PB	N66	N6	PMMA	SBR	PC	PS	PET	ABS	PVC
Prob. [%]	98.2	84.9	99.5	67.2	56.8	50.3	50.2	28.1	19.8	12.7	12.1	0.9

containing 11 polymers was used for calibration curves. PB was added to the user library of the F-Search MPs analysis software.

Results:

Figure 1 shows the obtained pyrogram of the bread packaging bag. Software analysis of the pyrograms of the bread packaging bag showed high qualitative match rates of about 90 % for PP, PE, and PB, as listed in Table 1.

For the quantification of these three polymers, the characteristic pyrolyzates, 2,4-dimethyl-1-heptene (C₉'), 1,20-heneicosadiene (C₂₁''), and 2,4-diethyl-1-octene (C₁₂') were used. The amounts of PP and PE were determined to be 31.9 µg and 8.81 µg, respectively, from the calibration curves obtained using MPCS-SiO₂, which corresponds to a mass composition ratio of 69.3 : 19.2 considering a sample mass of 46 µg. However, these determined values were outside the range of the calibration curves, with PP exceeding the upper limit (19.5 µg) and PE below the lower limit (14.3 µg). So, calibration curves were prepared for these three polymers using standard samples, and quantitative values were obtained for each.

Conclusions:

Table 2 summarizes the resulting mass composition ratios, which are close to the known composition ratios of the bread packaging

TABLE 2: Quantification results.

Polymer	PP	PE	PB
Mass composition ratio (formulated value) [%]	82.5	12.5	5.0
Mass composition ratio (quantified value) [%]	82.6	13.8	3.6
RSD (n=3) [%]	1.1	5.2	1.0

Mass composition (normalized to 100)

bag. The maximum relative standard deviation (RSD) of the quantitative values was 5.2 % (n=3), demonstrating excellent reproducibility and confirming the effectiveness of the F-Search MPs with a Microplastics Analysis Library for multilayer film analysis. ■

Reference

- (1) This technical content was developed by Frontier Laboratories Ltd. 4-16-20 Saikon, Koriyama, Fukushima, 963-8862 JAPAN. www.frontier-lab.com



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Implementation of EPA 8270E Using Solid-Phase Extraction and Hydrogen as Carrier Gas for GC/MS Analysis

Arielle Coccozza, Environmental R&D Chemist at UCT

This application note describes the extraction of SVOCs following EPA Method 8270E using a proprietary 8270 sorbent to selectively isolate acidic, basic, and neutral compounds. This sorbent is combined with an activated carbon cartridge to improve the retention of polar analytes during solid-phase extraction (SPE), prior to GC-MS analysis.

Introduction

EPA Method 8270E introduces solid-phase extraction (SPE) as an alternative to traditional solvent-based extraction methods, such as liquid-liquid or separatory funnel extraction for extracting SVOCs. SPE allows laboratories to significantly reduce solvent consumption, which lowers environmental impact and enhances laboratory safety by minimizing exposure to harmful chemicals. Additionally, the method now includes hydrogen as a carrier gas for GC-MS analysis, offering a cost-effective and environmentally friendly alternative to helium.

UCT Part Numbers:

EC82702M15: 2000 mg 8270 sorbent/15 mL cartridge **EU52113M6:** 3000 mg activated carbon/6 mL cartridge **ECSS25K:** Enviro-Clean® Bulk Sodium Sulfate Anhydrous, ACS grade, granular, 60 mesh **AD0000AS:** Cartridge adaptor **VMFSTFR12:** Large volume sample transfer tubes **VMF016GL:** Glass Block Vacuum Manifold System

SPE Procedure

Sample Prep: Dechlorinate the sample if needed. Adjust pH < 2

System Set-up: Connect the carbon cartridge to the end of the 8270-Cartridge

Condition: (a) 15 mL DCM (b) 15 mL MeOH

Equilibrate: 15 mL 0.05N HCl in water

Load: 1-L sample

Wash: 10 mL DI water

Dry: Dry for 10-15 minutes at full vacuum

Instrument Method

GC/MS:	Agilent 6890/5975C
Column:	Restek Rxi-5silMS, 30m x 250um x 2.5um
Inlet Temperature:	280 °C
Injection:	1 µL; Split 10:1
Liner:	Restek Topaz Splitless, 4mm
Oven:	40 °C (1 min) to 280 °C at 20 °C/min, 300 °C at 5 °C/min
Carrier gas:	Hydrogen at 1.2 mL/min constant flow rate
MS Parameters:	Source at 230 °C; Quad at 150 °C; transfer line temperature at 280 °C; Electron energy at 70 eV
Detector:	MSD Quadrupole, full scan mode (EI), 50-500 amu
Tune File:	DFTPP.U

Elute: (1) EC8270 cartridge elution: (a) 10 mL of 1:9 acetone: n-hexane (collection Vial A) and (b) 10 mL of 18:2:80

IPA:NH₄OH:DCM followed by 15 mL DCM (Collection Vial B). (2) Carbon cartridge elution: 15 mL of DCM

Dry & Evaporate: (a) Remove water using Na₂SO₄ (b) concentrate eluates at 40-45 °C to 1 mL final volume with DCM (c) Add IS

Analyze: By GC/MS

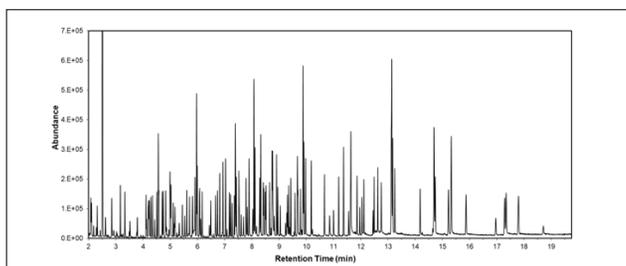


FIGURE 1: Total Ion Chromatogram (TIC) of the 1L fortified sample at 20 µg/L (with internal standards at 40 µg/L, 133 compounds)

Results

Table 1 presents quality control data at two concentration levels for the extraction of 133 compounds. Performance evaluation showed that 81 of the 133 compounds analyzed exhibited acceptable recovery (50-150%) and precision (RSD ≤ 20%) at a lower limit of quantification (LLOQ) of 1 µg/L. At a concentration level of 20 µg/L, only two out of 133 compounds failed to meet the acceptable recovery range: 1,4-phenylenediamine (34%) and hexachloropropene (41%). These compounds are either known to present extraction difficulties, as indicated in the method, or lack historical data for aqueous extraction techniques. ■

TABLE 1: QC Data: Initial demonstration of proficiency

Concentration	1 µg/mL, n=7	20 µg/mL, n=4
Recoveries	50% - 121%	56 - 121%
RSD	4% - 20%	2% - 19%

Reference

(1) U.S. Environmental Protection Agency. (2014). SW-846 Test Method 8270E: Semivolatile organic compounds by gas chromatography/mass spectrometry (GC/MS). U.S. Environmental Protection Agency.

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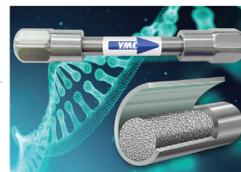
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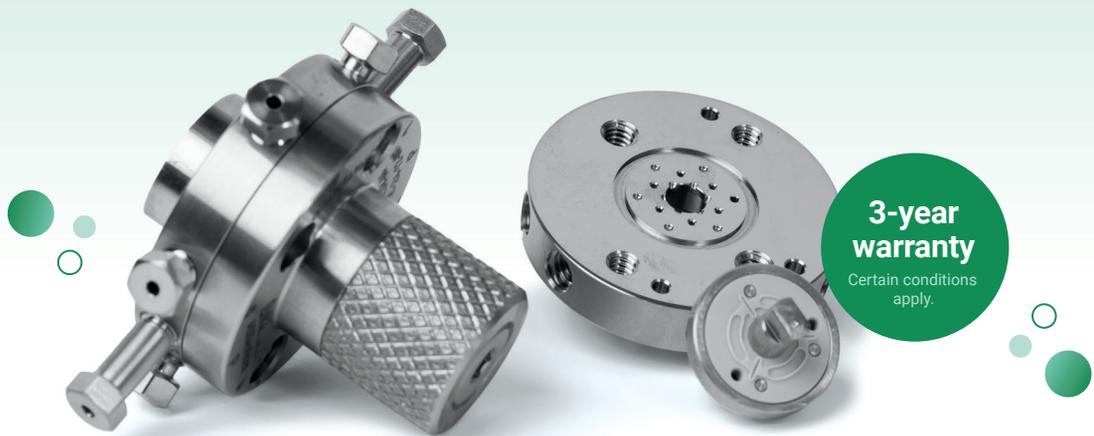
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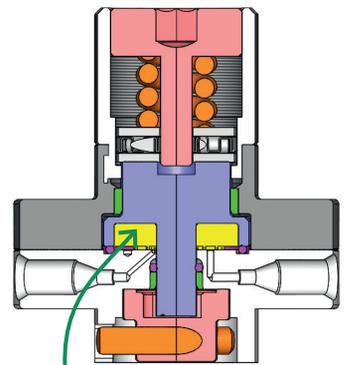
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