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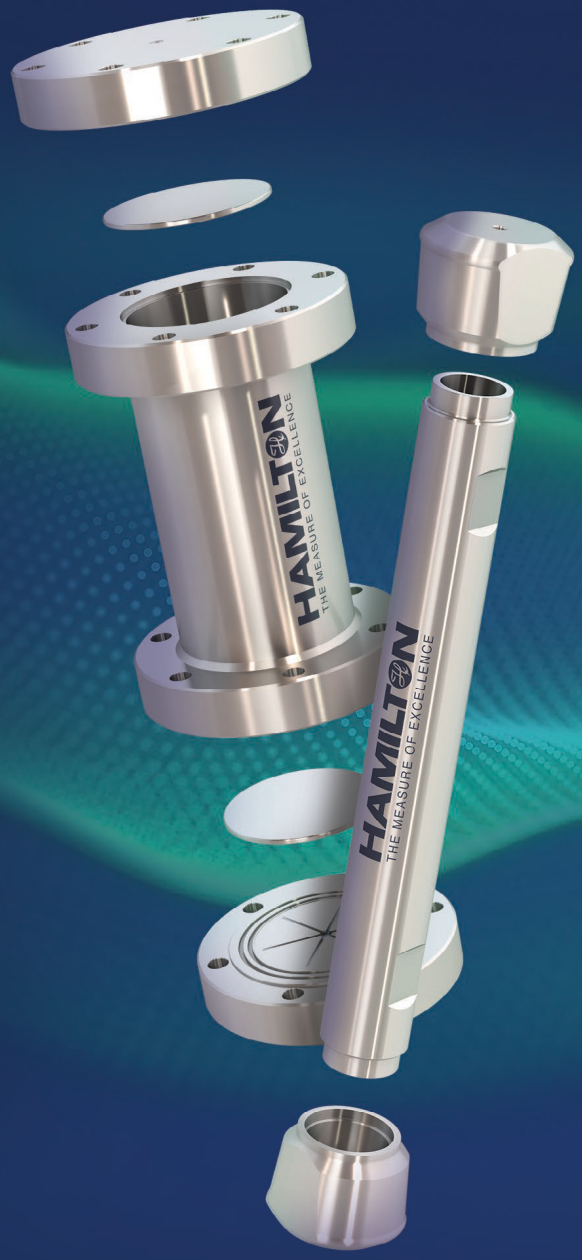

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HPLC 2024 Conference Recap

In this episode, podcast co-hosts Dwight Stoll and James Grinias discuss highlights and observations from HPLC 2024, which was held in Denver, Colorado in July.

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NOTE FROM THE CEO

I AM THRILLED to introduce the September 2024 issue of *LCGC International.* As we transition into the latter part of the year, our focus turns to some of the most compelling and timely topics in the field of chromatography. This issue is packed with insightful articles, cutting-edge research, and practical tips that I am confident will enhance your understanding and practice in this dynamic field.

As the use of two-dimensional liquid chromatography (2D-LC) becomes increasingly common in the pharmaceutical industry, it's crucial to ensure accurate interpretation of results. Our "LC Troubleshooting" column delves into the potential artifacts that can occur due to analyte degradation during the transfer between dimensions in 2D-LC, and provides practical experiments to help you determine if new peaks in your chromatograms are genuine or simply artifacts, ultimately increasing your confidence in your results.

Next, our "Column Watch" column (and cover story) brings you highlights from the 52nd International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2024) recently held in Denver, Colorado. Whether you attended the symposium or not, this article will catch you up on the cutting-edge developments that were discussed, giving you a glimpse into the future of liquid chromatography.

Often considered a straightforward step, splitless injection is actually a complex process that can significantly impact the reproducibility and precision of your results. In the "GC Connections" column, we break down the steps involved in a splitless injection, from sample introduction to the beginning of separation, and offer practical advice on how to optimize each phase to ensure the highest quality data.

Our "Questions of Quality" column addresses a critical update in the world of chromatography: the new requirements of *USP* <621>. We'll walk you through the key changes and their implications for your laboratory practices. Staying compliant with these new regulations is essential, and this article provides the clarity you need to navigate these updates with confidence.

Finally, we present a feature article that takes a deep dive into sustainability in chromatography. In "An Integrative Analytical Quality by Design (AQbD), Up-To-Date Greenness, and Whiteness Set of Tools for Evaluation of a Sustainable RP-HPLC Method for Regulated Products," our interview with Hemanth Kumar Chanduluru from the SRM Institute of Science and Technology in Chennai, India explores how analytical quality by design (AQbD) and other modern tools can be used to develop and evaluate more sustainable reversed phase–high pressure liquid chromatography (RP–HPLC) methods, making a positive impact on both your laboratory's efficiency and the environment.

This issue of *LCGC International* is packed with invaluable knowledge that will help you stay ahead in the ever-advancing field of chromatography. I hope you find these articles both enlightening and useful in your daily work.

Happy Reading! ■

Mike Hennessy, Jr.
President & CEO, MJH Life Sciences®

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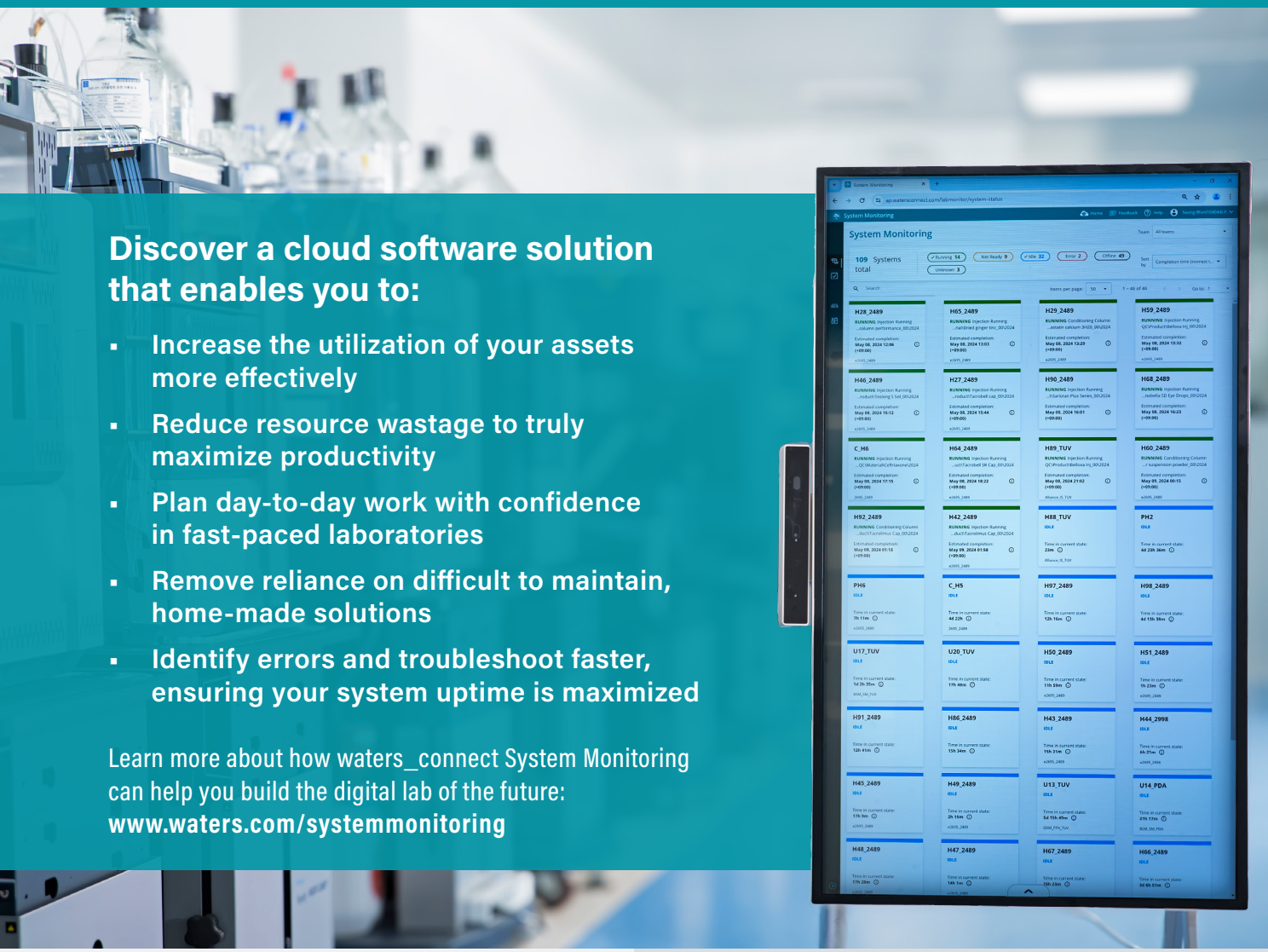

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In-Loop Analyte Degradation in Two-Dimensional Liquid Chromatography: Example and Solutions

Ziqing Lin, Qinggang Wang, and Dwight R. Stoll

The use of two-dimensional liquid chromatography (2D-LC) to assess peak purity is becoming common in the pharmaceutical industry. In this application space, it is critically valuable to demonstrate that all compounds eluted from a column during an LC assay are accounted for, and that no impurities are "hiding" under the peaks of known compounds. Although the addition of the second dimension (^{2}D) separation makes 2D-LC an exquisitely powerful tool, it also warrants additional care when interpreting the results to avoid misidentification of apparently new peaks as coeluted impurities, when in fact they are analytical artifacts. In this installment of "LC Troubleshooting," we describe one such possible artifact that can arise because of the degradation of compounds during the transfer of fractions of the first dimension (^{1}D) column effluent to the ^{2}D separation. We suggest simple experiments to determine whether new peaks observed in 2D chromatograms result from degradation, ultimately increasing our confidence in the interpretation of 2D-LC results.

IN RECENT YEARS, two-dimensional liquid chromatography (2D-LC) has gained popularity in pharmaceutical analysis. Although more work is still needed before this technique can be used routinely in Good Manufacturing Practice (GMP) laboratories, it has enabled many powerful methodologies in the drug development space across pharmaceutical modalities ranging from small synthetic compounds to large biomolecules. The applications include complex sample profiling, coupling separation modes in a single method, peak purity assessment, online desalting, trace analysis, and others (1). Among these, 2D-LC peak purity assessment is especially attractive in the development of synthetic drugs to help reduce risks arising from potential unknown organic impurities that could be coeluted with an active pharmaceutical ingredient (API) in conventional one-dimensional LC (1D-LC) analyses, and thus remain "hidden" to the analyst. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidelines (ICH) require peak purity assessment during LC method validation per *ICH Q2* to ensure chromatographic peak homogeneity (often referred to as "peak purity") (2). However, confirming the absence of coelution using traditional ultraviolet (UV) absorbance or mass spectrometric (MS) techniques can be challenging when impurities are present at low levels (for example, at a 0.05% reporting limit for unknown organic impurities per *ICH Q3A* [3]), or when dealing with compounds exhibiting high structural similarity, if not impossible (for example, stereoisomers having indistinguishable UV absorption and mass spectra). In comparison, 2D-LC provides unique advantages over conventional 1D-LC in this space by introducing a complementary dimension of separation. The addition of this highly selective separation stage can improve the likelihood of discovering an impurity coeluted from a ^{1}D separation, especially when coupled with MS for characterization.

Challenges Encountered in the Development of a 2D-LC Method for Peak Purity Assessment

There are many possible ways to carry out 2D-LC separations, involving a variety of different interfaces and means of transferring analytes from the outlet of the first column to the inlet of the second one (4). The transfer approach most used in practice involves one or more valves fitted with one or more open capillaries that are typically referred to as sample loops. In this case, the ^{1}D column effluent flows into a loop, and it is held there until the ^{2}D separation is ready to accept the introduction of this fraction of effluent into the ^{2}D column (that is, the injection). This transfer mode is compatible with both heart-cutting (LC-LC) and comprehensive (LC×LC) 2D-LC methods with

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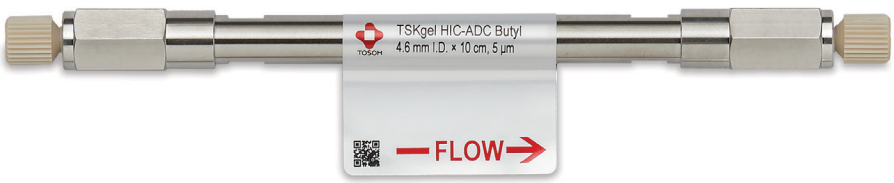

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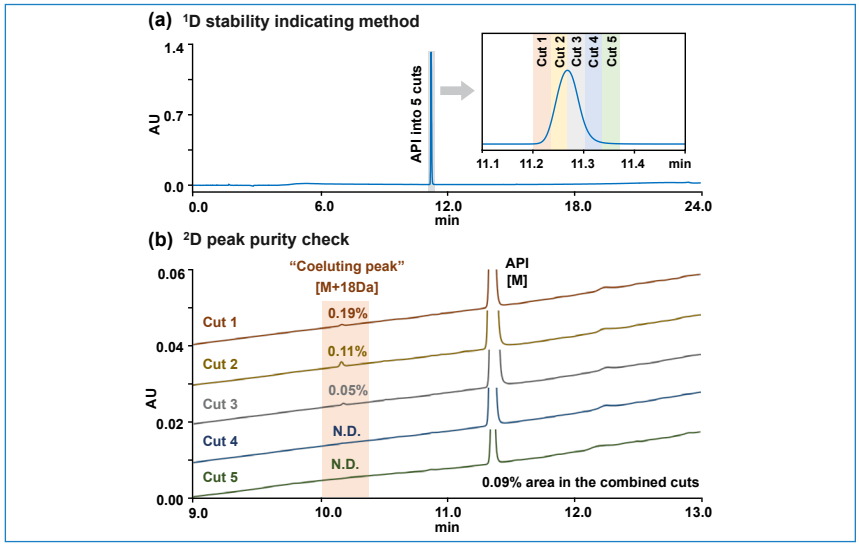


FIGURE 1: "Coeluting peak" observed by 2D-LC separation carried out with multiple fractions analyzed separately: (a) ^{1}D chromatogram obtained using the stability indicating method; and (b) ^{2}D chromatograms of five high-resolution cuts revealing a potential "coeluting" impurity. ^{1}D conditions: Injection volume - 5 µL; Column - Waters XSelect CSH C18, 150 mm x 4.6 mm i.d., 3.5 µm particle size; MPs (mobile phases) - 0.05% trifluoroacetic acid (TFA) in water: MeCN (acetonitrile) (A-95:5, B-5:95); Gradient - 0%B hold for 2 min, 0-100%B in 20 min, 100%B hold for 3 min, equilibration 0% B for 5 min; Flow rate - 1.0 mL/min; Column temperature - 30 °C; Detection – UV (ultraviolet) absorbance at 222 nm. ^{2}D conditions: Column - Agilent RRHD Zorbax Bonus RP, 100 mm x 4.6 mm i.d., 1.8 µm particle size; MPs - 10 mM NH_4OAc (ammonium acetate) in water: MeOH (methanol): MeCN (A-95:5:0, B-5:75:20); Gradient - 0%B hold for 1 min, 0-100%B in 15 min, 100%B hold for 1 min, equilibration 0% B for 3 min; Flow rate - 1.0 mL/min; Column temperature - 40 °C; Detection – UV absorbance at 222 nm. Interface: Loop size - 40 µL; 80% fill; Active solvent modulation enabled. API sample: 0.6 mg/mL in water: MeCN (50:50).

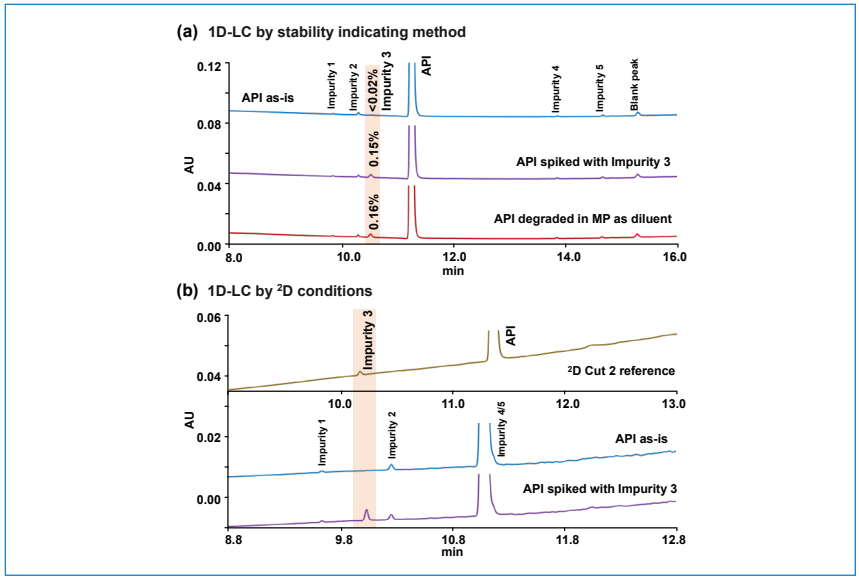


FIGURE 2: In-loop analyte degradation confirmed by 1D-LC. (a) chromatograms obtained by 1D-LC using the stability indicating method: (top) API as-is, (middle) API spiked with Impurity 3, and (bottom) API degraded in water: MeCN (acetonitrile): TFA (trifluoroacetic acid) (65:35:0.05, v/v/v) at 30 °C for 30 min.; (b) chromatograms obtained by 1D-LC, but using the ^{2}D conditions from the 2D-LC experiment: (top) chromatogram for Cut 2 from Figure 1b, (middle) API as-is, and (bottom) API spiked with Impurity 3. Other 1D-LC conditions were the same as those in Figure 1, unless stated otherwise.

either gradient or isocratic elution in one or both dimensions. Heart-cutting 2D-LC (reversed-phase [RP-RP]) with loop transfer has been routinely used at Bristol Myers Squibb to assess peak homogeneity for both APIs and synthetic intermediates as part of method development (5,6). In such applications, the method conditions in the first dimension are treated as fixed and based solely on the existing method for the compound at hand. These ^{1}D methods are often diverse in column selection (both chemistry and dimensions) and mobile phase (MP) composition (organic modifier, additives, and pH). As a result, the volume of a ^{1}D peak targeted for analysis can range from 50 µL for an ultrahigh-performance liquid chromatography (UHPLC) method to 600 µL for some high-performance liquid chromatography (HPLC) methods, depending on the physicochemical properties of a specific compound and the method of choice.

To achieve a quantitative 2D-LC peak purity assessment, two actions must be taken:

1. Choosing ^{2}D method conditions that complement those of the ^{1}D separation; and
2. Transferring the entire target peak to the second dimension for quantitation

A shallow ^{2}D gradient (with analysis time on the order of tens of minutes) is often used. Moreover, stationary and mobile phase chemistries [for example, methanol (MeOH) to complement acetonitrile (ACN), and use of pHs differing by several units to affect the ionization states of target analytes] are chosen to complement the selectivity of conditions frequently used in the first dimension. All these parameters are selected to maximize the likelihood that the ^{2}D separation can resolve compounds that are coeluted from the ^{1}D column.

The quantity of an impurity that is well-resolved by the second dimension could be underestimated or overestimated if the target ^{1}D peak is not quantitatively transferred to the second dimension. For example, an impurity eluted in the tail of the target ^{1}D peak could be missed or underestimated if only the middle portion of the peak is sampled. Conversely, an impurity with an

actual level below the 0.05% reporting limit could appear to be present at more than 0.05% (and thus reportable) if it is eluted in the tail of the ^{1}D target peak and only the tail is sampled. In such instances, the total mass of the main peak in the first dimension is underrepresented. Therefore, it is important to find a way to quantitatively transfer the entire target ^{1}D peak. One solution involves splitting the ^{1}D peak into multiple fractions that are sampled into separate loops. These separate fractions can either be analyzed separately using the second dimension (as in Figure 1b), or they can all be injected serially and then eluted once together from the ^{2}D column (this approach is referred to as "multi-inject" and was used to acquire the data shown in Figure 3b). For example, if the interface is fitted with six 40 µL loops and five are filled to 32 µL (80% filling to avoid analyte loss; one loop is frequently used as a bypass in case the fractions cannot be analyzed as soon as they are sampled) (7), the largest ^{1}D peak that can be quantitatively transferred is 160 µL. Of course, wider ^{1}D peaks can be accommodated by changing to a set of sampling loops with larger volumes. In cases where multiple fractions of a single target ^{1}D peak are analyzed separately, collected fractions may be held in the sampling loops for times up to several hours.

In-Loop Analyte Degradation is a Possibility That Should Be Considered During Method Development

When fractions are held in the sampling loops for long durations, it creates an opportunity for undesired in-loop degradation of analytes captured from the ^{1}D separation. A peak resulting from the detection of a degradant compound can be mistaken as a "coeluted impurity" when the main compound is unstable in the ^{1}D elution solvent or buffer on the timescale of tens of minutes, even if such degradation is not observed during 1D-LC analysis. For example, during the peak purity assessment for an HPLC stability indicating method (Figure 1a), the API peak (~150 µL peak volume) was divided into five cuts for 2D-LC analysis. An additional impurity peak was observed with a resolution better than ten relative to the API in the second dimension (Figure 1b). The impurity level was estimated at 0.09% area, above the 0.05% reporting limit, in the combined cuts. However, the relative peak areas (impurity or API) in individual cuts showed an increasing trend as a function of storage duration in-loop. The fractions that were analyzed last had higher levels (0.19% area in Cut 1 and 0.11% area in Cut 2) than those of the cuts analyzed earlier (not detected in Cuts 4 and 5). Note that the order of analysis for the fractions is dictated by the instrument software and is, in this example, the opposite of the order in which they are collected to avoid cross contamination of the contents of each of the loops. The MS spectrum of this "coeluted impurity" indicated an 18 Da mass increase relative to the API, which coincided with the mass of a known acid-induced hydrolysis degradant that is ordinarily well resolved from the API by the ^{1}D method.

During the development of this stability indicating method (1D-LC), MPs at different pHs were evaluated extensively. Acidic MPs with 0.05% trifluoroacetic acid (TFA) were deemed suitable

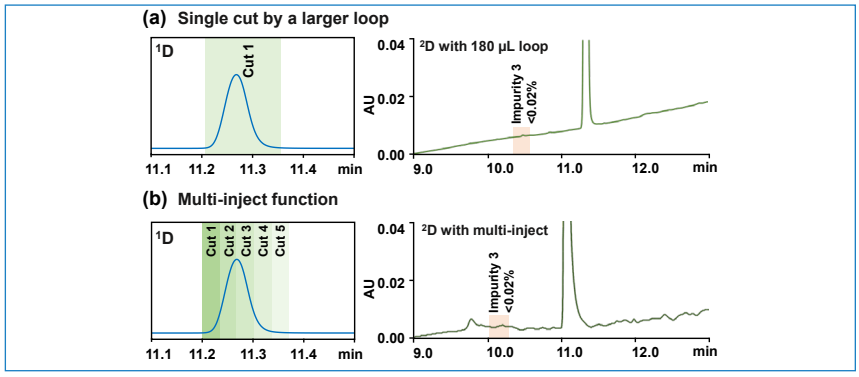


FIGURE 3: Solutions to mitigate undesired in-loop degradation. (a) use a single cut with a larger loop (180 µL) followed by immediate ^{2}D separation; or (b) apply the multi-inject approach to serially inject all ^{1}D fractions into the ^{2}D column before eluting the material with a single gradient elution program. 2D-LC conditions the same as those in Figure 1, unless mentioned otherwise.

to separate all specified impurities (Impurity 1-5) without causing significant on-column hydrolysis (Figure 2a). Neutral MPs, in contrast, did not provide sufficient resolution of Impurities 4 and 5 from the API regardless of stationary phase chemistry. The risk of on-column degradation because of the acidic MPs is well controlled under normal use of the 1D-LC method since the level of this hydrolysis degradant, namely Impurity 3, was always under 0.02% area throughout the robustness study.

Recommended Experiments to Check for In-Loop Analyte Degradation

Two experiments can be used to determine whether an apparent impurity peak observed in the second dimension is the result of in-loop analyte degradation. The first approach (Test 1) is to evaluate the in-solution stability of the compound that is the main component of the target ^{1}D peak, using the ^{1}D MP composition (estimated at the point of elution from the ^{1}D column) as the diluent, at the ^{1}D column temperature. In our case, the API was diluted in a water:MeCN:TFA (65:35:0.05, v/v/v) solution and maintained for 30 min at 30 °C before analyzing it using the ^{1}D method (Figure 2a). A degradation peak corresponding to Impurity 3 was observed at 0.16% area, a clear increase over the API as-is sample (<0.02% area). In this approach, Test 1 provided a positive control to confirm the possibility of in-loop analyte degradation, which is also consistent with the observation that the apparent "coeluted impurity" observed in the second dimension had the same MS profile as the known hydrolysis product of the API. The second approach (Test 2) is to carry out a 1D-LC analysis of the API as-is sample using the ^{2}D chromatographic conditions from the 2D-LC experiment. If the apparent impurity resolved by the ^{2}D separation is actually present in the as-is sample, it should also be resolved by the ^{2}D separation alone. Figure 2b shows that no peaks were observed in the API as-is sample in the retention time window expected for the apparent impurity based on the 2D-LC result (about 10.0–10.3 min.; see Figure 1b, Cut 2). The absence of the "coeluted impurity" in the as-is sample in Test 2 served as a negative control, suggesting that in fact the apparent impurity peak observed in Figure 1b was an artifact generated during the 2D-LC analysis. Results from these two experiments (positive for Test 1, negative for Test 2) can be used to assess whether an apparent impurity peak observed in the second dimension is because of in-loop degradation. On-column stability of the analytes of interest in ^{2}D MPs should be also considered before running the peak purity assessment by 2D-LC.

Suggestions to Mitigate the Risk of In-Loop Analyte Degradation

For compounds that are demonstrated to be stable on-column but unstable in a solution prepared using MP as the diluent, a practical solution to mitigate in-loop degradation is to minimize the fraction storage time by immediately analyzing a fraction in the second dimension once it has been collected in the loop. The simplest approach without modifying the 2D-LC configuration is to build a series of 2D-LC methods where each method involves a single cut but taken at different times across the width of the target ^{1}D peak, so that the entire ensemble of 2D-LC analysis yields data similar to what would be obtained from a single 2D-LC involving multiple cuts across a single 1D target (for example, as in Figure 1). However, the extent to which these two results are similar will depend on the retention time repeatability of the ^{1}D method. A slight retention time shift in the first dimension might cause a partial transfer of the main peak or a repeat analysis of some parts of the peak, and these variations could result in inaccurate quantitation of low-level impurities. Here, we describe two solutions that are accessible using commercially available hardware and software.

The first solution is to use larger loops for transferring the entire ^{1}D peak in one cut. For the instrumentation used in this study, the largest commercially available loop is 180 µL. This corresponds to a maximum ^{1}D peak volume of 144 µL (assuming 80% filling), which can cover main peaks encountered in most UHPLC methods, and some HPLC methods. To demonstrate this, the same API peak targeted in Figure 1a was transferred, captured using one loop, and analyzed using the same elution conditions as in Figure 1b. Figure 3a showed that the apparent impurity peak observed in Figure 1b was no longer observed above 0.02% area, again supporting the idea that the peak observed in Figure 1b was in fact the product of in-loop degradation.

The second solution is to implement the multi-inject feature described previously, which enables serial injection of up to five fractions into the ^{2}D column at once before eluting the material from the column using a single gradient elution program. To show the impact of this approach, five cuts of the target ^{1}D API peak (32 µL per cut) were all injected into the ^{2}D column following by a single gradient elution program (Figure 3b). In this case, the peak we attribute to in-loop degradation was not observed at more

than 0.02% area in the second dimension (with compound identity confirmed by MS). The multi-inject function enables quantitative transfer of larger volume ^{1}D peaks even without the need to change physical loops, which is convenient. Our experience with use of this mode has been that loops need to be washed prior to the next 2D-LC run to avoid carryover, so this is a tradeoff to consider.

Of course, it is also possible to combine the two approaches—that is, use larger loops and the multi-inject feature to accommodate all HPLC methods while mitigating potential in-loop analyte degradation. For instance, a five-cut multi-inject analysis using 180 µL loops could transfer a total of 720 µL peak volume to the second dimension at once. A practical concern is the potential for mobile phase mismatch to compromise the performance of the ^{2}D separation when such a large volume is transferred, even if an active solvent modulation approach is used to mitigate this risk (4).

Summary

Peak purity assessment is emerging as an important application of 2D-LC in the pharmaceutical industry. In-loop analyte degradation can occur in 2D-LC applications, which can lead to false positives. In the context of peak purity assessment, a false positive would lead one to conclude that the ^{2}D separation has separated an impurity from the main compound present in the target ^{1}D peak, when in fact the additional peak observed in the second dimension is an analytical artifact. Obviously, it is essential to understand when this might occur, carry out experiments to check for in-loop degradation when it is suspected, and make decisions when developing 2D-LC methods that minimize the occurrence of such artifacts in the first place.

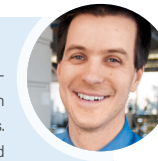
In this installment of "LC Troubleshooting," we have discussed a case study that used a real pharmaceutical API to demonstrate what in-loop degradation looks like when it occurs, and tests that can be performed to assess whether or not in-loop degradation is occurring. In cases where the extent of in-loop degradation depends on the time a fraction of ^{1}D effluent is held in a sampling loop, minimizing this holding time is critical. Two accessible ways to do this are to: 1) use large sampling loops so that entire ^{1}D target peaks can be quantitatively sampled using just one or two loops; or 2) use the multi-inject approach that enables serial injection of multiple fractions of ^{1}D effluent into the ^{2}D column at once following by elution using a single gradient program. In the case study discussed here, we have shown that both approaches are viable, however they both have advantages and disadvantages that users should consider prior to implementation. ■

Acknowledgment

We would like to thank Dr. Yehia Baghdady for his work in implementing large transfer loops.

This article has supplemental information and references only available online. **Scan code for link.**

ABOUT THE COLUMN EDITOR



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Highlights from the 52nd International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2024)

Cory E. Muraco

The 52nd International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2024), chaired by Susan Olesik, was held from July 20 to 25 in Denver, Colorado, USA. This "Column Watch" installment presents many of the highlighted topics and trends observed at this symposium.

THE 52ND INTERNATIONAL SYMPOSIUM on High Performance Liquid Phase Separations and Related Techniques (HPLC 2024) was held in Denver, Colorado, and chaired by Susan Olesik (The Ohio State University). Continuing a long-standing trend, the HPLC symposium is the premier gathering of separation scientists in liquid chromatography and related techniques. The conference was composed of high-quality oral presentations from leading scientists, engaging poster sessions with scientists and students from all levels, and numerous opportunities to network and (re)connect with colleagues and friends.

In this installment of "Column Watch," notable trends and highlights from the conference are described. Akin to previous symposia reviews (1–6), many colleagues in attendance were asked for their expert opinions on the conference, and what "stuck out to them" the most in terms of emerging, sustaining, or fading trends in the science. The article that follows is a distillation of these highlighted topics and some personal musings on the conference. For another perspective on the symposium, please also check out the "Analytically Speaking" podcast, where Dwight Stoll and James Grinias provide their analysis of the symposium (7).

Analysis of Oligonucleotide (and Other) Biopharmaceuticals

Oligonucleotides were by far the most discussed topic at HPLC 2024. The promise of this class of biomolecule being used to treat the most difficult diseases has driven the exponential increase in interest in the synthesis, development, and analysis of oligonucleotides. Many talks at the symposium centered around sample preparation, chromatographic analysis, and oligonucleotide purification. In addition, "legacy" research topics in the biopharmaceutical industry continued to be discussed at the show, including monoclonal antibody (mAb) analysis, antibody-drug conjugate (ADC) analysis, and peptide separations. Finally, several talks fell into the "omics" category, including proteomics, metabolomics, and glycomics. Indeed, the quest to use high-performance liquid chromatography to better understand the pharmaceuticals used to treat disease and the biochemistry occurring in our bodies was well-represented at HPLC 2024. A few of the talks on the topic of biopharmaceuticals and omics applications are presented here.

The first talk of the first day of the symposium made clear the importance of oligonucleotide analysis in the biopharmaceutical industry. Claus Rentel (Ionis Pharmaceuticals) delivered a comprehensive talk on oligonucleotide therapeutic analysis. After a review of the current critical quality attributes (CQAs) in oligonucleotide analysis, Rentel discussed the challenges in impurity analysis of oligonucleotide therapeutics. Several of the impurities that may be present in an oligonucleotide formulation are positional isomers or species that only differ by one dalton; these impurities cannot be resolved

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by mass spectrometry (MS) alone. To achieve resolution of these impurities, Rentel's work highlighted two-dimensional (2D) weak anion-exchange ion-pair reversed-phase chromatography (WAX-IP-RPC) methods used to resolve the species. Rentel also provided several strategies for characterizing silencing ribonucleic acid (siRNA) duplexes via size-exclusion chromatography (SEC), hydrophilic-interaction liquid chromatography (HILIC), ion-pair reversed-phase chromatography (IP-RPC), and strong anion-exchange (SAX). Finally, Rentel explained to the audience that in certain situations for Good Manufacturing Practices (GMP) in industry, spectral clarity and quality trumps chromatographic resolution, underscoring the fact that even though advanced methods and instrumentation may be available, ease of adoption into manufacturing and quality laboratories is more desired.

Characterizing biotherapeutic formulations for purity has been the central application driving interest in new methods and tools in protein chromatography. However, as noted in an interesting talk by Fred Regnier (Novilytic), appraising the function of the purified protein (mAb) is of equal importance. Regnier introduced the concept of mobile affinity selection chromatography (MASC) to examine the function of monoclonal antibodies (mAbs) in crude solution. In this mode of chromatography, an affinity selector is added to the mobile phase which binds to the Fc receptor of a mAb. The chromatographic trace then provides two pieces of data to the analyst: a sharp peak indicating efficient capture of the target antibody and a negative baseline peak indicating that the Fc function is working (as the Fc portion of the antibody is binding affinity selector). This technology, commercialized by Novilytic, was then applied to resolving mAb monomers from aggregates and fragments, and further extended to the analysis of different mAb subtypes and bispecific antibodies (bsAbs).

Popularized by the success of the COVID-19 vaccines, messenger RNA (mRNA)-based biotherapeutics were the subject of various presentations at HPLC 2024. Alexandre Goyon (Genentech) delivered such a presentation where he discussed strategies for analyzing mRNA and plasmid-based biotherapeutics. Plasmids can exist in several different forms, including supercoiled, open-circular, linear, or as multimers, thus leading to a complex characterization challenge. Certain modes of chromatography, like anion-exchange (AEX), can underestimate certain forms (i.e., open-circular); therefore, particular attention needs to be taken in developing the analytical method. Goyon documented how plasmids can degrade or be affected depending on the storage diluent. In addition, Goyon documented how certain impurities can affect therapeutic efficacy more than others, such as how open-circular forms affect knock-in of the plasmid into cells more than multimers. Nevertheless, larger multimers need to be detected, and Goyon highlighted how SEC can be used to characterize mRNA multimers ranging from 1000–5000 nucleotides (nts). Finally, in a novel use of multidimensional chromatography, Goyon demonstrated the use of columns with immobilized enzymes bonded to the stationary phase to perform online nucleotide mapping of mRNA species.

As the few highlighted talks above indicate, the biopharmaceutical industry is continuing to evolve and create more complex biotherapeutic modalities. These new formats require advanced characterization techniques that continue to drive innovation in LC instrumentation, detectors, and stationary phases. Future instances of this symposium are primed to witness continued advances in the field of separation sciences for biotherapeutics.

Sustainability Applied to Liquid Chromatography

Sustainability and the "greening" of liquid chromatography continued to be a trend at HPLC 2024. This observation was validated by the number of talks that either centrally focused on the concept of sustainability or applied aspects of green chemistry to their research.

Robert Kennedy (University of Michigan) delivered a talk on innovations in capillary liquid chromatography (LC). Kennedy used a 5 mm x 0.3 mm I.D. capillary column with electrospray ionization (ESI) MS for high-throughput analysis. According to Kennedy, one of the purposes of this paper was to reduce the amount of acetonitrile used in LC methods. Kennedy used a flow rate of 70 µL/min and employed a novel injection technique called "droplet injection," which can continuously inject samples in series. This technique allowed Kennedy to process hundreds of samples enabling real time monitoring of organic reactions. Each chromatographic run lasted only 6 s. At the end of the talk, Kennedy showcased how his method ranked in terms of greenness by utilizing a calculator from the American Chemical Society (ACS) (8). His Analytical Method Greenness Score was ranked high, indicating a method that is sustainable.

Continuing the trend of using capillary LC to minimize sample consumption and solvent use, Samuel Foster (Rowan University) presented results obtained with a portable capillary LC instrument. In addition to the sample and solvent savings of using capillary flow LC, additional "green benefits" of this method include the fact that the instrument is brought to the sample (on-site analysis) instead of sending the sample to the instrument (which entails emissions from transporting the sample to the laboratory). Foster noted that additional advances in instrument and detector design (from Axcend Corporation) will improve the sensitivity of the measurements.

Elia Psillakis (Technical University of Crete) delivered a comprehensive presentation on Circular Analytical Chemistry. This approach to defining sustainability builds upon the concept of Green Chemistry through 12 goals, as illustrated in Figure 1. Psillakis walked the audience through each goal, and she emphasized the benefits that could be achieved if end-users and vendors adopted these principles. Many attendees commented that the presentation was a welcome invitation to changing long-held mindsets in analytical chromatography and chemistry that is worth addressing.

Based on these few highlighted talks, and other discussions observed at HPLC 2024, the topic of sustainability is here to stay. Larger corporations are adopting internal policies to look for ways to become more sustainable, and the analytical chromatography community has several tactics that can be employed to facilitate the industry's

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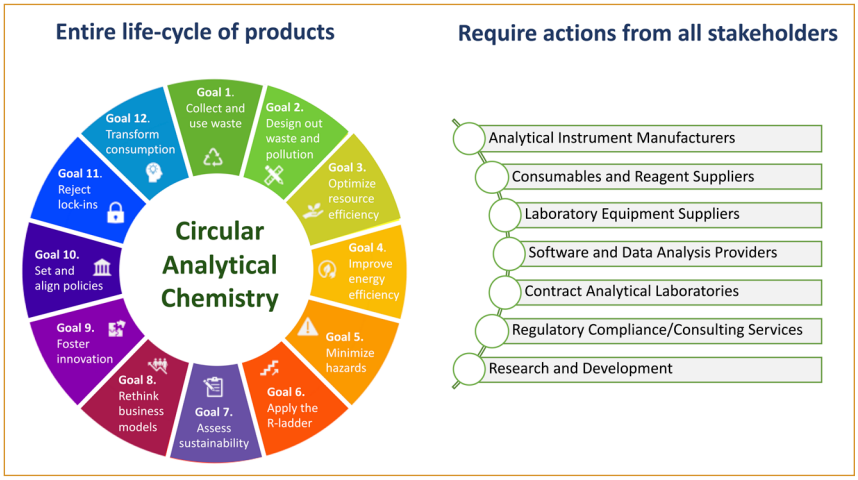

FIGURE 1: The twelve goals of Circular Analytical Chemistry.

transition to more sustainable practices.

Bioinert LC and LC Consumables

As was also highlighted in the HPLC 2023 review, the importance of bioinert instrumentation and consumables was featured in many conference contributions throughout HPLC 2024. Many of the talks discussing oligonucleotide analysis made use of bioinert instruments and hardware owing to oligonucleotides' proclivity to adsorb to stainless steel portions of the flow path. Several vendors have employed metal passivation strategies that minimize the presence of exposed stainless steel in the injection flow path. The industry has moved well past "simple" PEEK-clad columns and tubing, as noticed at HPLC 2024. As one attendee said, "If a vendor does not have bioinert consumables or instruments, we go elsewhere." As new, complex biotherapeutic modalities and increasingly intricate, matrix-rich samples for environmental, clinical, or food analysis become more common in testing, the capabilities of current bioinert coatings to conceal secondary interactions and adsorption will continue to be challenged. It will be intriguing to observe the innovative solutions that arise to address sample loss and chromatographic performance deterioration.

Other Conference Highlights

Poster Presentations

The poster presentations continue to be an important aspect of the HPLC symposiums. The breadth of topics and techniques on display at the poster presentations covered the entire spectrum of the chemical industry employing liquid chromatography (LC). In alignment with the oral sessions, many posters included topics focusing on biother-

apeutic analysis, including oligonucleotides, mAbs, ADCs, and others. Many posters also included supercritical fluid chromatography (SFC) as the analytical method of choice, which was an interesting approach to making methods more sustainable due to the limited use of solvent in the methods. It will be interesting to see if SFC continues to become more prominent at the HPLC symposiums in the future, especially with the renewed push for greener methods and with SFC instrumentation becoming more user-friendly.

HPLC Tube Competition

The HPLC Tube Competition is a scientific contest for the best video in which each author presents the impact of their research for society. The contest provided a fun and entertaining alternative approach to presenting scientific information whilst simultaneously highlighting the authors' creative talents. As noted by several attendees, the HPLC Tube was a conference highlight and continues to be an invigorating and amusing part of the overall symposium program.

Conclusions

The HPLC 2024 symposium was well-organized, informative, and engaging. The analysis of oligonucleotides and their related impurities was a driving force behind many of the presentations. Continued advances in this area will drive overall innovation in chromatography and enable the use of the separation sciences to solve ever increasing challenges. Sustainability was also a central topic at the symposium. Whether through reduction of organic solvent in the mobile phase, use of capillary LC columns and instruments to minimize sample consumption, or adopting completely new mindsets to analytical chemistry (Circular Analytical Chemistry), the separation sciences are examining how they can play a role in green chemistry adoption. Several other topics were also repeated throughout the conference, including the use of bioinert instruments and consumables for biomolecule analysis, using SFC to improve sustainability, and multidimensional LC, among others. Innovation continues to be shown at the HPLC symposium, and what was presented at this edition of the symposium continues to validate the claim that many at the show echo: "This is the premier conference for separation scientists."

The next HPLC meeting will be in Bruges, Belgium from June 15th–19th, 2025. ■

Acknowledgments

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Optimizing Splitless Injections in Gas Chromatography, Part 1: What It Is and What Happens When We Inject

Nicholas H. Snow

In gas chromatography (GC), injecting samples is seemingly among the simplest steps in the analysis. For trace quantitative analysis, the splitless technique is most used as an inlet capable of performing both split and splitless injections, and is standard equipment on most gas chromatographs. In this and upcoming installments, we take a closer look at splitless injection, which is not as simple as it seems. Beginning with the syringe and autoinjector, the sample is subjected to passage of the syringe needle through a septum, ejection from the needle, deposition onto a surface in the inlet, evaporation, transfer to the column, condensation of the sample vapor in the column, and finally, temperature programming. All of this typically occurs in the first 30–60 s of the separation. If unwanted chemistry occurs in any of these steps, the injection may not be reproducible enough for precise quantitation. As we proceed, we will discuss some straightforward optimizations that can be performed to assist in obtaining reproducible splitless injections.

THE INLET IS THE MOST critical and least understood part of a gas chromatograph. Although injecting liquid samples seems so simple, the processes involved in transferring samples from a syringe into a capillary column are very complex. Even the terminology confuses many chromatographers. In this article, and in the best usage, the term *inlet* refers to the device mounted on the gas chromatograph into which a syringe needle penetrates when a sample is injected. *Inject* (as a verb) and *injection* (as a noun) refer to the process of transferring a sample from a syringe or valve into the inlet. An *injector* refers to a device external to the gas chromatograph, usually an autoinjector, but sometimes the chromatographer themself, that performs the act of injection. We often use combined terms, such as *split injection*, *splitless injection*, or *on-column injection* to describe the entire process.

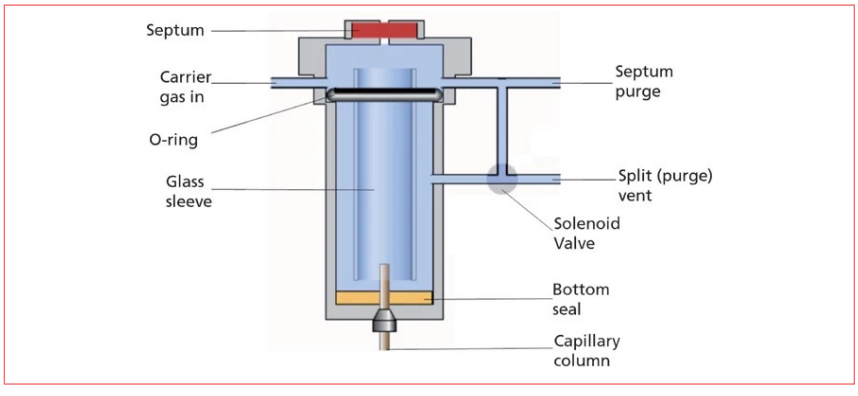

FIGURE 1: Schematic diagram of a split-splitless inlet.

An inlet capable of being used to perform both split and splitless injections is standard equipment on nearly all commercial laboratory gas chromatographs. When reporting results or writing methods, be careful to accurately describe the inlet and technique used. Very often, when reviewing literature, I see the injection technique or inlet described as "split/splitless" rather than "split" or "splitless." The inlet can only do one technique at a time, split or splitless, not both.

Before considering the details of splitless injection, it is useful to briefly discuss its origin. In his classic book, Grob, who performed the work, describes this in detail. In referring to this book, we see that split and splitless injections and inlets are complex

enough to require an extensive text (1). The split technique came first, and was among the original techniques used for injecting samples at the inception of capillary gas chromatography.

Figure 1 shows a schematic diagram of the inlet used for split and splitless injections, captured from *LCGC International*'s online learning platform, ChromAcademy (2). Ideally, split injection involves setting up the inlet so that the injected sample is rapidly vaporized inside the glass sleeve, and mixed with carrier gas at a high flow rate. From the glass sleeve, there are two ways out: the column, which has a low flow rate, for example, 1 mL/min, and the split vent, which has an adjustable, usually high, flow rate, for example, 50 mL/min. Ideally, in this example, a 50:1 ratio of sample out the split vent to sample in the column is generated.

Inlet splitting was thought to be necessary from the inception of capillary gas chromatography, as it was believed that too much injected solvent would cause the stationary phase coating to strip from the inside walls of the column. Note that today's fused silica columns with chemically bonded and crosslinked stationary phases did not exist then. Stationary liquid phases were coated on the inside walls of the capillary, with the capillary usually being made from glass. Columns of the day were much more fragile, difficult to manufacture, and expensive than they are today.

The first splitless injection was performed by accident in the late 1960s (3). At that time, a simple way to save carrier gas but keep the instrument running during downtime, today's "gas saver" feature, was to simply close the split vent when the instrument was not running. Most injections at that time were performed manually, and most data analysis was done using a strip chart recorder. Modern autoinjectors and data systems did not exist. Imagine then, injecting a sample and starting the recorder, but not opening the split vent beforehand. You look down at the instrument about a minute later, realize your mistake, and open the vent.

At this point, you expect that your analysis, and possibly your column, are ruined. The baseline is "maxed out" at the top of the paper, and it does not seem to come down. You are hoping that the recorder will eventually return to baseline, and that your stationary phase is still in the column. You step away for a short time, and return to a pleasant surprise: a pretty normal-looking chromatogram, but with a big solvent peak.

The signal returned to baseline relatively quickly, and the stationary phase was apparently not damaged. Interestingly, the analyte peaks are tall and mostly symmetrical; we would call these good peaks. Even this simple story describes what a splitless injection and inlet are, and why

FIGURE 2: Result of about 1 teaspoon of water being added to a cast iron skillet. Full evaporation required appromixately 10 s. Note that some droplets are colored due to contamination on the skillet surface. Reprinted from reference (4) with permission of the author.

we do them. Splitless is so interesting and useful because it uses the same hardware as split, so a split/splitless inlet is really two inlets in one. The split inlet is good for routine analysis of relatively high concentration (ppm and higher) samples and for complex mixtures requiring a very fast injection process and sharp peaks. The splitless inlet is useful for lower concentration analysis (ppm and lower). Both inlets have excellent performance in quantitative analysis when operated correctly. We will now explore some of the lessons from the initially surprising results of the first splitless injection seen in our story.

This story, undoubtedly embellished (as legends often are), demonstrates the key features of the splitless injection technique and inlet, teaching several lessons about good chromatography and science. As described, there are several variables involved in setting up for a splitless injection. For the inlet, we consider the inlet temperature and pressure, carrier gas flow rate, purge valve time (how long the split vent is kept closed following the injection), and geometry and surface chemistry of the inlet liner.

In the story, it was likely 1 min or more from the injection to the opening of the split vent, so it can be surmised that the analytes took the full 1 min to travel through the inlet. Yet the peaks we see in our chromatograms are much sharper than one minute. This suggests that there must be a focusing mechanism that happens in the column. Additional variables must therefore include column dimensions, stationary phase film thickness, the chemical nature of the stationary phase, sample solvent, and analytes.

Finally, in the story, the liquid sample was manually injected using a syringe. Especially in the days of manual injection, proper syringe handling techniques were required for reproducible injections. When I started graduate school with Professor McNair at Virginia Tech, our first lesson on injecting was to make ten consecutive manual injections of a simple solvent mixture with a less than 2% relative standard deviation on the peak height. Even with a short 3-min run time, this took all day for me to achieve. Today, we use fast autoinjectors, but the syringe and its operation remain a critical part of the injection process.

As we now see, the injection process includes several steps, beginning with drawing a liquid sample into the syringe, followed by inserting the syringe into the inlet, usually through a septum, depressing the plunger to eject the sample into the inlet, vaporizing the sample in the inlet, mixing it with the carrier gas, and transferring the sample vapor-carrier gas mixture into the column. For now, let's assume that we are using a fast autoinjector to inject a 1 µL liquid sample with a typical 10 µL syringe, the most common configuration. Let's assume for now that the autoinjector does its job, rapidly and quantitatively, injecting the sample into the inlet.

One challenge in thinking about the inlet is that we cannot see what is going on inside the glass liner when the sample is injected. There is an excellent set of videos that accompany the Grob text mentioned earlier, produced using an all-glass inlet that clearly show what happens under different injection conditions. Using some simple tools likely available in your kitchen, you can simulate what really happens in the inlet immediately following injection.

Figure 2, reprinted from an earlier installment of "GC Connections," shows the result of a very simple experiment (4). Originally, I termed this the "This is your brain on drugs" experiment, after a similar and famous public service television announcement in the United States (5). To repeat this experiment, obtain a heavy cooking pan, such as a cast iron skillet, a teaspoon, and some water. Heat the skillet on the stove over high heat, and make it hot. Using the spoon, pour a small amount of water into the skillet, and observe the results.

In Figure 2, we see that, even though the thermal mass of the skillet is much greater than the water and the skillet is heated well above the boiling point of water, the water does not immediately evaporate; it instead appears to dance around the surface of the skillet for several seconds or more before it all evaporates. This behavior when a small amount of liquid is placed on a hot surface has significant implications for liquid sample injections in gas chromatography, and it points out some common misconceptions about injections that are commonly described in textbooks and short courses.

Misconception #1: The liquid sample evaporates instantaneously upon injection. From Figure 2, we see that the liquid does not instantly evaporate. Remember that gases are very strong insulators; think about the double- and triple-paned glass windows commonly used in cold climates. The gas space between the panes provides excellent insulation. The insulating properties of gas within the inlet prevent the heated surfaces from quickly transferring that heat to the sample. *Reality: liquid samples require several seconds or more to evaporate.*

Misconception #2: The liquid sample evaporates quickly when it lands on the heated surface. The dancing water droplets seen in Figure 2 show that, even when the liquid strikes the surface directly, it still

does not immediately evaporate. When the liquid strikes the surface, the liquid touching the surface evaporates, leaving a small vapor space between the surface and the rest of the droplet. This then insulates the rest of the droplet, with movement of this heated vapor causing the droplet's movement. *Reality: The liquid evaporates slowly from the surface.*

Misconception #3: Mixing between the carrier gas and the sample vapor is rapid and homogeneous. Under splitless conditions, inlet liner flow is slow. With a typical volumetric flow rate of 1 mL/min and an inlet liner volume of about 1 mL, approximately 1 min is required for the full liner volume to be swept by carrier gas. Therefore, the evaporation and mixing process is slow and possibly non-homogeneous, especially if the inlet is dirty. *Reality: Evaporation and mixing with carrier gas in the inlet are slow.*

These realities demonstrate some characteristics of your own splitless inlets that you may have observed. First, the inlet is heated to facilitate sample evaporation. Second, the glass inlet liner most likely contains obstructions, baffles, or glass wool to provide greater heated surface area for evaporation and to prevent injected liquid from shooting straight to the bottom of the inlet. Third, the injection is slow, so there must be some focusing processes that occur after the sample reaches the column; this is why nearly all methods including splitless injection include temperature programming. In future installments, we will further explore setting up the inlet and column conditions for successful splitless injections. We will also share special techniques that can be used if traditional injection does not provide adequate peak shapes or quantitative reproducibility.

A splitless inlet is simultaneously simple and complex. Setting up the inlet and performing the injection is quite simple. However, there is much background chemistry going on that can become complex, which can be one of the major and most common causes of reproducibility and performance problems in gas chromatography. ■

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Are You Sure You Understand *USP* <621>?

Paul Smith and R D McDowall

The updated version of *United States Pharmacopoeia (USP)* general chapter <621> Chromatography has changes that impact chromatography parameters. In this column, we look at the current version and the update of *USP* <621> on high performance liquid chromatography (HPLC) that becomes effective 1st May 2025. Do you fully understand the changes and how they will impact your laboratory?

CHROMATOGRAPHIC ANALYSIS IN regulated Good Manufacturing Practice (GMP) laboratories is under the control of pharmacopoeial general chapters. These should define the qualification parameters for instrumentation and System Suitability Test (SST) parameters to demonstrate the analytical system is under control. The changes are a result of the harmonization process between *USP, Japanese Pharmacopoeia* (*JP*) and *European Pharmacopoeia* (*Eur. Ph.*). The harmonized *USP* <621> became official in December 2022; however, a few subsections of the SST section were delayed and will become official in May 2025 (1). Are you prepared?

We also look and revisit the use of SSTs for Analytical Instrument Qualification (AIQ) that were discussed in three earlier "Questions of Quality" columns.

Before providing an overview of the changes in *USP* <621>, we compare the structure of the chromatography general chapter against the *USP* spectroscopic general chapters shown in Figure 1.

USP Chromatography and Spectroscopy General Chapters

USP chapter numbers below 1000 are mandatory and applicable, and those between 1000 and 1999 are informational and strong guidance for the industry. These are shown in different colors in Figure 1. *USP* <621> currently covers many existing chromatography modalities in a single chapter with few exceptions, such as supercritical fluid chromatography (SFC) and Capillary Electrophoresis. Historically, the same approach was used with spectroscopic techniques originally featured in *USP* <851>. However, *USP* has now split each spectroscopic technique into separate technique and applicable chapters (*USP* <852> to <858>) along with informational chapters covering fundamentals and applications (*USP* <1852>–<1854> and <1856>–<1858>) as described by Burgess and Hammond (2). This is shown for ultraviolet–visible (UV–vis) and near-infrared (NIR) spectroscopy on the right of Figure 1.

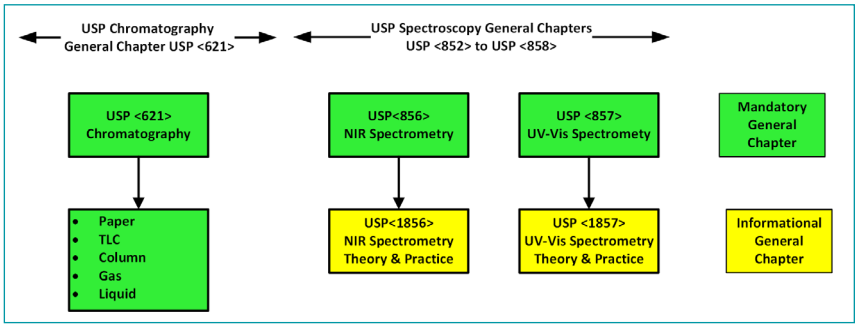

FIGURE 1: Structure of *USP* <621> and two spectroscopic General Chapters.

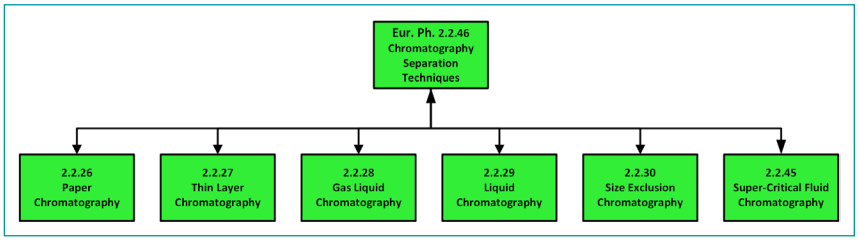

FIGURE 2: Structure of chromatography General Chapters in the *European Pharmacopoeia*.

In contrast, the *European Pharmacopoeia* has an overarching general chapter 2.2.46 (3) that is linked to six general chapters for specific chromatography techniques shown in Figure 2.

Given the importance of chromatography in pharmaceutical analysis, is the update of

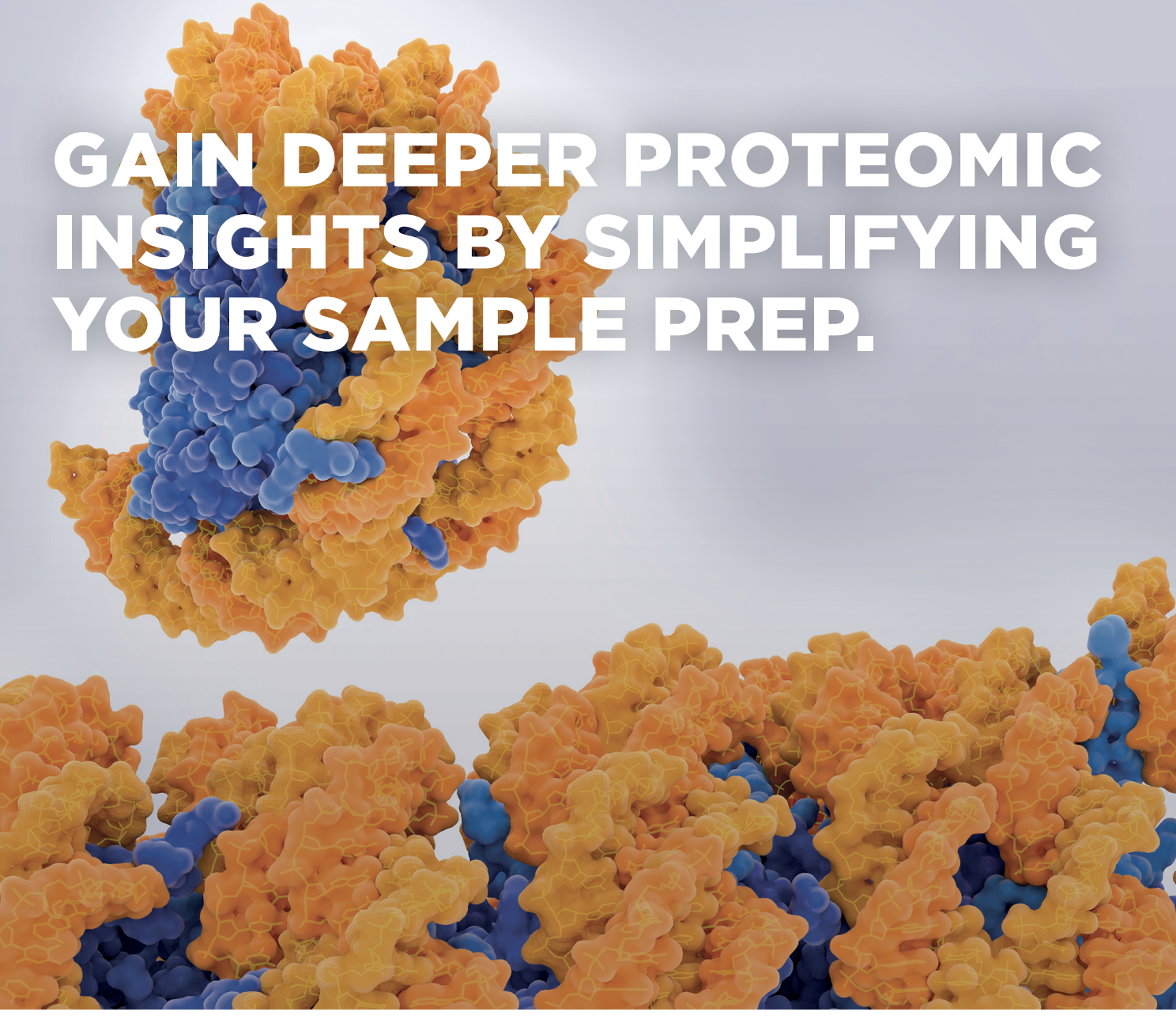

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TABLE I: Timeline of *USP* <621> changes published in *Pharmacopoeial Forum* 2002–2017.

Year	PF	Pages	Revision / High Level Nature of Change / Comments
2002	28(3)	4	Column Packing (Changes to packing L53)
2002	28(3)	1	Column Packing
2002	28(4)	6	Column Packing
2002	28(6)	6	Column Packing
2004	30(1)	6	System Suitability Test, Harmonization of Changes with EP (20 years ago)
2004	30(2)	11	Thin-Layer Chromatography Section
2004	30(3)	16	Column Packing, Interpretation of Chromatograms, Glossary of Terms
2004	30(5)	14	Introduction of Quantitation Limit (QL) Solution
2004	30(6)	15	Interpretation of Chromatograms, Glossary of Terms
2005	31(3)	17	System Suitability – Reference to <1226> Verification of Procedures
2005	31(6)	11	Column Packing
2006	32(2)	1	Indefinite postponement of QL/detector sensitivity
2006	32(3)	6	Column Packing
2006	32(4)	9	Remove <1226> Reference, Column packings moved to new section
2008	34(1)	4	System Suitability Test – Gradient - no change for Flow / Mobile Composition Column Diameter Changes, Changes in Flow Rate, Symbols
2008	34(3)	4	System Suitability Test – Broader scope and application
2008	34(5)	6	HPLC – Flow – 50%, Signal to Noise (S/N) Definition
2009	35(6)	14	Major Revision, Harmonizing with EP, Half Height of Peak Calculations (Resolution R, Number of Theoretical Planes (N), Implementation of Repeatability Table (from 2 % RSD)
2016	42(3)	11	SST – Remove High Particle Size Requirement, for GC, allowance for linear velocity adjustments
2017	43(5)	13	Stage 4 Harmonization with European Pharmacopoeia

USP <621> overdue? Division into separate technique general chapters similar to the EP and the *USP* spectroscopic general chapters could be more didactic and effective. In fact, this was proposed for the *USP* 2005–2010 cycle (4), but no progress has been made yet. Why?

Timeline of USP <621>

USP has included a general chapter related to chromatography since at least 1955, and the first publication of the chapter as *USP* <621> was in 1980. *USP* <621> is one of the most important and accessed *USP* general chapters for the pharmaceutical industry. The scale of *USP* <621> is evident when searching for "<621>" in the current *United States Pharmacopeia–National Formulary* (*USP–NF*) electronic edition, which yields 3,980 results. Therefore, the scale of the changes required if <621> was to be split (which is required to align with other instrumental related general chapters) could potentially explain why *USP* has been reluctant to implement the change.

Because of the broad scope of *USP* <621>, it has undergone many revisions and updates over the years. Between 2002 and 2017, 20 entries related to changes were published in Pharmacopeial Forum (PF). This included successive PF updates; for example, four updates were made in 2002 and five were made in 2004. Additionally, the changes included in the many PF versions of <621> can be difficult to trace into released updates of the general chapter, possibly compounding uncertainty over changes. To be clear, effective August 1st 2014, <621> mapped the "allowable adjustments" that can be made to *USP* monograph methods without having to re-validate the method. Prior to this, as introduced in 2009, changes to isocratic high performance liquid chromatography (HPLC) methods were only possible to achieve system suitability. Major changes over this time are summarized in Table I.

It is possible that a chapter or monograph goes to PF with changes but they are either not approved or it takes too long for the approval to take place. This means that not every change in PF will necessary become official in the *USP-NF*. This is why it is difficult to track draft proposals in PF to issued changes to <621>. Most of the changes between 2002–2017 resulted from the Pharmacopoeial Discussion Group (PDG) taking actions to finalize harmonization details.

In 2017, PF43(5), the *USP* published the Stage 4 Harmonization version of <621>, with it becoming official in the *USP-NF* on December 1st 2022. With this update, as was previously allowed for LC methods based on isocratic elution, modification to LC methods based on gradient elution could also be made without revalidation, provided that System Suitability requirements are met. Additional verification tests may still be required. Based on stakeholder feedback, the *USP* proposed postponing a few sections to allow the industry additional time to conduct risk assessment and evaluate the impact of the changes regarding two proposed new SST requirements. Below are examples are two Intent to Revise notices:

- **System Suitability Section Aug 26, 2022:**
  - The purpose of this revision is to delay the implementation of the sections titled "System Sensitivity' and "Peak Symmetry" under System Suitability: https://www.uspnf.com/notices-gc-621-ni-tr-20220826.
- **System Suitability Section Aug 25, 2023:**
  - Based on the comments received from stakeholders, the committee

considered it appropriate to introduce a change in the text under the subsections "System sensitivity" and "Peak Symmetry."

- *This new PF proposal would require an additional step to align the official date for the two sections proposed to be revised with the target official date of the PF proposal, namely changing it from December 1, 2023 to May 1, 2025. Chromatography* USP-NF *(uspnf.com).*

The version of <621> effective May 1st, 2025 includes these postponed changes (1).

Detailed Structure of *USP* <621>

As *USP* <621> has evolved, so have the sections contained within it. The new version includes the sections shown in Table II.

Based on the extensive chromatographic theory/explanation included in *USP* <621>, the different structure becomes more evident when compared with the modernised spectroscopy general chapters. There are now 19 pages for *USP* <621> compared to just nine pages for *USP* <857> on UV–vis spectrometry (5).

Interpreting *USP* <621>

When reading any pharmacopoeial General Chapter, it is important to understand that it does not exist in a vacuum. There is interaction between it, the General Notices (6) (the bit you skip over to get to an analytical general chapter), and the monograph for the substance or product being analyzed, as shown in Figure 3.

The hierarchy means that the general chapter must be followed unless there is a statement in the monograph that overrules the general chapter. You will often read the statement, "unless specified in the monograph," in a general chapter. Additional information for an analysis is given in the General Notices. For example, if the monograph says, "Weigh about 100 mg," Section 8.20 of the General Notices states, "'About' indicates a quantity within 10% (6)."

New System Suitability Changes in *USP* <621>

The two changes to SST sections are the inclusion of new requirements for system sensitivity (signal-to-noise ratio) and peak symmetry; the current and new definitions are shown in Table III.

Comparing the old and new definitions can give the impression of slight changes, but like regulations—and *USP* <621> is a mandatory general chapter—the devil is in the details. Here, the pharmacopoeia says what you must comply with, but there are individual interpretations of how a laboratory or company complies with it.

Let us look in detail at system sensitivity:

- **Does a Monograph Specify a Limit?** A corollary of Sod's Law is when all else fails, read the manual, or in this case, the monograph. Does it specify a reporting threshold? If not, don't measure the signal-to-noise (S/N) ratio. However, do not forget, this is strongly recommended to be established as control strategy for impurities procedures.
- **When to Measure (S/N)**: The first part of interpreting the new definition is to define when this SST requirement must be used. It should be self-evident that if you are measuring S/N, then you will be determining impurities at or near to limits of quantification. Apparently

TABLE II: Structure of *USP* <621> Chromatography, effective May 2025 (1).

Section	Contents	
INTRODUCTION		
GENERAL PROCEDURES	Paper Chromatography Thin-Layer Chromatography Column Chromatography	Gas Chromatography (GC) Liquid Chromatography
CHROMATOGRAPHIC COLUMNS	See USP-NF, Reagents, Indicators, and Solutions-Chromatographic Columns	
DEFINITIONS	Chromatogram Distribution constant (K_0) Dwell volume (D) Hold-up time (t_M) Hold-up volume (V_M) Peak-to-Valley Ratio (p/v) Plate height (H) Plate number (N) Reduced plate height (h) Relative retardation (R_{rel}) Relative retention (r) RRT Resolution (R_s)	Retardation factor (R_f) Retention factor (k) Retention time (t_R) Retention time (t_0) (unretained) Retention volume (V_0) (unretained) Separation factor(α) Signal-to-noise ratio (S/N) Symmetry factor (A_s) System repeatability Total mobile phase time (t_t) Total mobile phase volume (V_t)
SYSTEM SUITABILITY	System Repeatability System Sensitivity	Peak Symmetry
ADJUSTMENTS OF CHROMATOGRAPHIC CONDITIONS	Thin-Layer Chromatography Liquid Chromatography: Isocratic	Liquid Chromatography: Gradient Gas Chromatography
QUANTITATION	External Standard Method	Internal Standard Method
OTHER CONSIDERATIONS	Detector Response	

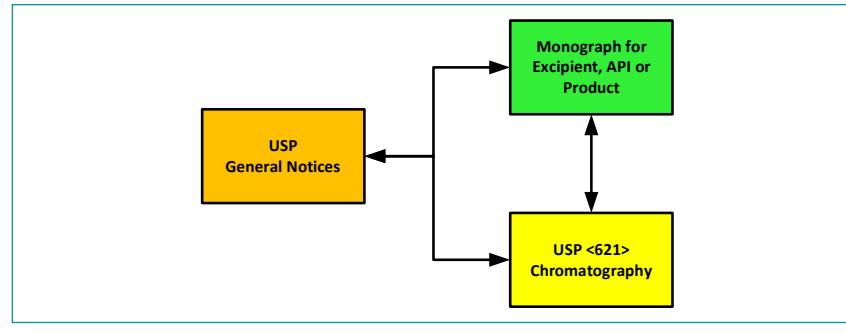

FIGURE 3: Interpreting a monograph, general notices, and a *USP* General Chapter.

For references, go to chromatographyonline.com/journals/lcgc-international

not! Therefore, the first part of the new definition is to make an explicit statement that system sensitivity is when you measure impurities. Consider, if you are measuring an active pharmaceutical ingredient, why on earth would you measure S/N? The main peak will be orders of magnitude above any noise. This is legislation for idiots.

- **S/N is an SST parameter**: The corollary is that sensitivity is not a test for instrument qualification because S/N is dependent on the analytical procedure used. We will discuss why SSTs are not a substitute for instrument qualification later in this column.
- **Instructions for Measuring S/N**: Here comes the interpretation. Your method validation experiments will determine the limit of quantification (LOQ), but not on a day of routine analysis. That is why a point-of-use measurement is essential to ensure the chromatography is fit for its intended purpose on the day and not subject to variances because of the instrument, column, mobile phase preparation, or other factors. Always use the pharmacopoeial reference standard for the measurement, never a sample. However, a laboratory still has to interpret other factors. How many injections? Are we looking at range of concentrations or just one? Should we determine S/N throughout the run, at the start and end, or just at the start?
- **Acceptance Criterion:** The LOQ is based on a S/N of 10 and is related to the monograph.

Although <621> applies to drug monograph testing, it is not unusual for laboratories to ask if chromatographic calculations performed during analytical instrument qualification, such as S/N, "comply" with *USP* <621>. The short answer is, of course, yes. This is because the <621> only applies to drug monograph testing.

However, this is an important subject, and at the heart of it is the need to understand the role of the tests performed during instrument qualification life cycle stages and SSTs during routine use. Typically, this question is most often asked in relation to the injection precision tests (see System Repeatability section in <621>). However, it should be noted that injection precision is analytical method-specific, so unless a drug monograph test is performed during an operational qualification (OQ), the OQ test is not designed to meet the stringent performance criteria expressed in <621> and does not need to satisfy this criterion. However, this is where the role of PQ rears its head again!

Historically, for impurity analysis, column-to-column variation meant that one of the best ways to ensure suitable chromatography, sensitivity of the system, and resolution of critical trace impurities eluting in the tail of a larger peak, was to prepare a designated "typical sample" and examine the integration and peak shapes as part of approving the SST of the methodology on the day. Use of inappropriate SSTs using sample injections and without acceptance criteria can find its way into a Warning Letter (7), as well as the requirement that SSTs are supported by validation report (8).

Unfortunately, the evolution of fraudulent data integrity practices also means that it is no longer acceptable to do this because it would be classed as trial injections and testing into compliance (9) (unless the material used was well characterised and could be readily differentiated from a "typical batch"). The FDA has a Q&A on trial injections on their website that will allow evaluation injections under strict criteria only using standard solutions–see Q17 (10).

Additionally, there is a regulatory expectation that failure to meet SST requirements (for example, at the end of a run) will be monitored and investigated because of data integrity and OOS risks. Peak integration and manipulation of SST injections to invalidate a failing run was discussed in an earlier "Questions of Quality" column (11).

TABLE III: Change in System Sensitivity and Peak Symmetry Definitions for *USP* <621>, effective May 2025.

Parameter	Current USP <621> (official chapter)	Proposed in PF49(6)*
System Sensitivity	The signal-to-noise ratio is used to define the system sensitivity. The limit of quantitation (corresponding to a signal-to-noise ratio of 10) is equal to or less than the reporting threshold. Note in our view use of a sensitivity test solution at the reporting threshold is significantly more robust than using LOQ (S/N > 3) or LOQ (S/N > 10) from the chromatogram, which would vary from run to run.	In impurity tests, when the procedure in a monograph prescribes the use of a reporting threshold, the signal-to-noise ratio is used to define the system sensitivity. To determine the signal-to-noise ratio, inject a reference solution of the analyte (official substance) at the concentration corresponding to the reporting threshold. The requirement is met if the limit of quantitation (corresponding to a signal-to-noise ratio of 10) is equal to or less than the reporting threshold.
Peak Symmetry	Unless otherwise stated, in a test or assay, the symmetry factor (tailing factor) of the peak used for quantification is 0.8–1.8.	Unless otherwise stated, in the Organic Impurities test, Related Substances test, or the Assay, the symmetry factor (tailing factor) of the peak in the standard solution used for quantification is 0.8–1.8. The peak symmetry requirement is waived if the determination of the %RSD is prescribed as part of the System Suitability Test.

* Publication in USP-NF 1st November 2024 and set to become official on 1st May 2025.

Adjustment of Chromatographic Conditions

Changes to <621> over time include evolution of the role of System Suitability for chromatography methods. Used to verify the reproducibility and resolution of the chromatography system, adjustments to analytical method operating parameters could be made to meet system suitability require-

ments. Historically, for non-monograph testing, there was always a risk that successive changes could result in the analytical method "drifting" outside of its registration and validation limits.

Over time, *USP* have addressed this subject in <621> through iterations of changes in PF. Originally, for HPLC, only changes could be made to isocratic methods, but the need to address the requirements to be able to transfer methods from traditional columns to more modern columns and ultrahigh-pressure liquid chromatography (UHPLC) systems led to the current situation as defined by clear guidance in the harmonized *USP* <621> published in 2022 (1).

A summary is shown in Table IV. This shows the maximum variance allowed (unless otherwise directed in the monograph). A change is allowed, provided SST requirements are met, and the selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent, though additional verification may be needed. Changes other than those indicated would require revalidation of the procedure. It should be noted that for some parameters, limits must be calculated (based on the equations provided in the chapter). It is worth mentioning that the decision to implement these changes is not made by an individual. A risk assessment needs to be conducted, and various departments and individuals should be involved and held accountable for the decision. Don't forget that when making changes of this nature, they need to be traceable, controlled, approved, and documented. Changes may impact the overall method performance, which may be identified when results are trended, and you'll need to identify the possible cause.

USP <621> Frequently Asked Questions

The *USP* provides a Frequently Asked Questions (FAQ) page for *USP* <621>, with examples of questions and responses being shown in Table V.

It should be noted that in addition to the provision that changes to monograph methods can be made within the ranges specified, there are some additional provisions which need to be understood (14):

- Multiple adjustments can have a cumulative effect on the performance of the system and must be properly evaluated by users.
- If adjustments are made to a pharmacopeial procedure, additional verification tests may be required.
- Compliance with the system suitability test criteria is required to verify that conditions for satisfactory performance of the test or assay are achieved.
- Adjustment of conditions with gradient elution HPLC is more critical than with isocratic HPLC, since it may shift some peaks to a different step of the gradient, potentially causing partial or complete coelution of adjacent peaks or peak inversion. Thus, this could lead to the incorrect assignment of peaks and to the masking of peaks or a shift, such that elution occurs beyond the prescribed elution time.

TABLE IV: Allowable changes to HPLC methods in *USP* <621> (1).

Mobile Phase	Isocratic		Gradient	
pH	± 0.2 Units[#1]			
Ratio of Components	Minor Component (<= 50%) ± 30% relative, cannot exceed ± 10% absolute. Can only adjust one minor component in a tertiary mixture		Principle peaks elute within ± 15% of the retention time of the original conditions. Does not apply if the column dimensions change. The composition of the mobile phase	
Salt Concentration	Within ± 10%, if pH Variance is met			
Column	**Isocratic**		**Gradient**	
Stationary Phase	No changes allowed[#2]			
Column Internal Diameter	Flexible			
Method Transfer Type#	**TPP to TPP**	**TPP to SPP[#3]**	**TPP to TPP**	**TPP to SPP**
Column Dimensions: Length (L), Particle Size (dp)	L/dp: -25 to 50%	L/dp: -25 to 50%	L/dp: -25 to 50%	See <621> -25 to 50%
Instrument Settings	**Isocratic**		**Gradient**	
Injection Volume	Adjust when changing column diameter (refer to *USP* 621 for equation)			
Flow Rate	With change in particle size and column dimension, calculate flow rate accordingly. (only for isocratic: ±50% with no column dimension change is permitted)			
Column Temperature	± 10 °C		± 5 °C	
Gradient Time	Not Applicable		Gradient adjustments based on particle size, column dimensions and flow rate	
Wavelength (UV-Vis)	No adjustments permitted[#4 #5]			

#: See notes below:

1: Accurately measuring the pH of mobile phase is difficult (12) and at best requires procedural control

2. This is one of the most critical factors involved in method scaling in our view. Guidance on LC columns selectivity is not provided in *USP* <621>. Often, critical LC column attributes that impact selectivity are not considered, as the trial-and-error approach is frequently used for column selection. Many scientists mistakenly believe that all C18 columns are equivalent. Silly people!
3. No limitation for changing porous particle (TPP) to superficially porous (SPP) or vice versa, except for the number of theoretical plates (N), to remain between -25 and +50% of the prescribed values.
4. <621> now simply states "no adjustments permitted." In the previous version of <621>, it stated, "Deviations from the wavelength specified in the procedure are not permitted. The procedure specified by the detector manufacturer, or another valid procedure, is used to verify that error in the detector wavelength is, at most, ± 3 nm." So, no deviations from the wavelength specified in the monograph are allowed, but no acceptance criteria are specified for UV-visible HPLC detector accuracy!
5. Evolution of the technique specific general chapters for spectroscopy allowed the inclusion of a specific section addressing instrument qualification requirements. *USP* <1058> (13) provides the life-cycle framework, but *USP* <857> (for UV-visible spectroscopy) specifies the qualification requirements; for example, "...the wavelengths selected for qualification must bracket the intended range for use" (5).

- These changes are acceptable, provided that system suitability criteria are fulfilled, and selectivity and elution order of the specified impurities to be controlled are demonstrated to be equivalent.

Therefore, caution is required.

USP <1058> Data Quality Triangle

USP <1058> on Analytical Instrument Qualification contains the Data Quality Triangle (13) consisting of four layers, stating at the bottom:

- **Analytical Instrument Qualification (AIQ):** This is the foundation of all analytical work and is a major contributor to data quality: do you believe your instrument? AIQ is independent of an analytical procedure *and* calibrated *and* traceable test equipment *and* reference standards. AIQ should enable efficient technology transfer as a chromatograph can be shown to be equivalent of another one.
- **Analytical Procedure Validation**: Using a qualified instrument, a procedure from sampling to a reportable result is developed and validated. The instrument control parameters for acquisition, processing (especially the peak integration [11, 15, 16]), calculation, and reporting are defined and validated. In addition, the specific SSTs to confirm that the system is ready to analyse are selected while the acceptance criteria are defined and verified.
- **System Suitability Tests**: To determine if the chromatographic system can analyse samples, an SST or point-of-use check is performed. The FDA's Out of Specification (OOS) guidance (17) specifically says that if SST injections are outside of their acceptance criteria, then the run can be disregarded. Be careful, as some "enterprising' chromatographers have used this to invalidate OOS results (11), which generally means that regulators will take a strong interest in runs which fail SST. Additionally, persistent SST failures may result in the performance of the method being challenged, and you may have a bigger problem–defending all the decisions you have made using this method.
- **Quality Control Checks**: These will not be discussed as the topic is not pertinent.

USP <621> Has No Instrument Qualification Criteria

The problem with *USP* <621> is that, unlike the technique general chapters, there are no instrument parameters with acceptance criteria to qualify a chromatograph. For a liquid chromatograph, there are no criteria for pump flow accuracy, gradient mixing (either high- or low-pressure), autosampler precision and accuracy, detector accuracy, and more. This is a failing that needs to be rectified in an updated *USP* <621>.

In contrast, if we look at *USP* <857> for UV–vis spectrometry, there is a section on Qualification that presents Installation Qualification (IQ) requirements and Operational Qualification (OQ) tests. The latter includes wavelength accuracy, absorbance, and stray light, with acceptance criteria that take up about half of the general chapter (5).

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TABLE V: *USP* <621> frequently asked questions.

Question	Answer
1. To what degree can a chromatographic procedure be modified and still be in compliance? Can column length, internal diameter, mobile phase composition be modified?	Chromatography General Chapter <621> contains a list of allowed adjustments to chromatographic systems. However, the user should verify the suitability of the method under the new conditions by assessing the relevant analytical performance characteristics potentially affected by the change (see section System Suitability under Chromatography <621>).
2. What brand of HPLC/GC column was used in the development and validation of a particular test? Is there an alternative chromatographic column for a particular test?	The most updated information on the brand name of the column used to validate any chromatographic procedure in *USP*-NF, together with possible alternatives, where applicable, are available at the following: www.uspchromcolumns.com.
3. How much deviation is allowed from a relative retention time prescribed in a monograph?	From <621>, the deviations of relative retention time values measured for the test substance from the values obtained for the reference compound and mixture should not exceed the reliability estimates determined statistically from replicate assays of the reference compound. Also, relative retention times may be provided in monographs for informational purposes only, to aid in peak identification. There are no acceptance criteria applied to relative retention times.

From: https://www.usp.org/frequently-asked-questions/chromatography

SSTs Are Not Instrument Qualification Tests

The SST and instrument qualification have different purposes. This was covered in some depth in three Questions of Quality columns in 2010–2012 (18-20), and key points are summarised below:

- SSTs are intended to demonstrate adequate performance of the whole analytical system (chromatograph, column, mobile phase, and data system) on the day of analysis.
- SSTs are not instrument qualification tests and are method-specific.
- Acceptance criteria for SSTs are verified and documented in the validation report for each analytical procedure.

You will notice from the data quality triangle that the AIQ and SST layers are separated from each other by the procedure validation because they have different functions. Qualifying an HPLC pump will use a calibrated digital flow meter and a recognised wavelength standard, such as holmium perchlorate solution, to check the wavelength accuracy of the detector. However, there will not necessarily be a check for S/N. This is because S/N is a holistic system check for any impurity analysis LOQ on the day.

Where SSTs can be used, in part, to indirectly confirm the correct operation of a chromatograph is in the Performance Qualification (PQ) phase of the instrument's life cycle. An example is the use of some SST parameters, such as retention time, to infer that the pump is performing as intended (21).

Summary

This article started out as a review of changes to *USP* <621> that become official on December 1st 2024 and the ones to become official on May 2025 (such as SST acceptance criteria for sensitivity and peak tailing check). However, as we investigated the subject, it became apparent that a more detailed discussion was required because of the criticality of *USP* <621> and potential confusion that surrounds appropriate interpretation of this general chapter. Because chromatography tends to be a focus area during regulatory inspections and audits, even this represents a regulatory risk which could potentially involve compliance issues.

Do you fully understand the changes and how they impact your laboratory?

Recent changes to the Definitions section are still relatively new and require careful understanding to ensure compliance. Use of new examples of chromatograms in *USP* <621> helps make these changes stand out.

A significant proportion of *USP* <621> covers explanation and theory–which now contrasts strongly with the structure of spectroscopy general chapters. Additional contrast and limitations are evident when Analytical Instrument Qualification requirements are considered for chromatography systems. The idea of splitting *USP* <621> into specific general chapters for each chromatographic technique is strongly recommended by the authors. Common theory and explanation could still be grouped into an appropriate overarching informational general chapter, so it remains a collective. This would enable focused clarification of instrument requirements and limits for each specific technique and allow inclusion of instrument qualification requirements to align with *USP* <1058>.

As pharmacopoeial chapters change, it is important that your CDS application is upgraded to incorporate and validate the new changes unless you want to have an entry into the Museum of Analytical Antiquities (22). ■

Acknowledgments

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This article has supplemental information and references only available online. **Scan code for link.**

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An Integrative Analytical Quality by Design (AQbD), Up-To-Date Greenness, and Whiteness Set of Tools for Evaluation of a Sustainable RP-HPLC Method for Regulated Products

Jerome Workman, Jr.

In this interview, we asked Hemanth Kumar Chanduluru from the SRM Institute of Science and Technology in Kattankulathur, India, multiple questions regarding sustainability in analytical separation methods. Within the context of developing chromatographic analytical methods, the concept of sustainability refers to designing methods that minimize the environmental impact while maintaining high efficiency and effectiveness in separation and analysis processes. This involves several considerations including reducing usage volume of solvents, energy efficiency, waste reduction, Green chemistry principles, and overall life cycle assessment for sustainability of the entire analytical measurement and equipment process. Along these sustainability efforts there are useful processes and metrics that may be used to objectively evaluate progress, including Analytical Quality by Design (AQbD), and up-to-date ChlorTox Scale, greenness, and whiteness score toolsets.

Q Could you elaborate on how Integrative AQbD (Analytical Quality by Design) principles were applied in the development of the RP-HPLC method for simultaneous separation of triple antihypertensive combination therapy (1)?

Integrative Analytical Quality by Design (AQbD) principles offer a systematic approach to method development, focusing on understanding the relationships between critical method parameters (CMPs) and critical method attributes (CMAs) to ensure robust and reliable analytical methods. When applied to the development of a reversed-phase high performance liquid chromatography (RP-HPLC) method for simultaneous separation of a triple antihypertensive combination therapy, several steps can be followed:

1. **Defining the Quality Target Product Profile (QTPP):** This involves specifying the desired characteristics of the analytical method, such as specificity, accuracy, precision, linearity, range, and robustness.

2. **Identification of Critical Method Attributes (CMAs):** These are the characteristics of the method that have a significant impact on its performance. For an RP-HPLC method, CMAs may include resolution, retention time, and peak shape for each component of the triple antihypertensive combination.

3. Selection of Critical Method Parameters (CMPs): These are the parameters of the analytical method that can influence CMAs. Examples of CMPs for RP-HPLC may include mobile phase composition, column type and dimensions, temperature, flow rate, and gradient program.

4. Design of Experiments (DoE): Using statistical design principles, experiments are conducted to systematically evaluate the effects of different levels of CMPs on CMAs. This helps in understanding the design space of the method and identifying the optimal conditions.

5. Risk Assessment and Mitigation: Potential risks to method performance are identified and addressed through risk assessment tools such as Failure Mode and Effects Analysis (FMEA). Strategies are developed to mitigate these risks, ensuring method robustness.

6. Method Development and Optimization: Based on the results of DoE and risk assessment, the method is developed and optimized to achieve the desired CMAs. This may involve adjusting CMPs within the established design space to maximize method performance.

7. Method Validation: Once the method is developed, it undergoes validation to demonstrate its suitability for its intended purpose. This includes assessing parameters such as specificity, accuracy, precision, linearity, range, and robustness, in accordance with regulatory guidelines.

8. Continuous Improvement and Lifecycle Management: After validation, the method is subject to continuous monitoring and improvement throughout its lifecycle. Any changes to method conditions or procedures are systematically evaluated to ensure that method performance remains within acceptable limits.

By following these steps, integrative AQbD principles can be effectively applied in the development of an RP-HPLC method for simultaneous separation of triple antihypertensive combination therapy, ensuring that the method is robust, reliable, and fit for its intended purpose.

Q What specific considerations were taken into account to ensure the sustainability of the RP-HPLC method during its development and validation process?

To ensure the sustainability of the RP-HPLC method during its development and validation process, several specific considerations can be taken into account:

1. Green Chemistry Principles: Integration of green chemistry principles involves minimizing the use of hazardous materials, reducing waste generation, and optimizing resource utilization. This can be achieved by selecting eco-friendly solvents, reducing solvent consumption, and employing efficient chromatographic conditions that minimize environmental impact.

2. Economic Viability: Considering the economic aspects of method development and validation is crucial for sustainability. This includes evaluating the cost-effectiveness of reagents, consumables, and equipment, as well as assessing the scalability of the method to ensure its feasibility for routine use in the laboratory.

3. Energy Efficiency: Optimizing method conditions to minimize energy consumption contributes to sustainability. This may involve reducing run times, optimizing column temperature, and selecting appropriate instrument settings to conserve energy while maintaining method performance.

4. Resource Conservation: Minimizing resource consumption, such as solvent usage and sample volume, helps conserve valuable resources and reduces waste generation. Techniques such as microextraction and miniaturization can be employed to reduce sample and solvent volumes without compromising method sensitivity or reliability.

5. Robustness and Reliability: Developing a robust and reliable method ensures its long-term sustainability by reducing the need for frequent method optimization and troubleshooting. Implementing quality control measures and conducting thorough method validation help establish the method›s reliability and ensure consistent performance over time.

6. Compliance with Regulatory Guidelines: Ensuring compliance with regulatory guidelines and standards is essential for the sustainability of the method, as it facilitates acceptance and adoption by regulatory authorities. Adhering to Good Laboratory Practices (GLP) and relevant regulatory requirements during method development and validation is critical for achieving regulatory approval and maintaining method sustainability.

7. Continuous Improvement and Optimization: Implementing a process of continuous improvement and optimization throughout the method lifecycle enhances its sustainability. This involves monitoring method performance, identifying areas for improvement, and implementing changes to optimize method efficiency, reliability, and environmental impact.

By incorporating these considerations into the development and validation process of the RP-HPLC method, sustainability can be effectively addressed, ensuring that the method remains environmentally friendly, economically viable, and operationally efficient throughout its lifecycle.

Q How does the use of a special C18 (2) column contribute to the sustainability aspect of the developed method compared to other column options?

The choice of the C18 column can contribute to the sustainability aspect of the developed RP-HPLC method compared to other column options in several ways:

1. Longevity and Durability: Our selected C18 columns are known for their robustness and longevity. These columns typically exhibit excellent column stability and can withstand a wide range of mobile phase conditions and sample matrices without significant degradation. As a result, they have a longer operational lifetime, reducing the frequency of column replacement and minimizing waste generation.

2. Reduced Solvent Consumption: Our C18 columnwas designed to provide efficient chromatographic separations at lower solvent volumes. Their high efficiency and resolving power allow for shorter analysis times and reduced solvent consumption per analysis. This not only saves solvent costs but also contributes to environmental sustainability by minimizing solvent usage and waste generation.

3. Lower Energy Consumption: Our C18 column used in RP-HPLC typically requires lower column temperatures for optimal performance compared to other column options. This can lead to reduced energy consumption during chromatographic runs, contributing to overall energy efficiency and sustainability.

4. Compatibility with Green Solvents: Our selected columns are often compatible with eco-friendly solvents, such as aqueous-organic mobile phases containing lower concentrations of organic solvents or alternative green solvents. By enabling the use of greener solvent systems, these columns help reduce the environmental impact associated with solvent usage in analytical laboratories.

5. High Analytical Performance: Our C18 columns are known for their high analytical performance, including excellent peak shapes, resolution, and reproducibility. Their superior chromatographic properties minimize the need for method optimization and troubleshooting, leading to more efficient and sustainable analytical workflows.

6. Versatility and Application Range: Our selected C18 columns are versatile and suitable for a wide range of applications, including pharmaceutical analysis, environmental monitoring, food safety testing, and more. Their broad applicability reduces the need for multiple column types, simplifying laboratory operations and inventory management, which in turn enhances sustainability by minimizing resource consumption and waste generation.

Overall, the use of the appropriate C18 columns in the developed RP-HPLC method contributes to sustainability by improving column longevity, reducing solvent and

energy consumption, enabling the use of green solvents, enhancing analytical performance, and simplifying laboratory operations. These factors collectively support environmentally friendly and economically viable analytical practices, aligning with sustainability goals in analytical chemistry.

Q Can you discuss the rationale behind selecting ethanol (EtOH) and KH_2PO_4 as components of the mobile phase, particularly in terms of their environmental impact and sustainability?

Ethanol can be derived from renewable resources such as biomass (for example, corn, sugarcane, or cellulosic materials). Unlike fossil-based solvents, which are finite resources, ethanol production can be sustainable when sourced from renewable feedstocks.

Ethanol is also biodegradable, meaning it can be broken down into harmless byproducts by microorganisms in the environment. This reduces its environmental impact compared to non-biodegradable solvents.

Third, ethanol is generally less toxic than some other organic solvents commonly used in chromatography, such as acetonitrile or methanol. Lower toxicity levels contribute to reduced environmental and health risks associated with solvent handling and disposal.

And finally, ethanol typically has lower volatile organic compound (VOC) emissions compared to certain other organic solvents. This is beneficial for air quality and reduces the environmental footprint associated with solvent evaporation during chromatographic analyses.

For KH_2PO_4, it serves as a buffer salt in the mobile phase, helping to control and maintain the pH of the solution. Phosphorus is an essential nutrient for plant growth, and its use in the form of KH_2PO_4 can contribute to the fertilization of soils when disposed of responsibly.

Second, KH_2PO_4 is biodegradable and does not persist in the environment, unlike some other buffer salts or additives commonly used in chromatography. Its biodegradability ensures minimal long-term environmental impact.

Third, potassium dihydrogen phosphate is generally considered to have low environmental toxicity. When disposed of properly, it poses minimal risks to aquatic organisms and ecosystems compared to certain other buffer salts or additives.

And finally, the production and use of KH_2PO_4 typically have a relatively low environmental footprint compared to some other buffer salts or additives. This is important for sustainability, as it reduces resource consumption and energy usage associated with manufacturing and transportation.

Q What strategies were employed to optimize the separation time while maintaining the environmental sustainability of the method?

To optimize the separation time while maintaining the environmental sustainability of the RP-HPLC method, several strategies can be employed:

1. Column Selection and Dimensions: Choosing an appropriate column type and dimensions can significantly impact separation time. Columns with smaller particle sizes and shorter lengths typically offer higher efficiency and faster separations. By selecting a column optimized for rapid separations, overall analysis time can be reduced, leading to lower solvent consumption and energy usage.

2. Mobile Phase Composition and Gradient Program: Optimization of the mobile phase composition and gradient program plays a crucial role in achieving fast and efficient separations. By carefully selecting solvent systems and gradient profiles, it is possible to improve peak resolution and shorten analysis time without compromising separation quality. Additionally, using eco-friendly solvents with lower environmental impact, such as ethanol, and optimizing gradient conditions to minimize solvent usage contribute to environmental sustainability.

3. Optimized Flow Rate: Adjusting the flow rate of the mobile phase can influence separation time without compromising chromatographic performance. Higher flow rates generally result in faster elution times but may compromise resolution. By optimizing the flow rate within the range that maintains adequate resolution while minimizing analysis time, overall solvent consumption and energy usage can be reduced, enhancing environmental sustainability.

4. Temperature Control: Proper temperature control of the chromatographic system can affect separation efficiency and analysis time. Maintaining a stable column temperature within an optimal range can improve chromatographic performance and reduce the time required for equilibration between runs. Additionally, optimizing column temperature can help achieve faster elution times without sacrificing resolution, contributing to shorter analysis times and reduced energy consumption.

5. Method Development using Quality by Design (QbD) Principles: Applying Quality by Design (QbD) principles during method development allows for systematic optimization of critical method parameters while considering environmental sustainability. By utilizing experimental design techniques, such as Design of Experiments (DoE), and conducting risk assessments, it is possible to identify the most influential factors affecting separation time and develop strategies to optimize them while minimizing environmental impact.

6. Continuous Monitoring and Improvement: Implementing a process of continuous monitoring and improvement allows for ongoing optimization of the method to further reduce separation time and enhance environmental sustainability. By regularly evaluating method performance and identifying opportunities for refinement, it is possible to achieve incremental improvements in efficiency and environmental impact over time.

By employing these strategies, it is possible to optimize the separation time of the RP-HPLC method while maintaining environmental sustainability, thereby reducing solvent consumption, energy usage, and overall environmental impact associated with chromatographic analysis.

Q Could you explain how the gradient elution system was designed to enhance both separation efficiency and greenness of the method?

Designing a gradient elution system in an RP-HPLC method to enhance both separation efficiency and greenness involves optimizing the mobile phase composition and gradient profile to achieve efficient analyte

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separation while minimizing environmental impact. Here's how this can be achieved:

- **Use of Eco-Friendly Solvents:** Selecting environmentally friendly solvents, such as ethanol (EtOH), as the primary organic component of the mobile phase contributes to the greenness of the method. Ethanol is derived from renewable resources and has lower environmental impact compared to some other organic solvents commonly used in chromatography.
- **Reduced Organic Solvent Content:** Minimizing the organic solvent content in the mobile phase helps reduce environmental impact by decreasing solvent consumption and emissions. By optimizing the ratio of organic solvent to aqueous component, it is possible to achieve efficient analyte separation with lower overall organic solvent usage.
- **Buffer Selection:** Choosing buffer salts that are environmentally benign and readily biodegradable, such as potassium dihydrogen phosphate (KH2PO4), further enhances the greenness of the method. These buffer salts minimize environmental toxicity and contribute to sustainability.
- **Gradient Shape and Duration:** Designing a gradient profile with appropriate shape and duration is critical for achieving efficient separation while minimizing analysis time and solvent consumption. Gradual changes in mobile phase composition help elute analytes sequentially, optimizing resolution and peak shape. By carefully adjusting gradient parameters, it is possible to achieve rapid separations without compromising separation efficiency.
- **Minimization of Solvent Steps:** Minimizing the number of solvent steps and transitions in the gradient profile reduces solvent consumption and waste generation. Simple gradient profiles with fewer steps are preferred from both efficiency and greenness perspectives.
- **Column Equilibration Time:** Optimizing the equilibration time between runs is essential for maximizing throughput and minimizing solvent usage. Balancing the need for thorough column equilibration with the desire for rapid analysis helps achieve efficient separations while minimizing solvent waste.
- **Real-Time Monitoring:** Imple-menting real-time monitoring of chromatographic parameters, such as pressure, retention times, and peak shapes, allows for immediate detection of issues that may impact separation efficiency or greenness. Continuous monitoring enables timely adjustments to gradient parameters to maintain optimal performance.
- **Automated Control Systems:** Utilizing automated control systems for gradient elution, such as programmable chromatography software, facilitates precise control over gradient parameters and ensures reproducible method performance. Automation minimizes human error and optimizes resource utilization, contributing to overall greenness.

By incorporating these design principles, a gradient elution system can be tailored to enhance both separation efficiency and greenness in an RP-HPLC method. Optimization of mobile phase composition, gradient profile, and control parameters enables rapid, efficient separations with reduced environmental impact, aligning with sustainability goals in analytical chemistry.

Q How does the use of a specific temperature (35 °C) during chromatographic analysis align with the concept of sustainability in the developed method?

The use of a specific temperature, such as 35 °C, during chromatographic analysis can align with the concept of sustainability in the developed method in several ways:

1. Energy Efficiency: Maintaining a constant temperature during chromatographic analysis helps optimize energy efficiency. By operating the chromatographic system at a moderate temperature, such as 35 °C, excessive energy consumption associated with extreme temperature settings can be avoided. This contributes to overall energy conservation and reduces the environmental footprint of the analytical method.

2. Column Stability and Longevity: Operating the chromatographic column at a controlled temperature helps maintain column stability and prolong its operational lifetime. Fluctuations in temperature can lead to column degradation and reduced chromatographic performance over time. By setting a specific temperature, such as 35 °C, within the optimal range for the column material, stability is enhanced, minimizing the frequency of column replacement and reducing waste generation.

3. Reproducibility and Robustness: Consistent temperature control ensures reproducible chromatographic results and method robustness. Temperature variations can affect analyte retention times, peak shapes, and resolution, leading to inconsistencies in analysis. By maintaining a constant temperature, method variability is minimized, enhancing the reliability and reproducibility of the analytical method, which is crucial for sustainable analytical practices.

4. Reduced Solvent Consumption: Stable chromatographic conditions, including temperature, contribute to efficient solvent usage. Fluctuations in temperature can impact solvent evaporation rates and elution times, affecting overall solvent consumption during chromatographic analysis. By optimizing temperature control, solvent usage can be minimized, leading to reduced waste generation and environmental impact associated with solvent disposal.

5. **Method Transferability and Standardization:** Setting a specific temperature for chromatographic analysis promotes method transferability and standardization across different laboratories and instruments. Consistent temperature conditions facilitate reproducibility of results and ensure comparability of data generated from different systems. This promotes efficient knowledge sharing and collaboration within the scientific community, contributing to sustainable practices in analytical chemistry.

Overall, the use of a specific temperature, such as 35 °C, during chromatographic analysis aligns with sustainability principles by optimizing energy efficiency, enhancing column stability, improving method reproducibility, reducing solvent consumption, and promoting method transferability. These considerations collectively support environmentally friendly and economically viable analytical practices, contributing to sustainable development in analytical chemistry.

Q Can you describe the role of the Analytical Method Greenness Score (AMGS) in evaluating the environmental impact of the developed RP-HPLC method?

The Analytical Method Greenness Score (AMGS) serves as a quantitative tool for assessing the environmental impact of an analytical method, such as the developed RP-HPLC method. It provides a systematic approach to evaluate various aspects of method design, operation, and waste generation, enabling researchers to make informed decisions to minimize environmental impact. Here's how the AMGS can be used to evaluate the environmental sustainability of the developed RP-HPLC method:

1. **Component Assessment:** The AMGS considers the environmental impact of individual components used in the analytical method, including solvents, reagents, buffers, and consumables. It evaluates factors such as toxicity, biodegradability, resource consumption, and emissions associated with each component. For the RP-HPLC method, the AMGS would assess the greenness of solvents (for example, ethanol), buffer salts (for example, KH_2PO_4), and other chemicals used in the mobile phase and sample preparation.

2. **Energy Consumption:** AMGS considers the energy consumption associated with method operation, including instrument usage, temperature control, and data processing. It evaluates the efficiency of energy usage and identifies opportunities to minimize energy consumption while maintaining method performance. For the RP-HPLC method, the AMGS would assess the energy usage during chromatographic analysis, including column heating, pump operation, and detector operation.

3. **Waste Generation:** The AMGS evaluates the amount and nature of waste generated during method operation, including solvent waste, consumable waste, and chemical waste. It considers factors such as solvent usage, sample volume, consumable consumption, and waste disposal practices. For the RP-HPLC method, the AMGS would assess the volume of solvent waste generated during chromatographic analysis, as well as the disposal practices for used columns, consumables, and chemicals.

4. **Life Cycle Analysis:** The AMGS may incorporate life cycle analysis principles to assess the environmental impact of the method throughout its entire lifecycle, from raw material extraction and manufacturing to method operation and disposal. It considers the environmental footprint associated with each stage of the method lifecycle and identifies opportunities to reduce environmental impact at each stage. For the RP-HPLC method, the AMGS would assess the environmental impact of column manufacturing, instrument operation, sample preparation, analysis, and waste disposal.

5. **Scoring and Ranking:** Based on the assessment of various environmental factors, the AMGS assigns a numerical score to the analytical method, indicating its overall greenness. This score allows for comparison and ranking of different methods based on their environmental impact. Researchers can use the AMGS to identify areas for improvement and implement strategies to enhance the environmental sustainability of the method. For the RP-HPLC method, the AMGS would provide a quantitative measure of its environmental impact, guiding efforts to minimize resource consumption, energy usage, and waste generation while maintaining analytical performance.

In summary, the Analytical Method Greenness Score (AMGS) plays a crucial role in evaluating the environmental impact of the developed RP-HPLC method by assessing factors such as component greenness, energy consumption, waste generation, and life cycle analysis. It provides researchers with a quantitative tool to assess and optimize the environmental sustainability of analytical methods, contributing to the development of greener analytical practices.

Q In what ways does the RP-HPLC method address concerns regarding solvent selection and waste generation, considering the principles of green analytical chemistry?

The RP-HPLC method can address concerns regarding solvent selection and waste generation in alignment with the principles of green analytical chemistry in several ways:

- **Use of Eco-Friendly Solvents:** The method utilizes environmentally friendly solvents, such as ethanol (EtOH), as the primary organic component of the mobile phase. Ethanol is derived from renewable resources and has a lower environmental impact compared to some other organic solvents commonly used in chromatography, such as acetonitrile or methanol.
- **Reduced Hazardous Solvent Usage:** By selecting ethanol as the organic solvent in the mobile phase, the method reduces the usage of hazardous solvents that pose risks to human health and the environment. Ethanol is generally less toxic and less hazardous than certain other organic solvents, contributing to safer laboratory practices and reduced environmental impact.
- **Optimized Solvent Consumption:** The method is designed to minimize solvent consumption by using efficient chromatographic conditions and optimized gradient profiles. By reducing the volume of solvent required for each analysis, the method minimizes waste generation and lowers the environmental footprint associated with solvent disposal.
- **Recycling and Reuse:** Where feasible, the method may incorporate strategies for solvent recycling and reuse to further reduce waste generation. Solvent recovery systems can be implemented to recover and purify used solvents for subsequent analyses, minimizing the need for fresh solvent procurement and waste disposal.
- **Method Optimization for Greenness:** During method development, green analytical chemistry principles are applied to optimize solvent selection, gradient profiles, and operating conditions to minimize environmental impact. Design of Experiments (DoE) and other systematic optimization approaches are employed to identify conditions that achieve efficient separations with minimal solvent usage and waste generation.

- **Greenness Evaluation:** The method undergoes evaluation using tools such as the Analytical Method Greenness Score (AMGS) to quantify its environmental impact and identify areas for improvement. Continuous monitoring and optimization throughout the method lifecycle ensure that greenness considerations remain a priority in method operation and development.
- **Adherence to Environmental Regulations:** By selecting environmentally friendly solvents and minimizing waste generation, the RP-HPLC method aligns with regulatory requirements and guidelines aimed at reducing the environmental impact of analytical practices. Compliance with regulations ensures that the method meets environmental standards and contributes to sustainable laboratory operations.

Overall, the RP-HPLC method addresses concerns regarding solvent selection and waste generation by the principles of green analytical chemistry by prioritizing the use of eco-friendly solvents, minimizing solvent consumption, optimizing method efficiency, and ensuring regulatory compliance. These efforts collectively contribute to the development of greener analytical practices that promote environmental sustainability.

Q Could you discuss how the concept of "whiteness" was integrated into the evaluation of the sustainability profile of the developed method?

The concept of "whiteness" in the evaluation of the sustainability profile of the developed RP-HPLC method likely refers to the consideration of aspects related to the environmental impact of materials used in the method, particularly in terms of their purity, cleanliness, and ecological footprint. Integrating the concept of whiteness into the evaluation of sustainability involves assessing the environmental impact of materials and processes involved in method development and operation, with a focus on promoting purity, cleanliness, and minimal ecological disturbance. Here's how this concept might be integrated into the evaluation of the sustainability profile of the developed method:

- **Solvent Purity:** Assessing the purity of solvents used in the method, such as ethanol, is essential for ensuring minimal environmental impact. High-purity solvents reduce the risk of contamination and minimize the release of impurities into the environment during use and disposal. Techniques such as distillation or purification processes may be employed to enhance solvent purity and promote whiteness in terms of cleanliness and ecological responsibility.
- **Chemical Reagents:** Evaluating the purity of chemical reagents and buffer salts used in the method is crucial for minimizing environmental contamination and waste generation. Selecting high-quality, pure reagents with minimal impurities reduces the environmental footprint associated with chemical synthesis, transportation, and disposal.
- **Analytical Method Cleanliness:** Ensuring cleanliness and minimal environmental impact in method operation involves optimizing chromatographic conditions, minimizing waste generation, and implementing efficient sample preparation techniques. Clean chromatographic separations with well-defined peaks and minimal baseline noise contribute to the whiteness of the method by reducing the need for repeat analyses and minimizing resource consumption.
- **Waste Reduction Strategies:** Implementing strategies to reduce waste generation during method operation, such as solvent recycling, sample volume minimization, and waste segregation, promotes cleanliness and ecological responsibility. By minimizing waste generation, the method contributes to a cleaner and more sustainable laboratory environment.
- **Resource Conservation:** Assessing the ecological footprint of the method involves evaluating resource consumption, energy usage, and waste generation throughout the method lifecycle. Strategies to minimize resource consumption, such as solvent optimization, energy-efficient operation, and waste reduction, promote whiteness by reducing the environmental impact of analytical practices.
- **Sustainability Assessment:** Conducting a comprehensive sustainability assessment of the method, considering factors such as greenhouse gas emissions, water usage, and biodiversity impacts, helps quantify its ecological footprint and identify opportunities for improvement. By integrating whiteness into sustainability assessment, the method aims to minimize ecological disturbance and promote environmental stewardship.

Overall, integrating the concept of whiteness into the evaluation of the sustainability profile of the developed RP-HPLC method involves assessing the purity, cleanliness, and ecological responsibility of materials and processes involved in method development and operation. By prioritizing purity, cleanliness, and minimal ecological disturbance, the method aims to promote environmental sustainability and contribute to a cleaner and more sustainable laboratory environment.

Q How does the application of the Chloroform-oriented Toxicity Estimation Scale (ChlorTox Scale) indicator contribute to assessing the environmental sustainability of the chemicals used in the method?

The ChlorTox Scale (3) is an indicator used to assess the environmental sustainability of chemicals based on their potential to generate chlorinated organic compounds and contribute to environmental pollution. It evaluates the chlorine content of chemicals and assigns a score based on their potential to form chlorinated by-products during use and disposal. The application of the ChlorTox Scale in assessing the environmental sustainability of the chemicals used in the developed method can provide valuable insights into their environmental impact and help identify opportunities for improvement. Here's how the ChlorTox Scale contributes to assessing the environmental sustainability of chemicals used in the method:

1. Quantification of Environmental Impact: By assigning a numerical score based on the chlorine content of chemicals, the ChlorTox Scale quantifies their potential environmental impact in terms of chlorinated

by-product formation. Chemicals with higher ChlorTox scores indicate a greater potential for generating chlorinated organic compounds and contributing to environmental pollution.

2. Comparison and Ranking: The ChlorTox Scale allows for the comparison and ranking of chemicals based on their environmental sustainability, enabling researchers to prioritize the use of less environmentally harmful alternatives. Chemicals with lower ChlorTox scores are preferred from an environmental sustainability perspective, as they pose reduced risks of chlorinated by-product formation and environmental pollution.

3. Guidance for Chemical Selection Incorporating the ChlorTox Scale into chemical selection criteria helps guide decision-making towards the use of environmentally sustainable chemicals in the method. By considering ChlorTox scores alongside other factors such as performance, cost, and availability, researchers can make informed choices to minimize the environmental impact of chemical usage.

4. Promotion of Green Chemistry Principles: The application of the ChlorTox Scale promotes the principles of green chemistry by encouraging the use of chemicals with lower environmental impact and reduced potential for chlorinated by-product formation. By selecting chemicals with lower ChlorTox scores, researchers contribute to sustainable chemical management practices and reduce the environmental footprint of analytical methods.

Overall, the application of the ChlorTox Scale in assessing the environmental sustainability of chemicals used in the developed method provides a systematic approach to evaluating their potential to generate chlorinated organic compounds and contribute to environmental pollution. By considering ChlorTox scores in chemical selection and method optimization, researchers can minimize the environmental impact of analytical methods and promote sustainable laboratory practices.

Q Can you elaborate on the significance of the Greenness Index tool through spider plots (radar charts) in evaluating the environmental sustainability of solvents and chemicals employed in the method?

The Greenness Index tool, often represented through spider plots, is a valuable tool for evaluating the environmental sustainability of solvents and chemicals employed in analytical methods, including the developed RP-HPLC method. Spider plots provide a visual representation of various environmental parameters, allowing for a comprehensive assessment of the greenness or environmental impact of different chemicals.

Here's how the Greenness Index tool through spider plots can be significant in evaluating the environmental sustainability of solvents and chemicals used in the method:

1. Multi-Parameter Evaluation: Spider plots display multiple environmental parameters simultaneously, such as toxicity, biodegradability, resource depletion, and energy consumption, allowing for a holistic evaluation of the environmental impact of solvents and chemicals. By considering various factors together, spider plots provide a comprehensive view of the overall greenness or sustainability of chemicals.

2. Comparative Analysis: Spider plots enable comparative analysis of different solvents and chemicals based on their environmental performance. By plotting multiple chemicals on the same graph, researchers can easily compare their greenness across various parameters and identify chemicals with superior environmental profiles. This facilitates informed decision-making in chemical selection and promotes the use of more sustainable alternatives.

3. Identification of Strengths and Weaknesses: Spider plots highlight the strengths and weaknesses of individual chemicals in terms of environmental sustainability. Each point on the plot represents a different environmental parameter, allowing researchers to identify areas where a chemical performs well or poorly relative to others. This information helps pinpoint specific areas for improvement and guides efforts to optimize chemical selection and method design.

4. Optimization of Method Components: By evaluating the environmental sustainability of solvents and chemicals using spider plots, researchers can optimize method components to minimize environmental impact. Chemicals with lower environmental scores or ratings can be replaced with greener alternatives, leading to the development of more environmentally friendly analytical methods. Spider plots guide the selection of chemicals that align with green chemistry principles and contribute to sustainable laboratory practices.

5. Communication of Sustainability Performance: Spider plots provide a visually intuitive way to communicate the sustainability performance of solvents and chemicals to stakeholders, including researchers, regulators, and the general public. The graphical representation of environmental parameters makes complex sustainability data more accessible and understandable, facilitating discussions and decisions related to chemical management and method development.

Overall, the Greenness Index tool through spider plots is significant in evaluating the environmental sustainability of solvents and chemicals employed in analytical methods by providing a multi-parameter evaluation, enabling comparative analysis, identifying strengths and weaknesses, optimizing method components, and facilitating communication of sustainability performance. Incorporating spider plots into sustainability assessments enhances the transparency, comprehensiveness, and effectiveness of efforts to promote green chemistry and sustainable laboratory practices. t. ■

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ABOUT THE INTERVIEWEE

Hemanth Kumar Chanduluru is a distinguished pharmaceutical scientist renowned for his groundbreaking research and prolific publications. With a Ph.D. in Pharmaceutical Analysis from SRM College of Pharmacy, his expertise lies in sustainable analytical method development and validation for pharmaceutical substances. Having authored numerous papers in respected scientific journals, Chanduluru's research contributions span eco-friendly solvents, stability studies of pharmaceuticals, and advanced tools in green analytical chemistry. His work has garnered recognition, including the prestigious Young Researcher Award by INSC.

Currently serving as an assistant professor of research at SRM College of Pharmacy, Chanduluru continues to push the boundaries of pharmaceutical sciences through his innovative research and collaborative projects. His dedication to advancing knowledge and promoting sustainable practices underscores his leadership in the field.

HIC Method Optimization for ADC Characterization

Tosoh Bioscience

Here we describe how mobile phase modifications can significantly enhance the efficacy of hydrophobic interaction chromatography (HIC) separations of an antibody-drug conjugate (ADC) with varying drug-to-antibody ratios. (DARs).

IN THE ANALYSIS OF biomolecules maintaining the native state of these complex structures is crucial. HIC addresses this by separating biomolecules under conditions that preserve their functional integrity. It uses a mobile phase with high salt concentrations to bind biomolecules, elution is achieved by gradually decreasing the salt concentration. This results in the early elution of more hydrophilic molecules and the later elution of more hydrophobic ones. Here we describe how modifications of pH and organic solvent content can enhance the separation efficacy.

Experimental Conditions

Column: TSKgel HIC-ADC Butyl (4.6 mm ID × 10 cm L)
Mobile phase: A: 0.05 mol/L sodium phosphate + 1.2 mol/L ammonium sulfate
B: 0.05 mol/L sodium phosphate
Variation of pH: pH 5- 7 (A & B) with 20 % 2-propanol in B
Addition of organic solvent to B: 0 - 20%
2-propanol at pH7 (A&B)
Gradient: 0 - 100 % B linear in 15 min, 100 % B for 5 min
Flow rate: 0.8 mL/min
Detection: UV @ 215 nm
Injection vol.: 10 µl
Temperature: 25 °C
Sample: ADC mimic (1 mg/mL)

Results

The influence of pH on the separation of an ADC mimic was determined from pH 5 to 7. At pH 5, the low DAR variants (DAR = 0, 2, and 4) of the ADC mimic elute earlier, and the peaks for high DARs are slightly less retained than at pH 6 and 7 (Figure 1). Peaks of high DAR species (DAR = 6 and 8) analyzed at low pH suffer from band broadening and decreased sensitivity. As the pH is increased, the chromatographic efficiency for high DAR species is significantly improved, as observed by the sharper peak shapes. As the molecule approaches the p*I* of the ADC mimic (close to 7), the molecule is less charged and more hydrophobic, leading to the observed higher retention.

The TSKgel HIC-ADC Butyl column features an increased ligand density tuned toward separating variably conjugated DAR isoforms. Adding organic solvents can modify hydrophobic interactions between analytes and the stationary phase, which are the main drivers of hydrophobic interaction separations. A comparison

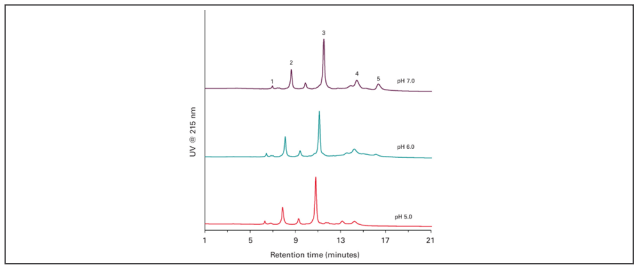

FIGURE 1: Impact of pH value on HIC separation of an ADC mimic.
Mobile phase pH is indicated in the graph. 1. DAR = 0; 2. DAR = 2; 3. DAR = 4; 4. DAR = 6; 5. DAR = 8

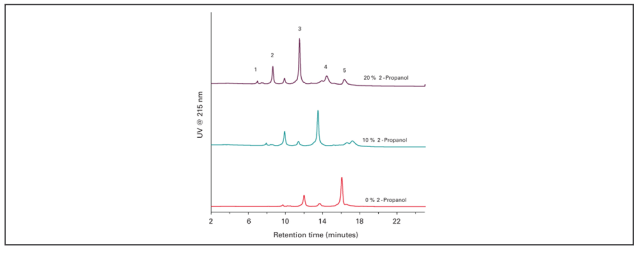

FIGURE 2: Impact of organic solvent addition on HIC separation of an ADC mimic.
The percentage of isopropanol in mobile phase B is indicated in the graph; DAR see figure 1

of separations with and without the organic modifier isopropanol (IPA) shows a faster and complete elution when IPA is added, while in the absence of IPA high DAR species (DAR=6, DAR=8) do not elute from the column. At higher isopropanol concentrations, high DAR variants elute as sharp peaks. In contrast, little or no addition of IPA to the elution buffer results in band broadening of hydrophilic variants and only partial elution of high DAR species.

Conclusion

Organic solvent composition and pH play crucial roles in HIC separations. A comprehensive understanding of how these factors interact allows for the systematic optimization of HIC methods, leading to more efficient and effective separations.

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Analysis of Tianeptine by Reverse-Phase SPE and LC-MS/MS

Emily Eng, Forensic Technical Specialist at UCT

This application note outlines an optimized solid phase extraction (SPE) method employing a polymeric Styre Screen® HLB SPE column, coupled with an LC-MS/MS analysis utilizing a SelectraCore® C18 column.

Introduction

Tianeptine is an antidepressant prescribed in some countries; however, it is not approved by the U.S. Food and Drug Administration (FDA) for medical use [1]. It is an emerging drug of abuse in the U.S., being falsely marketed in gas stations and online as a dietary supplement under names such as "gas station heroin", "Zaza", and "Neptune's Fix" [2]. Tianeptine is not scheduled under the Controlled Substances Act, but a few states such as Florida, Alabama, Georgia, and Mississippi, have already banned it [1]. A stand-alone analysis method for tianeptine was developed due to its unique amphoteric characteristics and abuse at high concentrations.

UCT Part Numbers:

SSHLB063: Styre Screen® HLB 60 mg, 3 mL
SCS27-C18521: SelectraCore® C18 Column 50 x 2.1 mm, 2.7 µm
SCS27-C18GDC21: SelectraCore® C18 Guard Column 5 x 2.1 mm, 2.7 µm
SLGRDHLDR-HPOPT: Selectra® Direct Connect Guard Holder

Instrument Method

LC-MS/MS System	Shimadzu Nexera LC-30AD with MS-8050
Column Temperature	40°C
Flow Rate	0.4 mL/min
Injection Volume	5 µL
Mobile Phase A	5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	5 mM ammonium formate + 0.1% formic acid in methanol
Gradient Program	Conc. B 5% (0 min) - 100% (6-7 min) - 5% (7.1-10 min)
Calibration Curve	20,50,100,200,500 and 1000 ng/mL

SPE Procedure

Sample Prep: In a test tube, add 200 µL sample + 2 mL of 100 mM phosphate buffer pH 6.0 + ISTDs. Mix and centrifuge
Condition: (a) 1 x 3 mL MeOH
(b) 1 x 3 mL 100 mM phosphate buffer pH 6.0
Load: Load sample at 1-2 min/mL

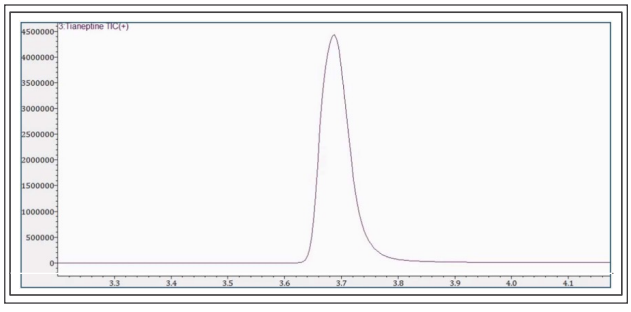

FIGURE 1: Chromatogram of an extracted standard at 75 ng/mL.

Wash: (a) 1 x 3 mL 100mM phosphate buffer pH 6.0
(b) 1 x 3 mL 10% MeOH
Dry: (a) Dry for at least 10 minutes at full vacuum or pressure
Elute: (a) 1 x 3 mL EtOAc:IPA:NH_4OH (78:20:2)
Note: Make elution solvent fresh daily
Evaporate: (a) Evaporate eluate at 40°C, starting at 5 psi and increasing the pressure slowly over 30 minutes
Reconstitute: (a) Reconstitute samples in 1 mL of 50:50 H_2O:MeOH or other appropriate solvent and volume

Results

TABLE I: Analysis of QC samples prepared at 25 ng/mL and 750 ng/mL.

n=5	Blood	Urine
Recoveries	87% - 89%	93% - 96%
Matrix Effects	(-12%) - 24%	(-15%) - (-19%)
RSD	4% - 5%	5% - 7%

*Recoveries were calculated using a pre- and post-spike sample technique. Matrix effects were calculated by comparing post-spike samples and solvent standards. Acepromazine not included.

References

(1) FDA Consumer Updates (February 2022) Tianeptine Products Linked to Serious Harm, Overdoses, Death. https://www.fda.gov/
(2) The Center for Forensic Science Research and Education (February 2024) Emerging Drug Alert: Tianeptine. https://www.cfsre.org

*To download the full application note, please visit;

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Fast lipidomic analysis with high resolution of the molecular species

Ann Marie Rojahn, *YMC Europe GmbH*

This application note shows how a less hydrophobic YMC Accura Triart C8 column with bioinert coating can be implemented into real-life lipidomic analysis with short runtimes and higher levels of sensitivity and recovery.

THE LIPID SIGNATURE of biological samples, or lipidome, is remarkably different between health and disease states. Therefore, lipids are good candidates to produce potent biomarkers. From an analytical point of view analysis of lipidomes deals with a large number of isomeric compounds, making comprehensive separation essential to generate a biologically informative dataset.

In order to achieve sufficient separation, the analysis times with standard methods, commonly using C18 stationary phases, tend to be longer and typically feature runtimes of >20 min. However, measurements of large cohorts of clinical samples (>200 per batch) require shorter runtimes to maintain overall reliability and improve cost-efficiency of the analysis. This example uses human plasma samples to demonstrate that separations under 10 minutes are also possible with real samples using a less hydrophobic YMC Accura Triart C8 column with bioinert coating to facilitate higher levels of sensitivity and recovery.

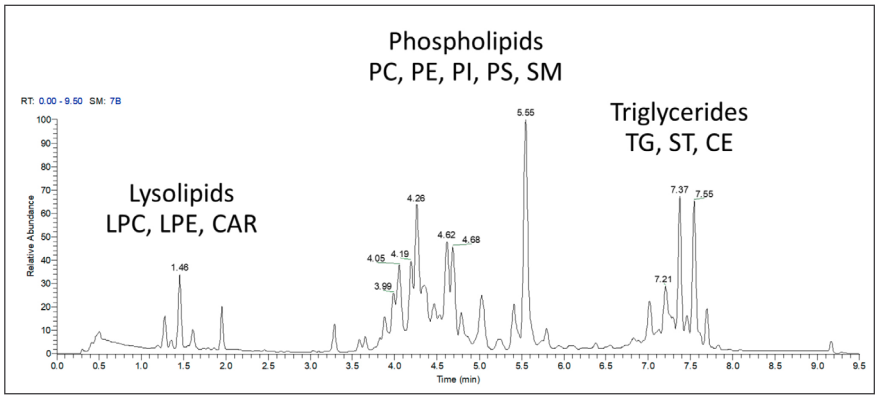

FIGURE 1: Successful lipidomic analysis of a human plasma sample using a YMC Accura Triart C8 column.

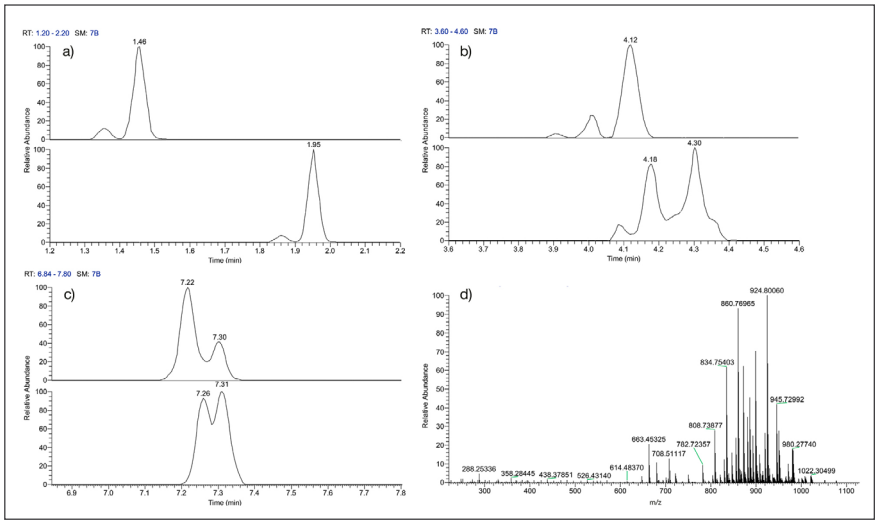

FIGURE 2: Chromatographic resolution of a) early eluting LPC/LPE (1.20–2.20 min), b) mid-gradient eluting PC/PE (3.60–4.60 min) and c) late eluting triglycerides (6.84–7.80 min) with d) a typical full scan spectrum at 7.27 min.

Experimental Conditions

A YMC Accura Triart C8 column is used equipped with a bioinert coating on the column body and frits, in order to achieve a short analysis time while ensuring high resolution and recovery. Human plasma extracted with 2-propanol with solvent to sample ratio of 4:1 was used as sample. Chromatographic conditions can be found in Table I.

Results

Figure 1 shows a well-defined separation between classes of lysolipids, phospholipids and triglycerides (Table II)- typical

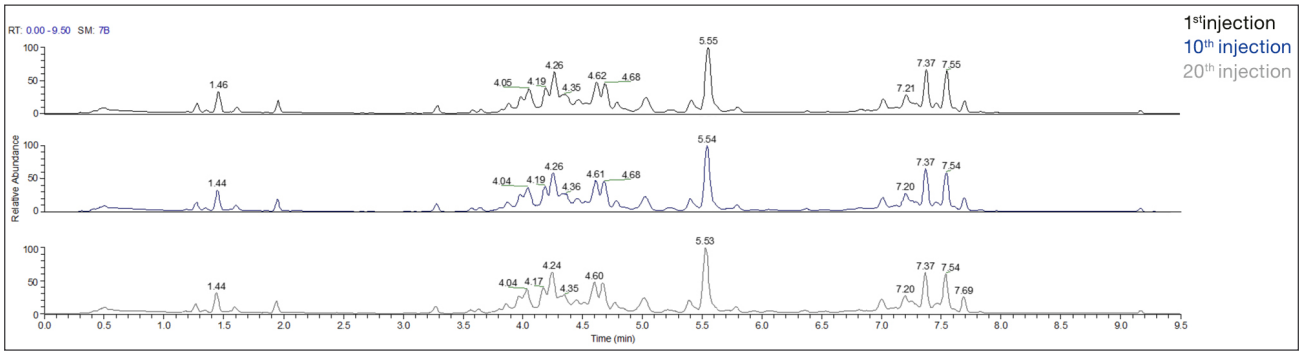

FIGURE 3: 20 consecutive injections show that high recoveries and good peak shapes are achieved from the first injection.
** Application data by courtesy of Sergey Girel, Institute of Pharmaceutical Sciences of Western Switzerland (University of Geneva), Geneva, Switzerland.*

TABLE I: Chromatographic conditions.

Column:	YMC Accura Triart C8 (12 nm, 1.9 µm) 100 x 2.1 mm ID
Part No.:	TO12SP9-10Q1PTC
Eluent:	A) 10 mM ammonium acetate in water/acetonitrile (50/50) B) acetonitrile/2-propanol (50/50)
Gradient:	10%B (0–0.5 min), 10–50%B (0.5–1.5 min), 50–99%B (1.5–7.5 min), 99%B (7.5–8.5 min), 99–10%B (8.5–8.6 min), 10%B (8.6–9.5 min)
Flow rate:	0.6 mL/min
Temperature:	50°C
Injection:	2 µL
Sample:	Human plasma extracted with 2-propanol (solvent to sample ratio of 4:1)
System:	ESI-MS

TABLE II: Evaluated lipids.

Lipid class	Lipid	Abbreviation
Lysolipids	Lysophosphatidylcholine Lysophosphatidylethanolamine	LPC LPE
Carnitins	Acylcarnitine	CAR
Phospholipids	Phosphatidylcholine Phosphatidylethanolamine Phosphatidylinositol Phosphatidylserin Sphingomyelin	PC PE PI PS SM
Triglycerides	Triglyceride Sterols Cholesterol esters	TG ST CE

for reversed-phase lipidomic analysis. Even with the less hydrophobic YMC-Triart C8 modification, molecular species of complex lipids can be feasibly separated in a fast run, as already visible from a TIC chromatogram. Hence, around 700 distinct molecules may be reliably determined during this fast analysis depending on the MS/MS performance of the spectrometer. The YMC-Triart C8 stationary phase has a very high specific surface area of 360 m^2/g, providing a sufficient loading capacity to accommodate the samples with a very high content of the analytes, such as plasma lipid extracts. As an example, figure 2 shows the chromatographic resolution of some early, mid-gradient and late eluting lipids. The early eluting LPC and LPE as well as the mid-gradient eluting PC and PE can be separated with appropriate resolution. This LC-MS method is also suitable for the characterisation of late eluting triglycerides with similar retention.

The used bioinert coated YMC Accura Triart C8 column ensures high recovery from the first injection (see Figure 3) since the lipids containing phosphate groups do not come into contact with metal surfaces, preventing adsorption. Twenty consecutive injections prove that the column provides reliable results from the first injection.

Conclusions

The YMC Accura Triart C8 column efficiently separates lysolipids, phospholipids, and triglycerides in a fast, high-capacity LC-MS run. Its high surface area and bioinert coating ensure reliable, reproducible results, ideal for complex lipidomic analyses.

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Efficient Method Development of Oligonucleotides by RP-IP

Shimadzu

This article describes how to efficiently achieve optimal separation of oligonucleotides and related impurities using LabSolutions MD, a dedicated software for supporting method development through initial screening and optimization phases.

NUCLEIC ACID DRUGS, such as antisense oligonucleotides, work by interacting with targets (genes and proteins) inside and outside of cells. These drugs are produced by chemical synthesis, but because the synthesis process can introduce impurities such as shorter and longer length of products and protection groups, proper separation of the target oligonucleotide is required. Reversed-phase ion-pair chromatography (RP-IP) is commonly used for LC separation, and the separation patterns can vary depending on the concentration of the ion-pair reagent and the composition of the organic solvent. Additionally, separation behaviour may differ depending on product length, nucleobase, and modifications.

Sample information

A target oligonucleotide and five related impurities that have different sequences are used as a model sample of synthetic antisense oligonucleotide (Table 1). The sample mixture included a full-length product (FLP), deletion sequences n-1(3′), n-1(5′), and n-3, an addition sequence n+1, and PO (modified from phosphorothioate to phosphate diester at 5′).

TABLE I: Sequences of Oligonucleotide and Related Impurities

Name	Sequence (x)	Length
FLP	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG[1]dA-dA-A*-T*-mC*-mC*-mC*	20 Mer
n-1(3′)	T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG[1]dA-dA-A*-T*-mC*-mC*	19 Mer
n-1(5′)	mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA[1]dA-A*-T*-mC*-mC*-mC*	19 Mer
n-3	T*-G*-dG-dT-dT-dA-dC-dA-dT-dG-dA-dA-A*- T*-mC*-mC*-mC*	17 Mer
n+1	T*-T*-mC*-T*-T*-G*-dG-dT-dT-dA-dC-dA-dT[1]dG-dA-dA-A*-T*-mC*-mC*-mC*	21 Mer
PO	FLP (modified from phosphorothioate to phosphate diester at 5′)	20 Mer

Note: * = 2′-O-methoxyethyl, m = 5-methyl, d = 2′-deoxy, PS (full)

TABLE II: Analytical Conditions for Initial Screening

System:	Nexera XS inert (Method Scouting System)
Column:	Shim-pack Scepter Claris (100 mm × 2.1 mm I.D., 3 µm, P/N: 227-31210-05*) *Shimadzu GLC product number
Temperature:	60 °C
Injection Volume:	2 µL
Mobile Phases:	Pump A – Line A: 100 mmol/L HFIP (*1) and 20 mmol/L TEA (*2) in water – Line B: 100 mmol/L HFIP in water – Line C: 200 mmol/L HFIP and 20 mmol/L TEA in water – Line D: 200 mmol/L HFIP in water Pump B – Line A: Acetonitrile – Line B: Methanol
Flowrate:	0.4 mL/min
Time Program (%B):	6% (0 min) →24% (36 min) → 50% (36–37 min) →6% (37–46 min)
Detection:	260 nm (SPD-M40, UHPLC inert cell)
System:	LCMS-2050
Ionization:	ESI/APCI (DUIS ™) negative mode
Mode:	SCAN (m/z 500-2000)
Nebulizing Gas:	2.0 L/min (N2)
Drying Gas:	5.0 L/min (N2)
Heating Gas:	7.0 L/min (N2)
DL Temp.:	200 °C
Desolvation Temp.:	450 °C
Interface Voltage:	-2.0 kV

*1) 1,1,1,3,3,3-hexafluoro-2-propanol
*2) Triethylamine

Initial screening of mobile phase

For initial screening, parameters affecting separation such as the HFIP and ion-pair reagent (TEA) concentration in the aqueous mobile phase and the ratio of acetonitrile and methanol in the organic solvent were considered. Specifically, two HFIP concen-

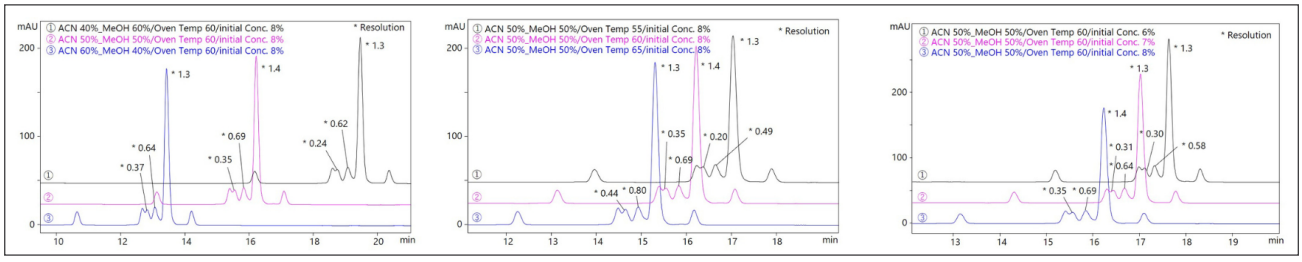

FIGURE 1: a) Chromatograms with Different Acetonitrile Ratio 40% (①), 50% (②), and 60% (③)
b) Chromatograms with Different Column Oven Temperature 55 °C (①), 60 °C (②), and 65 °C (③)
c) Chromatograms with Different Initial Concentration 6% (①), 7% (②), and 8% (③)

trations of 100 and 200 mmol/L, four TEA concentrations of 5, 10, 15, and 20 mmol/L, and three acetonitrile ratios of 0, 50, and 100% were evaluated, resulting in 24 patterns (2×4×3) to find the combination for the optimal separation of target oligonucleotide and impurities. LabSolutions MD facilitates the creation of an analysis schedule by setting parameters like mobile phases and column oven temperature. The mobile phase blending function automatically prepares phases with varying HFIP and TEA concentrations and acetonitrile/methanol ratios for automated screening. The full analytical conditions are shown in Table II.

Chromatograms of FLP and impurities were measured under different HFIP and TEA concentrations and acetonitrile/methanol ratios. The screening results showed that these factors significantly affect the separation of FLP and related impurities.

Finding the optimal condition

Because screening generates numerous chromatograms, evaluation to determine the optimal one is required. Manually checking all chromatograms is troublesome and time-consuming. LabSolutions MD uses an equation to quantitatively evaluate chromatographic separation by calculating an Evaluation Value, which is the number of peaks detected multiplied by the sum of resolution factors for all peaks.

(Evaluation Value) = P × (Rs1 + Rs2 +...+ RsP-1)

Optimization

Based on initial screening results, further optimization was done by adjusting acetonitrile ratio (40, 50, 60%), column oven temperature (55, 60, 65 °C), and initial concentration of gradient program (6, 7, 8%). Higher acetonitrile ratio, column oven temperature, and initial concentration generally improved peak resolution (Fig. 1 a–c).

Due to high similarity in UV spectra (>0.99) of impurities, peak tracking based on UV is difficult. LabSolutions MD uses m/z values obtained with LCMS-2050 for accurate peak identification, automatically tracking each peak through different LC parameters, thereby enhancing operational efficiency and reducing human error.

The optimal condition (point A) chromatogram showed resolution of n-1(3') and PO >0.7, and retention time of last eluting peak

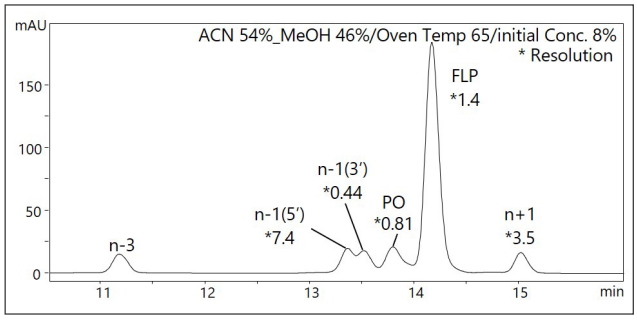

FIGURE 2: Chromatogram at Optimal Condition (100 mmol/L HFIP and 10 mmol/L TEA)

(n+1) <16, meeting optimization criteria (Fig. 2). LabSolutions MD maximizes resolution without relying on user experience, even for closely related structures like n-1(5') and n-1(3').

Conclusion

Oligonucleotide separation patterns depend on HFIP and TEA concentrations, acetonitrile/methanol ratios, column oven temperature, and gradient program concentration. LabSolutions MD can automate the workflow, including analysis schedule generation, mobile phase preparation, and data processing with features like automated peak tracking and chromatogram ranking by Evaluation Value, enhancing separation efficiency.

Reference:

(1) Application News, Shimadzu Corp. (01-00558-EN) April 2023

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Comprehensive Polar Metabolite Profiling with HILIC-LC-MS

Rongrong Cheng[1], Jianwei You[1], Wen Jiang[2], and Li Chen[1]
[1]*Shanghai Key Laboratory of Metabolic Remodeling and Health, Institute of Metabolism & Integrative Biology, Fudan University,* [2]*HILICON AB*

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 hydrophilic metabolites, such as small organic acids, amino acids, nucleosides, and sugars, meets challenges due to their poor retention in traditional 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 the hydrophilic metabolites despite the earlier works show it is more effective for a small set of metabolites (2). Thus, it's essential to achieve a balance between separation and coverage of biologically relevant metabolites in metabolomics studies.

In this application note, we describe a HILIC-LC-MS method that accomplished high coverage and satisfying separation of several hundred metabolites using a polymeric iHILIC-(P) Classic column in a single run. The importance of incorporating the 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 40:40:20 (v/v%) acetonitrile-methanol-H_2O dilution solution and stored at −80 °C. The final concentration was 10 µM.
2. 100 µL plasma was mixed with 400 µL ice-cold aforementioned dilution solution in an 1.5 mL tube by vortexing for 3-5 s. The mixture was then incubated overnight at -80 °C. Whereafter, the samples were centrifuged at 15,000 rpm for 15 min at 4 °C. Supernatants were transferred to sample vials for LC-MS analysis.
3. 293T cells after medium removal were extracted with 500 µL ice-cold aforementioned dilution solution for about 10 min. The cell extracts were transferred to 1.5 mL tubes and stored overnight at −80 °C. The protein removal was the same as that for plasma. The supernatant was used for LC-MS analysis.

LC-MS/MS system:

A Shimadzu ExionLC AC HPLC system was connected to a Triple TOF 6600+ mass spectrometer from AB Sciex. Electrospray ionization (ESI) in both ESI+ (positive) and ESI- (negative) mode were used for detection. The ESI source parameters: source temperature at 550 °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, iHILIC®-(P) Classic (P/N 160.152.0520, 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 I: 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 represent a significant advancement for HILIC separation at basic pH. With the simplified extraction technique and the optimized mobile phases, the columns allow to successfully separate several hundred hydrophilic metabolites in a single run at pH >9. This makes them an invaluable tool for untargeted metabolomics studies, especially when combined with ESI- mass spectrometry for enhanced detection and quantification. 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 293T cell, 300 in plasma, and 113 in both. We were able to use our dataset of 154 metabolite standards to identify 61 metabolites exclusively in 293T cell, 34 in plasma, and 59 in both. The detection method applied in this study is effective for capturing a broad spectrum of metabolites in different classes, especially for organic acids and their derivatives and nucleosides.

The detection sensitivity of the described HILIC-MS method for analyzing metabolite standards is depicted in Figure 2. It illustrates a range of detection limits for each metabolite, from the least sensitive (highest detection limit) to the most sensitive (lowest

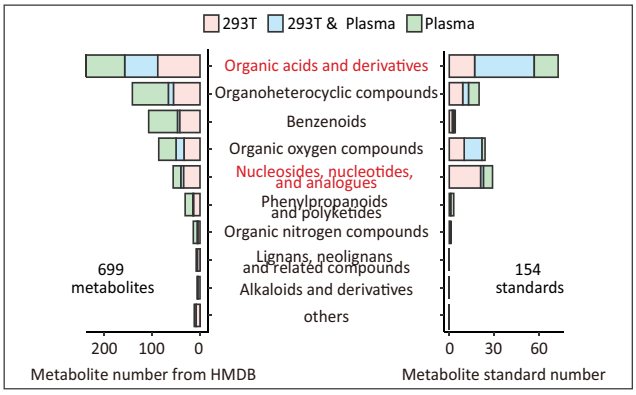

FIGURE 1: Untargeted profiling of hydrophilic metabolites in 293T cells and plasma with HILIC-MS method in ESI- mode.

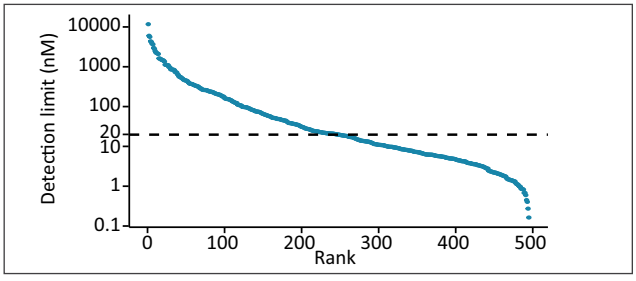

FIGURE 2: Distribution of detection limits for metabolite standards.

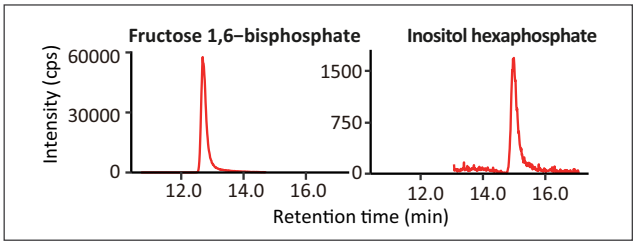

FIGURE 3: Extracted ion chromatograms of highly polar metabolite standards.

detection limit). The median detection limit is reported to be 19.7 nM, which indicates our method is highly capable of detecting a wide range of metabolites at low concentrations. Consequently, high sensitivity and versatility of this method ensure accurate and reliable quantification of metabolites in complex biological samples.

Furthermore, the HILIC-LC-MS method can separate and detect the phosphorylated metabolites that play important roles in cellular metabolism and signaling pathway. As shown in Figure 3, highly polar compounds with various levels of phosphates, such as Fructose 1, 6-bisphosphate and Inositol hexaphosphate are well identified. The effectiveness of the HILIC-MS method also enables superior separation of four biologically important metabolites and their isomeric forms, shown in Figure 4. Such separations are the foundation of the quantification and identification of metabolites for biological interpretation.

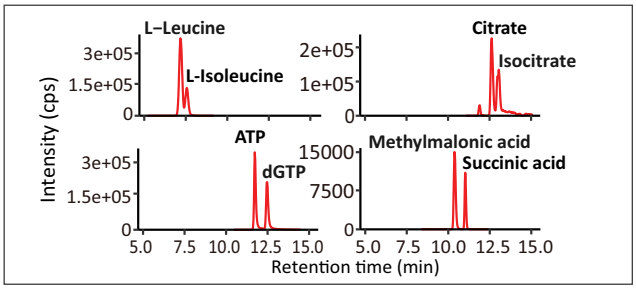

FIGURE 4: Extracted ion chromatograms of isomers from standards or samples. L-Leucine and L-Isoleucine in real samples; ATP: Adenosine triphosphate; dGTP: Deoxyguanosine triphosphate.

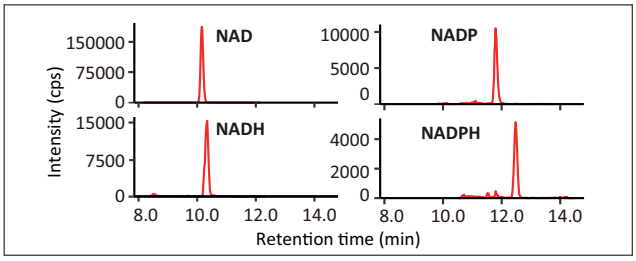

FIGURE 5: Extracted ion chromatograms of fragile metabolites in real samples.

It's worth emphasizing that our sample extraction methods without drying and reconstitution ensure the preservation of fragile metabolites, like nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADP). The detection of NADPH/NADP+ and NADH/NAD+ are presented in Figure 5.

Conclusion

In summary, the current HILIC-LC-MS method profiles 500+ polar metabolite standards across categories in metabolomics studies. Its excellent detection sensitivity and coverage of metabolites, along with straightforward sample preparation, enable the method being applied in comprehensive polar metabolite profiling of biological samples.

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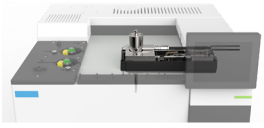
GC Detector

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



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Parkstrasse 2, CH-6214 Schenkon, Switzerland

UHPLC HILIC Columns

iHILIC-Fusion and iHILIC-Fusion(+) have two lines of 1.8 µm UHPLC HILIC columns with different surface chemistries. They provide customized and complementary selectivity, ultimate separation efficiency, and ultra-low column bleeding. The columns are particularly suitable for LC–MS based applications in the analysis of polar compounds.

HILICON AB

info@hilicon.com | www.hilicon.com

Tvistevägen 48 A, SE-90736 Umeå, Sweden

Parallel Peptide Purification Solution: Biotage® PeptiPEC-96

The Biotage® PeptiPEC-96 is a high-throughput, automated peptide purification solution designed for use with the Biotage® Extrahera workstation. Utilizing PurePep® EasyClean (PEC™) technology from Gyros Protein Technologies, it employs a catch-and-release methodology for purifying chemically synthesized peptides. Compared to sequential processing with RP-HPLC, it accelerates purification by up to 75% and reduces solvent use by up to 98%.

Biotage Sweden AB

info@biotage.com | www.biotage.com/peptiren-plates-peptipec-kit | Headquarters: Vimpelgatan 5, 753 41 Uppsala, Sweden

PREPSYSTEM-Chromatography Separation System

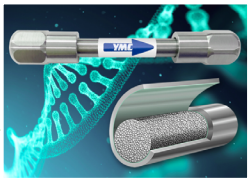
A compact high-end preparative binary HPLC system with an excellent price/performance ratio. Suitable for the most demanding and complex separations, including small molecules, cannabinoids, oligonucleotides, and peptides, the PrepSystem is particularly useful for applications requiring very precise and accurate gradient composition mixing., and is capable of an advanced automated purification method-stacking injections.

ECOM spol. s r.o.

info@ecomsro.cz | www.ecomsro.com/prepsystem-chromatography-separation-system | Chrastany, Czech Republic

Bioinert Coated IEX Columns

The new bioinert coated YMC-Accura BioPro IEX columns ensure exceptionally high recoveries. They provide very sharp peaks with high sensitivities due to an efficient bioinert surface coating of the column body and frits. YMC-Accura BioPro IEX columns are the first choice for high resolution analysis of antibodies, proteins, and oligonucleotides. Their non-porous hydrophilic polymer beads provide high efficiencies and high throughput. The superior lot-to-lot reproducibility guarantees reliable results for analytical and (semi)preparative separations as well as when coupled to MS.



YMC Europe GmbH

support@ymc.eu | https://ymc.eu/iex-columns.html | Schöttmannshof 19, D-46539 Dinslaken, Germany

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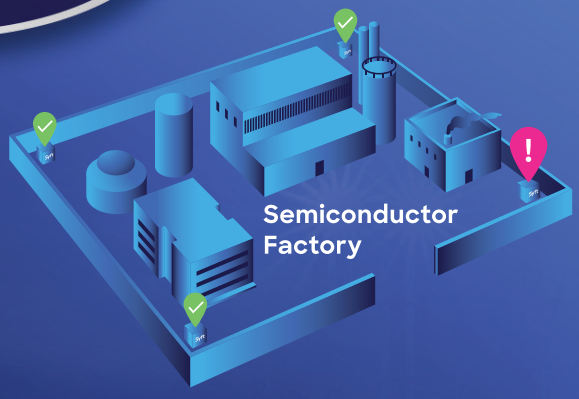

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