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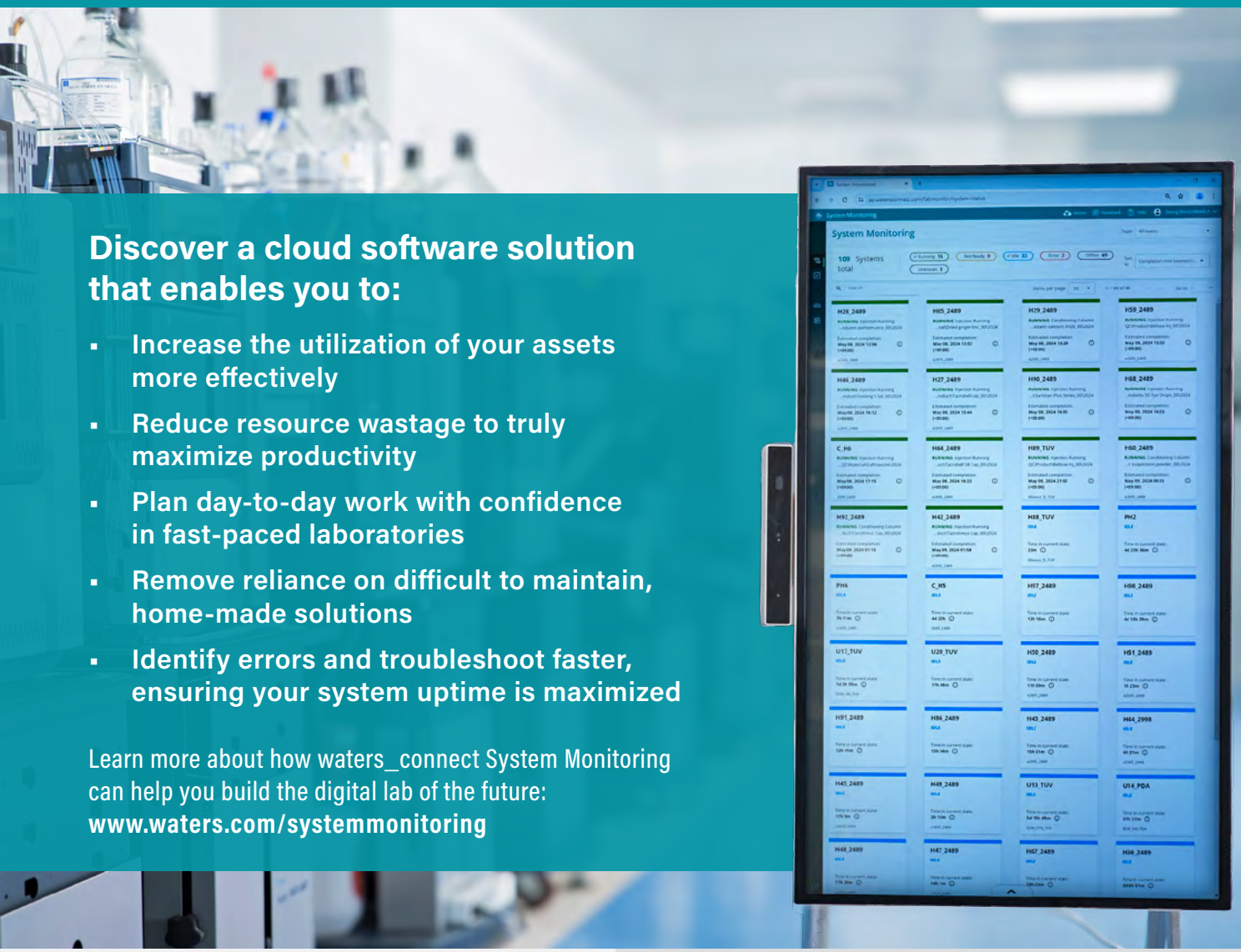

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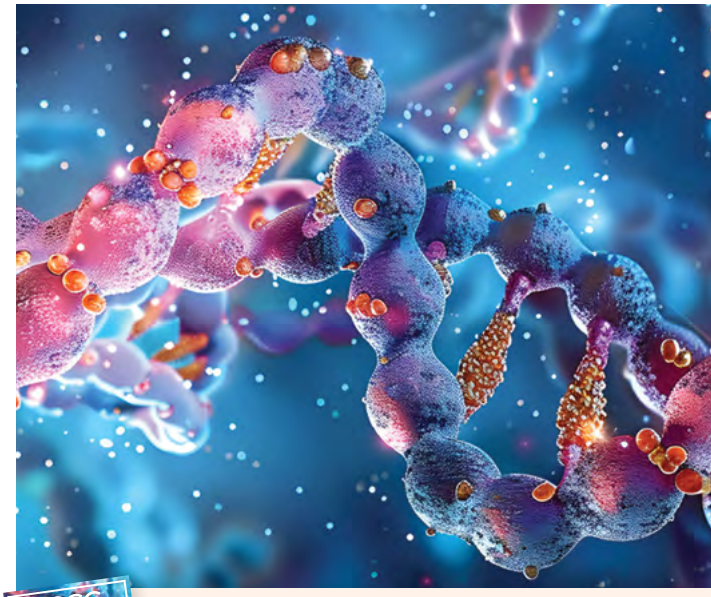

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Using HILIC–MS for characterization of oligonucleotides for genetic research.

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What Does Industry Want New Chemists to Know?

The results of a survey asking BA-level chemists working in industry what industry wants them to know, as well as potential future work that could fill in knowledge gaps identified by the survey, are discussed.

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NOTE FROM THE CEO

WELCOME TO THE OCTOBER 2024 issue of *LCGC International*! We're thrilled to present an exciting collection of articles showcasing the latest advancements in liquid chromatography and mass spectrometry techniques, with a special focus on oligonucleotides, therapeutic proteins, and biopharmaceutical analysis, with expert insights, in-depth discussions, and cutting-edge innovations designed to enhance your laboratory practices and deepen your understanding of analytical techniques.

Dwight Stoll kicks things off with his popular "LC Troubleshooting" and a comprehensive update on what's new in reversed-phase liquid chromatography selectivity. Stoll provides an enlightening discussion on how the Hydrophobic Subtraction Model (HSM) continues to evolve, focusing on more than 30 new stationary phases recently added to the HSM database. He also offers tips for using this updated information to optimize method development and troubleshoot issues in your LC methods.

Next, in our "Column Watch" series, a team of experts from Agilent presents "Systematic Evaluation of HILIC Stationary Phases for MS Characterization of Oligonucleotides," which dives into the advantages of using hydrophilic interaction liquid chromatography (HILIC) as an alternative to ion-pair reversed-phase chromatography (IP-RPLC) for oligonucleotide analysis. The authors explain how HILIC's compatibility with mass spectrometry (MS) can simplify instrument use while maintaining analytical performance.

In "Perspectives in Modern HPLC/UHPLC," Michael Dong shares "A Well-Written Analytical Procedure for Regulated HPLC Testing," offering practical guidance for developing a stability-indicating HPLC assay that meets regulatory compliance. Dong's tips are invaluable for ensuring clarity and efficiency in your testing procedures, with a particular focus on simplifying the execution for laboratory analysts.

"Biopharmaceutical Perspectives" presents an insightful feature titled "Detailed Glycosylation Analysis of Therapeutic Enzymes Using Comprehensive 2D-LC–MS," demonstrating the powerful capabilities of 2D-LC–MS for analyzing the glycosylation patterns in therapeutic enzymes, offering a step-by-step breakdown of how to apply this technique to therapeutic protein analysis.

Finally, we close with a feature article titled "Characterization of Product-Related Variants in Therapeutic Monoclonal Antibodies," which explores the critical process of identifying and quantifying size and charge variants in monoclonal antibodies—an essential step in ensuring the efficacy and safety of therapeutic antibodies.

We hope this issue inspires your next scientific breakthrough.

Enjoy the read! ■

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

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What's New in Reversed-Phase Liquid Chromatography Selectivity?

An Update from the Perspective of the Hydrophobic Subtraction Model

Dwight R. Stoll

It has been more than five years since the last update in this column on the evolution of the Hydrophobic Subtraction Model (HSM) of reversed-phase selectivity and characteristics of new stationary phases recently characterized using the model. In this time, nearly 50 new columns have been added to the public HSM database, and new perspectives on the limitations and use of the model have been published. In this installment, I discuss the continuing evolution of the model, and characteristics of the recently added columns, with an eye toward use of this information for troubleshooting and method development.

IN THE APRIL 2020 INSTALLMENT of "LC Troubleshooting," I summarized the findings reported by five different speakers as part of a symposium at the Pittsburgh Conference held in Chicago that year that was aimed at highlighting the success and evolution of the hydrophobic subtraction model of reversed-phase selectivity (HSM) (1). The HSM has been wildly successful by any measure, and the public database associated with the model is still the single largest compilation of characteristics of reversed-phase columns that is freely available, with data for more than 775 columns. The development of the model was initiated in the late 1990s by Lloyd Snyder, John Dolan (the long-time author of this column until 2017), Peter Carr, and many other collaborating research scientists from both academia and industry. This early work was supported by the Product Quality Research Institute, the National Institutes of Health, and the United States Pharmacopoeia, resulting in tens of research articles describing the work over the following two decades, and the public database of column characteristics as we know it today, which is maintained on two websites that will be referenced later in the article. Over the past decade, growth in the database has been quite steady, with additions at a rate of about two columns per month on average. Most of this growth has been supported by column manufacturers who provide the columns that are used to make the retention measurements needed to determine the column characteristics as described in the following section.

In my interactions with LC practitioners, I find that there is a wide range of ways that different scientists interact with the HSM and the columns database. On one hand, there are people very familiar with the model and database who describe numerous examples of ways that they have leveraged the model in their method development work, including using the database to address difficult situations. On the other hand, there is still a large population of scientists who have either never heard of the database, or do not have enough familiarity with it to leverage it effectively in their work. In this installment, I hope to provide something useful for both beginners and advanced users.

Basics of the Hydrophobic Subtraction Model of Reversed-Phase Selectivity

The basic principle of the HSM model was first described in a journal article by Snyder and coworkers in 2002 (3). Since then, many articles have been published on the topic, but two resources are particularly noteworthy for readers interested in learning more about the model. First, in 2012, Snyder and coworkers published a book chapter in *Advances in Chromatography* that is still the most comprehensive discussion of the model and its application that has been published to date (2). Second, a more recent article in *LCGC* provides more of an overview of the model and its application that may be an easier place to start for those that are completely new to the idea (4). The model, which was originally developed using retention data from alkyl phases (for example, C4, C8, and C18) bonded to high-purity type B silicas, assumes that RP selectivity (defined here as the ratio of retention factors for a compound of interest, such as ethylbenzene) can be described using the sum of five pairs

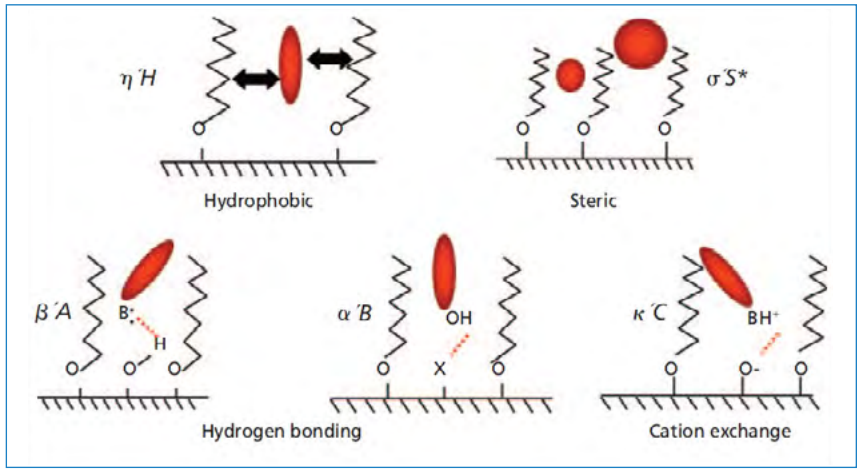

FIGURE 1: Conceptual illustration of the five major solute-stationary phase interactions accounted for by the HS model. Reprinted from reference (3).

of column and solute parameters that are related to different physicochemical interactions between solutes and the RP stationary phase. A view of the nature of each of these interactions is shown in Figure 1.

The mathematical expression of the model is shown in Equation 1, where the capital Roman letters H, S*, A, B, and C are column parameters, and the Greek small letters *η*, *σ*, *β*, *α*, and *κ* are solute parameters.

$$\log\left(\frac{k}{k_{EB}}\right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C \quad [1]$$

The column parameters are determined experimentally by measuring the retention times of 16 carefully chosen probe solutes in a mobile phase composed of acetonitrile and potassium phosphate buffer at pH 2.8, calculating the selectivity value for each compound (k/k_{EB}), and regressing those selectivities against the known solute parameters for the probe compounds (1). To date, parameters for 775 commercially available columns have been determined and are publicly available for free through two websites: 1) a site maintained by the United States Pharmacopoeia (https://apps.usp.org/app/USPNF/columnsDB.html); and 2) a site maintained by my research group (www.hplccolumns.org). The two primary uses of this database are finding columns that have similar selectivities (for example, identifying a backup column during method development), and finding columns that have very different selectivities (for example, identifying a set of columns to screen during method development). In recent years, the identification of highly complementary selectivities that can be used in the second dimension of 2D-LC platform methods to evaluate peak purity has become very important in the pharmaceutical industry (5), and the HSM can be used to guide this process.

New Entries in the HSM Database in the Last Five Years

Over the last five years, we have made 46 new entries for RP columns in the publicly available HSM database. A summary of the numbers of columns in different phase categories is shown in Figure 2. Some of these columns are truly new, both to the database, and to the commercial market. Others have been on the market for some time, but have been characterized only recently using the HSM. Here, we see a few interesting trends. First, it is remarkable how dominant the C18 group is in the most recent set of additions. I suspect there are at least a couple of major drivers for this. Many of the new columns in the 2019 to 2024 group are coming from relatively new manufacturers, and it makes sense that many of their first entries in the market would be in the C18 space, given that it is the dominant chemistry used for RP separations. The other factor that is definitely playing a role here is that we rarely see "plain vanilla" C18s these days. Although we often don't know what the details of the stationary

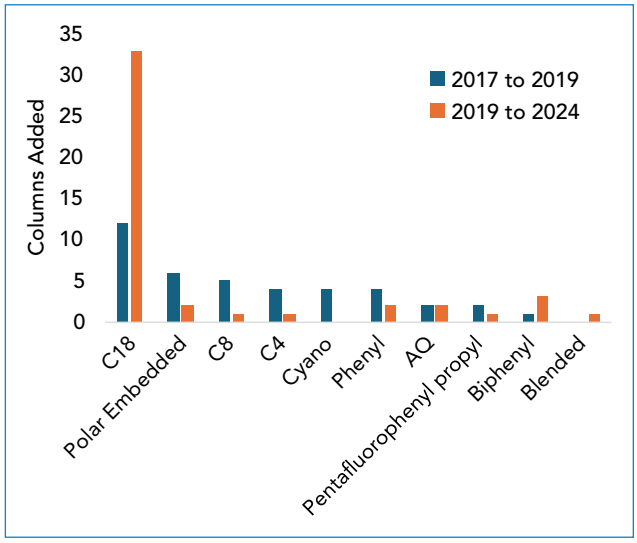

FIGURE 2: Numbers of columns of different stationary phase types added to the HSM database in the past five years compared to the prior two-year period.

phase chemistry are, it is often evident from the column name that, although the phase is referred to as a "C18," it really is probably some kind of hybrid phase with functionalities other than C18 that are influencing the selectivity of the phase in important ways. Other interesting trends we see here are that there hasn't been a single new cyano column (CN) introduced to the database in the last five years, and just one pentafluorophenyl propyl phase (PFP), but we may be seeing an increase in the popularity of biphenyl phases, with three new columns coming from three different manufacturers in the last five years.

New to the Database Versus New Chemistry

An interesting question to consider when thinking about the recent additions to the database is, "Which of these new database entries reflects selectivity that was not represented in the database previously, and which of the new entries is redundant in the sense that the new entry is effectively equivalent to another column that was already in the database?" Both types of entry are valuable, of course. Columns that truly reflect a new selectivity that was not represented in the database previously deepen the selectivity pool, giving analysts more options to choose from when looking for a column with different selectivity compared to what they already have. On the other hand, new entries to the database that are effectively equivalent to an existing entry add resilience to the RP column ecosystem. In the event of a supply chain disruption, for example, it is valuable to be able to quickly identify an alternate or "backup" column that can be used in place of the column normally specified for a method, such that a similar separation is achieved, and the results can be used with little to no method development effort. Over the years, I have had several highly experienced LC users tell me about situations like this where the HSM columns database has "saved the day" by quickly identifying an alternative column when they have experienced problems with the normally used column.

To identify "equivalent" columns, Snyder, Dolan, and coworkers have advocated for the use of a "similarity factor," F_S, which is a weighted distance between two columns in five-dimensional selectivity space (4). When F_S is calculated this way, columns for which $F_S < 3$ are considered "equivalent," meaning that they are effectively interchangeable and will produce very similar chromatograms for most applications. Of course, this must be verified experimentally for any particular application, but it is at least a good guideline. Columns for which $F_S > 100$ are considered very different, and looking for such columns may be useful when intentionally assembling a set of columns with very different selectivities. Interpretation of F_S values between 3 and 100 depends on the properties of solutes in a particular mixture of interest, and the degree of change in selectivity that is tolerable when trying to identify columns with similar selectivity (or "equivalent" columns). Figure 3 (Figures 3-5 are available online by accessing the QR code at the end of the article) shows a histogram of F_S factors calculated for each new entry in the database since 2019. We see that 27 of the new entries are effectively equivalent to an already existing entry, and eight more are very similar to existing entries ($3 < F_S < 5$). We see that the largest F_S factor for any of the new entries is just 12, meaning that none of the new entries are very different from the entries that existed prior to 2019.

Evolution of the Hydrophobic Subtraction Model Itself

The original HSM, as developed by Snyder and colleagues in the early 2000s, is still the only model of its kind for which a public database exists. However, my group has been working with Dr. Sarah Rutan over the past few years to think about how we might refine or update the model in light of both the proliferation of non-C18 phases and the much larger data sets now made available since the development of the original HSM. We have made two major steps in this direction. First, in 2021, we published a paper describing what I think of as a refinement of the original HSM, which we now refer to as HSM2 (6). The original HSM was built using retention data from alkyl silica stationary phases, and the fits of that model to data from non-C18 type phases have not been as good as the fits for C18-type phases. In the development of HSM2, we considered retention data for 551 phases, including all phase types. The improvement in the fit of the model for the full spectrum of stationary phase chemistries can be visualized by looking at histograms of the model residuals, which are shown in Figure 4 for both the original HSM and HSM2. What is particularly striking to me in these plots is the dramatic reduction in the number of very poor fits, as measured by residuals (that is, the difference between the experimentally determined selectivity and the model value) greater than 10%. For the original HSM, there are 230 such values, but for the HSM2, this number is reduced by approximately 90% to just 25. Although we do not maintain a public database for the HSM2, the column and solute parameters were published with the paper, so anyone interested in using those values locally for any purpose can do so.

Having realized how much the model could be improved by training it on a larger data set, more recently we began exploring the capabilities of an HSM-like model trained using data from a much larger solute set. We refer to this next iteration of the model as HSM3 (7). Whereas the original HSM was based on data from 16 solutes and several hundred columns, the HSM3 is based on data from 78 sol-

utes and 13 columns. In this work, we were particularly interested to evaluate the ability of such a model to capture the solute- and stationary phase-related factors that contribute to the selectivity needed for isomer separations. Figure 5 shows a dramatic example of this, where we find that differences between the hydrogen bond acidity (*a* parameter in Figure 5) for two isomers of dinitrophenol, complemented by the hydrogen bond basicities (*B* parameter in Figure 5) of different stationary phases, leads to dramatic differences in selectivities for the separation of these two isomers. For 12 of the 13 stationary phases studied in this work, the 2,4-dinitrophenol isomer is eluted before the 2,5-isomer, but for one of the phases, the elution order is reversed, and the model predicts this reversal accurately.

We are enthusiastic about what this work shows about the potential for future HSM-like models trained on large retention datasets. These models evidently not only have the potential to predict selectivities needed for challenging separations, but also provide insight into what drives these separations at the molecular level.

Summary

In this installment, I have reviewed the basic principles of the hydrophobic subtraction model (HSM) of reversed-phase (RP) selectivity. The HSM and the accompanying free database of column parameters is the single largest publicly available resource for comparing the selectivities of RP columns, which currently stands at over 775 entries. In the past five years, there have been 46 new entries, about half of which are effectively equivalent to already existing entries as measured by the HSM. In parallel with the steady growth of the HSM database, investigators are also considering refinement and evolution of the model itself. In the past five years, two new variants of the model have been discussed in the literature, which we refer to as HSM2 and HSM3. This work provides hints about what the future of these hydrophobic subtraction models might hold, including greater predictive accuracy and insights about the molecular-level drivers for difficult separations, such as the separation of isomers.

Acknowledgment

I want to thank Sarah Rutan for providing Figure 5, which was originally presented at the HPLC 2024 meeting in Denver in July of 2024. ■

This article has additional supplemental information only available online.
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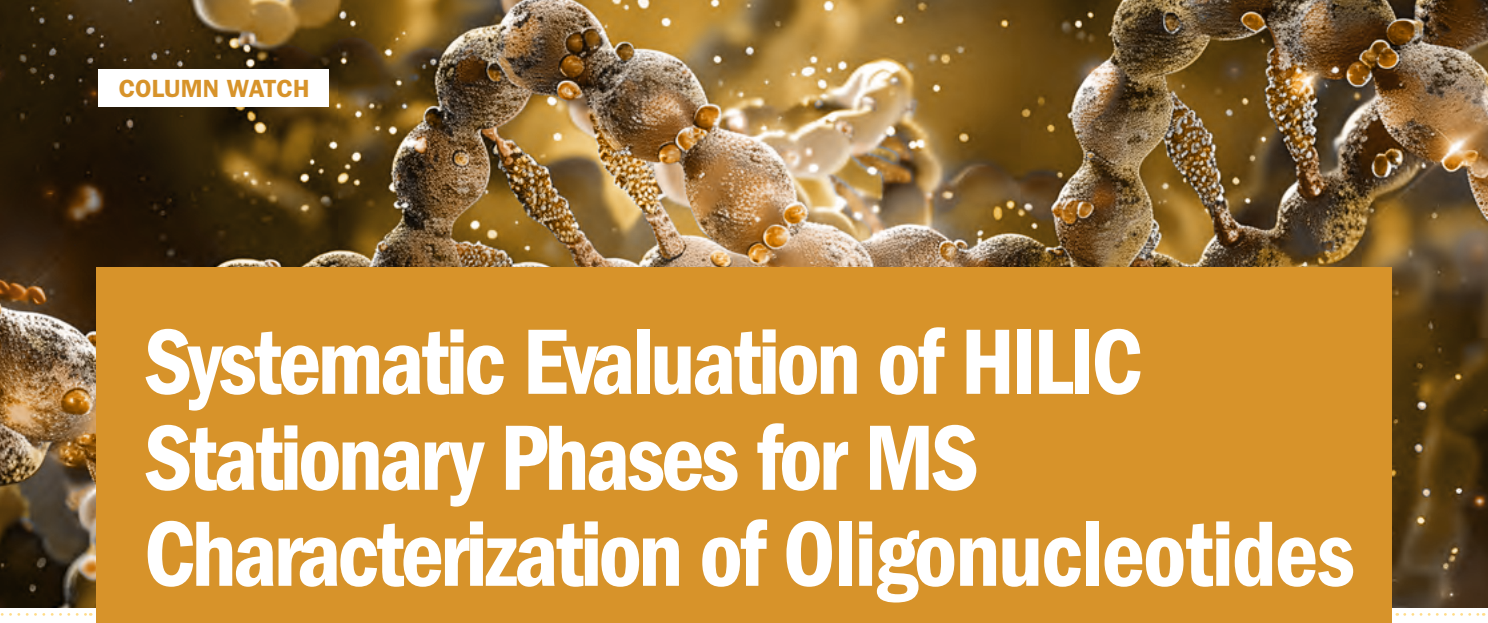

Systematic Evaluation of HILIC Stationary Phases for MS Characterization of Oligonucleotides

Jordy J. Hsiao, Lee J. Bertram, Alex Apffel, Andrea Angelo P. Tripodi, Andrew Coffey, Ta-Chen Wei, and Connor Flannery

Hydrophilic interaction chromatography–mass spectrometry (HILIC-MS) offers a flexible and efficient alternative to ion-pairing reversed-phase liquid chromatography (IP-RPLC) for oligonucleotide analysis, with column selectivity and mobile phase pH being key factors in optimizing retention and detection.

ION-PAIRING LIQUID CHROMATOGRAPHY (IP-RPLC) coupled to mass spectrometry (MS) represents the most common analytical approach for oligonucleotide analysis (1–3). Alternative separation methods are desired as alkylammonium ion-pair reagents force users to have dedicated instruments. Moreover, the use of highly fluorinated chemicals, such as hexafluoroisopropanol (HFIP), as mobile phase modifiers have been under scrutiny because of potential environmental and health-related issues (4). Although ion-exchange chromatography (IEX) represents a viable alternative technique because of its excellent selectivity for oligonucleotides based on their length and negatively charged phosphate backbone, it is not preferred because of its mobile phase incompatibility with MS detection. Hydrophilic-interaction chromatography (HILIC) is an analytical separation technique where analytes are retained because of their polarity as they partition between the organic-rich mobile phase and an aqueous layer created by the hydrophilic stationary phase. The polar nature of oligonucleotides lends itself well to this approach. Moreover, HILIC serves as a valuable alternative to IP-RPLC and IEX as HILIC mobile phases are compatible with MS, thus providing flexibility in instrument use.

A wide variety of HILIC stationary phases exist with different polar functional groups that can contribute to oligonucleotide interaction as the analyte partitions into the water layer near the particle surface. Lardeux and others previously evaluated seven zwitterionic HILIC columns from various manufacturers and provided valuable insights into the columns' retention characteristics that were dependent on the oligonucleotide's chemical makeup (5). In our study, five commonly used HILIC chemistries were evaluated. The columns evaluated include bare silica, poly-hydroxy fructan, zwitterionic, and two different amide chemistries. These five columns were initially screened with the Tanaka test, which is a well-established method to characterize HILIC separation mechanisms (6). This was followed by HILIC-MS analysis of DNA and RNA standards at acidic, neutral, and basic conditions to determine the impact of mobile phase pH on column selectivity and MS ionization for oligonucleotides.

The work presented in this article aimed to characterize the main attributes of the HILIC columns and determine the utility of these columns for MS detection of unmodified and heavily modified samples that mimic biopharmaceutical products. This study highlights the utility of HILIC for oligonucleotide analysis, as well as the critical parameters that need to be considered to optimize LC–MS performance.

Materials and Methods

HILIC Columns

Five HILIC columns (Agilent Technologies) were evaluated in this study. The columns included the Poroshell 120 HILIC (P120 HILIC), HILIC-OH5, HILIC-Z, Amide HILIC, and Glycan Mapping. The Glycan Mapping column represents a neutral amide stationary phase, whereas

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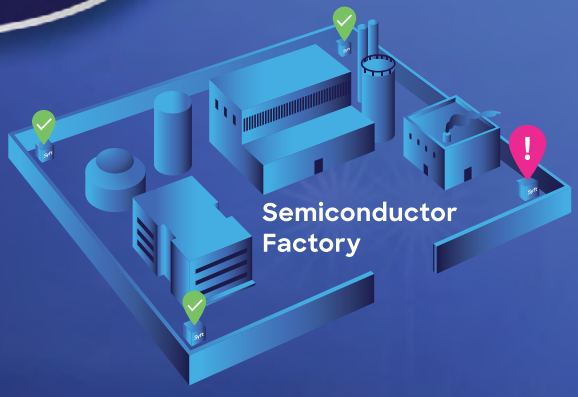

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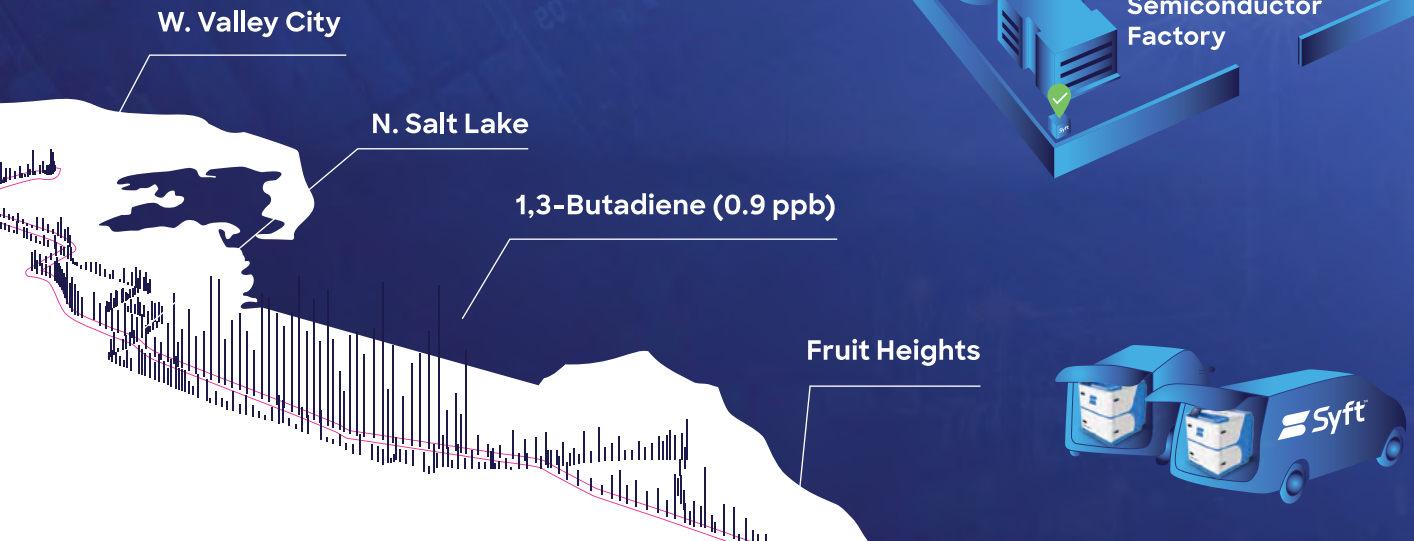

TABLE I: List of HILIC stationary phases evaluated for oligonucleotide separation

#	Column	Pore Size (Å)	Dimension (mm)	Particle Size (µm)	Surface Chemistry	pH Range	Pressure Rating (bar)	Max Temp (°C)
1	HILIC	120	2.1 x 150	2.7	Bare Silica	0–8	600	-
2	HILIC-OH5	120	2.1 x 150	2.7	Poly-hydroxy fructan	1–7	400	45
3	HILIC-Z	100	2.1 x 150	2.7	Zwitterionic	2–12	600	80
4	Glycan Mapping	120	2.1 x 150	2.7	Neutral Amide	2–7	600	40
5	Amide HILIC	300	2.1 x 150	1.8	Mixed Mode Amide	2–7	1200	80

TABLE II: The relative retention of key analyte pairs was used to calculate the indicated retention and selectivity characteristics. Toluene was used as t_0 marker with the following chemicals: uridine (U), 5-methyluridine (5MU), 2′-deoxyuridine (2dU), adenosine (A), vidarabine (V), 2′-deoxyguanosine (2d), 3′-deoxyguanosine (3d), sodium p-toluenesulfonate (SPTS), N,N,N-trimethylphenylammonium chloride (TMPAC), theobromine (Tb), and theophylline (Tp)

#	Parameter	Toluene	Analyte 1	Analyte 2	Description
1	k(U)	t_0	U		Uridine retention factor (k), hydrophilicity indicator
2	α(CH_3)	t_0	U	5MU	Methylene selectivity, hydrophobicity indicator
3	α(OH)	t_0	U	2dU	Hydroxy selectivity, water layer thickness indicator (partitioning)
4	α(V/A)	t_0	V	A	Configurational isomers selectivity
5	α(2d/3d)	t_0	2d	3d	Positional isomers selectivity
6	α(AX)	t_0	SPTS	U	Anion exchange selectivity
7	α(CX)	t_0	TMPAC	U	Cation exchange selectivity
8	α(Tb/Tp)	t_0	Tb	Tp	Acidic-basic nature of stationary phase

the Amide HILIC column represents a mixed-mode HILIC stationary phase with both amide and ion-exchange functionality. All columns contain 2.7 µm superficially porous particles (SPPs), except for the Amide HILIC column, which is a 1.8 µm fully porous particle. A summary of the columns used is described in Table I.

Tanaka Test

The Tanaka test method was adapted from Lardeux and others and applied to the five HILIC columns. Stock solutions of each compound were prepared at 1 mg/mL by dissolving 1 mg of analyte in 1 mL of the mobile phase. The Toluene solution (1 mg/mL) was used as the t_0 marker and prepared by diluting 1.5 µL of toluene in 1 mL of the mobile phase. The test mixtures of the key analyte pairs are listed in Table II. Isocratic separations were performed with analytes at 1 mg/mL, mixtures at 0.33 mg/mL, and an injection volume of 1 µL. A stock solution of 200 mM ammonium acetate adjusted to pH 4.7 with acetic acid was first made. Solvent A was prepared by mixing 100 mL of the stock solution with 900 mL of acetonitrile, which yielded a final concentration of 20 mM ammonium acetate (pH 4.7) in 90% acetonitrile. The flow rate was set at 0.5 mL/min and the column temperature was set at 30 °C. Absorbance data were acquired at 260 nm with five replicate injections per column.

HILIC–MS Analysis of Oligonucleotides

Stock solutions of 100 mM ammonium acetate with pHs of 4.4, 6.8, and 9.0 in water were first made. The acidic buffer was adjusted to pH 4.4 with acetic acid. The basic buffer was adjusted to pH 9.0 with ammonium hydroxide. Solvent A was prepared by mixing 100 mL of each stock solution and 900 mL of water, which yielded a final concentration of 10 mM ammonium acetate at the specified pH in water. Solvent B was made by mixing 100 mL of the appropriate stock solution with 900 mL of acetonitrile, which yielded a final concentration of 10 mM ammonium acetate at the specified pH in 90% acetonitrile. The flow rate was 0.25 mL/min, and the column temperature was set at 30 °C. For the analysis of the 18-mer anti-sense oligonucleotide (ASO), the column was held at 75% solvent B for 2 min before the gradient with solvent A was applied. The gradient elution profile was from 75% B to 55% B for 7 min, followed by washing with 55% B for 2 min. The column was

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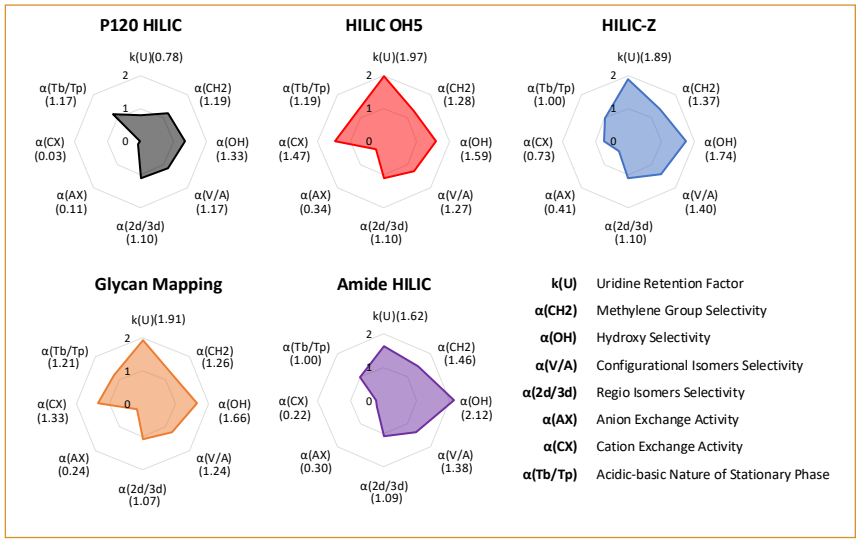

FIGURE 1: Radar plots for five different HILIC stationary phases, with the average of five replicate injections plotted.

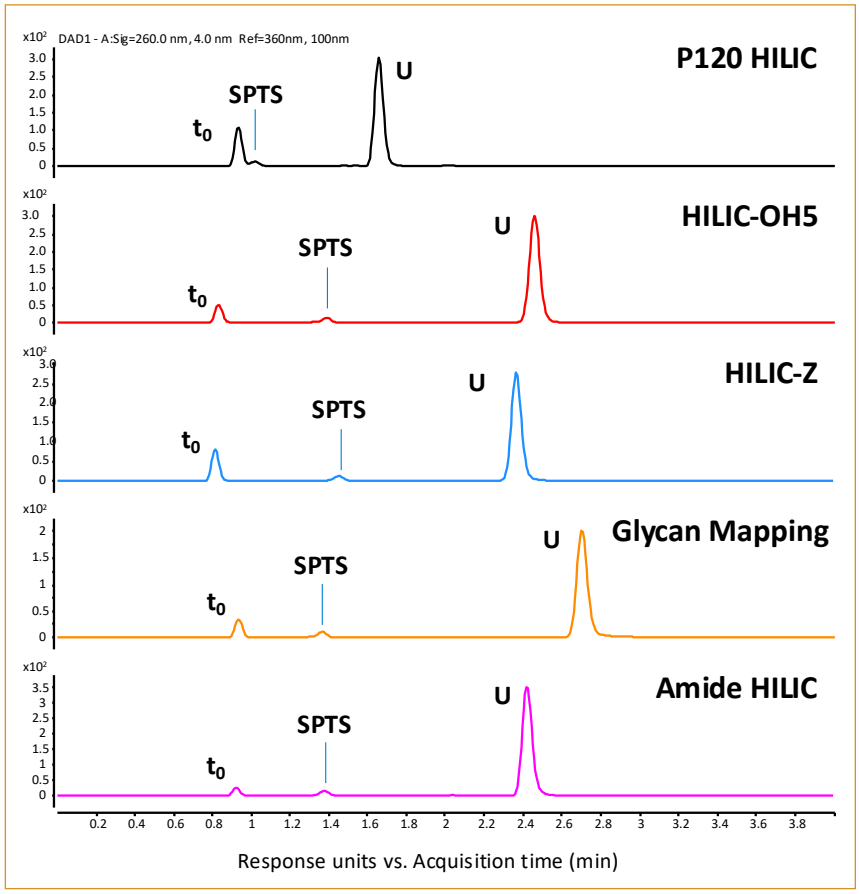

FIGURE 2: Separation of key analyte pairs to determine attributes that participate in the HILIC retention mechanism. The example shown illustrates the retentiveness of the HILIC stationary phases that can be estimated based on the retention factor of uridine, k(U). The relative retention of sodium p-toluenesulfonate (SPTS) vs. uridine (U) can be used to calculate the anion exchange selectivity, α(AX).

equilibrated with 75% B for 5 min prior to subsequent analysis, resulting in reproducible retention time from injection to injection. Full MS (MS1) data was acquired with a mass range of 300–3200 *m/z* and an acquisition rate of 1 spectrum/s on a 6545XT AdvanceBio LC/Q-TOF system (Agilent Technologies). The instrument was operated in negative ion mode.

Results and Discussion

Tanaka Test Results

The Tanaka test was initially performed to better understand the retention characteristics of each of the HILIC column chemistries and how they may contribute to the retention and selectivity of oligonucleotides. The goal was to try and use this information to understand why one HILIC column chemistry may perform better than another for oligonucleotide separation. The retention times collected from each column are used to calculate the retention factor (*k*) of each analyte. The ratio of key analyte pairs was then used to determine the selectivity values (Figure 1). The retention factor of uridine, k(U), can be used to estimate the HILIC stationary phases' retentiveness for hydrophilic molecules. Based on the results, the HILIC-OH5, Glycan Mapping, and HILIC-Z columns were the most retentive, followed by the Amide HILIC column. Not too surprisingly, the P120 HILIC bare silica column had the lowest retentiveness with the least uridine retention compared to the other stationary phases functionalized with polar groups.

In addition to polar interactions, hydrophilic partitioning also plays a significant role in HILIC-based separation. Hydrophilic partitioning is based on the thickness of the immobilized water layer on the stationary phase, which can be estimated using the α(OH) (7). The Amide HILIC column had the highest α(OH) value, followed by

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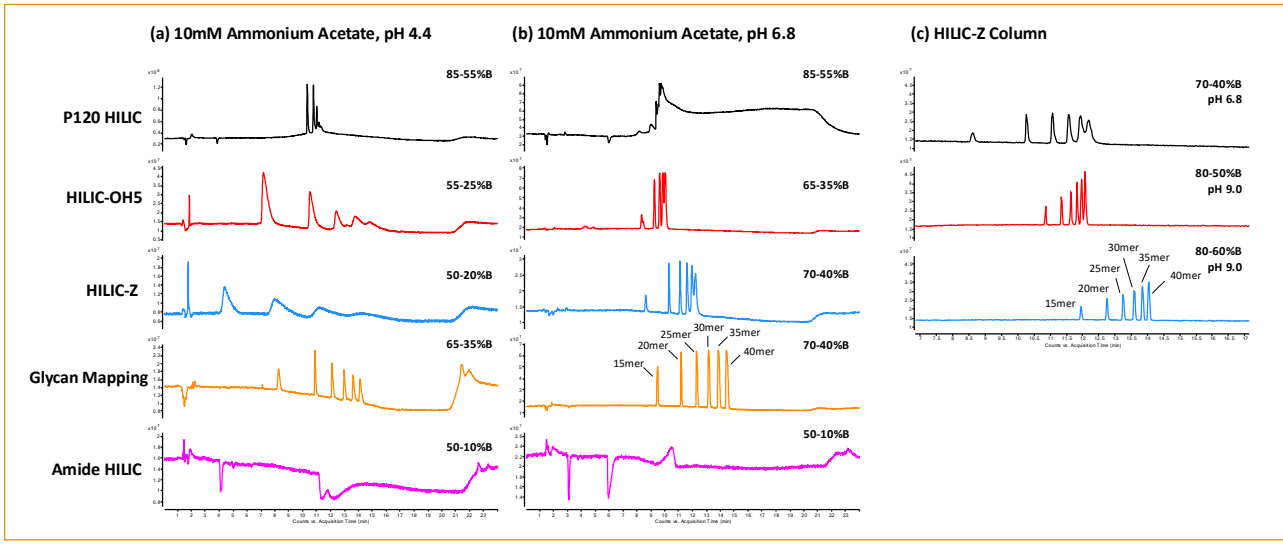

FIGURE 3: HILIC–MS analysis of 15-, 20-, 25-, 30-, 35-, and 40-mer DNA sample using stationary phases with varying chemical properties with 10 mM ammonium acetate buffer at (a) pH 4.4 and (b) pH 6.8. (c) The HILIC-Z column was additionally tested at pH 9.0. The gradient used for each individual column is listed in the top right corner of each chromatogram. The gradient was modified to improve peak resolution and adjust for retention time shifts when switching the pH of the mobile phase.

the HILIC-Z, Glycan Mapping, and HILIC-OH5 columns with less than 25% difference amongst the four functionalized columns. The P120 HILIC bare silica column had the lowest α(OH) value; thus, it had the thinnest adsorbed water layer on the stationary phase.

The selectivity for a methylene group, $\alpha(CH_2)$, can be used to determine the hydrophobic selectivity of the stationary phase. This separation mechanism is useful to resolve methylated impurities from target products, which is measured by the relative retention of 5-methyluridine and uridine. The Amide HILIC column had the highest $\alpha(CH_2)$ value, but less than 20% difference was observed across the columns (Figure 1). This indicated minimal hydrophobic selectivity differences amongst the columns screened in this study.

IEX interactions can be influential in HILIC separation, leading to drastic changes in selectivity. Because oligonucleotides are anionic molecules with a phosphate backbone that is negatively charged, anion exchange (AX) interactions play a stronger role in oligonucle-

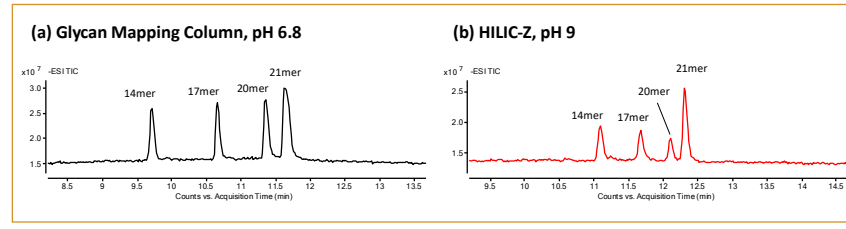

FIGURE 4: HILIC separation of 14-, 17-, 20-, and 21-mer RNA using the Glycan Mapping column at pH 6.8 and HILIC-Z column at pH 9.

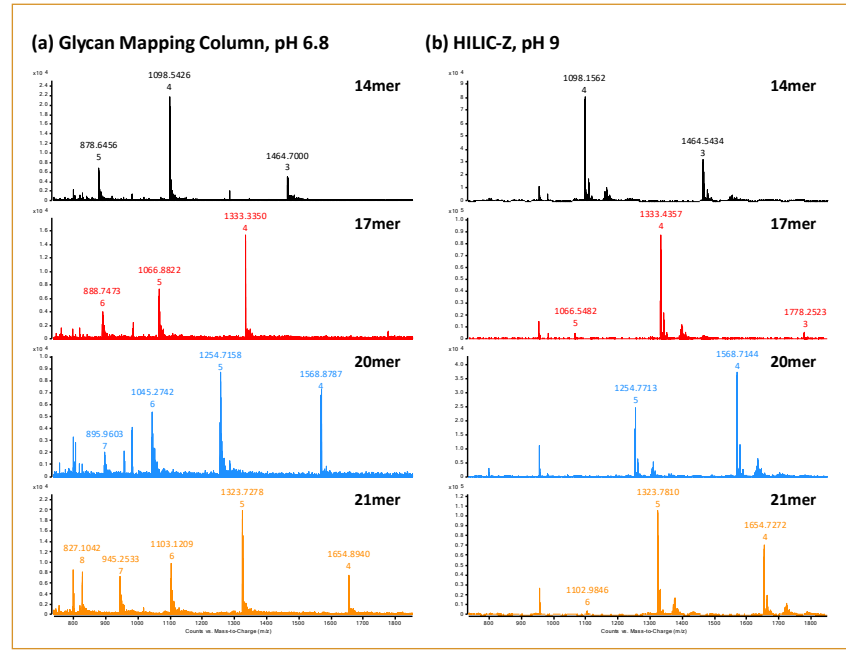

FIGURE 5: Full-scan MS1 mass spectra for 14-, 17-, 20-, 21-mer RNA.

otide retention relative to cation exchange (CX). The HILIC-Z column was found to have the highest α(AX) value followed by HILIC-OH5 and Amide HILIC columns. In comparison, the other columns have moderate to low AX capacities (Figure 1). A representative chromatogram for α(AX) and k(U) is shown in Figure 2 to illustrate the separation of SPTS and U relative to t_0 on each individual HILIC column. This figure also demonstrated the selectivity and retention time differences across the five different HILIC stationary phases. Of note, the lower-than-expected IEX activity collected for the bare silica and amide phases could be because of the use of higher buffer concentrations (20 mM). The higher buffer concentration could have negated the detection of both the cationic and anionic exchange properties, thus reducing the IEX activity. Future experiments using lower buffer concentration would be ideal in order to properly assess these columns' IEX selectivity.

Based on the k(U) and α(AX) values, columns may be categorized by their relative ability to retain negatively charged analytes, a particular interest for oligonucleotide analysis. HILIC-OH5 and HILIC-Z columns are expected to be the most retentive materials, followed by Glycan Mapping and Amide HILIC columns, with the P120 HILIC bare silica column being the least retentive material (Figure 2).

Evaluating HILIC Columns with Oligonucleotides

To assess the utility of the different HILIC stationary phases with oligonucleotides, the columns were evaluated at pH 4.4, pH 6.8, and pH 9.0, as the varying mobile phase composition could change the stationary phases' selectivity and thus alter oligonucleotide separation. Moreover, the mobile phase buffer pH may play a role in oligonucleotide electrospray ionization (8). Understanding and balancing the impact of buffer choice on retention, selectivity, and ionization efficiency is critical when developing optimized LC–MS methods.

To ensure a fair comparison across all columns, the same gradient slope (30% B linear change in 10 min) was used. In general, the columns performed better at pH 6.8 than the acidic pH 4.4 condition (Figure 3a and 3b). This is understandable given the benefits of elevated pH for oligo analysis because it promotes denaturing conditions that result in better retention, resolution, selectivity, and ionization for IP-RP (9). Moreover, the Glycan Mapping column yielded the best selectivity in resolving the varied oligonucleotides of different sequence lengths, followed by the HILIC-Z and then the HILIC-OH5 columns. The P120 HILIC column showed poor selectivity for the DNA oligonucleotides, which was anticipated based on the poor retention and ion-exchange activity determined through the Tanaka test. However, the Amide HILIC column's results were unexpected because changes to the mobile phase composition did not yield any detectable oligonucleotide peak. This may be because of the ion-exchange properties of the mixed-mode column, which is surprising given that this column did not show the most ion-exchange characteristics for the small molecule probes in the Tanaka test. This perceived difference may be reflective of the structural com-

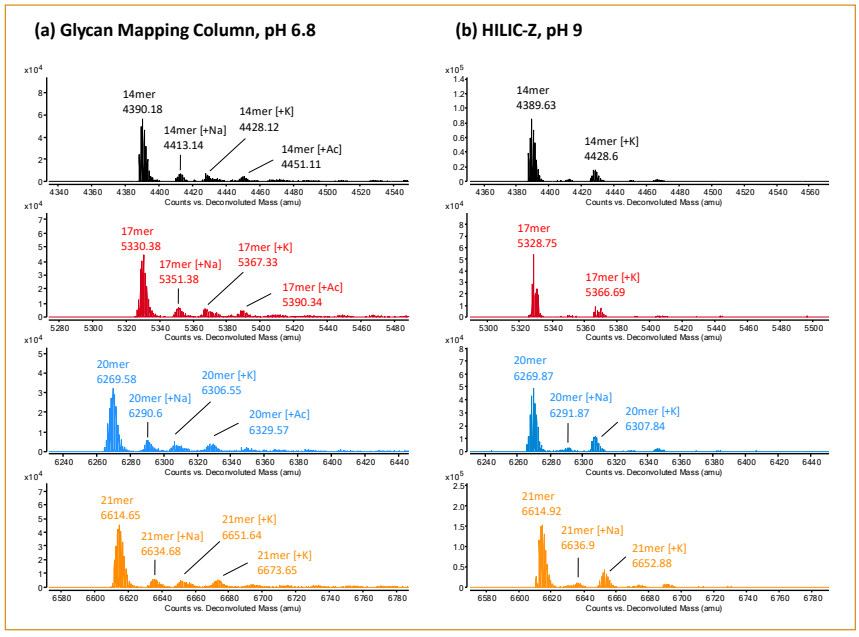

FIGURE 6: Spectral deconvolution of 14-, 17-, 20-, 21-mer RNA, showing the applicability of the LC–MS method for intact mass determination.

plexity of the oligonucleotides, as well as the positional location of the ion-exchange functionality on the different bonded HILIC phases. Further mobile phase and gradient optimizations such as an increase in salt concentrations are required to elute the oligonucleotides off the column.

Given the improved performance of the oligonucleotides going from acidic to neutral pH, the impact of elevated pH was further explored with the HILIC-Z column because of its stability at higher pH (10). The oligonucleotide sample was analyzed with the HILIC-Z column at pH 6.8 and pH 9.0 (Figure 3c). At pH 6.8, the HILIC-Z column yielded slightly broader peaks than pH 9.0 with lower resolution between the 35-mer and 40-mer oligonucleotides. At pH 9.0, the oligonucleotide peaks were noticeably sharper. Baseline separation could be achieved by flattening the gradient from 3% B/min to 2% B/min, with the potential to use an even shallower gradient to further resolve the oligonucleotides.

Based on the results shown in Figure 3, we decided to continue the downstream analysis with the Glycan Mapping column at pH 6.8 and the HILIC-Z column at pH 9.0. RNA samples composed of 14-, 17-, 20- and 21-mer lengths were analyzed on the two columns (Figure 4). Importantly, the 20 and 21-mer RNA samples were resolved with both columns, where separation of the n-1 oligonucleotide pair represents a critical attribute that is closely monitored for the incomplete synthesis of an oligonucleotide (11,12). A closer examination of the mass spectra revealed a wider charge state distribution for the oligonucleotides analyzed with mobile phase buffer at pH 6.8 than pH 9.0. Specifically, a range of 4- to 8-charge states were observed for the 21-mer RNA at pH 6.8 (Figure 5a). In contrast, a range of 4- to 6-charge states were observed for the RNA oligonucleotides at pH 9.0 (Figure 5b). As the length of the oligonucleotide sequence increases, the ability to detect higher charge states would become necessary to allow for detection within the mass range limitations of the mass spectrometer. Furthermore, a broader range of detectable charge states lends more confidence in the target analyte's identification. In addition to optimizing the chromatographic conditions for the columns, it's

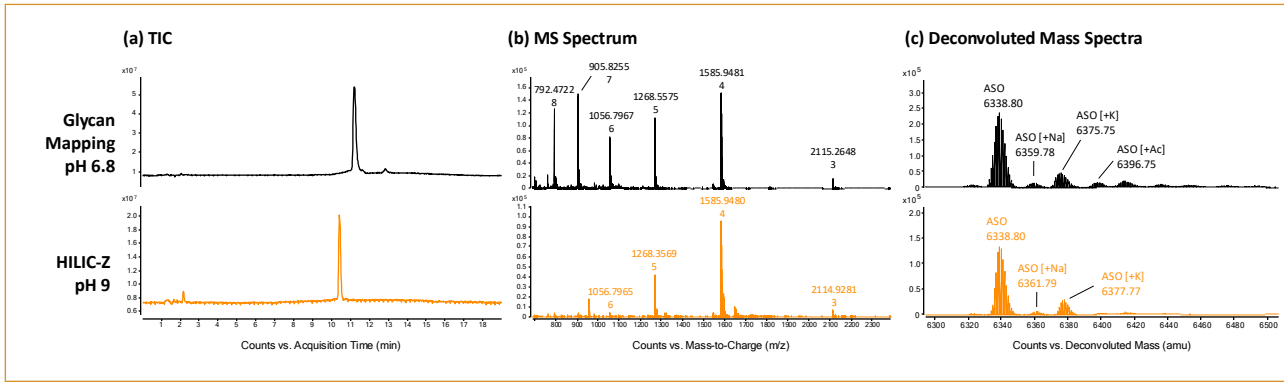

FIGURE 7: Comparison of the Glycan Mapping and HILIC-Z columns for characterization of an 18-mer antisense oligonucleotide.

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equally important to determine the method's compatibility to yield an optimal MS signal response. This allows users to confidently identify the peaks detected by LC–MS. This was demonstrated in Figure 6, where the spectral deconvolution of the mass spectra confirmed the masses for the 14-, 17-, 20-, and 21-mer RNA.

To evaluate our optimized HILIC-MS methods with a heavily modified oligonucleotide, an antisense oligonucleotide (ASO) with multiple phosphorothioate bonds and 2-methoxyethoxy (2MOE) modifications were analyzed using the Glycan Mapping and HILIC-Z column (Figure 7). The results showed both columns yielded a sharp peak for the ASO, with the Glycan Mapping column having better sensitivity relative to the HILIC-Z column (Figure 7a). As expected, the Glycan Mapping also showed a broader charge state distribution with 8-charge state being detected. In contrast, the HILIC-Z column's highest charge state detected was 6- for the ASO (Figure 7b). Lastly, the ASO's identity was confirmed through the deconvoluted mass spectra from both experiments (Figure 7c).

Summary

In this study, we have systematically evaluated five different HILIC stationary phases with the Tanaka test to help understand how the different functional groups contribute to different HILIC retention characteristics. It's clear that the attributes assessed with the small molecule probes don't exactly translate to the larger, more structurally complex molecules such as oligonucleotides. However, it was still insightful to understand the differences among the HILIC stationary phases. As part of our method development, we then aimed to optimize the mobile phase pH to yield better chromatographic peak shape, resolution, and MS signal for several oligonucleotide samples. We found that use of different mobile phase pH conditions can impact the charge state distribution of the analyte, which may be considered when working with longer oligonucleotides. The methods developed ultimately facilitated the LC–MS analysis of a heavily modified ASO. Our work confirms that HILIC chromatography serves as an approach that can be an attractive alternative to IP-RPLC for the analytical characterization of oligonucleotides. The methods could be applied to other exciting and growing oligonucleotide modalities including siRNA, aptamer, single-stranded guide RNA (sgRNA), and mRNA sequencing in the future. ■

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A Well-Written Analytical Procedure for Regulated HPLC Testing

Michael W. Dong

This paper describes the content of a well-written analytical procedure for regulated high-performance liquid chromatography (HPLC) testing. A stability-indicating HPLC assay for a drug product illustrates the required components for regulatory compliance, including additional parameters to expedite a laboratory analyst's execution.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) plays a significant role in the quality control of pharmaceuticals, and the development of stability-indicating assays is often the first key task for separation scientists in the pharmaceutical industry. The intricated method development process of these analytical procedures and regulatory expectations have been described in books (1–3), journal articles (4), and regulatory guidelines (5). This column focuses on the recommended contents of the analytical procedure as outlined in a United States Food and Drug Administration (FDA) guidance document published in 2015 (6). An HPLC assay method for a small-molecule drug product is used here as an illustrative example of the required regulatory compliance elements and suggested parameters that help the analyst for more straightforward method execution with better accuracy.

The Content of an Analytical Procedure

This section is extracted from the US FDA guidance document (6) on the expected content of an analytical procedure used in regulated testing:

"You should describe analytical procedures in sufficient detail to allow a competent analyst to reproduce the necessary conditions and obtain results within the proposed acceptance criteria. You should also describe aspects of the analytical procedures that require special attention. The analytical procedure may be referenced from FDA-recognized sources ([such as the] United States Pharmacopeia/National Formulary [USP/NF], [or the] Association of Analytical Communities [AOAC] International) if the referenced analytical procedure is not modified beyond what is allowed in the published method. You should provide in detail procedures from other published sources. The following is a list of essential information you should include for an analytical procedure."

Principle/Scope

A description of the basic principles of the analytical test/technology (for example, separation or detection); target analyte(s) and sample(s) type (for example, drug substance [DS], drug product [DP], or impurities or compounds in biological fluids).

Apparatus/Equipment

All required qualified equipment and components (including instrument type, detector, column type, dimensions, alternative column, and filter type).

Operating Parameters

Qualified optimal settings and ranges (include allowed adjustments supported by compendial sources or development or validation studies) critical to the analysis (such as flow rate, components temperatures, run time, detector settings, and gradient). A drawing with experimental configuration and integration parameters may be used as applicable.

Reagents/Standards

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- Source (for example, USP reference standard, qualified in-house reference material)
- Purity (for pure chemicals only), state (for example, dried or undried), and concentration
- Potencies (where required by Code of Federal Regulations [CFR], USP)
- Storage conditions
- Directions for safe use (as per current Safety Data Sheet)
- Validated or documented shelf life

Sample Preparation

Procedures (such as extraction method, dilution or concentration, desalting procedures, and mixing by sonication, shaking, or sonication time) for the preparations for individual sample tests. A single preparation for qualitative and replicate preparations for quantitative tests with appropriate units of concentrations for working solutions (for example, µg/mL or mg/mL) and information on the stability of solutions and storage conditions.

Standards Control Solution Preparation

Procedures for the preparation and use of all standard and control solutions with appropriate units of concentration and information on stability of standards and storage conditions, including calibration standards, internal standards, system suitability standards, etc.

Procedure

A step-by-step description of the method (equilibration times, and scan/injection sequence with blanks, placebos, samples, controls, sensitivity solution [for impurity method] and standards to maintain the validity of the system suitability during the span of analysis), and allowable operating ranges and adjustments, if applicable.

System Suitability

Confirmatory tests procedures and parameters to ensure that the system (equipment, electronics, and analytical operations and controls to be analyzed) will function correctly as an integrated system at the time of use. The system suitability acceptance criteria applied to standards controls and samples, such as peak tailing, precision and resolution acceptance criteria, may be required as applicable. For system suitability of chromatographic systems, refer to the FDA guidance for industry on *Validation of Chromatographic Methods and USP General Chapter <621> Chromatography*.

Calculations

The integration method and representative calculation formulas for data analysis (standards, controls, samples) for tests based on label claim and specification (such as assay, specified and unspecified impurities, and relative response factors). This includes a description of any mathematical transformations or formulas used in data analysis and a scientific justification for any correction factors used.

TABLE I: Qualified optimal parameter settings and ranges critical to the analysis

Parameter	Condition		
Column	ACE-3 C18 Column, 150 mm x 4.6 mm, 3 µm, or equivalent		
Column Temperature	30°C		
Flow Rate	1.0 mL/min		
Injection Volume	10 mL		
Recommended Needle Wash	20 mM Ammonium formate buffer, pH 3.7		
Detection Wavelength	UV at 280 nm DAD: Bandwidth: 4 nm, Reference Wavelength: Off		
Mobile Phase	A: 20 mM Ammonium formate buffer, pH 3.7 B: 0.05% Formic acid in acetonitrile (ACN)		
Mobile Phase Gradient Program	Time (min)	% A	% B
	0	95	5
	5	85	15
	30	60	40
	33	10	90
	35	10	90
	36	95	5
Run Time	36 min		
Post-Run Equilibration Time	6 min		

Case Studies from an Early-Phase Small-Molecule Development Project: Background Information

A case study from an early-phase small-molecule oncology drug development project was used to illustrate a regulated HPLC method's content and operating parameters. The new chemical entity (NCE) is a multi-chiral molecule with a complex synthetic scheme to ensure chiral purity, requiring the development of 40+ HPLC achiral and chiral methods to support process chemistry development (7,8). The NCE is a hygroscopic basic compound developed as a monochloride salt with partial crystallinity. The Phase I clinical trial material (CTM) DP was the

powder in a capsule (PIC) dosage form. Refrigeration and storage with a desiccant were required for DS and DP to eliminate moisture absorption of the hygroscopic active pharmaceutical ingredient (API).

Case Study: A Stability-Indicating Early-Phase HPLC Method

In this case study, a stability-indicating early-phase HPLC method illustrates the content and parameters of a well-written analytical procedure used in regulated testing. Comments are included as explanations, clarifications, or justifications for the inclusion of additional information or parameters.

Principle/Scope

To determine the assay (% Label Claim), related substances, and identity in G-1234 drug product capsules by HPLC.

Comments: *This method serves as an assay procedure for three critical quality attributes of the DP: % Label Claim (potency or the amount of the API), related substances (levels of impurities and degradants), and identification (by matching retention time of the main peak with that of a qualified reference standard). The sample is a drug product (5-mg capsule) and the technique is HPLC using reversed-phase LC with ultraviolet (UV) detection.*

Apparatus/Equipment

- HPLC system equipped with a binary or quaternary pump, auto-sampler, temperature-controlled column compartment, UV-detector, and electronic integrator or computer system capable of peak integration or equivalent
- HPLC Column: ACE 3 C18,150 mm x 4.6 mm, 3 µm (P/N ACE-111-1546), or equivalent
- Analytical balance capable of accurately measuring to 0.01 mg
- Top loading balance capable of measuring to 0.1 g
- pH meter
- Vortex mixer
- Sonicator
- Class A volumetric glassware
- Automatic delivery pipettes, or Class A volumetric pipettes
- Syringe Filter, 0.45 µm nylon.
- Disposable syringes.

Comments: *For better method portability or applicability of the analytical procedure across laboratories and countries using equipment from different manufacturers, their requirements should be kept "generic" if possible. The HPLC system must be qualified for Good Manufacturing Practice (GMP) applications (9,10). The exception is the HPLC column, whose description should be detailed and specific, including the part number, manufacturer, dimension, bonded phase, and particle size. An alternate column is often not found in stability-indicating assays, as column equivalency is challenging to demonstrate in complex separations.*

Operating Parameters

See Table I.

Comments: *The operating parameters should have sufficient details*

to allow duplication by another analyst, including parameters such as the composition of the needle wash solution and spectral bandwidth of the diode array detector (DAD). The full gradient program should be listed, including the post-run equilibration time. Including the expected initial column pressure is highly recommended to help in method troubleshooting. The maximum absorbance wavelength of the API is often used as the detection wavelength, as most related substances have the same chromophoric properties as the API. Far ultraviolet (UV) wavelengths may occasionally be selected to provide higher sensitivity to the API and its impurities. Including system dwell volume may be helpful for ultrahigh-pressure liquid chromatography (UHPLC) methods for complex samples.

Reagents/Standards

- Purified water, suitable for HPLC analysis, or equivalent.
- Acetonitrile (ACN): HPLC grade.
- Formic acid: ≥ 97%, or equivalent.
- Ammonium formate: LC/MS grade (e.g., high-purity grade from Sigma-Aldrich, P/N 516961, ≥99.995%).
- G-1234 reference standard.

***Comments**: Reagents and their specified grades or purity should be listed. A qualified reference standard (2, 8) is generally required in regulated pharmaceutical analysis to calibrate the testing system in the potency assay of the API and its identification.*

Sample Preparation

Preparation for 5 mg capsules: Approximately 0.50 mg/mL in diluent. Prepare in duplicate. For example, gently open and drop five capsules into a dry 50 mL wide-mouth volumetric flask. Add diluent and sonicate for at least 5 min to dissolve. Once the sample is dissolved, dilute to volume with the diluent and mix well. Pass an aliquot of the solution through a 0.45 µm nylon filter into an HPLC vial, discarding the first 0.5 mL.

***Comments**: For DS and DP analysis, a simple "dilute-and-shoot" method is generally adopted, with an extra filtration step for tablets and capsules using a disposable 22-mm i.d. membrane filter (2).*

Mobile Phase and Standards Control Solution Preparation

- Mobile Phase A Preparation (MPA): 20 mM ammonium formate buffer, pH 3.7 (For example, weigh 2.52 g ± 0.2 g of ammonium formate on a balance and transfer into 2 L of purified water and mix well. Mix in 1.3 mL of formic acid to arrive at the target pH 3.7 ± 0.1. If required, adjust the pH using additional formic acid. Do not filter.
- Mobile Phase B Preparation (MPB): 0.05% Formic acid in ACN For example, pipette 500 mL of formic acid into 1 L of acetonitrile. Mix well.
- Diluent: 20 mM ammonium formate buffer, pH 3.7
- Reference Standard Solution: Approximately 0.5 mg/mL G-1234 Reference Standard in diluent. Prepare in duplicate. For example, accurately weigh 25 mg of G-1234 Reference Standard and transfer to a 50 mL volumetric flask. Add sufficient diluent to dissolve, and mix thoroughly using a vortex mixer and/or sonication, if needed. Dilute to volume using diluent and mix well.
- System Suitability Sensitivity Check Solution: 0.05% Reference Standard Solution prepared in diluent. For example, pipette 50 mL of Reference Standard Solution into a 100 mL volumetric flask containing diluent. Dilute to volume with diluent and mix well.
- Retention Time Marker Solution: Approximately 0.5 mg/mL G-1234 toxicology lot prepared in diluent.

***Comments**: Detailed procedures for the preparation of MPA (the weaker aqueous MP) and MPB (the stronger organic MP) are included in this procedure. No MP filtration is required when a high-purity reagent such as an LC/MS grade ammonium formate is used (2). The Retention Time Marker Solution is prepared using a toxicologic DS lot (often called the Good Laboratory Practice toxicological evaluation lot) (10) containing some of the expected process impurities and degradation products. The solution is part of the System Suitability Testing (SST) Solution to ensure the executed method can provide adequate resolution between key analytes. Alternatively, this solution can be made by spiking the reference solution with synthesized reference standards of related substances. The use of this solution helps with the accurate identification of key analytes in release testing and stability studies.* Note that standard and sample solution stability data may be included if available.

Procedure

Before analysis, equilibrate the system and column by pumping the mobile phase at the set flow rate. Test injections may be performed until a stable baseline and/or acceptable response is obtained. Flush the column with a water-acetonitrile mixture or another suitable solvent if a clean baseline is not obtained with blank injections.

The suggested injection sequence is as follows:

Sample	Number of Injections
Blank	≥ 1
Sensitivity Check	1
Retention Time Marker	1
Reference Standard A	5
Reference Standard B	1
Sample	2 per preparation
Reference Standard A (Bracketing Reference)	1
Blank	1

Re-inject Reference Standard A (Bracketing Reference) after not more than 9 sample injections and again at the end of the sequence.

Comments: *The injection sequence table of nine initial injections followed by sample analysis and bracketed standards is typical in most regulated testing (2).*

System Suitability Test (SST)

- Evaluate the blank chromatograms for the presence and impact of any peaks that elute in the region corresponding to G-1234 or known related substances. There should be no significant interference from the blank.
- The signal-to-noise ratio (S/N) for the G-1234 peak in the Sensitivity Check Solution is ≥ 10.
- The % RSD of the G-1234 peak area in all Reference Standard A injections (including bracketing injections) is ≤ 2.0%.
- The % Recovery of the G-1234 peak in Reference Standard B against the average G-1234 peak area in the first 5 Reference Standard A injections is 100.0% ± 2.0%
- Report the USP tailing factor (Tf) of the G-1234 peak in the first Reference Standard A injection.
- Evaluate resolution using the Retention Time Marker Solution. USP Resolution (Rs) is ≥ 1.2 between the SRS peak and the G-1234 peak, and Rs is ≥ 1.5 between the G-1234 peak and the RRR diastereomer peak.
- The % RSD for the retention time of the G-1234 peak in all Reference Standard A injections, including bracketing injections, is ≤ 2.0%.

Comments: *The SST acceptance criteria are typical of those found in* USP *<621> (11). A tighter acceptance criterion of ≤ 0.73% RSD is more useful and realistic for the performance expectations of modern HPLC systems. The specified tailing factor is "report" in this method, even though most assays specify an acceptance criterion of ≤* 2.0. *Note that for many NCEs with multiple basic functional groups (for example, amines), a Tf of 3.0 is not unusual.*

Calculations

Label Claim Determination

Calculate the % Label Claim using equation 1:

$$\%\ Label\ Claim = \frac{(Peak\ Area_{sample})\ (PF)\ (SDF)\ (SF)}{\frac{(RF_{All\ ref\ std\ A})}{Label\ Claim\ (mg)}} \qquad [1]$$

where *PF* equals Purity Factor of the Reference Standard (expressed as a decimal); *SDF* equals Sample Dilution Factor (Volume of Sample [mL] / Number of Capsules; *SF* equals salt conversion factor (for example, for mono-HCl = 1.08); and $RF_{\frac{all\ ref}{std\ A}}$ equals the mean peak area of G-1234 peak in all Ref Std A inj. / Std A Conc. (mg/mL).

Calculate the average % Label Claim from *n* = 4 injections per test sample.

Report the average % Label Claim for all samples.

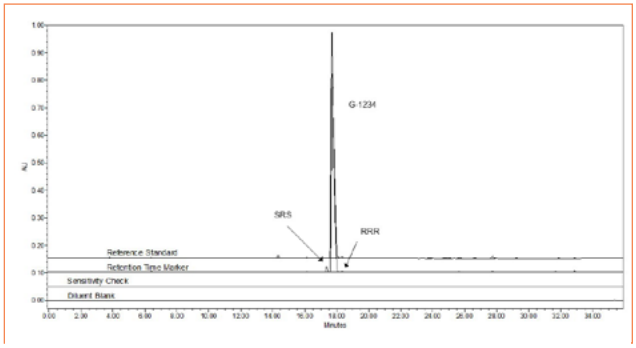

FIGURE 1: The full-scale overlaid chromatograms of the reference standard, retention time marker, sensitivity check, and diluent blank solutions. The full-scale chromatograms show the overall separation, including the API's peak shape and height. The optimum peak height of the API is kept at 1.0 to 1.5 absorbance units to prevent UV detector saturation while maximizing method sensitivity to ensure a QL of 0.05% can be reached.

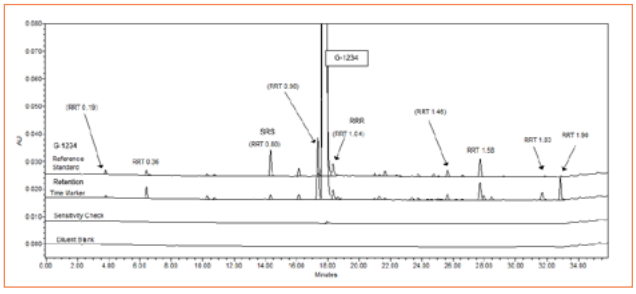

FIGURE 2: The expanded-scale overlaid chromatograms of the reference standard, retention time marker, sensitivity check, and diluent solutions are helpful to the analyst in providing a high-sensitivity view of the expected resolution of the retention time marker solution, the S/N ratio of the sensitivity check, and the presence of "blank" peaks in the diluent blank.

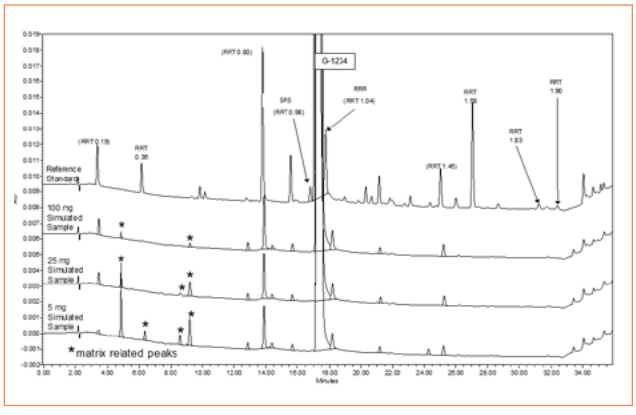

FIGURE 3: The expanded-scale overlaid chromatograms of the reference standard spiked with impurities (retention time marker) and several representative sample solutions of the drug products of different strengths.

Related Substances Determination

For each sample injection, integrate all peaks ≥ DL (0.02%), excluding those due to the blank and any matrix-related peaks. Calculate the amount of each individual related substance in the G-1234 drug product using equation 2:

$$\% \, Component = \frac{Peak\ Area_{Component}}{Peak\ Area_{Total}} \, x\ 100 \quad [2]$$

Calculate the average % individual related substances from n = 4 injections per sample.

Report related substance levels (Area %) for each individual related substance ≥ Quantitation Limit (QL. 0.05%), where each individual related substance is identified by its relative retention time (RRT) or compound name, if available. Report individual-related substances less than the QL but greater than or equal to the Detection Limit (DL) as "<QL."

Determine the Total Related Substances by summing each injection's individual related substances (≥ QL). Calculate and report the average (n = 4) as Total Related Substances.

***Comments**: The reporting and calculations of the Label Claim and related substances were discussed and explained in more detail in an earlier paper on Certificate of Analysis (CoA)(8). Note that a relative response factor (RRF) is used for late-phase methods if the molar absorptivity of the related substance varies more than 80-120% of that of the API.*

Example Chromatograms

Example chromatograms of the various solutions (in normalized and expanded scales of the retention time marker, reference, sensitivity, and sample solutions) should be included, such as those shown in Figures 1–3.

Conclusions and Summary

The analytical procedures for quality assessment and control of pharmaceuticals are critical tools, and these documents must be appropriately developed and written to allow for more straightforward implementation and transfer by analysts for release testing and stability studies. Well-written procedures are clear and include mandatory compliance components and sufficient details to minimize the risk of analyst errors caused by misinterpretation. Here, the 2015 US FDA guidance document's recommendations for the basic elements of an analytical procedure for regulated testing are described. In this installment, a DP method illustrates the best practice by documenting the mandatory regulatory components and additional operating parameters/chromatograms to aid the analyst in successfully performing the regulated assay.

Disclaimers

This paper discusses the recommended content of analytical procedures for regulated testing from the 2015 FDA guidance document and cites an actual case study example of an early-stage stability-indicating method (phase II) to illustrate the expected contents. The reader is referred to textbooks, reference articles, regulatory guidelines, and company-specific standard operating procedures for considering the specific stage-appropriate content and details of the analytical procedures.

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Detailed Glycosylation Analysis of Therapeutic Enzymes Using Comprehensive 2D-LC–MS

Koen Sandra, Kathleen Piens, Debby Bracke, Pat Sandra, and Wouter Vervecken

The use of comprehensive two-dimensional liquid chromatography (LC×LC) coupled to mass spectrometry (MS) for characterizing glycosylation of therapeutic enzymes is presented. Recombinant human acid α-glucosidase (rhGAA) was digested and resulting peptides were separated by reversed-phase LC (RPLC) at high and low pH in, respectively, the first and second dimension. Glycopeptide peaks were then selectively detected and identified by MS operated in all-ion fragmentation mode. The study of first generation rhGAA (myozyme), expressed in Chinese hamster ovary (CHO) cells, and next-generation glyco-engineered rhGAA, produced in yeast cells to finetune the mannose-6-phosphate (M6P) content, is described.

HUMAN ACID α-GLUCOSIDASE (hGAA) catalyzes the hydrolysis of glycogen to glucose in the lysosomes of the cell. There are around 50,000 people worldwide which have a deficiency of this enzyme, leading to glycogen accumulation in the lysosomes, a rare and fatal disorder known as Pompe disease (1–5). Pompe patients typically receive an enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (rhGAA) commercially known as myozyme or lumizyme. rhGAA is a heavily N-glycosylated protein with a MW of 110 kDa as expressed in Chinese hamster ovary (CHO) cells. The enzyme contains seven N-glycosylation sites which are occupied with complex and high mannose glycans (2–5). The former complex glycans are predominantly sialylated and, to a lesser extent, acetylated, the latter glycans contain mannose-6-phosphate (M6P) structures considered a critical quality attribute (CQA) as these are responsible for targeting the enzyme to the lysosomal compartment of the cell where it needs to be catalytically active and break down glycogen.

To study N-glycosylation of therapeutic enzymes, glycans are commonly liberated from the protein backbone using PNGase F, fluorescently labeled and separated using hydrophilic interaction liquid chromatography (HILIC) (6–9). While this methodology provides a wealth of information, site specific data is lost, such as which glycans are conjugated to which asparagine residue, and to which extent. To obtain the latter, peptide mapping is required. When digesting a therapeutic enzyme, hundreds of peptides are to be expected with varying physicochemical properties present in a wide concentration range. Two-dimensional liquid chromatography (2D-LC) is perfectly suited to tackle this complexity (10). In 2D-LC, two different chromatographic separation mechanisms are combined and material eluted from a first column is further separated on a second column which has an orthogonal separation behavior. 2D-LC comes in different flavors: (multiple-)heart-cutting (LC–LC), where one or a couple of peaks are transferred from first to second dimension, and comprehensive 2D-LC (LC×LC) where the entire first dimension chromatogram is sampled thereby maximizing separation power. The present manuscript demonstrates how LC×LC in combination with quadrupole-time-of-flight (Q-TOF) mass spectrometry (MS) operated in all-ion fragmentation mode comes in as a very powerful tool to study glycosylation of rhGAA.

Materials and Methods

Materials

Water, acetonitrile (ACN), methanol (MeOH) and formic acid were purchased from Biosolve. Dithiothreitol (DTT), 2-iodoacetamide (IAA) and ammonium bicarbonate were from Sigma-Aldrich. Tris-HCl pH 8 was purchased as a 1M solution from Thermo Fisher Scientific. Porcine sequencing grade modified trypsin was acquired from Promega and Rapigest from Waters. Myozyme was obtained from Sanofi-Genzyme. rhGAA was expressed in glyco-engineered *Yarrowia lipolytica,* essentially as described in Tiels et al (11).

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Sample Preparation

To a volume corresponding to 100 µg of therapeutic enzyme, 105 µL of 0.1% Rapigest in 100 mM Tris-HCl pH 8 was added followed by the addition of 100 mM Tris-HCl pH 8 to a final volume of 192.5 µL. The sample was subsequently reduced at 60 °C for 30 min by the addition of 5 mM DTT (2.5 µL of 400 mM DTT in 100 mM Tris-HCl pH 8) and alkylated at 37 °C for 1 h by adding 10 mM IAA (5 µL of 400 mM IAA in 100 mM Tris-HCl pH 8). Lyophilized trypsin (20 µg) dissolved in 100 mM Tris-HCl pH 8 (50 µL) was added in a volume of 10 µL, giving rise to a final sample volume of 210 µL and an enzyme to substrate ratio of 1/25 (w/w). Digestion proceeded for 16 h at 37 °C.

LC×LC–MS

LC×LC analyses were carried out on an Agilent 1290 Infinity 2D-LC system (Agilent Technologies). Two G4220A binary pumps, a G4226A autosampler with G1330A autosampler thermostat, a G1316C thermostatted column compartment and a G1170A valve drive with 2-position/4-port duo valve (G4236A), equipped with two 40 µL loops, were used. High-resolution accurate mass data were acquired on an Agilent G6530 Q-TOF equipped with a JetStream source (Agilent Technologies). The 2D-LC system was controlled by OpenLab CDS Chemstation with 2D-LC add-on software and the Q-TOF by MassHunter Acquisition software (Agilent Technologies). Data analysis was performed with GC Image LC×LC-HRMS Edition software (GC Image, LLC) and MassHunter Qualitative Analysis software complemented with BioConfirm (Agilent Technologies). LC×LC and MS method details are listed in Table I (Table I with method settings is only available online). To cope with the high flow rate, the effluent of the second dimension column was split. Therefore, a zero dead volume T-piece was connected to the second dimension column outlet via a stainless steel capillary (9 mm long, 0.12 mm internal diameter). The outlets of the T-piece were connected to the MS (directly on the ESI needle) with stainless steel tubing (34 cm long, 0.075 mm internal diameter) and to the waste with a stainless steel capillary (27 cm long, 0.12 mm internal diameter). In this manner, the necessary restriction is provided to direct the majority of the flow to the waste and only a small fraction to the MS.

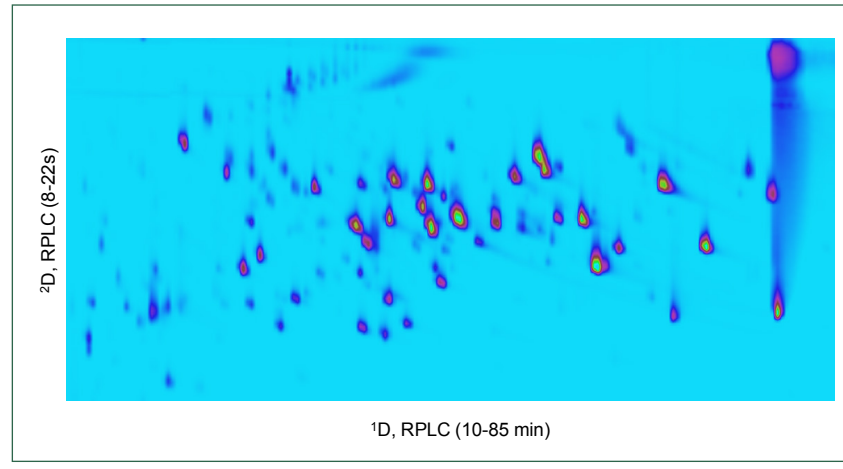

FIGURE 1: LC×LC–MS peptide map of rhGAA trypsin digest using RPLC at high pH in first (^{1}D) and RPLC at low pH in second dimension (^{2}D).

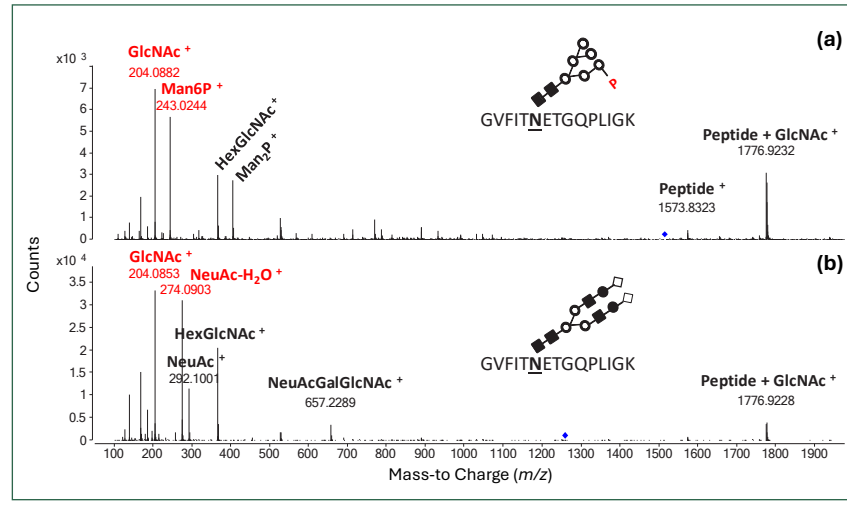

FIGURE 2: MS/MS spectra of tryptic peptide GVFITNETGQPLIGK decorated with phosphorylated high mannose (a) and sialylated complex N-glycans (b).

TABLE II: Abbreviations and symbols used.

Monosaccharide	Abbreviation	Symbol	Sugar Oxonium Ion (*m/z*)
N-acetylglucosamine	GlcNAc	■	204.0867
Mannose	Man	○	NA
Mannose-6-phosphate	Man6P	○–P	243.0264
Galactose	Gal	●	NA
N-acetylneuraminic acid	NeuAc	◆	274.0921

Results and Discussion

In this study, site-specific glycosylation of rhGAA was studied using LC×LC. Compared to one-dimensional LC (1D-LC), resolution in LC×LC is drastically increased as long as the two dimensions are orthog-


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onal and separation obtained in the first dimension is maintained upon transfer to the second dimension. To achieve the latter, effluent is collected in two loops installed on a 2-position valve which are alternately transferred to the second dimension column. The second dimension analysis of one loop takes place during the filling of the other loop. As such, there is a demand for fast second dimension separations to maintain the first dimension separation. The selectivity of the two separation mechanisms toward the peptides must differ substantially in order to maximize orthogonality and resolution. The combination of reversed-phase LC (RPLC) at pH extremes—high pH in first and low pH in second dimension, is very powerful in that respect (12–14). Orthogonality is mainly directed by the mobile phase pH and the zwitterionic nature of the peptides. Partial correlation that might exist is overruled by the high peak capacity in both dimensions. Using a shifting second dimension gradient with increasing elution strength in function of analysis time furthermore increases surface coverage.

Figure 1 shows the RPLC×RPLC–MS peptide map of the myozyme digest. Excellent chromatographic and mass spectrometric performance is obtained providing detailed structural insights. The MS/MS spectra of two representative N-glycosylated peptides are shown in Figure 2. Upon collision induced dissociation (CID), glycosylated peptides give rise to specific fragments originating from the glycan part. These sugar oxonium ions (Table II) can be used to selectively recognize glycosylated peptides in the data. For that, one can operate the mass spectrometer in the all-ion fragmentation mode in which all peptides are transferred into the CID cell where they are fragmented. By alternating all-ion fragmentation with regular MS acquisition, precursors giving rise to sugar oxonium ions can be revealed and a detailed study of glycosylation sites achieved. Figures 3a-d (figures 3-5 are available online by accessing the QR code at the end of the article) show the regular MS peptide map (a) and the all-ion fragmentation peptide map extracting the sugar oxonium ions at *m/z* 204.0867 (b), 274.0921 (c), and 243.0264 (d). The ion at *m/z* 204.0867 corresponding to N-acetylglucosamine is shared by all N-glycans and can be used as a general marker for N-glycosylation. Consequently, Figure 3b reveals all glycosylated peptides. Different clusters are observed in the peptide map which in fact correspond to the seven different glycosylation sites (Table III). Note that glycosylation site N334 is spread over two regions (1 and 1') because of the partial cyclization of the N-terminal amino acid glutamine (formation of pyroglutamate–pyroE–during trypsin digestion rendering glycopeptide more hydrophobic). The different spots within a cluster correspond to different glycans at a given glycosylation site. When extracting the oxonium ions at m/z 274.0921 and 243.0264 (Figures 3 c-d), one can specifically visualize peptides decorated with, respectively, sialylated (N-acetylneuraminic acid-NeuAc) and phosphorylated (M6P) N-glycans. The data reveals that three sites (N84, N177, and N414) are occupied with phosphorylated glycans while all sites are decorated with sialylated N-glycans (Table III). A remarkable separation is achieved based on site heterogeneity with glycopeptides eluting in the following order in the first dimension at high pH: di-phosphorylated < di-sialylated < mono-phosphorylated < mono-sialylated < neutral. In the second dimension at low pH, elution order is reversed: neutral < mono-sialylated < mono-phosphorylated < di-sialylated < di-phosphorylated.

While M6P is required for targeting the enzyme to the lysosomes, only three N-glycosylation sites decorated with the latter species are present in myozyme, resulting in poor cellular uptake (11). Several next-generation glyco-engineered variants with improved cellular uptake have been developed where M6P content is increased by (1) conjugating pre-synthesized phosphorylated oligosaccharides to oxidized sialylated complex N-glycans, or (2) by expression in glyco-engineered yeast cells that have been modified to produce phosphorylated high mannose N-glycans, and subsequent exposing of the phosphate and trimming terminal mannose residues to generate the desired N-glycan structures (11,15). Figures 4a-d presents the RPLC×RPLC–MS peptide map of the glyco-engineered variant produced in yeast cells in comparison to the first-generation product derived from CHO cell expression (full MS and all-ion fragmentation). It can be concluded that yeast-derived rhGAA is devoid of sialylated N-glycans and occupied with phosphorylated high-mannose N-glycans at all seven N-glycosylation sites. In-vitro measurements (Figure 5) furthermore demonstrate that the glyco-engineered variant gives rise to a 20-fold higher uptake in fibroblasts from Pompe patients with the increased M6P content responsible for this outcome. This represents a perfect illustration of how cutting-edge biology and state-of-the-art analytics go hand in hand.

Conclusion

The detailed study of glycosylation of the therapeutic enzyme rhGAA using LC×LC–MS has been demonstrated. The resolving power offered facilitates an in-depth structural characterization and using all-ion fragmentation, glycosylated peptides can selectively be recognized facilitating data interpretation. The method has successfully been applied to first and next-generation rhGAA and findings placed in a biological context.

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Characterization of Product Related Variants in Therapeutic Monoclonal Antibodies

Navin Rauniyar and Xuemei Han

The characterization of product-related variants in monoclonal antibodies involves identifying and quantifying the size and charge of variants that can impact the activity, efficacy, and safety of the antibodies. These variants represent distinct molecular forms that may arise from processes such as fragmentation, dimerization, aggregation, or post-translational modifications. The characterization of variants typically involves isolating the relevant species using a semi-preparative scale HPLC system and analyzing them using various analytical techniques and biological assays. The commonly used analytical techniques include size-exclusion and ion-exchange chromatography, light scattering, mass spectrometry, capillary isoelectric focusing, and capillary electrophoresis-sodium dodecyl sulfate with ultraviolet or laser-induced fluorescence detection, among others. Additionally, functional assessments are performed using cell-based assays and binding assays to assess the biological activities of the variants. Identifying product-related variants through characterization enables the recognition of impurities that compromise the quality and safety of the drug.

RECOMBINANT MONOCLONAL ANTIBODIES (mAbs) are biotherapeutics known for their high selectivity in binding to target antigens and inducing an immune response. Multiple structural variants may arise in mAbs due to post-translational modifications (PTMs) or processes like fragmentation, dimerization, or aggregation (1). The common modifications in mAbs include N-linked glycosylation, oxidation, deamidation, isomerization, glycation, cysteinylation, and C-terminal lysine cleavage, among others, leading to increased heterogeneity and diverse charge variants (Table I) (1–5). These product-related variants may form at any stage of the antibody manufacturing process, including cell culture, downstream recovery, or storage. These variants can impact quality attributes like stability, potency, and serum half-life, thereby limiting the product shelf-life (6).

According to the *ICH Q6B* Guidelines (7), product-related variants comparable to the desired product in terms of activity, efficacy, and safety are deemed product-related substances, while those deviating in these properties are labeled as product-related impurities. For instance, C-terminal lysine or N-terminal pyroglutamate variants are not expected to affect safety or efficacy, as these regions are highly exposed and not part of any ligand binding sites. In contrast, variants with deamidation and isomerization in the complementary determining region (CDR) can reduce antigen binding affinity and potency, categorizing them as product-related impurities (8–10). Similarly, oxidation in the Fc region may affect neonatal receptor (FcRn) binding, potentially influencing the drug's half-life in serum (10–12). Generally, modifications in the Fc region do not significantly affect Fab function; however, modifications in the Fab region, especially in the CDR region, are more likely to affect antigen binding and potency (13).

TABLE I: Common modifications that form acidic and basic charge variants in mAbs.

Acidic Species	Basic Species
Asparagine deamidation, Aspartate isomerization, Glycation, Sialylated N-glycans, N-terminal cyclization (pyroglutamate), Cysteinylation, Citrate or Succinate adducts, Trisulfide bonds, Non-classical disulfide linkage, Reduced disulfide bonds	C-terminal lysine, Aspartate isomerization, Succinimide intermediate (from Aspartate isomerization), Methionine oxidation, C-terminal amidation, Disulfide related, Variants with leader peptide, Aglycosylation, Leader sequence, Serine to arginine sequence variant, Aggregates

To ensure product quality, it is essential to comprehensively characterize, quantify, and closely monitor product-related variants throughout the product's lifecycle. The characterization of variants typically involves isolating the relevant species and extensively analyzing them using various techniques, which are discussed below.

Isolation of Product-Related Variants

Analytical assays, such as size-exclusion and cation exchange separations with high-performance liquid chromatography (SE-HPLC and CEX-HPLC, respectively), are used to monitor variants contributing to heterogeneity in drug substances (DS)

and drug products (DP). Figure 1 (available online by accessing the QR code at the end of this article) shows typical chromatograms of an IgG1 mAb product.

It is critical to understand the properties of product-related impurities and substances observed in SE-HPLC and CEX-HPLC assays, and their differences from the desired drug product. This is primarily because species like truncated forms, aggregates, and degradation products can impact activity, efficacy, and safety. Therefore, thorough product characterization helps establish individual and collective acceptance criteria for product-related substances and impurities. Additionally, it is a regulatory expectation that all peaks from chromatography release tests are identified (14).

To perform a comprehensive characterization of the variants, isolation of each variant with high purity (>80% enriched) and suitable quantities–often milligram levels–is required. This is challenging for biologic products in which the variant species are less than 1% of the total content in the product. Coupling SE- or CEX-HPLC, which use volatile salts, directly to native mass spectrometry is an emerging and promising technique for variant characterization (15). Despite this advancement, isolation of product-related variants through offline fraction collection remains necessary to obtain material for bioactivity assays, in order to determine the structure-function relationship. A semi-preparative scale HPLC system is often used to isolate size, charge, and hydrophobic variants in mAbs.

Characterizing variants observed in SE- and CEX-HPLC involves transferring these analytical HPLC methods to a semi-preparative scale for the collection of a sufficient quantity of each fraction. Method transfer, however, can be challenging due to differences in column dimensions, particle sizes, and flow rates. Moreover, variations in tubing lengths and components may contribute to band broadening caused by extra column volume, which may impact peak resolution. Therefore, successful scale-up requires careful optimization and adjustments at the semi-preparative scale to ensure a close match of the chromatographic profile to the analytical scale. These adjustments may involve modifying the gradient in analytical HPLC to facilitate variants' proper elution and separation with the large volumes of the semi-preparative column. Scaling up also requires larger volumes of solvents and additional consumables, highlighting the importance of balancing efficiency to manage costs effectively.

In addition, maintaining reproducibility across injections is critical when collecting fractions with semi-preparative HPLC, particularly when pooling fractions from multiple injections is required to obtain sufficient quantities of low-abundant variants. Once the semi-preparative HPLC method is optimized for reproducibility,

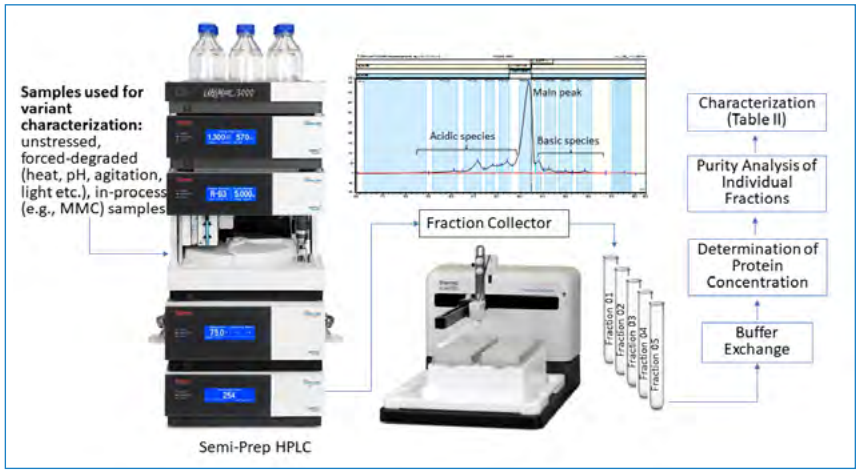

FIGURE 2: A semi-preparative HPLC fractionation workflow used for characterizing product-related variants.

fractions containing desired variants are collected, but it is crucial to collect individual peaks instead of grouping low-abundant peaks together. However, a single peak does not necessarily indicate a single variant, as it may contain one or more species. The success of variant characterization depends on the purity of the fractions. Fraction purity is assessed by analyzing each fraction alongside the unfractionated starting material using analytical HPLC. The chromatographic profiles are overlaid with the unfractionated samples, and their elution order is confirmed. In instances where co-fractionated species are present and might interfere with accurate characterization of the variants, re-fractionation of the collected fractions may be necessary to ensure the isolated fractions are pure and can be reliably characterized. Figure 2 shows a semi-preparative HPLC fractionation workflow commonly used for variant characterization.

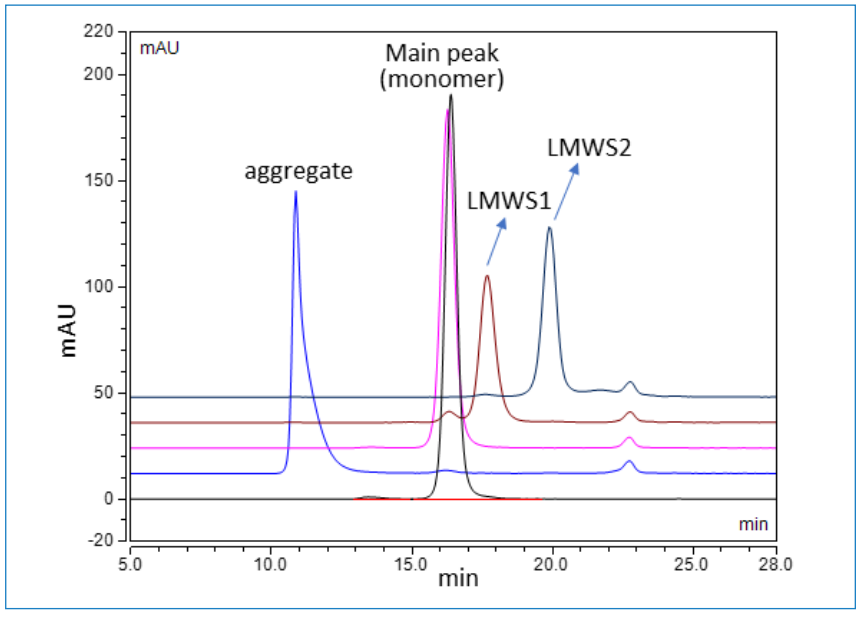

FIGURE 3: Overlaid SEC chromatograms of unfractionated mAb (black); and aggregate (blue), main peak (magenta), LMWS1 (maroon), and LMWS2 (dark blue) fractions collected from the stressed (65 °C for 7 days) mAb product.

The main peak, which is the desired drug product and elutes as a major peak, is collected, and analyzed alongside the variant fractions as a control. The main peak usually lacks C-terminal lysine in the heavy chains but is glycosylated with neutral oligosaccharides at the conserved asparagine residue in the Fc region. Figure 3 shows an example of overlaid SEC chromatograms depicting the unfractionated material and four fractions of a mAb product, where each fraction appears highly enriched compared to the original unfractionated sample. It is important to control the artifacts introduced during fraction collection and sample preparation. Modifications can occur during concentration, buffer exchange, pH variations, and freeze-thaw cycles. The side-by-side characterization of the main peak with each variant is recommended as an assay control (13). Modifications can also be lost during sample preparation. Succinimide is unstable under typical denaturation, reduction, alkylation, and enzymatic digestion conditions used for generating peptides for LC-MS analysis (13).

Although fraction collection is the initial step toward thorough characterization, determining the contribution of sialic acid to acidic species and C-terminal lysine to the basic species requires enzymatic removal under native conditions before fraction collection. Sialic acid can be removed using sialidase, and C-terminal lysine can be removed by carboxypeptidase B (CPB) without affecting antibody structures. In certain cases, treatment with CPB or sialidase may be necessary before fraction collection to enrich other variants overlapping with C-terminal lysine or sialic acid variant peaks. Furthermore, the collection of Fab and Fc fragment fractions obtained from enzymatic digestion can help localize the acidic or basic species associated with Fab or Fc.

Following successful fraction collection, various analytical techniques are used for characterizing the variants. Orthogonal methods, as outlined in Table II, are used to provide unambiguous characterization of the variants.

Forced Degradation for Variant Characterization

In-depth characterization of variants in mAbs DS and DP can be challenging due to their extremely low abundance. This challenge can be addressed by exposing DS and DP to various stress conditions to enrich these low-abundant species prior to isolation. The selection of stress conditions is based on an extensive understanding of the therapeutic protein's biophysical properties, degradation pathways, and the likelihood of exposure to those conditions during processing, packaging, shipping, and handling. Commonly applied forced degradation conditions include high temperature, freeze-thaw cycles, agitation, pH extremes, light exposure, and oxidation. The variants most frequently observed in these forced degraded samples include aggregate, fragment, oxidized, and deamidated species. Each stress condition tends to generate specific types of variants in abundance. For instance, incubation with hydrogen peroxide generates oxidized species. A major degradation pathway resulting from heat stress is the formation of aggregates, comprising both insoluble (precipitates and particles) and soluble aggregates of both covalent and non-covalent natures (14). Forced degradation conditions should be carefully selected to mainly modify the same sites that are present in those identified in the separated acidic and basic species. When modifications at other sites cannot be avoided, the impact of off-target modifications should be considered (16). In addition to stressed samples, various in-process samples, such as those from mixed mode chromatography (MMC) fractions, serve as a valuable source for enriched variants. Therefore, along with in-process samples, forced degradation studies designed to induce and identify product-related variants enhance our ability to comprehensively characterize and understand the diverse variants present in DS and DP.

Techniques for Characterization of Size Variants

Size variants represent distinct molecular forms of therapeutic proteins that may result from processes like fragmentation, dimerization, or aggregation during manufacturing or storage. Size variants are one of the CQAs for which specifications must be set for batch release.

SE-HPLC is a widely used technique for monitoring size variants. It separates proteins based on their hydrodynamic radius, where smaller molecules permeate column matrix pores and elute later than larger molecules that pass through more quickly, resulting in distinct chromatographic peaks. SEC primarily separates the monomeric IgG from dimers, trimers, or higher order aggregates (high molecular weight or HMW species) and fragments (low molecular weight or LMW species). This allows for the quantification and characterization of different size variants. Although SEC theoretically involves no analyte interaction with a stationary phase, secondary interactions based on charge or hydrophobicity are possible. To minimize these interactions, additives such as

TABLE II: Analytical methods used for variant characterization.

Characterization Method	Purpose
SEC-MALS	To obtain size distribution profile and molar mass information under native conditions
CEX-HPLC	To obtain charge distribution profile
RPLC-MS	To provide identification and molecular weight information
RPLC-MS/MS	To provide site-specific identification and quantification of PTMs
icIEF	To obtain charge distribution profile
CE-SDS-UV or CE-SDS-LIF	To obtain size distribution profile under denaturing conditions (reduced and non-reduced)
Target or Receptor Binding Assays ELISA, AlphaLISA, Surface Plasmon Resonance (SPR)	To determine binding affinities to various targets or receptors
Cell-based Assays Anti-Proliferation Assay, Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) assay, Complement-Dependent Cytotoxicity (CDC) Assay	To determine the biological activities

arginine or isopropyl alcohol are commonly used. Figure 1a provides a representative UV chromatogram example of size variants in a mAb monitored by SE-HPLC (17).

Mass spectrometry (MS) is a powerful and accurate method for determining the molecular mass of size variants. The use of enzymes such as PNGaseF for deglycosylation removes N-linked glycans, simplifying the MS peak profile. This facilitates the identification of truncated variants at the hinge region and localization of the cleavage site. Cleavage in the hinge region is a major degradation pathway for mAbs. In IgG1 mAbs, if all inter-chain disulfide linkages are conserved, cleavage generates Fc-Fab, Fab, and Fc fragments (Figure 4 [Figures 4-8 are available online by accessing the QR code at the end of this article]). The Fc-Fab fragment is an intact mAb lacking one Fab arm, potentially impacting potency if interaction with the target receptor requires both Fab arms. The Fab fragment lacks Fc-mediated effector function and exhibits a reduced circulation half-time in serum. In the presence of a reducing agent, the Fc-Fab, Fc, and Fab transform into HC, LC, 1/2Fc, and Fd species, with Fd representing the piece of the heavy chain included in the Fab (Figure 4c) (18). Additionally, deglycosylation of mAbs facilitates the identification of glycation variants wherein reducing sugars covalently links to amines of lysine residues. To locate the glycation site, a bottom-up peptide mapping approach is commonly used (19).

Size exclusion chromatography with multi-angle light scattering and refractive index detectors (SEC-MALS-RI) is another powerful analytical technique for characterizing size variants under non-denaturing conditions. In SEC-based separation, the elution position relies not only on the protein's molecular weight but also on its shape. Additionally, interactions with the column matrix can alter the elution position. However, the benefit of coupling MALS to SE-HPLC is that SEC-MALS is able to provide absolute molar mass irrespective of elution time, column calibration standards, molecular conformations, and non-ideal column interactions. SEC-MALS helps in identifying and determining the molecular weight of monomers, dimers, oligomers, and aggregate species, as well as truncated fragments at the hinge region.

Capillary electrophoresis with sodium dodecyl sulfate and ultraviolet or laser-induced fluorescence detection (CE-SDS-UV or CE-SDS-LIF, respectively) are orthogonal methods for the quantitative estimation of size variants under denaturing conditions, with or without reducing disulfide linkages. According to the monograph 129 of the United States Pharmacopeia (USP) (20), SEC-HPLC is considered a robust method for measuring monomer and HMW species, while CE-SDS provides reliable quantitation of LMW species of mAbs. CE-SDS under non-reducing conditions is used to monitor the purity of denatured intact antibodies, while CE-SDS under reduced conditions is used to monitor intact light chains, heavy chains, and non-glycosylated heavy chains (21). Figure 5 provides representative electropherograms of size variants in a mAb monitored by non-reduced and reduced CE-SDS. Together with SEC-MALS and RPLC-MS analysis, CE-SDS provides additional insights into identifying size variants and determining whether aggregates and dimers consist of covalent or non-covalent bonds.

Size variant fractions are analyzed using CEX-HPLC to evaluate their charge properties and gain a better understanding of product heterogeneity. While charge-based methods are effective for assessing dimers composed of several distinct species, they may not be as suitable for monitoring fragmentation or aggregation due to their sensitivity to chemical and structural modifications (1,2). In fact, it can be challenging to determine which peaks are a result of changes in size. In more complex scenarios, such as characterizing highly heterogeneous aggregate species, the CEX-HPLC method may show a broad and difficult-to-quantify peak in the basic region of the chromatogram. Low molecular weight fragments can produce acidic and basic variants (22).

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In addition to these analytical techniques, functional assessments of variants are performed using cell-based assays and various binding assays (Table II) to assess the impact on bioactivity including the Fab- and Fc-mediated functions. The target and Fc receptor binding assays that are used include ELISA, alphaLISA, and Surface Plasmon Resonance (SPR). Cell-based assays such as anti-proliferation assays and ADCC cytotoxicity assays are important to assess the product potency and effector functions. Figure 6 shows the potency assessment result of the aggregate, dimer, and LMWS1 fractions collected from a thermally stressed mAb product (Figure 3) using a cell-based proliferation inhibition assay, indicating that the isolated dimer species is as potent as the unfractionated mAb reference standard, whereas the aggregate and the low molecular species (fragments) have either completely lost their potency or are much less potent.

Techniques for Characterization of Charge Variants

Charge variants in mAbs primarily result from PTMs that modify their isoelectric point (pI) or charge distribution profile. Table I outlines key modifications contributing to acidic and basic variants commonly found in mAbs. Acidic species have a lower apparent pI, while basic species have a higher apparent pI when analyzed using isoelectric focusing (IEF)-based methods. In ion exchange chromatography-based methods, the retention times relative to the main peak define acidic and basic species. Acidic species elute before the main peak in CEX-HPLC or after the main peak in anion exchange-HPLC (AEX-HPLC). Given the basic pI of most human IgGs, CEX-HPLC is typically used to characterize the charge variants.

In ion-exchange HPLC, electrostatic interactions between the ionic groups of the stationary phase and those on the mAb surface form the basis of the separation. For example, in CEX-HPLC, separation is based on the interaction between positively charged analytes and a stationary phase with negatively charged functional groups. The antibody is subsequently eluted from the column by a salt gradient, pH gradient, or a combination of both. Basic variants with a net positive charge interact more strongly with the negatively charged stationary phase and elute later in the chromatogram. Conversely, acidic variants with a net negative charge experience weaker interactions and elute earlier. Figure 1(b) provides a representative example of a UV chromatogram of charge variants in a mAb monitored by CEX-HPLC.

The acidic and basic charge variants are characterized by multiple analytical methods outlined in Table II. High resolution MS is a powerful technique for identifying PTMs. The identification and quantification of modifications in the acidic and basic charge variant fractions often use bottom-up approaches, which involve enzymatic digestion and reversed-phase liquid chromatography-tandem mass spectrometry (RPLC-MS/MS) analysis. Tandem MS enables the determination of the position of the modifications within the peptide. Tandem MS also facilitates the identification of glycopeptides, the determination of glycosylation sites, and the quantification of various glycoforms (23). RPLC-MS/MS has been demonstrated to complement the HILIC-FLD (hydrophilic interaction chromatography with fluorescence detection) method for glycan analysis (23). Figure 7 shows the major PTMs in the charge variants of a mAb product identified by RPLC-MS/MS analysis of fractions collected from CEX-HPLC. In addition, the middle-down approach by RPLC-MS could also be used for the rapid assessment of oxidation in mAbs (24). This involves enzymatic cleavage below the hinge region followed by the reduction of inter-chain disulfide bonds, allowing the separation of the LC, Fd, and 1/2Fc fragments (Figure 8).

Imaged capillary isoelectric focusing (icIEF) is a high-resolution technique that separates variants based on their pI or net charge. In isoelectric focusing, a continuous pH gradient is established in the capillary by ampholytes upon the application of high voltage. A protein migrates along the gradient to the point at which the overall charge is neutral. The pI markers spiked in the sample help establish the pI of unknown proteins through interpolation. icIEF has become the industry standard technology for charge variant analysis due to its fast run time, high resolution, and compatibility with quality control processes (25).

As discussed earlier, functional assessments using cell-based assays and various binding assays are also important in assessing the impact of PTMs on the charge variants to the bioactivities. For instance, in a study by R.J. Harris et al, the isolated acidic fractions containing highly enriched deamidation in CDR, and basic fractions containing highly enriched isomerization in CDR, showed reduced potency compared to the main peak fraction collected from a mAb product (9).

Conclusion

Monoclonal antibodies commonly exhibit multiple product-related variants with differences in charge, molecular weight, or other properties. While the chemical nature of the main species is usually well-understood, characterizing the variants is integral for understanding their effect on safety, efficacy, and potency. There are many factors to consider in the characterization of the product-related variants, including variant isolation technique, starting material design and selection, and the availability of extended characterization methods. A comprehensive understanding of product-related variants in monoclonal antibodies not only enhances product development but also ensures regulatory compliance and supports the continuous improvement of manufacturing processes. Ultimately, it contributes to the production of safe and effective biopharmaceuticals. ■

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Analysis of Microplastics in Roadside Debris by Py-GC-MS

In this application note using Py-GC-MS and F-Search MPs 2.0 mass spectral search software (from Frontier Laboratories Ltd.), qualitative and quantitative analysis of MPs present in sand, soil, and other material that accumulates on the road shoulders were performed individually without pretreatment. F-Search MPs 2.0 supports easy identification and quantitation of unknown MPs in the environment.

SMALL PLASTIC PARTICLES with diameters up to 5 mm are known as microplastics (MPs). There are now concerns about the impact of such MPs on environmental pollution and human health. In recent years, identification and quantitation using pyrolysis-GC- MS (Py-GC-MS) have been considered to evaluate the chemical properties of MPs.

Experimental Conditions

An MP calibration reference sample containing the 12 types of plastic with the highest global production quantities (MPs-CaCO3 from Frontier Laboratories Ltd.) was used as a standard sample for qualitative and quantitative analysis. 0.4, 2.0, and 4.0 mg quantities of the MP calibration reference sample were placed in each sample cup, with quartz wool inserted to prevent scattering, and then analyzed.

Results

Real sample was measured, and similarity search for the detected peaks were performed. The results showed a 90 % or over similarity to 6 types of plastic, PMMA, N66, SBR, PET, PE, and PS. For the plastics with a 90 % or over similarity, quantitation values and their percent content were calculated based on the calibration curves created (Table 1). PE is the highest percent rate. It is assumed originated from container packaging materials, agricultural films, and other materials based. SBR is second and used in the tire tread (the part in direct contact with the ground), and presumably derive from tire wear.

TABLE I: Qualitative and Quantitative Analysis Results

Plastic	Retention Time (min)	Quantitated Value*2 (µg)	Rate*3 (%)	Similarity (%)
PMMA	3.77	(0.062)	0.66	98.3
N66	5.18	(0.47)	5.0	99.8
SBR	10.61	3.5	37	95.4
PET	12.82	(1.0)	11	90.8
PE	15.02	(4.2)	44	98.6
PS	19.05	(0.25)	2.6	97.9

***2 Values indicated in parentheses were calculated by extrapolation of calibration curve.**
***3 Calculated assuming the total sum of quantitation values for all plastics with a 90 % or over similarity is equal to 100 %.**

Conclusion

This article described qualitative and quantitative analysis of MPs accumulated on the road shoulders using Py-GC-MS. The calibration curves created from the MP calibration reference sample provided good results. Py-GC-MS and F-Search MPs 2.0 software enable qualitative and quantitative analysis of multiple MPs in environmental samples individually. This method improves the simplicity and efficiency of analysis without pretreatment steps.

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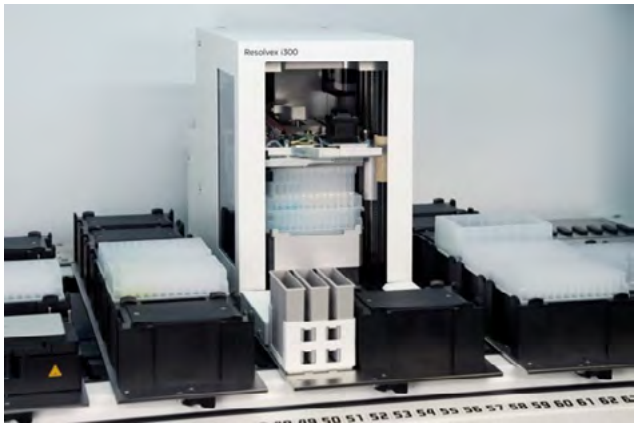
Solid Phase Extraction Of Fentanyl Analogues In Urine Using NBE™ HP-SCX Columns On The Resolvex® i300

The detection of synthetic opioids including fentanyl in biological samples is crucial in clinical and forensic toxicology. Analysis techniques, such as liquid chromatography-tandem mass spectrometry (LC- MS/MS) enable multiplexed detection of fentanyl analogues with high selectivity and sensitivity. However, achieving high throughput with accurate quantification requires sophisticated sample preparation. Automated solid phase extraction (SPE) applied to fentanyl extraction with HP-SCX sorbent, offering reversed phase and strong cation exchange chemistry, addresses this challenge. Here, we highlight an automated workflow using the Resolvex® i300 and HP-SCX Narrow Bore Extraction columns (NBE columns™) to extract 23 fentanyl analogues from human urine efficiently.



Resolvex i300 integrated onto Fluent®

Sample preparation and SPE method.

25 fentanyl compounds (23 compounds and 2 internal standards) (Cerilliant Corp.) were utilized to demonstrate the HP-SCX SPE method. Compounds were diluted to 20 ng/mL, resulting in 1 ng/mL concentration when spiked into human urine samples. The Tecan Resolvex i300 was used for solvent dispensing, positive pressure loading, and analyte dry down. 24 technical replicate samples were processed using NBE columns with 5 mg of HP- SCX (30 µm) sorbent (Tecan P/N: 427-0051R-NBE). Utilizing the automated i300 workflow, 96 samples can be fully processed in 75 minutes.

Data analysis.

Samples were analyzed on a Sciex® ExionLC™ coupled with the Sciex Zeno-TOF™ 7600 System. Mass spectra of fragment ions were acquired in MRM-HR mode. Two fragment ions for each precursor were monitored and fragment ion spectra were summed together to generate a single composite peak area per compound. The obtained peak area ratios of the SPE samples were compared to the peak area ratios of the standard, non-extracted samples to determine percent recovery of the compounds.

TABLE I: HP-SCX SPE protocol parameters:

Step	Solvent	Volume	i300 pressure profile	
Condition	0.3 M NH4O-Ac, pH4.8	500 µL	60 sec	18 psi
Sample load	Urine + 0.3 M NH4OAc, pH 4.8	200 µL urine + 600 µL buffer	75 sec	18 psi
Wash	DI Water	700 µL	60 sec	23 psi
Wash	2% NH4OH	300 µL	30 sec	18 psi
Wash	DI Water	200 µL	25 sec	18 psi
Wash	0.1 M HCl	300 µL	30 sec	20 psi
Wash	MeOH	700 µL	30 sec	18 psi
Wash	DCM	700 µL	60 sec	10 psi
Dry	N/A	N/A	5 min	80 psi
Elution	80:18:2 DC-M:IPOH:NH4OH	300 µL	45 sec	4 psi
Dry down	11.5 min, Evaporate at 40 °C, 40 L/min with pressurized air			

Results.

All 23 compounds resulted in recovery greater than 82%. Compounds showed reproducibility, with %CVs no greater than 11% across the 24 technical replicates.

Conclusion.

The HP-SCX SPE sorbent has been proven to effectively extract a panel of 23 fentanyl-related compounds from human urine, yielding recoveries greater or equal to 82% across all tested compounds with high quantitative performance. By utilizing the Tecan Resolvex i300, the SPE method is fully automated, resulting in a simple, but efficient and scalable extraction protocol.

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Analysis of 6PPD-Q & Alternative PPD-Qs in Fish Using QuEChERS

Arielle Cocozza, Environmental R&D Chemist at UCT

This application outlines a QuEChERS extraction of a subset of PPD-Qs in salmon, combined with push-thru cartridge clean-up using UCT's Quick QuEChERS®, C18, and LipiFiltr® in series to achieve the best level of sensitivity in the fatty matrix. The extracts are analyzed on UCT's SelectraCore® C18 HPLC column using LC-MS/MS.

Introduction

Tire degradation releases 6PPD, which reacts with ozone to form toxic 6PPD-quinone (6PPD-Q), threatening aquatic life, particularly juvenile coho salmon, at LC50 as low as 0.094 ng/g. This concern necessitates a sensitive and accurate sample analysis method.

UCT Part Numbers:

ECQUUS2-MP: QuEChERS Original Mylar Pouch (4000 mg $MgSO_4$ + 2000 mg NaCl), **ECPURMPSMC**: Quick QuEChERS Medium Cartridge (110 mg $MgSO_4$ + 190 mg PSA), **CEC18MC**: Clean-Up® C18 Medium Push-Thru Cartridge **LPFLTR01**: LipiFiltr® Push Thru Cartridge, **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, **SLC-1850ID46-5UM** UCT Selectra® C18, 50 × 4.6 mm, 5 µm

Instrument Method

HPLC System	SCIEX Exion LC
Column Temperature	45°C
Flow Rate	0.5 mL/min
Injection Volume	10 µL
Mobile Phase A	0.2% formic acid in water
Mobile Phase B	Acetonitrile
Gradient Program	Conc. B 5% (0 min) – 50% (1.6 min) – 100% (6.8-8.7 min) – 5% (8.8 – 11 min)
Calibration Range	0.073 – 73 ng/g

QuEChERS Procedure

1. Sample Pretreatment

a) If using fresh fish, freeze at -40°C overnight to break down cells
b) Thaw and homogenize the sample
c) Weigh 5.5 ± 0.1g thawed sample into a 50mL centrifuge tube
d) Spike extracted ISTD (EIS, 13-C6-6PPD-Q) prepared in acetonitrile into all samples and spike target analytes prepared in acetonitrile into QC. Be cautious, as CPPD-Q will fall out of solution in cold acetonitrile. Prepare solutions of CPPD-Q above 100 µg/mL in DCM and further dilute with acetonitrile for spike solutions. Sonicate the target analyte mixture for 15 minutes before adding it to the sample
e) Vortex to disperse. Let equilibrate for 5 minutes

2. QuEChERS Extraction

a) Add 4mL 1% formic acid in water and a necessary volume of acetonitrile to create a final volume of 14mL, accounting for EIS and spike solution added
b) Shake for 4 minutes on a Spex SamplePrep Geno/Grinder 2010 or equivalent at 1700 rpm
c) Add contents of QuEChERS Original Mylar Pouch (4000 mg $MgSO_4$ + 2000 mg NaCl) (ECQUUS2-MP)
d) Immediately shake for 4 minutes on a Spex SamplePrep Geno/Grinder 2010 at 1700 rpm
e) Centrifuge the sample at ≥ 5000 rcf for at least 5 minutes

3. Sample Cleanup

a) Attach the following in series, from top to bottom: Quick QuEChERS Medium Cartridge (110 mg $MgSO_4$ + 190 mg PSA) (ECPURMPSMC), Clean-Up® C18 Medium Push-Thru Cartridge (CEC18MC), LipiFiltr® Push Thru Cartridge (LPFLTR01)
b) Using a 6mL disposable syringe, take 3mL of supernatant, followed by filling the syringe with room air
c) Attach the syringe to the top of the push-thru cartridge series
d) Place a 15mL centrifuge tube in a collection rack
e) Slowly push the supernatant, followed by the 3mL of void volume in the syringe, into the 15mL centrifuge tube
f) Continue to push air through the cartridge with the syringe until ~2mL of extract is collected
g) Aliquot 500µL of extract into a 2mL polypropylene LC vial
h) Dilute with 500µL 2ng/mL ISTD (D5-6PPD-Q) in acetonitrile
i) Analyze on LC-MS/MS

Results

Calculated Results in 5.5g Atlantic Salmon Extract			
	Analyte Recovery (%)		
Analyte	0.073 ng/g (n=7)	3.64 ng/g (n=4)	18.2 ng/g (n=4)
6PPD-Q	111	91	91
7PPD-Q	71	99	97
CPPD-Q	94	101	95
The RSD values range from 3.1 to 23.9.			

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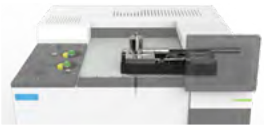

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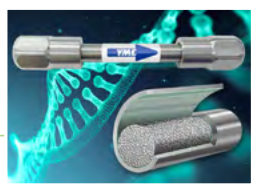

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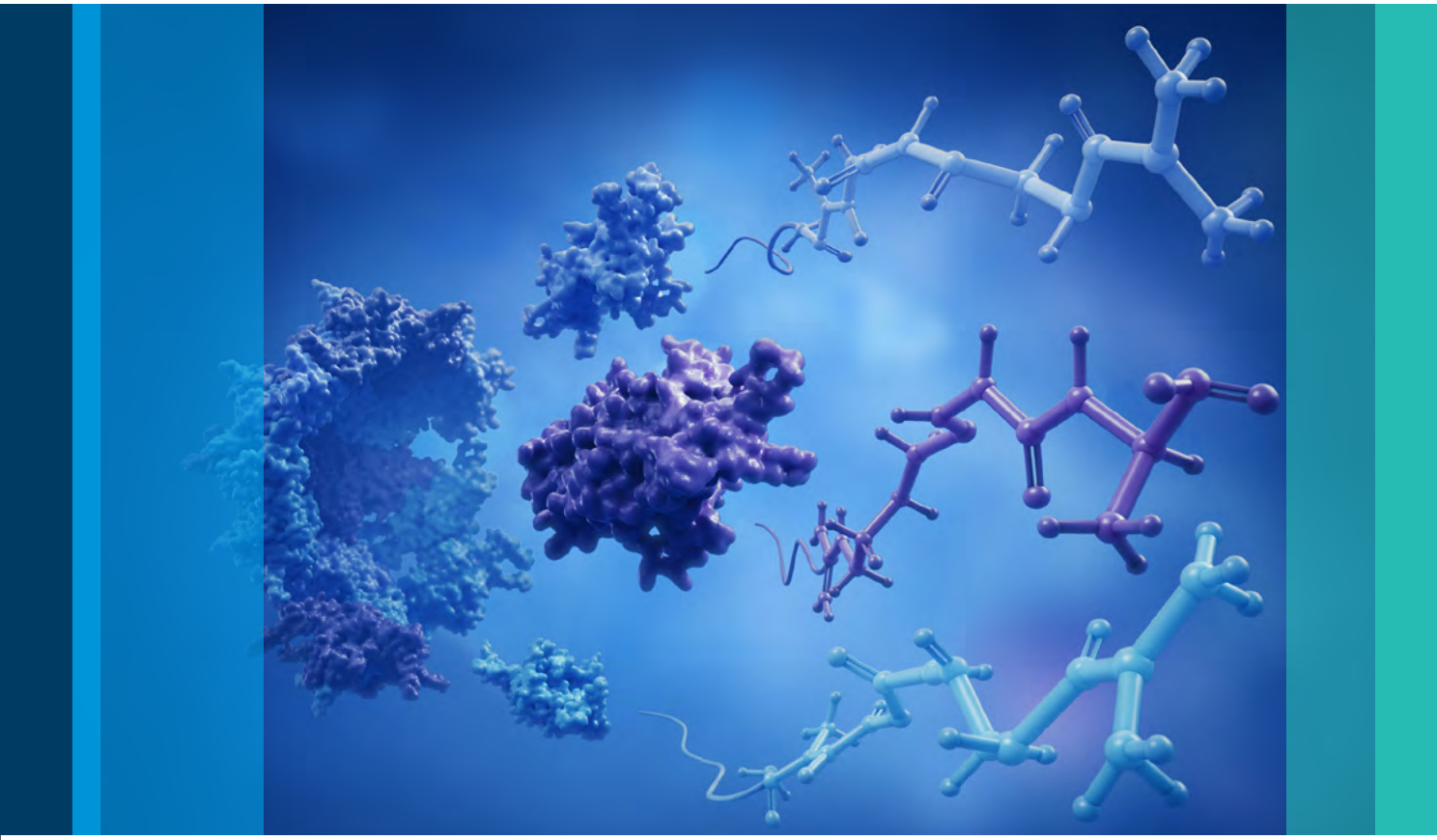

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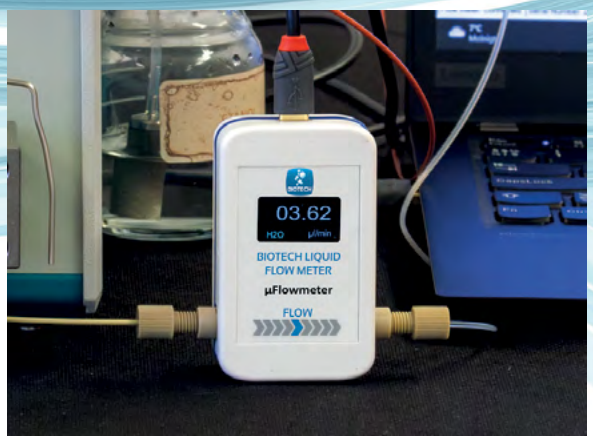

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