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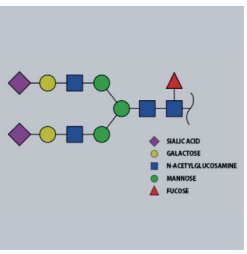

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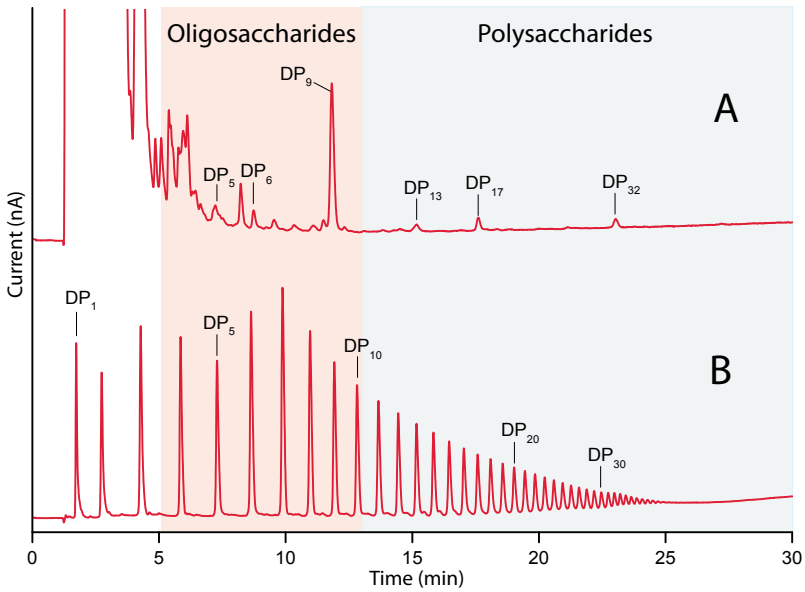

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A compilation of news stories, Q&As, and technical articles that spotlight the current and ongoing chromatographic research in the field of agriculture.

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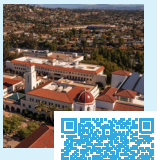

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Inside the Laboratory: The McCall Group at San Diego State University

Laura-Isobel McCall, an associate professor of chemistry at San Diego State University, discusses her group's most recent work using "chemical cartography" in order to perform effective liquid chromatography–mass spectrometry (LC–MS) data acquisition.

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NOTE FROM THE CEO

AS WE STEP into February 2024, I am delighted to present this issue of *LCGC International* which encapsulates the essence of excellence in chromatography. This edition is a dynamic blend of insightful columns, cutting-edge research, and the celebration of outstanding contributions to the field.

In the "LC Troubleshooting" column, Dwight Stoll explores the often-overlooked yet critical parameter of gradient delay volume in "The Gradient Delay Volume, Part II: Practice – Effects on Method Transfter." He offers practical insights for method development and transfer, explaining best practices and strategies for seamless transitions across different LC instruments.

The "Column Watch" article introduces a novel stationary phase for carbohydrate analysis. In "New Stationary Phase for the Separation of All Classes of Carbohydrates by HPAEC-PAD," Christian Marvelous, Daniel Vetter, Hendrik-Jan Brouwer, and associates unveil a unique polymeric anion-exchange stationary phase that promises rapid, high-resolution separations of carbohydrates with exceptional efficiency.

For those seeking ingenious solutions, R. D. McDowall's column, "Questions of Quality," provides a lighthearted yet informative take on "Ingenious Ways to Manipulate Peak Integration." A humorous, yet serious, dive into manipulating peak integration awaits you!

On the subject of biopharmaceutical analysis, Koen Sandra's article, "Quantitative Metrics to Properly Describe Solute Elution in Size Exclusion Chromatography," serves as a valuable tutorial. It addresses the nomenclature and metrics challenges in size exclusion chromatography, offering worthwhile guidance for practitioners.

Our peer-reviewed article and cover story, "Quantitative Determination of Four Lignans in *Phyllanthus niruri L.* by HPLC," by Jianzeng Xin and Sheng Liu, delves into the quantitative determination of lignans in *Phyllanthus niruri L.* plant samples, providing insights into their distribution and suggesting optimal collection times for anti-hepatitis B treatment.

Finally, in our feature article, Jerome Workman, Jr. presents "The 2024 Lifetime Achievement and Emerging Leader in Chromatography Awards." We congratulate Wolfgang F. Lindner and Martina Catani, winners of the 17th annual *LCGC* Lifetime Achievement and Emerging Leader in Chromatography Awards, respectively. Their exceptional contributions will be celebrated at the Pittcon 2024 conference in San Diego, California.

This issue is a testament to the vibrancy of chromatography, from practical insights and groundbreaking research to the acknowledgment of outstanding achievements. We hope you find inspiration and knowledge within these pages.

Happy reading! ■

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

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The Gradient Delay Volume, Part II: Practice – Effects on Method Transfer

Dwight R. Stoll

The gradient delay volume is one of the most important, yet least understood, parameters that affect how gradient elution separations in liquid chromatography (LC) work. This parameter has implications for method development and method transfer during the lifecycle of an LC method. In this installment, I illustrate the impact of different gradient delay volumes when transferring a method between instruments and discuss some strategies that can be used to mitigate these challenges.

IN MY INTERACTIONS with people learning about various aspects of liquid chromatography (LC), I find that the concept of the "gradient delay volume" (GDV) is one of the most difficult ideas to grasp and apply in practice. I find this to be the case both for true beginners—students who are just learning the basics of LC—and for more experienced scientists who have always dealt with GDV, knowingly or unknowingly, but are perhaps having to think about its impact on their work in new ways. The GDV concept has been important since the first times LC separations involving changes in the mobile phase composition—now known as "gradient elution" separations—were made during an analysis. However, given the various ways that GDV can impact the practice of LC, and that we continue to see changes in commercial instrumentation that affect the way we interact and think about GDV, I think a dive into the details is warranted here. In last month's installment of "LC Troubleshooting," I reviewed the basic elements of the GDV concept and discussed how we understand that GDV affects characteristics of LC separations from a theoretical point of view. In this month's installment, I discuss the implications of these ideas, with an emphasis on how the differences in GDVs between instruments can impact how a particular method will function on said instruments.

The GDV is commonly referred to by others as the "gradient dwell volume," or sometimes just "dwell volume." I prefer the inclusion of "gradient" to make it clear what we are talking about, and I prefer "delay" over "dwell" because "delay" communicates one of the most important impacts of GDV—that it delays the arrival of a programmed change in the mobile phase composition at the column inlet. Nevertheless, from my point of view, "gradient delay volume" and "gradient dwell volume" refer to the same thing.

In this two-part series on GDV, we discuss details in a way that assumes we are talking about the reversed-phase (RP) mode of LC. However, most of the discussed ideas are applicable to other modes of LC separation, such as hydrophilic-interaction chromatography (HILIC) and ion-exchange chromatography (IEC).

Finally, readers interested in learning more about GDV will not have a hard time finding good resources, and they are encouraged to consult them. A short list includes several articles in the *LCGC* magazine, and the book by Snyder and Dolan that is focused entirely on gradient elution LC (1). The relatively recent books edited by Stavros Kromidas have rich sections written by major instrument vendors that explain in some detail the software- and hardware-oriented approaches they have taken to effectively achieve variable GDV in their instruments (2,3). Searching the LC Troubleshooting Bible website (https://lctsbible.com/) for the keyword "dwell volume" will immediately return about a dozen articles from the last 20 years.

Potential Impacts of Gradient Delay Volume on Transferability

In last month's installment, I reviewed the essential elements of the theory of gradient elution with an emphasis on the primary variables that control analyte retention time, explaining how GDV influences these relationships. I repeat two of the most important relationships here for convenience. Equation 1 shows the relationship between the retention time (t_r) and the column dead volume (V_m), mobile phase composition used as the starting point in the gradient (ϕ_i), change in

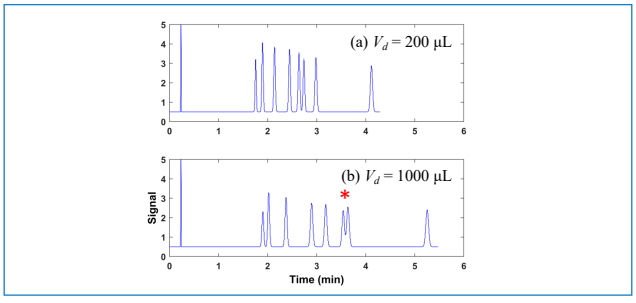

FIGURE 1: (a) V_d = 200 µL and (b) V_d = 1000 µL. Comparison of separations for a mixture of small molecules obtained on systems with different GDVs. Chromatograms are simulated (www.multidlc.org/hplcsim) using the following parameters: Stationary phase, C18; Column dimensions, 50 mm x 2.1 mm i.d. (1.8 µm particle size); Flow rate, 0.4 mL/min.; Temperature, 40 °C; Gradient elution from 20–45 %B from 0–6 min.; (a) solvent, water; (b) solvent, acetonitrile. The red asterisk indicates ethylparaben and nitrobenzene are coeluted. Other compounds are uracil, methylparaben, 3-phenylpropanol, benzonitrile, p-chlorophenol, acetophenone, and propiophenone.

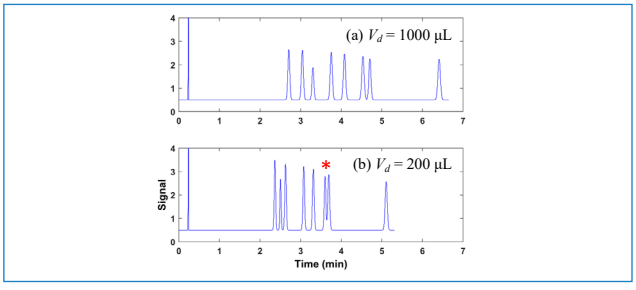

FIGURE 2: (a) V_d = 1000 µL and (b) V_d = 200 µL. Comparison of chromatograms obtained on systems with different GDVs, but with a method that had been developed using the system with the larger GDV. Conditions are the same as in Figure 1, except that the gradient was 15–40%B from 0–6.5 min.

mobile phase composition during the gradient ($\Delta\phi$), GDV (V_d), and the flow rate (F) (4). The parameter b is known as the gradient slope, given by Equation 2, where t_g is the gradient time. Finally, k_i is the retention factor of the analyte in the mobile phase used as the starting point in the gradient (that is, ϕ_i). The parameter S is analyte-specific, and depends on the mobile- and stationary-phase chemistries and temperature, and it is obtained from the slope of a ln(k) vs. mobile phase composition (ϕ, on a 0–1 scale, where 0 represents pure weak solvent, and 1 represents pure strong solvent). Note that Equation 1 assumes that the plot of ln(k) vs. ϕ is linear (that is, we are using the Linear Solvent Strength Theory here).

$$t_r = \frac{V_m}{F} + \frac{V_d}{F} + \frac{V_m}{b \cdot F} \cdot \ln\left[b \cdot \left(k_i - \frac{V_d}{V_m}\right) + 1\right] \quad [1]$$

$$b = \frac{S \cdot \Delta\phi \cdot V_m}{F \cdot t_g} \quad [2]$$

The challenges we face with the transferability of gradient elution methods arise from the relationship between the GDV (V_d) and other variables in Equation 1. V_d appears both inside and outside of the

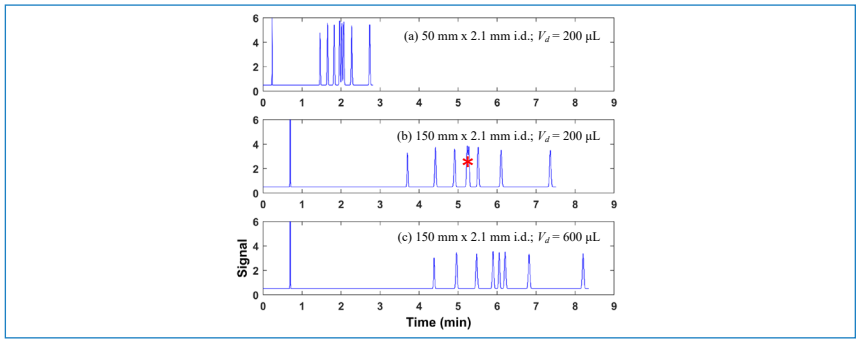

FIGURE 3: Illustration of the interactions between column dimensions and GDV, and their effects on resolution. Conditions are the same as in Figure 1, with the following exceptions: (a) gradient is 20–60% B in 0–3 min; (b,c) gradient is 20–60% B in 0–9 min. The red asterisk indicates the coelution of p-chlorophenol and ethylparaben.

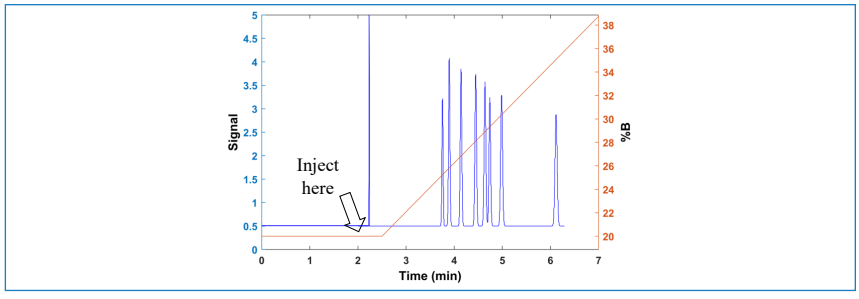

FIGURE 4: Simulated chromatogram for a system with GDV of 1000 µL, but with a delayed injection such that the effective GDV is 200 µL. Other conditions are the same as in Figure 1a.

log term, and also very importantly, in the numerator of the ratio V_d/V_m.

Impact of GDV on Transfer of a Method from One LC System to the Next

In contemporary LC practice, it is quite common for a method to be developed in one laboratory, but applied for many years in one or more other laboratories—sometimes on different continents, and sometimes in the laboratories of different companies. In these situations, it is inevitable that, at least at some point in the lifecycle of the method, the different LC systems used for development and application of the method will be from different manufacturers, with different designs and GDVs. This can lead to outcomes like those illustrated in Figures 1 and 2. In the scenario illustrated in Figure 1, a method has been developed using an instrument with a GDV of 200 µL, but it was applied using an instrument with a GDV of 1000 µL. Whereas the mixture of eight components is nicely resolved using the development system (Figure 1a), the resolution for the critical pair when applying the method is only approximately 1.0 (Figure 1b), which is clearly a major problem.

Figure 2 illustrates the opposite situation, where the method is developed using a LC system with a GDV of 1000 µL, and then applied using a system with a GDV of 200 µL. In this case, the conditions were adjusted to obtain a baseline resolution of the sample using the development system, even though the GDV is large at 1000 µL (Figure 2a), and the separation is adequate. However, when this method is run on the system with the lower GDV of 200 µL, again we see coelution, in this case for the same critical pair (ethylparaben and nitrobenzene) as in Figure 1b.

Impact of GDV on Transfer of a Method from One Column to the Next

It is also common during method development to do an initial screening of conditions (mobile and/or stationary phases) using a short column, and then move to a longer column for more efficiency and resolution once a small number of candidate conditions have been identified. Ideally, the columns of different lengths would have exactly the same selectivity so that the conditions worked out with the short column can be transferred to the longer column without any unexpected changes in selectivity. However, even if the columns do have exactly the same selectivity, unexpected decreases in resolution can occur when moving to the longer column because of the impact of the GDV on retention time. Panels A and B of Figure 3 show an example of this scenario. In Figure 3a, the mixture is nicely resolved in under 3 min using a system with a GDV of 200 µL and a 50 mm x 2.1 mm i.d. column. Now, when we move to a longer column, we should scale the gradient time accordingly if we want to realize the same selectivity observed with the short column. However, we see in Figure 3b that when the gradient time is tripled inline with the tripling of the column length, we have a serious coelution of p-chlorophenol and ethylparaben in the middle of the chromatogram. This coelution occurs because the ratio V_d/V_m inside the log term in Equation 1 changes if we change the column dimensions without changing the GDV. As soon as we increase the GDV by a factor of three so that the ratio V_d/V_m stays constant, we see in Figure 3c that the same selectivity is realized as in Figure 3a, but with increased resolution because the longer column provides more efficiency (that is, plate number). Therefore, the important takeaway here is that when changing column dimensions, we should be sure to change two other variables if we want to maintain the selectivity with the new column: 1) the gradient time should be adjusted in proportion to the ratio of the column volumes (more generally, the gradient volume should be adjusted; this is critically important if the flow rate is also changed); and 2) the GDV should be adjusted so that the ratio V_d/V_m stays constant (5).

Solutions to the Transferability Challenge

The challenge encountered in the second scenario discussed above where a method is developed using a system with a larger GDV and applied using a system with a smaller GDV (see Figure 2) is easier to address than the reverse scenario (see Figure 1). This is because the solution involves making the instrument with the smaller GDV

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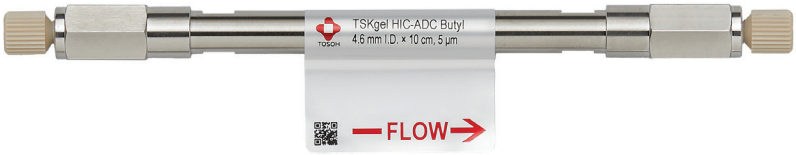

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behave as though it has a larger GDV. Instrument manufacturers have implemented a variety of creative technological solutions in recent years to address this, which involve software- or hardware-oriented solutions, or both. For example, a Chromatography Data System (CDS) could "know" that one way to increase the effective GDV is to simply program an isocratic hold into the beginning of the solvent gradient program that is executed by the pump. This would be an example of a software-oriented solution that does not require any physical changes to the instrument hardware. In this case, one could configure the instrument to always behave as if it had a specified effective GDV that is larger than the physical, and apply that behavior to all methods, rather than having to program the isocratic hold into each method. On the other hand, the physical configuration and behavior of the instrument can also be changed to adjust the effective GDV. For example, pieces of tubing with known volumes can be mounted onto a switching valve so they can be introduced into the flow path between the pump and column under software control. Alternatively, the effective volumes associated with components in pump heads and sampler syringes can also be adjusted by changing the positions of pistons in those devices. Again, all the examples mentioned here have been implemented by manufacturers in commercial instruments. Readers interested in learning more about these possibilities are encouraged to reach out to salespersons for the instruments they have in their laboratories.

Solutions to Consider When Moving to a System with a Larger GDV

There are two approaches that have been commonly used to address the challenge described in the first scenario illustrated in Figure 1 (6). The first is that the sample injection can be delayed relative to the start of the method (and thus, the start of the "clock" against which the gradient delay time is calculated). In this case, the physical GDV is not changed, but the effective GDV is reduced by the product of the injection delay time and the flow rate. This approach is illustrated in Figure 4. Most modern LC instruments support time-delayed injections, and one of the advantages of this approach is that the degree of adjustment is highly variable and software-controlled.

The second, and more widely implemented approach, is to deliberately add an isocratic hold to the solvent gradient program early on in method development, even if the physical GDV is rather small. The idea here is that if the added isocratic hold is chosen to be as large (in volume terms; $V_d = t_d*F$) as the GDV of any instrument the method could possibly be transferred to, then the method developed on the system with a low physical GDV can be transferred to any instrument with a large physical GDV. In this case, adjustment of the method would principally involve adjusting the length of the isocratic hold to account for the difference between the GDVs of the two instruments (that is, the development instrument and the application instrument). The upside of this approach is that it can be very effective. The downside is that the GDVs of instruments the method might be transferred to in the future have to be anticipated early on in the method development process.

Other Practical Factors

Two other practical factors that deserve mention here are the "rounding" of solvent gradient profiles, and the effect of pressure on GDV. In this installment, I have focused on the gradient delay volume and its impacts on retention, selectivity, and resolution. The volume associated with the fluidic components between the pump and the column is indeed the primary determinant of these effects. However, the geometries of these components (for example, lengths, diameters, and shapes) influence the "rounding" of the solvent composition profile that actually arrives at the column inlet. This is because of axial dispersion of the mobile phase components, which in turn is influenced by the geometries of the fluidic components. Although these effects are secondary, they can be quite important in some situations. Readers interested in learning more about this aspect are referred to the literature on the topic (7,8).

Finally, some older designs of LC pumps relied on "pulse dampeners" to smooth out pressure fluctuations in the mobile phase delivered by the pump. These dampeners involved compressible fluids whose volumes depended on pressure, resulting in a significant dependence of the observed GDV on pressure. Most modern LC pumps do not rely on such dampeners, but this is something to be aware of when working with older instrumentation.

Summary

In this installment of "LC Troubleshooting," I've discussed the impact of gradient delay volume (GDV) on the transferability of gradient elution methods between instruments with different GDVs, and with columns of different dimensions. We can run into trouble moving in both directions—that is, moving from a system with a small GDV to a system with a larger GDV, or vice versa. Fortunately, these challenges can be mitigated using software- or hardware-based approaches to change the effective GDV of an instrument available in most modern LC instruments. Transferring methods from modern instruments to older ones with much larger GDVs can be challenging, but is facilitated by building in method features early in the development process that anticipate the need to transfer the method later on in the method lifecycle. Understanding the theoretical influence of GDV on retention, selectivity, and resolution, and the implications of these effects in practice give us a rational means of coping with the GDV during method development and troubleshooting. ■

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Separation of All Classes of Carbohydrates by HPAEC-PAD

Christian Marvelous, Daniel Vetter, Hendrik-Jan Brouwer, Martin Eysberg, Nico J. Reinhoud, and Jean-Pierre Chervet

High performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is a potential method of choice for the analysis of carbohydrates.

CARBOHYDRATES ARE ESSENTIAL to a wide range of industries, including food, pharmaceuticals, and consumer goods. Within the food industry, carbohydrates stand out as one of the key factors in determining the nutritional value of a product. Consequently, the analysis of carbohydrates has become an indispensable tool in the food industry. Various techniques are available for carbohydrate analysis, each with its own merits and disadvantages (1). This article focuses on high performance anion-exchange chromatography in combination with pulsed amperometric detection (HPAEC-PAD) as a preferred technique for carbohydrate analysis. HPAEC allows the separation of complex mixtures of carbohydrates, for example between mono-, di-, oligo-, and polysaccharides. Furthermore, isomeric sugars such as epimers, or disaccharides with different linkage positions, are known to be separated using HPAEC (2,3). The use of pulsed amperometric detection (PAD) in combination with HPAEC enables the direct analysis of carbohydrates, eliminating the necessity for derivatization (2,3). Additionally, PAD enables sensitive detection of carbohydrates down to pico- or femtomole levels (2).

The history of carbohydrate analysis using HPAEC-PAD started in the late 1950s when the ionization of hydroxyl groups of carbohydrates in alkaline conditions was shown, revealing the potential for carbohydrate separation using anion-exchange chromatography (AEC) (4). At that time, the lack of strong and commercially available anion-exchange resins capable to withstand the harsh alkaline conditions limited the practical use of this discovery. It was not until 1983 that Rocklin and Pohl introduced the first AEC for carbohydrates using a 10-µm particle coated with a monolayer anion-exchange latex (5). Since then, the development of carbohydrate analysis using HPAEC-PAD has significantly progressed through improvements in both the chemistry of the anion-exchange resins and the reduction in particle sizes.

The separation capability of a stationary phase depends on several factors, such as the type of porous resin (microporous, macroporous, or super macroporous), particle sizes (substrate and latex bead diameters), crosslinking degree of the substrate and latex beads, and type of anion-exchange group. For instance, the particle sizes have evolved from 10 µm to smaller dimensions, such as 8.5 µm, 6 µm, 5.5 µm, 4 µm, and sub-4-µm particles, to improve separation efficiencies and shorten analysis time (6–8). Using smaller particle sizes with improved chemistries and stationary phase architecture enabled fast, high-resolution anion-exchange separation of complex carbohydrate samples (6–8). Nevertheless, it is evident that smaller particle sizes will give rise to higher column back pressures, especially the use of columns with sub-5-µm particles, which puts some limitations on the metal-free ion chromatography (IC) instrumentation that can be used for fast, high-resolution HPAEC-PAD analysis. The construction materials of the equipment, capillaries, and column blanks should have a sufficiently high maximum pressure rating to operate with such columns. Therefore, a novel agglomerated pellicular anion-exchange stationary phase for carbohydrate analysis has been developed and evaluated. The new stationary phase is based on a monodisperse 5-µm resin of a highly crosslinked poly(divinylbenzene-co-ethylvinylbenzene) copolymer coated with quaternary amine functionalized latex nanoparticles. A 200 × 4 mm i.d. column packed with these highly uniform 5-µm resin particles produces relatively low column back pressures, reaching only approximately 130 bar under typical separation conditions (0.7 mL/min, 12 mM NaOH, 30 °C). The schematic in Figure 1 illustrates the particle architecture of the new stationary phase, and the monodispersity of the particles is evident from the provided scanning electron microscope (SEM) image. The monodisperse particle size of the resin should enable

Supplied by Jean-Pierre Chervet/ modified by Helena Coppola

high-resolution separation in contrast to resins with a larger particle size distribution. In this article, we demonstrate the performance of this new stationary phase in the separation of all classes of carbohydrates, ranging from mono-, di-, and trisaccharides to oligo- and polysaccharides. The separation was performed using a 200 × 4 mm i.d. analytical column packed with this new stationary phase. The specifications of the column are shown in Table I.

TABLE I: Specifications of a column packed with the new stationary phase.

Parameter	Specification
Type	Latex agglomerated pellicular resin
Particle diameter	5 µm
Material	Poly(divinylbenzene-co-ethylvinylbenzene)
Crosslinking (%)	80%
Functionality	Quaternary amine groups
Column dimensions	200 x 4 mm i.d.
Ion exchange capacity	86 µeq
Organic solvent limit	0–100% of any common solvent (cleaning)
Temperature limit	5–60 °C (recommended 10–40 °C)
pH range	0–14
Pressure rating	Max. 300 bar/4500 psi
Typical back pressure	Around 130 bar (0.7 mL/min, 12 mM NaOH, 30 °C)

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich, Carbosynth, or Alfa Aesar unless stated otherwise. All carbohydrate standards were of analytical grade. Sodium hydroxide solution (50% w/w), high performance liquid chromatography (HPLC) grade sodium acetate trihydrate, and LC–mass spectrometry (LC–MS) grade acetonitrile were purchased from Fisher Scientific. Ultrapure water was obtained using a Merck Synergy Water Purification UV System (resistivity 18.2 MOhm/cm, TOC ≤5 ppb). All mobile phases were manually prepared, sparged, and blanketed with nitrogen 5.0 (nitrogen ≥ 99.999%) to minimize the build-up of carbonate ions and to ensure a reproducible analysis.

General Methods

All analyses were performed using the ALEXYS Carbohydrate Analyzer (Antec Scientific). This metal-free, bio-inert analyzer consists of a quaternary low-pressure gradient (LPG) pump, autosampler, column

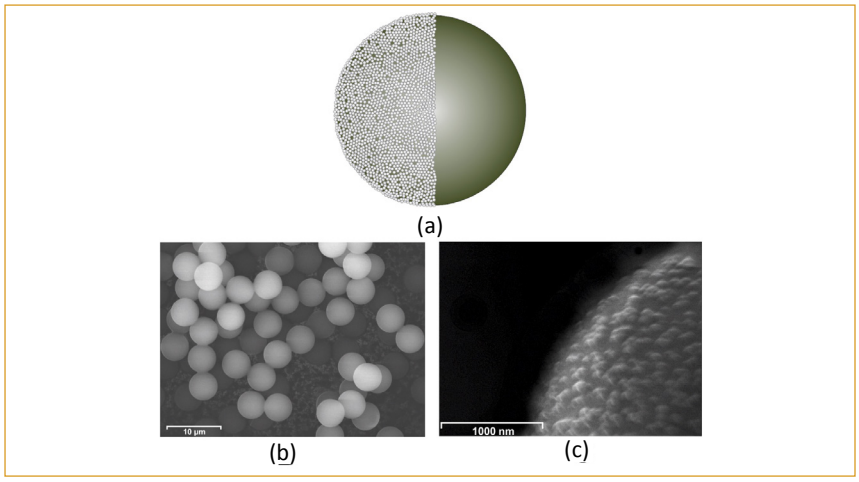

FIGURE 1: (a) Schematic of the individual resin particle of the new stationary phase (SweetSep AEX200). The particle consists of a 5-µm non-porous poly(DVB-co-EVB) core (green) coated with latex particles (white) with quaternary amine anion-exchange groups (for clarity, only half of the nano-beads are shown). (b) SEM picture of monodisperse resin particles, scale bar 10 µm. (c) SEM picture of the latex agglomerated surface of the monodisperse resin particles, scale bar 1000 nm.

thermostat, eluent tray, and electrochemical detector. During the preparation of the mobile phase, borate ions may be present in low parts-per-billion (ppb) concentrations, which can lead to peak tailing of some carbohydrates such as fructose and lactulose. Therefore, as a precaution, a borate ion inline trap column (50 × 4 mm i.d., Antec Scientific) was installed between the pump and the injector. A 200 × 4 mm i.d. analytical column packed with the new stationary phase (SweetSep AEX200) was used for all experiments. The separation temperature was set to 30 °C and an injection volume of 10 µL was used in all applications. For pulsed amperometric detection, the SenCell electrochemical flow cell was used (9). This flow cell has a confined wall-jet design and consists of a gold working electrode (WE), HyREF (Pd/H_2) reference electrode (RE), and stainless-steel auxiliary electrode (AE).

The flow cell has an adjustable spacer and was set to position 2, which corresponds to a 50-µm spacing and a 160-nL working volume. A four-step potential PAD waveform was applied for detection: E_1, E_2, E_3, and E_4 were +0.10, –2.0, +0.6, and –0.1 V, respectively, with pulse duration of t_1 = 400 ms, t_2 = 20 ms, t_3 = 10 ms, and t_4 = 70 ms. The signal (cell current) is acquired for 200 ms with a sampling rate of 10 ms during t_1 between t = 0.20–0.40 s. The signal output is the average cell current in nA measured during this 200-ms time period. The data rate of the signal output is 2 Hz, which corresponds to the 500-ms pulse time duration of the applied four-step potential waveform. This particular four-step waveform has several benefits: (1) long-term reproducible response factor for all analytes and (2) minimal electrode wear (10). The detection temperature was set to 35 °C. The stock solutions of the individual standards were prepared in 95:5 (v/v%) water/acetonitrile with a concentration of 10 mM. Acetonitrile was added to prevent fast degradation and minimize bacterial or fungal growth. The stock solutions of the standards were stored in the freezer at –20 °C and were stable for more than a month. The working standard mixes were prepared by serial dilution of the stock standards with deionized (DI) water.

Evaluation of Column Performance And Long-Term Stability

Separations of a mix of 10 sugar standards were performed on the aforementioned analytical column to evaluate its performance and long-term stability. The mix of standards consists of fucose, arabinose, galactose, glucose, sucrose, fructose, allolactose, lactose, lactulose, and epilactose in DI water. The final concentration of the mix was 10 µM. The separation was based on a step gradient. During the first 20 min, the sugars are eluted under isocratic conditions using 12 mM NaOH as the mobile phase at a flow rate of 0.7 mL/min. The isocratic elution step was followed by a column clean-up step using 100 mM NaOH for 5 min at 0.8 mL/min and equilibration to the starting conditions for 17.5 min, resulting in a total run time of 42.5 min. The long-term stability of the column was assessed by repetitive 10 µL injections of the standard mix solution for about 4 months, resulting in more than 2600 chromatographic runs.

Application 1: Sugars in Honey

A 10 µM mix of 14 sugars in DI water commonly found in honey (trehalose, glucose, fructose, isomaltose, sucrose, kojibiose, gentiobiose, turanose, palatinose, melezitose, raffinose, 1-kestose, maltose, and erlose) was used as the working standard for this application. A wild honey obtained from a Swiss beekeeper was used as a sample. The honey sample was harvested during the summer season of 2023. The honey samples were prepared by weighing 100 mg of the honey and dissolving it in 100 mL DI water to achieve a concentration of 1 g/L. Subsequently, the samples were filtered over a 0.22-µm polyethersulfone (PES) syringe filter (GVS Filter Technology) into the vials for injection. The separation was performed on the HPAEC-PAD system described above, using the following step-gradient program: isocratic elution at 0.7 mL/min using 68 mM NaOH for 25 min, followed by a 5-min column clean-up step using 100 mM NaOH + 100 mM NaOAc (sodium acetate), and equilibration to the starting conditions for 15 min. The total run time of each run was 45 min. For quantification purposes, the honey sample was diluted into concentrations of 0.1 g/L and 0.01 g/L using serial dilution with DI water.

Application 2: Profiling of Fructooligosaccharides (FOS)

Inulin from chicory was used as a sample to obtain the fructooligosaccharides profile. The sample was prepared by dissolving a known amount of inulin powder in DI water, followed by filtration over a 0.22-µm PES syringe filter and dilution to the final concentration of

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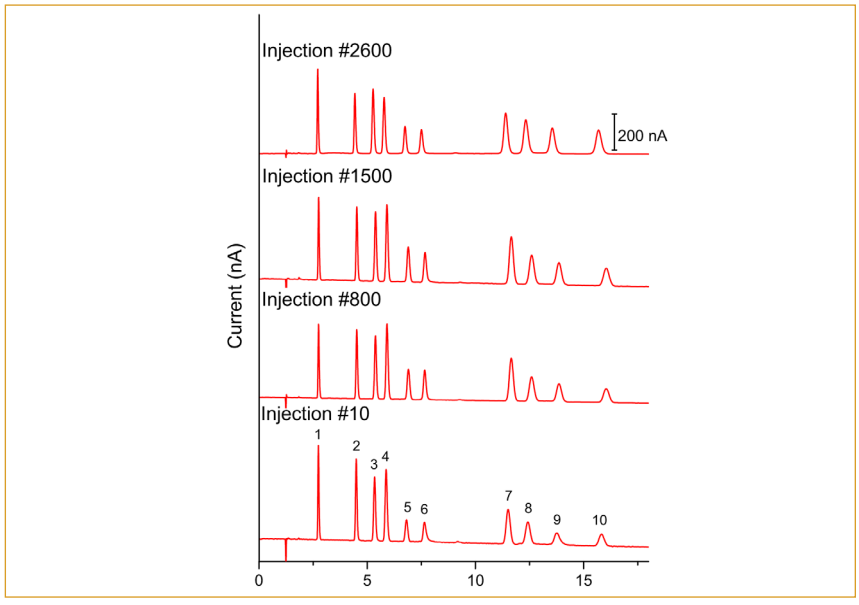

FIGURE 2: Overlay of injections #10, #800, #1500, and #2600 after 4 months of continuous injections of a carbohydrate mixture. Peak labels: (1) fucose; (2) arabinose; (3) galactose; (4) glucose; (5) sucrose; (6) fructose; (7) allolactose; (8) lactose; (9) lactulose; and (10) epilactose.

TABLE II: Column performance test results, 200 x 4 mm i.d.

No.	Compound	*Tr* (min)	*k*	*N*	*Rs*	Symmetry/ Tailing	HETP (µm)	*h*
1	Fucose	2.75	1.17	12312		1.11	16.2	3.2
2	Arabinose	4.33	2.41	18494	14.01	1.23	10.8	2.2
3	Galactose	5.08	3.00	16981	5.25	1.14	11.8	2.4
4	Glucose	5.59	3.40	17322	3.18	1.08	11.5	2.3
5	Sucrose	6.36	4.01	16455	4.18	1.07	12.2	2.4
6	Fructose	7.08	4.57	17748	3.50	1.07	11.3	2.3
7	Allolactose	10.56	7.31	18375	13.33	1.09	10.9	2.2
8	Lactose	11.43	8.00	16661	2.61	1.12	12.0	2.4
9	Lactulose	12.53	8.87	18538	3.08	1.09	10.8	2.2
10	Epilactose	14.29	10.25	19375	4.53	1.09	10.3	2.1

200 ppm. The separation was performed on the same HPAEC-PAD system as mentioned earlier, using a gradient program with a flow rate of 0.8 mL/min. The gradient program started with 100 mM NaOH, and a linear gradient to 100 mM NaOH + 180 mM NaOAc was applied until t = 12 min. Subsequently, a more gentle linear gradient to 100 mM NaOH + 450 mM NaOAc was applied until t = 60 min. The system was equilibrated to the starting condition for 15 min, resulting in a total run time of 75 min. Initial peak assignments were based on the elution pattern of glucose (G), fructose (F), sucrose (GF), 1-kestose (GF_2), nystose (GF_3), and fructosyl nystose (GF_4). Because of the lack of commercial standards for sugars with a high degree of polymerization (DP), further assignments were based on the assumption that the retention of a homologous series of carbohydrates increases as the DP increases.

Results and Discussion

Column Performance and Long-Term Stability

The column performance and long-term stability assessment of a 200 × 4 mm i.d. column were conducted based on the separation of 10 sugars. The 10 sugars were carefully chosen to cover a range of molecular structures of saccharides: (1) monosaccharides (glucose) or disaccharides (sucrose); (2) isomers (allolactose and lactose); (3) epimers (galactose and glucose); (4) hexose (fructose) or pentose (arabinose) for monosaccharides; and (5) deoxy sugars (fucose). The separation of the 10 sugars on the analytical column was achieved under isocratic elution condition with 12 mM NaOH at the flow rate of 0.7 mL/min. Under this condition, all 10 sugars were baseline separated (resolution ≥1.5). The symmetry and tailing factors for the 10 sugars were excellent, with a value of approximately 1.1 for most sugars except for arabinose (1.2). The plate numbers of all sugars range between about 12300 to 19300, and the reduced plate heights (h) for most of the sugars were close to the ideal value of 2.0 for a 200 mm column with a 5-µm particle size. All column parameters are provided in Table II.

The long-term stability of the 200 × 4 mm i.d. column was assessed using the same 10-sugar mix under the same conditions. The overlay chromatograms of several selected injections over four months, during which more than 2600 injections were conducted, are provided in Figure 2. The retention times for all 10 compounds remained stable over this period. The small variations in peak height and peak area were caused by differences in the manual preparation or aging (degradation) of the standard mix. The long-term stability of the column was also assessed based on the loss of the plate numbers and changes in tailing factors. There was no observed loss in plate numbers nor increase in tailing factors, which indicates that columns packed with this resin result in very stable columns with outstanding lifetime.

Sugars in Honey

Honey is a complex natural substance with a promising potential for various health benefits, and it consists of approximately 80% carbohydrates (11). Because of its economic appeal, honey is susceptible to food fraud and adulteration. For instance, in 2021 the value of imported honey was 2.32 €/kg, whereas commonly used adulterants such as rice syrups cost approxi-

mately 0.40–0.60 €/kg in the European Union (EU) (12). The composition and definition of honey in the EU are regulated by the EU Honey Directive 2001/110/EC (13). The directive specifies the criteria for unadulterated honey products including the threshold of sugars in honey. Therefore, HPAEC-PAD is an attractive method that can quantify sugars in honey to check the authenticity of honey samples.

The separation of the 14 sugars commonly found in honey using the aforementioned column is depicted in Figure 3. Out of the 14 sugars, two are monosaccharides (glucose and fructose), eight are disaccharides (trehalose, isomaltose, sucrose, kojibiose, gentiobiose, turanose, palatinose, and maltose), and four are trisaccharides (melezitose, raffinose, 1-kestose, and erlose). All sugars were eluted within 25 min, and most of the sugars were baseline separated (R ≥1.5), except for palatinose and melezitose (resolution 1.1 and 1.2, respectively). The peak efficiency for all sugars ranged between 8000–16000 plates, and all peaks exhibited no significant tailing (tailing factor between 1.0–1.2).

The presented method was validated by testing the linearity, repeatability, and determining the limits of detection (LODs). The linearity of the method was investigated in the concentration range of 0.01–50 µM. In this concentration range, the linearity is excellent with the correlation coefficients (r) >0.999 for almost all sugars except for turanose (r = 0.9986). A total of 10 repetitive injections of the 10 µM standard mix in DI water were performed to assess the repeatability of the method. Excellent repeatability was found as shown by the very small relative standard deviations (RSDs) of the retention times, peak heights, and peak areas (<0.3%, <0.5%, and <0.6%, respectively). The LODs were calculated based on the International Council for Harmonization (ICH) guidelines (that is, LODs were calculated as the analyte response corresponding to 3× the ASTM noise, with an average peak-to-peak baseline noise of 10 segments of 0.5 min). The excellent sensitivity of the method is evident from the low detection limits for all sugars (<70 nM).

To demonstrate the applicability of the method, a summer honey sample obtained from the Swiss beekeeper was tested. The chromatogram of the 10 µL injection of the honey sample is shown in Figure 3. All 14 sugars were detected in the honey, with glucose and fructose being the most dominant sugars. Quantification of the sugars shows that the glucose, fructose, sucrose, and maltose contents are 27.4 g, 31.9 g, 0.1 g, and 0.8 g per 100-g honey products, respectively. These values align with the specified criteria of unadulterated honey defined by the EU Honey Directive 2001/110/EC (13). Overall, the presented method shows the outstanding separation of sugars using the new stationary phase and sensitive detection of the sugars in honey using pulsed amperometric detection.

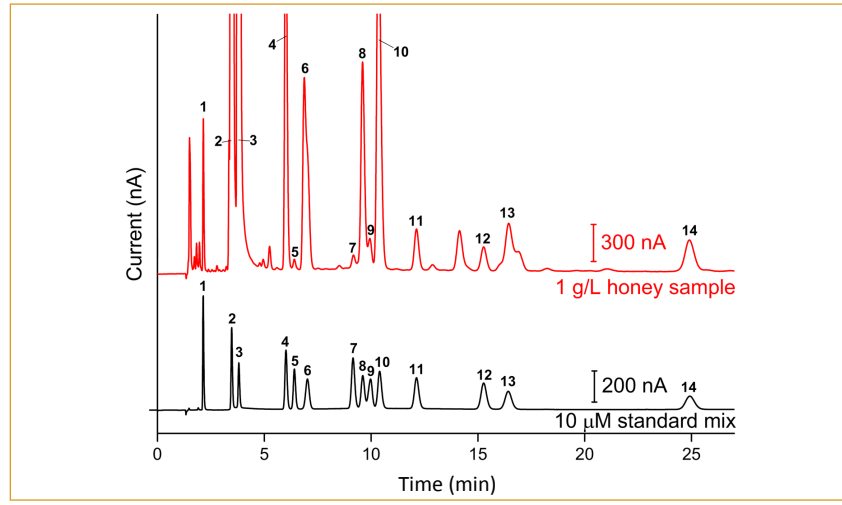

FIGURE 3: Overlay chromatograms of 10 µL injections of 10 µM standard mix of 14 sugars commonly found in honey (black lines) and 1 g/L honey sample obtained from Swiss beekeeper (red lines). Peak labels: (1) trehalose; (2) glucose; (3) fructose; (4) isomaltose; (5) sucrose; (6) kojibiose; (7) gentiobiose; (8) turanose; (9) palatinose; (10) melezitose; (11) raffinose; (12) 1-kestose; (13) maltose; and (14) erlose.

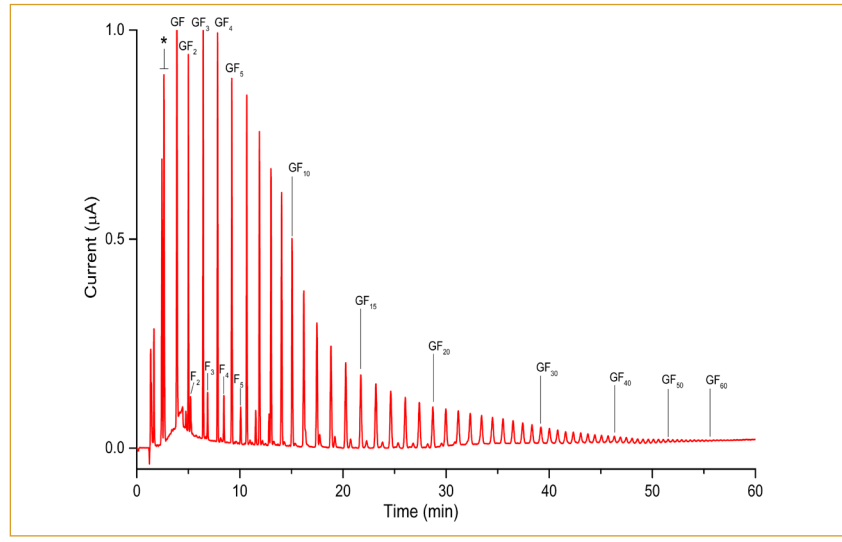

FIGURE 4: Chromatogram of 10-µL injections of 200 ppm inulin from chicory. Monosaccharides (glucose and fructose) are labeled with an asterisk.

Profiling of Fructooligosaccharides (FOS)

Fructooligosaccharides are polymers consisting of fructose found widely distributed in nature as plant storage carbohydrates. Fructooligosaccharides are a form of dietary fiber, and they can serve as an energy source for the gut microbiota (14). Many

plant species, including wheat, onion, bananas, garlic, and chicory, contain inulin-type fructooligosaccharides (ITF). ITF exists as a blend of polymers with degrees of polymerization (DP) ranging from 2 to 60 subunits (14). Some ITFs in plants have a glucose unit at the reducing end, while others do not include a glucose residue at all. Therefore, all ITFs can be described with the generic chemical structure GF_n (with G as optional glucose, F as fructose, and n indicating the number of fructose moieties).

The column described earlier was employed to obtain a fructooligosaccharides profile in inulin from the chicory sample. A chromatogram in Figure 4 illustrates the profile of fructooligosaccharides from the sample. Based on the chromatogram, inulin predominantly consists of GF_n-type fructooligosaccharides ranging from DP_3 (GF_2) to approximately DP_{61} (GF_{60}). Additionally, this sample contains a substantial amount of free sugars (glucose, fructose, and sucrose). Although the GF_n and F_n type fructooligosaccharides are baseline separated until GF_7 and F_7, they exhibit slightly different retention behavior. Consequently, they unavoidably overlap, leading to the coelution of components starting from GF_8 and F_8. The GF_{12} and F_{12} were observed to be baseline separated again until approximately GF_{22} and F_{22}. Further, F_{23} onwards was not observed, whereas GF_n-type was still detected up to approximately GF_{60}. It is important to note that the chain-length distribution should only be interpreted qualitatively because the response factor decreases with increasing chain length, and therefore, it does not represent the exact quantitative distribution. Overall, the presented method demonstrated excellent separation of inulin-type fructooligosaccharides.

Conclusion

A new anion-exchange stationary phase based on 5-µm particles was developed, and it enables fast, high-resolution separation of carbohydrates at moderate column back pressures. A 200 mm × 4 mm i.d. analytical column based on the stationary phase demonstrated a superior performance with reduced plate heights for nearly all sugars close to the ideal value of 2.0. The column showcased great stability in retention times, peak efficiencies, and tailing factors over an impressive span of 2600 injections. The versatility of this new stationary phase was evident in its ability to achieve high-resolution separation of carbohydrates from mono-, di-, tri-, oligo-, up to polysaccharides. In conclusion, the newly introduced column provides high-resolution separation of all classes of carbohydrates using HPAEC-PAD and will help to achieve accurate identification and quantification of carbohydrates in food products, including detection of adulteration and fraud. ■

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

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are members of Antec Scientific.

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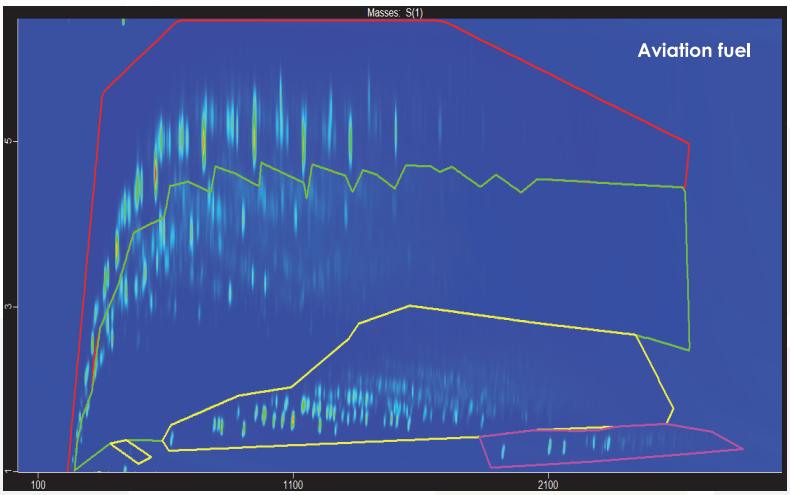

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HOW A CHROMATOGRAPHY data system (CDS) can be involved in data falsification in regulated good practice (GXP) environments is public knowledge since the 2005 Able Laboratories fraud case (1). Chromatograms were reintegrated multiple times, but the most creative falsification was chromatographic titration using this sequence file: *sample weights were changed until a passing result was obtained* (1). FDA missed the fraud (as they focused on paper records), but a whistleblower alerted the local field office, and the rest is history. Ironically, all data manipulation was recorded in the CDS audit trail. The Able fraud case has resulted in three updates of the *FDA Compliance Policy Guide 7346.832 for Pre-Approval Inspections* (PAIs) since 2010 (2–4); the last two updates were discussed in my "Focus on Quality" ("FOQ") column in *Spectroscopy* (5,6).

A "Questions of Quality" ("QOQ") column discussed the role of CDS in fraud and falsification and described the 10 compliance commandments (7). If implemented, these commandments should help prevent many poor data management practices such as shared user identities, roles with conflicts of interest, deleting records, shredding printouts, selective reporting, invalidating out of specification (OOS) results because of human error, manual integration, turning the audit trail off and back on, or failing to turn it on in the first place.

Let us see how the CDS Class of 2023 have failed when inspected by the FDA. I would like to thank Paul Smith for providing the 13 483 citations from 2023. I have extracted the CDS-related citations from the 483s, classified them, and lightly edited them to fit in Table I. Be warned that regulatory authorities have received training in CDS applications from software vendors.

The key question? Have companies learned and implemented earlier CDS compliance lessons? Spoiler alert...No! Given the attention that regulatory agencies have given chromatography since 2005, you would have thought that companies would have made some progress to eliminate common problems, but no. On the plus side, this provides me more data to highlight the stupidity of some organizations to keep current with guidance and regulatory actions. In this column, we look at the current ways of poor data management practices and data falsification including peak integration—again! This is the third time the subject has been discussed in a "Questions of Quality" column (8,9).

The focus of this column is on the CDS, but don't forget that much of the preliminary sample preparation is manual, and, in principle, laboratories need to perform a risk assessment to determine if the process needs to be witnessed (10).

Warning Lights Are Flashing in Quality Control

Since Able, we have seen several ways chromatographers can attempt to pass product batches:

- **Test and Prep Samples:** The old school approach to testing into compliance created a directory called "Test, Wash, Demo or Prep," and sample injections were made to see if the batch would pass or not (24–26). One such folder contained over 3300 data files (27). All are easy to detect, and this has been phased out in favor of other options to cheat. Ideally, your laboratory should only allow one location to store CDS data for any run.
- **Conflicts of Interest:** Another old school non-compliance that should have been resolved by now is to give users privileges where they can do activities they should not be able to do. Table I shows that the practice is not extinct, and users still can delete or manipulate data if they are given the privileges. My view is simple—no user should have deletion privileges.
- **Audit Trail Not Turned On:** Table I also has an instance where the system audit trail has not been turned on (12). Simple stupidity...either not thinking about data integrity, or not bothering to understand

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Robust PFAS extraction for **EPA Methods 537.1, 533 and 1633**

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POLYMERIC WEAK-ANION EXCHANGE

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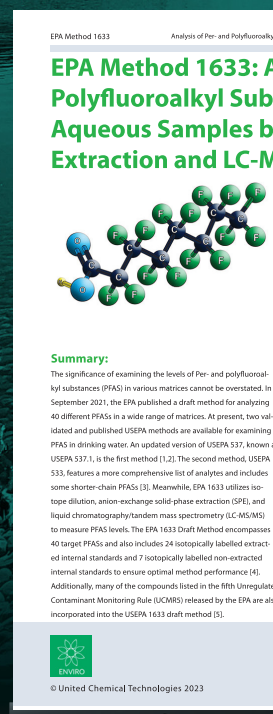

EPA Method 1633 — Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Aqueous Samples by Solid-Phase Extraction and LC-MS/MS

EPA Method 1633: Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Aqueous Samples by Solid-Phase Extraction and LC-MS/MS

UCT Part Numbers

ECWAX156-P* ENVIRO-CLEAN® WAX 500 mg, 6 mL cartridge, PE frits	**VMF016GL** Complete 16 Position Glass Block Manifold
RPP00150P Empty Polypropylene Reservoirs	**SCS27-C18521** SelectraCore® C18 HPLC Column (50 × 2.1 mm, 2.7 µm)
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SLC-18SOID46-5UM Selectra® C18 Delay Column (50 × 4.6 mm, 5 µm)	***ECWAX126-P** 200 mg, 6 mL cartridge Available for extraction of 100 mL samples

Summary:

The significance of examining the levels of Per- and polyfluoroalkyl substances (PFAS) in various matrices cannot be overstated. In September 2021, the EPA published a draft method for analyzing 40 different PFASs in a wide range of matrices. At present, two validated and published USEPA methods are available for examining PFAS in drinking water. An updated version of USEPA 537, known as USEPA 537.1, is the first method [1,2]. The second method, USEPA 533, features a more comprehensive list of analytes and includes some shorter-chain PFASs [3]. Meanwhile, EPA 1633 utilizes isotope dilution, anion-exchange solid-phase extraction (SPE), and liquid chromatography/tandem mass spectrometry (LC-MS/MS) to measure PFAS levels. The EPA 1633 Draft Method encompasses 40 target PFASs and also includes 24 isotopically labelled extracted internal standards and 7 isotopically labelled non-extracted internal standards to ensure optimal method performance [4]. Additionally, many of the compounds listed in the fifth Unregulated Contaminant Monitoring Rule (UCMR5) released by the EPA are also incorporated into the USEPA 1633 draft method [5].

This application note outlines the analysis of 40 distinct PFASs in aqueous samples as per EPA 1633 3rd draft method by utilizing UCT's Enviro-Clean® polymeric weak-anion exchange (WAX) SPE cartridges. A SelectraCore® C18 HPLC analytical column was employed for LC-MS/MS analysis, while a short (5 cm) C18 delay column was used to reduce potential of PFAS contamination from the HPLC system. SelectraCore® column was able to achieve a baseline separation for many critical analytes, some of which are branched vs linear isomers like PFHxS and PFOS. A calibration curve specific to each analyte was performed according to the guidelines written in the method. All compounds were linear, with R^2 values >0.99. The extraction method was evaluated by spiking aqueous samples with PFAS's at high and low fortification levels, as indicated in the results table in this application note. Recoveries of all analytes were within a range of 80-110% and RSD values <10%.

WHEN RESULTS MATTER

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TABLE I: FDA 483 citations for chromatography data system non-compliance

Citation	CDS Non-Compliance
Roles with Conflicts of Interest	• Owners and chemist supervisor are ... administrator ... able to ... to delete projects, delete data, modify integration parameters and delete results (11) • Chemist supervisor can adjust integration parameters (11) • CDS permission of Save Results and Calibration in Review as ON ... allowing the processing of manually integrated data. The firm stated that they do not perform manual integration (12).
Audit Trail Off	• ..software has not been programmed to capture a system audit trail (11)
Invalidating Results	• Investigations concluded that the chromatographic system, column resolution and integration parameters were the most likely caused of the failed outcomes.... the data was invalidated as it was run on a system that does not provide the expected quality (13).
Lack of Complete Data	• ...HPLC technician to review injected data prior to processing the method. This review is not identifiable on an audit trail as you do not save the review (12). • Analysts and reviewers have ... permission to see the peak area counts in the review window. .... change integration while seeing how it affects the area count, without requiring them to save the changes (14) • Only initial and final electropherograms are submitted for review and approval. Analysts use manual integration and generate multiple versions of the electropherograms, but the review process does not evaluate all versions .. and determine whether changes are appropriate. 33 versions were generated for one electropherogram (15). • Test injections are performed <redacted> and they do not get submitted for review (16).
No SOP for Peak Integration	• Your firm doesn't have a set SOP ... for chromatographic integration (17). • There is no procedure .... to ensure manual integration events require explanation (18). • no procedure to describe how to correctly perform integration to ensure consistency from analyst to analyst and from day to day (15). • Your firm does not have an SOP on <redacted> integration for the <redacted> HPLC <redacted>. ... technicians are allowed to perform .... Integration of the product peak, if required (19). • No written procedure in place that restricts analysts from retesting samples or from performing inappropriate manual integration. ... performing manipulations that may affect the analytical result (manual integration) (20)
Failing to Follow Peak Integration SOP	• Changes must be approved by the laboratory director ... before any changes occur. There was no evidence that the chemist supervisor informed ... prior to adjusting the integration parameter (11). • Procedure ... requires the analyst to attempt to optimize the automatic integration parameters before applying manual integration, but review of the electronic files showed no documented results demonstrating attempts to optimize the integration parameters (14).
Peak Integration and Parameters	• Integration parameters differ between suitability and samples analyzed during related substances testing of <redated> tablets (21). • In addition, assay and impurity methods use the integration parameter of "reset baseline" which can alter the integration of assay and impurity peaks (21). • All chromatograms, including assay control and test samples, contained <redacted> integrated peaks with reason "integration" (18). • Actual "inhibit integration" ... should only be utilized to remove interference of blank/placebo peaks that are present in the sample chromatograms as recommended in the respective analytical procedure. ... unknown peak ... was absent in blank and system suitability injections. QC supervisor ... deviated from the SOP by applying peak integration function. (e.g inhibit integration) (22). • Different processing methods with inconsistent peak integration parameters used to integrate peaks of the standards and different lots of the product. The lab used three different processing methods ... to integrate ... standards and ID 4206 for the product (23). • The practice of valley-to-valley integration parameters at the beginning of analyses during impurity analyses for finished products, APIs and raw materials resulted in uncounted areas under two or more co-eluting peaks (16).
Mid Run Test Injections	• Your firm was unable to explain the rationale for test injections post system suitability and standard injections (20).

the CDS technical controls available versus the regulatory requirements (28,29).

- **Selective Reporting or Lack of Complete Data:** There are several citations for the lack of complete data in Table I. This can vary from only saving the original file and the "final" reintegrated file to ignoring failing results and only reporting results within the specification. Table I shows an example where a sample was reintegrated 33 times to obtain the correct result (15). Integrating into compliance?
- **Hiding Peaks by Printing:** One way to hide poor impurity peak shape is to print the main peak as 100% full scale deflection, allowing the impurities to disappear into the baseline. A reviewer would need an electron microscope to see the peaks on the printout. Ignore printouts and review chromatograms on screen, as discussed later.
- **Instrument "Problems":** Has a chromatograph suffered a malfunction that invalidates the run? If so, the impacted chromatograms should have evidence of the problem; for example, a leaky piston seal should result in poor peak shape and longer retention times. Create a pump leak, then (shame!) the run must be invalidated and the analysis repeated so the batch passes. The falsified data hide in plain sight. Refer to the instrument logbook to see that the problem is documented, resolved along with any applicable requalification, and then refer to the CDS so as to check that the data file date and time stamps are consistent with the logbook entries. Table I has a real gem with instrument problems but also invalidating an OOS result: *the data was invalidated as it was run on a system that does not provide the expected quality (13).* The obvious question is: Why start an analysis using a chromatograph that was allegedly not fit for purpose? System suitability test (SST) acceptance criteria, anyone?
- **Short or Aborted Runs:** Another way to hide falsification is to use short or aborted runs of one or two injections using samples to see if they pass or not. Searches

of the database should be able to identify short and aborted runs. The question, then, is: why was the run aborted? This must be linked with the instrument problems discussed above. We discuss how to evaluate if the system is ready to run later in this column using guidance from the FDA website.

- **Manual Injection:** Accessing the quality of samples by manually injecting "outside" of the CDS, using the control panel on the chromatograph.
- **IT Help Desk:** Another way that file deletion has occurred has been through logging requests and changes through the IT Help Desk, which may not necessarily have the same awareness of the regulatory impact of the changes. Does your laboratory have a quality agreement with IT (28)? The FDA have audited IT Help Desk systems to check for this.

To highlight both instrument problems and short or aborted runs, a 483 citation for Aurobindo noted that the message center of a CDS logged 6337 (yes, really!) error messages from July 1 to August 1, 2022 (30). Analysis identified the following:

- 411 instrument failure messages;
- 13 messages of sequence stopped because of error or sequence stopped by user; and
- 20 failed to get the newest information of the batch queue because of the communication failure messages.

For further reading, a "QOQ" column discusses orphan data (31), and an "FOQ" column looked at the role of an instrument logbook (32).

The remainder of this column discusses peak integration and cover procedures, including failure to follow one and the use of various integration parameters to help or hinder your journey to data integrity nirvana.

Controlling Peak Integration

My experience is based on small molecule analysis, so please interpret my comments for more complex separations. There seems to be a great deal of confusion over terminology, in regards to what labels to use for different kinds of integration "activity" and what is allowed and not allowed. Earlier "QOQ" columns discussed the requirement for automatic peak integration to be applied to all chromatograms the first time, every time (8,9). It also differentiated manual intervention (changing automatic integration parameters with automatic baseline placement) from manual integration (manual reportioning of the baselines) (8). Manual intervention should be applicable to any chromatogram (such as for peaks outside expected retention windows, or increasing minimum area to reduce the impact of a noisy baseline), but the changes must be applied to all files in the sequence. From quality and regulatory perspectives, testing samples from a validated manufacturing process on a qualified instrument using a validated analytical procedure raises the question—why would you need to change the integration? This is why changes to integration for product assay methods would be interpreted suspiciously. Manual integration must only be allowed in specific cases

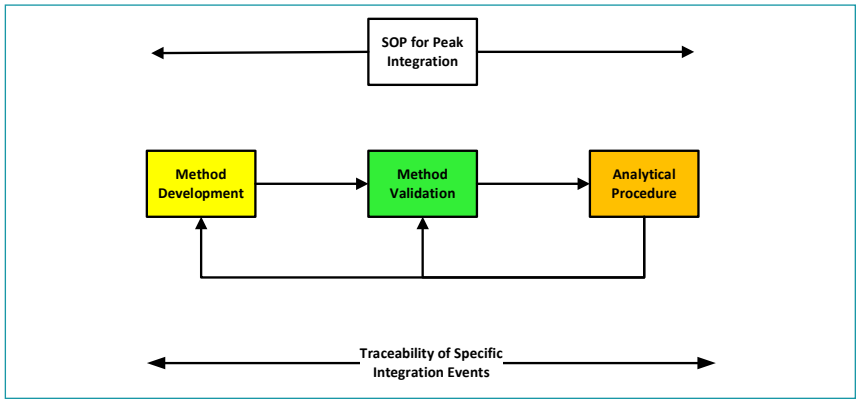

FIGURE 1: Ensure specific integration requirements are incorporated in an analytical procedure and are traceable.

such as impurities or complex separations, with the rationale documented in the method validation report.

As part of the laboratory controls, you need a procedure and training in peak integration. One citation in Table I stated: *No procedure to describe how to correctly perform integration to ensure consistency from analyst to analyst and from day to day* (15). It is not just having a procedure; it is about how a laboratory ensures consistency across the whole user base.

- **Peak Integration Standard Operating Procedure (SOP):** It is imperative that there is an SOP for peak integration. You have got one, haven't you? Some FDA warning letters required laboratories to have an SOP for manual integration. In my view, this is wrong and is not scientifically sound as required by 21 *CFR* 211.160(b) (33). An imperative is an SOP that covers all peak integration, highlighting what must be done and especially what must not be done. It is not a treatise on the fundamentals of integration, and the book by Dyson (34) is the best source of integration knowledge. Where permitted, all integration changes made by an analyst must be justified and documented, typically in the audit trail. CDS applications provide the means to have user-defined reasons for change, and this feature should be used. The journey from the first to the last integration of any file must be traceable, complete, and with reasons for change to ensure data integrity. The peak integration SOP must be linked to individual analytical procedures where specific integration requirements must be defined.
- **Peak Integration Training:** What training in the integration procedure was carried out and what were the results? It is not just training individual chromatographers; it is also ensuring integration consistency throughout the laboratory. One laboratory has a series of standard chromatograms that all must integrate after training. Interestingly, results for assay had excellent agreement across the laboratory, but could vary with impurities close to the limits of quantification. Using automatic integration first time, every time, resolved much of the problem.
- **Analysts Reviewing Other's Work:** This is important so that they gain experience of reviewing chromatographic data from an independent quality perspective and therefore better understand the risks and implications for changing integration.

You Have an SOP – Follow It!

It's all very well having a procedure with effective training, but does a laboratory follow the SOP? The obvious answer is yes. Again, there are two examples in Table I where the integration SOP has not been followed (11, 14). In addition, there is another citation:

> *Procedure SE/BQC/00165 Interpretation of Chromatograms requires manual integration be documented clearly stating the reason the manual integration was performed and the initials of the section head for approval. But when analysts manually enter integration events to force the software to integrate in a specific way, there is no similar documented justification and approval process* ... (35).

As the old saying goes, you can take a horse to water …..

Linking Integration Events to an Analytical Procedure

Closely allied with an integration SOP is the necessity to incorporate any pertinent integration requirements into each analytical procedure to avoid citations such as:

> *The method does not include how to properly apply these <redacted> integration events to process the analytical data and no procedure was provided on how to report <redacted> integration within the result sets* (18).

Figure 1 shows a peak integration SOP that provides overall control of integration and what is allowed, what is not allowed, and what is applicable throughout the analytical procedure lifecycle (36). However, such a procedure cannot go into detail for all chromatographic methods, and this is the role of each analytical procedure:

- If the procedure is for an assay, can manual integration be performed at all? Many would argue that a method is out of control if you have to manually integrate the main peak.
- Example chromatograms should also include any specific requirements for integration parameters such as integrate inhibit and baseline zero, and be scientifically sound and justified. As shown in Figure 1, these parameters should be traceable to the development and validation reports.
- Only test "equivalent" samples on the same chromatographic run—in particular, segregate the analysis of stability samples from runs containing current production samples. Integration requirements and the impurity profile of the stability samples may mean different integration parameters are required.

To help scientific justification, any integration parameters from the analytical procedure should be traceable back through the validation report to the development report shown in Figure 1. You know it makes sense, but will you be allowed time to do this? Also

shown in Figure 1 are the return loops from the analytical procedure to development and validation phases of the lifecycle. If there is a problem with the analytical procedure, modification and revalidation may be required.

Cunning Chromatographers and Insidious Integration

Always remember that chromatography is a comparative and not absolute analytical technique. Therefore, all injections in a run must be integrated with the same method. Table I has an example where three processing different methods were used for the standard samples and yet another one for the samples (23).

This fact provides more subtle means of data manipulation by treating samples differently from standards. Reviewers must be aware of this and ensure that any attempts to manipulate peak integration is identified before results are calculated.

A more insidious approach was identified in the Intas Pharmaceuticals 483 (35):

- *… Additionally, the 6-month accelerated time point for the same lot <redacted> was* ***integrated manually by adding a fronting sensitivity and a tailing sensitivity factor to the peak for impurity <redacted> but not for the standard of the same impurity.*** *This reduced the area of the impurity compared and gave a result of <redacted>% compared to a limit of <redacted>%. When the fronting and tailing sensitivity factors are removed to ensure integration of the impurity compared with the standard, the reportable result changes to <redacted>%, a value that would have required an investigation.* This is one 483 citation—there are several other integration issues in the 483 (35).

Reviewers and quality assurance (QA) must be aware that standards and samples must be integrated using the same parameters and check to ensure that this has occurred.

System Suitability Test Failures

A short diversion is required to discuss the FDA's present to the industry in the *Guidance on Investigation of Out Of Specification Results*. Under the "Responsibilities of the Analyst" section, there is the following:

Certain analytical methods have system suitability requirements, and systems not meeting these requirements should not be used. For example, in chromatographic systems, reference standard solutions may be injected at intervals throughout chromatographic runs to measure drift, noise, and repeatability. ***If reference standard responses indicate that the system is not functioning properly, all of the data collected during the suspect time period should be properly identified and should not be used.*** *The cause of the malfunction should be identified and, if possible, corrected before a decision is made whether to use any data prior to the suspect period (37,38).*

This is key to some peak integration problems and interprets the section above in a way the FDA did not intend. This also links with the instrument problems discussed earlier in this column. If the SSTs fail throughout the run, the files are still part of the complete data of the analysis but should not be used. Complete data (21 *CFR* 211.194(a) (33) is the regulatory requirement and the term that was discussed in earlier article (39). What most analysts forget is that the reason for the problem must be investigated and corrected.

How does this section impact peak integration malpractice? One way to invalidate a run is to integrate the SST injections to ensure that the acceptance criteria are out of limits. The material to use for SST injections is discussed on the FDA's web site in the section on Laboratory Controls, Question 16 (40).

Is My Chromatogram Big Enough?

Second person review is critical to confirm chromatography has been performed correctly and that there is no data manipulation. To achieve this, forget printing chromatograms. Instead, implement electronic signatures, and, if you really must, only print a summary of the analysis. You must review chromatograms and the associated metadata, including audit trail entries on screen. Please understand that this section should not be used to justify the purchase of a 55-inch 4k internet-enabled screen, but you do need to have a large enough screen or dual screens to perform an adequate second person review. Have the chromatograms in one window and the audit trail or other run information in another so that you can cross reference both easily.

One function of a CDS that is critical for review, audit, or inspection is chromatogram overlay. Chromatograms can be plotted superimposed on each other to assess retention time and peak shape consistency throughout the run. Alternatively, to get a better picture, especially for impurities, the overlay offset function enables an easier comparison. A larger screen or screens enables the chromatograms and the applicable audit trail entries in a second window to be correlated simultaneously, rather than laboriously switching between windows with a small single screen.

Five Rules of Peak Integration

An integration SOP was discussed earlier to help understand what should be in it and the associated training. There are five rules to consider (9), summarized here:

- **Rule 1: The main function of a CDS is not to correct your poor chromatography.**
  Good chromatography requires a robust analytical procedure with good peak shape and separation. Know and control the factors that influence separation and ensure that automatic peak integration is the norm not the exception. This is especially true for pharmacopoeial methods that never work as written. See Stage 1 of the *USP* <1220> [36] rather than the abysmal *ICH* Q2(R2) and Q14 guidance document [41, 42] that are not integrated and have large gaps in the final versions that were not corrected from the draft versions [43].
- **Rule 2: Never use default integration parameters.**
  Using a default or generic method results in an excessive need for manual integration to name and calculate peaks. Without exception, peak integration and result processing must be defined and validated for each method so that all peak windows and names are established. Where necessary, any system peaks are identified. If used, integrate inhibit must be scientifically justified and be traceable to method devel-

opment and validation reports. Unlike Dr Reddy's, who received a 483 citation for the incorrect use of the integrate inhibit function to mask an unknown peak in samples that was not present in blank or standard samples coupled with no investigation of the problem (22).

- **Rule 3: Always use automatic integration as a first option and control manual integration.**

  Remember that the use of manual integration is a regulatory concern, and its use needs to be scientifically sound. Manual integration slows down processing, so see Rule 1 to get the right method, depending on the sample matrix and peaks of interest.
- **Rule 4: Understand how the CDS works and how the numbers are generated.**

  This requires basic training in the principles of peak integration and how a CDS works. The problem is that with company mergers or acquisitions that encourage experienced analysts to retire and employ younger workers, skills are being eroded, and a CDS can be looked at as a black box that always gives the right answers. Learn and understand how the basics of a CDS works.
- **Rule 5: Use your brain—think.**

  This rule is sometimes difficult to follow, but it builds on Rule 4. You can have what appears to be a perfect separation and peak integration, but look at the peak start and end placement; do they look right? Does there appear to be a coeluted peak? Use the zoom and overlay functions of the CDS to see if the standards and samples have the right peak shape. The analyst has the responsibility to execute applicable procedures correctly, which includes correct peak integration. The reviewer also has a role to ensure that all integration (whether automated or manually placed) follows the guidance for placing baselines as the SOP and analytical procedure describe. Significant peak area manipulation should be easily noticed by an experienced reviewer.

Can I Use System Evaluation Injections?

Good scientific sense is to check that the chromatograph and column is equilibrated and ready for analysis. However, some laboratories are fearful of doing this, as they might be accused of testing into compliance with sample injections. Help is at hand from an FDA Q&A on laboratory controls on their website (40). Question 17: *Is it ever appropriate to perform a "trial injection" of samples?*

- *No.*

  This is unofficial testing disguising testing into compliance which is a violation of GMP and is unacceptable.
- *Column conditioning does not involve injecting a sample from a lot and is not considered a trial injection. When its use is scientifically justified,* ***column conditioning should be fully described in the method validation package*** *as to the conditions needed to make the measurement (that is, based on data from the method validation) and should be* ***clearly defined in an approved and appropriate procedure.***
- *Consistent and unambiguous injection nomenclature should be used, and all data from the column conditioning, including audit trail data, should be maintained and subject to review.*

Note the wording in the second bullet point that the use of conditioning injections should be traceable to the method validation or verification report with criteria to determine if the system is ready to start analysis. You must use the correct terminology for injections and conditioning injections (not Test or Prep!), as they are part of complete data (33) or raw data (44) for any run.

Will AI Solve My Integration Problems?

I know what some of you are thinking, that all I have written here is tosh, and artificial intelligence will solve all my problems. In your dreams; the clue is in the name *intelligence.* You must train an AI application, and this means you must know what you and the CDS are doing (see Rules 4 and 5), and you need good chromatography for good integration (see Rule 1).

Workman has provided a good overview of the background and essentials of AI in analytical chemistry (45). Trawling the internet will not capture a scientifically sound integration method for a specific separation. Different CDS applications have different algorithms for peak integration, and, while you may get a separation on one system, another one may integrate the same chromatogram differently; peaks areas could be the same, but rarely identical. It is imperative that you have good quality data sets for training the AI application (46).

You may be better off waiting until your CDS supplier has developed an AI module for you to train to help you integrate peaks.

Summary

Chromatography data systems continue to be the source of many poor data management and falsification practices found in regulated laboratories. Often, these are repetition of poor practices that are well known, and steps should have been taken to avoid them. Ensuring the data quality and data integrity relies on culture and ethics as well as a procedure and training for peak integration. Reviewers, QA, auditors, and inspectors are aware of these, and they will be checking them. Looking on the bright side, failure to learn or improve will be a continuing source of future "Questions of Quality" columns.

Acknowledgement

I would like to thank Paul Smith for providing the 13 483 citations that form the basis of this column and for his review comments. ■

This article has additional supplemental information only available online.
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Reimagined Lab Management for the Lab of the Future

Kate Wearden, Principal Product Marketing Manager, Waters Corporation

Discover an easy to implement alternative to LIMS that combines data management, workflow, and sample management.

FOR YEARS NOW Lab Information Management System (LIMS) software has been the tool of choice for digital lab management, unsurprising given the benefits they provide in effectively managing samples and associated data. Employing a LIMS can be very useful as a business system to extract discreet result values like retention time, weight, etc., however it often fails to support labs with their wider data management needs. Labs on their digital transformation journey are often looking for software that acts as a lab system and enables them to manage the complete workflow of data as it enters the lab through to when it exits. Historically, LIMS software has also been complex, costly, and time-consuming to deploy and integrate with the other business systems in the lab.

Often LIMS purchases are also made because of the large number of functions they offer such as invoicing, customer relationships management (CRM), key performance indicators (KPIs) and more depending on the LIMS software purchased. However, the reality is many customers who buy LIMS are only able to use a small set of these tools. The decision often taken to limit the scope of the deployment is typically made as a result of the lengthy time it takes to gather all of the master data needed to successfully implement these functions, which can take months and sometimes years. As a result, the fate of many LIMS implementations ends with shelf-ware, lost time, and money.

However, all is not lost for labs looking for an easy to implement lab and data management solution that provides usable functionality and flexibility. Software solutions such as NuGenesis Lab Management System (LMS) combine high impact functionality with a high degree of flexibility, readily adapting to labs' existing Informatics environments, enabling software integration and standardization without the complex, costly, and time consuming deployments often encountered with traditional LIMS solutions.

NuGenesis LMS provides five key functions of lab management:

- Sample Management (inclusive of Stability Study Management),
- Lab Execution,
- Inventory Management (Consumables and Instruments),
- Electronic Lab Notebook (ELN),
- The use of Empower Software as a chromatography data system,
- Scientific Data Management System (SDMS).

By combining all six of these key functions in one, easy to deploy solution, NuGenesis LMS provides a holistic approach to lab management, providing automation where labs need it most while also ensuring complete and compliant records. After two years of implementation of NuGenesis LMS, Shandong Loncom Pharmaceutical found that NuGenesis LMS:

'has greatly improved the accuracy of the original data and test results. The software automatically captures various data through the uploaded electronic map, and then automatically calculates and generates the results through the pre -set formula. The errors we used to see that were caused by incorrect data copying and formulating have reduced greatly and vastly improved the accuracy of experimental data.'

Shandong Loncom Pharmaceutical also benefited from how easy to implement the software is, commenting that they were able to get up and running with the software very quickly:

'I encountered no difficulties learning to use the software, it is very simple and convenient to operate. For our experiments, we can get started quickly, and the information is accurate, which greatly reduces errors.'

NuGenesis Lab Management System is a Lab System created to capture, not just discreet result values; it also captures the whole data set from the report to the RAW data files. Not only is it easier to capture the data from instruments and data sources, but it also scales easily, allowing labs to add new systems as data contributors as their laboratory grows.

Shandong Loncom Pharmaceutical also hopes to take advantage of this as they discuss how they anticipate their use of NuGenesis will evolve:

'We hope to further expand the coverage of electronic records, while continuing to optimize the use of features related to data reporting. Based on the two years of use, we plan to further realize the data mining and analysis of production quality in the future to increase product quality.'

NuGenesis LMS automatically catalogues data enabling easy searching and the re-use of all of the information captured, whether for Sample Management, Inventory Management, Lab Protocol execution, or use with other business system applications such as SAP. The advanced smart searching feature also allows users to apply parameters such as exclusion to streamline their data search and optimize the efficiency of their data management.

In summary, more and more labs like Shandong Loncom Pharmaceutical are opting for alternative solutions to LIMS software, such as NuGenesis LMS, that not only provide more comprehensive, automated lab and data management, but are also much easier to implement and integrate within their current lab informatics ecosystem. ■

Kate Wearden

Principal Product Marketing Manager

Waters Corporation

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Quantitative Metrics to Properly Describe Solute Elution in Size-Exclusion Chromatography

Szabolcs Fekete, Mateusz Imiolek, Mingcheng Xu, and Matthew Lauber

Many different terms and definitions are used to explain the elution and the rate of retention/exclusion/retardation of an analyte in a chromatographic phase system. Size-exclusion chromatography is probably the most challenging chromatographic mode in terms of nomenclature, terms and metrics, with different terms sometimes being used incorrectly. The purpose of this short tutorial article is to review the terms and official nomenclatures for size-exclusion separations and to provide some guidance and recommendations for practicing chromatographers. The interconversion between the different metrics is explained and some examples are presented.

SIZE-EXCLUSION chromatography (SEC) is unique among the separation techniques, being driven by non-retentive, differential particle pore penetration (solute exclusion). As no interactions with the packing material are required, this chromatographic mode is extremely mild and well-suited for analysis of biological molecules in their native state; as such, it is widely used for antibody-based therapeutic products (1). Size distribution and aggregation are key quality attributes for emerging new modality drugs that are based on increasingly complex polymeric biomolecules and their assemblies (lipid nanoparticles, nucleic acids, viral vectors etc.). Consequently, size-exclusion separations are predicted to be of growing importance and constitute the backbone of quality control testing (2,3). It is thus imperative to use proper quantitative metrics to describe and understand solute elution.

Unlike most liquid chromatography methods, SEC is an entropy-controlled process rather than enthalpy-controlled (4), which necessitates a different approach for an accurate description. In this spirit, one may wonder whether a commonly used term for peak characterization, "retention time", or its proxy, "retention volume", (5) are appropriate in a situation where there is not physicochemical retention (ideal SEC separation), and presumably because of this, sometimes, "elution time" can be found instead (6). Neither allows for straightforward communication between system/column comparative studies, which requires further terminology creation. Since efforts to extend the theoretical models in column chromatography to these separations are relatively recent (7), the applied nomenclature is often non-uniform. Hence, the aim for this article is to be a clarifying and unifying guide for SEC practitioners.

Unclear Definitions and Terms: Is There "Retention" in SEC?

It is important to understand the meaning of retention in chromatography in order to accurately describe analyte elution. Historically, volume-based units were used (i.e. retention volume, elution volume), but today, time-based units (i.e. retention time, elution time, hold-up time) are preferred, simply because time can be directly and intuitively read from a chromatogram. In the case of a non-compressible mobile phase system, time and volume measures are interchangeable and transferable.

Various definitions of retention time can be found in handbooks, guides and articles. Here, we list a few of them: a) "Retention time is the time that a solute spends in a column"; b) it can be defined as the time spent in the stationary and mobile phases"; c) "retention time is a measure of the time taken for a solute to pass through a chromatography column"; d) "retention time is the amount of time a compound spends on the column after it has been injected" (8). Considerations become a bit more complex upon reviewing the definition of retention volume (and/or time) provided by International Union of Pure and Applied Chemistry (IUPAC) (9): "The volume of mobile phase entering the column between sample

injection and the emergence of the peak maximum of the sample component of interest, or the corresponding time. It includes the hold-up volume (or time)." Thus, we need to know the meaning of the hold-up volume. The hold-up volume (sometimes referred to as "void-volume" or "dead-volume" and denoted as V_M) is defined as: "The volume of the mobile phase required to elute the unretained compound from the chromatographic column" (10,11). Please note that this volume is the sum of the interstitial (external, or interparticle) and pore (internal, or intraparticle) volumes of a column. In SEC, however, the compounds elute before the hold-up volume. Therefore, some chromatographers have delineated "exclusion chromatography" from any form of "adsorption chromatography". "Exclusion chromatography is based mainly on exclusion effects, such as differences in molecular size and/or shape or in charge. The term "size-exclusion chromatography" may be used when a separation is based only on molecular size" (9,10).

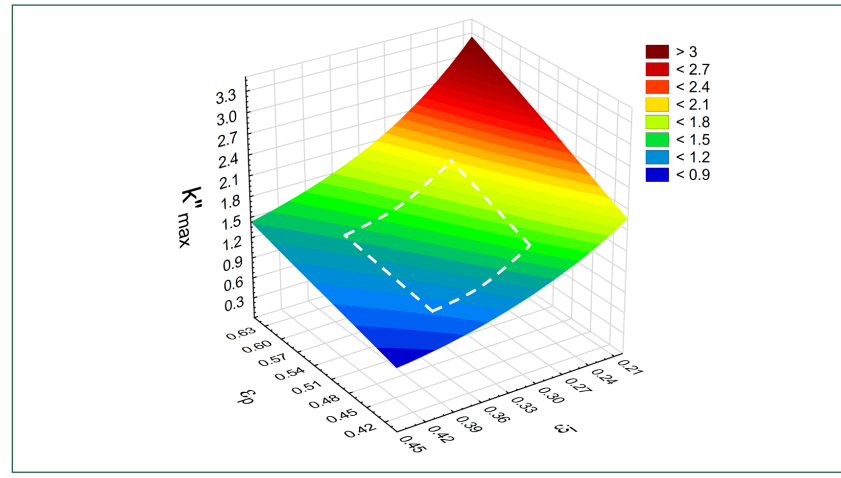

FIGURE 1: The change of the relative length of elution window (k''_{max}) as function of ε_p and ε_i. The area bracketed by the white dashed lines corresponds to the porosity characteristics of most commercial SEC columns.

Nevertheless, many chromatographers associate "retention" with physicochemical interactions occurring between a solute and a stationary phase. However, in an ideal SEC separation, molecules do not bind to any component within the column. Therefore, we can quickly run into another problem related to the definition of the "stationary phase". One of the most

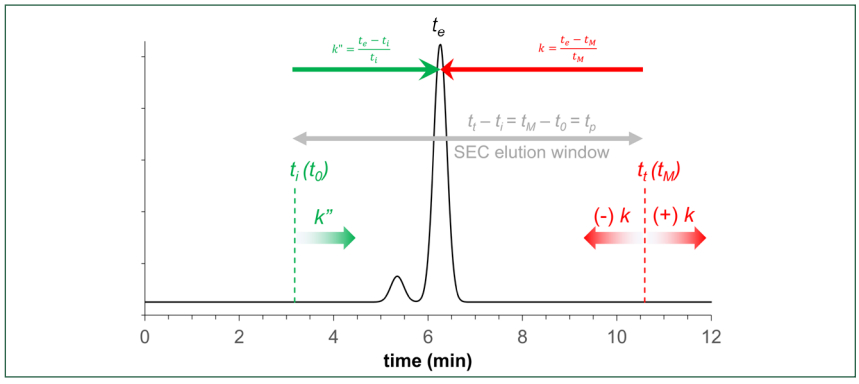

FIGURE 2: Illustration of the directly readable time-based units and measures of an SEC separation.

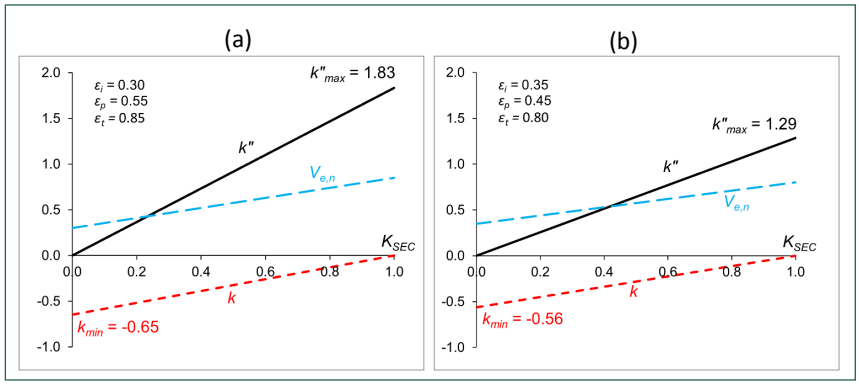

FIGURE 3: k, k'' and $V_{e,n}$ as functions of K_{SEC} for (a) a column with ε_i = 0.30, ε_p = 0.55 and ε_t = 0.85, and for (b) a column with ε_i = 0.35, ε_p = 0.45 and ε_t = 0.80.

common definitions states that "the stationary phase is the part of a column that interacts with the target compound". This definition suggests that the stationary phase is the "active" part of a column, which is responsible for the adsorptive interactions, and thus, the component that contributes to solute retention. In this manner, it is sometimes referred to as a "bonded phase" (9,10), while the whole solid matter inside a chromatography column is often termed as the "chromatographic bed" or "packing material".

Due to the ambiguous definitions of "retention" and "stationary phase", a fundamental question quickly arises: is it correct to say "retention" and "stationary phase" when describing an SEC separation? Many chromatographers agree that neither "retention" nor "stationary phase" are correct terms for SEC. Instead, it is better to simply refer to "elution" and to "column" or "packing material" or "chromatographic bed". While others (often found in fundamental SEC studies) accept the concepts of retention and stationary phase in SEC, they argue that there is a certain distribution of solutes that are partitioning between the "moving" and "stagnant" phases of the SEC phase system, regardless of the separation mechanism, which is technically accurate. (Please note that here the "moving" refers to the liquid phase–eluent–located in the interstitial column volume, while "stagnant" refers to liquid phase located in the internal pores.) To conclude, both concepts can be applied. The important thing is the selection of a reference point, like "total exclusion", "total penetration", or "hold-up" volume/time, so that there is relevance to defining solute elution and/or the migration rate of an analyte.

Measures to Describe Solute Elution in SEC

At any rate, we do not contradict any definition if we simply use "elution time" (t_e) as a directly readable measure. It refers to the time between the moment of sample injection and the appearance of the peak apex.

The elution of a compound can be expressed in volume units which has the advantage of being independent of flow rate (F). The elution volume (V_e) is the product of t_e and F:

$$V_e = t_e \cdot F \quad [1]$$

It is even more beneficial to use the ratio of V_e and the empty (superficial) column volume (V_c). Such a "normalized elution volume" ($V_{e,n}$) is independent of both flow rate and column dimension. Therefore, this dimensionless number ($0 < V_{e,n} < 1$) can be used to compare SEC measurements performed on different column sizes and at different flow rates. The normalized elution volume can be written as:

$$V_{e,n} = \frac{V_e}{V_c} \quad [2]$$

The equilibrium constant related to the partitioning of a solute between the solid and liquid phases is regularly called the "partitioning coefficient", "equilibrium constant", "distribution constant", or "equilibrium partition constant", and is often denoted as K or K_{SEC} (12,13).

$$K_{SEC} = \frac{V_e - V_i}{V_p} = \frac{V_e - V_i}{V_M - V_i} = \frac{t_e - t_i}{t_M - t_i} = \frac{t_e - t_i}{t_p} \quad [3]$$

where V_i is the interstitial volume of the column (external porous volume) and t_i is the corresponding time, V_p is the internal pore volume of the column (internal porous volume) and t_p is the corresponding time, and V_M, t_M are the column hold-up volume and time, respectively. The value of K_{SEC} ranges between 0 and 1; 0 corresponds to total exclusion, while 1 corresponds to total penetration through the internal pores.

The most used dimensionless unit of solute retention in liquid chromatography is the retention factor (*k*, *k'*). The retention factor is a measure of the time the sample component resides in the stationary phase relative to the time it resides in the mobile phase. In other words, it expresses how much longer a sample component is retarded by the stationary phase than it would take to travel through the column with the velocity of the mobile phase (9). Therefore, when analytes are excluded from the pores of a column, they elute with negative retention factors (that is, they only penetrate through a fraction of the column bed). The retention factor is written as:

$$k = \frac{V_r - V_M}{V_M} = \frac{t_r - t_M}{t_M} \quad [4]$$

Where V_r and t_r are the conventional retention volume and time, respectively. In SEC–formally–V_r and t_r can be substituted by V_e and t_e. The value of the retention factor is $-1 < k \leq 0$. Please note that the official symbols of a column's hold-up time and volume are t_M and V_M, but in many research papers and handbooks, the hold-up time and volume are denoted as t_0 and V_0.

According to the USP's definition, in SEC, "V_0 is the retention volume of a component whose molecules are larger than the largest gel pores (11). It may be calculated from the retention time of an unretained compound (t_0) and the flow rate:

$$V_0 = t_0 \cdot F \quad [5]$$

Therefore, V_0 is determined by the interstitial column volume (or in other words by the total exclusion volume) and thus $V_0 = V_i$.

Similarly to equation 4, a relative measure of solute elution can be introduced considering the interstitial column volume (or time) as the reference point. This measure is often called the "zone retention factor" (*k"*), which is an elution factor with respect to the elution time/volume of a non-permeating marker. It expresses the ratio of probabilities of the solute staying in the stagnant mobile phase inside the pores or in the moving mobile phase of the interstitial volume. The zone retention factor is often used when studying bulk, intraparticle, and effective diffusion of a solute. Here, we encourage the readers to formally use the zone retention factor in SEC and call the "elution factor". The *k"* is defined as:

$$k'' = \frac{V_e - V_i}{V_i} = \frac{t_e - t_i}{t_i} \quad [6]$$

It is worth noting that in SEC, *k"* is bound by the above, and it reaches its limit (k''_{max}) if a solute penetrates all pores (if $V_e = V_M$ then $V_e - V_i = V_p$):

$$k''_{max} = \frac{V_p}{V_i} = \frac{t_p}{t_i} = \frac{\varepsilon_p}{\varepsilon_i} \quad [7]$$

where ε_p is the pore (internal) porosity and ε_i is the interstitial (external) porosity of a column. The sum of the two gives the total porosity ($\varepsilon_t = \varepsilon_p + \varepsilon_i$). It is worth mentioning too, that consequently, analytes elute in a "limited elution window", as determined by the ratio of pore to interstitial porosity. The k''_{max} is a good measure of the elution window; the higher the k''_{max}, the broader the elution window. A wider elution window provides a higher probability of separating compounds. Figure 1 shows the change of k''_{max} as a function of ε_p and ε_i. As expected, the combination of low-interstitial and high-pore porosity results in a wide elution window. The k''_{max} value ranges between 1.1 and 2.1 for most commercial SEC columns.

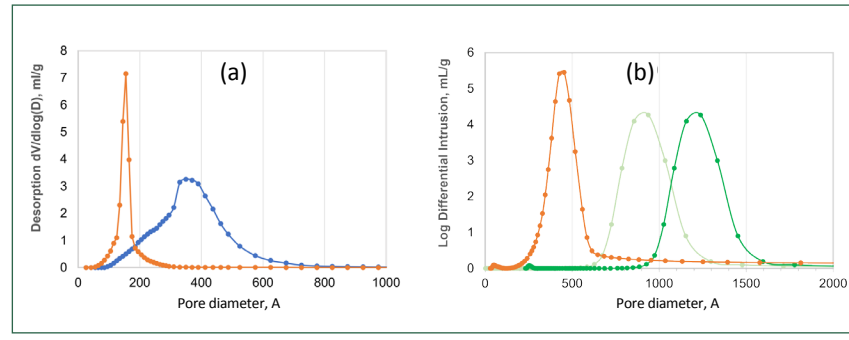

FIGURE 4: Typical results of pore size, PSD and pore volume measurement of SEC packing materials: (a) Pore volume (*V*) vs pore diameter (*D*) obtained from nitrogen BET porosimetry for a narrow (orange) and a wide pore (blue) material, and (b) mercury intrusion porosimetry for a wide (orange) and two ultra-wide pore (grey and green) materials.

To help practicing chromatographers, Figure 2 shows and explains the directly readable time-based measures on an SEC chromatogram.

Interconversion of the Different Measures

The various metrics discussed in the above section can be easily interconverted. Some of the important conversions are explained here. When rearranging and combining equation 1 and equation 3, one can express the elution time as the function of porosity, equilibrium constant and flow rate:

$$t_e = \frac{V_i + K_{SEC} V_p}{F} \quad [8]$$

Such a formula is very useful when studying the dependence of t_e on V_i, V_p or K_{SEC}.

When combining equation 3 and equation 6, a simple conversion between *k"* and K_{SEC} can be written:

$$K_{SEC} = \frac{V_i(1+k'') - V_i}{V_p} = \frac{t_i(1+k'') - t_i}{t_p} = \frac{\varepsilon_i(1+k'') - \varepsilon_i}{\varepsilon_p} \quad [9]$$

Time-based, volume-based, or porosity-based formulas can equivalently be used.

When combining equation 4 and equation 6, the conventional retention factor (*k*) can be expressed as a function of *k"*:

$$k = \frac{V_i(1+k'') - V_t}{V_t} = \frac{t_i(1+k'') - t_t}{t_t} = \frac{\varepsilon_i(1+k'') - \varepsilon_t}{\varepsilon_t} \quad [10]$$

K_{SEC} can be written as a function of k when combining equation 3 and equation 4:

$$K_{SEC} = \frac{V_t(1+k)-V_i}{V_p} = \frac{t_t(1+k)-t_i}{t_p} = \frac{\varepsilon_t(1+k)-\varepsilon_i}{\varepsilon_p} \quad [11]$$

And finally, K_{SEC} can also be expressed from normalized elution volume and column volume by combining equation 2 and equation 3:

$$K_{SEC} = \frac{V_{e,n}V_c-V_i}{V_p} = \frac{V_{e,n}-\varepsilon_i}{\varepsilon_p} \quad [12]$$

As an illustration, Figure 3 shows the conversion between the dimensionless measures (k, k'', $V_{e,n}$ and K_{SEC}).

Column Characteristics Affecting Solute Equilibrium Partition Constant (K_{SEC})

The pore size of the packing material and its distribution play critical roles in SEC separations, since they determine the analyte's accessibility to the internal pores, and thus the equilibrium constant and the solute's elution time/volume. Pore volume has a negligible effect on separation of large molecules that are fully excluded from the pores but affect the separation of smaller molecules that can diffuse (penetrate) into the pores.

Pore size, pore size distribution (PSD), and pore volume are often measured by porosimetry that is mostly performed by nitrogen BET and mercury intrusion methods. Based on the physical adsorption of nitrogen on the surface of the packing material at 77 K, nitrogen BET analysis is used to measure mesopores, which covers the pore size range from 2 nm to 50 nm. The Brunauer-Emmett-Teller (BET) equation is used to calculate the pore volume, pore size and its distribution (14). Mercury intrusion porosimetry is used to analyze macropores (>50 nm) and the wider part of mesopores (7.5 nm to 50 nm). The method is based on the penetration of mercury into the pores as a function of the applied pressure. The amount of pressure required for mercury to intrude into the pores is inversely proportional to the size of the pores. The pore size, PSD, and pore volume is calculated from the pressure versus intrusion data by using the Washburn equation, which describes capillary flow in a bundle of parallel cylindrical tubes (15). Please note that the Washburn method gives an estimation, since in reality, pores are not necessarily cylindrical and parallel, but instead possess a complex random and heterogeneous network.

Typical experimental results of pore size and PSD are illustrated in Figure 4.

Conclusion

Understandably, chromatographers have acquired a range of terms and definitions to explain their separations. A special set of descriptors has arisen for size exclusion chromatography. This form of chromatography solicits the most varied opinions. There are debates on the use of retention, exclusion, or elution, as well as packing material versus stationary phase. That this debate at all exists confirms that SEC is a prevalent approach and that chromatographers are applying their skills and intellectual power. SEC has been an impactful technique for many different fields, ranging from polymer chemistry to biological pharmaceuticals. The value of SEC is evident. Everyone need not agree to using the exact same vernacular; however, if we had our druthers, we might prefer to use the terms "elution time" and "packing material" when discussing SEC. Our preference for these terms over the use of "retention time" and "stationary phase"can be attributed to the years we have spent researching and developing SEC surfaces with weaker and weaker secondary interactions. Accordingly, we are sensitive to connotations of adsorption while discussing idealized SEC behavior. On the other hand, we also recommend using the limit of the zone retention factor (k''_{max}, the ratio of pore and interstitial porosity) as a measure of the width of the SEC elution window, which is an important characteristic of the SEC phase system.

Strong opinions or not, we look forward to more years of advancing the theory and implementation of SEC techniques.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. ■

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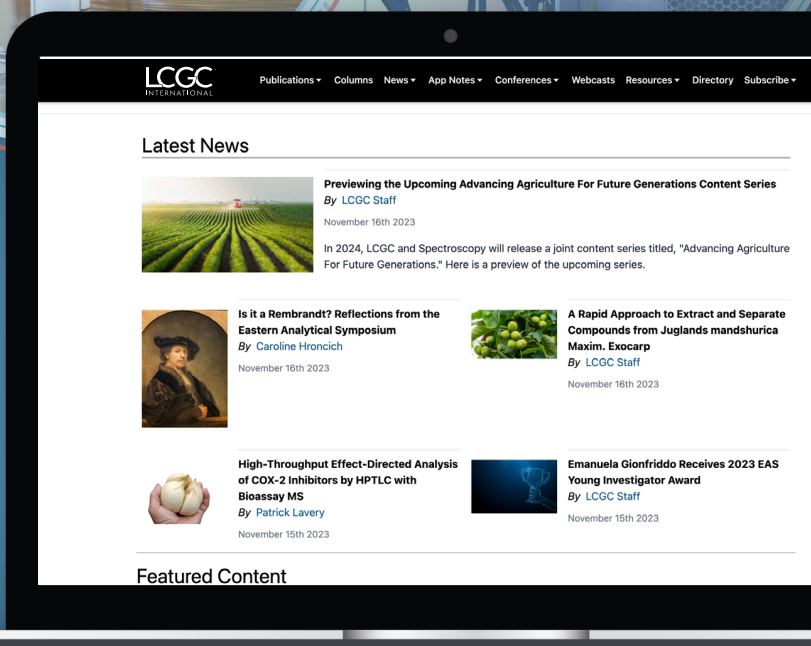

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Replacing 40+ Year-Old Analytical Techniques

Pharmaceutical science has used slow analytical quality testing techniques for several decades. Dr. Fred Regnier, CTO of Novilytic and one of the world's top analytical chemists, explains how a new method is being utilized to expedite the molecular analysis of antibodies and how to eliminate sample prep, protein A purification, and reduce reliance on mass spectrometry.

Dr. Fred Regnier, Ph.D.
CTO and Professor Emeritus at Purdue University
Novilytic

LCGC: What is your background?
DR. FRED REGNIER: I grew up in Nebraska on a farm, which means there is a lot to manage. You have to harvest grain and keep the farm running even if your equipment breaks down. During university, I worked in the library shelving books and found some on chromatography and electrophoresis. I was amazed at the similarities between harvesting grain and harvesting molecules. This interest carried me through graduate school and into my postdocs at Harvard and the University of Chicago, where my love for proteins and HPLC took off and eventually led me into the corporate world.

The six companies I started are still successful, which is a lot of fun. The other thing I had fun with is that all of them were based on advancing chromatography and finding solutions to problems that would impact society. Probably the most important one of these that I ever did was what we're up to now at Novilytic. It turns out that these therapeutic proteins that are now manufactured are massive in that they're literally curing cancer and impacting all kinds of diseases in society. We know from COVID, that society is very concerned about the safety and efficacy of these new protein miracles that are happening – that's what we're doing, providing assurance and confirmation, in minutes, that medicinal batches are safe and efficacious.

LCGC: What are some drawbacks of pharmaceutical science using the same analytical testing techniques for 40+ years?
REGNIER: What is interesting about all fields of science, particularly both drug discovery and large-scale manufacturing, is it takes a bunch of people to succeed, who are often in their own silos. One group is dedicated to cell growth, the other genetics, and so forth. As it turns out, pharmaceutical companies often don't have a chromatography silo, in particular analytical chromatography, that you would need for the analysis of proteins. They then turn to research-based academic literature. The problem there is the interest in how fast things can be done with serial assays. Research didn't think about the industry need for decision-dependent data and never provided it to pharmaceutical companies.

The industry wants an answer to whether their monoclonal antibody is therapeutic. They don't care about how many peaks or plates are in a chromatogram. Also, samples can be rather large with 2,000 to 10,000 proteins in them. The peak capacity in a column is in the hundreds, so there is a conflict between what they want and what we want, so we try to disguise the presence of proteins.

For something to be therapeutic, it must have all the right critical quality attributes and turns out that there can be 50 to 100 of those in a protein. What we do at Novilytic is turn that around, so we only have to look at two or three things to know if the molecule is efficacious and not toxic. This drastically cuts down on time and gets pharmaceutical companies the information that they actually need.

LCGC: Why is this new technology going to replace Protein A usage and save hours of time?
REGNIER: Protein A is an affinity chromatography method the world has used for over 20 years now. It is a small protein that you can immobilize on the surface of a chromatography particle with a high specificity for binding antibodies. When you take a culture medium from a fermentor and put it into a protein A column, it purifies the monoclonal antibodies.

Almost all proteins are like a human family. There are common features that everybody has and there are variable features. Like a family, it doesn't mean that everybody has the same skills. With a monoclonal antibody, there are some monoclonals that have more skills than others. You can't put 10,000 proteins in a mass spectrometer; you purify them down until they are relatively pure. It takes a lot of time. What people have been doing with protein A is purifying the family of proteins in one chromatography column, and then take another chromatography column to go off and do a separation of the proteoforms or the peptides. Finally, mass spectrometry is used.

Our approach is totally different in that it looks only at the intact molecule itself. What we do to have the same effect as mass spectrometry is take a particular feature in the molecule necessary for the protein to be efficacious and attach a molecule to it by molecular recognition. It recognizes that structural feature and attaches a fluorescent dye to that feature. Now, we can detect that molecule and analyze a couple thousand proteins within three or four minutes.

LCGC: How does the Proteometer-L Kit that you've created at Novilytic solve this problem and modernize analytical chemistry?
REGNIER: To look at a molecule, you need an instrument platform that executes certain features. Breaking down the name of this kit, a cell contains many proteins, which is referred to as the proteome. This instrument system is a meter for proteoforms. The reactor we use is the Proteometer-L Reactor, and its job is to analyze particular features of proteoforms that make monoclonal antibodies effective.

LCGC: Will this testing work for bi-specific and multi-specific antibodies?
REGNIER: Yes. As I mentioned, features exist within a molecule that must be there. Mass spectrometry allows us to take the molecule apart and look for those features. As an example, our body does not have a mass spectrometer, but it identifies hundreds of proteins through molecular recognition. It's similar to our sense of smell and taste. We can decide within seconds if we like something or not.

We built sensor molecules that go in, recognize, and bind to a particular feature in the monoclonal antibody. Secondly, we made the molecule fluorescent so that it has completely different spectral properties than anything else. When we add that molecular recognition phase to our mobile phase, it goes in and binds to the monoclonal antibodies. As the monoclonal antibodies go through the MASC reactor, they separate from each other, and as they come out, they run through a fluorescence detector. If that antibody fluoresces, it means that particular molecule is there. We don't need to tear the molecule apart to do it. Molecular recognition is simply copying what all biological systems have done forever by recognizing each other.

LCGC: Will this work on other types of samples besides antibodies?
REGNIER: Of course. Let's examine hemoglobin A1C. It has a critical structure attribute in a disease, a solution to a disease, or a molecular recognition element. It may not be a monoclonal antibody, but you simply make a molecular recognition agent that notices a feature in that molecule that allows it to do what it's supposed to do. When you run it through the separation system, there may be multiple forms of it. The separation system can separate those multiple forms and you will see them in the presence of thousands of other proteins.

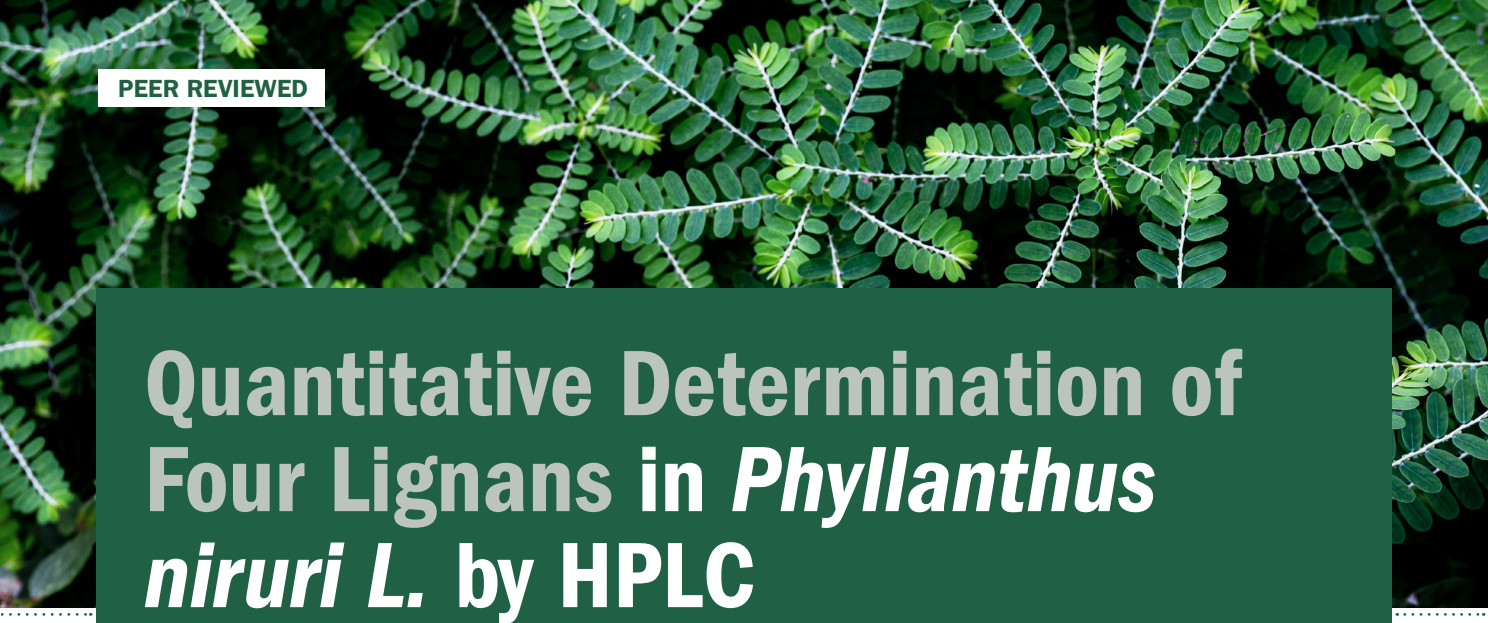
What you achieve with this molecular recognition is what people in large pharma and medicine want to see, as I discussed earlier. They want to be able to look at a chromatogram and know that what they want is there. Our chromatography is the same except that we use this third phase with the MASC reactor.

LCGC: What types of applications does your solution have and where does it fit within the process?
REGNIER: One worry at any time is regarding host cells being used to produce a therapeutic protein. They are supposed to make things in a certain ratio and for some reason or other, they may get sick. The temperature may not be right, or the nutrient input, and so forth. This leads to molecules that are not biologically active monoclonal antibodies or ones that are toxic or immunogenic. Knowing that right away in a manufacturing, or a drug discovery, process means a remediation of the problem without throwing away money and antibodies that take two weeks to make.

Another reason for rapid analysis is clone selection. When looking at cell lines, you have all kinds of cells with different amounts of genetic manipulation. The interest lies in the cell lines that will deliver the product. This technique then is valuable in clone selection because you can tell right away which clone delivers the highest quality product—all within 10 minutes.

Molecule purification is another place for this technology. Sometimes during purification, molecules aggregate together, and those aggregates are toxic. As you're doing large-scale property chromatography, you can tell whether you're getting aggregation or if you're losing particular types of molecules. After this purification, you want to know immediately if the molecule is still therapeutic. A new version of the Proteometer will launch this year to do just that!

LCGC: What are the components of this kit and who is it designed for?
REGNIER: It comes with a reactor, buffers, and a fluorescent-labeled molecular coding reagent and is designed to run on any LC (Waters, Agilent, Shimadzu, etc.). It can be utilized in drug discovery, clone selection, high throughput screening, as well as process R&D and manufacturing.



PEER REVIEWED

Quantitative Determination of Four Lignans in *Phyllanthus niruri L.* by HPLC

Jianzeng Xin and Sheng Liu

A simple analytical method using HPLC with ultraviolet (UV) detection was developed for the simultaneous determination of four main lignans, Niranthin, Nirtetralin B, Hypophyllanthin, and Phyltetralin, in *Phyllanthus niruri L.* plant samples from Guangxi province, China. The aim was to validate the accumulation rule in the growth periodicity of the active lignans for anti-HBV. The results indicated that the active lignans Niranthin and Nirtetralin B were mainly distributed in the leaves of *Phyllanthus niruri L.*, and the highest total content was found in the end of July to August (full fruiting period). Therefore, the *Phyllanthus niruri L.* of Guangxi province (China) should be collected for antihepatitis B treatment on the full fruiting period (the end of July to August), and its leaves might be the main part for collection. The results of the study provided a strong support for the use of the traditional Chinese herb *Phyllanthus niruri L.* from Guangxi province, China.

MOST TRADITIONAL Chinese medicines come from plants, and their chemical components are actually the secondary metabolites of these medicinal plants, such as artemisinin, a medium anti-malaria drug, and paclitaxel, an anti-cancer drug (1–3). The study of the change process of the secondary metabolites in plant growth periodicity could help to know the accumulation rules of medicinal components. Furthermore, it also benefits the full utilization of the medicinal plant resources (4,5).

Phyllanthus niruri L. (*Euphorbiaceae*), widely distributed in the south of China, is known as a traditional Chinese herb used in treating fever, colic, and diarrhoea, liver protection, and antihepatitis B (6,7). Previously, on the basis of its reputation in liver protection and antihepatitis B, we initiated a series of studies to investigate the bioactive constituents of the genus *Phyllanthus niruri L.* from Guangxi province, China. A series of lignans were discovered, in which some were shown to possess significant anti-HBV activity (8–10). Lignans niranthin, nirtetralin, nirtetralin A and nirtetralin B showed significant anti-HBV activity, while lignans hypophyllanthin and phyllanthin, despite similar structures, showed little activity. These lignans were secondary metabolites of *Phyllanthus niruri L.*, and the similar skeleton structure suggested a similar biosynthesis pathway in the plant. It was hypothesized that, in some period of the *Phyllanthus niruri L* growth cycle, the active lignans are produced in greater quantity than the inactive lignans. So the research on the bioavailability of the active lignans in the growth cycle of the plant is of great significance.

Similar studies were reported (11,12) which focus on developing and improving the analytical method of the lignans in *Phyllanthus niruri L.* from Malaysia. There were no related reports about the new lignans we isolated from the herb from Guangxi province, China. The regional disparity of the herb may result in different structures within active constituents, as well as the accumulation rule of the active lignans in the growth cycle of the plant. Thus, a study on accumulation rule of the active lignans of this herb from Guangxi province was also necessary for providing a support for making full use of the herb from this region.

Hence, the focus of this study was to validate the accumulation rule in the growth periodicity of the main lignans of *Phyllanthus niruri L.*. The lignans have ultraviolet (UV) absorption in the UV terminal, so in this study, the HPLC-UV method was developed for the analysis of the lignans, which were representatives of root, stem and leaves.

Materials and Methods

Standards, Samples and Chemicals

Four lignans, as shown in Figure 1a, Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyltetralin (4), isolated from *Phyllanthus niruri L.* according to the protocol established described previously (8–10), were used as standards for quantifying

the plant samples. Briefly, the powder of air-dried *Phyllantthus niruri L.* (100 kg) was extracted with 80% ethanol (3×300 L) for 2 days. The pooled extractions yielded 10.2 kg of crude residue after being evaporated under a vacuum. This residue was dispersed in water and extracted with petroleum ether to yield 3.1 kg petroleum ether extract residue. This residue, dissolved in ethanol and water, was introduced to the ethanol solution in a ratio of 3:2 (ethanol:water, v:v). After precipitant was removed, the aqueous ethanol solution was evaporated to provide a residue (450 g). This residue (450 g) was subjected to chromatography on a silica gel column (200–300 mesh, 10×100 cm, 10000 g) with a gradient eluting increase of ethyl acetate in petroleum ether (0, 4, 10, 20, 30, 40 and 50 %, each 50–100 L and offered fraction 1 (105 g), fraction 2 (95 g), fraction 3 (85 g), fraction 4 (50 g), fraction 5 (45 g), fraction 6 (30 g). The 6 fractions were merged according to thin layer chroma-

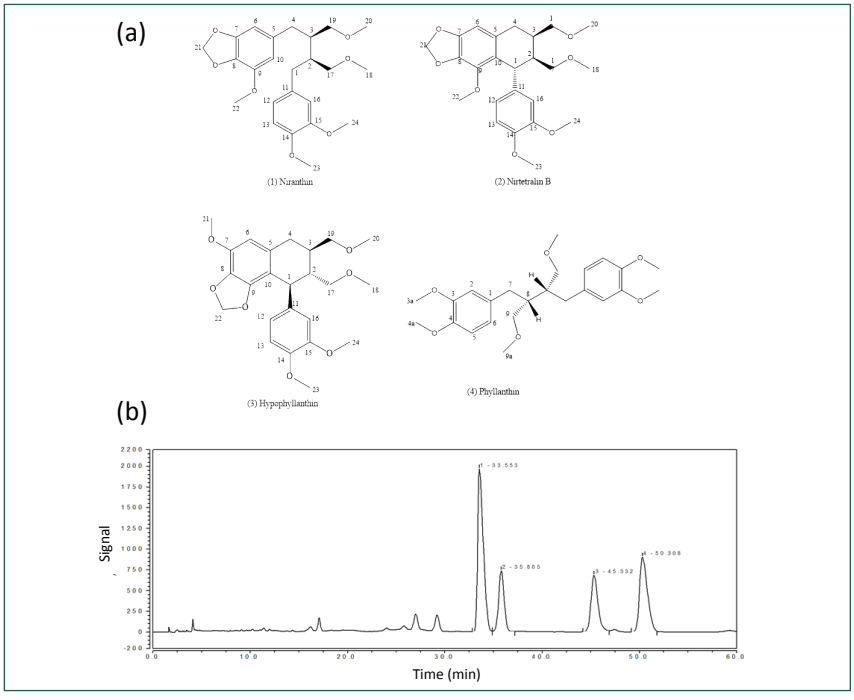


FIGURE 1: Chemical structures of lignans isolated from (a) *Phyllanthusniruri L.* and (b) their HPLC chromatogram.

TABLE I: Calibration results, LOD and LOQ values of the lignans by HPLC-UV detection.

Reference Standards	Calibration Curve	R^2	Linear Range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Niranthin (1)	y = 287.08*x*-14.831	0.999	16.2-11810	0.52	16.2
Nirtetralin B (2)	*y* = 291.78*x*+1.9174	1.000	10.768-7850	0.51	10.768
Hypophyllanthin (3)	*y* = 362.1*x*+6.0711	0.9998	7.984-5820	0.41	7.984
Phyllanthin (4)	*y* = 187.31*x*-8.413	0.9998	1.367-21360	0.80	1.367

TABLE II: Accuracy values for niranthin (1), nirtetralin B (2), hypophyllanthin (3) and phyllanthin (4).

Analytes	Original (µg)	Spiked (µg)	Found (µg)	Recovery[a] (%)	RSD (%)
	13.060	0.486	13.540	98.7	2.1
Niranthin (1)	13.060	4.374	17.393	99.1	1.8
	13.060	39.367	52.432	100.0	2.3
	5.532	0.323	5.850	98.5	1.5
Nirtetralin B (2)	5.532	2.907	8.412	99.1	0.9
	5.532	26.167	31.62	99.7	1.6
	6.236	0.239	6.479	101.7	1.1
Hypophyllanthin (3)	6.236	2.156	8.371	99.0	0.8
	6.236	19.400	25.610	99.8	2.6
	35.212	0.068	35.279	98.5	2.0
Phyllanthin (4)	35.212	1.709	36.902	98.9	1.9
	35.212	42.720	77.999	100.2	1.3

[a]Recovery (%) = [(found-original)/spiked] × 100.

tography (TLC) and yielded fraction A1, A2 and A3. Fraction A1 was subjected chromatography on a silica gel column with ethyl acetate-petroleum ether 2:1 (v:v) to afford 1 (13.6 g). Fraction A2 was subjected chromatography on a silica gel column with ethyl acetate-petroleum ether 2:1 (v:v) to afford 2 (6.8 g). Compound 3 (25.6 g) and 4 (25.6 g) were yielded from fraction A3. The purity of the compounds was confirmed to be more than 95% by a Thermo Fisher UltiMate 3000 HPLC system. Their structures were confirmed by comparison of the NMR and MS spectra reported previously (8–10).

Samples for analysis comprising the root, stem and leaves of *Phyllanthus niruri L.* were collected from Guangxi province, China, and identified by Dr Shifeng Ni, Northwest University, Xi'an, China. A voucher specimen was deposited in the Department of Chemistry, Guangxi University. The HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Deionized water for HPLC was redistilled water.

Instrumentation and Optimized Analytic Procedure

The HPLC-UV system was Thermo Fisher UltiMate 3000 equipped with a UV detector. A Thermo Hypersil Gold C18 column (2.1×250 mm, 5 µm) was used with the following analytical conditions: a mobile phase of acetonitrile-water (55:45, v:v) with a flow-rate of 1 mL/min at a column temperature of 30 °C. UV detection was at 230 nm. The sample injection volume was 10 µL.

Preparation of Standard Solutions

The four reference standards were weighed accurately and dissolved in methanol in a volumetric flask to form a stock solution of standards, the concentration of each compound was: (1) Niranthin, 11.810 mg/mL; (2) Nirtetralin B, 7.850 mg/mL; (3) Hypophyllanthin, 5.820 mg/mL; (4) Phyllanthin, 21.36 mg/mL. Working standard solutions were prepared from the stock solution by further dilution with the appropriate volume of methanol. Working solutions of 1 were prepared at the following concentrations: 16.2, 48.6, 145.8, 437.4, 1312.2, 3936.7, and 11810.0 µg/mL, of 2 at 10.8, 32.3, 96.9, 290.7, 872.2, 2616.7and 7850.0 µg/mL, of 3 at 8.0, 23.9, 71.9, 215.6, 646.7, 1940.0 and 5820.0 µg/mL, and of 4 at 1.4, 6.8, 34.2, 170.9, 854.4, 4272.0 and 21360.0 µg/mL to cover the range of sample concentrations. All the solutions were stored in a refrigerator at 4 °C prior to analysis.

Preparation of *Phyllanthus niruri L.* Samples for Analysis

The quantification of lignans in *Phyllanthus niruri L.* plant was carried out in two parts. The first part involved the quantification of the lignans in the petroleum ether extracts from the whole plant, whereas the second part involved the

quantification of the lignans in the petroleum ether extracts from the different parts (root, stem and leaves) of the plant.

After collection, the samples were dried at room temperature and then pulverized by mechanical comminution. Samples of 2.0 g each were accurately weighed and put into a 50-mL conical flask, and then extracted with 3×20 mL petroleum ether in an ultrasonic bath (ultrasonication frequency 40 kHz) for 30 min. The combined extracts were evaporated to dryness under a nitrogen flow. The residue was reconstituted in 5 mL of methanol. The sample solution was filtered through a 0.45 µm filter prior to analysis.

Method Validation

The analytical parameters of selectivity, linearity, limits of detection (LODs) and quantification (LOQs), accuracy, precision, expressed as repeatability, and stability were evaluated. The established procedures were validated following SANTE/11813/2017 validation criteria for quantitative analytical methods.

Results and Discussion

Development of the Chromatographic Method

The HPLC-UV conditions were investigated in this study, with different mobile phase compositions (water-methanol and water-acetonitrile), column temperatures (25, 30, 35 °C), injection volume (5, 10, 20 µL), and water-acetonitrile rates (50:50, 55:45, 45:55, 40:60, v:v) being tested and compared. Compared to water-methanol as a mobile phase, water-acetonitrile achieved a better shape of the peaks and separation of the four lignans. Retention time was too large in the condition of water-acetonitrile (50:50, v:v) whereas the four lignans could not be separate completely when the water-acetonitrile ratio was 45:55 or 40:60 (v:v). So, water-acetonitrile (55: 45, v:v) was finally used as the mobile phase, because it effectively improved the shape of the peaks and a better separation of the four lignans was achieved. Thus, the optimal chromatographic conditions were as follows: column temperature, 30 °C; flow rate, 1 mL/min; mobile phase, acetonitrile-water (55:45, v:v); injection volume, 10 µL; UV detection wavelength, 230 nm. Thus, well-resolved peaks of the four major lignans, 1, 2, 3 and 4 from *Phyllantthus niruri L.* were achieved with a total run time of 60 min (Figure 1b). The corresponding retention time of standard Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyllanthin (4) were 50.308 min, 45.332 min, 35.805 min and 33.553 min, respectively.

Validation of the HPLC Method

Calibration curves, limits of detection and quantification: The reference compounds

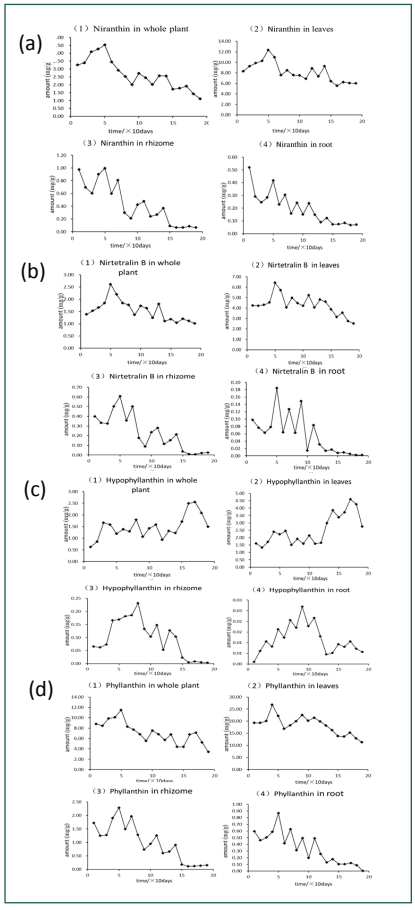


FIGURE 2: (a–d) Concentrations of Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyllanthin (4) in *P. niruri L.* with samples collected from different times.

were dissolved in methanol and stored in a refrigerator at 4 °C. Seven levels of standard solutions of the four lignans were used, in the high and low working ranges. For the HPLC-UV detection method, the data provided a linear function for all reference lignans following the equation: $Y = aX + b$ with Y being the value of the peak area, and X being the value of the amount of the reference lignans injected into HPLC. The signal/noise ratios of 3:1 and 10:1 were used to establish limits of detection (LOD) and limits of quantification (LOQ) of the four lignans under the present chromatographic conditions, respectively. The calibration results, LOD, and LOQ values of the lignans were shown in Table I (all tables are accessible through the QR code at the end of the article). The equations were obtained using the least squares method and showed excellent coefficients of determination ($R^2 \geq 0.99$), indicating good adherence to the linear model for all the lignans studied.

Accuracy: Accuracy was assessed through recovery experiments. An appropriate amount of reference standards was weighed and spiked with a known amount of each reference compound, then prepared and analysed in accordance with the methods mentioned earlier. The experiments were performed at three concentrations to cover two values belonging to each of the linear ranges applied in the determination of the lignans. The mean recovery values were calculated for five replicates and are listed in Table II.

Precision: The precision of the method was evaluated with standard solution by within-laboratory repeatability under the selected optimal conditions five times. The repeatability experiments were executed on the same day by the same analyst. The relative standard deviation (RSD(%)) was taken as a measure of precision. As shown in Table III, the RSD values were less than 4.5% in all cases, which comply with the policies established by the European Commission (EC, 2002) and the criteria of the AOAC International (2016).

Stability: The stability of the method was evaluated with a sample solution of *Phyllantthus niruri L.* on a specified period (No.1) under the selected optimal conditions. 10 µL of the sample is injected and analysed at 0, 2, 4, 6, 8 and 10 h. The value of the peak area of Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyllanthin (4) were recorded with the RSDs were all less than 4%, indicating that the sample solution was stable within 10 h. The results were shown in Table IV.

Repeatability: To further evaluate the repeatability of the assay, the leaves of *Phyllantthus niruri L.* were analysed in five replicates, as described earlier. The RSDs were all less than 2%, indicating that the method has a good repeatability. The results were shown in Table V.

Sample Analysis

The established HPLC-UV method was applied to the simultaneous determination of the four main lignans Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyllanthin (4) in the whole plant and the different parts (root, stem and leaves) of *Phyllantthus niruri L.* of Guangxi province, China. The sample solution of different growth period of *Phyllanthus niruri L.* and its root, stem and leaves were analysed in three replicates under the selected optimal conditions described earlier.

Samples for analysis were collected for the first time on June 20, with an interval of 10 days for further collection. The concentration changes of lignans Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyllanthin (4) of *Phyllantthus niruri L.* during its growth period were shown in Figure 2. The lignan profiles from the different parts of the plant can also be differentiated. The major lignans were found in all parts of the plant, while the highest total content for lignans 1–4 was found in the leaves throughout the whole growth cycle of the plant. The lowest total content for lignans 1–4 was found in the root. For the concentration changes of Niranthin (1) in *Phyllantthus niruri L.* during the whole growth period, the concentration of Niranthin (1) increased from June to the end of July, namely, the seedling stage, flowering stage and early fruiting period. The highest total content for Niranthin (1) was found in the full fruiting period, followed with the decreasing trend in August till withered. The variation trend of Niranthin (1) in the leaves was in keeping with that in the whole plant, whereas the variation trends of Niranthin (1) in the root and stem were observed to show some different. In the root and stem, the concentration of Niranthin (1) decreased from seedling period to flowering period, then increased from early fruiting period to full fruiting period. The highest total content was also found in the end of July, then decreased after August. The variation trends of Nirtetralin B (2) and Phyllanthin (4) were similarly with Niranthin (1). However, the concentration of Hypophyllanthin (3) was increased from seedling period to the end of November (maturation period), and the

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TABLE III: The results of the precision experiment.

Reference Standards	Peak Area					RSD (%)
	1	2	3	4	5	
Niranthin (1)	3403.597	3415.001	3405.426	3410.131	3400.085	0.17
Nirtetralin B (2)	2293.485	2295.565	2285.169	2303.213	2288.805	0.30
Hypophyllanthin (3)	2105.585	2113.385	2120.625	2096.985	2108.505	0.42
Phyllanthin (4)	4001.928	4021.115	4006.521	4009.715	4015.028	0.19

TABLE IV: The results of the stability experiment.

Peak Area of Samples	Time (h)						RSD (%)
	0	2	4	6	8	10	
Niranthin	473.87	473.993	470.553	461.557	476.157	473.870	1.22
Nirtetralin B	288.107	291.844	290.216	310.457	305.407	288.107	3.38
Hypophyllanthin	230.931	232.436	243.954	241.982	235.682	230.931	2.43
Phyllanthin	652.991	652.558	647.829	652.616	662.616	652.991	0.83

TABLE V: The results of the repetition experiment.

Samples	Time (h)					RSD (%)
	1	2	3	4	5	
Niranthin	844.617	861.960	866.085	854.587	864.210	1.03
Nirtetralin B	557.695	560.714	564.110	566.715	569.011	0.81
Hypophyllanthin	486.706	484.005	487.086	489.016	485.726	0.39
Phyllanthin	1319.491	1311.257	1321.822	1317.623	1315.982	0.30

highest total content was also found in the end of November. The variation trend of Hypophyllanthin (3) in the leaves was in keeping with that in the whole plant, whereas the highest total content in the root and stem were found in the end of August (full fruiting period).

Conclusion

The lignans from *Phyllantthus niruri L.* were proved to be the active constituent for its anti-HBV activity. Among the four main lignans Niranthin (1), Nirtetralin B (2), Hypophyllanthin (3) and Phyllanthin (4) of *Phyllanthus niruri L.* from Guangxi province, China. Lignans Niranthin (1) and Nirtetralin B (2) were proved to show significant anti-HBV activity, while lignans Hypophyllanthin (3) and Phyllanthin (4) showed inactivity for HBV. Our study was focused on the accumulation rule of the four main lignans in the whole growth cycle of the *Phyllantthus niruri L.* The results indicated that the active lignans Niranthin (1) and Nirtetralin B (2) were mainly distributed in the leaves of *Phyllantthus niruri L.*, and the highest total content was found in the end of July to August (full fruiting period). Therefore, the *Phyllanthus niruri L.* of Guangxi province (China) should be collected for antihepatitis B treatment on the full fruiting period (the end of July to August), and its leaves might be the main part for collection. The results of the study provided a strong support for the use of the traditional Chinese herb *Phyllanthus niruri L.* from Guangxi province, China. ■

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Declaration of Competing Interest

The authors declare no competing financial interests.

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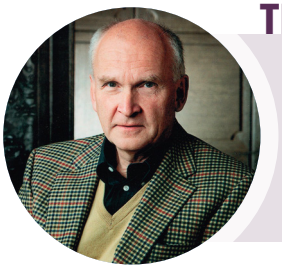
The 2024 Lifetime Achievement and Emerging Leader in Chromatography Awards

Jerome Workman, Jr.

Wolfgang F. Lindner and Martina Catani are the winners of the 17th Annual *LCGC* Lifetime Achievement and Emerging Leader in Chromatography Awards, respectively. These awards honor the work of talented separation scientists at different stages in their career. The award winners will be honored in March in an oral symposium at the Pittcon 2024 conference in San Diego, California.



The Lifetime Achievement Award

Wolfgang Lindner

is the 2024 Lifetime Achievement in Chromatography Award recipient. The award honors an outstanding and seasoned professional for a lifetime of contributions to the advancement of chromatographic techniques and applications (Table I).

WOLFGANG F. LINDNER, the 2024 winner, is chair of Analytical Chemistry at the University of Vienna. Lindner is a prominent figure in the realm of chiral separations, particularly in analytical and preparative liquid chromatography (LC), as well as in contemporary separation methods such as capillary electrophoresis (CE), capillary electrochromatography (CEC), and supercritical fluid chromatography (SFC). Rather than solely focusing on plate counts, Lindner's expertise lies in enantioselective molecular recognition, especially concerning polar and ionic species. His notable contributions encompass the development and licensing of various chiral stationary phases, which have found widespread use among researchers and within the pharmaceutical industry. His work extends to the application of enantiomer separations in the fields of chiral drug pharmacokinetics and the metabolomics of endogenous compounds, including amino and hydroxyl acids. "I am known best for the field of enantioselective chromatography," Lindner said.

At the core of Lindner's research is a deep understanding of non-covalent binding interactions between analytes and chiral stationary phases, as well as the roles of accompanying mobile phase components. His innovations expand into the realm of highly selective mixed-mode and hydrophilic interaction chromatography (HILIC) phases, along with arginine modifications tailored for liquid chromatography–mass spectrometry (LC–MS) analysis of highly basic peptides. Throughout his work, Lindner remains committed to fundamental physico-chemical principles, striving to create exceptionally selective separations.

As a teenager, Lindner became fascinated by chemistry. "Curiosity drove me my whole life and still does it today," he said. Beginning his academic research journey in the late 1970s, Lindner entered a landscape where chiral separations were an art, but his efforts, combined with those of other leaders, have transformed it into a routine practice.

"I became specifically fascinated by chirality as a property and shape descriptor of molecules and thus by the concepts to enable resolution of chiral molecules with chromatographic, electrophoretic, and crystallization technologies," Lindner said.

Notably, he introduced O,O-diacyltartaric acid anhydrides as chiral derivatizing agents in the mid-1980s, a contribution that continues to impact the analysis and production of enantiomerically pure drugs. In the mid-1990s, he introduced cinchona-based chiral anion exchange columns, followed by a chiral cation exchange column. However, his most significant breakthrough involved the development of chiral zwitterionic stationary phases capable of separating positive, negative, and ampholytic compounds.

This ingenious approach combined a chiral amidosulfonic acid with quinine and quinidine scaffolds. Many of these columns have been licensed and commercialized, significantly influencing the pharmaceutical industry. Lindner's enduring legacy is evident in the transformation of chiral separations from an art to a routine science.

"The success of modern HPLC is driven by its power of selectivity (multidimensional), efficiency, speed, scalability (nano to process), productivity, hyphenation (with mass spectrometry), and applicability (even for complex samples)," he said.

Main Research Topics

Lindner's major research interests have focused on the power of selectivity in LC.

"My research interests were always driven by the elucidation of the fundamentals of the interactions of molecules to each other," he said. Lindner's passion has culminated in the development of diverse and useful methodological concepts for LC systems. The areas of particular interest to Lindner include:

- Reversed-phase separation of optical isomers using chiral metal chelate additives
- Strong detrimental effect of enantiomeric impurities on enantioselectivity
- Liquid chromatographic separation of enantiomeric alkanolamines
- Quinine and quinidine derivatives as chiral selectors
- Elucidation of chiral recognition mechanisms of cinchona alkaloid carbamate receptors
- Chiral recognition of peptide enantiomers by cinchona alkaloid derived chiral selectors
- Direct high-performance liquid chromatographic separation of peptide enantiomers
- Novel strong cation exchanger type chiral stationary phase
- Synergistic effects on enantioselectivity of novel zwitterionic chiral stationary phases
- Chiral monolithic columns for enantioselective capillary electrochromatography.

Major Research Contributions

Lindner's transformative journey in separation science has been characterized by groundbreaking contributions that have revolutionized the field. As a postdoctoral researcher in the late 1970s, at Barry Karger's lab in Boston, Lindner's work introduced a pioneering concept of chiral analyte separation using chiral selectors in the mobile phase via chiral ligand exchange chromatography (CLEC) (1). This experience set the stage for his remarkable career in chiral chromatography, where he skillfully harnessed his strong organic chemistry background to design novel chiral systems with exceptional selectivity, achieving impressive alpha values as high as 100 (2).

Together with luminaries such as Pirkle, Armstrong, and Okamoto, Lindner is revered as an expert in the liquid chromatographic separation of enantiomers. Their collective efforts catalyzed the widespread adoption

of analytical and preparative chiral columns, playing a pivotal role in drug synthesis and chemical research. The significance of this impact is exemplified by the 2021 Nobel Prize in Chemistry, acknowledging the role of chiral chromatography in enabling advancements like asymmetric catalysis. This prize was awarded to Benjamin List and David W. C. MacMillan "for the development of asymmetric organocatalysis." Major pharmaceutical companies have been empowered by efficient facilities for racemic drug separation on a preparative scale and the screening of individual enantiomers to decipher their pharmacological profiles. Continuing his trajectory of direct enantiomer separation, Lindner introduced diacyl tartaric acid anhydride as a chiral derivatizing agent in the mid-1980s, enabling excellent separation of diastereomeric species (3). This patented approach, even three decades later, continues to impact drug production, as seen with (S)-Timolol. HCl, a potent antiglaucoma medication.

But Lindner's most enduring legacy resides in his transformative contributions to chiral stationary phases, particularly ion exchangers. His pursuit of chargeable chiral selector species led him to the fundamental alkaloids, quinine and quinidine, in the 1990s. These pseudo-enantiomeric diastereoisomers, characterized by a chiral tertiary amino group within the quinuclidine moiety, possess remarkable stereochemical capabilities. Lindner's meticulous studies, both spectroscopic and chromatographic, culminated in the addition of a carbamate group near the chiral centers, facilitating highly specific hydrogen bonding that significantly enhanced chiral recognition (4–7).

The grafting of the chiral selector onto porous silica packing led to the pioneering enantioselective anion exchanger, which has now been commercially available for more than 20 years (4). His subsequent innovation in 2008, incorporating a negatively charged chiral sulfohexoylcarbamate scaffold into a positively charged quinine or quinidine selector motif, created the widely recognized zwitterionic chiral stationary phase (8), permitting comprehensive chiral separations of anions, cations, and ampholytes, with applications spanning amino acids and peptides (9).

Lindner's far-reaching contributions extend beyond liquid chromatography into capillary electrophoresis (10), capillary electrochromatography (11), supercritical fluid chromatography (12), and affinity chromatography (13,14). His focus on leveraging chemistry for selectivity has expanded stereoselectivity beyond enantioselectivity, with his ion-exchange columns serving as effective mixed-mode (15) and hydrophilic interaction chromatography (16) stationary phases.

Key Awards and Honors

Lindner believes that when working in research-focused environments, scientists must accept a collegial-like competition among colleagues on scientific achievements.

"It affords a constant engagement with the latest developments in the field," he said. Lindner himself has been selected by many colleagues for various scientific awards. Below, you can find a list of all the major awards he has won.

1991 Jubilee Award of the British Chromatography Society

1995 Tswett Memorial Medal (Russia)

1995 Belgium Pharmaceutical Society Award (Inaugural)

1996 Hoechst Prize in Medicinal Chemistry (Denmark)

2003 Honorary Membership, Hungarian Society for Separation Sciences

2007 Halasz Medal, Hungarian Society for Separation Sciences

2008 Chirality Medal (International Conference on Chirality)

2009 A.J.P. Martin Gold Medal, British Chromatographic Society

2012 ACS Award in Chromatography (USA)

2013 CASSS Award for Outstanding Separation Science (USA)

2013 No. 8 on List of Most Powerful Analytical Scientists

2014 Nernst-Tswett Award of EUSSS (Europe)

2015 Lochsmitt Medal of the Austrian Society of Chemistry

2016 Pregl Medal of the Austrian Society of Analytical Chemistry

Service to the Scientific Community

Lindner's contributions are substantial and multifaceted. His academic impact is evident through a prolific publication record, encompassing 590 peer-reviewed papers, with an h-index of 71 and over 22,100 citations. Notably, he holds 17 patents, several of which have been licensed for commercial products. His influence also extends to education, having mentored over 100 Master's and PhD students, as well as more than 15 extended-stay postdoctoral researchers, many of whom have achieved distinguished careers in separation science globally. Examples of his notable mentees include Michael Lammerhofer, Kevin Schug, Alexander Leitner, and others. Lindner has found that individual and personalized mentoring of coworkers and students is a particularly challenging and a rewarding task.

Lindner's prominence is demonstrated by his extensive involvement in scientific conferences. He has delivered over 100 plenary, keynote, and research lectures at major symposia, in addition to participating in more than 100 named lectureships and lectures at universities and industries. His support for young scientists is evident through his mentorship and more than 100 posters presented by students and collaborators at significant symposia. Furthermore, his role as chairman in various scientific meetings, such as the International Symposium on Liquid Chromatography and the International Symposium on Chromatography, showcases his dedication to advancing the field.

His contributions to scientific journals are substantial; he served as editor for the *Journal of Chromatography B* from July 1995 to June 2006, and he remains active on several Editorial Advisory Boards for esteemed journals including *LCGC*, *Chirality*, *Chromatographia*, *Journal of Chromatography B, Journal of Pharmaceutical and Biomedical Analysis, International Journal of Bio-Chromatography,* and *Journal of Analytical and Bioanalytical Chemistry.*

Lindner's commitment to nurturing the next generation of analytical scientists is palpable through his establishment of the Austrian Summer Training in LC–MS

and the Austrian Young Analysts Forum, providing platforms for skill development and networking. His leadership within science societies is also evident, having served as President of the Austrian Society of Analytical Chemistry and currently serving as its Honorary President.

In addition to his prolific publication record, Lindner's advances have been widely commercialized and adopted, significantly transforming chiral separations from an art to a routine procedure. His mentorship has paved the way for students who have embarked on major careers in separation science, while his leadership in international meetings and committee service underscores his commitment to advancing the separation field (17). As an indisputable candidate for the *LCGC* Lifetime Achievement Award, Wolfgang Lindner's exceptional impact resonates throughout the chromatographic separation science arena, shaping its trajectory with innovation and excellence. ■

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The Emerging Leader Award

Martina Catani

is the 2024 recipient of the Emerging Leader in Chromatography Award, which recognizes the achievements and aspirations of a talented young separation scientist who has made strides early in their career toward the advancement of chromatographic techniques and applications.

MARTINA CATANI is an assistant professor in Analytical Chemistry at the University of Ferrara in Italy. She received her PhD in chemistry in 2018 in Chemical Sciences from the University of Ferrara. Previously, she was a research associate in the Department of Chemistry and Pharmaceutical Sciences at the University of Ferrara and a postdoctoral research fellow at the Department of Chemistry and Applied Biosciences at the Institute for Chemical and Bioengineering at ETH Zurich in Switzerland. She was also a postdoctoral research fellow at the Department of Chemistry and Pharmaceutical Sciences at the University of Ferrara in Italy.

Catani received her bachelor's degree in chemistry in 2011, magna cum laude, and master's degree in chemical sciences in 2013, magna cum laude, from the University of Ferrara. Her PhD was completed in 2018 under the supervision of Alberto Cavazzini.

The University of Ferrara is one of the oldest universities in the world, founded in 1391. "Among the famous alumni who studied in Ferrara, it is worth mentioning Nicolaus Copernicus and Paracelsus," she said.

Catani's work primarily focuses on the fundamental aspects of kinetic and thermodynamic phenomena in both chiral and achiral high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC). Notably, she has made significant contributions to the field of separation science, earning the Csaba Horvath Award in 2018 at the HPLC meeting for the best research presented by a young scientist. Her research delves into the impact of kinetics, thermodynamics, and particle geometry on the efficacy of chiral and achiral columns in HPLC and SFC. Her studies have reinvigorated discussions on optimal particle characteristics for high-efficiency separations. Furthermore, she has actively developed advanced purification techniques for therapeutic bio-macromolecules using both single-column and continuous multicolumn preparative liquid chromatography.

Catani's research group has a long tradition in the study of theoretical aspects of chromatographic techniques from many perspectives. "These perspectives range from the stochastic description of the chromatographic process to the study of competitive nonlinear systems by means of deterministic models," she said.

In the past year, Catani's group has started working on challenging research projects to reduce the environmental impact of chromatographic technologies. "We are working to replace the toxic solvents commonly used in LC (firstly, acetonitrile) with others that are more eco-friendly," she said.

Career Highlights

Catani's career in chromatography is marked by her significant contributions and practical applications in the field. She stands out for her collaborative research approach, working closely with industrial and academic partners to address real-world analytical challenges. Scientific discussions and idea-sharing are paramount. "The possibility to meet in person other colleagues and exhibitors from industries is, I believe, a fundamental and irreplaceable part of our work," she said.

Catani has collaborated with major pharmaceutical companies like Fresenius Kabi iPSUM and Merck. Her work has involved the development of continuous purification methods for therapeutic peptides and the purification of carrier proteins for vaccines. These collaborations have had a meaningful impact on industry practices. In addition to her pharmaceutical collaborations, Catani has ventured into cannabinoid research in partnership with HPLC technology companies, such as ChromSword, Restek s.r.l., and Chiral Technologies. This expansion into new exploratory fields demonstrates her adaptability and willingness to explore emerging topics.

Catani has also filed a European patent application in collaboration with Fresenius Kabi iPSUM, demonstrating her innovation for advancing chromatography technology. Catani has also played a role in organizing scientific conferences, such as her participation in the organizing committee for HPLC 2019 in Milan, contributing to the success of the event.

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With an impressive publication record of 53 articles and 3 book chapters, according to Scopus, Catani has consistently shared her knowledge and research findings with the chromatography community. As an active presenter at scientific conferences, she has delivered 35 presentations, including both invited and contributed talks, and has been a member of the Early-Career Board of Analytical Chemistry since 2022, reflecting her standing in the field. Her dedication to excellence was acknowledged with the Best PhD Thesis Award in Chemical Sciences at the University of Ferrara in 2018.

Catani is teaching, or has taught courses, named Instrumental Analytical Chemistry, Separation Methods, Analytical Chemistry Laboratory I and II, and Analytical Chemistry.

Most Influential and Cited Research Work

Catani's significant contributions in separation science are exemplified through five notable research works. In reference (18), Catani and associates examine the kinetic performance of columns packed with sub-2 µm C18 fully porous particles of narrow particle size distribution (nPSD). Their findings revealed that columns containing these particles exhibit low eddy dispersion and comparable longitudinal diffusion and mass transfer kinetics to other fully porous particles. Reference (19) showcases a study on chiral columns with Pirkle-type chiral selectors packed with either fully porous or core-shell particles. Notably, the authors achieved ultrafast separation of enantiomers within a second on a short column, emphasizing the correlation between kinetic performance and chiral selector loading.

Reference (20) reports a breakthrough in chiral separation efficiency using enantioselective supercritical fluid chromatography (SFC). By modifying a commercial SFC instrument, the authors harnessed the turbulent regime for unmatched kinetic performance, building on fundamental studies by chromatography pioneers. In reference (21), Felletti and associates explain the phenomenon of convex-upward van Deemter curves in chiral chromatography. The study elucidates this behavior's origins in cases of strong analyte adsorption and negligible solid-phase diffusion. Reference (22) highlights a groundbreaking purification method for the peptidomimetic Icatibant using multicolumn countercurrent solvent gradient purification (MCSGP). This approach yielded remarkable gains in yield and productivity while reducing solvent consumption. These works collectively underscore Catani's pivotal role in advancing separation science through innovative studies on kinetics, thermodynamics, and purification techniques, enhancing both understanding and practical applications in the field.

Catani's studies include both chiral and achiral high performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC), which began in her PhD research studies. Her research aim was to unravel mass transfer phenomena through packed beds made of particles with different geometries and physico-chemical characteristics as well as to understand the thermodynamics of adsorption (adsorption isotherm determination) through nonlinear chromatography. "This work has been conducted in strict cooperation with Prof. Francesco Gasparrini's group from University of Rome 'La Sapienza,'" she said. Catani's studies have culminated with the realization of ultrafast separations, "where the enantiomers of some compounds of pharmaceutical interest could be separated in less than one second with extremely high efficiency."

Most Recent Research

The most recent work published by Catani and coworkers focus on a diverse range of topics in separation science. They include studies on the purification of Cannabigerol (CBG) from cannabis extracts using simulated moving bed chromatography, investigations into the retention behavior of small molecules and amino acids on chiral stationary phases, an exploration of rare convex-upward van Deemter curves in chiral liquid chromatography, the analysis of pesticide residues in water resources using multidimensional gas chromatography, and a review of the challenges and prospects for purifying cannabinoids from cannabis extracts. Additionally, Catani's research extends to the synthesis of molecular hybrids with potential therapeutic applications, an examination of the effects of storage conditions on the phytochemical composition of garlic, and an overview of hydrophilic interaction liquid chromatography (HILIC) as an analytical technique. These recent articles collectively contribute to the advancement of separation science and its applications.

In Catani's most recent publication, her research focused on purifying cannabigerol (CBG), a minor cannabinoid found in *Cannabis sativa L.*, known for its various therapeutic properties (23). In this work, simulated moving bed chromatography was used to purify CBG from a cannabis extract efficiently and in an environmentally friendly manner. The method resulted in a CBG extract that was free of psychoactive tetrahydrocannabinol (THC) with a high recovery rate of 100% and a final purity of 97%.

In another recent article, Catani and coworkers explored the retention behavior of small molecules and N-protected amino acids on a zwitterionic teicoplanin chiral stationary phase (CSP) with different organic modifiers (24). This research explains that the choice of modifier significantly impacted efficiency and enantioselectivity. Methanol improved enantioselectivity, while acetonitrile allowed for high efficiency even at high flow rates.

Catani and colleagues investigated the rare occurrence of convex-upward van Deemter curves in chiral liquid chromatography (25). This work focuses on the behavior of chiral sulfoxides on a polysaccharide-based chiral stationary phase and explored the strong, localized adsorption of one enantiomer. The study explores correlations between molecular properties and specific interactions on the chiral stationary phase (CSP).

A chiral stationary phase (CSP) is a crucial element in chiral chromatography, involving a technique used to separate mirror-image isomers known as enantiomers. Enantiomers possess identical physical properties, but exhibit distinct chemical and biological behaviors. The

CSP is typically composed of a chiral compound covalently bonded to a solid support or adhered to the surface of small particles within a chromatography column. Acting as the stationary phase, it remains fixed as samples containing enantiomers pass through. Enantiomers interact differently with the CSP due to their unique three-dimensional structures, resulting in varying retention times or elution sequences. This differential interaction enables the separation of enantiomers during chromatographic analysis.

In another study, Catani addressed analyzing the presence of pesticide residues in water resources due to agricultural and non-agricultural pesticide use (26). Her research team developed a method for analyzing 53 pesticides and their metabolites in surface and groundwater using multidimensional gas chromatography. The method was validated and applied to real-world water samples.

Multidimensional gas chromatography (MDGC) is an advanced analytical technique employed for the separation and analysis of complex mixtures of volatile compounds. It involves the sequential use of multiple chromatographic columns with different selectivity and separation mechanisms. In MDGC, the sample is first injected into the first-dimension column, where compounds are separated based on their volatility and polarity. The effluent from the first column is then transferred to a second-dimension column, often via a modulator, where further separation occurs, resulting in improved peak resolution. This multidimensional approach allows for the separation of closely eluting compounds and provides enhanced analytical specificity and sensitivity. MDGC is particularly valuable in applications such as environmental analysis, food and flavor profiling, and the characterization of complex mixtures in various scientific and industrial fields.

With the legalization of cannabis in many countries and increased research on its therapeutic properties, there is a growing need for highly purified cannabinoids. A review by Catani and team discusses the challenges and opportunities for purifying major and minor cannabinoids from cannabis extracts, especially for use as reference materials and in clinical trials (27).

The purification of major and minor cannabinoids from cannabis extracts holds significant importance for several reasons. Firstly, it allows for production of standardized and highly pure cannabinoid compounds, which are crucial for scientific research, clinical trials, and the development of pharmaceutical products. These purified compounds serve as reference materials, enabling accurate and reproducible experiments to assess the therapeutic properties and potential medical benefits of cannabinoids. Additionally, the precise purification of cannabinoids ensures compliance with regulatory requirements for pharmaceutical and therapeutic applications, where quality control and consistency are paramount.

Moreover, the purification process removes unwanted impurities and psychoactive components like THC, making the resulting cannabinoid extracts safer for both research and medical use. The purification of major and minor cannabinoids not only facilitates scientific advancements, but also enhances the safety and reliability of cannabinoid-based therapies and products.

Another recent research article by Catani and associates explores the synthesis of molecular hybrids by connecting bioactive molecules with a metabolizable linker. Specifically, it describes the lipase-catalyzed condensation of ascorbic acid with ketone bodies, which results in novel compounds with potential for treating neurodegenerative diseases and cardiac injuries (28). This study focused on creating molecular hybrids by connecting bioactive molecules using a metabolizable linker for the treatment of complex diseases. It successfully synthesized two novel compounds, 6-O-acetoacetyl ascorbic acid and 6-O-(R)-3-hydroxybutyryl ascorbic acid, using a lipase-catalyzed method. The research not only provided efficient synthesis pathways but also characterized the compounds and explored their antioxidant properties, offering valuable insights for potential therapeutic applications.

Catani and coworkers investigated the effect of storage conditions on the phytochemical composition, biological effects, and shelf-life of Voghiera garlic, a Protected Designation of Origin (PDO) variety (29). Different storage conditions were considered, and changes in organosulfur compounds, total condensed tannins, flavonoids, phenolic compounds, and antioxidant activity were monitored. The study also assessed the impact of storage on the garlic's bioactive effects. The research conducted by Catani and colleagues holds significant importance as it addresses the crucial issue of preserving the quality and bioactive properties of Voghiera garlic. By systematically investigating various storage conditions, the study offers insights into the optimal methods to retain the garlic's phytochemical composition, including vital organosulfur compounds, tannins, flavonoids, and phenolic compounds. Moreover, by assessing changes in antioxidant activity and bioactive effects during storage, the research provides valuable guidance for maintaining the garlic's bioactive potency, shelf life, and overall quality, which is essential for its culinary and potential therapeutic applications. This information contributes to the preservation and utilization of a valuable agricultural product, benefiting both consumers and the garlic industry.

A book chapter by Catani provides an overview of hydrophilic-interaction chromatography (HILIC), highlighting its characteristics and applications (30). The chapter discusses the fundamentals of retention mechanisms in HILIC, and the materials used for stationary and mobile phases, emphasizing HILIC's role as a complementary chromatographic technique for separating polar molecules. Hydrophilic-interaction chromatography (HILIC) is a chromatographic technique of great importance in analytical chemistry for several reasons. Firstly, it enables the separation of highly polar and hydrophilic compounds, such as small polar metabolites, peptides, and glycoproteins, which are often challenging to separate using traditional reversed-phase chromatography. This capability is crucial in fields like metabolomics and proteomics, where the analysis of these compounds is essential for understanding biological processes and disease mechanisms. Secondly, HILIC is valuable in environmental and pharmaceutical analysis, where the separation of polar analytes is common. Additionally, HILIC can be used in tandem with other chromatographic methods, enhancing its versatility in comprehensive analyses. HILIC plays a vital role in expanding the range of analytes that can be effectively separated and detected in various scientific and industrial applications, contributing significantly to advances in analytical chemistry.

A Bright Future

Martina Catani has demonstrated remarkable potential for a bright future in the field of chromatography and separation science. Her ability to quickly grasp complex concepts, as evidenced during her research visits to leading institutions, showcases her adaptability and determination.

"Sustainable development is the most important challenge that we have to face in all fields," she said. "I may anticipate that there will be an increasing interest in 'green' chromatography. This will embrace both the study of the fundamentals of separation and the development of new applications to satisfy different requests."

Catani's career is characterized by her pragmatic approach to chromatography, her impactful collaborations, and her dedication to advancing the field. Her contributions, both in research and practical applications, continue to make a meaningful impact on chromatography. Catani's strong network and active involvement in collaborative projects and academic events underline her dedication to advancing the field. She has already achieved an impressive research output, with an h-index of 13 and numerous presentations at international conferences. Her enthusiasm, presentation skills, and sociable nature contribute to her standing as a promising scientist and future leader in chromatography. Her impactful research in macromolecule and optical isomer separation, garnering attention from the pharmaceutical industry, further solidifies her potential for excellence in the field. Catani's comprehensive knowledge and dedication make it highly likely for her to continue to significantly contribute and lead the advancement of separation science. ■

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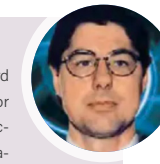

Jerome Workman, Jr.
serves on the Editorial Advisory Board of Spectroscopy and is the Senior Technical Editor for LCGC and Spectroscopy. He is the co-host of the Analytically Speaking podcast and has published multiple reference text volumes.

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Topics

08:30 What is Analytical Pyrolysis : Theory

09:30 Applications using Evolved Gas Analysis and Multi-step Pyrolysis

10:30 Additional Applications : TD, DHS, Reactive Py

11: 30 Data Analysis : Pyrolysis Database of Polymers

12:30 End

Registration

Presented by Karen Sam, Lab Manager of CDS Analytical, with over 15 years of PY-GC-MS experience

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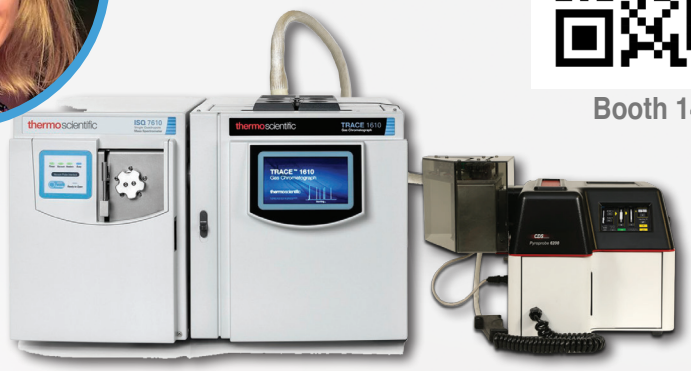

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SFC Separations with iHILIC-Fusion and iHILIC-Fusion(+) Columns

Stefan Bieber[1], Wen Jiang[2] and Thomas Letzel[1], AFIN-TS GmbH1, Augsburg, Germany, Hilicon AB[2]

SUPERCRITICAL FLUID chromatography (SFC), so-called "chromatography with carbon dioxide," is one of the "green chromatographic" techniques that have been studied in the past decades. The high separation efficiency, combined with fast mobile phase gradients and a broad selectivity, make SFC an interesting complementary technique to liquid chromatography (LC) in many analytical fields. Recent studies also show that the range of separable compounds by SFC is significantly broader than that by reversed-phase LC (RPLC) or hydrophilic interaction liquid chromatography (HILIC) (1) alone. SFC can easily be used with UV and mass spectrometry (MS) detection to provide great benefits when aiming to separate compounds in complex samples. However, the retention mechanisms in SFC are more complex than in other LC modes (2). This often makes it necessary to screen different columns in method development and find the most suitable one for the given task. To compare the basic characteristics of various columns and evaluate the applicability for SFC, AFIN-TS GmbH has established a column screening model. Based on a generic separation method, columns can be investigated for their capability to retain and separate compounds with a broad range of polarity. The results from such a screening are provided in a specific certificate that is intended to give guidance when choosing columns for SFC method development.

In this study, iHILIC-Fusion and iHILIC-Fusion(+) from Hilicon were evaluated for SFC separations.

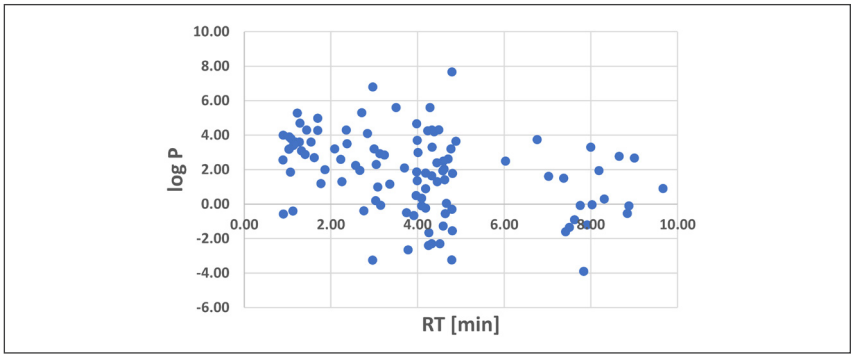

FIGURE 1: Retention time vs. logP plot of the analyzed standard compounds with the iHILIC®-Fusion column.

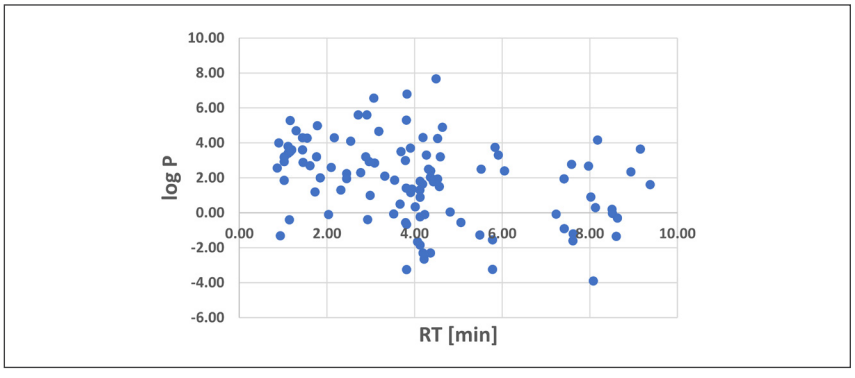

FIGURE 2: Retention time vs. logP plot of the analyzed standard compound with the iHILIC®-Fusion(+) column.

TABLE I: Gradient profile for SFC separations

Time (min)	B(%)
0	5
1	5
8	40
9	40
9.5	5
10	5

Experimental

A mixture of 114 standard compounds in a polarity range from logP -3.90 to +7.67 was used to evaluate the applicability of the columns for SFC. The final

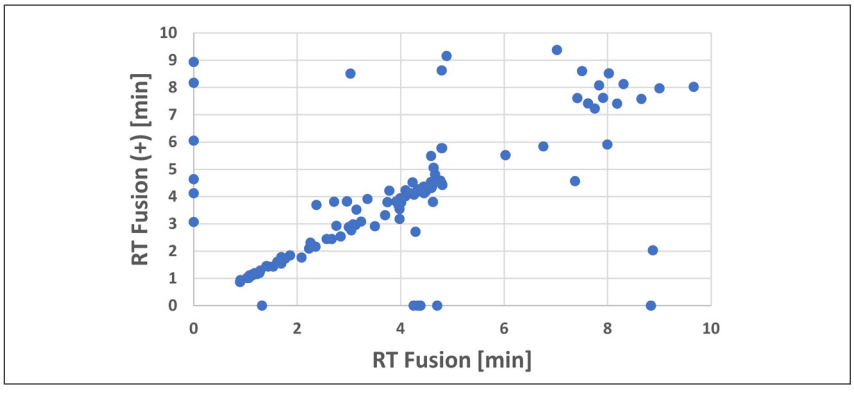

FIGURE 3: Orthogonality plot of the retention times of standard compounds with iHILIC®-Fusion and iHILIC®-Fusion(+) column.

concentration for all compounds was 2 µmol/L in 50/50 (v/v) acetonitrile/H_2O solution. Please contact HILICON to get details on the used compounds. **SFC–MS/MS System:** An Agilent 1260 Infinity I SFC system was used for the separations. The system consisted of a binary pump with solvent selector valve, an autosampler, a column oven with a column selection valve, a UV detector, and a back-pressure regulator. The SFC system was connected to an Orbitrap Exploris 120 MS (Thermo Fisher Scientific), which used a heated electrospray ionization (HESI source with positive/negative switching. The source and gas parameters were set as follows: ion spray voltage at +3.5 kV/ -3.0 kV, sheath gas flow rate at 50 arb units, aux gas flow at 8 arb units, ion transfer tube temperature at 320 °C, vaporizer temperature at 350 °C. Data was acquired in full scan mode with data dependent MS2 acquisition. **SFC Separations:** *Columns*: 1) 100×3 mm, 3.5µm, iHILIC®-Fusion (P/N 114.103.0310, HILICON) 2)100×3 mm, 3.5 µm, iHILIC®-Fusion(+)(P/N104.103.0310, HILICON) *Eluents*: (A) Carbon dioxide (3.5 grade);(B) Isopropanol(gradient grade); *Flow Rate*: 1.50 mL/min; *Column temperature*: 40 °C;Back pressure: 100 bar; Injection volume: 10 µL.

Results and Discussion

Selectivity in SFC is driven by several factors, including not only mobile phase composition and stationary phase chemistry but also the system pressure and temperature (2). To compare the columns, a generic separation method was used for screening and assessing their applicability in SFC separations. Figure 1 and Figure 2 show the retention time vs. logP plots of iHILIC-Fusion and iHILIC-Fusion(+) column, respectively. In contrast to RPLC, the hydrophobicity of compounds seems not to be the key parameter to dominate the retention in SFC. As a result, there is no strict correlation between retention time and logP.

When comparing the retention times of compounds on both iHILIC columns, which is shown in Figure 3, there is a linear correlation for many compounds. However, the iHILIC-Fusion(+) tends to show stronger retention for some later eluting compounds than the iHILIC-Fusion column. Furthermore, both columns could separate compounds exclusively (points on *y*- or *x*-axis, respectively), which indicates a certain degree of complementarity between these two types of columns. This could be helpful in method development where slight differences in selectivity can be used to optimize separations of critical peak pairs. The range of separable compounds with the investigated columns includes the whole spectra of tested compounds from polar to non-polar compounds. Figure 4 shows the extracted ion chromatograms of four exemplary compounds with the iHILIC columns.

Both iHILIC-Fusion and iHILIC-Fusion(+) are very well suitable for SFC separations.

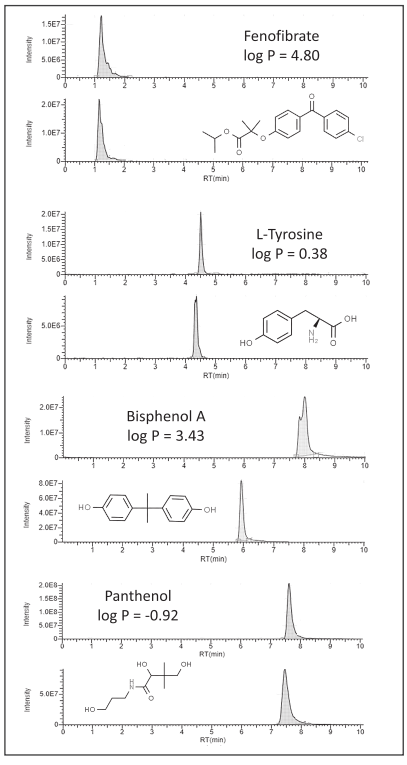

FIGURE 4: Extracted ion chromatograms of four standard compounds, separated with iHILIC-Fusion (top, trace with name) and iHILIC-Fusion(+) (bottom, trace with structure).

A broad range of separable compounds make both columns qualified for screening applications. In addition, the differences in selectivity between the iHILIC columns are valuable for specific SFC method developments.

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