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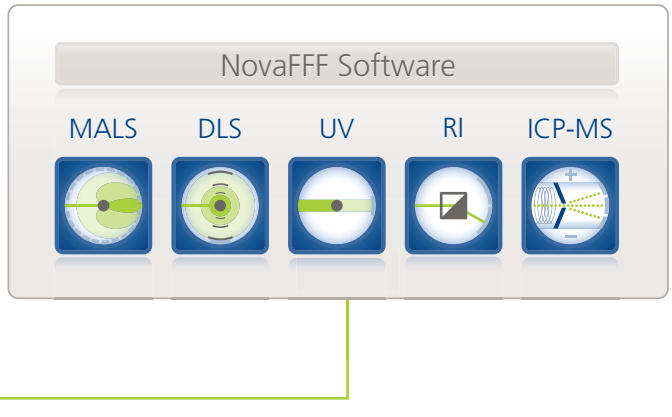

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PEAKS of Interest

Daniel W. Armstrong Named the 2020 Winner of the *LCGC* Lifetime Achievement Award

LCGC is proud to announce that Daniel W. Armstrong is the winner of the 13th annual *LCGC* Lifetime Achievement in Chromatography Award. Armstrong will be honored in a symposium as part of the technical program at the Pittcon 2020 conference in Chicago on March 3, 2020. The Lifetime Achievement in Chromatography Award honors an outstanding professional for a lifetime of contributions to the advancement of chromatographic techniques and applications.

Armstrong is the R.A. Welch Distinguished Professor of Chemistry and Biochemistry at the University of Texas, in Arlington. He has worked on an extremely broad range of separation techniques, including high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical fluid chromatography (SFC), micellar liquid chromatography, thin-layer chromatography, countercurrent chromatography, capillary electrophoresis (CE), and field flow fractionation, among others. He developed the theory and mechanistic background behind many of the practical advances in these techniques. Furthermore, he advanced the use of separations techniques as a means to obtain important physico-chemical data. His most recent work in ultrafast separations and signal processing is driving fundamental changes in the field.

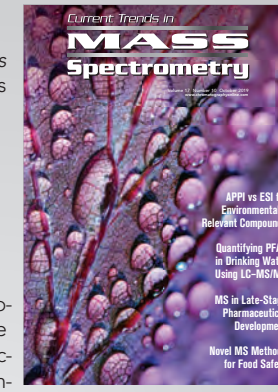
Another important contribution is Armstrong's development of comprehensive solvation and characterization models for room-temperature ionic liquids as stationary phases in GC. In his 1999 paper in *Analytical Chemistry,* it was shown that ionic liquids exhibit a unique "dual nature" retention selectivity toward polar and nonpolar molecules. In 2002, he published a comprehensive model that relates the solvation properties of ionic liquids to their unique structural features that comprise both the makeup of the cation and anion within the ion pair. This was quickly followed by the development of methods to design analyte-specific stationary phases that could be employed at high temperatures. These stationary phases have subsequently been commercialized and have been an important contribution to the field, particularly in multidimensional GC, where ionic liquids exhibit very unique selectivity compared to most other commercially available stationary phases.

Armstrong's group was the first to introduce macrocyclic glycopeptide chiral selectors in HPLC, SFC, and CE, as well as cyclofructan chiral selectors in HPLC, GC, SFC, and CE. Chromatographic columns possessing these stationary phases were commercialized and adopted as the leading chiral stationary phases as they exhibited wide chiral selectivity for a broad set of chiral molecules. In 2014, the European Space Agency's Rosetta mission soft-landed its Philae probe on comet 67P/Churyumov-Gerasimenko, and one of the instrument packages on the lander contained a chiral GC column (Chiraldex G-TA) invented by Armstrong. This column proved invaluable in the specific mission to separate small chiral molecules representing potential organic precursors in the search for life on the comet.

Armstrong's impact results not only from his research, but also from the over 175 former graduate students, post-doctoral fellows, and visiting scientists who have studied and trained under his guidance. Armstrong's former students are making contributions in academia in six countries and at pharmaceutical companies worldwide, as well as in petrochemical/polymer companies and federal agencies. He is the long-time Separations Associate Editor of the ACS journal *Analytical Chemistry*.■

SPECIAL ISSUE HIGHLIGHTS

CURRENT TRENDS IN MASS SPECTROMETRY



The latest issue of *Current Trends in Mass Spectrometry* presents articles on the following topics:

- **Ionization Efficiency for Environmentally Relevant Compounds Using Atmospheric Pressure Photoionization Versus Electrospray Ionization**
  Atmospheric pressure photoionization is compared to the default ionization method, electrospray ionization, for solution-phase samples.
- **Recent Advances in Hyphenated Chromatography and Mass Spectrometry Techniques and Their Impact on Late-Stage Pharmaceutical Development**
  A new generation of high-resolution mass spectrometers and ion mobility mass spectrometers have greatly increased the ability to resolve impurities and increase the level of analytical information gained from a single analysis.
- **Quantitative Analysis of PFAS in Drinking Water Using Liquid Chromatography Tandem MassSpectrometry**
  Per- and polyfluoroalkyl substances (PFAS) are found in fire-fighting foams and consumer products. They are ubiquitous in the environment and are an emerging human health concern. This work compares the 2009 and 2018 revised US Environmental Protection Agency (EPA) LC–MS/MS methods of analysis for PFAS in drinking water.
- **Novel Methods Using Mass Spectrometry for Food Safety—From Contamination to Nutrition**
  This article highlights three events that required the development of new mass spectrometry methods, including the detection of pesticides (such as fipronil and glyphosate), and the detection and quantification of fat-soluble vitamins.

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LC TROUBLESHOOTING

Effects of Flow Rate on UV Detection in Liquid Chromatography

If I increase the flow rate of my separation when using UV absorbance detection, should I expect peak area to change?

Dwight R. Stoll

In my personal experience with troubleshooting my equipment in my laboratory, and in thinking about topics for this column, I have found that effective troubleshooting skills and techniques are built on a solid foundational understanding of how the system under study (which is broken, if we are troubleshooting) is supposed to work. On a number of occasions, I have found myself thinking about and discussing with students and liquid chromatography (LC) practitioners the impact of flow rate on characteristics of chromatograms and peaks. For this month's "LC Troubleshooting," I've decided to dig into this basic, but very important, topic, with the intention that a deeper theoretical understanding of what should happen will help diagnose problems that may be related to flow rate when something does not look right.

Fundamentals

It is instructive to start a discussion of the effect of flow rate on LC separations with a kind of inventory of possible effects, along with a comparison of the predictions of simple theory and observations from real experiments (supported by more elaborate theories).

In this article, I am going to focus on the last two rows of Table I, because I have found through discussions with a variety of people that some confusion originates from these topics. Readers interested in the topics addressed in the second and third rows are referred to the references cited there for more information.

Relevant Background on Principles of Detection by Absorption of UV-Visible Light (UV Detection)

When thinking about the effects of flow rate on UV detection, it is critically important to recognize that we refer to UV detection as a type of *"concentration-sensitive" detection*. Concentration-sensitive detection is fundamentally different from "mass-sensitive" detection. Readers interested in the differences between these types of detection, and which LC detectors fall into which category, are referred to a recent educational article focused on this topic by Urban (4). Briefly, concentration-sensitive detectors respond to changes in analyte concentration presented to the detector (that is, moles/L, or mg/mL), whereas mass-sensitive detectors usually respond to changes in the mass of analyte presented to the detector over time (for example, pg/s). In the case of UV detection in particular, the detector reports absorbance values (A) in response to changes in analyte concentration (c) arriving at the detector. These absorbance values can be related to analyte concentration using the Beer-Lambert law:

$$A = \varepsilon bc \quad [1]$$

where ε is a measure of the absorptivity of the analyte, and b is a measure of the length of the light path through the detector flow cell. Readers interested in more details associated with the inner workings of UV detectors are referred to a recent article by Dong and Wysocki in *LCGC North America* (5).

Details Related to the Effect of Flow Rate on Peak Height (UV Detection)

To understand the effects of flow rate on peak height and area, we need to start with a model of chromatographic peaks. In the simplest case, we use a Gaussian distribution as a model of the peak shape, which expresses the dependence of analyte concentration in the LC column effluent arriving at the UV detector on time.

$$C_{\text{detected,i}} = \frac{\text{moles of } A}{\sqrt{2\pi}\sigma_v} \exp\left(\frac{-(t_i - t_R)^2}{2\sigma_t^2}\right) \quad [2]$$

Here, $C_{\text{detected,i}}$ is the concentration of the analyte arriving at the detector at time i, "moles of A" is the number

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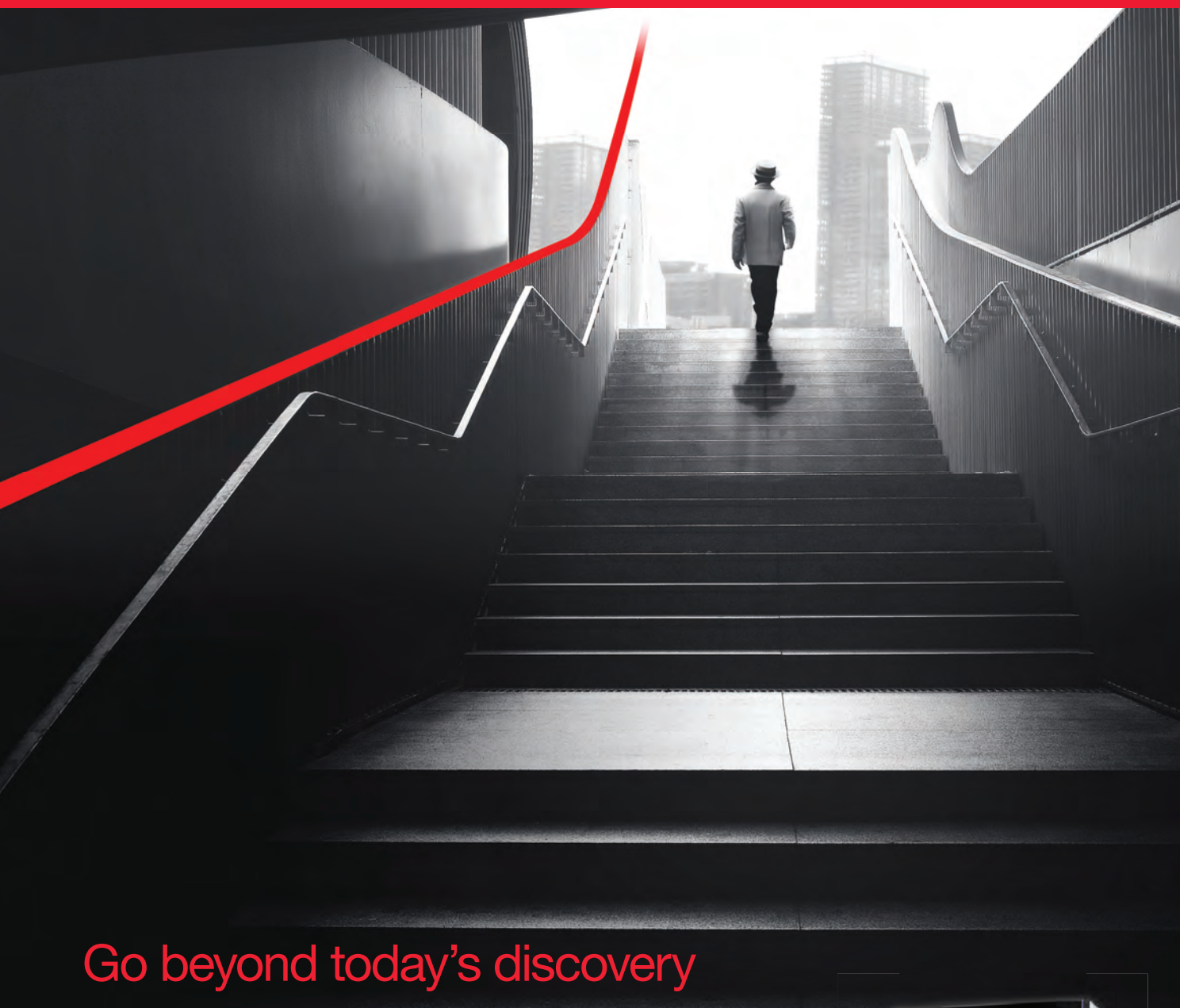

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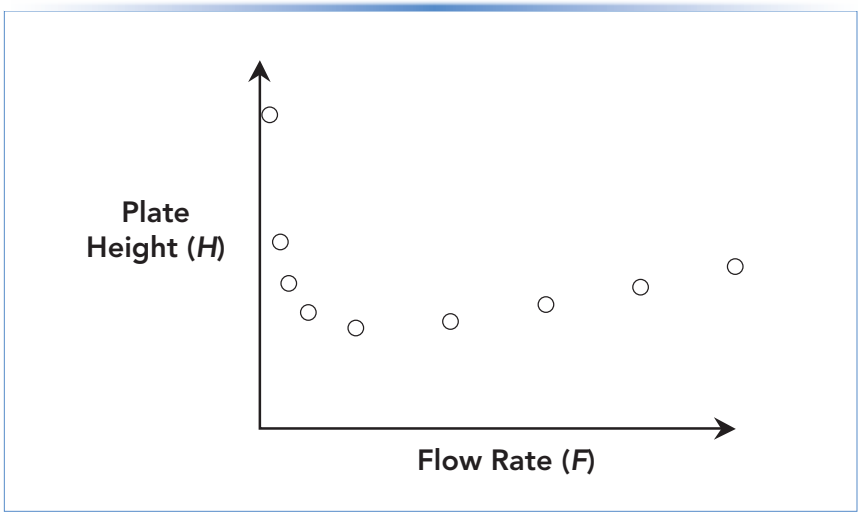

FIGURE 1: General shape of the dependence of plate height on flow rate that results from a van Deemter-type relationship $(H = A + \frac{B}{F} + C*F)$.

of moles of the analyte injected into the column, t_i is a time point in the chromatogram, t_R is the retention time of the analyte, and σ_v and σ_t are the standard deviations of the distribution (that is, a measure of the peak width) in volume and time units, respectively. At the apex of a chromatographic peak, $t_i = t_R$ and we have exp(0) = 1. Thus, the concentration of the analyte at the peak apex, and therefore the peak height, is entirely determined by the pre-exponential term:

$$\frac{\text{moles of } A}{\sqrt{2\pi}\sigma_v} \quad [3]$$

Now, the moles of analyte injected are not affected by the flow rate, nor is $\sqrt{2\pi}$. Although there is no explicit dependence of σ_v on flow rate, the flow rate will affect the peak height whenever the flow rate affects the plate height (H) of the column in use, which is almost always the case. The relationship between plate height and σ_v is shown in equations 4 and 5, where N is the column efficiency or plate number for the column, and V_R is the retention volume of the analyte ($V_R = t_R * F$).

$$H = \frac{L}{N} \quad [4]$$

$$N = \left(\frac{V_R}{\sigma_v}\right)^2 = \left(\frac{t_R}{\sigma_t}\right)^2 \quad [5]$$

From a theoretical point of view, we know quite a bit about the dependence of plate height on flow rate through relationships such as the van Deemter equation (6). The general shape of this type of dependence is shown in Figure 1.

The details of these relationships are not important here. The important fact is that, for relatively small changes in flow rate, the changes in plate height and σ_v, and therefore peak height, will be relatively minor, as shown by the experimental data discussed below. Readers interested in learning more about the dependence of plate height on flow rate are referred to the literature, which is a rich source of material on this topic (7).

Details Related to the Effect of Flow Rate on Peak Area (UV Detection)

Whereas the peak height is determined entirely by the pre-exponential term in equation 1, the peak area is determined by the integral of this equation, where the limits of integration are the time points that define the "start" and "end" of the peak. Indeed, when we talk about peak area, we sometimes refer to the "area under the curve." Now, if we consider a chromatographic peak obtained with a specific set of conditions and think

TABLE I: Inventory of some expected effects of flow rate on LC chromatograms and peaks.

Effect of Flow Rate (F) on...	Prediction of Simple LC Theory	Observations from Experiments and More Detailed Theory
Retention Time	Retention time increases in proportion to 1/*F*	-----
Retention Factor	No Effect	Use of high flow rates and pressures can lead to conditions where retention factors appear to depend on flow rate; this is more likely an outcome of a change in column temperature due to viscous heating (1), or a dependence of retention factors on pressure (2).
Column Inlet Pressure (P)	Pressure increases in proportion to *F*	Deviations from our expectations will occur if column temperature changes due to viscous heating (1), or if turbulent flow develops in connecting capillaries, or both (3). Both of these effects could lead to an apparent nonlinear dependence of P on F.
Peak Height (UV Detection)	No Effect	If the variation in flow rate is sufficient to have a measurable effect on the plate height of the column, then the peak height will change as a result of a change in peak variance.
Peak Area (UV Detection)	Area increases in proportion to 1/F	-----

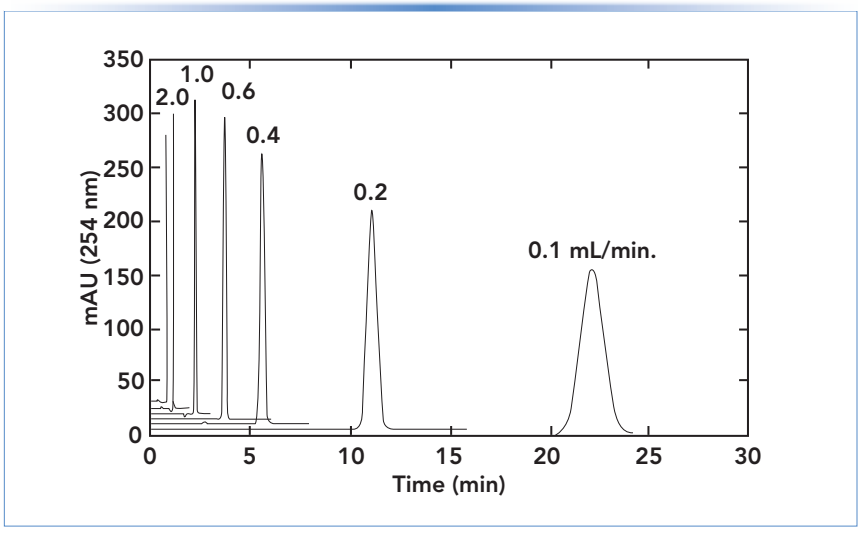

FIGURE 2: Effect of flow rate (indicated at the top of each peak; the label for 3 mL/min is not shown for clarity). Starting from 0.1 mL/min. the baseline of each chromatogram is offset by 5 mAU to facilitate visualization. Chromatographic conditions: column, Agilent SB-C18 (50 mm x 4.6 mm i.d., 5.0-µm); mobile phase, 50:50 acetonitrile:water; temperature, 40 °C; injection volume, 1 µL; data acquisition rate, 40 Hz; analyte: acetophenone at 0.5 mg/mL in acetonitrile. The retention factor of acetophenone is about 2 under these conditions.

about what happens when we double the flow rate, we will observe that the width of the peak decreases by about a factor of two. The degree of decrease would be exactly a factor of two in a case where the plate number is not affected by flow rate, because the ratio of t_R and σ_t is dictated by the plate number, as in equation 5. However, in most real situations, the plate number is affected by flow rate as discussed above, and the degree of change in width will be slightly different accordingly.

The net effect of flow rate on peak area in the case of UV detection is a consequence of two things happening at the same time: 1) the peak width changes in time units, expanding or contracting the integration window; and 2) the peak height is independent of flow rate, such that even if the peak becomes wider, time is added to the window over which the analyte is detected at a high concentration. In other words, the analyte flows through the UV detection cell at a finite velocity. The time over which the analyte can absorb photons is determined by the length of the light path the analyte travels through, and the velocity through that path. As the flow rate is reduced, the velocity through the detection path decreases, the residence time increases, and there are more opportunities for photons to be absorbed. Following this logic, we would expect to observe that peak area will increase in proportion to the inverse of the flow rate (that is, $A \propto \frac{1}{F}$).

Let's Look at Some Data

To illustrate the key points made above, I've made some experimental measurements of peak height and area at different flow rates, all under isocratic conditions. Figure 2 shows a series of chromatograms obtained at different flow rates in the range of 0.1 to 3.0 mL/min for the analyte acetophenone. From these chromatograms we see two clear trends: 1) the peak height varies slightly across these flow rates, but not in a simple linear way; and 2) the area under each peak obviously increases dramatically as flow rate is reduced.

Figure 3 shows a more quantitative view of peak height (a) and area (b) results from the chromatograms

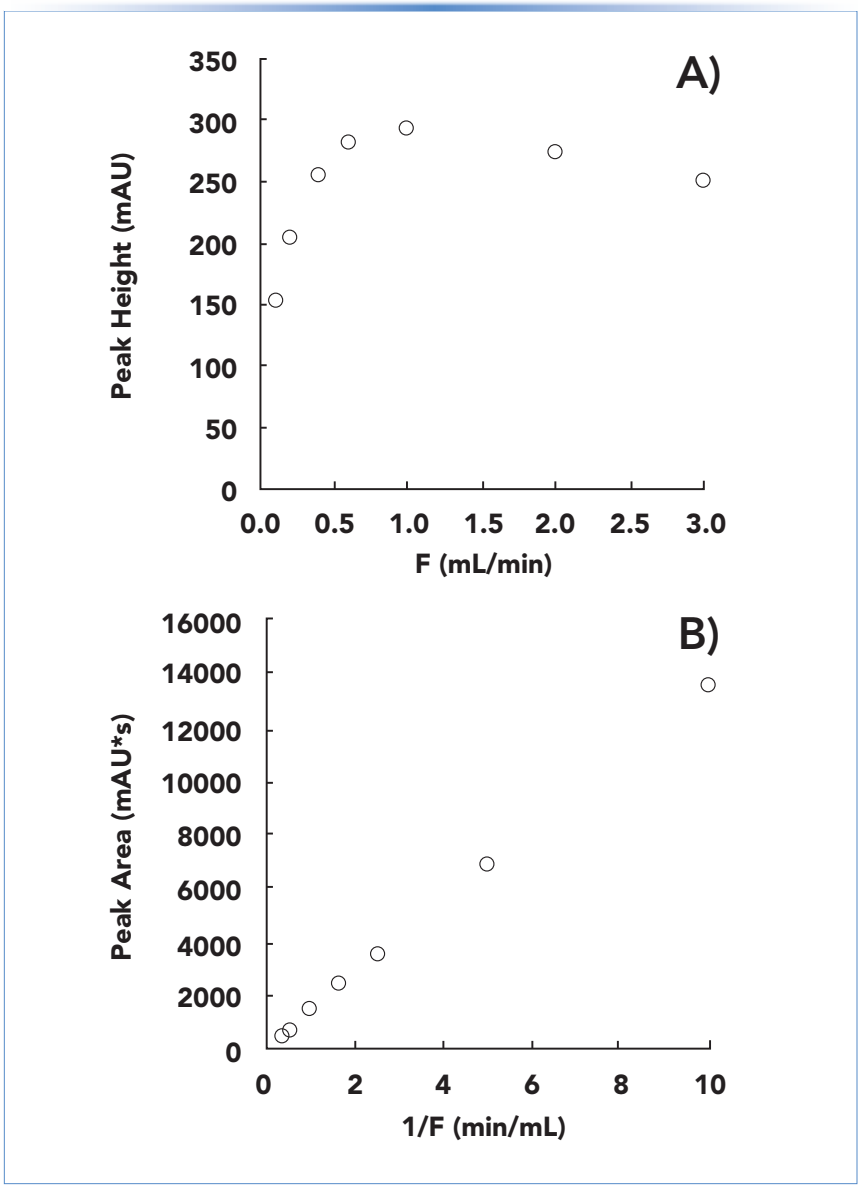

FIGURE 3: Quantitative view of the dependence of peak height (a) and peak area (b) on flow rate for the separations shown in Figure 2.

shown in Figure 2. We see that the shape of the dependence of the peak height on flow rate is the inverse of the shape of the plate height versus flow rate curve shown in Figure 1. Whereas there is a minimum in the *H* versus *F* curve in Figure 1, there is a maximum in the dependence of peak height on *F* around 1.0 mL/min. in Figure 3a. This is expected because of the inverse relationship between $C_{detected}$ and σ_v.

On the other hand, the dependence of the peak area on flow rate (Figure 3b) is very different. We see that the peak area increases in direct proportion to the inverse of the flow rate. This is because each part of the peak moves through the detection flow cell more slowly at a lower flow rate, the residence time in the detection zone is longer, and each analyte molecule contributes more to the measured absorbance.

Closing Thoughts

Our effectiveness in troubleshooting problems with LC separations improves as we deepen our basic understanding of how the separations work. In this article, we have examined the dependence of peak height and area on flow rate when using UV detection. Whereas peak height is only weakly dependent on flow rate, the peak area is strongly dependent on *F*, and decreases significantly as flow rate is increased. The extent of the expected decrease is important to know when troubleshooting problems with quantitation. For example, a leak between the injector and detector could also lead to decreases in peak area at higher flow rates (and consequently higher pressures).

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ABOUT THE COLUMN EDITOR

Dwight R. Stoll is the editor of "LC Troubleshooting." Stoll is a professor and co-chair of chemistry at Gustavus Adolphus College in St. Peter, Minnesota. His primary research focus is on the development of 2D-LC for both targeted and untargeted analyses. He has authored or coauthored more than 60 peer-reviewed publications and four book chapters in separation science and more than 100 conference presentations. He is also a member of LCGC's editorial advisory board. Direct correspondence to: LCGCedit@mmhgroup.com

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PERSPECTIVES
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Chromatography Data Systems: Perspectives, Principles, and Trends

This installment is the last of a series of four articles on high-performance liquid chromatography (HPLC) modules, covering pumps, autosamplers, ultraviolet (UV) detectors, and chromatography data systems (CDS). It provides a technical overview of CDS design, historical perspectives, the current marketing landscape, instrument control, data processing practices, and future trends.

Robert P. Mazzarese, Steven M. Bird, Peter J. Zipfell, and Michael W. Dong

Chromatographic analysis, including high-performance liquid chromatography (HPLC), gas chromatography (GC), ion chromatography (IC), supercritical fluid chromatography (SFC), and capillary electrophoresis (CE), constitutes a major portion of testing performed in analytical laboratories. All of these instruments have one thing in common: They all require the use of a chromatography data system (CDS), which plays a pivotal role in instrument control, data processing, report generation, and data archiving.

In laboratories performing regulated testing for quality control, pharmaceutical development, or manufacturing, the CDS is likely a validated client-server network designed to provide data security and integrity. Our observations indicate that laboratory scientists in regulated laboratories tend to spend as much time performing data processing as in front of a chromatographic system. Thus, to have a better understanding of improved analytical practices, it is critical to have an in-depth knowledge of the role of a CDS in both instrument control and data processing.

A modern CDS is a complex software system that is used in many rapidly changing analytical science fields to control instruments, gather and process data, and generate reports. A literature search revealed surprisingly few overviews of CDS and related topics in textbooks (1–2), book chapters (3–6), and journal articles (7–8). Nevertheless, detailed information is available from manufacturers on specific CDS, and can be found in websites, brochures, and manuals (9–12).

In this installment, we strive to provide a general overview of CDS and its pivotal role in the analytical workflow, focusing on client-server networks. We review historical developments of CDS, and describe the operating principles on instrument control and data processing (data acquisition, peak integration and identification, calibration, and report generation), as well as the current marketing landscape, and modern trends.

Glossary of Key Terms and Acronyms

Key Terms

- **21 *CFR* Part 11:** The Code of Federal Regulations that defines the criteria under which electronic records and signatures are considered trustworthy, reliable, and equivalent to paper records.
- **A/D Converter:** An analog-to-digital converter that takes the analog voltage from a detector and converts it into a digital signal.
- **Algorithm:** A process or set of rules to be followed in calculations or other problem-solving operations, typically performed by a computer.
- **Analytics:** Systematic analysis of data using metrics and statistics.
- **Audit Trail:** A historical record or set of records that enable data and their associated events to be accurately reconstructed.
- **Business Continuity:** The process of creating systems of prevention and recovery to deal with potential threats to a company. In addition to prevention, the goal is to permit ongoing operation before and during the execution of disaster recovery.
- **Calibration:** A process for the quantitation of analytes in a sample by comparing peak areas of identified analytes with those from reference solutions with known concentrations.
- **CDS:** A chromatography data system, which is used to acquire, integrate, quantitate, and report data produced by a chromatography instrument.
- **Citrix:** A program that allows a client personal computer (PC) to access a server-based "virtualized" instance of the client software remotely and securely, thus avoiding a local installation of the software.
- **Client-Server Network:** A client-server network is designed for end-users, called *clients*, to access resources such as files and programs from a central computer called a *server*. A server's purpose is to serve as a central repository

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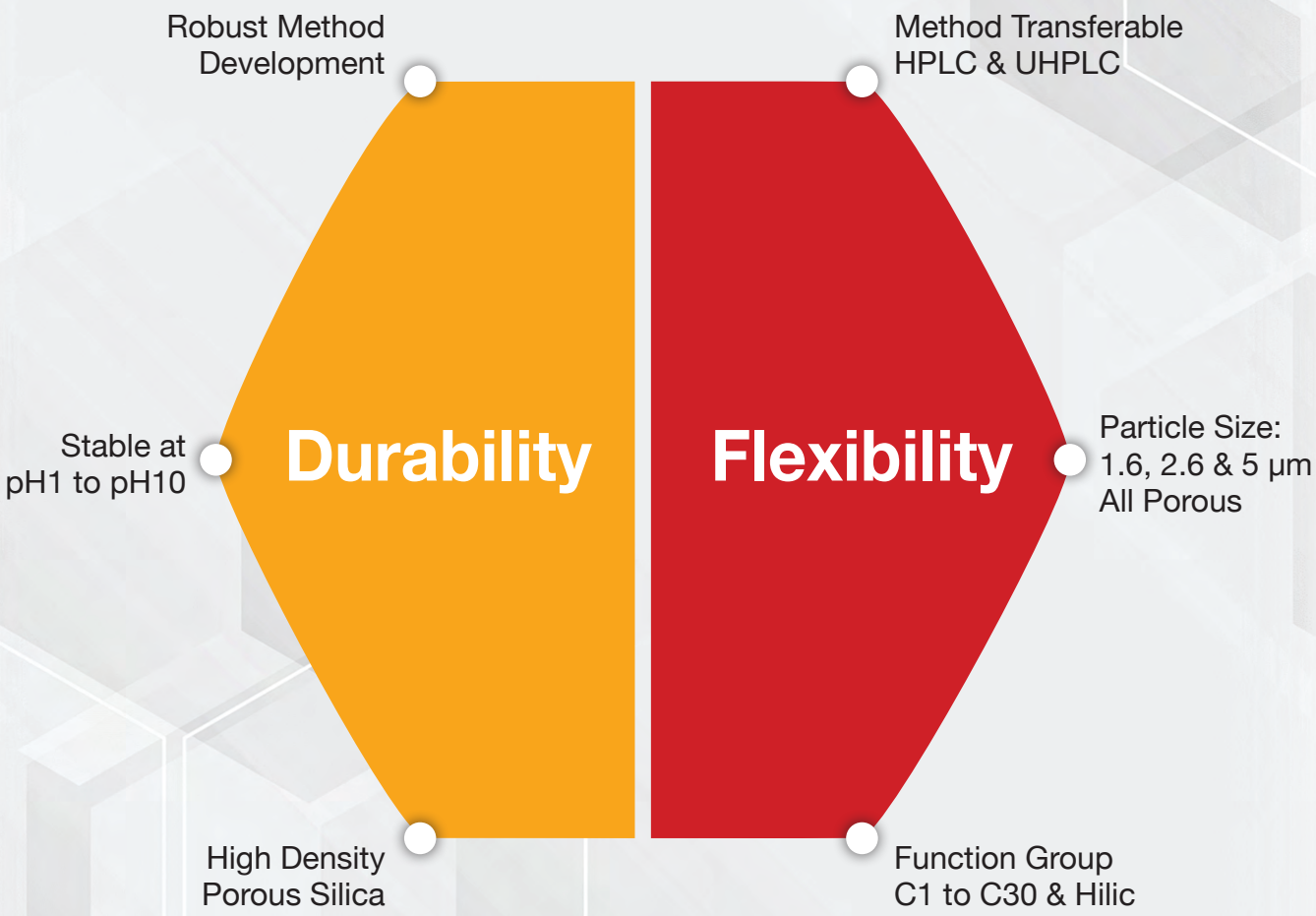

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TABLE I: Fundamental requirements and desirable characteristics of a network CDS

Requirements and Functionalities
• **Data Acquisition:** Acquires raw data from the detectors from one or more chromatography systems. Data acquisition commences with the start of the sample injection. • **Data Processing:** Includes processes such as peak integration, identification, calibration, report regeneration, and data archival in a highly automated and customizable fashion. • **Instrument Control:** Provides single-point instrument control of all instruments (such as an HPLC: pump, autosampler, column oven, and detector) for one or more chromatographic systems in the network. • **Regulatory Compliance:** Provides data security, traceability, and integrity in compliance with GMP and 21 *CFR* Part 11 regulations. CDS must be validated to allow the release of GMP results.
Desirable Characteristics

• Multi-vendor, Multi-instrument, Multi-lab, Multi-detector, and Multi-language Connectivity:
  • Controls chromatography instruments (for example, HPLC, UHPLC, GC, IC , CE, and SFC) and detectors from different manufacturers.
  • Processes and displays data from information-rich detectors such as diode array detector and MS
  • Scalable system platform: with expandability from a single workstation to a global multisite network for thousands of users and instruments.
  • Supports multiple languages (English, Spanish, Chinese, German, and so on).
• Networking and Operating System:
  • Compatible with common operating systems, including Windows 7, 8.1, and 10.
  • Remote access to instruments/data in the network using internet browsers (such as Internet Explorer 11, Microsoft Edge, Google Chrome) and includes access through mobile devices such as smartphones and tablets.
  • The CDS network server can be deployed and managed on premise, or at a remote location, or managed by a third-party service provider in the cloud.
  • Tools for network failure protection and disaster recovery.
• Data Security, Flexibility and Archival
  • Uses common relational databases (Oracle or SQL Server) for structural data management/archival and rapid retrieval.
  • Allows third-party data reviews and sign-off with electronic signature.
  • Supports data processing from single or multiple sample sequences or queues.
  • Supports custom calculations and reporting.
  • Provides data audit trail and archive of method, data, result, and information with version and date stamps.
  • Provides exporting functionality with the ability to automate data exportable in common formats (pdf, xls, csv, doc, txt, aia, gaml, xml, Allotrope, and so on) and other software.
• Other Enhancements and Links to Informatics Systems
  • Easy-to-use user interface with customizable tooling for system administrators and expert users.
  • Supports tools and display for instrument diagnostics, performance monitoring, and service notifications.
  • Provide tools for CDS validation and document support for IQ, OQ, and PQ.
  • Low software licensing fee, hardware, and maintenance cost.
  • Supports automated interfacing with ELN, LIMS, workflow solutions, e-mails, and artificial intelligence software.
  • Supports other software such as GPC, Simulated Distillation, HPLC method development, and validation.
  • Supports interfaces to ERP, Digital Lab, MES, LotF, and so on.
  • Cloud deployment, compatibility, and virtualization.

IQ, OQ, PQ: Installation, Operation, Performance Qualification; ELN: Electronic Laboratory Notebook; LIMS: Laboratory Information Management System; GPC: gel-permeation chromatography; ERP: Enterprise Resources Planning; MES: Manufacturing Executive Systems; LoTF: Lab of the Future

of computing programs and data archival. The server can be located on-site, off-site, or in the cloud.

- **Cloud Computing:** The practice of using a network of remote servers hosted on the internet to store, manage, and process data, rather than using a local server or a personal computer.
- **Cloud Storage:** In cloud storage, data are maintained, managed, backed up remotely, and made available to users over a network, typically via the internet.
- **Disaster Recovery:** A set of policies, tools, and procedures to enable the recovery or continuation of vital technology infrastructure and systems following a natural or human-induced disaster.
- **Instrument control:** A key function of a CDS is instrument control where all the parameters of each module (such as an HPLC: pump, autosampler, column compartment, and detectors) are controlled from a single instrumental method in the CDS.
- **Integration:** A process that uses a mathematical algorithm to transform raw data from a detector into processed data consisting of peak retention times and peak areas. Integration algorithms are classified as "traditional," using slope thresholds or second derivatization of the raw data.
- **Metadata:** A set of data that describes and gives information about other data, including raw data, sample data, or analyst data. For a CDS, metadata are all of the associated data describing the raw data and their calculated results, such as instrument conditions, errors generated, integration and calibration parameters, user information, review, and approval.
- **Metrics:** Measurements to help evaluate performance or progress.
- **Raw Data:** Chromatographically derived digital data obtained from the chromatographic detector acquired by the CDS before any data processing or transformation. For regulatory testing, the raw data cannot be deleted or altered.
- **Relational Database:** A collection of data items that have predefined relationships, which are organized as a set of tables with columns and rows.
- **Report:** A visual arrangement of information about a sample and the associated results that is typically generated at the end of data processing by the CDS, either automatically or by manual processing of data from a sample sequence. In most cases, reports contain information such as the amount or concentration of each identified peak, sample information, a chromatogram, and a spectrum. A summary report contains reported data from a set of samples and may contain

statistical evaluation data such as peak area precision. A report can also contain details about whether the system suitability, assay, and sample acceptance criteria are met or not. Information reported is dependent on assay type and organizational requirements.

- **The Quality Unit, QA, QC:** A quality unit reporting to the head of a production or development facility is mandated in good manufacturing practice (GMP) regulations. Quality Assurance (QA) is responsible for the overall Quality System and equipment qualification. Quality Control (QC) is the laboratory branch responsible for the actual analytical testing.

Acronyms

- **IaaS:** Infrastructure as a service
- **PaaS:** Platform as a service
- **SaaS:** Software as a service. (IaaS, Paas, and Saas are types of cloud computing setups that replace varying degrees of on-premise computing.)
- **CE:** Capillary electrophoresis
- **CoA:** Certificate of analysis
- **DAD:** Diode array detector
- **ELN:** Electronic laboratory notebook
- **GLP:** Good laboratory practice (21 *CFR* Part 58)
- **GMP:** Good manufacturing practice (21 *CFR* Part 211
- **HRMS:** High-resolution mass spectrometry
- **IC:** Ion chromatography
- **LIMS:** Laboratory information management system
- **LMS:** Laboratory Management System
- **LoTF:** Laboratory of the future
- **MS:** Mass spectrometry
- **SDMS:** Scientific data management solutions
- **SFC:** Supercritical fluid chromatography
- **SQMS:** Single-quadrupole MS
- **SST:** System suitability testing.
- **TQMS:** Triple-quadrupole mass spectrometry.

Requirements and Desirable Characteristics of an Enterprise CDS

Table I summarizes the requirements and desirable characteristics of a modern CDS network for regulated laboratories. These requirements and the operating principles are further discussed in later sections. Our goal is to increase the understanding of the fundamentals of CDS by the laboratory scientist, thus leading to more efficient laboratory practices.

A Historical Perspective

Let us start with a brief historical review of the evolution of CDS. Figure 1 shows four devices for chromatography data handling since the 1970s.

Strip Chart Recorders

A strip chart recorder plotted analog signals from chromatography detector(s) (in volts or millivolts) on a long roll of moving chart paper to generate chromatograms of detector response versus time. Chart recorders were the primary data handling

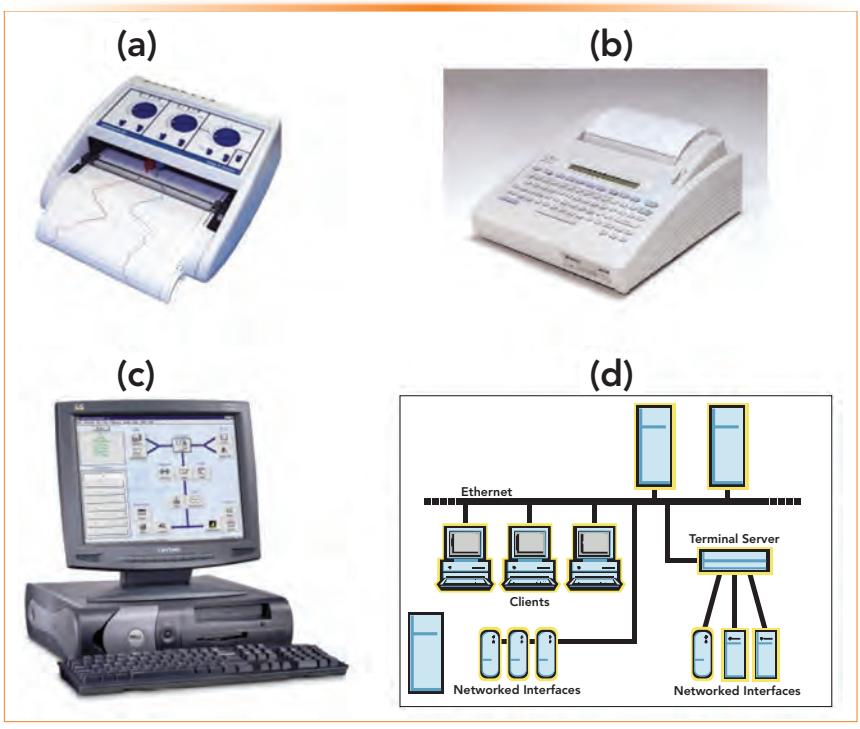

FIGURE 1: Four images illustrating the key evolution of CDS from (a) strip chart recorder, (b) electronic integrator, and (c) PC workstation to (d) client-server network.

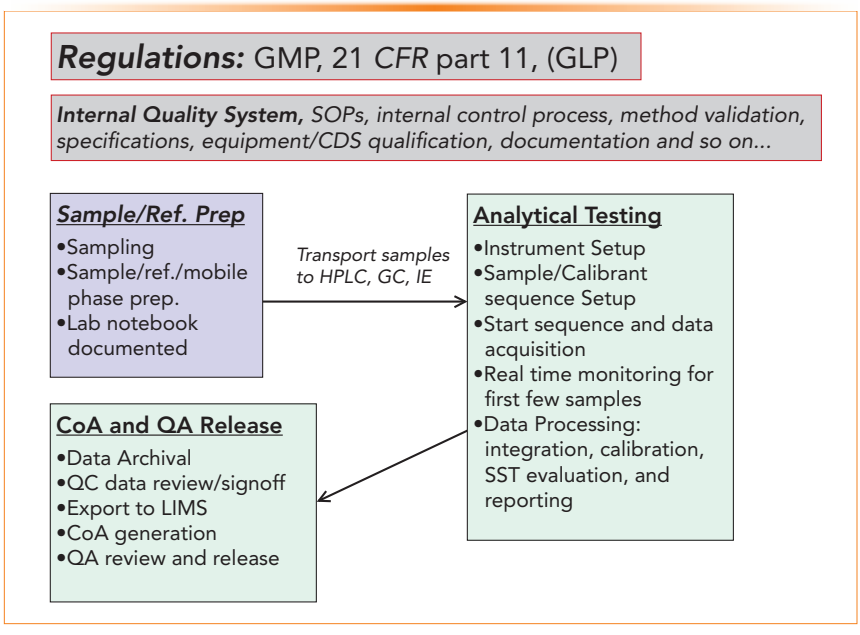

FIGURE 2: The many steps of a chromatographic analysis workflow in a regulated laboratory. Today, it can be a complex process because it must comply with various regulations and internal quality systems and SOPs shown inside the upper rectangles. The actual analytical workflow starts from sampling, and sample/reference/mobile phase preparation before transporting the samples for analytical testing; which is comprised of instrument and sample sequence set up, data acquisition, result calculation, and report generation. Finally, the data is reviewed by the QC manager and signed-off, exported to LIMS, and merged with other analytical data to generate a Certificate of Analysis (CoA). QA then reviews for transcription accuracy and audit trail before releasing the batch for production or clinical use. CDS is heavily utilized in the automation of the analytical workflow and plays an increasingly important role in post-analysis processes.

devices for early chromatographs in the 1960s and 1970s. Quantitation was estimated using manual measurements of peak heights or peak areas using a "cut-and-weigh" of the peak area or via a triangulation calculation approach (peak height times peak-width-at-half-height). Today, these recorders are rarely used, except in preparative chromatography (3).

Electronic Integrators

The age of the "electronic revolution" heralded in the electronic integrator for chromatography (with Hewlett-Packard's HP-3380A in the mid-1970s, and Shimadzu's C-R1A in the early 1980s). These were capable recorders with thermal paper printers and built-in A/D converters, LCD, internal storage memory, and firmware for automated peak integration, calibration, quantitation, and report generation. Some offered calculations for system suitability testing (SST) parameters and provided BASIC programming for customization. These were relatively inexpensive devices that were light years ahead of the simple chart recorders at the time.

Their use was short-lived, as they were quickly supplanted with the advent of the personal computer (PC) in the 1980s, which offered greater flexibility and infinite possibilities in data handling and instrument control. Nevertheless, a few models still linger on today, such as the Shimadzu C-R8A Chromatopac Data Processor, because of its low cost and easy operation for small laboratories.

PC Workstations

In the 1980s, analytical instrument manufacturers began adopting microprocessor technologies in the design of all analytical instruments, which led quickly to the use of the PC workstation as the preferred controller and data handling device.

One of the most successful PC-based workstations for chromatography was launched by Nelson Analytical in Cupertino, California, in the early 1980s, followed by a highly successful CDS network called TurboChrom. The early adoption of the Windows operating system was an important part of the success of TurboChrom. Nelson Analytical was acquired

by PerkinElmer in 1989, and TurboChrom continued to dominate the early client-server based CDS market for many years until strong competitors debuted in the mid-1990s (6,13).

Network and Client-Server CDS

The first commercial chromatography network CDS was likely the HP-3300 data acquisition system launched in the late 1970s by Hewlett-Packard, and installed in many large chemical and pharmaceutical laboratories. It was a mini-computer–based system capable of acquiring data from up to 60 chromatographs through A/D converters (4).

The Windows-based PC-workstations and client-server CDS networks became dominant in the 1990s for small and large laboratories, due to their versatility, convenience, and the ability to provide compliance to 21 *CFR* Part 11 regulations (4,13–14).

In the client-server model, adding a PC as a client to the network increases the processing power of the overall system (4,7). The client typically provides the graphical user interface, instrument control, temporary data storage, and some of the data processing in a distributive computing system. The server maintains the databases and manages data transactions with the clients. A critical responsibility of the server is to have central control of the applications as well as to safeguard data integrity and security. The client/server model has several major advantages such as a highly scalable system design (for small laboratories to global multisite installations), a reduction in issues related to system maintenance, easier sharing of data and methods for all users, and the ability to support remote access using web browsers on PCs or mobile devices (tablets and smartphones) (4, 7)

Current Marketing Landscape for CDS

The current market size for HPLC has been estimated to be at approximately 5 billion USD, with four major manufacturers, Waters, Agilent, Thermo Fisher Scientific, and Shimadzu, consistently responsible for >80% of the global HPLC market in recent years (15–16). The market size of CDS,

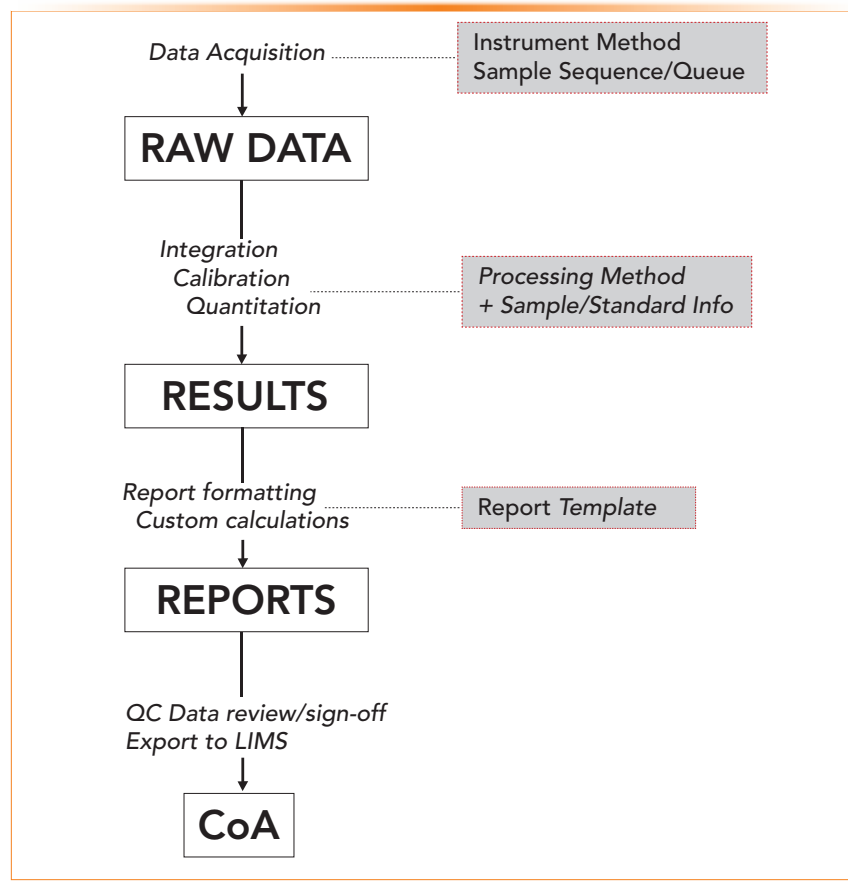

FIGURE 3: Schematic diagram showing the analytical data workflow in a specific CDS (Waters Empower CDS) and the type of methods used: 1. instrumental setup for the acquisition of the raw data using instrument method and a sample sequence; 2. data processing to generate results using a processing method; and 3. Generation of formatted reports using a reporting method. The report is then reviewed by QC management and signed-off directly in the CDS, where they can often be exported automatically to a LIMS for the generation of a CoA.

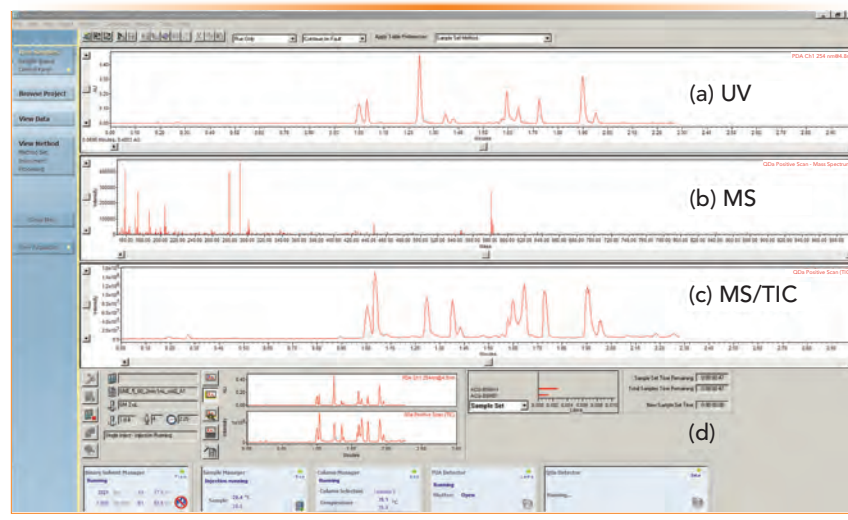

FIGURE 4: Instrument control screen with real-time data monitoring of multiple detector signals: (a) UV chromatogram, (b) mass spectrum, and (c) MS total ion chromatogram (TIC) shown from a Waters Empower 3 CDS. The instrument status of various HPLC modules and the sample and sequence status are shown in (d) the lowest panel.

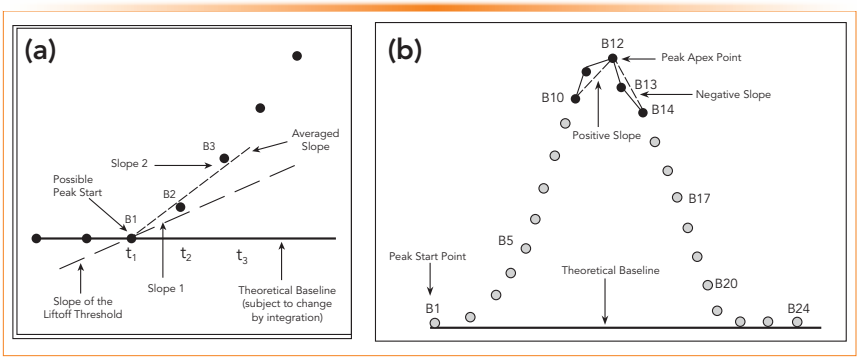

FIGURE 5: (a) Illustrates how a traditional algorithm compares changes in signal slope to determine the start of a peak; (b) Illustrates how the algorithm determines the retention time of the peak being integrated. Figures adapted from reference (4).

according to a survey by Top-Down Analytics, is estimated at approximately $700 million USD (17), with $425 million USD for HPLC and $275 million USD for GC. The top three providers are Waters, Thermo Fisher Scientific, and Agilent.

Waters has held a prominent CDS position since its first introduction of Millennium software on an Intel-486 microprocessor PC with an Oracle database in 1992. With continual improvements to its current Empower CDS (current version 3), Waters has attained wide acceptance from regulators, while establishing a very strong position within the pharmaceutical industry.

Thermo Fisher Scientific has become one of the leading CDS providers with its Chromeleon software platform (launched in 1996), which brings extensive compliance coverage and global networking capabilities that now include control, data acquisition, and data processing for high-resolution MS instruments. Known for its multi-vendor instrument control, Thermo Scientific Chromeleon CDS provides control for chromatography and single-quadrupole MS, triple-quadrupole MS, and HRMS instruments, leading to its popularity in both routine and development labs.

Agilent's HPLC instruments are popular in research laboratories where scientists embrace its ChemStation CDS with an easy-to-use instrument control interface. The most recent revamped version of Agilent's OpenLab CDS (version 2.4) has advanced data processing and regulatory compliance capabilities that enhance its competitiveness in QC laboratories. Agilent still offers the OpenLab ChemStation edition for specialty applications such as 2D-LC.

Shimadzu HPLC and GC instruments have a strong presence in the food, environmental, pharmaceutical quality control, and industrial markets, and the company offers LabSolutions, a network CDS, for their GC, HPLC, and secondary ion MS systems.

The rest of the CDS market belongs to manufacturers that cater to smaller installations or controllers and data devices for their own brands of chromatography or purification instruments. Examples of these are Clarity (DataApex), Chromperfect (Justice Lab Systems), CompassCDS (Scion Instruments), PeakSimple (SRI Instruments), ChromNAV 2.0 (Jasco), and Chromera/TotalChrom (PerkinElmer).

CDS have continued to improve in capability, reliability, and ease of use over the past three decades through advances in software, computers, and network implementations. Current features and desirable characteristics of modern network CDS are listed in Table I. With rapidly evolving technologies and a diversity of product features catering to different market segments and instrumentation, it is challenging to give accurate general statements or descriptions of CDS. The reader is therefore referred to the manufacturers' websites and brochures for more technical details on specific systems.

Next, we focus on the role of CDS in the analytical workflow and review the principles of instrument control, data acquisition, peak integration, and data processing, with illustrations from specific CDS for UV and MS instruments.

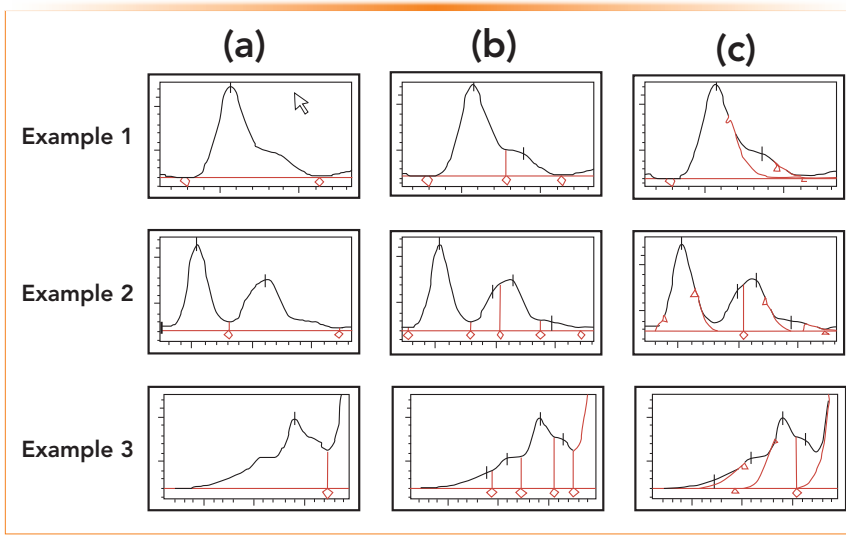

FIGURE 6: Illustrates the ability of the Waters ApexTrack Integration algorithm, which can easily identify and quantitate shoulders versus the results obtained with a traditional integration algorithm (three examples for each integration type are shown). (a) Traditional, (b) ApexTrack with detect shoulders event, and (c) ApexTrack with detect shoulders and Gaussian skim events. Figures adapted from reference (4).

Chromatography Analysis in a Regulated Environment: The Role of CDS

Today, performing regulated HPLC release testing of a pharmaceutical sample requires considerable resource allocation for regulatory compliance in the laboratory. Equipment validation, personnel training, and method validation take a significant amount of time and energy. Also, the laboratory must adhere to internal quality systems and processes, and standard operating procedures (SOPs), as listed in the analytical workflow example in Figure 2 (3). The role of the CDS during the analytical testing steps is summarized in a case study on a specific CDS implementation (Figure 3) (4).

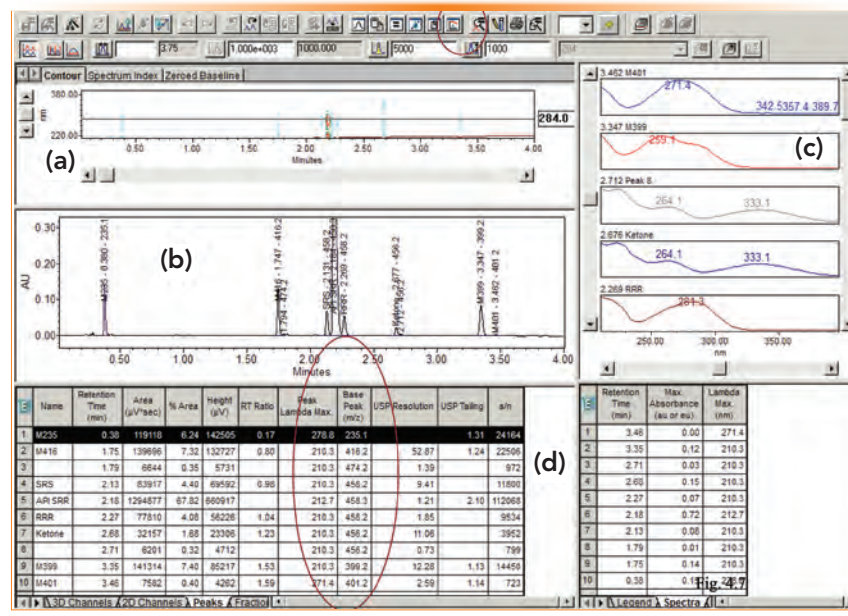

FIGURE 7: Showing (a) a UV contour map; and (b) the graphical user interface (GUI) of Waters Empower 3 CDS showing a result from the injection of a retention marker solution into an HPLC-UV–MS system displaying a chromatogram at 284 nm; (c) shows UV spectra; and (d) displays a peak table showing various extracted UV and MS parameters.

Regulations and Quality Systems of the Organization

Figure 2 shows the various processes of a pharmaceutical analytical testing workflow in and outside of the operation under external regulations and internal quality system processes (shown above the workflow schematics in Figure 2).

First, the laboratory, laboratory equipment, and analytical procedures and processes must follow GMP regulations (21 *CFR* part 211) (18) and handling of data both inside and outside of the laboratory 21 *CFR* Part 11 (14). Note that other facilities such as contract research organizations (CROs) often operate under GLP regulations (21 *CFR* part 58) (19) for nonclinical studies such as toxicology evaluations or bioanalytical studies.

Second, the laboratory analyst must be thoroughly trained and follow the company's internal quality system (3,20) and already defined SOP, and must document all pertinent data in a laboratory notebook (paper-based or electronic laboratory notebook (ELN)) (19,21). All critical laboratory equipment, including the CDS, must be qualified, and the analytical method used must be qualified and/or validated (2-3).

Sampling and Sample Preparation

The laboratory analysis workflow starts with a sampling step to obtain a representative sample from a batch of drug substance or drug product, followed by a sample preparation step that includes the preparation of the sample solution(s), reference solutions, mobile phases, and system suitability solutions that verify the system's sensitivity, precision, or peak tailing performance, and its ability to achieve sufficient resolution of all key analytes (3). These sample vials are then transported

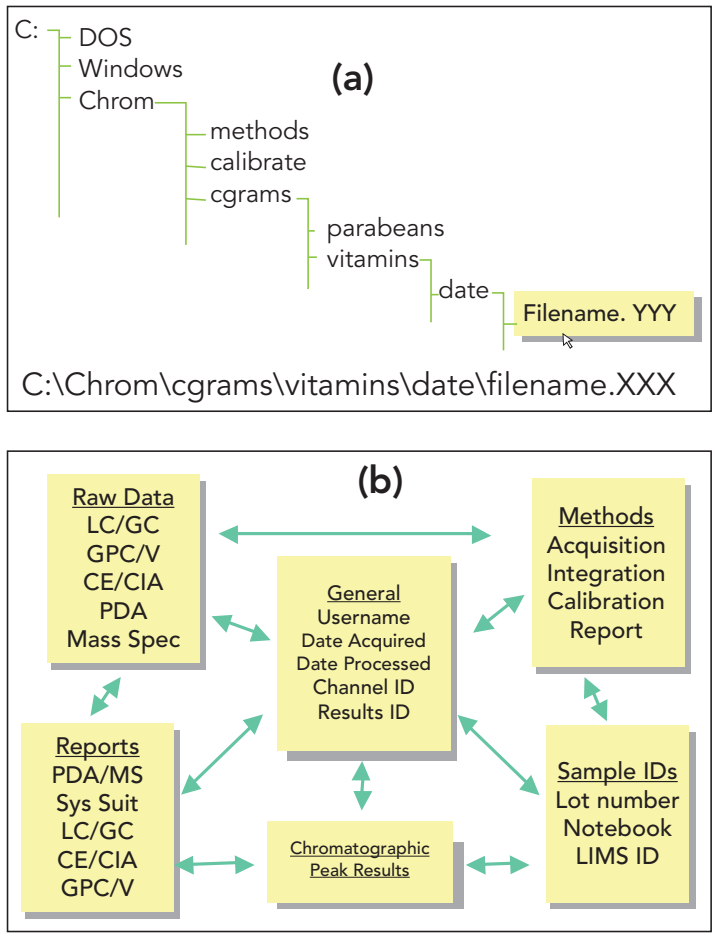

FIGURE 8: (a) Typical directory structure found in a "flat file" data system; and (b) the relationship of the different tables of data within a CDS based on a relational database.

to the HPLC system, and placed inside the autosampler tray. According to GMP regulations, all pertinent information of the samples, reagents, instruments, columns, and mobile phases must be recorded appropriately for traceability in a regulatory audit (3,18).

Analytical Testing

During the next analytical testing phase, the CDS plays a major role in the instrument control and data processing steps to generate results and reports, as summarized in the data flow schematic diagram in Figure 3 (4).

Instrument Setup

HPLC instrument control can be a complex process with many precisely engineered modules of the HPLC system that must work together to produce accurate results (3). For an HPLC method to perform correctly, all modules (pump, autosampler, column oven, and detector) must be set up properly with the correct column, mobile phases, samples, and standards. All of these instrument parameters are typically "choreographed" or coordinated by the CDS workstation or network, which allows a single-point control of all the modules, which are typically connected via Ethernet or USB cables, using an instrumental method (or an instrumental control section of a CDS method) (4). A CDS network allows flexibility for a user to control any instruments in the network using a client or terminal in the lab, or remotely from a PC in the office or home.

Setup of Sample Sequence

Most active pharmaceutical ingredient (API) quantitative analyses use a reference standard and the external standardization technique to quantitate the main components and all key analytes (3). A sample sequence is typically set up, indicating the names, vial positions, and injection volumes of the samples, references or SST solutions, and blanks. Most CDS systems allow the analyst to use different injection volumes in a single run, although most quality control methods require that the injection volume remain constant throughout. Moreover, before the results from any regulated sample analysis can be accepted, the HPLC system must pass acceptance criteria for SST to ascertain the readiness of the system to obtain accurate and precise results. Resolution, sensitivity, tailing factor, and retention time or area precision are common parameters to determine the suitability of the system for the chromatographic assay (3,21–22).

Data Acquisition and Real-Time Monitoring of Detector Signals

Before starting any sample analysis, it is important to prepare the HPLC system by purging and equilibrating the system and column with the mobile phases to ensure that the system pressure and detector baseline are stable (3). The analyst can perform these functions at the HPLC instrument using the instrument controller (a keypad) or an adjacent PC terminal in the laboratory. These functions can also be performed in the office remotely using a CDS, though no direct observations can be made for situations such as column leaks or mobile phase reservoir misplacements.

The sample sequence or queue is then started from the CDS, and data acquisition from the detector(s) is initiated immediately after the sample is injected from the autosampler. An analyst typically uses real-time monitoring at the CDS client to observe the chromatographic signals for the first few injections, and monitors the pertinent system parameters (pressure, baseline noise, peak retention time, and so forth) to ensure that the sample sequence is

running as expected before moving onto other tasks.

Figure 4 shows a screenshot of Waters Empower 3 CDS during real-time monitoring of a sample injected to an HPLC-UV–MS system. The top window displays the real-time signals from the UV and single-quadrupole MS total ion chromatogram with the active mass spectra displayed in the middle panel. The bottom window shows the status of the sequence and pertinent parameters of the operating modules.

Data Processing (Integration, Calibration, and Report Generation)

Data processing typically commences on completion of the entire sample sequence or the following day using an approved processing method, which includes appropriate peak integration (area threshold for peak start), peak identification (expected analyte retention time window), and calibration parameters (weight and concentration of samples and reference standards). In a CDS, information and instructions are contained in the processing method. A new processing method is created during method development, and can be revised later to optimize all parameters. Most analysts use the manual processing function in CDS (for example, in batch processing), unless the sample analysis becomes so reproducible that reports can be generated automatically. During the development of the processing method, the data processing step can be an iterative process, as the integration and calibration and quantitation parameters are optimized, particularly necessary for complex chromatograms. It is, therefore, important that the CDS records the different versions of the processing method during this procedure before the final processing method is used for reporting. No raw data or metadata can be erased or overwritten, as required by 21 *CFR* Part 11 regulations. Complete data traceability is a mandatory requirement for today's CDS.

Setting Integration Parameters

The built-in integration algorithm of a CDS is used to transform chromatography raw data into an integrated chromatogram (often called a *result file*) with peak retention time and peak area or height data (4). Figure 3 offers an example of the general process used in a typical CDS in the transformation from raw data to result.

The analyst first defines the integration start and end time, the narrowest expected peak width, the peak start threshold, and the detector noise level. This is typically using a "wizard" interface. The traditional integration algorithm tracks the detector baseline and looks for an increasing baseline "lift-off" to indicate the peak start of an emerging peak (Figure 5a) (1,4). It does so by comparing the slope of the data against a user-input threshold or slope sensitivity value.

Similarly, a change from a positive to a negative slope may indicate the apex or top of a chromatographic peak (Figure

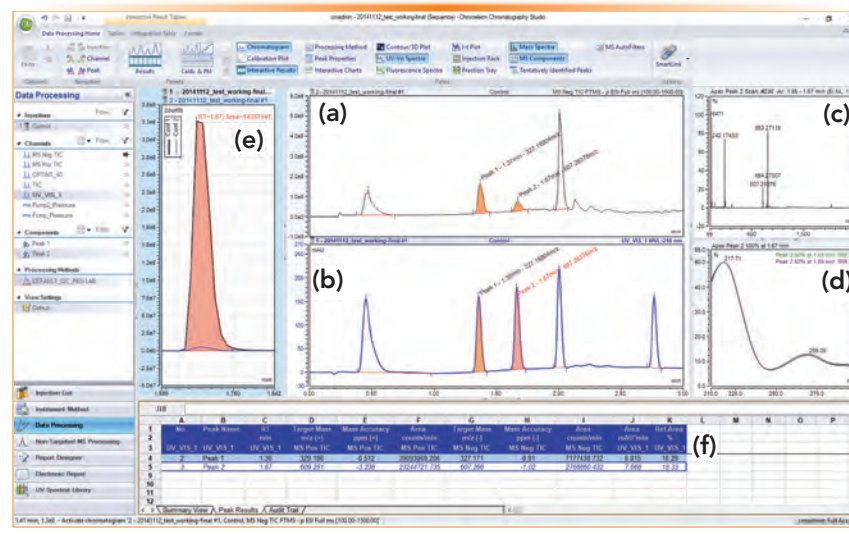

FIGURE 9: Screenshot from Chromeleon 7 CDS showing targeted screening for known components of interest using both HRMS (Thermo Scientific Q Exactive Quadrupole-Orbitrap Mass Spectrometer) and DAD. This CDS can process data from both MS and UV detectors and simultaneously view, analyze, and report HRMS and 3D UV data. The screen shows (a) the MS, and (b) UV channels, (c) MS and (d) UV spectra, (e) an overlay of the confirming ions, plus (f) the relevant peak results. (Figure courtesy of Thermo Fisher Scientific.)

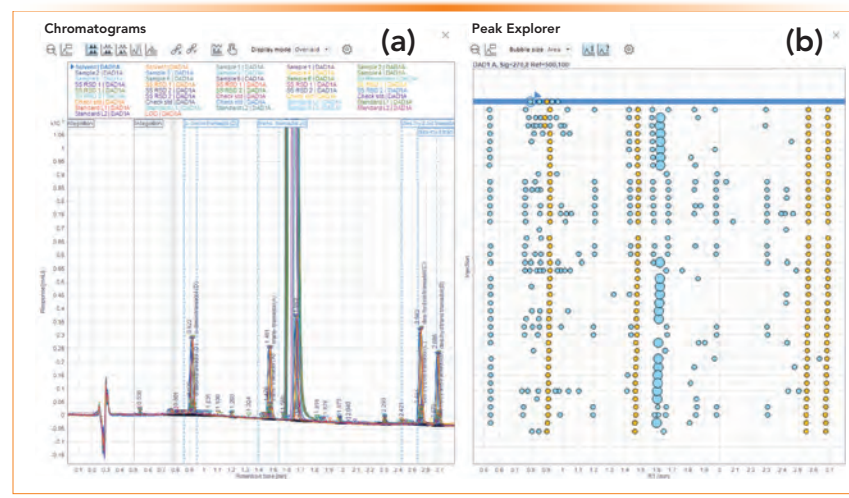

FIGURE 10: Screenshots from OpenLab CDS showing the display (a) of a large number of samples in the overlaid chromatograms view, and (b) Peak Explorer view. The latter allows easier visual detection of patterns, artifacts, outliers, and anomalies in a large sample set. (Figure courtesy of Agilent Technologies.)

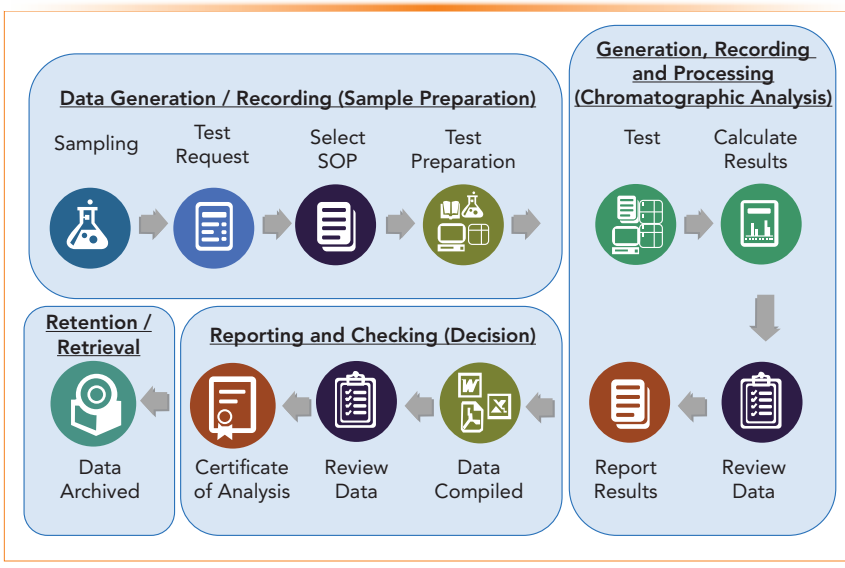

FIGURE 11: The total chromatographic workflow from sample and solution preparation, through the analyses and data review, creation of the CoA, and finally, the archiving of the data. The block on the right represents the typical analysis and CDS processes. The blocks before and after, represent the work performed by ELNs, paper notebooks, LIMS, inventory systems, and data management systems. It is through the seamless communication and transfer of information that we begin to realize the vision of the digital LoTF.

5b). Tick marks and projected baselines can be used to visualize how the CDS integrates the raw data. Because peaks broaden with retention time under isocratic conditions, raw data points are generally "bunched" to allow the appropriate settings of the lift-off thresholds (1,4).

While this traditional integration algorithm can work reasonably well for simple chromatograms, it may require substantial fine-tuning and optimization for a complex chromatogram with many merging peaks or sloping baselines. Most CDS offer options such as "valley-to-valley," "tangential skim," or "Gaussian skim" for these situations. Most CDS offer a "manual integration" option, but regulatory agencies discourage this somewhat subjective process, which can become problematic when the integrated peak is near specification limits. An improved algorithm using a second derivative approach, such as ApexTrack in Empower CDS or Cobra in Chromeleon CDS, appears to work well for both simple and complex chromatograms without user intervention (See examples in Figure 6) (4).

System Suitability Testing (SST)

The first section of the sample sequence in regulated testing is generally reserved for SST, which typically involves ten injections of SST solutions consisting of a blank, sensitivity verification, retention marker solution, reference standard A (2 injections), and reference standard B (5 injections) (3,21–22). The average response factors of the two reference standards must come within 2% to demonstrate the proper weighing of the reference materials. The peak area precision of the five repetitive injections should be set to <0.73% RSD to demonstrate system precision, as suggested by the *United States Pharmacopeia* (3,22), even though most laboratories still routinely use an acceptance criterion of 2.0% RSD. The tighter criteria are more appropriate because most HPLC systems can routinely achieve a precision level of 0.2–0.5% RSD, which is required for release testing of drug substances with potency specifications of 98.0–102.0%. An HPLC system with peak area precision of only 2.0% RSD will lead to many erroneous out-of-specification results just from the variability of the measurements.

Sample results cannot be used or reported for regulatory testing if there is a failure to meet any of the SST criteria defined (such as resolution, sensitivity, peak tailing, precision), (3). In this scenario, the analyst must document the results and investigate the root cause for SST failure, enforcing any remedial actions, and repeating the analysis.

Peak Identification, Calibration, and Quantitation

Peak identification is more commonly accomplished in HPLC-UV methods by matching the peaks in the sample with those in the reference standard within a stated retention time window (for example, <2% of the retention time of the reference peak). There are three types of commonly used quantitation approaches in HPLC: normalized peak area percent, external standardization, and internal standardization (3). Normalized area percent is often used for reporting impurities during early pharmaceutical development (3,23). External standardization using a single-point calibration of a reference standard is used for potency assays of drug substances and drug products (3,4). Internal standardization is used by spiking the sample with an internal standard to compensate for loss during sample preparation. For bioanalytical testing using LC–MS/MS, an isotopically labeled internal standard is typically used to correct for both MS ionization suppression and sample preparation recovery.

A response factor calculation (such as peak area or amount) is generally used for external standardization, assuming that the response factor is the same for a specific analyte in the reference standard and the sample. A bracketed calibration standard approach is used after a certain number of injections (for example, ten samples) in a long sequence in regulated testing (3,21).

Result Table and Inclusion of both UV and MS Spectral Data

An important time-saving feature of a modern CDS is the integration of spectral data from both diode array detector (DAD) and MS instruments and the ability to automate the insertion of such useful

information into a peak table (4). Figure 7 shows the screen display of a result file from a processed sample injection of a retention marker solution using DAD and MS detection. The displayed chromatograms include automated annotation of peak names, retention time, and the parent MS peak (M+1) of each analyte, and a 2D contour map from the DAD detector with UV spectra in the right-hand panel. The peak table includes data such as peak name, retention time, area, height, and area%, plus additional spectral data of λ_{max} and parent MS peaks, and calculated parameters such as relative retention time (RT ratio), USP resolution and tailing factor, and signal-to-noise ratio (S/N) (3). A modern CDS allows customization of the result table with a display of the correct number of significant figures, as shown in Figure 7.

Generation of Formatted Reports

The final data processing step can be the generation of a report of a sample or the entire sample sequence for data review and archival. A reporting template is generally used, and the final report can be customized to generate the information required by the company or regulatory agency, which may include specialized calculations (for example, custom fields). A final report may include sample information (batch number, sample i.d., analysis date, result and sequence i.d., method i.d.), peak tables, chromatograms (full scale and expanded scale), spectral data (UV and MS), and pass or fail sample status against specifications.

Another type of CDS report is a summary report that extracts results from a group of samples and performs a calculation or statistical evaluation (such as repeatability of injections for peak area). Most CDS supports the use of standard report templates to facilitate report regeneration of routine assays.

Data Archiving, Data Review and Sign-off, Export to LIMS, and CoA Generation

All raw and metadata from a CDS for regulated testing must be archived, backed up and secured in compliance with 21 *CFR* Part 11 regulations with a high degree of data security, traceability, and integrity (2,7). Raw data cannot be deleted, overwritten, or altered. Critical metadata such as methods and processed data (results) cannot be deleted but can be revised with the date and version stamps to allow traceability. The CDS reports are reviewed and signed-off by the designed reviewers or approvers (such as the QC manager).

An electronic signature process is more commonly used after the review process of the CDS data in regulated testing laboratories, typically during the review and sign off of the laboratory notebook. An approved report should not be deleted.

Many CDS have automated exporting functions that export the approved chromatographic data to a LMS or LIMS, which can then generate an official CoA of the sample after merging data from other sources (3). The CoA of the drug substance or drug product sample is then further reviewed by QA for the official release of the batch for further development, clinical trials, or the market. Data are retained according to regulations and the corporate quality SOPs.

Recent Trends in CDS Technologies

Modern CDS networks are sophisticated informatics systems incorporating 40 years of advances in software, networking, and database technologies. Most leading CDS have desirable features and characteristics that are listed in Table I. Some recent prominent trends are described here.

Database Technologies

Early CDS used a directory structure called flat-file systems such as those used in MS-DOS operating system with folders and sub-folders in a hierarchical organization (Figure 8a). Although this file system worked well for small deployments, it proved inadequate for larger installations. The potential issues surrounding accidental deletion, data being overwritten, data traceability, and disaster recovery were significant. This was especially true when raw data were reprocessed multiple times with modified versions of the processing method, creating multiple result files derived from the same raw data.

One solution is the use of a relational database, which was first pioneered by Waters Corporation with the introduction of Millennium CDS in 1992, a predecessor to Empower CDS (4). Currently, all leading CDS manufacturers such as Waters, Thermo Fisher, Agilent, Shimadzu, and Justice Laboratory Software (Chromperfect) support the use of database technologies (Oracle, SQL server, or both).

Using relational database technology (Figure 8b) brings three significant benefits:

- Databases can "date and time stamp" all information. This makes accidental overwriting of raw data and methods less likely.
- The relational database ties all "metadata" together, covering all aspects of data acquisition, data processing, result generation, review, and approval. It provides a necessary audit trail as methods are modified, data reprocessed, and system settings changed.
- They provide faster and simpler mechanisms for data retrieval and management.

Instrument Control and Diagnostics

Most instrument manufacturers have moved away from proprietary control protocols and have begun using communication protocols like Ethernet to provide full, bidirectional instrument control capabilities to the CDS analyst. This enables laboratories to have a true single-point, single-keyboard control of their chromatographic systems while also providing enough data bandwidth to accommodate information-rich detectors like DAD and single-quadrupole MS (see example in Figures 4 and 7). For most CDS vendors, high-resolution MS, such as time of flight (TOF) instruments, still require their own control and data-handling software or workstations.

CDS can also provide enhanced analytics for instrument diagnostics, maintenance, troubleshooting, and service information, including online manuals, videos, and links to web resources. As modern analytical instruments are designed with sophisticated onboard diagnostics, many CDS are capable of identifying problems and even problem remediation by real-time actions, such as stopping a running

sequence and shutting down the instrument, if necessary.

Improved Integration of UV and MS Data

Another active area in CDS development is the improved integration of UV and MS data by many CDS manufacturers. UV detection, the standard for pharmaceutical analysis, can be effectively supplemented by MS detection during method development and sample analysis for definitive peak identification. Many modern CDS support the seamless control of their own brands of single-quadrupole MS with displays of spectral and ion current signal from the MS (total and selective ion) in addition to automatic annotations of parent ions in the UV chromatograms and result tables (case studies shown in Figures 4 and 7). This is particularly important as newer MS systems are becoming more compact and easier to use by chromatographers without requiring specialized MS training.

There is a growing trend for CDS to include support for triple-quadrupole MS and HRMS, and as such, these MS instruments often require their specialized data systems (such as the Waters MassLynx and Agilent MassHunter, which also have their own HPLC instrument control software). However, Chromeleon CDS has made significant advances in this area, providing the ability to acquire, process and report data from triple-quadrupole MS, HRMS, and chromatography instruments with a single software platform solution.

Figure 9 illustrates the growing trend of incorporating MS capabilities with Chromeleon CDS displaying both high-resolution accurate mass and UV spectral data.

More Efficient Data Review

Given that separation systems have enjoyed major advances that have significantly reduced chromatographic run times, they allow for larger amounts of chromatographic data to be collected. As a result, laboratories now process and review very large chromatography data sets, which sometimes contain thousands of peaks. Data review tasks typically rely on manual interpretation of chromatograms, peak integration baselines, calibration curves, and calculated results to ensure they fall within specifications. Further, any incident or anomaly that negatively affects production requires immediate investigation of these data to allow fast problem resolution. When presented correctly, the human eye is powerful in its ability to identify anomalies in large data sets. As shown in Figure 10, Peak Explorer, an OpenLab CDS data analysis capability, is specifically designed to present chromatographic data in a format optimized for visualization by the human eye. By presenting chromatographic data and results in a single helicopter view, users can easily and rapidly detect artifacts, outliers, and patterns.

Links to Software Tools and Informatics Systems

HPLC method development is a time-consuming task that demands considerable skills and efforts from an experienced scientist using the one-factor-at-a-time approach (3,24). Popular HPLC method development software (such as Fusion QbD from S-Matrix, ChromSword Developer from ChromSword, or ACD/AutoChrom) often works together with CDS to expedite or automate the method development process. For instance, Fusion QbD can utilize a design of experiments (DoE) approach to expedite a systematic method development process and work directly with many CDS (Empower, Chromeleon CDS, and OpenLab) by creating and downloading a sequence of methods of varied parameters. After the sequence result data are processed, the software can import the results back from the CDS and perform further statistical analysis to display the optimum separation conditions (24).

Similarly, software to expedite method validation is available such as Empower Method Validation Manager (25) from Waters. This is a workflow-based tool that manages the entire method validation process, from protocol planning to the final reporting. This software tool displays the status of ongoing validation studies, tracks corporate requirements, and acceptance criteria while flagging any out-of-specification results. All statistical calculations are performed within Empower 3, eliminating data transcription errors.

The ICH Method Validation Extension Pack, offered by Chromeleon CDS, can also be used to expedite the method validation process, providing the user with predefined templates and customizable workflows that have been developed in accordance with the guidelines and specifications outlined by The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH).

For laboratories performing frequent method development and validation studies, these automated tools can have a significant impact on productivity by saving time and documentation efforts.

Cloud Computing

Cloud computing is one of the most active areas of development for today's CDS manufacturers. Most readers of this article are probably using a CDS product that is running locally or in your company's data center. This is referred to as "on-premises." In this model, your information technology (IT) organization manages the server hardware, the laboratory hardware (acquisition devices and PCs), and the application, including all support and product upgrades. Cloud services are categorized as IaaS, PaaS, or SaaS, with increasing computing, operating system, networking, and archiving activities conducted in the cloud. As organizations try to reduce capital expenses for computers and infrastructure, there is also a big push toward business agility.

Companies like Thermo Fisher and Waters already offer CDS products that can be deployed using cloud services from Amazon (AWS) and Microsoft (Azure). Some of the other key benefits are dynamic scalability, easier access to remote sites, greater levels of security, and a level of disaster recovery that is difficult to attain with an on-premises deployment (26).

The Paperless Laboratory, Laboratory of the Future, Artificial Intelligence and Machine Learning

One often wonders if the Paperless Laboratory (27), Laboratory of the Future (LoTF) (28), Smart Laboratory, and Artificial Intelligence are truly attainable goals.

Today, we are much closer than ever to succeeding in these projects and achieving a true digital laboratory of the future. A recent, multi-year study performed by Gartner Research estimates that, by 2022, 40% of the top 100 pharmaceutical companies will establish digital technology platforms for R&D (28). Core to a LoTF strategy is treating laboratory-related information as an asset. This will be accomplished by linking laboratory data and activities across platforms and diverse business processes.

As more companies rely on technologies like Electronic Lab Notebooks (ELN), scientific data management solutions (SDMS), inventory systems, and of course, LMS/LIMS, the CDS remains a key focal point of the laboratory. When you consider the increased focus on data integrity, data review and approval, laboratory analytics, data lifecycle management, and reducing infrastructure complexity, we see some important changes coming. If you look at the last few years of CDS evolution for companies like Waters, Thermo Fisher, Agilent, or Shimadzu, you see some common themes. Multi-vendor instrument control has become a necessity for most organizations, as it is impractical for many laboratories to standardize on instrumentation from a single vendor. Of growing importance is the need for integrated laboratory solutions that go beyond the simple chromatographic workflow of the CDS alone. (See Figure 11,)

Integrating the CDS workflow into the broader laboratory process is not a new concept. LIMS vendors have been doing this for many years by transferring sample work lists to the CDS and retrieving the results after the analyses are complete. What has been missing is all of the valuable metadata that surround key laboratory activities like sample and solution preparation, balance and pH meter calibrations, adherence to approved SOPs, and compiling all of the non-CDS data that may be required to approve and release the final product. ELN vendors have also been busy trying to improve their integration with CDS as a way to better document the entire laboratory workflow, reduce the amount of peer review required, and improve the overall data integrity of the analyses being performed. In recent years, you may have heard terms like *right first time* and *review by exception*. Both terms point to the need for better laboratory process control and streamlining data review, all with the goal of preventing common errors, increasing laboratory efficiency, and improving overall data quality and data integrity. The major pitfalls to universal implementation remain a lack

of common standards in ELN, CDS, and LMS/LIMS, plus a tendency for underestimating the difficulties to obtain consensus between different departments in a global organization.

The last few years have seen significant activity from Agilent, Thermo Fisher, and Waters to try to address these issues. These vendors have looked at their product portfolios, and either made product acquisitions or tailored their existing products to more effectively connect with their own CDS. These newly created solutions significantly enhance the basic capability of their standalone CDS. All of these integrated solutions revolve around delivering four key benefits to the laboratory and the business:

- Extend the chromatography workflow to include sample management, sample and solution preparation, adherence to approved SOPs, improved data review and approval, reporting, and data archiving.
- Provide complete traceability for the entire process, not just the chromatography. This greatly simplifies the auditing and troubleshooting in the laboratory.
- Provide an improved user experience with functionality such as simple dashboards or landing pages that help guide the laboratory analyst.
- Provide data review tools (such as data visualization, trending analysis) that facilitate the real-time identification of areas in the process that may be out of specification or out of trend, and require immediate attention (example in Figure 10).

These product enhancements are the direct result of an ever-changing laboratory. All industries are experiencing greater demands on productivity, more stringent regulations for the laboratory, more complex analyses, and an increasing focus on quality. The move towards the digital LoTF is now becoming a reality. Utilizing artificial intelligence (AI) and machine learning within a cloud infrastructure enhances data integrity, data review, and approval. This type of modern architecture also provides the framework for improved laboratory analytics and data lifecycle management, all while dramatically reducing infrastructure complexity.

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This installment is the last of a series of four articles on HPLC modules, covering HPLC pumps, autosamplers, UV detectors, and CDS (29–31), which present updated overviews to the reader. The decision to collaborate with experts from manufacturers was necessary and pivotal to have an insider's view of these sophisticated modules. The process of working with scientists with a different perspective was challenging at times but proved to be rewarding. I would like to give special thanks to the first authors of the series: Konstantin Shoykhet of Agilent (pumps), Carsten Paul of Thermo Fisher Scientific (autosamplers), and Robert Mazzarese (CDS), formerly with Waters, for spending many hours on these comprehensive articles.

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FOCUS ON FOOD ANALYSIS

Studying Migration of Packaging Components into Food

The potential of food packaging components to migrate into food is an important health concern. Perfecto Paseiro Losada and his group at the University of Santiago de Compostela, in Spain, have been investigating the migration kinetics and actual migration of such compounds into a variety of types of food. They also have been carrying out studies to estimate dietary exposure. Paseiro recently spoke to *LCGC* about this work.

Laura Bush

You conducted a study on the identification of intentionally added substances (IAS) and non-intentionally added substances (NIAS) in plastic food packaging materials and their migration in food products (1). First, for those unfamiliar with the terms, can you explain the difference between these two types of substances? How do you determine what substances are important to quantify?

Both terms have their origin in the current European legislation on plastic materials for food contact (Regulation 10/2011). Only the substances included in the European Union (EU) list of authorized substances (such as monomers or additives) may be intentionally used for the manufacture of food contact materials, and these substances are commonly known as IAS. An example of an IAS is bisphenol A, used for the synthesis of polycarbonate or erucamide used as a slip additive in polyolefins. In principle, safety of IAS is evaluated before authorization, and they are subject to restrictions, so that their migration into food does not endanger human health.

NIAS are defined as "an impurity in the substances used or a reaction intermediate formed during the production process or a decomposition or reaction product." NIAS are not in included the EU List, but they may be present in the plastic materials, and must be assessed in accordance with internationally recognized scientific principles of risk assessment.

In the study, you used a non-targeted approach with gas chromatography–mass spectrometry (GC–MS) to identify compounds in the plastic packing materials. Why did you use GC–MS, and why a non-targeted approach? Are you able to detect and quantify the most important compounds using this method?

We focused the research on trying to detect any volatile or semivolatile substances present in plastic packaging samples, and GC–MS is the most appropriated technique. We use two approaches; dynamic headspace sampling and purge-and-trap for volatiles, and splitless injection mode after sample liquid extraction for semivolatiles.

The two approaches complement each other, and they give a very complete view about what volatile and semivolatile substances are present in the packaging, thus obtaining very useful information on what substances could potentially migrate to food. About 100 volatile and semivolatile compounds were detected using the two techniques.

Migration tests were carried out using Tenax and isooctane. Through those tests, 27 compounds were detected, and their relative amounts were estimated against an internal standard.

What performance were you able to achieve with the methods used in the study, in terms of limits of detection and repeatability? How did you verify this?

Method performance was very good, in terms of linearity, recovery, repeatability, and limits of detection and quantification. Recovery in foods (corn snacks, potato snacks, cookies, and cakes) was nearly 100% for most of the selected compounds, with a range between 82.7 and 116.1%, and the relative standard deviation (RSD) derived from the replicate concentrations measured in spiked foodstuffs ($n = 6$) was less than 9% for most of compounds, with range of 2.22–15.9%. Most of LODs were less than 0.003 mg/L.

What did you find in terms of which compounds migrated into food, and at what levels? Why are these compounds important as related to human health? Are these compounds known to be harmful?

All compounds selected—bis(2-ethylhexyl) phthalate (DEHP), diethyl phthalate (DEP), diisobutyl phthalate (DIBP), dibutyl phthalate (DBP), butylated hydroxytoluene (BHT), acetyl tributyl citrate (ATBC) and benzophenone (BP)—were previously identified in packaging materials. All compounds were found in at least some of the 34 analyzed food samples. ATBC was the most common (in 94% of the samples), and BHT the least common (in 12% of the samples).

The highest concentrations were found were in corn and potato snacks: ATBC (7.09 µg/g), DEP (1.44 µg/g), DEHP (0.57 µg/g), BP (0.2 µg/g), DBP (0.77 µg/g), DIBP (1.51 µg/g) and BHT (6.58 µg/g). DEP and DEHP were the most frequently detected phthalate

compounds in the food samples; the specific migration limit (SML) of 0.3 mg/kg established in Regulation 10/2011 was exceeded for DBP in one sample.

Phthalates are a group of chemicals of current concern for human health. They are known to be endocrine disruptors that affect the production of hormones, especially testosterone, and some studies associate them with infertility, obesity, and asthma.

ATBC is a widely used plasticizer to substitute for phthalates. BHT is an antioxidant also used as food additive, and BP is a photoinitiator, but, at the levels found, they do not represent a health concern.

You carried out a related study of the nontargeted analysis of IAS and NIAS and their migration into food simulants, using purge-and-trap GC–MS for volatile compounds, and extraction with organic solvents followed by GC–MS for semivolatile compounds (2). Why are food simulants used in a study like this? And what food simulants did you choose and why? How do you know that such studies correlate closely with the real-world conditions for packaged foods?

A *food simulant* is a test medium imitating food; in its behavior, the food simulant mimics migration from food contact materials. Simulants are much simpler analytical matrices than food; their use facilitates identification and quantification of migrants, and more reliable information is obtained about what substances migrate, or may migrate, to food. The nontargeted analysis we carried out would be very difficult to execute in the various food matrices.

Tenax is a food simulant for testing specific migration into dry foods, and isooctane is one of the well-known food simulant substitutes for fatty foods.

What conclusions have you drawn from your studies of the migration of packaging compounds into foods, and the perceived potential harm these of compounds to human consumers?

Many volatile and semivolatile chemicals are present in the analyzed packaging samples, including both IAS and NIAS, some of which are of very high concern (such as 2,4 and 2,6 toluene diisocyanate), although most of them did not migrate to the selected food simulants. In our opinion, the levels found in the studied foods and food simulants were low. However, for a complete estimation of chemical exposure other types of packaging and food must be considered and assessed by experts in risk assessment.

You have also studied the migration of two common components of UV-curing inks—known as photoinitiators—into food simulants (3). Why are these compounds of particular concern in terms of their health risks?

The Rapid Alert System for Food and Feed (RASFF) has reported many cases of different components of UV curing inks in foodstuffs in recent years. Photoinitiators are constituents of many printing inks applied on the non-food-contact side of food packaging. Photoinitiators may reach the food contact side, among other ways, by set-off (such as transfer of wet ink from the substrate film to another surface of the plastic film that comes in contact with the food). During storage these inks may also penetrate, by diffusion, into the internal film layer intended to come into contact with foods, which is usually made of PE. After the internal film layer has come in contact with food, the photoinitiators may migrate into the food.

The main reason for this research was to characterize the process of migration of two common UV ink components from PE into food simulants.

What type of mathematical modeling did you use in the study? Why was this specific approach selected?

We used a model based on Fick's second law, specifically the solution proposed by Crank for diffusion in a plane sheet from a stirred solution of limited volume. This solution is broadly accepted as a model for the migration of a substance from a plastic layer into a well-mixed liquid.

What did the study reveal about the migration kinetics of these compounds at the four storage temperatures studied? Does the food composition itself determine the migration kinetics?

We determined key parameters of migration (diffusion coefficients and partition coefficients) for 4-methylbenzophenone (a photoinitiator) and ethyl-4-(dimethylamino) benzoate (a coinitiator) from LDPE by fitting the experimental data with the mathematical model based on Fick´s second law. The model may be used to predict the migration process of those migrants. Furthermore, key parameters of the Arrhenius equation (activation energy and pre-exponential factor) were estimated and they can be used to cal-

culate diffusion coefficients at any temperature between 40 °C and –4 °C.

We also studied the migration at freezer storage temperature (–18 °C) into 50% and 90% ethanolic simulants, because very scarce information on migration kinetics at that temperature has been reported. Results showed that migration also happen in a great extension into both simulants.

In this study, foods were not included the composition and physico-chemical properties of each food affect the migration kinetics, especially the partition coefficient (polymer/food). On the other hand, for many foods, the diffusion coefficient of the migrant into the food should be estimated and included in the model, a circumstance that in the case of simulants is not considered significant.

You have also carried out research to estimate dietary exposure to packaging contaminants among the Spanish population from cereal-based foods (4). What foods and compounds did you study, and why? Is packaging compound migration a key health concern, more so than contamination of foods from pesticide residues?

Cereal-based foods are among the most consumed among the population groups studied. Representative food sample pools for each age group were prepared by combining amounts of rice, bread (toasted and not), and alternatives to bread, pasta, and breakfast cereals, according to consumption data obtained from Spanish national dietary survey on children and adolescents (ENALIA).

The chemicals selected comprise a wide range of substances, all of them previously identified in food packaging, mainly plasticizers such as phthalates (dibutyl phthalate, diisobutyl phthalate, diethyl phthalate, and benzyl butyl phthalate). Other substances include citrates (ATBC), adipates (DEHA), UV stabilizers (octocrylene), and slip agents (erucamide).

Chemical migration from packaging to foods is an inevitable process. The key point is to ensure that the amounts of substances that migrate to food do not endanger human health. When this principle is not achieved, then there is a health concern. The important thing is that the type and amount of chemical migrants from packaging, pesticides, or other chemicals in foods do not generate a health concern.

What were the analysis conditions of the GC and LC methods used in the study?

For GC, a ZB-5MS (30 m × 0.25 mm x 0.25-µm) column, splitless injection mode and oven temperature from 40 to 300 °C. For MS, full scan mode (*m/z* range of 35–500). For HPLC, a Kinetex biphenyl column (100 mm × 3 mm x 2.6-µm) at 30 °C with a mobile phase composed of methanol and water, both containing 0.1% (*v/v*) formic acid, gradient elution method from 30% water and 70% methanol to 100% methanol was used. For MS/MS, positive ESI mode and for each compound precursor ion was selected and two product ions, one for quantification and other for qualification purposes.

How did you optimize the sample preparation or extraction procedures for both the GC–MS and the LC–MS/MS methods?

In comparative extraction studies, acetonitrile got better recoveries for all analytes, and it was selected as the extraction solvent; the extraction time and solvent concentration steps also were optimized, especially the latter to avoid irreproducible results and loss of some analytes.

What further research would you propose in the field of migration of packaging chemicals into foods? Would you propose a healthier form of packaging and would food companies be receptive to changing their packaging methods?

From an analytical point of view, it is necessary to develop methodologies that facilitate the detection, identification, and quantification of any substance that migrates to food, especially for non-volatile compounds. There is still a lot of research to be done.

Many chemical substances of unknown or variable composition, complex reaction products, and biological materials (UVCB substance) are used for the manufacture of food contact materials (plastics, coatings, inks, adhesives, paper and paperboard, etc.), among them many resins containing prepolymers with reactive oligomers with a MW less than 1000 Da, therefore they are chemical hazards of concern.

In the framework of EU plastics regulation, prepolymers are authorized generically if they are used as starting substances and are synthesized from monomers already included in the EU list. These substances have not been evaluated before authorization, and they are not included as other not-listed substances (such as NIAS or aids to polymerization) to be assessed in accordance with internationally recognized scientific principles on risk assessment and they may migrate to foods without specific restrictions.

The positive identification, quantification, and safety assessment of hundreds, probably thousands, of unknown substances is a huge challenge in this field, because for most of them there are no in-standard MS-libraries and analytical standards are not available.

Polymeric materials used in food contact packaging have solved many food safety problems of the past, but they have also generated new problems related to the migration of chemical substances to food. At present, there is no realistic alternative to the use of polymeric materials in food packaging. Updated legislation is needed that does not generically authorize substances that have not been previously evaluated; we also need enforcement to ensure compliance with the legislation.

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Determination of PAHs in Combustion-Related Samples via Multidimensional Chromatographic Methods

Normal-phase liquid chromatography (LC) retention behavior was investigated for 239 polycyclic aromatic hydrocarbons (PAHs) on an aminopropyl (NH_2) stationary phase. Retention behaviors were used to develop a normal-phase LC fractionation procedure for complex combustion-related samples prior to analysis with gas chromatography-mass spectrometry (GC–MS). Reversed-phase LC with stop-flow fluorescence (FL) and constant energy synchronous fluorescence spectroscopy (CESFS) capabilities were explored to determine PAH isomers of molecular mass (MM) 302 g/mol in normal-phase LC fractions. The combination of these analytical methods allowed for the determination of PAHs in three combustion-related standard reference materials (SRMs): SRM 1597a (coal tar), SRM 1991 (coal tar/petroleum extract), and SRM 1975 (diesel particulate extract).

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Polycyclic aromatic hydrocarbons (PAHs) are a large class of environmental pollutants originating from natural and anthropogenic sources. PAHs with a molecular mass (MM) of 302 g/mol are of particular concern, due to their potential carcinogenic and mutagenic properties along with low biodegradability characteristics, namely, dibenzo[*a,l*]pyrene (DBalP), the most carcinogenic PAH tested to date. Unambiguous determination of DBalP and other MM 302 PAH isomers is imperative for accurate ecotoxicological assessment. PAHs have been identified in a variety of complex environmental matrices, such as coal tar, urban particular matter, and marine sediment, among others. The National Institute of Standards and Technology (NIST) provides an assortment of PAH-containing natural matrix standard reference materials (SRMs) such as SRM 1597a (coal tar), SRM 1991 (mix coal tar/petroleum extract), and SRM 1975 (diesel particulate extract). These SRMs are well-characterized, and often used for validating current and new analytical methodologies for PAHs.

Methods for the separation and identification of PAHs typically employ reversed-phase liquid chromatography (LC) with fluorescence detection (FL) or gas chromatography–mass spectrometry (GC–MS). Excellent PAH separation has been observed using polymerically bonded octadecylsilane (C18) and 50% phenyl columns for reversed-phase LC (1) and GC–MS (2) conditions, respectively. However, structural isomers having similar retention times pose significant challenges, especially with GC, when these isomers have virtually identical mass fragmentation patterns. Normal-phase LC is another separation technique known for excellent separation of isomeric groups; however, low resolution of isomers groups with normal-phase LC makes accurate determination of specific PAHs a challenging task (3). Chemically bonded and polar functionalized stationary phases, such as aminopropyl (NH_2), have been a practical approach for a class (number of aromatic carbons) fractionation prior to a secondary separation step (4). Table I summarizes the MM 302 PAH isomers identified in SRM 1597a (5), SRM 1991 (6), and SRM 1975 (7) via the traditional analytical methods described above. Herein, we explored the benefits of normal-phase LC fractionation as a sample clean-up step prior to further isomeric PAH separation and determination by both reversed-phase LC-FL and GC–MS (8–11). The combination of these analytical techniques provides a multidimensional approach to eliminating coelution and spectral interferences for PAH analyses.

Materials and Methods

Authentic PAH reference standards, SRM 1597a, SRM 1991, and SRM 1975 were obtained from multiple sources summarized elsewhere (8,9). Complete normal phase LC retention behavior was investigated for 239 PAHs, containing two to seven aromatic rings. Retention index values were determined for each PAH based on triplicate injections under normal-phase LC conditions. Separations were carried out on an NH_2 analytical column, purchased from Waters, with the following characteristics: 25.0 cm length, 4.6 mm inner diameter, and 5 µm average particle diameters. An isocratic mobile phase of 98% *n*-hexane, 2% dichloromethane (DCM), and a 1.0 mL/min flow rate was utilized.

Normal-phase LC fractionation was completed using a Varian 9012 LC system (Agilent) with a Jasco UV-1570 Intelligent UV-vis detector. Normal-phase LC separations were carried out on an NH_2 semipreparative column (Waters) with the following characteristics: 250 mm length, 10 mm inner diameter, and 5 µm average particle diameters. SRM 1597a was injected using a 250 µL sample loop with a mobile phase of 98% *n*-hexane, 2% dichloromethane (DCM), and a 4.0 mL/min flow rate. Fourteen fractions were collected from SRM 1597a, SRM 1991, and SRM 1975 using an in-house system over a 90 min separation interval, and evaporated with N_2 to match injection volumes.

All SRM normal-phase LC fractions were analyzed directly using a

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6890 series GC instrument (Agilent Technologies) using an HP 5973 quadrupole mass spectrometer with electron ionization (EI) (Agilent). GC separations were completed on a SLB-PAHms 50% phenyl stationary phase column with the following characteristics: 0.25-µm film thickness with a temperature maximum of 360 °C. PAH isomers were determined in each of the 14 fractions using selected-ion monitoring (SIM) mode. Peak identification in normal-phase LC fractions was determined by retention times and predominant mass ion peaks of authentic reference standards. The oven was temperature programmed to be isothermal at 100 °C for 1 min, 45 °C/min to 200 °C, 2 °C/min to 310 °C for 130 min, 45 °C/min to 325 °C, and isothermal at 325 °C for 60 min.

All reversed-phase LC-FL and reversed-phase LC-constant energy synchronous fluorescence spectroscopy (CESFS) analyses were carried out on an Ultimate 3000 Dionex HPLC system (Thermo Scientific) using an online degasser, a pump, and a UV-vis detector, along with a FL detector. Separations were performed on a Zorbax Eclipse polymeric PAH C18 column (Agilent) with the following characteristics: 250 mm length, 4.6 mm diameter, and a 5-µm average particle diameter. SRM 1597a fractions containing MM 302 PAH isomers (as determined by GC–MS) were separated using a mobile phase of 100% acetonitrile with a 1.5 mL/min flow rate. FL and CESFS spectral collection was obtained by using a stop-flow parameter in the instrumental software at the apex of each PAH chromatographic peak, which takes roughly 10–20 s per analyte. Quantitative reversed-phase PLC-CESFS measurements were performed using a linear regression model for synthetic mixtures of 13 targeted MM 302 PAH isomers prepared in toluene at concentration levels similar to Table I for SRM 1597a.

TABLE I: Mass fractions (mg/kg) of the MM 302 PAH isomers listed in the three SRM certificates of analysis

PAH	Abbreviation	Coal Tar		Diesel Particulate
		SRM 1597a (1)	SRM 1991 (6)	SRM 1975 (7)
Benzo[*a*]perylene	BaPer			
Benzo[*b*]perylene	BbPer	9.04 ± 0.99		
Dibenzo[*a,e*]pyrene	DBaeP	9.08 ± 0.39	0.105 ± 0.007	
Dibenzo[*a,h*]pyrene	DBahP	2.57 ± 0.30	0.027 ± 0.005	
Dibenzo[*a,i*]pyrene	DBaiP	3.87 ± 0.34		
Dibenzo[*a,l*]pyrene	DBalP	1.12 ± 0.17		0.010
Dibenzo[*e,l*]pyrene	DBelP	2.72 ± 0.17		
Dibenzo[*a,k*]fluoranthene	DBakF	3.21 ± 0.31		
Dibenzo[*b,e*]fluoranthene	DBbeF	0.98 ± 0.02		0.041
Dibenzo[*b,k*]fluoranthene	DBbkF	11.2 ± 0.80	0.122 ± 0.006	0.027
Dibenzo[*j,l*]fluoranthene	DBjlF	6.5 ± 1.40		0.011
Naphtho[1,2-*a*]pyrene	N12aP			
Naphtho[1,2-*e*]pyrene	N12eP			
Naphtho[2,1-*a*]pyrene	N21aP	10.2 ± 0.90		0.011
Naphtho[2,3-*a*]pyrene	N23aP	4.29 ± 0.89		
Naphtho[2,3-*e*]pyrene	N23eP	4.31 ± 0.44		
Naphtho[1,2-*b*]fluoranthene	N12bF	8.6 ± 2.00		0.058
Naphtho[1,2-*k*]fluoranthene	N12kF	10.7 ± 1.20		0.027
Naphtho[2,3-*b*]fluoranthene	N23bF	3.52 ± 0.30		
Naphtho[2,3-*j*]fluoranthene	N23jF			0.027
Naphtho[2,3-*k*]fluoranthene	N23kF	2.07 ± 0.06		

Results and Discussion

The schematic diagram for the analytical approach utilized in this work is detailed in Figure 1. SRMs were separated first by normal-phase LC and collected into 14 fractions. The normal-phase LC chromatogram for SRM 1597a with labeled fractions (F) is shown in Figure 1a. Each fraction was qualitatively analyzed by GC–MS in SIM mode to identify PAHs containing two to seven aromatic benzene rings (9). Quantitative GC–MS results after normal-phase LC fractionation have been published elsewhere by NIST (5–7), and was not a focus of this study. The MM 302 PAH isomers present in the three SRMs eluted in F10, F11, and F12. Figure 1b details the GC–MS chromatograms for F10, F11, and F12 for SRM 1597a, with the nearly identical mass spectra for DBalP and dibenzo[*j,l*]fluoranthene (DBjlF) shown in Figure 1c. After a comprehensive GC–MS evaluation of the isomers present in SRM 1597a, the three fractions containing MM 302 PAH isomers were then analyzed by reversed-phase LC with fluorescence detection (Figure 1d). The reversed-phase LC mobile phase was stopped at the apex of each chromatographic peak of interest for excitation/emission and synchronous emission spectral collection. Spectra collected in F10, F11, and F12 were compared with authentic reference standards for identification (Figure 1e). The reversed-

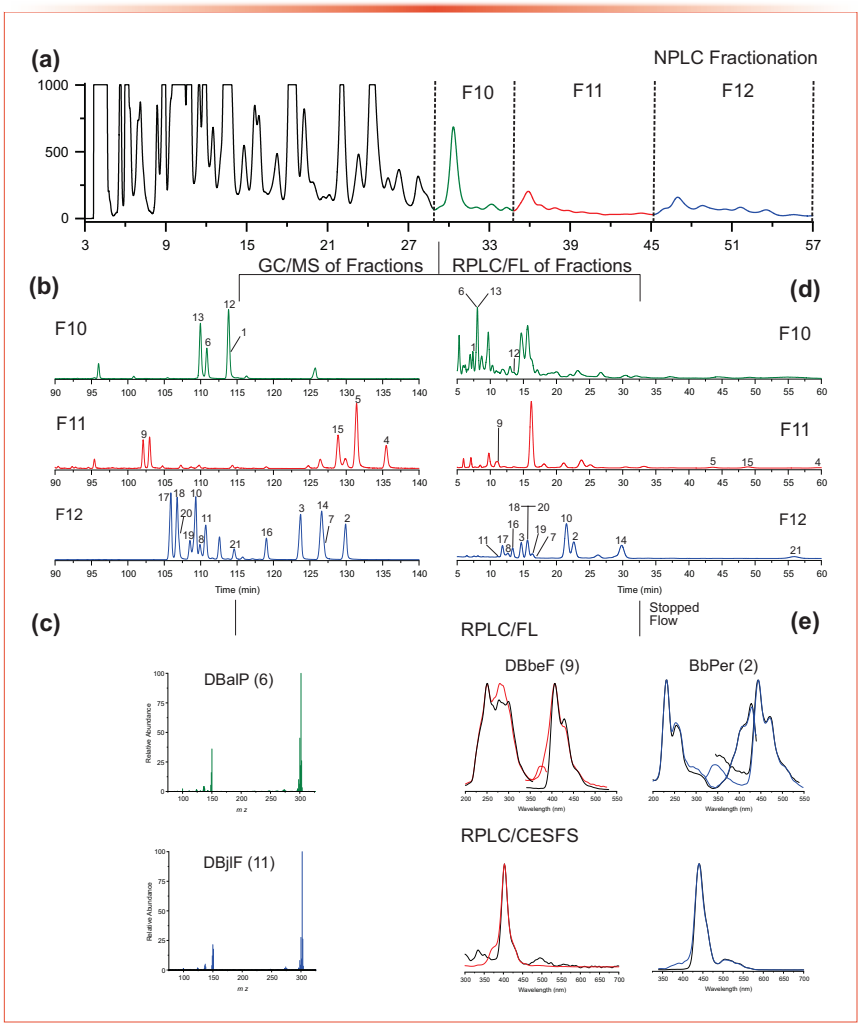

FIGURE 1: Schematic diagram for the separation and identification of MM 302 PAH isomers in SRM 1597a. The numbers correspond to the specific MM 302 PAHs in Table II and their molecular structures are published elsewhere (11). (a) Three fractions (F10, F11, and F12) were collected during the normal-phase LC separation of SRM 1597a. (b) All three fractions were qualitatively analyzed by GC–MS for the presence of MM 302 PAH isomers. (c) Mass spectra for DBalP and DBjlF. (d) All three fractions were further analyzed quantitatively by reversed-phase LC-FL. (e) Under stop-flow conditions, excitation/emission and synchronous emission spectra were collected during the reversed-phase LC-FL separation. Spectra for DBbeF and BbPer in SRM 1597a (colored line) were compared with authentic reference standards (black line). Chromatograms shown in (a) and (b) are published previously (9). Chromatograms and spectra shown in (d) and (e) are also published previously (11).

phase LC-CESFS method was then quantitatively evaluated for thirteen MM 302 PAH isomers in SRM 1597a.

Normal-Phase LC

The isolation of isomeric PAH groups under normal-phase LC conditions has long been popularized by the efficiency of a semi-preparative NH_2 column to separate PAHs based on the number of aromatic carbon atoms (8). The retention data for 239 PAHs are reported elsewhere (8). As seen in Figure 2, the retention of PAHs generally increases with increasing number of aromatic carbons. As the number of aromatic carbons increases for each isomeric group, the π-π interactions between the analyte and stationary phase strengthen, thus increasing the retention (8). Differences in retention behavior within each of the PAH isomer groups can mostly be attributed to differences in molecular thickness (non-planarity). Non-substituted PAHs with a thickness value of 3.90 are generally considered to be planar. As the molecular structure begins to deviate from planarity, PAH isomers are retained less on an NH_2 column (8). As shown in Figure 3, the elution order of the five selected MM 302 PAH isomers is a direct representation of the decrease in thickness. DBalP is one of the least retained MM 302 PAH isomers due to the corkscrew nature of the molecular structure whereas dibenzo[*e,l*]pyrene (DBelP) is more retained with a planar shape. The planarity of the PAH also increases the overall π-π interactions with the NH_2 stationary phase which increases column retention. Of the 25 studied MM 302 isomers, 6 of the 8 non-planar isomers elute before the remaining 17 planer isomers. The final two non-planar PAHs showed the largest affinity for the NH_2 stationary phase due to the presence of *bay*-regions in their molecular structures. Similarly, *bay*-regions in other isomeric groups of different MM increased the affinity for the NH_2 stationary phase (8). The normal-phase LC retention behavior was used to separate and isolate isomeric PAH groups in SRM 1597a, SRM 1991, and SRM 1975 into a total of 14 fractions over a 90 min time interval. It is important to note that the MM 302 PAH isomers were separated and collected in normal-phase LC F10, F11, and F12, as shown in Figure 1a.

GC–MS

PAHs were determined in the normal-phase LC fractions of SRM 1597a, SRM 1991, and SRM 1975 using GC–MS programmed in selected-ion monitoring (SIM) mode. GC–MS analyses of MM 302 PAHs in F10, F11, and F12 of SRM 1597a are shown in Figure 1b. The number of MM 302 PAH isomers identified in F10, F11, and F12 were 4, 4, and 13, respectively, based on normal-phase LC retention behavior and GC retention times of authentic reference standards (9). The comparison

of identified MM 302 PAH isomers in the three SRM certificates of analysis (COAs) and in the normal-phase LC fractions are listed in Table I and Table II, respectively. The analysis of SRM 1991 showed the greatest improvement by identifying 18 additional MM 302 PAHs when compared to those that are listed in the COA (6,9). The challenges with unambiguous identification by direct GC–MS analysis lie in the similarities in chromatographic behavior and molecular structures of these PAH isomers. One critical example includes the identification of DBalP, which is coeluted with DBjlF in the GC–MS separation of SRM 1597a without normal-phase LC fractionation. Mass spectra for these isomers are shown in Figure 1c, and have virtually identical mass fragmentation patterns making the unambiguous identification nearly impossible. Direct GC–MS analysis without fractionation increases the likelihood of misidentification of coeluted isomers, which limits the accuracy of the ecotoxicological assessment of the sample. However, DBalP and DBjlF are separated in normal-phase LC F10 and F12, respectively, due to the differences in structural non-planarity (9).

TABLE II: MM 302 PAH isomers identified in the three SRM NPLC fractions

			Coal Tar		Diesel Particulate
PAH	Fraction	Peak Label	SRM 1597a	SRM 1991	SRM 1975
BaPer	F10	1	x	x	
BbPer	F12	2	x	x	
DBaeP	F12	3	x	x	
DBahP	F11	4	x	x	
DBaiP	F11	5	x	x	
DBalP	F10	6	x	x	x
DBelP	F12	7	x	x	
DBakF	F12	8	x	x	
DBbeF	F11	9	x	x	x
DBbkF	F12	10	x	x	x
DBjlF	F12	11	x	x	x
N12aP	F10	12	x	x	
N12eP	F10	13	x	x	
N21aP	F12	14	x	x	x
N23aP	F11	15	x	x	
N23eP	F12	16	x	x	
N12bF	F12	17	x	x	x
N12kF	F12	18	x	x	x
N23bF	F12	19	x	x	
N23jF	F12	20	x	x	x
N23kF	F12	21	x	x	

Reversed-Phase LC-FL and Reversed-Phase LC-CESFS

SRM 1597a was selected for the reversed-phase LC-FL investigations, based on the increased number of isomers identified by GC–MS. The use of reversed-phase LC-FL for compound identification is normally limited to comparisons of retention times of unknowns with authentic reference standards (10). The reversed-phase LC-FL chromatograms for F10, F11, and F12 are shown in Figure 1d. Unfortunately, accurate identification of PAHs based on retention times with coeluted concomitants may require additional chromatographic methods due to a lack of spectral information. Traditionally, reversed-phase LC fractions are collected, preconcentrated, and analyzed by spectrophotometric instrumentation for spectral identification or GC–MS (12). Here, stop-flow fluorescence detection was used to collect room-temperature fluorescence spectra of suspected PAH peaks (based on matching retention times) during reversed-phase LC separation of F10, F11, and F12. Spectral collection was obtained by stopping the mobile phase flow at the apex of each suspected chromatographic peak for immediate excitation and emission spectral collection. Total time for implementing stop-flow conditions and spectral collection was roughly 10–20 s, depending on the length of the analyte's spectral profile. The spectra obtained from the fractions were compared to authentic reference PAH standards for identification. Reversed-phase LC-FL spectra obtained for DBbeF (F11) and BbPer (F12) in SRM 1597a fractions (colored) and authentic reference standards (black) are shown in Figure 1e. Full spectral profiles provide an additional layer of identification in complex matrices with minimal time of analysis. Of the 21 known MM 302 PAH isomers in SRM 1597a, 18 were identified based on reversed-phase LC retention times and spectral profiles with 8 having moderate to significant spectral interference (10).

While reversed-phase LC-FL with stop-flow capabilities proved beneficial for the analysis of complex environmental samples, the broad nature of room-temperature fluorescence invites significant spectral interference from coeluting concomitants. Here, we explore the benefits of CESFS in stop-flow conditions for the

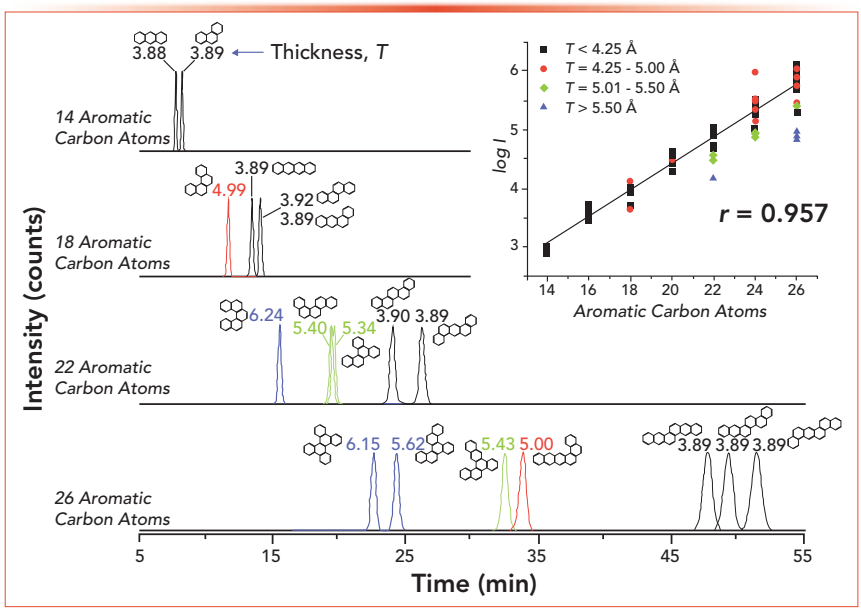

FIGURE 2: Normal-phase LC retention behavior for various PAH isomeric groups detailed through chromatograms of selected PAHs of increasing number of aromatic carbons.

reversed-phase LC analysis of F10, F11, and F12. CESFS reduces spectral overlapping by simplifying excitation and emission spectral profiles into a single synchronous emission peak. Spectral simplification is achieved by scanning the excitation and emission monochromators simultaneously at a fixed wavelength distance (Δλ) that is unique for each PAH isomer (11). Reversed-phase LC-CESFS spectra obtained in stop-flow conditions for DBbeF and BbPer in SRM 1597a fractions (colored) and authentic reference standards (black) are shown in Figure 1e. Of the 21 MM 302 PAH isomers known to be in SRM 1597a, 19 isomers were confirmed via reversed-phase LC-CESFS in F10, F11, and F12 with only three having spectral interferences (11).

Since reversed-phase LC-CESFS provides excellent qualitative information with little to no spectral interferences, the quantitative capabilities were explored for methodology validation. Thirteen MM 302 PAH isomers were selected for quantitative evaluation of reversed-phase LC-CESFS by comparison to their assigned mass fraction values in the current COA for SRM 1597a. Signal intensities for synchronous emission peaks of the 13 PAH standards were used to construct external calibration curves to quantify these isomers in the SRM 1597a NPLC fractions. Of the 13 MM 302 PAH isomers, 10 showed statistically equivalent mass fraction values by both reversed-phase LC-FL and reversed-phase LC-CESFS when compared to the reported COA values (see Table III) (11). The three PAH isomers that did not provide statistically equivalent mass fraction values were lower than the reported COA values. It was concluded that the signal loss may be due to inner filter effects caused by coeluted interferences of unknown concomitants (11).

Conclusion

The results presented in this work showcase the multidimensionality of chromatography for the separation and determination of PAHs in complex matrices with minimal coelution and little to no spectral interferences. Normal-phase LC retention behavior was used to develop a normal-phase LC fractionation procedure coupled with GC–MS for the determination of over 200 PAHs in environmental samples. Analyzing individual normal-phase

TABLE III: MM 302 PAH mass fraction values determined in SRM 1597a fractions by reversed-phase LC-CESFS (11)

PAHs	Certified Mass Fraction (mg/kg)[a]	Reference Mass Fraction (mg/kg)[b]	Reversed-phase LC-CESFS Mass Fraction (mg/kg)	t-Test Value[c]	Statistically Equivalent
N12bF		8.60 ± 1.00	6.56 ± 0.15	4.34	No
DBakF		3.21 ± 0.16	2.81 ± 0.06	0.66	Yes
N23eP		4.31 ± 0.22	4.13 ± 0.12	0.29	Yes
DBaeP	9.08 ± 0.20		8.55 ± 0.38	0.74	Yes
N12kF		10.70 ± 0.60	8.49 ± 0.27	3.69	No
BbPer		9.04 ± 0.50	8.93 ± 0.07	0.20	Yes
DBbkF	11.20 ± 0.40		8.66 ± 0.26	3.99	Yes
N23bF		3.52 ± 0.15	3.27 ± 0.08	0.41	Yes
N21aP		10.20 ± 0.45	10.41 ± 0.56	0.28	Yes
DBaiP		3.87 ± 0.17	4.03 ± 0.39	0.22	Yes
N23aP		4.29 ± 0.45	4.22 ± 0.45	0.29	Yes
N23kF		2.07 ± 0.03	2.04 ± 0.09	0.04	Yes
DBahP	2.57 ± 0.15		2.04 ± 0.19	0.80	Yes

[a] NIST certified values are based on combined measurements of two or more independent analytical methods.
[b] NIST reference values are based on measurements of one or more non-independent analytical methods.
[c] Statistical t-test value of 2.78 (α = 0.05)

LC fractions by GC–MS significantly reduced the coelution of structural isomers having virtually identical mass fragmentation patterns in SRM 1597a, SRM 1991, and SRM 1975. In addition to GC–MS, reversed-phase LC with stop-flow fluorescence detection capabilities was also explored for the analysis of SRM 1597a normal-phase LC fractions to improve GC–MS misidentification due to coeluted structural isomers. Obtaining excitation and emission profiles for reversed-phase LC chromatographic peaks of interest added a new level of identification for MM 302 PAH isomers in SRM 1597a. Similarly, CESFS was utilized in stop-flow conditions to record synchronous emission profiles for MM 302 PAHs in SRM 1597a fractions with the goal of eliminating spectral interferences. To evaluate the accuracy of the reversed-phase LC-CESFS methodology, 13 MM 302 PAH isomers in the COA of SRM 1597a were investigated. The CESFS approach provided statistically equivalent mass fraction values for 10 of the 13 isomers showcasing the validity of this nondestructive methodology. The combination of normal-phase LC, GC–MS, and reversed-phase LC with collection of additional spectral features provides a multidimensional approach for the identification of PAH isomers in complex environmental samples.

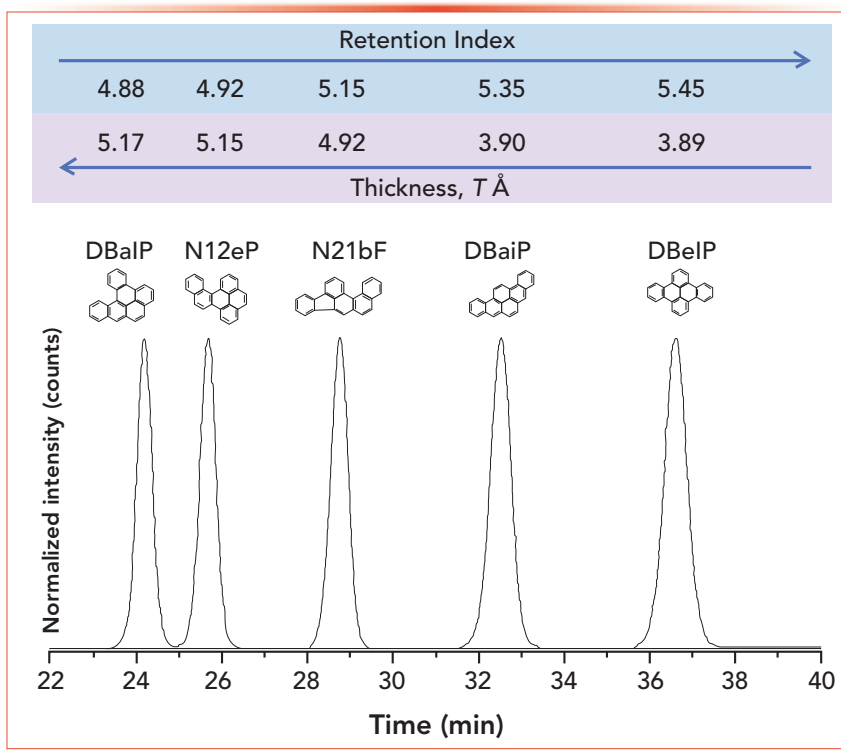

FIGURE 3: NPLC retention behavior for MM 302 PAH isomers. Retention on the NH_2 column increases with increasing structural planarity. DBalP has an earlier retention time due to a larger thickness value while DBelP has a longer retention time with a smaller thickness value.

Acknowledgments

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Disclaimer

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Clinical Metabolomics: Expanding the Metabolome Coverage Using Advanced Analytical Techniques

Metabolomics, the comprehensive analysis of all metabolites and intermediate products of reactions present within a biological system, is a promising field to enable precision medicine. Clinical metabolomics faces two main challenges at the bioanalytical level. The first is the need for high resolution to obtain maximum metabolome coverage. This is exemplified by the latest version of the Human Metabolome Database (HMDB), which reports more than 110,000 metabolites and endogenous compounds. The second is the high-throughput needed to enable the analysis of a large number of samples typically encountered in large-scale cohort studies. Reversed-phase liquid chromatography (LC)—at regular or ultrahigh pressures—combined with high-resolution mass spectrometry (HRMS) has long been considered the "gold standard" in metabolomics. However, these conventional reversed-phase LC–MS approaches are no longer sufficient to analyze the vast variety of polar compounds, as well as discriminate closely related compounds such as isomers or enantiomers. This review article discusses the novel separation and detection strategies that are considered promising in clinical metabolomics to enhance the metabolome coverage. It includes hydrophilic interaction chromatography (HILIC), supercritical fluid chromatography (SFC), multidimensional LC approaches, as well as ion-mobility mass spectrometry (IM-MS) and data-independent acquisition (DIA) analysis methods.

Rob Haselberg, Bob W.J. Pirok, Andrea F.G. Gargano, and Isabelle Kohler

Metabolomics, first formally introduced in the early 2000s and described as the comprehensive analysis of all metabolites present within a biological system, has attracted a growing interest over the last decade in clinical research. Together with other "omics" approaches, such as genomics and proteomics, metabolomics plays a key role in the implementation of personalized medicine. Two approaches are typically considered in metabolomics. In targeted metabolomics, known metabolites from given biochemical pathways are measured in a quantitative manner. Untargeted approaches, on the other hand, focus on the global and unbiased analysis of the highest number of compounds included in the metabolome, leading to qualitative and semiquantitative information (relative differences between populations). Both approaches have been increasingly used over the last couple of years in personalized medicine and drug discovery, in the aim of finding new metabolite biomarker candidates for earlier and more accurate diagnosis, for improving the prognosis and staging of diseases, and to increase the global understanding of pathophysiological processes via the discovery of novel biomolecular pathways (1,2).

In 2017, the fourth version of the Human Metabolome Database (HMDB 4.0) covered more than 110,000 fully annotated metabolites. This is a threefold increase compared with the previous release of HMDB 3.0 in 2013 (3). The human metabolome is very complex, and comprises a large diversity of compounds, including amino acids, organic acids, nucleosides, lipids, small peptides, carbohydrates, biogenic amines, hormones, vitamins, and minerals. Moreover, xenobiotics such as drugs, cosmetics, contaminants, pollutants, and their respective phase-I and phase-II metabolites are also part of this metabolome. The (ideally) comprehensive analysis of the metabolome is therefore linked to several analytical challenges due to (1) the large differences in physicochemical properties (polarity, solubility, pK_a values, molecular mass); (2) the broad dynamic range needed to analyze both trace compounds and highly abundant metabolites (up to nine orders of magnitude difference);and (3) the presence of multiple isomers with structural similarities but significant differences in their biological activities (lipid-based signalling molecules) (4).

Overall, this complexity highlights the need for state-of-the-art analytical approaches capable of tackling such challenges and enabling a qualitative and quantitative assessment of the metabolome. This should be done with the highest possible metabolic coverage via high resolving power and selectivity. In this context, metabolomics has strongly benefited from the latest developments in the fields of both chromatography and mass spectrometry (MS) over the past two decades. The use of reversed-phase liquid chromatography (LC) columns equipped with sub-2-µm fully porous particles (ultrahigh-pressure liquid chromatography [UHPLC]) or

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sub-3-µm superficially porous particles (core–shell technology) are now considered well-established methods in metabolomics owing to the dramatic improvements in resolution and throughput obtained with such phases compared with conventional high performance liquid chromatography (HPLC) (5–7). On the other hand, recent liquid-based chromatographic and MS innovations, notably within hydrophilic interaction chromatography (HILIC), supercritical fluid chromatography (SFC), multidimensional LC, ion-mobility mass spectrometry (IM-MS), and data-independent acquisition (DIA) approaches, are not widely used in metabolomics, despite the significant improvement in metabolite coverage expected with such techniques. Therefore, in this review article, the latest developments in the above-mentioned fields of chromatography and MS are discussed with a focus on their ability to increase the metabolome coverage.

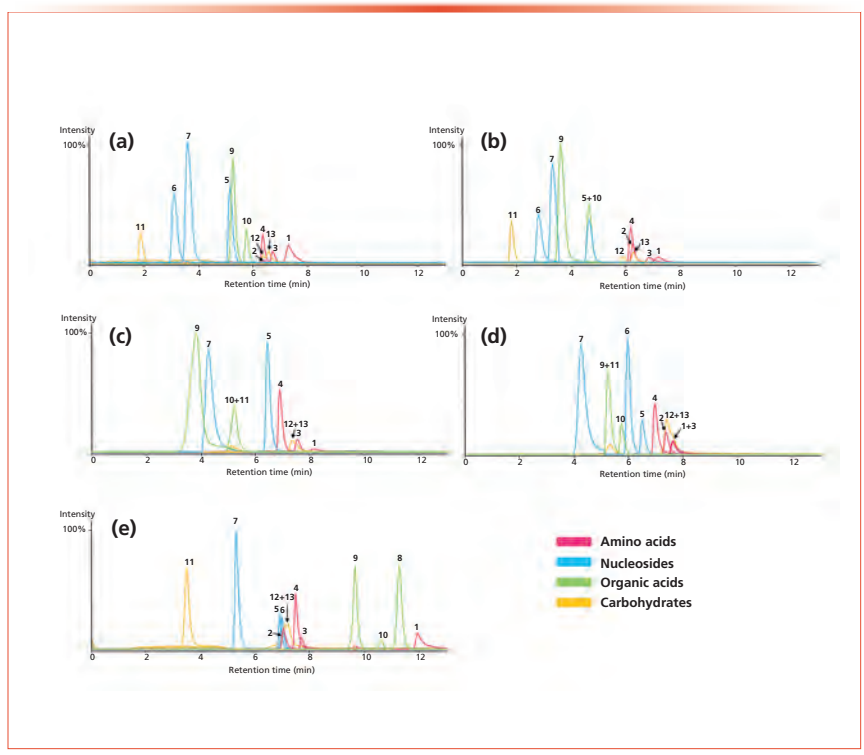

FIGURE 1: Illustration of the differences in selectivity observed upon modifications of the experimental parameters, that is, stationary phase and mobile phase composition, for the separation of a representative set of metabolites belonging to different classes—amino acids, nucleosides, organic acids, and carbohydrates—using HILIC. (a) Luna HILIC (cross-linked diol groups) column, 20-mM ammonium formate at pH 3.5; (b) Luna HILIC (cross-linked diol groups) column 20-mM ammonium acetate at pH 6.0; (c) ZIC-HILIC (sulphobetaine) column, 20-mM ammonium formate at pH 3.5; (d) ZIC-HILIC (sulphobetaine) column, 20-mM ammonium acetate at pH 6.0; (e) Luna NH2 column, 20-mM ammonium acetate at pH 9.0. Analytes: (1) aspartic acid, (2) proline, (3) threonine, (4) tyrosine, (5) guanosine, (6) inosine, (7) adenine, (8) malic acid, (9) hippuric acid, (10) nicotinic acid, (11) rhamnose, (12) trehalose, and (13) maltose. Experimental conditions: see reference 16. Adapted with permission from reference 16.

Improvement of Metabolic Coverage: Chromatographic Innovations

Hydrophilic Interaction Chromatography

Reversed-phase LC-based methods have long prevailed in metabolomics because of the large variety of column chemistries available, the ease of use, and retention time reproducibility. However, a large number of polar or ionizable metabolites, such as amino acids, small organic acids, nucleosides, phosphate derivatives, or saccharides, are not well-retained using reversed-phase LC. Still, many of these polar metabolites play an essential role in multiple (patho) physiological processes, showing the need for alternative approaches. HILIC, a technique first proposed by Alpert in 1990 (8), is well-suited for the analysis of polar compounds. Retention is based on a multimodal separation mechanism between a polar stationary phase and a relatively hydrophobic mobile phase composed of an aqueous–organic mixture with a high organic proportion. With a concentration of 5 to 40% of water in the eluent, a water-enriched layer is formed at the surface of the stationary phase, facilitating analyte partitioning between this stagnant phase and the bulk mobile phase. The exact mechanisms involved in retention and separation are not fully understood, but mostly rely on hydrophilic partitioning, dipole-dipole interaction, hydrogen bonds, and electrostatic interactions (depending on the stationary phase chemistry) (5,9).

A large diversity of phase chemistries based on silica or polymer material modified with polar functional groups (such as aminopropyl and amine, amide, diol, triazole, sulfobetaine, phosphorylcholine, hydroxyethyl, and sulfoethyl) are nowadays commercially available for HILIC analysis. Whereas, for reversed-phase LC analyte, retention can be easily predicted, helping to facilitate method development, this remains difficult in HILIC. The chromatographic selectivity is also strongly dependent on the stationary phase chemistry and composition of the mobile phase, as illustrated in Figure 1. A careful and extensive screening of different

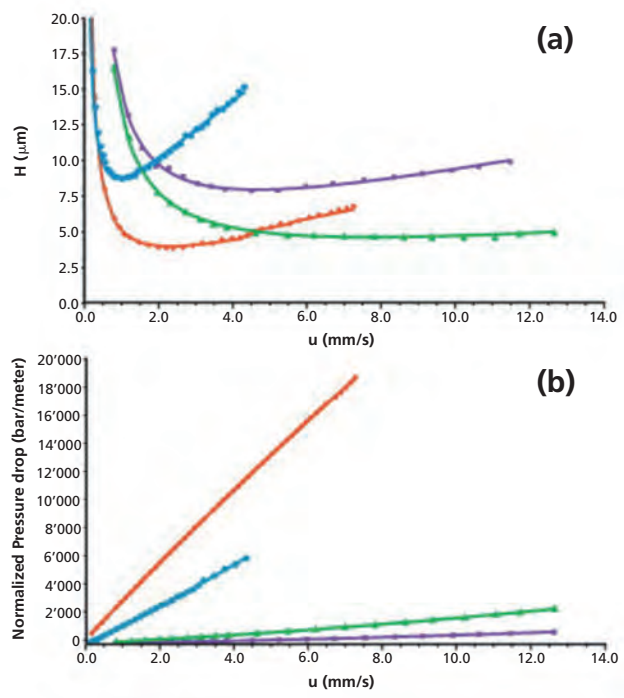

FIGURE 2: Improvements in plate height, mobile phase velocity, and pressure drop observed with supercritical fluid chromatography (SFC) compared to high performance liquid chromatography (HPLC) using columns packed with 3.5-µm particles, as well as ultrahigh-performance SFC (UHPSFC) versus ultrahigh-pressure LC (UHPLC) using columns packed with 1.7-µm particles. (a) Van Deemter curves obtained for butylparaben using a 50 mm × 4.6 mm, 3.5-µm XTerra RP18 (blue dots, HPLC), a 50 mm × 2.1 mm, 1.7-µm Acquity Shield C18 (red diamonds, UHPLC), a 100 mm × 3.0 mm, 3.5-µm Acquity UPC2 BEH 2-EP (purple squares, SFC), and a 100 mm × 3.0 mm, 1.7-µm Acquity UPC2 BEH 2-EP (green triangles, UHPSFC). (b) Corresponding generated column pressure drop normalized to 1 m of column. The optimal plate height is similar between UHPLC and UHPSFC, while the optimal velocity is 4× higher in UHPSFC vs. UHPLC (10 vs. 2.3 mm/s). Because of the low viscosity of supercritical fluids, the pressure drop is much lower in (UHP) SFC compared to (UHP)LC. This shows the benefits of (UHP)SFC in metabolomics, where excellent efficiencies can be obtained at higher throughput. Experimental conditions: see reference 23. Adapted with permission from reference 23.

conditions during method development using a large set of representative metabolites is therefore recommended to obtain an adequate metabolite coverage. The help of modern computer-assisted method development strategies, such as the predictive elution window shifting and stretching (PEWS^2) approach (10), could be useful here to speed up method development. Numerous studies comparing the different stationary phases for metabolomics applications showed that diol, amide, and zwitterionic phases usually give the best results in terms of metabolite coverage, therefore representing a good starting point in the method development process (11,12). Small organic acids, sugar phosphates, and nucleosides are difficult to analyze with reversed-phase LC. Using HILIC mode, these compounds are better retained, especially with polymeric zwitterionic phases, which allows analysis at high pH (pH 9–10) thanks to the polymeric nature of the stationary phase (13). Adding phosphate at micromolar concentrations to the mobile phase has also shown to further improve the peak shape and sensitivity when analyzing such metabolites with a zwitterionic phase (14). Next to the stationary phase chemistry, the composition of the mobile phase strongly influences the selectivity and quality of the separation. Acetonitrile is the optimal organic solvent because it is water-soluble and aprotic. Protic solvents such as methanol, isopropanol, and ethanol are not recommended, due to competition with water for the solvation of the stationary phase, which may lead to lower analyte retention (15,16). In HILIC, the high proportion of acetonitrile in the mobile phase decreases its viscosity compared with the mobile phase mixtures used in reversed-phase LC, which offers additional advantages such as the possibility to use longer columns (leading to higher efficiencies), a higher electrospray ionization (ESI) sensitivity, and higher volatility (15). The buffer composition (that is, salts concentration and pH) has a strong impact on both selectivity (Figure 1) and retention time reproducibility. The buffer concentration (commonly ≤50 mM to avoid salt precipitation in acetonitrile) influences the thickness of the water layer and thus the hydrophilic interaction, and plays an essential role in electrostatic interactions. Ammonium formate and acetate buffers are commonly used because of their MS compatibility. They also give better peak shapes than the corresponding acid solutions (16). Trifluoroacetic acid is not recommended in HILIC–MS because it leads to strong ion suppression in the range of compounds studied. Finally, an adequate and repeatable buffer pH is crucial in HILIC to ensure reproducible analyses. Changes in buffer pH will lead to a higher retention variability, showing the importance of repeatable procedures when preparing the buffer solutions.

Despite all the above mentioned advantages and the improved metab-

olite coverage that can potentially be obtained using the technique, HILIC remains sparsely used in metabolomics, mostly confined to untargeted studies (16,17). The complex mechanism of HILIC separation, the longer equilibration times, the attention required to ensure reproducibly prepared mobile-phase buffers, and the challenges in finding an adequate sample injection solvent might explain why this technique has not been widely adopted yet. However, there are now numerous excellent reviews available discussing these challenges, offering solutions and providing guidelines for state-of-the-art HILIC analysis (1,9,15,16,18). This will hopefully foster the use of HILIC in routine metabolomics.

Supercritical Fluid Chromatography

Although not new (the use of fluids in their supercritical state was first reported in the 1960s), SFC has shown a spectacular comeback in the last decade. This is due to the introduction of a new generation of instruments capable of performing robust, reproducible, reliable, and quantitative analysis. Similar to what has been observed in conventional LC, these new instruments have also fostered the development of columns packed with sub-2-µm fully porous (ultrahigh-performance SFC, UHPSFC) and sub-3-µm superficially porous particles specially designed for SFC analysis. Moreover, the new source designs recently developed for interfacing SFC with MS have also strongly contributed to developing the use of SFC in bioanalysis, including metabolomics (19,20). The metamorphosis of the technique has transformed UHPSFC–MS into a very competitive separation approach, complementary to UHPLC–MS, as underlined in the first ever inter-laboratory study. Between the 19 participating laboratories, similar or even better repeatability and reproducibility using SFC was shown for the determination of impurities in pharmaceutical formulations compared with conventional LC methods (21).

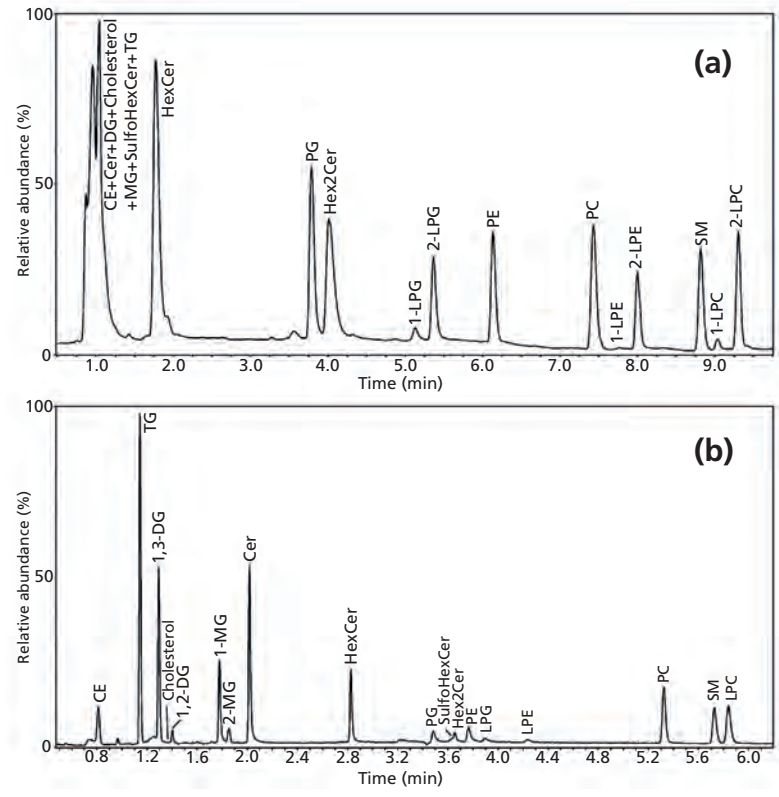

FIGURE 3: Comparison of lipidome coverage obtained with hydrophilic interaction chromatography (HILIC) and ultrahigh-performance supercritical fluid chromatography (UHPSFC). (a) HILIC analysis of lipid internal standards using a 150 mm × 2.1 mm, 1.7-µm Acquity UPLC BEH HILIC column. (b) UHPSFC analysis of lipid internal standards using a 100 × 3 mm, 1.7-µm Acquity BEH UPC2 column. Peak annotation: CE, cholesteryl ester; Cer, ceramide; DG, diacylglycerol; Hex2Cer, dihexosylceramide; HexCer, hexosylceramide; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; MG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; SM, sphingomyelin; SulfoHexCer, sulfohexosylceramide; and TG, triacylglycerol. Adapted with permission from reference 25.

Supercritical fluids have unique properties that take advantage of both gas and liquids, with viscosity and diffusivity very close to those of a gas, while their density and solvating power is close to those of a liquid. Overall, these inherent characteristics enable high separation efficiency at high mobile phase velocity with a low back pressure generated, and good solvation and fast transportation of the analytes (22). Carbon dioxide has been considered the solvent of choice as a result of the low critical temperature and critical pressure (31 °C and 74 bar, respectively), as well as its low toxicity, low flammability, and environmentally friendly properties. However, the low polarity of pure CO_2 limits its application to the analysis of rather nonpolar or hydrophobic compounds such as lipids. The addition of a miscible co-solvent (referred to

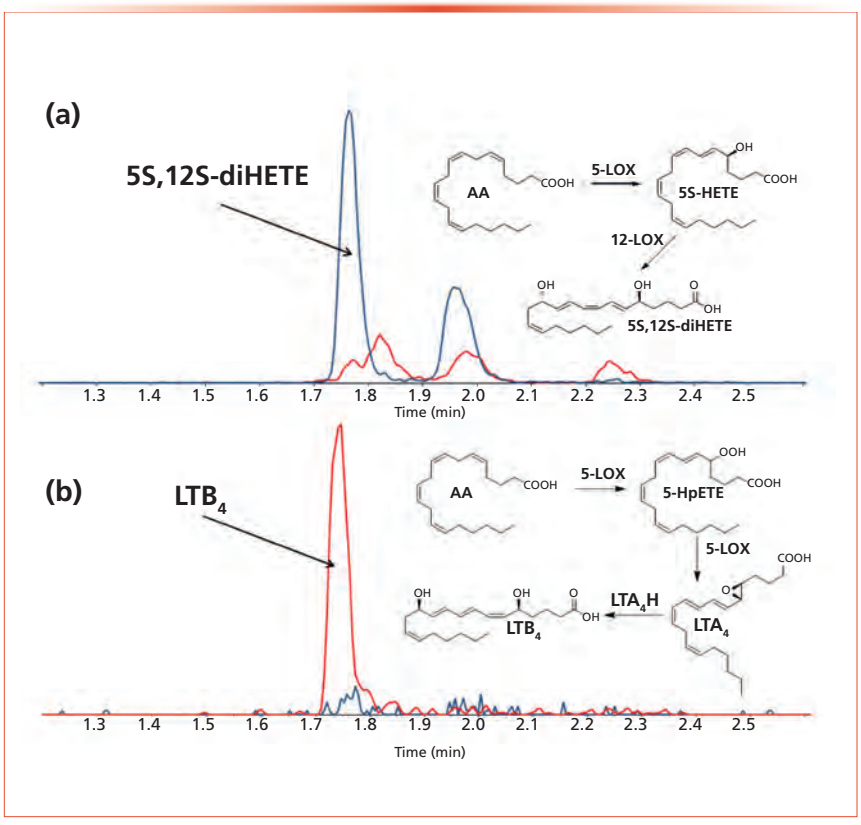

FIGURE 4: Additional selectivity obtained with differential mobility spectrometry (DMS) combined with micro-LC–MS/MS to separate the two lipid diastereomers 5S,12S-diHETE and LTB4 in murine peritoneal cell ethanol extracts. Both compounds coelute when using conventional C18 stationary phases, but can be separated when using a different compensation voltage in DMS. (a) Signal observed for 5S,12S-diHETE in the control mice population, (b) Signal observed for LTB4 in the challenged mice. Red trace, signal obtained at a compensation voltage of 17.9 V, corresponding to LTB4; blue trace, signal obtained at a compensation voltage of 20.3 V, corresponding to 5S,12S-diHETE. AA, arachidonic acid; 5S-HETE, 5(S)-hydroxyeicosatetraenoic acid; 12-LOX, 12-lipoxygenase; 5HpETE, 5-hydroperoxy-eicosatetraenoic acid; LTA4, leukotriene A4. Unknown: undefined isomer in the trace of 5S,12S-diHETE. Adapted with permission from reference 37.

as *modifier,* typically methanol) to the mobile phase is an adequate strategy to enable the retention of polar compounds. The use of an organic co-solvent influences the solvating power of the mobile phase, its hydrogen-bonding donor and acceptor properties, its density, the interaction between analytes and mobile phase, as well as the adsorption of analytes on the stationary phase (20,22). Yet, adding a modifier to this supercritical fluid increases the critical temperature and pressure of the fluid. In current applications, the pressure is commonly maintained over its critical point while the temperature is below its critical value. In this case, the fluid is in a subcritical state, showing a chromatographic behavior close to LC.

The addition of acids (formic acid, citric acid), bases (trimethylamine, isopropylamine), or salts (ammonium acetate, ammonium fluoride) at low concentrations in the modifier also increases the range of compounds that can be analyzed using SFC, especially ionizable compounds such as polyacids, aliphatic amines, and other polar metabolites (20,22). These additives also increase the separation efficiency and peak shape by acting as ion-pairing agents and by covering active sites on the stationary phase, leading to less tailing and better elution of polar compounds. The latest trend in SFC is the use of water as an additive in a CO_2–methanol mobile phase to improve peak shape (at a proportion of 1–5%, miscible in the mobile phase) or to enable the elution of very polar compounds (up to 30%, forming a ternary mixture) (20,22).

A number of SFC-specific stationary phases have been developed and commercialized in the past years, including 2-ethylpyridine, 4-ethylpyridine, pyridine amide, amino phenyl, 2-picolylamine, diethylamine, diol, and 1-aminoanthrocene. In addition to SFC-specific columns, reversed-phase LC- and HILIC-type stationary phases (ethylene-bridged silica, C18, fluorophenyl, amide) can also be used in SFC.

Most of the SFC-specific columns are also available in sub-2-µm format. However, the extracolumn band broadening of the state-of-the-art UHPSFC instruments currently on the market are still higher than the corresponding values obtained on UHPLC systems (namely, 85 μL^2 versus 2 to 20 μL^2), hindering the use of typical UHPLC column dimensions (50 mm × 2.1 mm, 1.7-µm) with these systems. On the other hand, 4.6-mm internal diameter (i.d.) columns require flow rates above the system limits (22). Therefore, most of the current state-of-the-art SFC applications are performed using 100 mm × 3.0 mm columns packed with sub-2-µm fully porous and sub-3-µm superficially porous particles, which represents an adequate compromise and can lead to excellent kinetic performance with a low pressure drop, as illustrated in Figure 2 (23).

Modern UHPSFC–MS analysis has recently started to gain more attention from the metabolomics community, not only in the fields of lipidomics but also as a complementary technique to UHPLC–MS to increase the metabolome coverage. Multiple metabolite classes, including amino

acids, bile acids, cannabinoids, fatty acids, saccharides, steroids, and tocopherols, have been successfully analyzed using UHPSFC–MS (24). A good example of the potential of UHPSFC was described by Holcapek and co-workers, who demonstrated the comprehensive and quantitative analysis of different lipid classes (25). Figure 3 shows the chromatogram obtained for the analysis of lipid internal standards with UHPLC–HILIC–MS (Figure 3a) and with UHPSFC (Figure 3b), both coupled to a quadrupole–traveling-wave ion mobility–time-of-flight (TOF) mass analyzer. Both HILIC and UHPSFC enable the separation of lipid classes without the typical overlap that is seen when using conventional reversed-phase LC approaches. Nonpolar lipids (cholesterol esters and triglycerides) as well as species with one hydroxyl group (ceramides, diglycerides, monoglycerides, and cholesterol) show poor retention in HILIC and elute in the void volume (Figure 3a). On the other hand, all lipids are retained using UHPSFC (Figure 3b). Whereas the positional isomers 1,2-DG/1,3-DG and 1,2-MG/1,3-MG are well resolved in UHPSFC (Figure 3b), the positional isomers of the more polar lysolipids 1-LPG/2-LPG, 1-LPE/2-LPE, and 1-LPC/2-LPC are well resolved using HILIC (Figure 3a) (25). This example illustrates the complementary nature of the different chromatographic modes, where no single analytical technique currently enables a comprehensive coverage of the metabolome.

Despite the excellent performance that can be reached with modern UHPSFC–MS, it remains little used in metabolomics, and only by a limited number of research groups. This reluctance might be explained by the large diversity of stationary phases currently available, together with the flexibility offered in the composition of the mobile phase (modifier, additives, gradient composition). Indeed, the method development step a priori may be seen as very cumbersome and time-consuming. However, it can be facilitated by using column classification maps to help select adequate column chemistries (26) and by using method optimization work (27).

Overall, there is no doubt that the multiple advantages of modern SFC will foster its use in metabolomics in the coming years as a complementary chromatographic approach to expand the metabolome coverage. The advantages of SFC in metabolomics include (i) its application range versatility, with a large range of metabolites with very diverse physicochemical properties that can be analyzed within a single run (as shown in reference 27), (ii) the sample compatibility with the mobile phase used in SFC, (iii) the excellent sensitivity of UHPSFC–MS, comparable or superior to that of UHPLC–MS, and (iv) the flexibility offered with the state-of-the-art instruments, which allows for both UHPLC and UHPSFC analysis within one single system and an unlimited combination of solvent and stationary phases. SFC technology has faced the same reluctance as HILIC a decade earlier, but both techniques are promised to rise further in the field of metabolomics.

Multidimensional Chromatographic Separations

A straightforward approach to increase the metabolome coverage of very complex samples or closely related metabolites is to add another separation dimension to provide additional selectivity.

Similar to what has been observed in the field of SFC, on-line two-dimensional liquid chromatography (2D-LC) is far from being a new concept but has seen a significant breakthrough in the last couple of years thanks to significant advances in theory and instrumentation. In on-line 2D-LC, two individual LC separations are combined, typically using a four-port duo valve or 10-port valve with two sampling loops, or connected to special valves with multiple sample parking loops.

Capturing all peaks—or a large number of fractions—from the first dimension into the second dimension is referred to as comprehensive 2D-LC (also called LC×LC), while multiple heart-cutting 2D-LC (also called LC-LC) is used when one or few d*istinct fractions are collected* from the first dimension and are subjected to a high-resolution analysis in the second dimension. Selective comprehensive 2D-LC (sLC×LC) is an intermediate approach where a series of fractions across one or more regions in the first dimension chromatogram are transferred to the second dimension (28,29). The comprehensive LC×LC approach appears particularly interesting in untargeted metabolomics, where hundreds of features can be profiled during one single analysis.

A large diversity of chromatographic modes can be combined in 2D-LC, including reversed-phase LC, HILIC, normal-phase LC, ion-exchange chromatography (IEX), ion-pairing chromatography, and porous graphitized carbon (PGC) columns. Different stationary phase chemistries and mobile-phase compositions can be employed, aiming for the highest orthogonality of separation between the two dimensions. The selection of the two separation dimensions depends on the analytes, the compatibility and miscibility of the mobile-phase solvents, the compatibility with the detector, and the selection of a faster technique (that is, based on UHPLC conditions) for the second dimension (4,28).

With the recent advent of state-of-the-art instruments for 2D-LC analysis, the number of experimental parameters that can be optimized during method development has dramatically increased. Indeed, setting up a complete 2D-LC method requires optimization of multiple parameters, including column dimension, stationary phase, particle sizes, mobile-

phase composition, gradient conditions, sample loop volume, injection volumes, flow rates, and modulation times. This can lead to a rather cumbersome and lengthy method development. Moreover, state-of-the-art 2D-LC analyses usually require a dedicated instrument (even though one-dimensional [1D]-LC systems can be upgraded to 2D-LC with only minor investment). Finally, hyphenating 2D-LC to MS adds another challenge, since the insertion of an additional LC dimension may induce a significant dilution of the effluent injected to the MS system (28,30).

Overall, this might explain the reluctance in using this technique in metabolomics, despite the remarkable promises 2D-LC holds in significantly expanding the metabolome coverage. This reluctance is similar to the one observed for HILIC and SFC in metabolomics, where inexperienced users are struggling to get reproducible data. Moreover, they might lack sufficient theoretical and practical knowledge to get the best out of those techniques. However, 2D-LC is currently a very dynamic field, and a number of excellent guidelines and tutorials have recently been published by experts in the field, guiding the inexperienced user through this method development (28–31). The recent developments in instrumentation, including the use of active-modulation techniques to alleviate the MS detector sensitivity problems and minimize effects from poorly compatible mobile phases, software tools to support method development, as well as continuous improvements in the algorithm available for processing 2D chromatograms, will certainly foster its use in clinical metabolomics. Most of the applications reported so far have been mostly based on heart-cutting approaches and proof-of-concept studies rather than clinical applications. However, the results presented highlighted the potential of 2D-LC in metabolomics, showing for instance, a twofold increased coverage of intracellular energy metabolites using a combination of reversed-phase LC with PGC (32) and the acquisition of both metabolomic and lipidomic information in a single analysis using heart-cutting 2D-LC (33).

Improvement of Metabolic Coverage: MS Developments

Ion-Mobility Mass Spectrometry

Among all recent developments discussed here, IM-MS is probably the one that has already been largely accepted by the metabolomics community even though it remains a relatively young technique. IM-MS adds an orthogonal separation dimension between chromatographic separation and MS detection without impacting the analysis time. IM-MS separation occurs in a timescale of milliseconds, which makes this technique fully compatible with both fast LC and high-throughput MS approaches (especially TOF mass analyzers, which offer fast duty cycles) (34).

IM-MS is a gas-phase technique separating ions driven through an ion mobility cell under an electric field in the presence of an inert buffer gas. Ions are separated according to their mobility or drift time, which is intrinsically linked to their size, shape, and charge. Assuming that the experimental parameters (for example, drift-tube length, gas pressure, temperature, and electric field) are constant, the ion drift time is proportional to the rotationally averaged collision cross-section (CCS) value, which represents the effective area involved in the interaction between an ion and the gas present in the ion mobility cell. The CSS value is not only highly reproducible but also unique for each analyte, and reflects its chemical structure and three-dimensional configuration. This shows the power of IM-MS in metabolomics, especially in untargeted metabolomics, where CCS values can be used in addition to conventional parameters typically reported in libraries (retention time, mass-to-charge ratio, fragmentation pattern) for metabolite characterization and to increase the confidence in metabolite identification (4,35).

Different IM-MS technologies are currently commercially available, namely, (1) drift-tube ion-mobility spectrometry (DTIMS); (2) traveling-wave ion-mobility spectrometry (TWIMS); (3) field-asymmetric ion-mobility (FAIMS), also called differential-mobility spectrometry (DMS); (4) differential mobility analyzer (DMA); and (5) confinement-and-selective-release ion mobility, also called trapped ion mobility spectrometry (TIMS). They differ amongst each other in terms of applied electric field and state of the buffer gas. DTIMS and TWIMS belong to the time-dispersive methods, where all ions drift along the same pathway and have a different drift time. FAIMS and DMA are space-dispersive methods that separate ions following different drift paths, based on their mobility difference. In TIMS, the ions are first trapped in a pressurized region before being selectively released based on their mobility differences. By using DTIMS instruments, CCS values can be directly derived from the drift time while other approaches require the use of calibrants with known CCS values to calculate the CCS value from the drift time of an unknown (35,36).

IM-MS is able to improve the metabolome coverage by enhancing the selectivity and resolution between metabolites, but one of its major impactful applications probably lies in the field of lipidomics. Indeed, lipid analysis remains exceptionally challenging because of their structural diversity and the multiple lipid isomers that can be present in a biological sample. Contrary to conventional MS/MS approaches, IM-MS enables the discrimination between lipid isomers that differ only in the position of the acyl chain or the double bond, or with a different double bond geometry. An example is shown in Figure 4 with the two lipids 5S,12S-diHETE and LTB4 , both of which arise from

different pathways and have different biological activities. As 5S,12S-diHETE and LTB4 are diastereomers and geometrical isomers, they therefore show identical mass spectra and similar retention behavior using conventional LC–MS/MS analysis. However, adding IM-MS (in this example DMS) enables a baseline separation of these two compounds using two different compensation voltages (37).

Beside lipid analysis, IM-MS has also demonstrated its usefulness for the analysis of polar metabolites in various body fluids. Most of these applications were untargeted, as discussed in references 34 and 36.

Despite its promising contribution to improve the metabolome coverage and metabolite annotation using the CCS value, IM-MS still faces important challenges linked to data interpretation. Indeed, in an LC–IM-MS workflow, the potential in-source fragments, dimers, and adducts will also be separated in the ion mobility cell. A correct regrouping and assignment of these signal features adds another layer of complexity, which is currently not completely tackled by the software available, especially in untargeted metabolomics workflows (35).

Data-Independent Acquisition

Another MS-based strategy used to improve the metabolic coverage is data-independent acquisition (DIA), which allows for the detection and identification of lower abundant metabolites otherwise not recorded with conventional data-dependent acquisition methods. DIA approaches are not new but they have gained more attention since the advent of SWATH-MS approaches. In DIA, precursors selection windows are defined in the first quadrupole (MS1) of a tandem mass spectrometer; all ions are then fragmented in the collision cell and collected into a composite spectrum in the third quadrupole (MS2). Several DIA techniques have been reported so far, including MSEverything (MS^E), all ion fragmentation (AIF), MSX, and SWATH (38). In MS^E and AIF, all coeluted precursor ions in the whole selected mass range are fragmented to acquire MS2 spectra. MS^E alternatively acquires the full MS1 scan with low collision energy (full MS spectrum) and MS2 scan from all precursor ions with high collision energy (MS/MS spectrum). In AIF, all precursor ions are transmitted into a higher energy collisional dissociation cell for fragmentation. Both AIF and MS^E acquisitions generate highly complex multiplexed MS2 spectra. SWATH, which stands for Sequential Window Acquisition of all Theoretical fragment ion spectra and was first described in 2012, has been developed to reduce this data complexity by using a narrow isolation window (39). In SWATH-based DIA techniques, implemented on quadrupole-time-of-flight (QTOF) or less frequently Q-Orbital trap instruments, all precursors ions are sequentially fragmented in a serial of quadrupole isolation windows (Q1 windows). The complete "snapshots" of all metabolite ions and their product ions in MS2 are recorded through the whole chromatogram. The full mass range can be covered in one cycle depending on the selected MS1 scan range and the width of the isolation window. The SWATH windows can be both fixed (typically 25 Da) or variable (that is, the window width is not uniform), and are selected depending on the selectivity required and the cycle time (as short as possible if combined with UHPLC). The complexity of the multiplexed MS2 spectra is therefore decreased by reducing the number of simultaneously fragmented precursor ions, which also improves the overall quantitative performance (37,38).

SWATH-MS is now widely used in proteomics, and has emerged as a powerful technique in other clinical applications because of its reproducibility, speed, compound coverage, and quantitation accuracy. The great performance observed in proteomics fields has also attracted the attention of the metabolomics community looking to expand the information gathered on the metabolome within a single run. A number of metabolomics and lipidomics applications of LC–SWATH-MS have already been reported in the literature. For example, UHPLC–SWATH-MS was used to investigate the changes in the urinary metabolome of rat models upon administration of vinpocetine. Information on both drug metabolism and endogenous metabolite expression changes were gathered, with the simultaneous detection of 28 drug metabolites as well as altered endogenous compounds (40). Using a combination of SWATH-MS and selected reaction monitoring (SRM), Zha and associates developed a two-step workflow to discover potential biomarkers for colorectal cancer. In this method, SWATH-MS was first used to acquire the MS2 spectra for all metabolites in one pooled biological sample. In the second step, a large set of SRM transitions was acquired, targeting both known and unknown compounds (around 1000–2000 metabolites). This approach increased the coverage in targeted metabolomics analysis, where more than 1300 metabolite were profiled in one run in colorectal cancer tissues (41).

Further developments of SWATH technology are still required, particularly in the data analysis pipeline. Indeed, in a DIA-based dataset, the direct connections between precursor and product ions are missing, rendering the metabolite identification very challenging. Chromatographic ion profiles can be used to reconstruct these connections, but coelution and co-fragmentation of precursor ions makes it complicated. Several software tools have been recently devel-

oped to overcome the challenges related to DIA-based data analysis (39). The open-source software MS-DIAL, for example, uses a mathematical deconvolution of fragment ions to extract the original spectra and reconstruct the link between precursor and product ion, allowing for compound identification, annotation, and quantitation. It also implements additional functions typically used in untargeted data processing, namely, peak alignment, filtering, and missing value interpolation (42).

Overall, SWATH-MS represents a great tool to expand the metabolome coverage and obtain both qualitative and quantitative information within a single run. The complexity of the generated data remains a challenge since the reconstructed spectral quality impacts both the confidence in metabolite annotation and quantitation accuracy. The addition of IM-MS in LC–SWATH-MS workflows might help to decrease the spectral complexity by adding an additional separation of the precursor ion to help facilitate the spectral deconvolution, as well as providing CCS values to help in metabolite identification, but also increase the need for adequate data processing software tools.

Conclusions

The last decade has seen a tremendous amount of technological developments in liquid-phase chromatography and MS techniques, developments initially for other applications but showing a considerable potential in metabolomics. Modern clinical metabolomics applications rely on two essential aspects, namely, high-throughput analysis and comprehensive metabolome coverage. The latter is crucial in the quest for the Holy Grail, that is, the discovery of new biomarkers that could ultimately lead to a better understanding of (patho)physiological conditions, an earlier disease diagnosis, a better prognosis evaluation, and an individualized prediction of treatment response. The more comprehensive the metabolome coverage is, the higher the chances are of finding specific metabolites or metabolite fingerprints.

The chromatographic and mass spectrometric innovations presented here have also largely demonstrated their relevance in expanding the metabolome coverage. Most of those techniques, however, are still in their infancy in the field of clinical metabolomics and are rarely used for large-scale studies, where reversed-phase LC–MS and gas chromatography (GC)–MS remain the gold standard techniques. Very few studies have reported the use of HILIC–MS for the analysis of hundreds of samples, while the robustness of SFC and 2D-LC needs to be further investigated, as well as the potential of these two novel techniques in large-scale metabolomics applications. One of the main obvious reasons is the lack of practical background knowledge of non-experienced users, who struggle to get repeatable and reproducible results. In this context, leading experts in these fields and professors play a crucial role and are strongly encouraged to share their knowledge with the younger generation of scientists. Moreover, further technological improvements are needed to ensure the batch-to-batch reproducibility of SFC and HILIC chromatographic columns, which currently remains a clear bottleneck in metabolomics. As an example, acceptable repeatabilities can be obtained in HILIC with >1000 injections (depending on the stationary phase chemistry) of pretreated biological samples using standard procedures (including adequate column re-equilibration time). However, it is much more difficult to reach such repeatability when using HILIC columns from different batches, rendering the use of HILIC in large-scale studies much more challenging than reversed-phase LC.

Increasing the metabolome coverage does not stop at discriminating metabolites with close physicochemical properties. An important aspect often overlooked in clinical metabolomics is the distinction of optical isomers (stereoisomers such as enantiomers). An excellent example is 2-hydroxyglutarate (2-HG), the first oncometabolite (cancer-causing metabolite) ever reported. Both D- and L- stereoisomers of hydroxyglutaric acid are normal endogenous metabolites found in human body fluids. D-2-HG—not L-2-HG—is produced in the presence of gain-of-function mutations of isocitrate dehydrogenase, causing a cascading effect in the cell that leads to genetic perturbations and malignant transformation. Typical routine analytical techniques only measure 2-HG, which strictly speaking corresponds to the sum of both D- and L-forms. Since the endogenous serum levels of L-2-HG have shown to be equal or even exceed the levels of D-2-HG in healthy individuals, it is essential to use state-of-the-art analytical techniques to discriminate between the two stereoisomers (43). Some of the advanced techniques discussed here, mostly SFC, are applicable to chiral analysis and are therefore expected to play a crucial role in next-generation metabolomics.

Overall, despite the technological improvements within each of the discussed techniques, none of the state-of-the-art analytical techniques is currently capable of exhaustively assessing the metabolome. SFC will certainly become a gold standard chromatographic technique complementary to reversed-phase LC and HILIC because of the versatility and flexibility offered (convergence chromatography) and the experimental conditions, where a large diversity of metabolites can be analyzed without strong variations of the operating parameters. Moreover, the higher throughput obtained with UHPSFC is also a clear advantage in clinical metabolomics, notably with the next generation of instruments, allowing

for higher back pressure to be generated, which is still a limitation in the instruments currently on the market.

The future of metabolomics probably relies on the combination of different separation dimensions in an on-line format, as demonstrated with 2D-LC approaches. The first 2D-LC-SFC application has been reported in the literature for simultaneous achiral-chiral analysis of pharmaceutical compounds (44), a multidimensional approach that might be further investigated for metabolomics-based applications. The combination of multidimensional LC with IM-MS has a promising future in metabolomics, showing the remarkable advantage of improving the metabolome coverage while keeping similar throughput. Alternative approaches based on miniaturization of conventional LC techniques and the use of micro-pillar array columns instead of columns packed with porous particles will also probably help to further expand the metabolome coverage, as already shown in lipidomics where structural lipid isomers were chromatographically baseline resolved using micro-pillar array columns (45).

One should also keep in mind the challenges associated with a substantial improvement of the metabolome coverage. First, the development of cutting-edge analytical instruments should not forget the importance of sample preparation, which should be as simple and generic as possible while providing sufficient clean-up to lower the occurrence of matrix effects. Moreover, an increased number of metabolites in quantitative targeted metabolomics means an increased number of internal standards, which raises the overall costs. Finally, enhancing the number of metabolite features measured in a studied population requests a much higher number of samples and subjects included in the study design to keep a sufficient statistical power, which in turn substantially increases the costs and the number of samples analyzed. A compromise between all aspects is definitely needed to achieve successful results in the field of clinical metabolomics.

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THE ESSENTIALS

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The Basics of HPLC Peptide Analysis

Much information is available when biomolecules are analyzed at the protein level, such as molecular weight, structural integrity, charge variants, aggregation, and post-translational modifications (PTMs). However, identification of PTM modification sites, as well as other critical quality attributes such as the glycoprofile, requires digesting the protein into representative peptides using a suitable proteolytic digestion enzyme.

The digested peptide-containing solution is then chromatographed, commonly using a generic reversed-phase liquid chromatography (LC) methodology that consists of an acidic mobile phase, a steeper gradient over a wider range, and a longer alkyl chain stationary phase (such as C18, for example) as compared to the method employed to analyze an intact protein.

A typical peptide map of a digested monoclonal antibody (mAb) is shown in Figure 1. It is considerably more complex than those generated for intact proteins, due to the number of peptides liberated and the artifacts that arise from the digestion process, such as residual reagents and missed cleavages.

Great care and consideration are required during the digestion process, as the proteolytic enzymes used and the conditions employed (pH, temperature, even storage time) not only affect the overall number of peptides liberated, but also the stability of associated PTMs, and can even introduce protein modifications of their own.

Broadly speaking, the digestion process can be broken down into three separate steps: reduction, alkylation, and digestion.

The first stage in the reduction step is to denature the mAb. This is commonly accomplished with an acid-labile surfactant that removes the higher order structure of the protein and exposes many otherwise internal disulfide bonds. These disulfide bonds are then ready for reduction, which is achieved using dithiothreitol. The pH is maintained at physiological levels throughout the process using buffers. To prevent reformation of disulfide bridges across the thiol groups of the cysteine (C) residues, the protein is then incubated with an alkylating agent such as 2-iodoacetamide, once again at physiological pH. The final stage is the addition of a proteolytic agent, which is capable of site-specific protein digestion. Table I details these enzymes and highlights their specific cleavage sites. Typically, fewer cleavage sites leads to larger, and therefore, fewer resulting peptides, and vice versa.

Due to the precise and predictable nature of the hydrophobic retention of reversed-phase LC, estimates as to where the modified peptide will elute in relation to the native, unmodified variant can be made (Table II). This can be a helpful tool when trying to identify and assign unexpected peaks. Asparagine deamidation can produce both pre- and post-peaks, due to deamidation occurring via the succinimide intermediate, iso-Asp (pre-peak) and Asp (post-peak) in a 3/4: 1 ratio.

TABLE I: Common proteolytic digestion enzymes and their specific cleavage sites

Enzyme	Site of Cleavage
Trypsin	Lys, Arg (C)
Chymotrypsin	Phe, Trp, Tyr (C)
Asp-N protease	Asp, Glu (C)
Pepsin	Leu, Phe, Trp, Tyr (N)
Elastase	Ala, Gly, Ser (C)
Cyanogen bromide	Met (C)
Endoproteinase Lys C	Lys (C)

TABLE II: Peptide PTM RPLC peak prediction relative to the unmodified parent peptide

Modification	Reversed-phase LC Peptide
Aspartate isomerization	Pre-peak
Asparagine deamidation	Post-peak + pre-peak
Oxidation	Pre-peak
PyroGlu from Glu (-H_2O)	Post-peak
PyroGlu from Gln (-NH_3)	Post-peak
Succinimide	Post-peak
Sugar	Pre-peak
C-terminal lysine	Pre-peak
Fragmentation	Variable

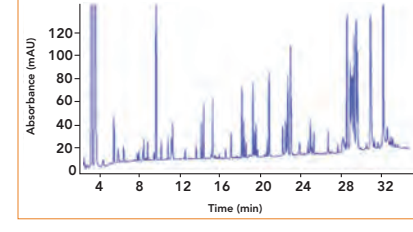

FIGURE 1: Typical reversed-phase chromatogram of a mAb peptide map.

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