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Covalent Organic Frameworks Analyzed Using New Solid-Phase Extraction

Scientists from Wuhan Textile University and the University of Oklahoma tested a new solid-phase extraction system for analyzing covalent organic frameworks (COFs).

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Podcast co-hosts Dr. Dwight Stoll and Dr. James Grinias talk with Dr. Andre de Villiers, Professor of Chemistry at Stellenbosch University in South Africa.

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NOTE FROM THE CEO

WELCOME TO THE June 2024 issue of *LCGC International*! We're excited to bring you a collection of insightful articles that explore the latest advancements and challenges in the field of separation science. This issue features five articles, each providing valuable knowledge from academic and industry experts.

Leading off, in our "LC Troubleshooting" column, Dwight Stoll discusses how the application of two-dimensional liquid chromatography (2D-LC) is expanding due to its remarkable flexibility and ability to address complex analytical challenges, and candidly addresses the common hurdles and uncertainties faced by users, offering practical solutions that will undoubtedly empower you to leverage 2D-LC to its full potential.

Michael W. Dong's "Perspectives in Modern HPLC" column, "Certificate of Analysis (CoA) and Calculations for Small-Molecule Drugs," meticulously explains the components and calculations involved in CoAs, a pivotal document in ensuring the quality of drug substances and products for small-molecule drugs, highlighting their critical role in maintaining quality across the supply chain. This column is essential reading for those engaged in drug development and production.

Virus-like particles (VLPs) pose significant analytical challenges because of their size and structural complexity. In our "Focus on Biopharmaceutical Analysis" column, Anurag Rathore provides an in-depth analysis of the techniques used to characterize VLPs, emphasizing the effectiveness of HPLC-based methods. His discussion on overcoming the difficulties associated with VLP analysis is both insightful and invaluable for biopharmaceutical professionals.

Bob Pirok addresses a persistent issue in chromatography in his Data Analysis Solutions column: "Why is Peak Integration Still an Issue?" Accurate peak integration is vital for reliable quantitative analysis, yet it remains a complex challenge. Bob explores the factors that complicate peak integration and offers strategies to improve accuracy and robustness. This column is a must-read for anyone striving to enhance their analytical precision.

Finally, we feature a compelling article by Ansgar T. Kirk, Tim Kobelt, Maximilian J. Kueddelsmann, and Stefan Zimmermann titled "Gas Chromatography and Ion Mobility Spectrometry – A Perfect Match?" This article examines the synergy between gas chromatography (GC) and ion mobility spectrometry (IMS), as well as the strengths and weaknesses of IMS and its alignment with GC capabilities. The authors provide practical advice for setting up GC-IMS couplings, and share exciting insights into future developments in this dynamic field.

I hope you find this issue as engaging and informative as we intended it to be. Each article offers practical insights and innovative solutions designed to keep you at the cutting edge of chromatography. We always welcome your feedback and look forward to continuing to bring you the highest quality content in future issues. ■

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

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Eyes on the Prize: Overcoming Uncertainty to Realize the Power of 2D-LC Separations

Dwight R. Stoll

Two-dimensional liquid chromatography (2D-LC) is a technique that extends the separation capabilities of conventional liquid chromatography by adding a second separation step to resolve compounds that are coeluted from a first column. This approach holds tremendous potential to solve difficult separation challenges in fields ranging from pharmaceutical analysis to biofuel characterization. Currently, method development is a significant bottleneck impeding more widespread implementation of 2D-LC methods, particularly for new users who are uncertain about how to proceed. In this month's column, I highlight some of the primary considerations we face in method development and point to resources that can help users overcome uncertainty and develop highly effective 2D-LC methods.

TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY (2D-LC) holds tremendous potential to impact many areas of science that rely on liquid chromatography (LC) to move those fields forward. For example, 2D-LC can be used to rapidly resolve all the enantiomers of a molecule with multiple chiral centers, which can be very difficult to achieve with any single conventional 1D-LC method (1). Over the last 20 years, the development of 2D-LC as a technique itself has been impressive, including important advances in our theoretical understanding of the benefits and limitations of the technique, as well as the proliferation of commercially available instrument hardware and software that makes 2D-LC easier to implement in practice. In a recent review article, we noted that 2D-LC is slowly but steadily making its way out of academic research laboratories and into industrial ones, as indicated by the increasing number of peer-reviewed papers in the 2D-LC space with co-authors from industry (2). These developments are attracting new 2D-LC users that have no experience with the technique whatsoever, which is exciting in the sense that these new folks broaden the user community and enrich the range of compelling use cases for 2D separations. However, it can also be challenging, in the sense that new users need support in their development of new methods and applications, even through there are many research questions that need to be more fully resolved before we can provide advice that is as complete as we would like it to be.

In this installment of "LC Troubleshooting," I briefly touch on several important aspects of the method development process for 2D-LC where I sense a high degree of uncertainty among new users about how to proceed. Overcoming these uncertainties is important for accelerating the development of effective 2D-LC methods and increasing the number of confident 2D-LC users in the separations community.

Selection of Stationary Phases and Optimization of Mobile-Phase Conditions

One of the essential principles of effective 2D-LC separations is that stationary phases and elution conditions must be chosen such that the selectivities of the first and second dimensions are complementary (3). Specifically, we aim for conditions that enable separation in the second dimension of compounds that are partially or fully coeluted from the first dimension (^{1}D) column. If we are unable to realize this complementarity for any reason, then the overall prospects for the 2D-LC method will not be very good because we end up just repeating a separation with a given selectivity twice.

Resources that Can Guide the Selection of Stationary and Mobile-Phase Chemistries

In the interest of focusing this part of the discussion, I am largely restricting it to situations where reversed-phase (RP) separations are used in both dimensions. Other combinations of separation modes are very important in some application areas, such as protein characterization. Readers interested in a more detailed discussion of column selection in those contexts are referred to other resources (4).

The Hydrophobic Subtraction Model of reversed-phase selectivity (5) is the basis of a freely available database of characteristics for more than 750 commercial RP columns (www.hplccolumns.org). This data set has been a rich source of information supporting discussion about sets of RP columns for

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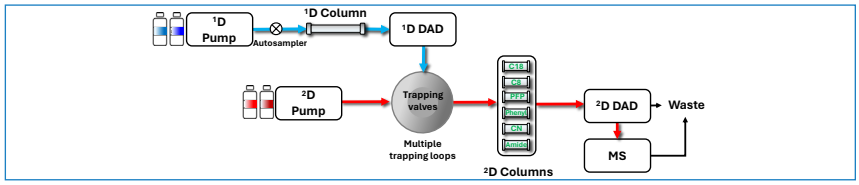

FIGURE 1: Illustration of a 2D-LC system hosting an array of different ^{2}D column chemistries. These columns can be mounted on a column selection valve, such that the specific ^{2}D column that is used for a particular analysis is controlled by a method setting. This enables automated, unattended screening of different stationary phases with one or more samples. Adapted from reference (9).

use in multidimensional LC separations for more than a decade (6–8). Theoretical studies that have considered the complementarity of thousands of different potential combinations of stationary phases drawn from this dataset invariably identify less frequently used phases (for example, graphite-like phases [8]) as having the most potential to yield the best 2D separations. However, these types of less frequently used phases are unlikely to be included in the pool of candidate stationary phases considered by large laboratories working with relatively short lists of preferred columns informed by decades of experience with them in the context of 1D-LC. Thus, it is instructive to look at the lists of columns that have been used in published experimental work that describe the use of arrays of ^{2}D columns to systematically screen different selectivities in the context of impurity detection for small pharmaceutical molecules.

In 2013, Zhang, Chetwyn, and associates described a 2D-LC setup similar to that shown in Figure 1 involving an array of ^{2}D columns mounted to a column selection valve (9). More recently, Wang, Regalado, and associates built on this concept through the addition of a column selection valve in the first dimension and mobile-phase selection valves in both dimensions (10). Such a system enables automated screening of hundreds of potential combinations of first and second dimension mobile- and stationary-phase chemistries. Most recently, in a paper published in this magazine last year (11), Lawler, Breitbach, and associates used an array of ^{2}D columns (all from the same manufacturer) that included C8, C18, RP-amide, PFP, cyano, phenyl-hexyl, and biphenyl phases. They developed 2D-LC methods and used them to screen the different combinations of first (C18) and second dimension columns with an eye toward resolution of an API and its impurities. This group applied the screening approach to several different small molecule APIs and their associated impurities. Interestingly, although several of the ^{2}D stationary phases yield good results, very often the best 2D method involved C18 phases in both the first and second dimensions. The authors concluded that using a different mobile-phase pH in the second dimension (compared to pH ~2 in the first dimension) was more important for resolving the APIs and their impurities than dramatically changing the stationary phase chemistry.

Over the past two years, Petersson, Euerby, and associates published a series of papers focused on identification of short lists of stationary and mobile-phase chemistries to include in screening 2D-LC method conditions for the specific purpose of detecting impurities related to therapeutic peptide APIs (12–14). After evaluating tens of different stationary- and mobile-phase chemistries by using performance metrics, including peak shape, general selectivity, and isomer selectivity, they were able to recommend short lists of stationary- and mobile-phase chemistries for use in 2D-LC screening systems that both leverage the complementarity of modern column chemistries (such as mixed-mode phases) and prioritize compatibility with mass spectrometric detection in the second dimension.

Resources that Can Guide the Selection or Refinement of Mobile-Phase Conditions

Once the stationary- and mobile-phase conditions have been chosen for the second dimension of a 2D-LC separation, one must decide how to go about choosing elution conditions—that is, isocratic or gradient, and if gradient, what program? Choosing a steep gradient that starts with minimal strong solvent (for example, mostly aqueous) and ends with mostly organic solvent provides the best chance at both getting some retention for the analytes of interest and ensuring that the compounds are eluted from the ^{2}D column before the end of the analysis. However, such steep gradients are also the least likely to yield adequate resolution, particularly when attempting to separate closely related compounds as is very often the challenge in the ^{2}D separation. Several studies have been published in recent years that describe the use of retention models in the context of non-comprehensive (that is, multiple heartcut, and selective comprehensive) 2D separations to help optimize ^{2}D elution conditions. In my own research group, we have been focusing recently on the development and use of an iterative modeling approach that enables the use of any endpoint of the optimization workflow, including isocratic conditions. This is particularly challenging when working with molecules that are highly sensitive to mobile-phase composition, such as peptides and oligonucleotides, and this iterative modeling approach provides a means to develop such conditions in an efficient and systematic manner (12,15).

Choosing Conditions to Deal with Mobile-Phase Mismatch

One of the biggest challenges we encounter in 2D-LC is quite different from the situation in two-dimensional gas chromatography, is that in 2D-LC, the mobile phase from the ^{1}D separation can seriously negatively impact the quality of the ^{2}D separation. This is especially acute in cases where the ^{1}D mobile phase contains a high concentration of a component that acts as a strong solvent in the second dimension. The most clear example of this is found in the use of hydrophilic-interaction chromatography (HILIC) in the first dimension, which relies on a high (typically greater than 80%) concentration of acetonitrile, and RP in the second dimension, where high concentrations of acetonitrile lead to very low retention. Unsurprisingly, the seriousness of this problem has attracted a great deal of attention from researchers over the years, and several potential solutions have been explored, such as evaporation of the strong solvent between the two dimensions, use of trapping cartridges between dimensions, and addition of a weak solvent diluent between dimensions. They are too numerous to describe here, but interested readers

are referred to other resources that describe these approaches in detail (16). Recently, Pardon, Cabooter, and associates carried out a systematic study of the effects of parameters involved in the approach known as active solvent modulation (ASM) for managing the mobile-phase mismatch problem (17). A valuable product of this study was an easy-to-follow flow chart that guides the analyst through a series of decisions that can impact the effectiveness of the ASM approach. I strongly encourage newcomers to 2D-LC to consider using this flow chart when thinking about how to manage the mobile phase mismatch problem in 2D-LC generally, and when developing a method specifically involving ASM.

Summary

In this installment, I have addressed some of the most important sources of uncertainty for new users approaching the development of a 2D-LC method. As is the case with conventional 1D-LC, the fact that we have so many choices of stationary- and mobile-phase chemistries is both a blessing and a curse. Having a large number of choices provides tremendous opportunities to tailor the separation chemistry to the problem at hand; however, it can also be paralyzing if we don't have a way to prioritize a small set of columns and mobile phases to try in actual experiments. Fortunately, several experimental studies in the past five years have demonstrated success in the development of short lists of conditions to use. Moreover, multiple groups have demonstrated the use of these conditions in 2D-LC systems set up with valves that enable automated selection of different mobile and stationary phases under software control. It seems likely at this stage this type of approach will be the cornerstone of 2D-LC method development for the foreseeable future. Finally, I briefly touched on recent work aimed at developing a systematic approach to managing the mobile phase mismatch problem that can be a major obstacle to implementation of effective 2D-LC methods. Taken together, guidance from these recent studies can help new 2D-LC users overcome uncertainty and realize the tremendous potential of 2D-LC for solving difficult separation challenges. ■

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

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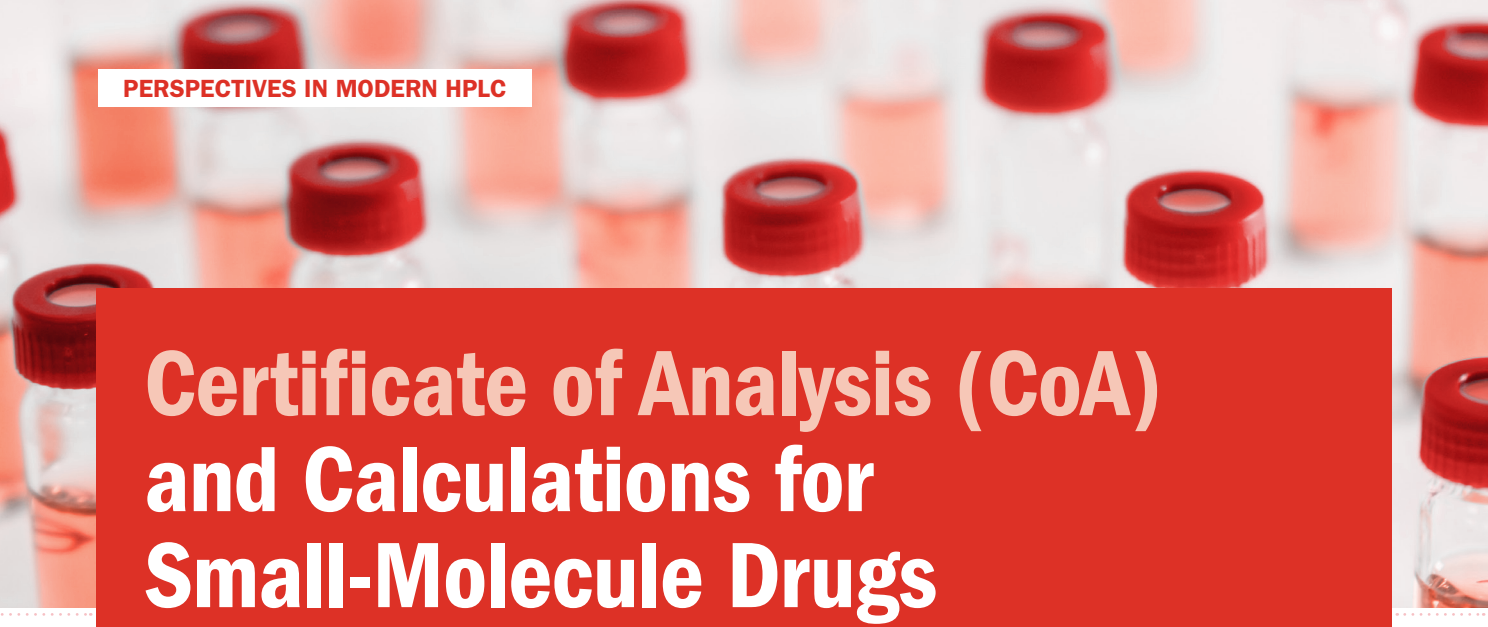

Certificate of Analysis (CoA) and Calculations for Small-Molecule Drugs

Michael W. Dong

The Certificate of Analysis (CoA) is a crucial Good Manufacturing Practice (GMP) document for a batch of drug substances or products in development or production. It contains essential quality information and a summary of specifications and testing results. This article describes the contents and associated calculations of CoAs for small-molecule drugs. It explains how these documents help to ensure drug quality in the supply chain.

THIS INSTALLMENT IS the seventh article in the white paper series on "The Pharmaceutical Industry for the Analytical Chemist," focusing on separation science in drug development, pharmaceutical analysis, regulatory compliance, and method development. The first six papers, published in 2022–2023, were overviews of the pharmaceutical industry, drug discovery and development processes, regulations, public standards, and internal compliance processes (1–6). Papers #7 and #8 will be on Certificates of Analysis (CoAs) and how to generate a well-written analytical procedure for regulated testing, respectively.

A CoA is a drug quality document for excipients, drug substances (DS), drug products (DP), and packaging components used in drug development and production. A CoA contains critical information on identity, origin, production/expiry date, specifications, storage conditions, and testing methods or results. The CoA of a drug is likened to the passport of a traveler in that it certifies that the batch is of sufficient quality to allow its shipment in the supply chain of clinical trial materials (CTM) or final products. This article describes the content of COAs and associated calculations for DS and DP. It explains the intent of CoAs for Good Manufacturing Practice (GMP) compliance (7) to ensure the safety and efficacy of the final drug products. Table I is a list of common acronyms used in the text (all tables are accessible through the QR code at the end of the article). Test method terms are categorized as those for identification, safety and efficacy assessments, solid-state characterization, performance, and others. The testing methods and their use are further elaborated in the text.

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

A case study from my experience as a chemistry, manufacturing, and controls (CMC) analytical team lead supporting an early-phase small-molecule oncology drug development project was used to illustrate how CoAs expedite quality documentation of testing methods and results. The new chemical entity (NCE) is a multichiral molecule with a complex synthetic scheme to ensure chiral purity, requiring the rapid development of more than forty high-performance liquid chromatography (HPLC) achiral and chiral methods to support process chemistry development (3, 8–10). The NCE is a hygroscopic basic compound (10) developed as a monochloride salt with partial crystallinity. The Phase I CTM DP was the Powder in a Capsule (PIC) dosage form. Refrigeration and storage with a desiccant were required for DS and DP to eliminate moisture absorption of the hygroscopic active pharmaceutical ingredient (API).

CoA of a Reference Standard of an NCE

During early development (Phase 0), a highly purified reference standard of an NCE is prepared (for example, ~50 g for the first process scale-up API batch) to serve as a calibration reference standard for identity and potency assays of subsequent batches (8,9). The reference standard undergoes substantial characterization and laboratory testing to establish the compound's identity unequivocally and a purity factor (such as, for example, on an anhydrous free-base basis). This reference standard batch is stored under specified conditions with appropriate packaging, and is requalified periodically. Table II shows an example of the CoA of a reference standard, which includes batch information, test methods, test results, and a calculated purity factor using a mass balance approach. The content of the CoA and explanations of the methodologies and calculations are in the commentary section.

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The Content of Reference Standard CoA

The CoA of a reference standard contains batch information and test results of several categories of methods, typically found in a CoA of a DS batch. Note that the testing included in CoAs may vary based on the relevance of the materials.

1. **Batch information:** Batch number, date of manufacturing, retest date, manufacturing location, storage, and packaging requirements.
2. **Compound Information:** Chemical formula and molecular weight of the NCE.
3. **Identity:** Visual, Fourier transform infrared spectroscopy (FT-IR) (attenuated total reflection/reflectance [ATR]), nuclear magnetic resonance (NMR), mass spectrometry (MS).
4. **Safety:** Purity (by HPLC and chiral liquid chromatography [LC]), heavy metals (limit test), heavy metals (catalysts) (inductively coupled plasma atomic emission spectroscopy [ICP-AES]), residual solvents (headspace gas chromatography [HSGC]), ROI.
5. **Solid State Characterization:** X-ray powder defraction (XRPD) and glass transition temperature (Tg) (differential scanning colorimetry, [DSC]).
6. **Others:** Water (Karl Fischer [KF]), counter ion (for salts by ion chromatography [IC]).
7. **Elemental analysis:** A traditional combustion test serves as a cross-check.

Commentaries

1. **Batch information:** The manufacturing location is not included here, as the batch is synthesized internally. Other information, such as material name, company item number, CoA number, contract manufacturing organization (CMO) name and number, and safety precautions, can be included.
2. **Compound Information:** The purported chemical formula and molecular weight must be confirmed and verified by high-resolution accurate mass MS and NMR (characterization test).
3. **Identity:** FT-IR (ATR) is a QC test for DS, while 2D-NMR for protons and C-13 is used by a subject matter expert (SME) who assigns every H and C atom in the molecule. The structure elucidation SME typically signs off on the identity report of every pivotal batch of DS, which may or may not be included in the CoA or regulatory filings.
4. **Safety: Potency, Purity, and Impurities** (by HPLC): Both potency and impurity content data typically come from a single stability-indicating method: reversed-phase liquid chromatography (RPLC) with UV detection) that separates all impurities from the API (8). HPLC Area% rather than %w/w data is considered sufficient for early-phase methods.
5. **Enantiomer Content by Chiral LC:** The % of the enantiomer of the API is determined by a chiral LC method, while the diastereomer contents for multi-chiral NCE are determined by the primary RPLC-UV (RPC/UV) stability-indicating RPLC-UV method. Heavy metals (limit test): Heavy metals are determined by *USP* <231>, a wet chemistry limit test (11). *USP* <231> is currently replaced by a modernized *USP* <232> and <233>.
6. **Metals Used as Catalysts (ICP-AES):** Metals used in the synthesis process are safety concerns and must be controlled below 20 ppm levels. Today, ICP-MS is generally preferred for this test.
7. **Residual Solvents (HSGC):** Residual solvents used in the synthesis and final crystallization step are safety concerned and must be kept <0.5% for most common solvents using headspace GC. Loss on drying (LOD) can be used for class 3 solvents below 0.5%.
8. **ROI: Residue on Ignition** is a wet chemistry gravimetric test for the general determination of inorganics is an organic compound sample. Any levels >1.0% may require further investigation on its origin.
9. **Solid-State Characterization:** The XRPD pattern is used to characterize the material's crystalline structure and the degree of crystallinity or amorphous content. Tg can be a useful guide for selecting stability storage conditions to prevent solid form transitions.
10. **Others:** Water content (by KF titration using coulometric detection) is required; Counter ion determinations are also needed for any API, which is a salt typically performed by IC (or microtitration for halides).

Approval and review signatories are redacted in this CoA. Note that a salt correction factor (SC) is used for purity factor calculations. For instance, the theoretical % of counter ion in a 100% monohydrochloride salt of the API = (wt of Cl ion/formula weight of the monochloride salt) x 100% = 35.5 / 494.5 x 100 % = 7.2% w/w. However, the measured values of the chloride counter ion could be higher or lower. The SC of the monochloride salt is, therefore, [1 - 0.01(%w/w of the counter ion)] or [1 - 0.01(7.2%)] or 0.93 to convert monochloride to free base. Alternately, the SC can be 1.08 for conversion in the reversed direction.

How to Calculate the Purity Factor

The purity factor or potency of the reference standard is determined as an anhydrous free base basis using the formula shown below via a mass balance approach to eliminate the contributions to the weight of the compound from water, residual solvents, ROI, and counter ion content (all non-efficacious content). The assigned purity factor to this reference standard is 90.6%.

Purity factor calculation = % purity by HPLC x [1 - 0.01(% water + % residual solvents +% ROI + % salt)]

Purity factor = 99.5% x (1 - 0.01(1.4% + 0.03% + 0% + 7.5%) = 90.6%

CoA of a DS Batch in Early Development

The CoA content of a DS batch shown in Table III is similar to that of a reference standard in Table II. Here, a comparative HPLC method using the reference standard as a calibrant is used to identify and determine API (potency) and impurities (8). Acceptance criteria (specifications) are listed and must be met for the batch to be acceptable for the intended use

(for example, as a CTM). Specifications for early development are less stringent than those from the International Conference for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines (12), and recommendations from the IQ Consortium or the US FDA can offer sound guidance in setting realistic specifications for early-phase CTM (9, 13–15). Many quality attributes can have acceptance criteria, such as "report," to ensure initial data collection for early batches, which allows appropriate specifications to be set later.

Calculations of Assay and Impurities in DS

The equation for DS Assay w/w value (as is) as a monochloride salt is shown below.

$$\text{Assay \% w/w (as is)} = \frac{A_{Samp}}{W_{Samp}} \times \frac{W_{Std} \times PF}{A_{Std}} \times \frac{1}{[1-0.01\ (\%\ \text{Counter ion})]} \times 100 \quad [1]$$

The salt correction factor is required because the purity factor of the reference standard in the assay is expressed on an anhydrous free base basis. At the same time, the samples are weighed out "as is," containing the monochloride form with water and other impurities.

The reported assay% w/w (anhydrous solvent-free) in the CoA required another correction step to eliminate the contributions of water, residual solvents, and ROI from the DS batch as the monochloride salt of the API.

$$\text{Assay \%w/w (anhydrous solvent-free)} = \frac{\text{Assay\% w/w(as is)}}{[1-0.01\ (\%\text{water} + \%\ \textit{residual solvents} + \%\textit{ROI})]} \quad [2]$$

Nevertheless, this DS batch's purity factor on an anhydrous free-base basis (often required for formulation development) required an additional salt correction factor.

Purity factor as an anhydrous free base = Assay% w/w (anhydrous solvent free) x salt correction factor = [1 - 0.01(theoretical wt% of Cl in the compound x relative% of counter ion found as decimal)]

Purity factor or potency as anhydrous free base = 99.0% x [1 - 0.01(7.2% x 0.97)] = 92.1%

Alternatively, one can use the absolute mass balance approach shown in Table I to calculate the purity factor.

Purity factor calculation as anhydrous free base = % purity by HPLC x [1 - 0.01(%water + %residual solvents +%ROI + %counter ion)]

Calculation and Reporting of Impurities in the DS Batch

Calculating impurities in the DS batch is straightforward using HPLC area % are under the curve (AUC) in early-phase development. According to ICH Q3A (R2)(13), the reporting threshold is 0.05% in DS, which should be the minimum achievable limit of quantitation (LOQ) of the HPLC/UV stability-indicating method. The assumption here is that the impurities or degradation products of the API (related substances) should have a similar UV response or a relative response factor (RRF) near 1.0 (8). This is not a flawed supposition since, in most cases, the chromophore of the related substance is preserved in most degradative pathways (the λ_{max} of the API is typically the monitoring wavelength). For Phase 2 methods, where synthetic reference standards of key impurities are available, the analyst can measure the RRF of the impurities vs API for more accurately determining some impurities with different chromophoric properties (e.g., conversion of an alcohol into a ketone). It is also customary to name the unidentified impurity as its relative retention time (RRT), indexed to the API (for example, $RRT_{API} = 1.00$) (8).

CoA Sign-Off Process

While the laboratory analyst performs the testing under a GMP environment, and the results are checked and signed off by the quality control (QC) manager, most CoA are prepared and signed by quality assurance (QA). The level of GMP compliance oversight is stage-appropriate, meaning scrutiny and cross-checking increase in late-stage development.

Example of CoA of a DP in Early Development

Table IV shows the CoA of a DP (100-mg capsule) in early development. In addition to identity, potency, and impurities testing, additional tests include dissolution (performance), content uniformity, and microbial limit tests. Typical safety tests, such as heavy metals or residual solvent or solid-state characterization tests, such as XRPD and DSC, are unnecessary, since the DP is manufactured from released DS batches in which these attributes have been controlled or within specifications.

Calculations of Potency as a Percentage of Label Claim

A composite assay of 10–20 units is typically used to minimize unit-to-unit variation for oral dosage forms such as tablets or capsules. Alternately, a portion of the composite ground powder equal to the average tablet weight (ATW) may be extracted and assayed. Quantitative extraction of the API from the solid formulations is critical.

Equations for potency calculations as % label claim (LC) for DP are shown below.

$$\%\ LC = \frac{A_{Samp}}{A_{Std}} \times \text{Std conc.} \times \frac{V_{Samp}}{N_{tab}} \times \frac{100}{\text{Label Claim}} \quad [3]$$

Where: $\text{Std. Conc.} = \frac{W_{Std}\ (mg)}{V_{Stock}\ (mL)} \times PF$

% LC = Percentage label claim of active pharmaceutical ingredient (API) per tablet or capsule
V_{Samp} = Volume of the sample solution, N_{tab} = Number of tablets or capsules tested,
100 = Conversion factor to %, V_{Stock} = Volume of stock solution of the reference standard,

Disclaimer

The case study and examples of CoAs came from an early-phase development candidate to illustrate the methodologies and results. They also reflect the critical quality attributes of that particular NCE, which may be less or not applicable to other small-molecule drugs.

Conclusions and Summary

This installment describes the Certificate of Analysis (CoA), one of the most common and critical quality documents in the quality control of pharmaceuticals; examples are provided to illustrate the contents, test methods, specifications, storage conditions, and test results of a reference standard, drug substance, and drug product batch. ■

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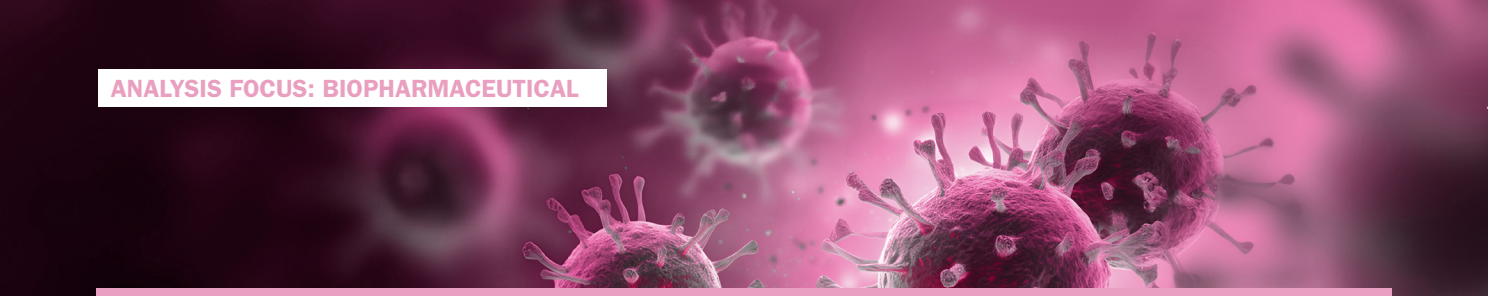

Challenges in the Analytical Characterization of VLPs Through HPLC-Based Methods

Anurag S. Rathore, Vineela Peruri, and Jared Auclair

Characterization and quantification of virus-like particles (VLPs) through high performance liquid chromatography (HPLC)-based methods are challenging because of their large size, structural complexity, internal structural heterogeneity, and instability. Analytical techniques are essential to monitor morphology and internal structural heterogeneity at each process stage. Common analytical tools used in VLP characterization are microscopic techniques (such as transmission electron microscopy [TEM], atomic force-field microscopy [AFM], cryo-electron microscope [cryo-EM]), biochemical techniques (SDS-PAGE, western blotting), and light scattering techniques (such as dynamic light scattering [DLS], nanoparticle tracking analysis [NTA], and size-exclusion chromatography coupled with multi-angle light scattering [SEC-MALS]). However, these techniques are semi-quantitative and do not address morphology and internal heterogeneity. Therefore, HPLC-based techniques are sensitive, robust, and offer better resolution. The purity and titer of VLPs at any process stage can be monitored by reversed-phase chromatography and morphology, and stability-related issues can be monitored by the combination of HPLC and light scattering techniques like SEC-MALS. Challenges in HPLC-based methods are choosing columns with the right pore size and surface chemistry and effective sample preparations, as VLPs are very unstable and prone to fragmentation at process stages and the low titre of the VLPs. This article discusses the challenges and effective solutions for HPLC-based analytical characterization of VLPs.

VIRUS-LIKE PARTICLES (VLPS) ARE nanoparticle-sized, multimeric, self-assembled protein complexes. They are non-infectious, as they lack the genetic material of parental viruses (1). VLPs trigger both cell-mediated and humoral-mediated immune responses, and are safer and more effective than attenuated or inactive vaccines, as there are fewer chances of reversion. Therefore, VLPs are emerging as potential vaccine candidates. Apart from vaccines, VLPs are also used as gene carriers in gene therapy (2). The size of VLPs ranges from 22–200 nm. VLPs can be expressed in different host systems, such as bacteria, yeast, insect cell lines, plant cell lines, and mammalian cell lines (3). Determining the identity, purity, and potency of VLPs is a crucial step towards regulatory approval, as they hold potential as vaccine candidates. Analytical methods play a critical role in ensuring quality control, monitoring stability during long-term storage, and thereby managing the critical quality attributes (CQA) in vaccine development.

Commonly Used Analytical Techniques for VLP Characterization

Microscopic techniques such as transmission electron microscopy (TEM), atomic force-field microscopy (AFM), cryo-electron microscope (cryo-EM), biophysical or light scattering techniques like dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS), asymmetric flow field fractionation coupled with multi-angle light scattering (AF4-MALS), and analytical ultracentrifugation (AUC) are commonly used to measure the morphology of VLPs (4). Biochemical techniques like sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting are commonly used for composition analysis of VLPs, and are quantified through densitometry. Other common techniques used for the quantification of VLPs are bicinchoninic acid assay, Bradford assay, and Lowry assay. Circular dichroism (CD) and differential scanning calorimetry (DSC) are also used to monitor thermal stability of VLPs (5) (Figure 1).

Challenges in VLP Characterization

Artifacts are introduced by microscopic methods such as TEM and AFM because they require drying for sample preparation. AFM offers superior resolution compared to TEM. Nevertheless, artifacts can be minimized when VLPs are scanned in their natural state utilizing cryo-EM. However, in general, microscopic methods are non-quantitative, and require laborious sample prepara-

tions. Biophysical techniques like DLS only work with monodisperse samples; however, DLS suffers from a major flaw in that the signal is disproportionately impacted by larger particles. Therefore, if there is a broad particle distribution, accuracy of measuring smaller particles is significantly compromised. Other biophysical techniques like SEC-MALS, AF4-MALS and electrospray-differential mobility analysis (ES-DMA) offer size separation before size measurement, which is more accurate and robust, but these techniques too are semi-quantitative. SDS-PAGE and western blotting are time-consuming and tiresome. Quantification through bicinchoninic acid assay, Bradford assay, and Lowry assays is sensitive to the use of detergents like urea, and also give total protein quantification rather than VLP structural proteins (6,7) (Figure 2).

Why HPLC-Based Methods?

Chromatography has been the most widely used technique for analytical characterization and quality control monitoring of biotherapeutics for decades. Reasons for this include the simplicity of operation, high resolution, robustness, sensitivity, and continued innovation in development of chromatography media that facilitates protein separation based on a variety of physicochemical characteristics, such as charge, hydrophobicity, and size (8). Beginning in the early 1980s, attempts were made to isolate influenza viral proteins using a variety of chromatographic techniques, including size-exclusion chromatography (SEC), reversed-phase chromatography (RP-HPLC), and ion exchange chromatography (IEX). However, significant challenges are faced when performing chromatographic characterization of VLPs.

Challenges Faced During HPLC-Based Characterization of VLPs

Multimeric State Through SEC

In comparison to other biotherapeutic proteins, VLPs are very large in size. Furthermore, most of the HPLC columns are designed for analysis of therapeutic proteins (such as monoclonal antibodies). As a result, when used for VLP characterization, the VLPs simply are eluted into void volumes of these columns. Very few bigger pore-size columns are available commercially for identification and quantification of intact VLPs. VLPs can be found in several molecular states in in-process samples. For instance, human papillomavirus (HPV) VLP is known to exist in three different states: monomeric (55 KD), pentameric (capsomeres), and completely assembled (19 megadaltons). It is impossible to identify and accurately measure each of these three states in a single HPLC analy-

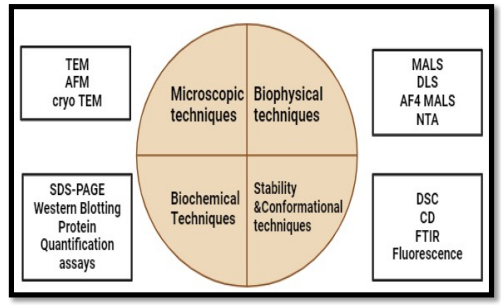

FIGURE 1: Analytical techniques for VLP characterization.

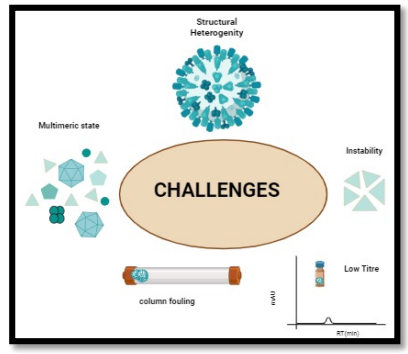

FIGURE 2: Challenges in analytical characterization of VLP through HPLC-based techniques.

sis. Recent development of two-dimensional liquid chromatography (2D-LC) has made it possible to use multiple pore-size SEC columns to better characterize the different molecular states. Researchers have coupled a 2D-LC system with refractive index (RI) and a MALS detector and effectively separated, disassembled—intact—and aggregated VLP species in a single run using SEC columns in both dimensions (9). In the first dimension, a 250 Å SEC column was used and the peaks eluted in void volume were transferred to the second dimension through a heart-cut method. The second dimension SEC column has a pore size of 2000 Å, an ideal column used for intact VLP separation (9).

Molecular Heterogeneity Through RP-HPLC

VLPs are also structurally heterogeneous, as they are composed of many structural proteins, and, in certain instances, structural proteins of the virus combine with the proteins of the host cell to generate irregularly shaped VLPs. Therefore, using SEC alone to characterize VLPs may be incomplete. A better approach in such a case would be to use chromatographic techniques that separate based on other physiochemical properties like hydrophobicity. However, VLPs must be reduced from a fully assembled state to a monomeric state to perform reversed-phase chromatography. It is possible to convert completely formed VLP into a monomeric state by efficient sample preparations with the optimum amount of denaturants and reducing agents. Selecting the appropriate carbon chain column and pore size together yields improved resolution and accurate quantification than SDS-PAGE. The molecular weight of the desired species can be confirmed using reversed-phase chromatography by coupling LC with mass spectrometry. By reducing VLPs and digesting the resultant peptides with the trypsin enzyme, reversed phase chromatography in combination with tandem mass spectrometry (MS/MS) provides sequence coverage of the molecule with the database sequence (known as *peptide mapping*). In a recent publication, we have demonstrated efficient sample preparation for any process stage for characterization of HPV VLP (10). Crude samples were precipitated with 30% ammonium sulfate solution and the pellet was dissolved in 8M guanidine hydrochloride (GuHCl) and 100 mM dl-dithiothreitol (DTT) to effectively reduce the 19 megadalton molecule into its monomeric forms of 55 kilodaltons. The reversed-phase chromatography method was validated to identify HPV VLP L1 proteins in 20 min.

Column Fouling

In many cases, nucleic acids act as scaffolds for assembly of viral structural proteins. The use of optimal mobile phases that preserve the native state of VLPs and reduce the interactions of nucleic acids with columns help in achieving better resolution. Often, SEC mobile phases constitute higher NaCl concentrations than usual, along with other components like ethylenediaminetetraacetic acid (EDTA) to minimize sample and resin interactions. In reversed-phase chromatography, enveloped viruses exert column interactions due to their outer lipid membrane, and zwitterionic detergents are used to solubilize surface envelop glycoproteins and to also minimise the column interactions. In the case of chikungunya virus VLP, researchers have incubated samples with Zwittergent 3–12 detergent before analysis (7). The same strategy can be followed to release hemagglutinin (HA) proteins of influenza VLP. Other researchers have incubated influenza whole virus (inactivated vaccine) with 1% zwittergent 3–14 for 10 minutes at room temperature before analysis through reversed-phase chromatography (11).

Detection

VLP samples typically have lower titres than monoclonal antibody samples. When detecting at 280 nm UV wavelength, it is advantageous to inject highly concentrated samples or to use UV wavelengths of 220 nm in addition to 280 nm. Using more sensitive detectors, like fluorescence detectors, would be the best choice.

Stability

Because of significant instability that is inherent in VLPs, they frequently fragment or agglomerate to create irregularly shaped particles. VLPs of asymmetrical shapes are not very potent. Therefore, maintaining ideal pH conditions and using optimum amounts of EDTA can preserve the VLP's native state and size. DTT and EDTA have been used to reduce the SV40 VLP's ability to withstand mechanical stress, leading to populations of varying sizes (12). Any negatively charged polymer, like DNA, promotes interpentameric (capsomers) interactions that lead to homogeneous populations.

HPLC in Conjunction with MS

HPLC in conjunction with MS provides detailed information on VLP components, primary structure, glycosylation patterns, post-translational modifications (PTMs), and also chemical modifications. With the advent of several sensitive MS techniques like orbital trap MS and charge detection–mass spectrometry (CD–MS), even knowing the molecular weight of fully assembled VLPs has been made possible.

Electrospray Ionization (ESI)-MS

VLPs are highly heterogeneous, and very large in size. This limits the application of MS in analyzing intact (fully assembled) VLPs. Electrospray ionization (ESI) is the most commonly used ion-generating technique for proteins. It can be coupled to liquid chromatography (LC) or capillary electrophoresis (CE) for protein separation. ESI can generate ions directly from solution, and thereby produce multiply charged ions, enabling determination of intact mass up to 1 mega Dalton. In some cases, there can be signal overlap and deviations in observed ionic mass from actual analyte mass, due to multiple charged ions and adduct formation. This complicates the interpretation of mass spectra, especially in heterogeneous ions, because of poorly resolved signal distribution.

Matrix-Assisted Laser Desorption Ionization (MALDI)-MS

MALDI-MS produces a singly charged state. Sometimes, ions may be multiply charged, especially in the case of larger molecules, and the low extent of multiple charging requires the use of the time-of-flight mass analyzers. Also, the adduct formation leads to deviation in ionic mass from the neutral analyte. MALDI-MS is not compatible with either LC or CE. Hence, it is poorly suited to native MS and requires chemical linkers for analyzing non-covalent interactions.

Ion Mobility–Mass Spectrometry (IM-MS)

IM-MS plays an efficient role in characterization of heterogeneous molecules up to a few kilodaltons. It resolves gas phase ions based on differences in their collision

cross-section. However, it is incompatible with large heterogenous molecules (13).

Charge Detection Mass Spectrometry (CD–MS)

CD–MS plays a prominent role in determining the mass of large heterogenous molecules where conventional techniques fail because of overlapping signals from complex ion distributions. CD–MS is a single ion approach that measures mass by estimating both mass-to-charge ratio (*m/z*) and charge of the ion. This gives an accurate mass of molecules over a broad size range. CD–MS resolves overlapping signals by transferring ions to a lower charge state. In CD–MS, ionization occurs through ESI or MALDI and the ion passes through a conducting tube, *m/z* is measured by the passage time or oscillation frequency, and *z* is determined from the magnitude of the charge. This technique is highly sensitive, as it measures the *m/z* and charge of a single particle and therefore requires very little sample. Researchers have used CD–MS to analyze the mass of a live attenuated virus (RotaTeq), a VLP (Gardasil 9), and a complete viral vaccination as an inactivated polio vaccine (IPOL) (14). In this study, ions were generated through nano-ESI and multiple phases of differential pumping were employed to thermalize and concentrate positively charged ions, accelerating them to an energy of 100 *eV/z*. An electrostatic linear ion trap (ELIT), which traps ions for a duration of 100 milliseconds, received a narrow energy distribution from a dual hemispherical deflection energy analyzer. A charge-sensitive amplifier picked up the oscillating ions' signal, which were then converted to digital form and examined using quick Fourier transforms. The researchers reported that CD–MS is a reliable, sensitive method that can quickly ascertain the mass of large, heterogeneous molecules (14).

Conclusion

VLPs are large heterogenous molecules and their characterization poses significant challenges, due to their large size, heterogeneity, low titres, and instability of intermediate molecular states. Traditional methods of analytical characterization include a wide variety of microscopic, biochemical and biophysical techniques. However, although all of these techniques do offer useful information about morphology or structural composition, they are semi-quantitative, tedious, and time-consuming. In contrast, HPLC offers the possibility of a robust, simple, and sensitive analysis. Most commonly used chromatographic techniques include size-exclusion chromatography for assessing morphology and molecular state (fully assembled, capsomere, or monomeric state) and reversed-phase chromatography for purity and structural composition. However, there are significant challenges that are faced when performing HPLC-based characterization of VLPs. These exist because of the large size (and, at times, improper assembly) of viral structural proteins. Furthermore, column fouling (because of strong binding or the instability of the VLP) can also be a hurdle. In this article, we reviewed the challenges—as well as solutions—that researchers have proposed. Intact VLP characterization through ESI-MS is not possible because of multiply charged states that would lead to overlapping signal and deviation because of adduct formation. CD–MS is the ideal choice, as it is a single ion approach and gives both charge and *m/z* ratio. ■

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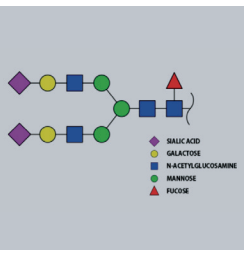

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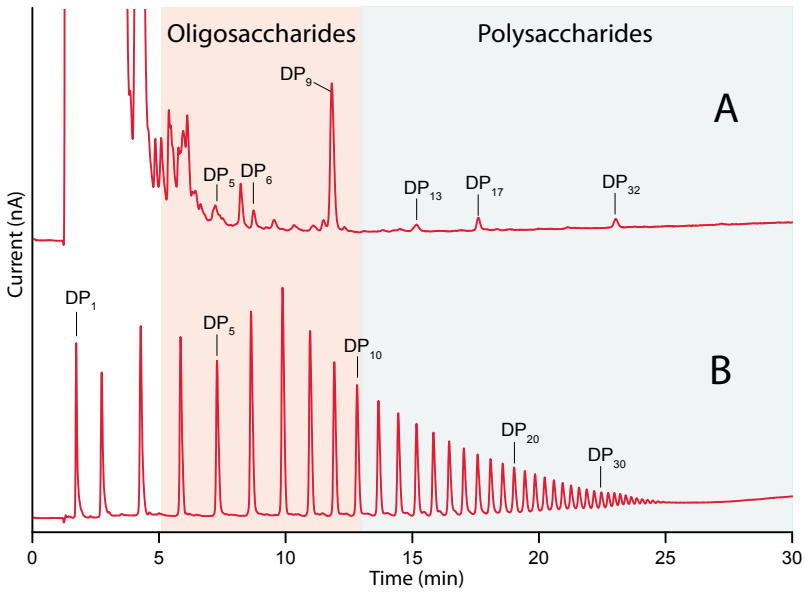

Analysis of honey (A) containing oligo- and polysaccharides and (B) reference maltodextrin (DE4-7), SweetSep AEX200 column 4.0 x 200 mm, 5 µm

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Resolving Separation Issues with Computational Methods, Part 2: Why is Peak Integration Still an Issue?

Bob W. J. Pirok

OUR ATTENTION is typically focused on the chromatography in the laboratory. Yet ultimately, the liquid chromatograph (LC) or gas chromatograph (GC) only produces a signal—the chromatogram—from which information still must be distilled. In this series, we will discuss different aspects of data analysis and learn how to extract different bits of information from our chromatograms. We start with simple, yet important, bits of information, which we will tie together in future articles. In this instalment, we establish why peak integration still poses challenges, and at the same time, see some of the computational techniques in action that we learn to use ourselves in future installments.

Signal Components of a Chromatogram

Any raw signal, such as those encountered in analytical separation science, is comprised of several components or frequencies. Frequency here refers to the rate at which the signal is changing over time. A large number of sudden changes within a limited time are of high frequency, whereas a slowly and gradually changing signal is of low frequency.

For chromatography, a signal can be roughly categorized as follows: (i) a high-frequency component that contains the noise; (ii) a low-frequency component that captures the baseline drift; and (iii) a medium-frequency component that usually includes the chromatographic peaks of interest. This is illustrated in Figure 1.

The integration of a peak is thus the result of several steps of data pre-processing in which the peaks (that is, the medium-frequency component) are first isolated. In future articles, we will learn in more detail how to perform these individual steps. For now, it suffices to understand that this isolation process poses a challenge in itself with significant risks of altering the actual area of a peak. For instance, errors may be introduced by excessive smoothing of the noise or by wrongly recognizing sections of (co-eluted) peaks as baseline. This may seem less relevant for relatively simple separations, where detection possibilities allow for clear chromatograms to be obtained. However, it is critical for complex separations, especially when trace concentrations are expected or the number of peaks is too large for manual curation of each individual analyte.

Impact of Peak Integration Strategy for Co-Eluting Peaks

Assuming that signal pre-processing has successfully been conducted, we can now focus our attention to the actual integration of a peak. Mathematically, this is the full integration of the peak from its start and end point, analogous to the zeroth moment (0th) as discussed in the previous installment (1). This is a relatively simple exercise for prominent, isolated peaks. However, it quickly becomes difficult for co-eluted signals.

Figure 2 shows an example, with peaks 2 and 3 partially co-eluted. The challenge now becomes to establish the true areas of these two peaks. At this stage, it is important to acknowledge that we are dealing with a chromatographic-resolution problem for which we now seek a computational solution. In essence, we must computationally solve the lack of resolution. Different strategies are now possible, and some of them are, for this reason, also known in literature as resolution-enhancement methods.

We will learn about these different strategies in future articles. In this article, we focus on the impact of the choice of strategy. Figure 2a shows a strategy based on local-maxima peak detection. This approach is probably best known to chromatogra-

phers. Here, the two co-eluted peaks are split at their saddle point, the minimum between the two signals. A vertical line (shown in pink) is drawn between the saddle point and the baseline. Everything to the left side is then considered to be the area of peak #2, and all of the area to the right would belong to peak #3.

Another strategy involves computationally deconvoluting the two peaks by fitting mathematical distribution functions through each (such as a Gaussian) and then integrating these individually. This is shown in Figures 2b and 2c, with the blue lines depicting the individual fitted peaks. The purple line depicts the sum of the blue lines, and the blue dots represent the original data. In panel b, Gaussians are fitted to the peaks, and in panel c, a modified Pearson VII function is used that can capture the tailing of chromatographic peaks.

Table I shows the resulting peak areas for each of the four peaks using the different strategies. The first two approaches suggest that peak #2 is smaller than peak #3, whereas the third approach suggests that the two peaks are equal in area. We also visually see in Figure 2c that the two fitted peaks are now similar in width, which is more consistent with what we would expect from a chromatographic perspective. The numbers by the third approach are in agreement with the composition of the created sample.

Peak Integration and Novel Methods Using Machine Learning

The choice of integration strategy thus significantly impacts the resulting number. The advantage of the first strategy is its simplicity and robustness. Splitting the peaks may not make much sense from a chromatographic perspective, but surely will produce consistent numbers.

Deconvolution methods may provide more accurate numbers, but they hinge on several factors, such as the severity of co-elution, as well as parameters, such as the choice of function that is fitted through the peaks. For real complex separations, it is impossible to manually inspect the results for each individual signal, and thus, improved robust strategies are of high interest. Multi-channel detectors, such as mass spectrometers, alleviate this problem somewhat, but also introduce new challenges with respect to signal consistency (for example varying ionization efficiencies across the peak as the degree of co-elution changes).

It is not surprising that groups around the world have devoted significant attention to alternatives including multivariate methods and machine learning (for example, [3,4]). ■

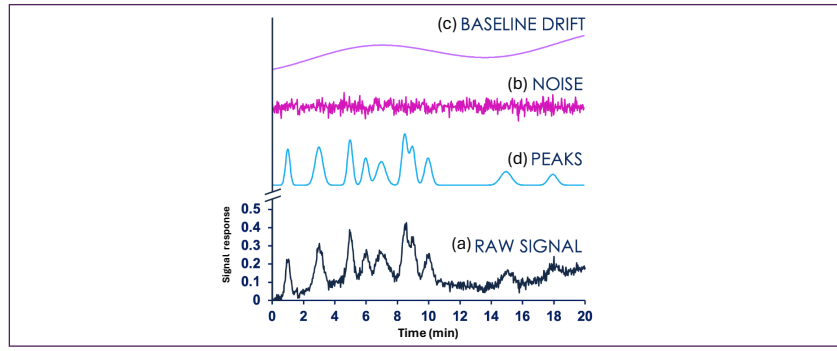

FIGURE 1: (a) A raw chromatographic signal decomposed into different frequency components: (b) the high-frequency noise, (c) the low-frequency baseline drift, and (d) the medium-frequency chromatographic peaks.

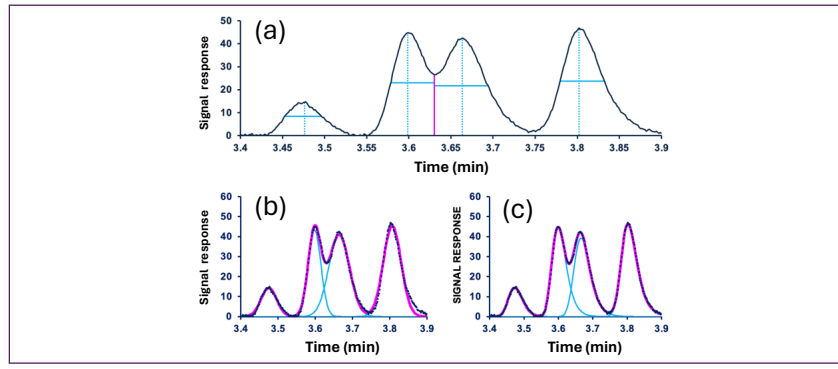

FIGURE 2: (a) Peak detection and characterization by the local-maxima strategy. The dotted lines indicate the center of the peak, the horizontal blue lines their half height, and the pink vertical line the border between the co-eluting peaks. (b) Deconvolution through curve resolution by fitting Gaussian distributions through the peaks. (c) Identical to (b), but now using modifier Pearson VII distribution functions. In the latter two chromatograms, the original data are depicted as dots, with the four individual distribution functions (light blue) and their sum (pink). Adopted from reference (2) with permission.

TABLE I: Determined peak areas for the peaks in Figure 2 using different peak detection and deconvolution strategies. Adopted from reference (2) with permission.

Peak Areas			
Peak No.	Local Maxima	Curve-Fit (Gaussian)	Curve-Fit (Mod. Pearson VII)
2	2.044	1.849	2.368
3	2.435	2.831	2.361

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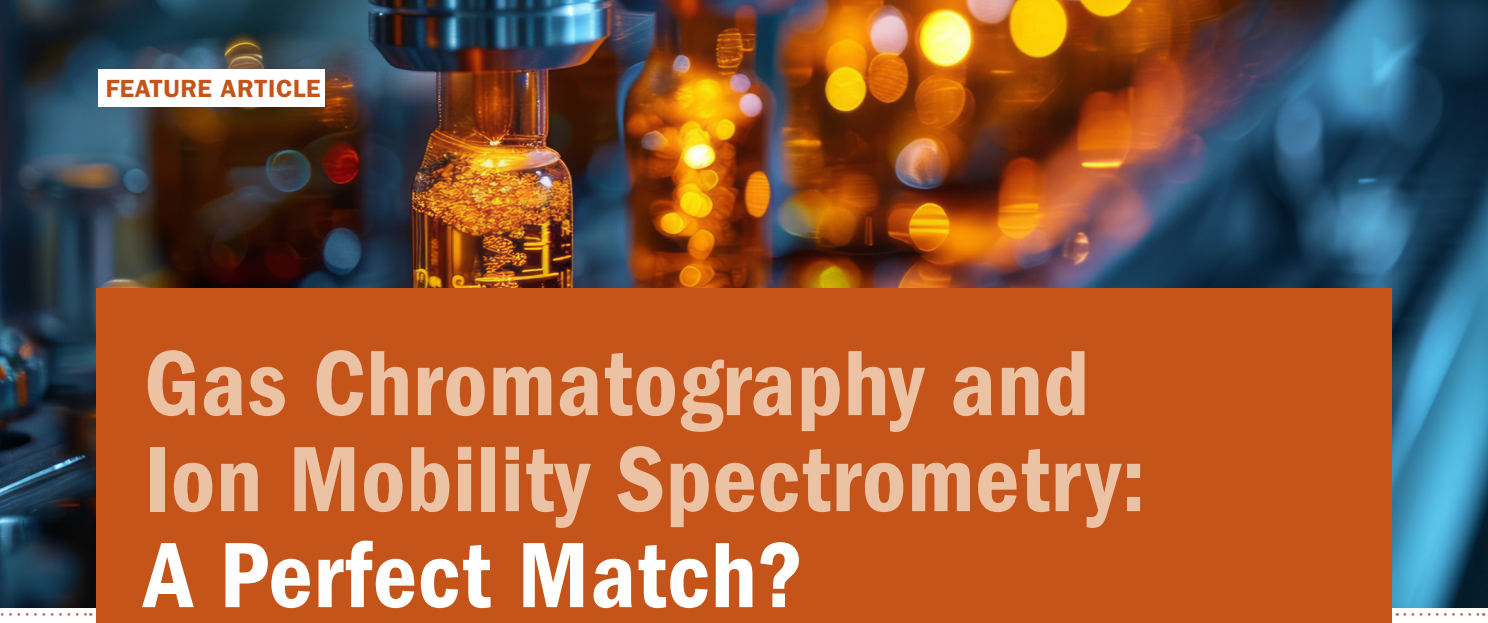

Gas Chromatography and Ion Mobility Spectrometry: A Perfect Match?

Ansgar T. Kirk, Tim Kobelt, Maximilian J. Kueddelsmann, and Stefan Zimmermann

Over the past few decades, their outstanding sensitivity and quick response times have allowed ion mobility spectrometers (IMS) to become increasingly popular detectors for gas chromatographs (GC). In this manuscript, we discuss the basic operating principle of IMS, its resulting strengths and weaknesses, and why both perfectly align with the capabilities and requirements of gas chromatographs. This is combined with basic advice for setting up GC-IMS couplings and an outlook on some future developments.

ION MOBILITY SPECTROMETERS (IMS) separate and characterize ions based on their motion through a neutral gas under the influence of an electric field. Thus, one could shrewdly argue that the first GC-IMS coupling was operated by James Lovelock when introducing the electron capture detector (ECD) (1,2). In the ECD, initial high-energy electrons create free thermalized electrons, which can be captured by analyte molecules with high electron affinity contained in the gas stream sweeping through the detector cell, forming negatively charged analyte ions. This electron capture process is extremely efficient, leading to the well-known excellent sensitivity of the ECD especially for halogenated analytes (3). A simplified schematic with parallel electrodes for better visibility is shown on the left in Figure 1. When a voltage pulse is applied between the two electrodes, the extremely mobile electrons follow the electric field to the detector, while the less mobile analyte ions either are carried out of the ECD by the gas stream or are lost to recombination with positive ions. This way, any electron-capturing analyte molecule present in the gas stream reduces the current measured by the current amplifier attached to the detector electrode. Thus, an ECD actually performs a primitive mobility measurement, being only able to separate the extremely mobile electrons from the much less mobile ions. Using this rather unusual point of view, the working principle and advantages of IMS can be easily understood.

Although there are dozens of different IMS variants (4,5), we will use a drift tube IMS with a field switching ion shutter (6) as shown on the right in Figure 1 for the comparison. Compared to the parallel plate ECD, it essentially adds the yellow drift region, which is continuously swept by a clean drift gas to prevent further reactions. Inside the drift region exists a constant electric field generated by a resistive voltage divider between the ionization region and detector electrode. However, this seemingly simple addition leads to a major difference in operating principle. By using a higher and longer voltage pulse, all ions and electrons that have been previously generated are injected into the drift region. They traverse the drift region at their characteristic drift velocities, and the ion current arriving at the detector is plotted over the time elapsed since the injection, giving the ion mobility spectrum. Containing several Gaussian peaks that mark the arrival of the different ion species, its appearance is similar to a gas chromatogram, and was, in fact, originally called a plasma chromatogram (7). Therefore, the IMS is able to differentiate various ion species from each other, and thus leads to three major advantages over the ECD while maintaining the excellent sensitivity allowed by the ionization principle. Over the past decades, several research groups have compared the analytical performance of IMS and ECD in various applications (8–10).

- IMS add a second separation dimension that is orthogonal to the GC and operates on a millisecond timescale. This provides 2D spectra as shown in Figure 2 (IMS drift time over GC retention time), similar to those from GCxGC. Because of the high repetition rate of the IMS, all peaks that are eluted from the GC can be continuously analyzed, allowing for easy reconstruction of the peaks in GC direction. Typically, even several IMS spectra can be averaged together to improve the signal-to-noise-ratio.
- As the mobility difference between protons (or more accurately protons clustered with water molecules) and positive ions is much less than the mobility difference between electrons and negative ions, it is not possible to build a variant of the ECD that detects analytes with high proton affinity instead of analytes with

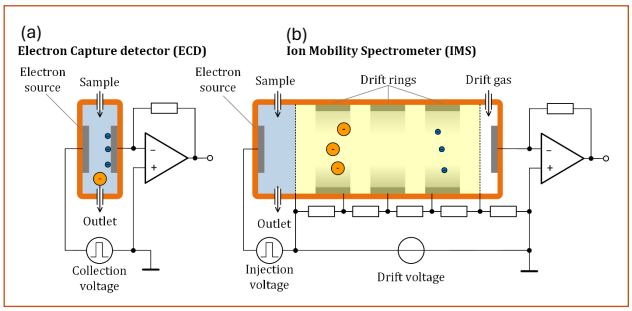

FIGURE 1: Comparison between the concepts of an electron capture detector (a) with parallel electrodes; and (b) a drift tube ion mobility spectrometer with a field-switching ion shutter. Note that neither design represents the most common variants in practice, but allow for an easily understandable comparison. See Figure 3 for a more detailed discussion of IMS design.

high electron affinity. IMS are able to resolve this much smaller mobility difference, and can therefore detect both analytes with high proton affinity and analytes with high electron affinity with the sensitivity known from the ECD.

- Unlike in ECD, where any electron capturing analyte causes a signal, electron capturing gases can even be used as carrier gases in IMS (8). Such gases have little effect on IMS sensitivity, as the ions formed from electron capturing carrier gases still ionize the target analyte, and the IMS can then separate the different ion species as explained before.

IMS can reach impressive resolving power, which is defined as the ratio between drift time and the full width at half maximum (fwhm), ranging from maybe 50 for small but sophisticated instruments to over 1000 for ultra-high-end devices (5). However, the ion mobilities of many volatile analytes fall into a rather small range of less than a factor of three, as can be seen by the *y*-axis in Figure 2, diminishing the separation performance. Nevertheless, the resulting peak capacities for a sub-second separation still range from 35 to 650 for different IMS (11). Combined with the advantages listed above, one might conclude that IMS should be able to analyze quite complex mixtures without any pre-separation. However, this assumption would lead to a rather unpleasant surprise, as one of the biggest strengths of IMS is also their biggest weakness—the extremely sensitive chemical ionization process in the gas phase is notoriously susceptible to matrix effects as shown in many studies (12–14). Ions tend to form clusters with water molecules, changing their reactivity based on humidity. Furthermore, ions and molecules from different analytes may react with each other. In the end, some analytes can be measured extremely well with IMS, even in mixtures, while others are extremely dependent on the background and might be completely suppressed. For example, despite naphthalene and pyrene having similar sensitivities when measured separately, in a mixture they only show similar signals at a concentration ratio of 100.000 to 1! (12)

Early IMS were designed for searching for reactive analytes, such as many chemical warfare agents or explosives (15), where this discrimination is not a problem at all, but an advantage as they stand out from the background. "Selective" ionization is often further

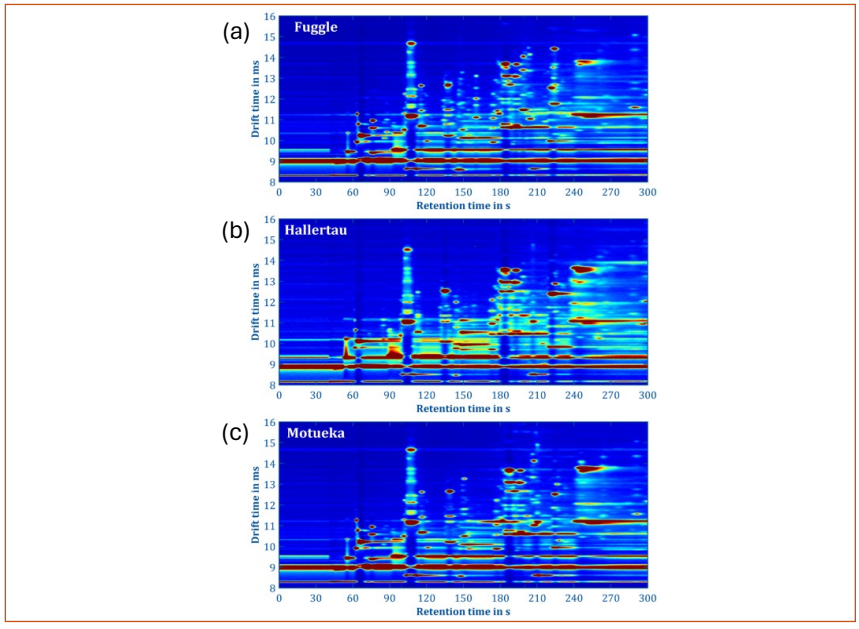

FIGURE 2: (a–c) Exemplary 2D spectra (positive ions recorded) generated from measuring the headspace of untreated hops samples using an experimental GC-IMS system with an IMS resolving power of 100 developed at the Institute of Electrical Engineering and Measurement Technology in Hanover. The GC column is a 530 µm x 1 µm x 30 m Rtx-Volatiles. At each retention time (*x*-axis), the analytes eluting from the GC are analyzed in drift time (*y*-axis). Color indicates signal intensity.

enforced through the use of dopants, ensuring that only the most reactive analytes are visible in the spectra (16,17). However, when facing complex mixtures containing the information (for example, when working in quality control or breath gas analysis), this will likely cause loss of relevant features in the spectra. Thus, even the highest resolving IMS cannot fully reveal the composition of a sample containing compounds not amenable for simultaneous ionization. This is the reason why IMS also need gas chromatography—the preceding separation from the GC breaks down complex samples into smaller chunks containing less components competing for ionization. In the combination of a GC-IMS, both techniques can bring their biggest strengths to bear.

Barring the initial quip regarding Lovelock's work, the first actual GC-IMS system was reported by Karasek and various associates in the 1970s (7,18,19), as an operational mode for a GC-IMS–MS coupling. In the following decade, research on IMS drift tubes dedicated as GC detectors followed, most importantly in the works of the Hill group at Washington State University (20–22). An overview on these developments can be found in the excellent, though now slightly outdated review by Kanu and Hill from 2008 (23).

Setting Up a GC-IMS Coupling

Generally, because of the mentioned advantages, setting up a GC-IMS system is rather straightforward:

- IMS need no consumables apart from the drift gas, which can be, for example, clean air or nitrogen and may therefore already be available in many labs, for example from systems for supplying flame ionization detectors (FIDs). Because of the mentioned effects of humidity, an additional moisture trap in front of the drift gas inlet may be advisable.
- The separation principle in IMS offers good orthogonality to many different stationary phases.
- IMS drift times are fast enough to analyze all GC peaks multiple times, allowing for easy reconstruction of the peaks in GC direction. Only hyperfast GC with only a few ten millisecond peak widths (24) could prove a limitation here.
- Both standard heated transfer lines and IMS fitting directly onto detector ports have been reported as suitable solutions to avoid cold spots in the thermal design of the setup (22). The required temperature for eluting sample components with very high boiling point may be a limitation, as the complexity of an IMS makes a high temperature design more challenging, but drift tube temperatures as high as 200 °C are possible (21).

However, despite these advantages, one key challenge remains: IMS share the fate of any other GC detector with considerable interior volume, causing excessive peak broadening if not properly designed (25). Having previously developed a holistic model for predicting both the resolving power and signal-to-noise-ratio of a stand-alone IMS (26), we are currently working on extending this model to a complete GC-IMS system. The effect of the IMS on the number of theoretical plates achieved in GC dimension is of special interest here. First studies concerning these effects have been published in joint works from Kirk, Kobelt, and Kueddelsmann and colleagues (27,28), showing that the quantity of interest for the GC–MS is the ratio of the internal volume of the IMS reaction region to the internal volume of the GC column. This leads to a more or less constant number of plates across a surprisingly wide range of linear velocities in the column. Depending on the ratio between volume of the IMS and volume of the column, a significant amount of makeup gas may be necessary to prevent peak broadening. This can, even though typically not explicitly discussed, be seen in the practice of setting up GC-IMS systems.

On the one hand, this ratio can be optimized through higher internal volume of the GC column. This has led to GC-IMS systems using either larger diameter columns, typically 320 µm or 530 µm, with high internal volume but also high transfer to mass resistance or using multi-capillary columns (MCC) consisting of multiple capillaries, combing even higher internal volume with low transfer to mass resistance (29). On the other hand, this ratio can be optimized through lower internal volume of the IMS reaction region. Starting from the first dedicated GC-IMS systems, this has led to specialized drift tube designs with low internal volume and optimized gas flow designs (20,21). The left hand side of Figure 3 shows an IMS with a reaction region volume of about one milliliter, unidirectional drift gas flow and direct axial sample introduction as described by St. Louis and associates (21).

Here, the drift gas sweeping the reaction region acts as an additional make up gas flow already forming a laminar flow profile, while the eluent from the GC is introduced into its center. The right hand side of Figure 3 shows an IMS based on an optimized ion injection method by Kirk and colleagues (6,26), allowing to reduce the effective reaction region volume to around a hundred microliters. Combined with a focusing gas flow from one or two sides, an even faster laminar flow through the reaction region is achieved (26,30,31), allowing measuring GC peak widths of half a second (32). This could be pushed even further using miniaturized drift tubes with this flow scheme (33).

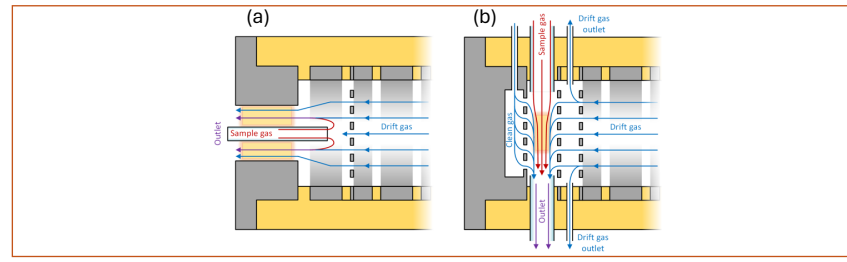

FIGURE 3: Gas flow schemes inside IMS drift tubes optimized for use as a GC detector. (a) Direct axial sample introduction utilizing the drift gas as make-up gas; and (b) focused sample introduction with a laminar flow utilizing the drift gas for focusing. The glowing rectangles mark the areas of ion generation.

Future Developments

A large part of today's IMS research is geared towards IMS–MS systems for analysis of high mass analytes such as biomolecules. However, because of these target applications, these devices are not necessarily suitable to be used as GC detectors, where the target analytes are often lower mass. As an outlook on the possible future of GC-IMS instrumentation, we would like to present some IMS specifically geared towards chromatographic detection currently under investigation in Hanover.

First, IMS can measure both analytes with high proton affinity and analytes with high electron affinity, and, typically, a complex sample will contain both kinds of analytes. As running the same sample twice through the GC needs twice the time, it is desirable to perform both IMS measurements in a single GC run. While splitting the flow between two IMS, one measuring positive ions and the other measuring negative ions, is certainly possible, it is rather inconvenient, especially due to the issues of limited sample flow from the GC discussed previously. Thus, the preferred solution would be a single IMS able to measure both polarities simultaneously. We have realized two different dual polarity IMS concepts. On the one hand, a single reaction region can be combined with two drift tubes, meaning that when the

injection pulse is applied, positive ions will be injected into one drift tube and negative ions into another, for example opposite, drift tube. Such an instrument and its coupling to a GC has been published recently by Lippmann and associates (34). On the other hand, IMS are operating on a millisecond timescale and thus significantly faster than GC as discussed before. Thus, it is possible to reverse all voltages of the IMS periodically during one GC run to record spectra in both ion polarities using a single but ultra-fast polarity switching IMS. Such an instrument and its coupling to a GC has been published recently by Hitzemann and colleagues. (35).

Second, the fact that both GC and IMS can be readily miniaturized has led to a possible realization of the long dream of handheld multidimensional analysis, where the loss of performance because of miniaturization is mitigated by the orthogonal separations (36,37). A handheld GC-IMS system, though requiring an additional gas bottle, was the first to meet this definition in 1993 (38). However, no further developments on this system were reported and in the following years, no further reports on hand-held GC-IMS appeared in the literature. Recently, Ahrens and associates published a compact GC-IMS system about the size of a shoebox that combines a miniaturized drift tube with a 7 x 1 m multi capillary column (MCC) (33,39). This system can even be operated with ultrafast polarity switching to quasi simultaneously measure spectra in both ion polarities (35).

Third, IMS are, of course, not limited to being detectors for GC, but can also be used in conjunction with other chromatographic techniques such as high performance liquid chromatography (HPLC) (40,41). Here, the sample is introduced into the IMS via electrospray ionization (ESI). Using a special ion shutter to allow efficient injection of large molecules (42,43), high-performance IMS has been successfully coupled to chip-HPLC (44) or electrochromatography (45). However, further requirements such as efficient desolvation of the ions after spraying the solvent need to be kept in mind for these applications.

In the end, one can conclude that the combination of IMS and chromatography has not only been extremely successful in the past decades, but is continuing to grow through the development of better instruments. With these, old applications can be performed more efficiently and new applications become possible. ■

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Safer AAV Full/Emtpy Analysis with Non-Toxic AEX Method

Tosoh Bioscience

We present an innovative anion-exchange chromatography method that replaces toxic TMAC with non-toxic choline-Cl, ensuring safety and efficiency in AAV capsid analysis, applicable across multiple AAV serotypes.

ANION-EXCHANGE (AEX) chromatography is a powerful method for determining the proportion of empty and filled AAV capsids. The empty/full ratio is a critical quality attribute for AAV biotherapeutics. However, traditional AEX methodologies rely on toxic eluents such as tetramethylammonium chloride (TMAC) for separation. We developed a novel AEX method utilizing a non-toxic choline-based alternative mobile phase for effective and safer AAV capsid separation[1].

Experimental Conditions

Column: TSKgel Q-STAT (4.6 mm ID × 10 cm L, 7 µm)
Mobile phase: A: 20 mmol/L Tris-HCl, pH 9.0; B: 20 mmol/L Tris-HCl, pH 9.0, 1.0 mol/L choline-Cl
Gradient: 10 - 35 % B linear in 20 min, 100 % B for 5 min, 10 % B for 5 min
Flow rate: 1 mL/min
Detection: UV @ 260 nm & UV @ 280 nm, Fluorescence Ex: 280 nm; Em: 350 nm
Samples: Purified AAV5 (Virovek, Hayward, CA); (+/- 4.7 kb ssDNA payload) at 2.0 × 1013 vc/mL.

Results

Initial experiments (data not shown) evaluated several alternative salts to replace TMAC in the gradient when using the TSKgel Q-STAT column for separating empty and full AAV5 capsids. Among these, choline chloride demonstrated the best separation efficiency, comparable to TMAC, and was selected for further experiments. A study on assay sensitivity revealed a limit of detection (LOD) of ~3.9 ×10^8 virus capsids (VC) for both empty and full AAV5 capsids using fluorescence detection, in line with values using TMAC-based measurements (Figure 1). Figure 1B indicates a strong linear response across a wide concentration range, confirming the assay's sensitivity and effectiveness. Figure 1C shows the enlarged chromatogram for the empty and full AAV5 capsids at the lowest injection amount (3.9 × 10^8 VC) where both peaks are still visible. The method's applicability to four AAV serotypes (AAV2, AAV5, AAV6, and AAV8) was confirmed, with all showing clear separation of empty and full capsids (data not shown).

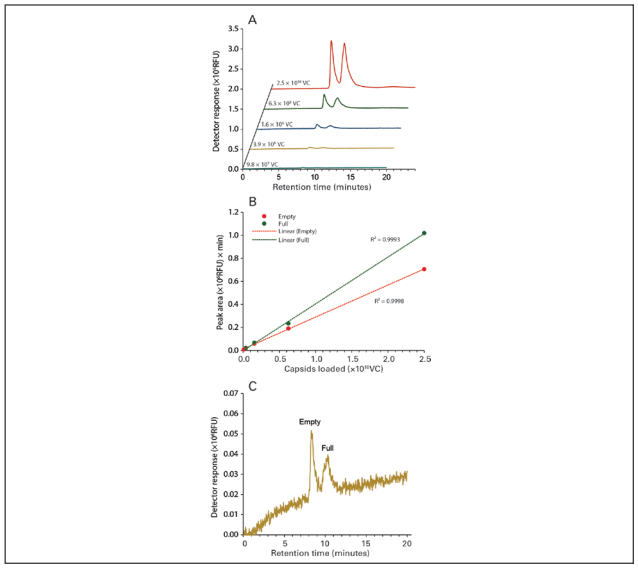

FIGURE 1: Limit of detection determination for AAV5 using a choline-Cl gradient.

Conclusion

This novel AEX method using a non-toxic choline chloride based mobile phase eluent separates empty and full AAV capsids for several serotypes. It enhances the safety for AAV critical quality attribute analyses while preserving the desired separation performance exhibited by its toxic counterpart, TMAC. Further method development, such as isocratic hold steps (data not shown), can be implemented to enhance serotype-dependent analytical quantitation.

Reference

(1) Kurth et al. Analytical Biochemistry, Vol. 686, 2024, 115421; https://doi.org/10.1016/j.ab.2023.115421

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Non-targeted profiling of polar metabolites in human plasma

Kirstin Arend & Ann Marie Rojahn, *YMC Europe GmbH*

This application note shows the non-targeted screening of polar metabolites in human plasma using a bioinert YMC Accura Triart Diol-HILIC column. The bioinert coated stainless-steel hardware is essential to achieve the highest sensitivity and chromatographic resolution of endogenous isomers.

THE FASCINATING ASPECT about non-targeted LC analysis is the presence of unknown compounds, for example in biomarker research. A particular chromatographic challenge is to find the balance between sensitivity and covering a broad spectrum of substances. Hydrophilic liquid chromatography (HILIC) is therefore especially suitable for such analyses containing polar compounds. It is a perfect match for mass spectrometry (MS) and has very good chromatographic performance. With this combination, non-targeted analysis is well suited to the screening of polar metabolites in human plasma.

Experimental Conditions

For this analysis proteins contained in the plasma were precipitated with acetonitrile (3:1 v/v). After decanting and drying the sample, it was re-dissolved with acetonitrile/water (70/30) to the original concentration.

Chromatographic conditions can be found in Table 1.

Results

Due to the complex matrix a pre-conditioning step of the stationary phase can be necessary. By using the bioinert YMC Accura Triart Diol-HILIC column conditioning is already achieved after 4 runs (see Figure 1). Furthermore, an excellent retention stability is provided after conditioning. The combination of the robust YMC-Triart Diol-HILIC stationary phase with a cleaning step after each injection and an optimal equilibration step leads to a robust and reliable HILIC method.

Figure 2 shows the non-targeted screening of a human plasma sample and selected chromatograms of polar

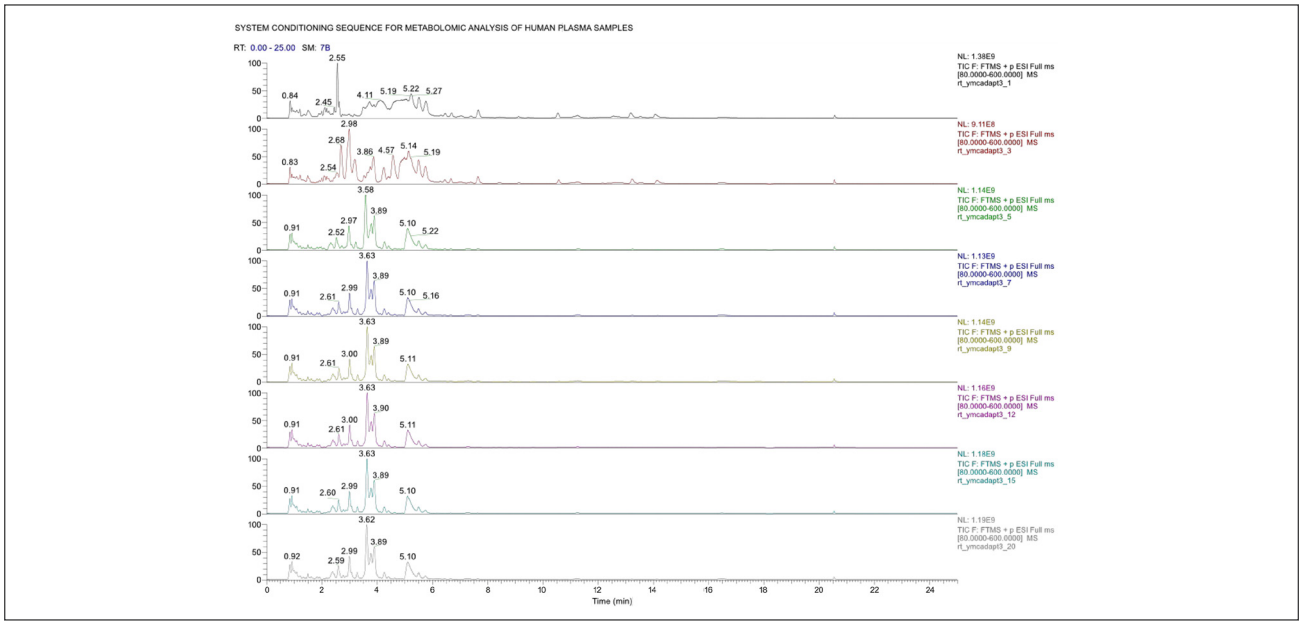

FIGURE 1: Rapid conditioning of the YMC Accura Triart Diol-HILIC column and the subsequent excellent reproducibility.

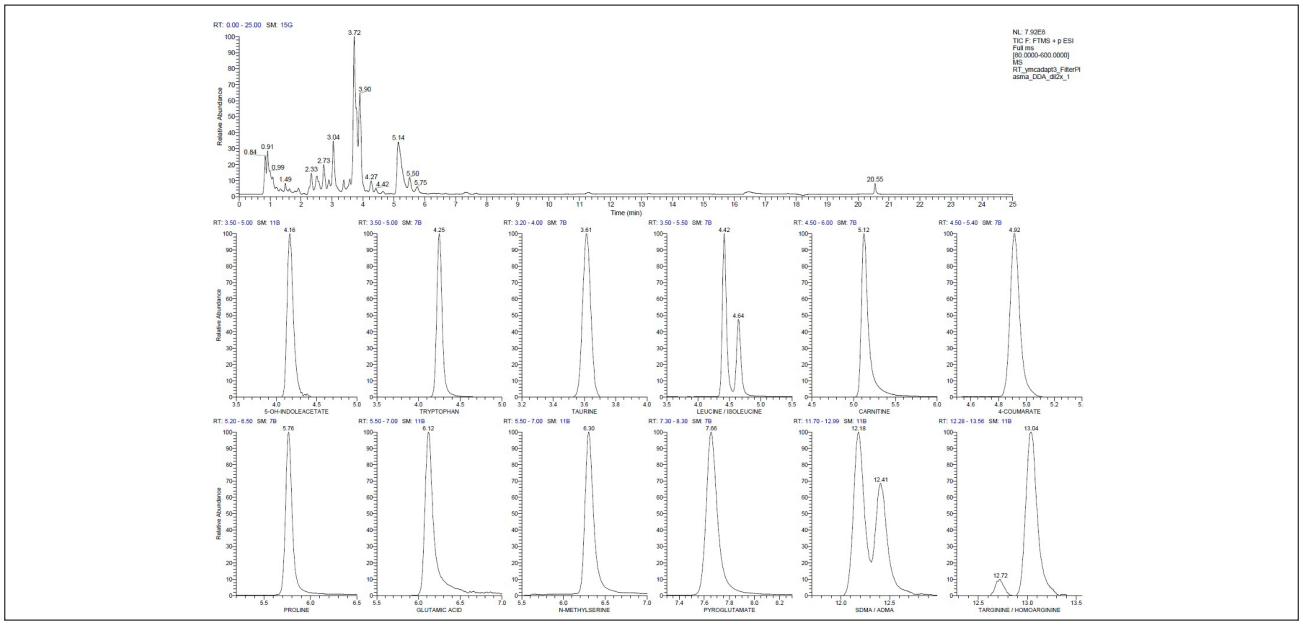

FIGURE 2: Non-targeted HRMS analysis of human plasma: total ion current of the plasma sample (top) and selected ion chromatograms of annotated metabolites < 5 ppm (bottom).

Application data by courtesy of Sergey Girel, Institute of Pharmaceutical Sciences of Western Switzerland (University of Geneva), Geneva, Switzerland.

TABLE I: Chromatographic conditions.

Column:	YMC Accura Triart Diol-HILIC (1.9 µm, 12 nm) 150 x 2.1 mm ID
Part No.:	TDH12SP9-15Q1PTC
Eluent:	10mM ammonium formate in acetonitrile/water (9/1) + 0.1% formic acid 10mM ammonium formate in acetonitrile/water (1/1) + 0.1% formic acid
Gradient:	1–38%B (0–15 min) [Cleaning step] 99%B (15.1–17 min [Equilibration] 1%B (17.1–24.9 min)
Flow rate:	0.4 mL/min During equilibration 0.65 mL/min (18–24.8 min)
Temperature:	30°C
Detection:	ESI positive, Orbitrap Exploris 120, R=60k@mz200 full scan, R=15k DDAtop4
Injection:	1µL
Sample:	Human plasma (protein precipitated) in 70% acetonitrile
System:	Thermo Vanquish Horizon

metabolites with low concentrations of < 5 ppm. The developed HILIC method using a bioinert YMC Accura Triart Diol-HILIC column covers a wide range of polar compounds with an excellent peak capacity (baseline peak width of 0.25 min on average). In addition, the method achieves resolution of important critical pairs such as leucine and isoleucine as well as asymmetric and symmetric dimethylarginine (ADMA and SDMA respectively). Together with the simultaneous high sensitivity, this method ensures a reliable generation of biological hypotheses.

Conclusions

The bioinert YMC Accura Triart Diol HILIC column provides rapid conditioning as well as sensitive and reliable analysis for non-targeted profiling of polar metabolites in human plasma.

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Comprehensive Polar Metabolite Profiling with HILIC-LC-MS

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

Hydrophilic interaction liquid chromatography (HILIC) offers a different retention mechanism that is advanced for straightforward separations of the hydrophilic metabolites despite the earlier works show it is more effective for a small set of metabolites (2). Thus, it's essential to achieve a balance between separation and coverage of biologically relevant metabolites in metabolomics studies.

In this application note, we describe a HILIC-LC-MS method that accomplished high coverage and satisfying separation of several hundred metabolites using a polymeric iHILIC-(P) Classic column in a single run. The importance of incorporating the medronic acid in HILIC separations (3) and a straightforward sample extraction procedure are also touched.

Experimental

Sample preparation:

1. Metabolite standard samples were prepared in 40:40:20 (v/v%) acetonitrile-methanol-H_2O dilution solution and stored at −80 °C. The final concentration was 10 µM.
2. 100 µL plasma was mixed with 400 µL ice-cold aforementioned dilution solution in an 1.5 mL tube by vortexing for 3-5 s. The mixture was then incubated overnight at -80 °C. Whereafter, the samples were centrifuged at 15,000 rpm for 15 min at 4 °C. Supernatants were transferred to sample vials for LC-MS analysis.
3. 293T cells after medium removal were extracted with 500 µL ice-cold aforementioned dilution solution for about 10 min. The cell extracts were transferred to 1.5 mL tubes and stored overnight at −80 °C. The protein removal was the same as that for plasma. The supernatant was used for LC-MS analysis.

LC-MS/MS system:

A Shimadzu ExionLC AC HPLC system was connected to a Triple TOF 6600+ mass spectrometer from AB Sciex. Electrospray ionization (ESI) in both ESI+ (positive) and ESI- (negative) mode were used for detection. The ESI source parameters: source temperature at 550 °C, ion source gas 1 and 2 at 60 psi, curtain gas (CUR) at 35 psi, ion spray voltage floating (ISVF) at 5.5 kV or -4.5 kV for positive or negative modes. Mass spectrometer was set at TOF masses of 70–1200 Da.

HILIC separation:

Columns:

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

Eluents:

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

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

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

Results and Discussion

Polymeric iHILIC-(P) Classic columns represent a significant advancement for HILIC separation at basic pH. With the simplified extraction technique and the optimized mobile phases, the columns allow to successfully separate several hundred hydrophilic metabolites in a single run at pH >9. This makes them an invaluable tool for untargeted metabolomics studies, especially when combined with ESI- mass spectrometry for enhanced detection and quantification. Figure 1 demonstrates the untargeted metabolomics measurements for real biological samples. After excluding metabolites belonging to "lipid or lipid-like" classes, a dataset containing 699 unique hydrophilic metabolites was obtained. Among them, 286 were found exclusively in 293T cell, 300 in plasma, and 113 in both. We were able to use our dataset of 154 metabolite standards to identify 61 metabolites exclusively in 293T cell, 34 in plasma, and 59 in both. The detection method applied in this study is effective for capturing a broad spectrum of metabolites in different classes, especially for organic acids and their derivatives and nucleosides.

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

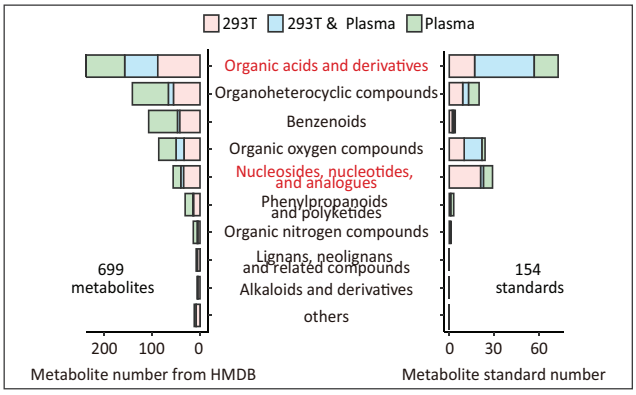

FIGURE 1: Untargeted profiling of hydrophilic metabolites in 293T cells and plasma with HILIC-MS method in ESI- mode.

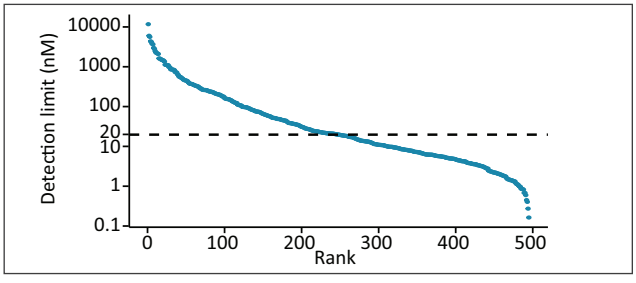

FIGURE 2: Distribution of detection limits for metabolite standards.

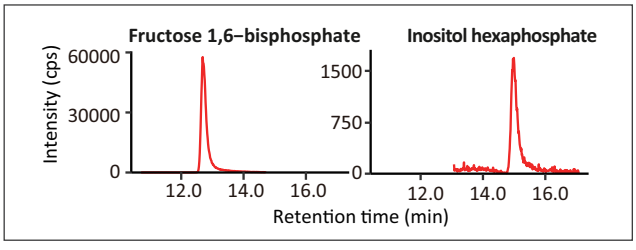

FIGURE 3: Extracted ion chromatograms of highly polar metabolite standards.

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

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

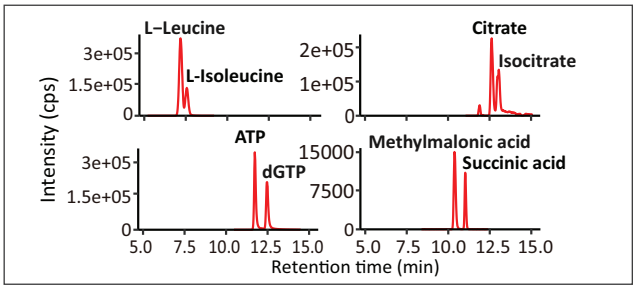

FIGURE 4: Extracted ion chromatograms of isomers from standards or samples. L-Leucine and L-Isoleucine in real samples; ATP: Adenosine triphosphate; dGTP: Deoxyguanosine triphosphate.

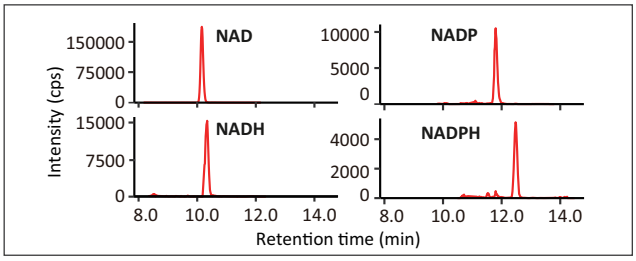

FIGURE 5: Extracted ion chromatograms of fragile metabolites in real samples.

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

Conclusion

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

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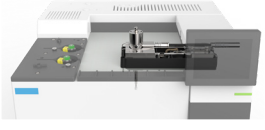

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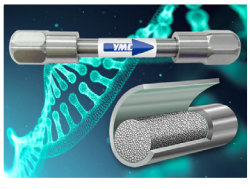

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