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Cover Story

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Rising Stars of Separation Science

A new series of interviews with early career researchers

Cover Story

Rising Stars of Separation Science: Mariosimone Zoccali 34 In the first of the series, Mariosimone Zoccali, discusses the development of a powerful analytical method to analyze important bioactive molecules using online SFE-SFC-QaQ-MS.

Features

LC–MS/MS for the Quantification of Tau and Phosphorylated Tau in Alzheimer's Disease Patients' CSF

Christopher Hirtz¹, Sylvain Lehmann¹, Laura Fichter¹, Stephane Moreau², & Jerome Vialaret¹, ¹University of Montpellier, France, ²Shimadzu Europe Quantification of Tau proteoforms in cerebrospinal fluid (CSF) could be useful to diagnose neurodegenerative diseases. LC-MS/MS results can help explain the underlying molecular mechanisms of disease evolution.

Method Robustness and Reproducibility for Monoclonal 10 **Antibody Purity Analysis**

Brian Rivera, Ivan Lebedev, and Chad Eichman, Phenomenex The benefits of a robustness assessment for the analysis of a NIST mAb using a wide pore C4 LC column are described.

Quantifying AAV Quality Attributes Using SEC-MALS 16

Sophia Kenrick, Anatolii Purchel, and Michelle Chen, Wyatt Technology SEC-MALS can measure important adeno-associated virus (AAV) guality attributes to ensure the safety and efficacy of gene therapy applications.

Regulars

9 News

The latest research and company news.

- Incognito: Analytical Laboratory Training: Far From Ideal! 21 Incognito considers the future of laboratory training, guestions the guality of current practices, and assesses the "easy wins" that can be learned across industries.
- **Tips & Tricks GPC/SEC: Separation Range and Resolution** 26 Daniela Held and Wolfgang Radke, PSS Polymer Standards Service GmbH This instalment describes the interplay between column length, pore size distribution, and particle size to optimize GPC/SEC separations.
- The LCGC Blog: Celebrating HPLC Pioneer Elmar Piel 31 James Grinias¹ and Ron Majors², ¹Department of Chemistry and Biochemistry, Rowan University, New Jersey, USA, ²Guest Editor for LCGC Elmar Piel's pioneering work was pivotal to the development of modern-day high performance liquid chromatography (HPLC).
- **Training Courses and Events** 38
- 40 Staff



К

Tips & Tricks GPC/SEC

LC–MS/MS for the Quantification of Tau and Phosphorylated Tau in Alzheimer's Disease Patients' CSF

Christophe Hirtz¹, Sylvain Lehmann¹, Laura Fichter¹, Stephane Moreau², and Jerome Vialaret¹, ¹University of Montpellier, France, ²Shimadzu Europe

Quantification of tau proteoforms in cerebrospinal fluid (CSF) could be useful to help diagnose Alzheimer's disease (AD), other neurodegenerative diseases, and brain damage. In this article, LC–MS/MS results for tau and phosphorylated tau (pTau) are compared with in vitro diagnostic (IVD)-qualified immunoassay kits to distinguish between AD patients and control patients (1).

Kenrick *et al*.

Training & Events

Cerebrospinal fluid (CSF) is a colourless biological fluid directly in contact with the brain. The analysis of CSF proteins is a potential indicator of abnormal states of the central nervous system such as inflammation, infection, neurodegeneration, and tumour growth (2–4). While CSF protein monitoring is

34 Rising Stars

The LCGC Blog

useful for diagnostic and prognostic purposes, it also provides valuable information about neurodegenerative diseases' pathophysiologies. Tau protein is mainly expressed in the central nervous system and as microtubule-associated protein, the role of which is to stabilize tubulin polymerization,



an essential mechanism for efficient intracellular transport and axonal growth. In the adult brain, 6 tau protein isoforms are translated from a single gene located on chromosome 17, and their expression is regulated by an alternative splicing mechanism. Moreover, tau proteoforms are highly phosphorylated, with about 80 putative sites scattered within the entire protein (5) and notably at position 181.

In Alzheimer's disease (AD), this hyperphosphorylation will promote its aggregation and thus the formation of neurofibrillary tangles in the brain, which are one of the histological hallmarks of the disease (6). Incidentally, tau is released in the CSF following cell damage, through secretion pathways which still needed to be fully characterized.

Importantly, AD is associated with an increase in tau and phosphorylated tau (pTau) (Serine 181) levels in CSF (7) and their quantification is included in the diagnostic criteria for AD (8). The use of tau and pTau increases diagnostic precision in cases of atypical AD states and helps in refining differential diagnoses. Furthermore, these biomarkers seem to have a predictive value on cognitive decline and disease prognosis (9). Their clinical use is implemented in many clinical laboratories (10) which used in vitro diagnostic (IVD) immunoassays

gualified kits that are conformed to the requirements of the clinical norm ISO15189.

The Clinical Proteomics Platform of Montpellier, France developed two complementary mass spectrometry (MS) approaches: a quantitative shotgun method using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS), and a guantitative targeted method using triple guadrupole mass spectrometry in multiple reaction monitoring mode (LC-MRM).

In this work, the scientific team used MS approaches (LC-MRM and LC-HRMS) and compared with IVD immunoassay results for CSF tau and pTau residue quantification.

Method

Human samples: CSF samples were obtained from patients, with follow-up by the Montpellier Neurological and Clinical Research Memory Centers (CMRR) for cognitive or behavioural disorders. Patients gave their informed consent for research and for the storage of their sample in an officially registered biological collection (#DC-2008-417) of the certified NFS 96-900 biobank of the CHRU University Hospital of Montpellier (Ref: BB-0033-00031, www.biobangues.eu) The cohort consisted of 19 control patients (CTRL) and 49 patients with AD.



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Hirtz et al.

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Figure 1: General workflow of an automated immunocapture was performed using automated liquid handling with LC–MS/MS analysis using a triple guadrupole mass spectrometer and a guadrupole time-of-flight mass spectrometer.

Enzyme-linked immunosorbent

assay (ELISA) tests: CSF tau and tau phosphorylated at Thr181 (pTau) were quantified using IVD commercially available Innotest sandwich ELISA assays (Innotest hTAU ag, Innotest_Phospho-Tau [181P], Fujirebio) according to the manufacturer's instructions.

Standards and controls: 15N recombinant tau protein (441) was obtained from Dr. Guy Lippens (UMR8525, Lille Pasteur Institute, France). Lyophilized standards were resuspended at 1 mg/mL with ammonium bicarbonate 50 mmol/L. The solution was then aliquoted into 50 µL in LoBind tubes and stored at -80 °C

until use. Standards were diluted with 50 mmol/L ammonium bicarbonate and 1 mmol/L BSA. Human CSF samples with tau concentrations determined by Innotest sandwich ELISA assays were mixed to obtain 3 CSF pool with low tau concentration (LTC, t-tau around 193 pg/ml), medium tau concentration (MTC, t-tau around 448 pg/ ml) and high tau concentration (HTC, t-tau around 615 pg/ml).

CSF tau immunoprecipitation and **digestion:** After thawing, 250 µL of CSF were incubated with 250 µL of incubation buffer (40 mM Tris) (Trisbase, Sigma Aldrich) at pH 7.4, 137 mM sodium chloride (Sigma Aldrich), 2 mM ethylenediaminetetraacetic acid (EDTA) (VWR), 10 mM Guanidine (Sigma Aldrich) and 2% NP-40 (Sigma Aldrich) and with 20 ng of 15N-tau-441 (1 µg/ ml) and 2.5 µg of biotinylated anti-tau HT7 antibodies (100 µg/mL) at 4 °C with overnight shaking. Tau immunocapture was performed using AssayMAP Bravo platform (Agilent Technologies) and AssayMAP streptavidin cartridges. The sample was eluted with 25 µL of elution buffer (12 mM NaCl/100 mM HCl, pH 2), neutralized with 20 µL of 100-mM Tris buffer at pH 8.5 and digested with 1 M of urea, 10% of acetonitrile + 0.5 μ g of Trypsine-LysC mixture for 6 h at 37 °C.

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QUALITY

After the desalting step, samples were transferred to LC–MS polypropylene vials and frozen at -80 °C before analysis.

LC-MS/MS acquisition: Two

complementary approaches of LC–MS/MS analyses were carried out (LC-MRM and LC-HRMS) and Skyline 19.1.0.193 version

was used to conduct data treatment. Data was acquired with both methods in the positive ion mode.

LC-MRM: Sample separation was performed using micro-liquid chromatography (µLC) with a Zorbax high performance liquid chromatography (HPLC)

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Hirtz et al.

column SB-Aq column, 1.0 mm × 150 mm, 3.5- μ m (Agilent Technologies). MS analysis of tau peptides was carried out on a triple quadrupole system (LCMS-8060, Shimadzu Corporation), equipped with an electrospray ionization (ESI) source. The applied mobile phase B gradient increased from 0 to 20% in 51 min, from 20 to 90% in 1 min and from 90 to 0% in 1 min, and 0% up to 60 min. The total acquisition time is 60 min.

LC–HRMS: Sample separation was performed using nano-liquid chromatography (nLC; nanoElute, Bruker) with a nanoElute 40 cm column, C18X75IDX 1.9 µm (Bruker) (Acclaim PepMap column). MS analysis of tau peptides was carried out on a quadrupole time-of-flight (QTOF) system (Impact IITM; Bruker), equipped with an ESI source. The applied mobile phase B gradient increases from 2 to 11% in 55 min, from 11 to 20% in 35 min, from 20 to 95% in 10 min, and 95% up to 110 min. The total acquisition time is 110 min.

Data processing: Skyline (64 bit) 19.1.0.193 version was used to conduct the data treatment. Based on an identified peptide list, coelution with N15 labelled peptides or aqua peptides, and retention time, masses were extracted from raw data obtained from profile analysis. **Statistical analysis:** Descriptive analysis, statistical tests and graphs were performed using Excel software (Microsoft). Statistical tests were performed on RStudio (v1.1.456). Normality was controlled with Shapiro-Wilk test on log transform data. Analysis of variance (ANOVA) was conducted to compare CTRL and Alzheimer groups with MS and immunoassays results.

Method validation: Three CSF pools with low (193 pg/ml), medium (448 pg/ml), and high (615 pg/ml) tau concentration with 15N recombinant tau protein (441) internal standard were used to evaluate inter-day variability, intra-day variability, and to perform calibration curves. Intra-day variability was evaluated by an analysis of six replicates of each quality control sample, and inter-day variability was evaluated over five different batches required to analyze the entire sample pool hailing from the cohort.

Conclusion

Fully automated LC–MRM and LC–HRMS workflows were set up and performances were compared to IVD immunoassay qualified kits (Figure 1).

Mass spectrometry allowed the quantification of several tau protein peptides, giving access to molecular

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Hirtz *et al*.

Staff

mechanisms occurring in the full-length protein such as truncations or post-translational modifications (PTMs). Monitored tau peptides are located between D24 and K223 on the tau protein sequence (tau 441).

Calibration performances were established based on different levels of tau protein in CSF. Coefficient of variation (CV) were between 7–41% depending on the peptide. The R-squared on the calibration curves were between 0.48 and 0.73.

Obtained concentrations were correlated between ELISA and MS (GAAPPGQK) to tau protein with a Pearson coefficient of 0.8803, and a coefficient of 0.7595 to pTau (T181). Graphical comparison was performed on box-plot (Figure 2). Statistical differences were observed between AD and CTRL patients.

A one-way ANOVA between disease groups was conducted to compare the capacity to separate CTRL and Alzheimer groups with MS and ELISA measures of tau and pTau.

All the results allowed, to a significant degree, to separate disease groups with a p-value < 0.05 level [F(1, 66) = 53.14, p = 4.94E-10]. Post hoc comparisons using the Welch test indicated that all the parameters were statistically relevant to separate control and Alzheimer groups (Figure 2).

ELISA and MS techniques were able to discriminate AD and CTRL patients based on pTau or tau with closed performances.

To conclude, ELISA IVD gualified kits and MS analysis gave close performances to quantify tau and pTau, both allowing to distinguish AD patients from CTRL patients. However, LC–MS/MS results have the additional advantage of being able to monitor multiple peptides and phospho-peptides along the protein sequence, which allows monitoring of specific molecular mechanisms impacting this protein during disease evolution.

Currently, the scientific team is developing the same LC-MS/MS approaches in human plasma (less invasive) to avoid CSF collection and consequently increase the accessibility of these tests. The hope is that such tests will allow, in a clinical context, a more precise follow-up of AD biomarkers, and provide a valuable clinical tool for prevention and personalized medicine.

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Christophe Hirtz is a fully tenured Professor in Biochemistry and Clinical Proteomics and specializes in clinical assays and R&D activities in the field of clinical proteomics (87 publications, ISI h-index 24).

Hirtz heads the Clinical Proteomics Platform, aiming to exploit the latest technological developments in MS and immunoassay for the discovery, validation, and use of biomarkers in various human pathologies (proteins, RNA, DNA, metabolites). He is involved in multiple scientific projects such as PI (France Alzheimer 2020) and work package leader (ANR SILK road, Proteocovid 2020).

Sylvain Lehmann was trained as an M.D. (1991, Strasbourg University, School of Medicine, France) and gained a Ph.D. in Neurobiology in 1992. He was the recipient of a Howard Hughes fellowship for physicians and spent four years in Washington University, USA, as a postdoctoral fellow and a research assistant professor (1993–1996). There he started working on Prion and AD using cell culture and cell biological tools. From 1997–2002 he was an **INSERM Senior Researcher and in 2003** obtained a position of professor of Pathology at the Medical School of Montpellier, France. As a Qualified Medical Biologist/Pathologist from The French National Medical Council (CNOM), his clinical unit at the University Hospital Center (CHU) of

Montpellier oversees the biological diagnosis of neurodegenerative disorders and he recently set up a reference Neurological biobank. Lehmann's laboratory hosts the "Clinical Proteomics Platform" of the CHU and is involved in innovative programmes on biomarker discovery and detection using mass spectrometry and immunoassays. In January 2021, he became Director of the Institute for Neurosciences of Montpellier (INM).

Laura Fichter has been an engineer specializing in mass spectrometry and proteomics at the clinical proteomics platform, since 2019, following the completion of a Master's degree in **Biotechnologies.** She is in charge of proteomics projects on the detection and characterization of tau proteoforms and is also involved in various projects on protein analysis in mass spectrometry, including characterization of the Spike protein of SARS-CoV-2.

Stephane Moreau obtained his diploma from INSA in fine chemistry and engineering with a specialization in chemical process engineering, in **1994.** He then started his professional career in laboratory equipment

distribution before joining the brand new Shimadzu France subsidiary in 2002. Since then, Moreau has held various positions to develop the MS range business. Since September 2013, he has been product manager for the MS range with Shimadzu Europe. Jerome Vialaret is an expert in proteomic, holding positions at Pierre Fabre Laboratories (Oncology center, France), EPFL (Lausanne, Switzerland), INRA (Montpellier, France), and Montpellier hospital, France. He is the team leader of the clinical proteomics platform (https://ppcmontpellier.com/index.php/accueilen/) including the technical part, the scientific communications, and the team management. Vialaret has great experience in large-sscale proteomics (proteome, protein-complex, phosphoproteome, and other PTMs) with dedicated quantitative methods (silac and label-free). In 2011 he began clinical chemistry development on protein quantification using targeted mass spectrometry in a clinical environment.

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2022 HTC INNOVATION AWARD Presented by LCKCC

LCGC Europe and HTC-17 Launch 2022 HTC Innovation Award

LCGC Europe, a leading multimedia platform of peer-reviewed technical information in the field of chromatography and the separation sciences, is now accepting nominations for the **2022 HTC Innovation Award** through to **June 30, 2021**.

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For more information and to submit a nomination, click here: https://bit.ly/3wcb3dW

Non-Targeted Chemical Fingerprinting of Phytotoxins in Environmental Matrices

Researchers from the University of Copenhagen (Denmark) have developed a novel, sensitive, and reliable analytical method to analyze phytotoxins in environmental matrices using reversed-phase liquid chromatography with electrospray ionization high-resolution mass spectrometry (RPLC–ESI-HRMS).

Phytotoxins have been classified as chemicals of emerging concern (CECs) (1). This class of secondary plant metabolites has gained attention because of their impact on the environment and potential adverse affects on human health. The development of new analytical methods to analyze these compounds is therefore highly desirable, and new methods for the targeted and non-targeted screening analysis of phytotoxins in environmental samples are in demand. The researchers from Copenhagen developed a non-targeted RPLC–ESI-HRMS method to identify five major groups of phytotoxins—steroids, alkaloids, flavonoids, terpenoids, and aromatic polyketides—in environmental matrices.

A novel, sensitive method for the targeted and non-targeted screening of phytotoxins was developed. This new non-targeted screening method was 40 times more sensitive than previous methods, according to the researchers, and allowed more than 30 phytotoxins to be identified from soil and water samples. The researchers suggested that for a balance between sensitivity, number of compounds detected, high-throughput, and peak capacity, a mobile phase consisting of 5 mM furmic acid at pH 3.0 with a gradient of 0.95% acetonitrile over 30 min should be used for both ESI + and ESI – with a column temperature of 25 °C.

In this study, the researchers also established that the negative ionization of phenols was assisted by the number of hydroxyl groups present on the ring rather than on their substitution position. This new RPLC–ESI-HRMS method will help to understand the fate of phytotoxins in the environment and assist in developing guidelines to monitor phytotoxins for public health, according to the researchers.

16

38

Kenrick *et al*.

Training & Events

Reference

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Method Robustness and Reproducibility for Monoclonal Antibody Purity Analysis

Brian Rivera, Ivan Lebedev, Chad Eichman, Phenomenex Inc., Torrance California, USA

Intact and subunit analysis by reversed-phase liquid chromatography (RPLC) is a common method for purity assay of monoclonal antibodies (mAbs). Monitoring these data in a quality control environment requires a reliable method to ensure any critical quality attributes are identified during development and production. This article describes the robustness assessment for the analysis of National Institute of Standards and Technology (NIST) mAb, intact and reduced, using a wide pore C4 LC. Method parameters such as temperature, acidic modifier, and gradient slope are assessed to determine their effect on method performance.

Kenrick et al

Training & Events

Monoclonal antibodies (mAbs) are well-established therapeutics, with a variety of different analytical methods associated with purity assays. These include many liquid chromatography (LC) and non-LC related techniques. However, one particularly attractive analytical technique common for mAb purity is reversed-phase high performance liquid chromatography (RP-HPLC). This analysis can be performed in a fairly short time, and is relatively high resolution, capable of separation of hydrophobic variants including oxidation, glycoforms, and lysine variants. As such, intact reversed-phase methods can be implemented early during development, for example, during lead selection and optimization. Coupling RP-HPLC to high

> 21 Incognito 40 Staff

Figure 1: Separation of intact NIST mAb using a 2.6-µm wide-pore, superficially porous particle. Shallower gradient of 0.38% B/min improves separation of pre-peak, as measured by USP Resolution, when compared to the steeper 0.76% B/min. Gradient programme length is 5 min for both examples.

resolution mass spectrometry (HRMS) facilitates in the characterization of these unknown variants, which is beneficial because the method transitions to downstream for stability and quality control lot-release testing.

Consequently, it behooves the analytical method developer to ensure that the RP-HPLC method is robust. This is especially true if a method originates from a platform method; that is, one which is applicable to many different analytes.

This is most certainly a viable strategy during early development, and one that is encouraged with intact RP-HPLC However, to have a transferable method that can withstand the rigours of routine testing and consistently separate out critical variants, the platform method likely will fall short. As well as for clarification for analytical method robustness, which may still get conflated with ruggedness or reproducibility. As defined in the ICH Q2B guidelines (1), method robustness is the

Rivera et al.

Figure 2: Separation of reduced NIST mAb. Heavy chain shows significant presence of pre-peak

purposeful variation of method parameters to assess their impact. In the case of intact RP-HPLC for proteins, the assessment of hydrophobic and hydrophilic variants is typically the method result in question. The question then becomes what method parameters should be adjusted, and what considerations need to be made.

One primary method parameter to vary for method robustness assessment in any RP-HPLC separation, intact or otherwise, is the gradient programme. That is, the time/length of the gradient programme,

and how shallow the gradient slope is, as measured by a percentage of strong solvent per column volume. Gradient programme robustness assessment commonly assesses length, for example, differences in method performance between 8 min vs. 10 min gradient programmes. However, given the limitation in gradient programmes to be as short as possible to minimize on-column degradation, the more prudent assessment is to vary starting and ending organic concentration, as this can effectively investigate gradient slope and

overall method effect. Figure 1 shows the difference between 25-45% B and 30–40% B. Resolution of the pre-peak is 0.96 USP with the shallower gradient slope, and any variability may lead to a decrease in resolution resulting in improper integration and thus misreporting of hydrophilic variants. Alternatively, flow-rate might be modulated to demonstrate the same. It is important to note the use of superficially porous, silica based particles for this example. Wide-pore (that is, >200 Å nominal pore diameter), superficially porous particles have been used for decades to improve the poor mass-transfer kinetics associated to macromolecules (2). However, an undervalued aspect of wide-pore, coreshell columns are the ability to run at faster linear velocities without an appreciable drop in chromatographic efficiency. The example in Figure 1 demonstrates separations being performed at 0.8 mL/ min using a 2.1-mm i.d., column which represents a linear velocity of 0.4 cm/s. For context, most intact RP-HPLC methods are much slower; 0.1–0.2 cm/s being the most common. For clarity, the intent is not to increase throughput but to allow for shallow gradient slopes without excessively long gradient programmes.

Gradient optimization seems the most apparent for a reversed-phase separation,

however, one method development parameter that must be explored for intact RP-HPLC is column temperature. Indeed, temperature may be the single most critical aspect of the reversed-phase separation of mAbs and mAb fragments. Although temperature improves diffusion and mass transfer for large molecules, it has been demonstrated that high temperature is often imperative for separation of mAb and mAb fragment segments by RP-HPLC because recovery can be impacted depending on the mAb physicochemical properties (3). Further, temperature may affect selectivity of RP-HPLC separations of mAb fragments (4), with most methods requiring temperatures exceeding 70 °C for an optimal separation.

Figure 2 shows the separation of reduced NIST mAb, using a temperature of 80 °C. Being a somewhat degraded sample, it shows the level of detail that one might obtain using intact RP-HPLC for fragments, in this instance, heavy chain and light. Figure 3 shows a different sample set, investigating the impact of purity analysis with the variation of temperature +/-3 °C from the method starting point of 80 °C.

Although the light chain shows similar % peak areas, there is some variation in pre-peak and main peak for heavy chain, with post-peaks not being present Figure 3: Reduced NIST mAb, run using identical method conditions but varying temperature. Percent peak areas for light chain are similar in both instances (28.3% and 28.1% for 77 °C and 83 °C, respectively). However, post-peak areas for the lower temperature (1.8% total peak area) are not observed with 83 °C.

with increased temperature. Again, since temperature can impact selectivity especially for mAb fragments, temperature should be explored not only during development for optimal selectivity but also modulated during robustness studies to assess impact to the chromatographic separation.

The next critical method parameter to evaluate for robustness would be the amount of acidic modifier added to the mobile phase. Trifluoroacetic acid (TFA) is the most common for RP-HPLC because it is an ion-pairing agent, acting

as a counter-ion to the positive moieties associated to proteins. This enhances the hydrophobic retention of the protein but because TFA is a strong acid, it decreases mobile phase pH thus minimizing unwanted secondary, electrostatic interactions with the inherent negative silanols present in any silica based reversed-phase media. Further, TFA is not only critical for an optimal separation, but also for protein recovery (5). A concentration of 0.1% TFA (v/v) is commonly used for intact RP-HPLC. This makes for facile mobile phase

preparation, with the addition of 1 mL of acidic modifier to the 1 L of HPLC-grade water and acetonitrile. However, even slight variation in the addition of TFA can have potentially drastic effects on overall retention and separation of large molecules. Although the effect of TFA concentration has mostly been studied with peptides (6), mAb fragments are subject to a similar behaviour chromatographically. That is, increases in TFA concentration can lead to overall increases in retentivity, thus changes in selectivity which must be explored during method robustness assessment. Figure 4 demonstrates the impact of TFA concentration on NIST mAb fragments, comparing method using 0.09 and 0.11% TFA in mobile phase A and B. As anticipated, there is a significant increase in retention, which can be problematic especially if the method has a retention time specification. More important though, is the variation in peak areas reported for mAb fragments. Although peak areas for light chain are similar, the heavy chain profiles vary significantly, with improved pre-peak recovery and separation of additional, later eluting variants. The difference of 200 µL added to mobile phase seems feasible, especially given variance in pipetting technique, as well as age and quality of TFA, and so on. Further, with

Figure 4: Reduced NIST mAb, run using identical method conditions but varying TFA concentration in mobile phase. Increases in TFA result in marked increase in retention, as well as differences in heavy chain percentage by peak areas, 67.4% with lower concentration and 56.3% with higher **TFA** concentration.

increases in retention and generally better separation with higher concentrations of TFA, this example demonstrates the case for implementing an expanded design of experiment wherein TFA concentration exceeds the 0.1% TFA which is commonly used. This has again been demonstrated with peptides (7) and could therefore provide utility in intact RP-HPLC.

A final note must be made on variation of column batches. Although batch-to-batch performance and even intra-batch variability should be assessed, it should be important to note what variation one might expect with intact RP-HPLC methods. Advances in silica sol gel have certainly improved particle morphology for more consistency in particle size and pore diameter. This

is especially true with the superficially porous wide-pore particles which are becoming more common for downstream applications wherein method transferability is a priority. However, because wide-pore particles have relatively low surface areas, sometimes as low as 25 m²/g, overall hydrophobicities between different column batches may be much more apparent. As such, one should expect a certain level of variation in overall capacity factor which may impact selectivity and resolution. This variation will then be amplified by any variance in method parameters that directly impact retentivity; namely gradient slope and TFA concentration.

Conclusion

In summary, intact RP-HPLC is an attractive method for purity analysis. Method robustness should be assessed to ensure method transferability and sustainability, especially to support downstream applications such as quality control lot release. Specifically, the gradient programme is very important: The starting and ending concentrations of strong solvent, temperature, and acidic modifier are three critical method development parameters to explore during development but also to assess during method robustness testing.

Rivera et al.

Incognito

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Quantifying AAV Quality Attributes Using SEC–MALS

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Adeno-associated virus (AAV) is an attractive delivery vehicle for gene therapy because of its mild immune response and ability to deliver the therapeutic gene into a wide range of host cells. To ensure the safety and efficacy of the viral vector-based drug product, it is essential to implement robust and reliable characterization tools throughout research, development, production, and manufacture. This article describes a method to measure three important AAV quality attributes: 1. total number of viral capsid particles; 2. relative capsid content, for example, ratio of full to total capsids; and 3. percentage of aggregates. The method uses size-exclusion chromatography (SEC) coupled to multi-angle light scattering (MALS), UV, and differential refractive index (dRI) detectors. Orthogonal methods based on high-throughput dynamic light scattering (HT-DLS) and field-flow fractionation (FFF) are reviewed and compared.

Over the last forty years, significant strides in capsid and gene optimization have made recombinant adeno-associated virus (AAV) a highly sought-after gene therapy vector, resulting in over 200 clinical trials to date and three marketed drugs (1). As engineering of

capsids and transgenes improves transfection efficiency and evasion of neutralizing antibodies, and candidates advance through product and process development, clinical trials, and full production, better analytical tools are required to characterize and quantify these novel

News

9

The LCGC Blog

Rising Stars

Rivera et al.

therapeutic agents. Light scattering provides unique capabilities for AAV quantitation and characterization in a single precise assay.

Size-exclusion chromatography with multi-angle light scattering (SEC-MALS) is a robust technique that is commonly used in the development and release assays of a large variety of biopharmaceuticals. Combining detection by MALS, UV absorption at 280 nm and 260 nm, and differential refractive index (dRI), SEC-MALS quantifies multiple AAV quality attributes (QAs) simultaneously: total capsid concentration (Cp), full-to-total ratio (Vg/ Cp), and purity (aggregation) (2). SEC–MALS eliminates the need to rely on multiple assays for capsid quantitation and characterization and is suitable for use throughout process and product development. Importantly, SEC-MALS provides significantly improved precision and accuracy compared to other techniques. For example, McIntosh et al. reported determination of total capsid concentration via SEC–MALS with <5% error relative to known values, as compared with SEC-UV (A260/A280 ratio), which resulted in >50% error, depending on the capsid content (Vg/ Cp) (3). Similar low precision has been reported by other techniques, such as enzyme-linkedimmunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qPCR) (3,4).

The enhanced performance of SEC-MALS stems from its absolute biophysical nature.

With no reliance on reagents or indirect reporters such as fluorescence, SEC-MALS determines key parameters directly, while adding deep characterization at no additional cost in time or effort. For example, combining MALS with dRI establishes molecular weight, confirming the identity of each eluting peak which might include monomers, oligomers, fragments, or contaminants; this serves as an internal control for data analysis and run-to-run comparisons. Aggregate quantitation by dRI eliminates uncertainties in UV quantitation related to scattering. Combining online UV and dRI further enables precise measurement of extinction coefficients, which are required for accurate analysis of AAV concentration and full-to-total ratio. In order to simplify rapid guantitation in guality control (QC) release assays, deep characterization can be dropped and the workflow is streamlined with analysis by just MALS and UV (260 nm and 280 nm). All of these analyses require no adjustment for different serotypes.

Complementary light scattering techniques, including field-flow fractionation with MALS (FFF-MALS) and high-throughput dynamic light scattering (HT-DLS), enable extended characterization of AAV samples and can be implemented as orthogonal methods to SEC-MALS (Table 1). FFF-MALS enables guantitation and characterization of large aggregates that may be sheared or removed by a SEC column;

Table 1: AAV analyses by three complementary light scattering techniques			
	SEC-MALS	FFF-MALS	High-throughput DLS
Separation	AAV SEC column	FFF channel	N/A
AAV concentration range	5×10 ¹⁰ to 1×10 ¹⁵ cp/mL	1×10 ¹¹ to 1×10 ¹⁵ cp/mL	6×10 ¹⁰ to 1×10 ¹⁵ cp/mL
Typical volume	1–30 µL*	1–30 µL	1536 well plate: 4–6 μL 384 well plate: 20–50 μL
Attributes measured	 Total concentration Full-to-total ratio Aggregates⁺ Identity (molar mass and size) 	 Total concentration Full-to-total ratio Aggregates⁺ Identity (molar mass and size) 	 Total concentration Aggregates⁺ Size and size distribution
* Assumes optimal SEC column with 5 μm silica particles, 500 Å pore size, and overall dimensions of 300 mm × 4.6 mm i.d. † Large aggregates are subject to shear or removal by the SEC column, but not by FFF or DLS.			

it is typically used to determine if process changes result in aggregate formation or other impurities (5). HT-DLS measurements rapidly characterize size, low-resolution size distributions, and total capsid count, and help assess thermal and colloidal stability (6).

Experimental

SEC-MALS data were collected using a standard high performance size-exclusion chromatography (HP-SEC) setup and following the procedures laid out in the SOP Guidance Manual: Critical Quality Attributes of AAV by SEC-MALS (Wyatt Technology Corporation) (7). The hardware requirements, sensitivity specifications, and typical method parameters are summarized in Table 1. Briefly,

the setup consisted of a high performance liquid chromatography (HPLC) pump, autosampler and diode array detector (Agilent Technologies), plus DAWN MALS detector and Optilab dRI detector (both from Wyatt). MALS, UV, and dRI detectors were plumbed downstream of an SEC column for separation with 5 µm silica particles, 500 Å pore size, and overall dimensions of $300 \text{ mm} \times 4.6 \text{ mm}$ i.d. (Wyatt). Pump control and data collection were performed with Astra software while data analysis was carried out with Astra's Viral Vector Analysis module (Wyatt).

Results and Discussion Total Capsid Concentration (Cp): SEC-MALS provides accurate quantitation of the total

Figure 1: Sensitivity and linearity of SEC–MALS method. (a) Total number of AAV guantified by SEC-MALS agrees well with expected values across three orders of magnitude. (b) Comparison of total physical titre (Cp) SEC-MALS compared to ELISA, provided by PTC Therapeutics.

number of virus particles over a wide range of concentrations. Run-to-run precision is typically within 5%, regardless of the starting concentration of AAV, and may be as good as 1% under optimal conditions. Figure 1(a) shows typical linearity, accuracy, and precision for capsid concentrations ranging from 8×10¹⁰ cp/ mL to 8×10¹³ cp/mL, with injection volumes varying from 1 μ L to 50 μ L. Equally high precision was observed upon injecting the same total number of capsids, using different combinations of volume and concentration, as for replicate measurements of the same sample. These results compare favourably with more traditional methods, such as ELISA, as shown in

Figure 1(b). Considering its automation and lack of complex sample preparation, SEC–MALS is suitable for routine measurements throughout the product and process life cycle.

Full-to-total Ratio (Vg/Cp): Since empty and full AAV have the same hydrodynamic radius, SEC does not separate empty from full viruses; however, separation is not required to quantify full-to-total ratio (Vg/Cp). Rather, data acquired simultaneously from multiple detectors are used to calculate the amount of protein and amount of nucleic acid in each eluting data slice and from these, the ratio Vg/ Cp. To generate the data in Figure 2, empty

and full AAV controls at a total concentration of 5×10^{12} particles/mL were mixed together to create Vg/Cp ratios from 0.03 to 0.97. For each mixture, the measured Vg/Cp is within ±0.03 of expected value, representing a level of precision and accuracy comparable to droplet digital PCR (ddPCR) and analytical ultracentrifugation (AUC).

Molar mass measurement by MALS further enhance the Vg/Cp quantitation. As shown in Figure 2(b), SEC–MALS quantifies not only the total molar mass but that of the protein and DNA components. Regardless of Vg/Cp, the protein molar mass measured by MALS reflects the expected capsid molar mass. The

measured protein molar mass, therefore, can act as an internal control, ensuring consistent analysis and complete AAV characterization; similar consistency checks are not available with simple SEC–UV measurements. Deviations from the expected capsid molar mass may indicate incorrect assembly, aggregation, fragmentation, or other degradation. These data, combined with physical titre and capsid content, are useful in assessing the consistency of recombinant AAV produced at multiple sites or evaluating cell lines and manufacturing strategies (8). DNA molar mass measurements can reveal lot-to-lot variability or changes in packing

efficiency (Figure 2b). AAV samples with high

Figure 3: SEC-MALS identifies aggregate species by their root mean square (RMS) radius. Large species may scatter UV and cause overestimation of aggregate content by UV peak area.

packing efficiency exhibit measured DNA molar masses at or near the molar mass of the transgene whereas empty AAV result in negligible DNA molar mass. AAV that pack multiple copies of a single transgene (such as in the case of self-complementary DNA) will result in DNA molar mass that is correspondingly larger than expected. By directly measuring the DNA packing, SEC-MALS can provide more accurate quantitation of Vg/Cp than gPCR which may report vast errors in titre in the presence of self-complementary DNA (4).

Aggregation and Extended

Characterization: SEC readily separates AAV

monomers from oligomers and fragments, and SEC-MALS is essential for characterizing oligomers, aggregates, and other impurities. By measuring the molar mass and radius of each eluting species, SEC–MALS identifies dimers, trimers, larger aggregates, and fragments (Figure 3) and quantifies the relative amount of each species. In conventional SEC–UV, large aggregates may scatter some of the incident UV light, causing overestimation of aggregate content by UV peak area. In contrast, MALS and dRI provide alternative quantitation of large particles and a more accurate counting of these aggregates. SEC–UV–MALS-RI data further identifies peaks that do not contain AAV,

such as free DNA released during accelerated stress studies (3).

Complementary, orthogonal light scattering measurements can confirm sample purity and assist in establishing system suitability of the SEC-MALS method. Batch DLS measurements. for example, are rapid, low volume, and nondestructive and may be used both in process development and QC to measure size distribution and to estimate total particle concentration (6). MALS, UV, and RI detection combined with field-flow fractionation (FFF-MALS) provides extensive characterization and quantitation of large species that may be retained or sheared by the SEC column. The combined light scattering toolkit, therefore, provides multiple methods for complete analysis of AAV QAs.

Conclusion

SEC-MALS measures AAV QAs in a single, rapid, automated method, with a high level of precision that analytical SEC or conventional techniques like ELISA and PCR cannot achieve (3,4). Additionally, SEC–MALS does not require special reagents, sample preparation, or adaptation to different serotypes, which allows easy transfer from development into QC and across production sites. Thus, SEC-MALS is becoming an important tool for analytical characterization and guantitation in AAV gene therapy applications.

Acknowledgements

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Kenrick et al. Rivera et al. Hirtz e*t al* News Tips & Tricks GPC/SEC The LCGC Blog **Rising Stars** Training & Events

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Hirtz e*t al*

Tips & Tricks GPC/SEC

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News

Solutions for **Viral Vector** Characterization

Analyze AAV attributes with SEC-MALS-DLS

Training & Events

Size-exclusion chromatography combined with multi-angle and dynamic light scattering (SEC-MALS-DLS) is a standard tool for biophysical characterization of biopharmaceuticals. Recent developments have highlighted its importance for adeno-associated viruses as vectors in gene therapy, where SEC-MALS-DLS determines multiple critical quality attributes, simultaneously.

SEC-MALS-DLS combines size-based separation, using standard HPLC equipment, with independent determination of molar mass and size by a DAWN® light scattering instrument. In combination with UV absorbance data, the system characterizes key CQAs: aggregation, relative capsid content and AAV physical titer.

To learn more about Wyatt's unique solutions for AAV characterization visit wyatt.com/GeneTherapy

Rivera et al.

Rising Stars

Analytical Laboratory Training: Far From Ideal!

In an increasingly digital world, Incognito considers the future of laboratory training and development, questioning the quality of current practices and assessing the "easy wins" that can be learned from other industries. With the end-goals of improving compliance, quality, and laboratory efficiency, as well as uplifting staff morale, Incognito shows that the entire industry can benefit.

I can say with some certainty that employers within Analytical Chemistry bemoan the skills and capabilities of graduates emerging from our academic systems. I can also say, with some insight, that the training provided to these graduates, once in industry, typically varies from poor to mediocre. Rarely have I seen examples of outstanding practice in workplace learning and development.

How many of us still have a large pile of standard operating procedures (SOP's) that must be "read and understood" as part of the on-boarding training process? Seriously, how much of this information do we think is retained by the recruit? Do we even bother to measure how much "understanding" has taken place?

Once the on-boarding process is complete, does the training quality improve? How

38

Kenrick et al.

Training & Events

many of us have designated trainers who have been assessed capable and gualified to train in a particular test or technique? Of these trainers, how many of them have had any degree of training in "how to train" the so-called *Train the Trainer* paradigm? Without some education and practice in andragogical practice and the most effective ways to deliver and assess training, we can assume that our training programmes are sub-optimal. Without an appreciation of cognitive and kinesthetic taxonomic levels, training designs for achieving maximum information retention and skills development or effective training techniques, then how can we expect our trainers to be effective? How much do we rely upon accreditation of prior learning (APL), in assuming that someone coming from a good university

> 21 Incognito 40 Staff

possesses some fundamental knowledge or skills which are beyond their capabilities? How much do we assume that recruits with previous laboratory experience are skilled in the basics such as gravimetric or volumetric measurement? Even if we have well developed training programmes, perhaps using dedicated test articles to assess if a trainee can produce the "correct" concentration for example, how much do we focus on the production of this "correct result" rather than the processes and underpinning knowledge associated with various stages of the analysis? How often is training based on "grandfathering" of knowledge from the more experienced team members—regardless of their knowledge or understanding? I think we all know that inherent misunderstandings or poor practice can be very quickly amplified under this type of system.

I believe we can do very much better in training analytical laboratory staff and below are some thoughts which I hope will inspire you to question your current training practice and develop your training programmes to meet the standards we associate with other industries.

Training Methodologies

First on my mind, some over-arching points relating to training methodologies. The new

zeitgeist for successful learning indicates that educational events should be: social, engaging, personalized, and inclusive. I've read this list several times lately, but most recently it was quoted on a call with LinkedIn Learning (previously Lynda.com) and I'll talk more about digital learning in a while. For now, let me just pose the question, "could your learning events be described using the list above?" I'll be honest and say that my own learning events often fall short, in all of these categories.

The folks who we use as "designated trainers" within the laboratory, will typically have reached a certain degree of knowledge and experience with a technique or test methodology that we have the confidence that they will be able to answer trainee questions, show good technique, and have a deep understanding of methodologies and equipment involved. I say "typically"; I should have said "demonstrably" because we really ought to be using subject matter or technique experts as our trainers, who can consistently show that they truly have the have required skills and knowledge. But how many of these folks will have been trained in the art and skill of training? Within larger organizations, who may have dedicated teams of trainers, this may well be the case, but how many smaller organizations could say the same? The way training is

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designed, delivered, assessed, and then transferred back into the workplace is of fundamental importance. Knowing how to prepare for training, the best ways to deliver an event to those with different learning styles, ensuring training techniques are used to maximize retention of information, and then properly assessing and ensuring the learning is transferred to daily practice is a specialist field and needs to be a primary consideration when building an effective work-based training and learning programme. This cannot simply be ignored because we work in a scientific discipline and these aspects feel like "soft skills", and therefore not applicable to our highly rigorous environment. Trainers need to be taught to be effective, and there is nothing that would convince me otherwise, and it is a fundamental truth that expertise does not give anyone, by right, the skills to be an effective trainer.

Of course, by taking this approach we are somewhat shooting ourselves in the foot with regards to the effectiveness of our training programme. I'm guessing the availability of staff with the required expertise who have been trained in the andragogical aspects of effective training design and delivery will be few and far between within any organization. Therefore, we butt heads against the evergreen

problem of "trainer availability". Laboratory Scientist A needs to be trained and "signedoff" prior to undertaking an analysis for which there is currently high demand, and therefore the training is "urgent", however there are no trainers available for scheduling in the next few weeks, and we have ourselves a problem. Well, here is where we need to get very creative, and I'll talk about the flexibility that can be afforded by digital learning in a little while. Many businesses will link their HR or quality management systems (QMS) to the training of individuals and it will be possible to schedule both onboarding and on-the-job training events well into the future, but real life rarely follows even the best planned schedule, and there must be enough capacity within the training system to flex with business requirements.

Grandfathering

In many organizations I see the processes of Grandfathering of knowledge and skills. This can even be the case where a good training documentation system exists, but where trainers very readily make statements such as, "the training documents say we do this, but for a while now we have been doing it this way" or "the training documents say we do this, but I always find it works better when I do it this way". The age-old question of who polices the police is a gnarly

one, but just ask yourself the questionswho keeps your training documentation up-to-date, and who checks that your trainers are delivering the information to the correct levels? Further, who decides what knowledge and skills can be covered with APL, when someone enters our business from another reputable company? Do we even bother to assess whether that PhD level chemist can use a positive displacement pipette properly? I could write a book about this subject but I suspect it wouldn't sell many copies and so for now, I'll leave you with the questions to ponder.

Assessment of Understanding

The last of my training methodology comments is around assessment and the so-called "transference of training" into the workplace. Again, there is so much I could write, but I'll take just a few examples to highlight the major points. The principle of "read and understand", I would contend actually means "read and acknowledge that you read", as very often there is little assessment of understanding. Why is this? Is it because it takes too much time and effort to design an effective assessment of the subject material? Is it because we need to get policies or test methods into circulation swiftly and there isn't time to design and deploy an effective assessment? Is it because

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having someone read the policy, procedure or test method is better than doing nothing at all? Or is it really because this approach is enough to cover ourselves from a quality perspective to tick a box to be able to state that "everyone was trained in the document"? Is it really too much trouble to use one of the really nice software based e-learning assessment tools to generate a meaningful "quiz" to ensure that the basics of the subject matter really are understood? I'm sure there are many examples when "read and understand" really requires some higher taxonomic level of understanding such as analysis or evaluation, where concepts need to be applied to new situations or used to draw conclusions from evidence presented. Can these levels of understanding be assessed using software tools or do we need to be more creative in order to evaluate the effectiveness of training and the assurance that we have tools to ensure learnings will be carried into the workplace. Assessment methods will need to be more comprehensive and rigorous coaching and evaluation employed on an ongoing basis to ensure the learnings are effectively transferred into daily working practice. What means do you have available to ensure this is happening? How do you assess the acquisition of new skills? Typically by "observation of the trainee" making

up a mobile phase, using a pH meter, using a pipette, and so on. I guess this is somewhat effective but read on as I pick up on the points of confirmation bias and the value of failure. Some laboratories will assess a "whole process", such as measuring the "correct" concentration for a pre-characterized test article using a chromatographic technique, which is very much a step in the right direction in terms of assessing at higher taxonomic levels, but how much confirmation bias is there in this process? What drivers are there for a trainer to observe impartially whilst a trainee makes mistakes and ultimately arrives at the "incorrect" answer for the test article concentration? How tempting is it for the trainer to intervene when they see an issue, to steer the trainee to get the right result at the end of the assessment process, because who wants to go through the training and assessment process all over again? Well, the temptation will be less if your trainers have been properly educated as educators, since they will know the positive benefits of being allowed to fail, and then deconstruct the failure for the benefit of improved understanding, and if there is an effective and flexible training scheduling programme in place, because there will be time to allow the learning through failure process to happen.

Continuing Professional Development (CPD)

An interesting question to ask, when assessing the quality of a training system, is, "to what level is knowledge, in its purest form, a fundamental aspect?" I believe that—understandably given the maturity of our science and the industrial context—we train "to do". Our training courses are based around SOP's, analytical methods, and instruments. I grant that there may be sections in these documents on "Background" or "Theory" but they are often scant and lacking in enough detail for them to be useful in, say, troubleshooting or method optimization. You may be thinking, "well, this is something which builds with a chemist over their career or, surely this should part of ongoing CPD activities", and to a large extent, I'd agree with you. But are we good at providing opportunities for CPD, do we consider it necessary for our profession? If the answer is yes, then the next question must surely be, why have our professional bodies not mandated that this is part of our ongoing training and career development. I grant you there have been welcome moves in this direction over recent years, but I don't see a clamour for the CPD points certificate at the end of external training courses, conferences, or symposia in analytical

chemistry, that I always see in the clinical diagnostics and toxicology conferences.

Digital Training

I've recently been studying how to better Video training resources, more specifically

At this point I should admit that I've been leading this discussion in a particular direction, in fact to two very important aspects that I believe point to the future of training learning in our industry. The first is simple, a single word in fact, digital. use digital learning technologies in staff development which involved a review of what is being done in allied industries. Below are just a few examples with explanation of why I think they are such good ideas. videos of experts demonstrating skills, can be highly useful in solving many of the problems highlighted above. The quality of the training is "fixed", our experts do the right things and their techniques can be reviewed by senior staff or external peers for the avoidance of doubt. The scheduling issues with training can be overcome—digital resources are accessible anytime and anywhere the trainee has internet access. Here's the killer part of implementation though—in a case study that I have followed, a peer group of trainees then gather to train each other, based on what they have learned online.

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Their skills demonstrations are filmed using GoPro type cameras or simply using their mobile phones, the videos being uploaded to a site for review and feedback from the subject matter expert trainers. However, it gets better, the trainees are then required to use their wearable or mobile tech to record the same operations during "daily" work over a set period or number of events, and these videos are then uploaded for review to ensure training transference into the workplace. This approach is evolution rather than revolution, but it has so much that is positive going for it. Preparing to teach others to do something, is in fact the best way to learn. So the "cohort learning" aspect is perfect in this regard, and it ticks so many of the boxes from our original list of ideal training requirements: social, engaging, personalized, and inclusive. The results from this study are already showing that the effectiveness of training in this manner is much higher than with traditional laboratory training approaches.

Measurable Delivery of **Knowledge-based Learning** and Assessments

Of course, digital delivery methods also allow measurable delivery of knowledge-based learning, such as that available from LCGC's very own CHROMacademy (1), or a host of

instrument manufacturers' websites, and a wide variety of other internet resources. The advantage of the CHROMacademy platform, is the number of assessments available and the way that these assessments are built to measure key facets of the learning with varying question sets—trainees are not learning the assessment answers, because the assessments change each time. Again, this improves the availability and breadth of the knowledge-based learning, and also the rigour of the assessment process, to a standardized level. For those who are sceptical of the value of digital learning, please move over and allow the Digital native generations to educate you on the amount of information that, within their daily lives, they derive from digital and online sources.

Combine all of these digital assets, learning and assessment opportunities into a digital platform (learning management system [LMS]) and one has the foundation of something which could be transformational in laboratory training and development. Heck, if we can then combine the confirmation of transference into the workplace with digital badges (the millennial equivalent of football stickers as far as I can tell!), then, with some standardization, we could perhaps begin to build a digital curriculum that could be used on an industry wide basis, which is the second important

proposal I wanted to lead us towards. Why can't we have a post graduate industrial training standard in analytical chemistry, just like so many other allied industries?

Barrier Breaking for The Greater Good

OK, so let's just pause to consider some barriers to the implementation of such a system. Who is going to build the digital assets and record the training videos? Someone still needs to arrange, and where appropriate oversee, the practical sessions where trainees are involved with "whole process" training. Someone will cry "foul" because the production of an industry wide curriculum for learning seems too democratic (why should we pay to help bring up the standards of our competitors) or that the material isn't prescient to the "way we do things here". Are we not grown up enough as an industry to overcome these barriers? With the exception of the most complex process related tasks, could our trainee cohort, given their prior exposure to digital learning and demonstrations, not be trusted to work in a safe and organized manner? Are there not a tonne of digital resources from vendors and reputable providers to lighten the burden of video production to at least give this approach a try? Are we not big enough to admit that "the way we do

things here" isn't always the best way? Isn't it about time we had some standardization and expectation setting for our analytical laboratory staff? Do we not have enough differentiators within our businesses to render analytical skills to the lesser leagues of "why we win"?

Conclusion

I'll leave everyone who made it thus far to reflect on what, in an increasingly digital world, might just be the future of laboratory training and development. We may want to ask ourselves if we are truly delivering a great industrial training and learning experience to our staff, and how anything we do to improve the effectiveness of laboratory training, might pay us pack very quickly in terms of improved compliance, quality, and laboratory efficiency. Not to mention the morale uplift in our staff who feel more "invested in" and are able to proudly display their digital badges which are collecting nicely towards their Registered Analytical Chemist certification.

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Tips & Tricks GPC/SEC: Separation Range and Resolution

Daniela Held and Wolfgang Radke, PSS Polymer Standards Service GmbH, Mainz, Germany

Whereas the elution time in interaction chromatography can be widely varied by applying a suitable eluent composition or gradient slope, the separation in GPC/SEC is restricted by the interstitial and the void volumes of the column. At the same time, the molar mass separation range of a GPC/SEC column or column combination is restricted by the column's pore size distribution. Because GPC/SEC is an isocratic method GPC/SEC resolution cannot be tuned by gradient slope, nor does eluent selection provide many options to optimize separation. Therefore, a proper choice of the GPC/SEC column or column combination is mandatory to achieve high resolution separations in GPC/SEC. This instalment describes the interplay between column length, pore size distribution, and particle size to optimize GPC/SEC separations.

Kenrick *et al*

Training & Events

In macromolecular science, GPC/SEC is a major separation technique. GPC/SEC separates individual macromolecules based on their hydrodynamic sizes in solution. A mixture of macromolecules of different sizes is injected into the chromatographic column, filled with porous particles. As the macromolecules migrate through the column, they pass pores of different sizes. Molecules larger than the pores cannot diffuse into the pores, while for molecules much smaller than the pores there is no substantial differentiation. With a size comparable to the pore size, macromolecules can migrate into the pores and are separated based on their residence time in the pores, which depends on their size in solution. Since macromolecules of the same chemical structure but varying in molar masses exhibit

Figure 1: Effect of non-optimal selection of pore size on chromatogram for a broadly distributed sample.

different sizes, they can be separated by GPC/SEC according to molar mass.

It then follows that a column providing only one pore size can only separate a limited molar mass range. As a rule of thumb, a single pore size column allows separating macromolecules differing in molar mass by approximately two orders of magnitude (1,2,3). If samples of broad molar mass distribution have to be separated, either columns providing a mixture of pore sizes or combinations of columns having different pore sizes need to be applied.

While the molar mass range that can be separated by GPC/SEC is governed by the

pore size distribution of the column packing, the resolution of two molar masses to be separated is additionally influenced by the total pore volume of the column or column combination and the particle size. The latter parameter needs to be taken into account, as it affects band broadening.

The first part of this instalment of Tips & Tricks will discuss the effect of pore size, pore size distribution, and column length on the ideal separation of two molar masses, while the second part will elucidate the effect of band broadening, in particular due to particle size on the resolution in GPC/SEC.

Effect of Pore Size on Molar Mass Separation Range

As mentioned above, the molar mass range that can typically be separated on a single pore size column covers approximately two orders of molar mass. Since the molar mass distribution of typical macromolecular samples often extends over several orders of magnitude (4), the column's upper exclusion limit or the low molar mass separation limit might be reached if the molar mass range of the single pore size column is not well adjusted to the molar mass range of the sample.

Figure 1 schematically depicts the effects of selecting different single pore size columns to the separation of a broadly distributed sample. On the column characterized by the black calibration curve, the sample is well separated, as the whole molar mass distribution is covered by the nearly linear range of the calibration curve. In contrast, on the column characterized by the blue calibration curve, corresponding to a column of smaller pore diameter, the sample reveals a rather narrow high molar mass peak followed by well separated oligomers. The steep increase at low elution volume is due to the very high molar mass fraction of the material approaching the exclusion limit of the column. Because the high molar mass fraction is not effectively separated any longer, the high molar mass molecules elute in a very narrow elution volume range, piling up their concentrations

which finally results in a sharp exclusion peak. Such exclusion effects can result in additional shoulders or even additional peak maxima. which are, however, not a characteristics of the sample's molar mass distribution, but originate from an unsuited molar mass separation range of the column. The chromatogram obtained on the column characterized by the red calibration curve reveals a well-separated high molar mass fraction, while the oligomers are not resolved and elute as a non-separated single peak. The lack of separation of the oligomers results from the steepness of the calibration curve at high elution volumes, which render the oligomers to elute very close to each other thereby merging into a single peak. It should be noted that the molar mass corresponding to the weight average molar mass of the sample is within the separating range of each of the three calibration curves. Thus, selecting a column based on published calibration curves considering only the weight average molar mass of the sample, without taking into account the width of the molar mass distribution, can result in selecting an unsuitable column.

Effect of Pore Size Distribution

The width of the molar mass ranges covered by typical macromolecular samples in conjunction with limited separation range of a single pore size, often necessitates the provision of not only one specific pore size but

Figure 2: Schematic comparison of molar mass separation range for a single pore size column (blue) and a mixed bed column (turguoise).

a distribution of pore sizes. Providing a wider range of pore sizes in a column or column combination extends the molar mass range that can be separated. Columns containing not only a single pore size, but a range of different pore sizes are referred to as "mixed bed", "linear", or "multipore" columns.

Since a single column packed with particles with a broad pore size distribution extends the molar mass range that can be separated, but does not alter the elution volume range over which the separation occurs, a steeper calibration curve results, as compared to a single pore size column. This is evident in Figure 2.

Here the two molar masses indicated will be separated by a lower elution volume range

on a mixed bed column of broad pore size distribution, as compared to the separation on a column providing only a single pore size.

Effect of Column Length

Instead of packing a single column with a material of broad pore size distribution, it is possible to couple several columns, each of which having a narrow pore size distribution. Such a column combination or column bank provides a large molar mass separation range, due to the different pore sizes present. In addition, such a column bank has a large pore volume. Thus, the volume range over which the separation takes place is extended as compared to a single column filled with

the same pore size mixture (mixed bed column). In both approaches the same pore size distribution and thus the same molar mass separation range exists. However, the separation volume for the column bank is larger than of the single column. Therefore, the slope of the calibration curve is less steep for the former, and two molar masses elute further apart, as on a single column. This is visualized in Figure 3. This enhanced separation is achieved, however, at the cost of longer analysis times and larger solvent consumption.

It is also possible to couple several linear or mixed bed columns to a column bank, maintaining the same separation range and increasing the separation volume, thereby decreasing the slope of the calibration curve. However, mixed bed or linear columns are

usually not well adapted to the molar mass distributions of the specific samples to be analyzed but are designed as "general purpose" columns. Therefore, linear or mixed bed columns often do not only provide pores required for the specific separation problem but also pore sizes which are of no particular advantage, due to being either too large or too small. Since these pores contribute to the total pore volume, linear or mixed bed columns usually increase analysis time and solvent consumption without providing the full benefit regarding sample separation.

Effect of Particle Size

As mentioned above, chromatographic resolution is determined by two factors: the difference in elution volume between the

two components that need to be separated and by band broadening effects, which deteriorate the separation of the two ideally separated peaks.

While the spacing between the different components in GPC/SEC is determined by the slope of the calibration curve and the available column volume, as explained above, several factors contribute to band broadening, for example, a finite injection and detector volume, broadening/mixing within the capillaries and detectors, and column band broadening. In general, smaller particles reduce column band broadening. Band broadening effects are typically quantified by injecting a monodisperse compound and determining the plate count (N_{+}) from peak width (w_{+}) and elution volume at peak maximum (V_{z}) , with L being the column length in cm:

$$N_{th} = \frac{554}{L \ [cm]} \left(\frac{V_p}{W_{1/2}}\right)^2$$

Due to the different diffusion and transport phenomena occurring in a chromatographic system, band broadening and thus plate counts depend on variety of parameters. Despite this, it is good practice to determine the number of theoretical plates regularly under identical conditions as it provides an easy tool to track the system performance. A significant decrease in plate count might indicate problems with the instrument and/or the separating column. However, as mentioned before, plate count is but one factor to achieve good GPC/SEC resolution. The other factor is the slope of the calibration curve (5).

The interplay of these parameters is schematically shown in Figure 4. Here we

investigate the separation of two molar masses present in the sample. When comparing the elugrams resulting from a column providing a flat (a) and a steep calibration curve (b) at the same instrument performance (peak width) the resolution between the two peaks is clearly higher for the column of lower slope. This is due to the distance between the two peaks being higher on the column providing the lower slope.

If we compare the chromatograms resulting from columns of identical slope but different plate counts (b, c), the resolution between the two peaks is diminished for the column of lower plate count (broader peaks, c). Such broader peaks might either result from aging of the column, which might lead to increased peak broadening, or from two columns having the same pore size distribution but differing in particle size.

It is not uncommon for SEC/GPC users to conclude from a low slope of the calibration curve to the existence of a well separating GPC/ SEC system. However, the above discussion showed that the slope of calibration curve is but one factor determining resolution. Some national and international standards stipulate a minimum for the specific resolution within the required separation range, which can only be realized using column combinations. The calculation of the specific resolution takes into account both, the slope of the calibration curve and the influence of band broadening. More details can be found in the Tips & Tricks instalment on "How to Test GPC/SEC columns" (5). As mentioned above, lower particle sizes are beneficial from the perspective of band broadening. However, macromolecules, especially of high molar mass are prone

to shear. Shear might result in stretching of the molecules and lead to elution, not determined any longer by the coil size of the macromolecule. Therefore, high molar mass polymers should be analyzed by GPC/SEC using larger particles. On the other hand, if oligomers or samples producing narrow peaks such as proteins need to be separated, band broadening should be reduced as far as possible, to obtain good resolution. Thus, lower particle size columns are recommended. The effect of particle size on band broadening and thus on oligomer separation is shown in Figure 5. Clearly the oligomer resolution is better the smaller the particles are.

Large particles are preferred for the separation of high molar mass molecules, whereas smaller particles are preferred for the resolution of oligomers. This seems to suggest the columns should be filled with mixtures containing both, large particles with large pores and also small particles with small pores, if broad molar mass samples need to be analyzed.

However, instead of combining the advantages by packing small particles with small pores and large particles with large pores in a single column or column combination, such an approach combines the drawbacks of both, as discussed in a previous instalment of Tips & Tricks for GPC/SEC (6).

Summary

- Resolution in GPC/SEC depends on the slope of the calibration curve and on plate count.
- The distance between two molar masses separated by GPC/SEC depends on the slope of the calibration curve.
- The molar mass range that can be separated by a single pore size column in GPC/SEC is restricted.
- Broad pore size distributions of a column's packing material provide a large molar mass separation range at the cost of a steeper calibration curve, as compared to an individual pore size column.
- The identical molar mass separation range obtained using a column bank provides a less steep calibration curve, thus, larger elution volume differences.
- Separations for large macromolecules require large pore and particle sizes.
- Oligomer separations should be performed on columns providing small pore and particle sizes.
- Column combinations of large and small

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Tips & Tricks

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James Grinias¹ and Ron Majors², ¹Department of Chemistry and Biochemistry, Rowan University, Glassboro, New Jersey, USA, ² Chromatography and sample preparation consultant, and Guest Editor for LCGC, USA

In this LCGC Blog, James Grinias invites Ron Majors to join him in writing about scientist, Elmar Piel. Perhaps unfamiliar to many chromatographers, Piel's pioneering work on early high performance liquid chromatography (HPLC) was pivotal to the development of our modern-day HPLC.

Ron Majors was the 2020 recipient of the U.S.-based Chromatography Forum of the Delaware Valley (CFDV) Award, which is given to those who have provided exceptional service to the Forum in addition to outstanding contributions within the field of chromatography. Readers of *LCGC* are well aware of his nearly 60 years of research and leadership in this area (1), but few outside the Delaware Valley region know of his decades of membership on the CFDV Executive Committee, including two terms as president. As part of this well-deserved honour, Ron gave a (remote) address to

the organization in October 2020, detailing his many accomplishments in the field and summarizing the current state-of-the-art in high performance liquid chromatography (HPLC) column technology (2). However, it was his introduction describing the early days of HPLC that stood out to me, specifically a name I had not heard before: Elmar Piel. For this month's instalment, I invited Ron to join me in writing a bit more about this scientist, who may be unfamiliar to many chromatographers.

Hirtz et al. Tips & Tricks GPC/SEC

The LCGC Blog

News

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34 Rising Stars

Rivera et al.

The Research We Know

Leslie Ettre was well known for his work

Figure 1: Separation of dyes on an alumina column with centrifugal driven mobile phase flow. Reprinted with permission from (5). Copyright 1966 American Chemical Society.

in capillary gas chromatography (GC), but also for his love of the history of modern chromatography. From 1999 to 2008, he contributed the "Milestones in Chromatography" series to LCGC (3),

and expanded on these articles in his 2008 book Chapters in the Evolution of Chromatography (4). This book is a must-read for chromatographers, as it carefully details a century of research in the field based on both published scientific literature and personal accounts of the work. Full chapters of the book are dedicated to the key developments in liquid-phase separation instruments from the mid-1900s: (A) the work of Stein, Moore, and Spackman, and later Hamilton, on ion exchange chromatography (IEC), (B) the work of Moore and the Waters Corporation in gel permeation chromatography (GPC), and (C) the development of the modern HPLC by Horváth, Preiss, and Lipsky. Ettre then goes on to highlight other key researchers that are often tied to the early development of HPLC: Giddings, Pretorius, Knox, and Locke on the theoretical side, and Huber, Kirkland, Snyder, and Scott on the experimental side. Most of these names are very familiar to chromatographers, and many scholars in the field are easily able to point to specific papers from these authors that demonstrate key advances in the field. A number of foundational papers written by these authors cite an Analytical Chemistry paper from 1966 entitled, "Accelerated Microparticulate Bed Liquid

Chromatography", written by Elmar V. Piel (5). Far fewer modern chromatographers are aware of this paper, however. What work does it actually describe?

The Work of Elmar Piel on Early HPLC

In his paper, Piel describes early approaches to the use of microparticulate-packed columns for liquid-phase separations. Of the roughly three dozen citations the paper has received, only a half dozen have come in the past two decades, with two from Ron himself in LCGC. In 2015, Majors detailed the overall focus of the paper in reference to pivotal works that led to modern HPLC (6):

"The first is a little known 1966 paper by Piel written as a short contribution to Analytical Chemistry where the author slurry-packed finely ground silica, calcium carbonate, or alumina particles into 1or 2-mm i.d. glass columns. The mobile phase was driven by centrifugation or with a high liquid pressure differential using a pump capable of 3500 psi operation. The particles used varied from less than 1-μm to 0.012-μm. The centrifugal driving force was 1000–1500 times gravity and was applied for 5 min while the pump's full pressure was applied. The beds varied

Figure 1.)

from 1.6–4.0 cm in length. Samples included various dyes as well as a spinach extract. Separations took only a few minutes for both operations. Piel claimed 'excellent resolution and high capacity of microparticulate beds' and showed the results in his accompanying figures." (See

Earlier in 2006, Ron was discussing the early development of silica particles designed for HPLC columns in the early 1970s, and infers why the work may have not received significant attention when first published (7):

"At the time, column researchers knew that small porous particles (less than 20 µm) would provide even better efficiency and maintain the high capacity of the earlier porous packings. Some earlier work by Piel in 1966... showed promise, but the particles that [he] used were commercially available Cabosil (Cabot, Billerica, Massachusetts, USA) fumed-silica packings that were sub-1.0 µm sizes, were fairly inert, were difficult to handle, and required extremely high pressures to operate. In short, these particles were unsuitable for the current needs at the time."

Thus, although my first realization of the impact of this paper did not come until late 2020, Ron had been discussing

this paper nearly fifteen years ago when I was a student in the classroom rather than the professor! During his award talk, Ron mentioned that Piel patented the first centrifugation-driven technique reported in the paper (8), but history might have turned out differently had it been the pump-driven operation that he pursued—the instrument we now know as the high pressure liquid chromatograph! A cursory search of the literature indicates that his subsequent papers concentrated on education-focused laboratory tools, although he did publish one additional paper in GC (9) two years before he published the liquid chromatography (LC) paper discussed here. While investigating this post, I also learned that Elmar was part of the Piel Brewery dynasty from Brooklyn and that his father was a brewmeister there—a family legacy in chemistry! (10)

Digging Deeper into Chromatography History

Efforts in maintaining the history of a technical field like HPLC has often relied on those individuals interested in the topic (such as Ettre), trade publications like *LCGC*, and local discussion groups (including CFDV). However, the details provided here were primarily obtained

Hirtz et al.

Tips & Tricks GPC/SEC

through a search of the scientific literature, which is fairly accessible to most practicing chromatographers. What other interesting papers and stories are waiting out there to be discovered?

Acknowledgements

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News

The LCGC Blog

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Training & Events

Alumnus of the Year Award, and the 2020 Young Investigator Award from the Chinese American Chromatography

Ron Majors (ChromPrep LLC) is currently a chromatography/sample prep consultant after having retired from Agilent Technologies as a senior chemist in late 2013. He was also the editor of "Column Watch" and "Sample Preparation Perspectives" for LCGC for over 30 years, retiring as a monthly columnist in 2015.

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Rising Stars of Separation Science: Mariosimone Zoccali

Kenrick et al.

In the first in our series of interviews with early career researchers we spoke to Mariosimone Zoccali, assistant professor of analytical chemistry at the University of Messina, Italy about his innovative work to develop a powerful analytical method using online SFE–SFC–QqQ-MS to analyze important bioactive molecules.

—Interview by Alasdair Matheson

Q. When did you first encounter chromatography and what attracted you to the subject?

A. I took my first steps into chromatography during my second year of Pharmaceutical Chemistry and Technology degree course in 2007 at the University of Messina (Italy). At that time. I had the chance to attend the analytical chemistry laboratory directed by Professor Luigi Mondello. I still remember the day I saw a two-dimensional (2D) chromatogram acquired by Professor Peter Tranchida using the longitudinally modulated cryogenic system (LMCS) modulator. I was immediately struck by the impressive 2D map of a cod liver oil fatty acid methyl ester

34. Rising Stars

The LCGC Blog

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graphics!

thesis?

Much of my experimental work has been

A. During the Ph.D. course, my research work has been focused mainly on the development of multidimensional chromatography techniques for complex food analysis. performed in the field of gas chromatography (GC), even though I also practiced liquid chromatography (LC). Moreover, I have been involved in theoretical and practical research in the field of cryogenically-modulated comprehensive two-dimensional gas

sample, which I associated with video game

Q. Can you tell us more about your Ph.D.

Mariosimone Zoccali is an assistant professor of analytical chemistry in the Department of Mathematical and Computer Science, Physical Sciences and Earth Sciences at the University of Messina, in Italy. His research is focused on the development of multidimensional chromatographic instrumentation and software (GC×GC, LC–GC, LC–GC×GC, SFE–SFC),

coupled to state-of-the-art MS for the study of complex matrices constituents and contaminants. Mariosimone has authored or co-authored more than 50 articles and book chapters. He has given award-winning presentations at multiple international meetings. He has been directly involved with the development of award-winning instrumentation. In particular, he assembled a five-dimensional instrument consisting of a liquid chromatography system combined with a comprehensive two-dimensional gas chromatography-triple guadrupole mass spectrometry instrument. Zoccali received recognition for his achievements in 2018 with the Young Researcher Award, on behalf of the Interdivisional Separation Science Group (Italian Society of Chemistry). He has pursued and won multiple travel awards to better disseminate his research to the broader scientific community. He is also leader of the working group three of the EuChemS-DAC Sample Preparation Study Group.

chromatography ($GC \times GC$) culminating with the introduction of a new optimization parameter: "gas velocity at the point of reinjection".

Q. What chromatographic techniques have you worked with?

A. Under the authoritative mentorship of Prof. Mondello and Prof. Tranchida, the techniques used during my Ph.D. have been: fast gas chromatography tandem mass spectrometry (fast GC-MS/MS), online LC-GC, off-line liquid chromatography coupled to comprehensive two-dimensional gas

chromatography mass spectrometry (LC// GC×GC-MS), and GC×GC–MS. The methods developed have been successfully applied to untargeted and targeted food analysis. The use of such powerful analytical instruments enabled detailed information to be obtained. particularly related to food safety. In this regard, different kinds of contaminants were investigated, such as mineral oil saturated hydrocarbons and pesticides, both topics of high relevance in recent years.

It is also worth mentioning the four-dimensional combination of LC and $GC \times GC - MS$, an approach that could open new analytical doors in the near future, especially for the elucidation of very complex samples, and for trace-analysis.

After achieving my Ph.D. degree, I also had the opportunity to work with supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) systems coupled with triple quadrupole MS (QqQ/MS).

Q. You recently published a paper on the determination of carotenoids and apocarotenoids in human blood samples by online supercritical fluid extraction supercritical fluid chromatography tandem mass spectrometry (SFE-SFC-MS/MS). Why is this type of analysis important?

A. Carotenoids are naturally occurring pigments, usually consisting of a C₄₀ backbone, associated with beneficial health properties. Apocarotenoids result from oxidative and enzymatic cleavage of the parent carotenoid, which are very important bioactive molecules in plants (1) and have also been reported in humans where they may exert unique biological activities (2). For example, it has been suggested that β -apocarotenoids function as naturally occurring antagonists of retinoic acid receptors, with possible implications as modulators in cardiovascular diseases and cancer prevention. Despite the great interest, only a limited number of studies

are available in the literature on human blood apocarotenoids. Carotenoids and apocarotenoids detected in very low amounts in human blood samples could represent useful biomarkers in large clinical or epidemiological studies. Consequently, we undertook the development of an analytical platform consisting of SFE–SFC with QqQ/MS, as a rapid and sensitive comprehensive evaluation tool.

O. You decided to use SFC-MS/MS as the separation technique for this application. Why was SFC chosen rather than, for example, LC–MS/MS?

A. Given my enormous curiosity, in 2016, as soon as the instrument was installed in the laboratory where I work, I immediately decided to learn how to use it. My initial idea was to develop a method that could attract a broader interest beyond the scientific community. The main practical advantages of SFC over high performance liquid chromatography (HPLC) are the low viscosity of the mobile phase and high-diffusion coefficients, allowing for high linear velocities to be employed, and high-efficient separations to be achieved. Additionally, supercritical CO₂ is non-polar and is ideally suited for the extraction and separation of hydrophobic compounds. MS/MS obviously allows high selectivity and sensitivity detection.

> Incoanito Staff

The idea was to develop a rapid screening method for bioactive compounds, such as carotenoids and apocarotenoids in biological samples, by combining the benefits of these techniques.

Q. What advantages did online SFC offer over other sample preparation techniques? When is it useful to use SFE?

A. In general, SFE offers a series of compelling advantages in terms of volume and toxicity of solvents employed, the amounts of waste produced, and a reduction in occupational exposure hazards. Apart from the significant savings in time and associated costs, further advantages of SFE over conventional extraction consist in its selectivity and easy removal of residual CO₂, by easy disposal of the expanded gaseous CO after depressurization. Moreover, hyphenated SFE–SFC systems are easily implemented and offer advantages in terms of solvent and energy consumption, separation efficiency, and speed of analysis. Online SFE-SFC offers a series of clear benefits, consisting in minimum sample preparation and associated risks of cross-contamination, suitability for analytes prone to oxidation or degradation, improved run-to-run precision, easy setting of batch-type applications, and the likelihood for complete automation of the whole analytical system.

Interestingly, by adding different amounts of a more polar solvent to the CO₂ mobile phase (the so-called *modifier*), the range of SFE–SFC-amenable compounds may be conveniently increased, to more polar analytes.

Q. What is novel about this approach from an analytical perspective and what were your main findings?

A. The online SFE–SFC–QqQ-MS method developed has shown to be a powerful analytical tool for rapid screening of bioactive molecules in intact human blood samples. The true significance of this research lies in the demonstration that proper operation of new analytical systems allow for the detection of bioactive molecules never reported previously. The latter could be identified in very small aliquots (only 10 µL) of biological samples, and without any sample pre-treatment. Moreover, the development of these methods fits perfectly into the context of green chemistry, reducing both the use of chemical solvents and the associated wastes.

Q. Do you think SFC is gaining wider acceptance and, if so, in what types of applications?

A. SFC was introduced in the 1960s and had a rapid rise as a new separation topic in the late 1980s and 1990s, before starting a slow decrease and almost waning over a

20-year span, standing behind the scenes of separation techniques. The last 15 years have witnessed a renewed interest in this technique, as the introduction of a new generation of user-friendly commercial instruments has given new propulsion to the development and application of SFC-based methodologies.

In the past, the SFC technique was largely used for the analysis of lipophilic compounds, mainly lipids. Nowadays, there is a growing trend towards the analysis of molecules with higher polarity—such as monosaccharides, saponins, flavonoids, and plant metabolites—with promising pharmacalogical effects. Another category, which is recently attracting a great deal of attention, is cannabinoids, often occurring in both medical and forensic applications.

Q. You also used SFC to analyze free apocartenoids and apocartenoid esters in human colostrum (3). What were your main findings here?

A. It was a very interesting study performed in collaboration with the Department of Human Pathology in Adult and Childhood of my University in Messina, Italy. The presence of non-retinoid apocarotenoids in human colostrum was reported for the first time. The formation of free apocarotenoids may be related to their potential contribution in the regulation of various cellular functions,

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as well as to homeostasis. The occurrence of the esterified forms is probably related to a more stable form of storage, as is the case of vitamin A deposited as retinyl esters in human tissues. Their occurrence in human colostrum has probable implications for newborn health status because colostrum represents the only form of food during the very first days of human life.

Q. What projects are you working on next?

A. I will certainly continue my research work based on the SFE-SFC technique; currently we are implementing a method for the extraction and characterization of chiral pesticides in hemp seeds. At the same time, returning to my first "love", in collaboration with Professor Synovec's group, we are testing the

capabilities of a newly developed software for comparing large GC×GC data sets. For this work, we are analyzing the volatile fraction of Sicilian autochthon wines by headspace solid-phase microextraction comprehensive two-dimensional gas chromatography timeof-flight mass spectrometry (HS-SPME-GC×GC–ToF-MS). Untargeted analysis of GC×GC-MS data is undoubtedly the most profitable strategy for exploring the volatile food metabolome, however, given the enormous amount of data generated, the use of statistical software has become essential for fast and reliable elaboration.

Q. What do you think are the most exciting emerging trends in chromatography?

A. The world is becoming greener and

smarter, and this trend is also clear in the field of separation science. In general, new developments are based on faster, simpler, and cost-effective approaches. An increasing trend to sustainable methods is recognized considering both sample preparation and analysis. "Intelligent" and fully automated systems are continuously improved, reducing the need for manpower and the associated error sources. I can foresee that the near future will witness these intelligent systems based on machine learning, allowing for rapid sample assessment by predictive and chemometric models, and automated targeted regulatory testing, as well as non-targeted studies.

References

1. D. Giuffrida, M. Zoccali, S.V. Giofrè, P. Dugo, and

- (2018).

The Column, will be running a series of interviews in 2021, featuring the next generation of separation scientists. If you would like to nominate a "rising star" for consideration, please send the name of the candidate and why they deserve recognition to Alasdair Matheson, Editor-in-Chief, LCGC Europe at amatheson@mjhlifesciences.com

Rising Stars of Separation Science

L. Mondello, Food Chem. 231, 316-323 (2017). 2. M. Zoccali, D. Giuffrida, F. Salafia, S.V. Giofrè, and L. Mondello, Anal. Chim. ACTA 1032, 40-47

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Rising Stars of Separation Science

Training Courses

GC

GC Introduction

Website: www.chromacademy. com/channels/gc-training-courses/ principles/gc-introduction

GC Troubleshooter

Website: www.chromacademy. com/channels/gc-training-courses/ troubleshooting/gc-troubleshooter

GC Fundamentals Onsite training Website: www.crawfordscientific. com/training-consultancy/gc-training/ gc-fundamentals

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Onsite training Website: www.crawfordscientific. com/training-consultancy/hplctraining/hplc-fundamentals

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com/channels/hplc-training-courses/ principles/hplc-introduction

HPLC Troubleshooter On-line training from **CHROMacademy** Website: www.chromacademy. com/channels/hplc-training-courses/ troubleshooting/hplc-troubleshooter

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SAMPLE PREPARATION **Fundamentals of Solid-Phase Extraction (SPE) Mechanisms** On-line training from CHROMacademy

Website: www.chromacademy.com/ channels/sample-preparation/technique/ fundamentals-of-spe-mechanisms

Practical Essentials of Sample Preparation: Module 7–Thermal Desorption (TD) Module 8–Pyrolysis (Py) 23 April 2021

On-line (total of 7.5 hours) The Open University, Milton Keynes, UK Website: www.anthias.co.uk/trainingcourses/practical-essentials-samplepreparation

MISCELLANEOUS

Coping With COVID-19: Remaining Productive and Safe in the Analytical Laboratory On-line webcast from CHROMacademy

Website: www.chromacademy.com/ channels/basic-lab-skills/technique/ coping-with-covid-19-remainingproductive-and-safe-in-the-analyticallaboratory

Introduction to IR Spectroscopy

On-line webcast from CHROMacademy Website: www.chromacademy. com/channels/infrared/principles/ introduction-to-infrared-spectroscopy

Applied Fourier-transform InfraRed spectroscopy (FT-IR) 12 April 2021

On-line (total of 6.5 hours) The Open University, Milton Keynes, UK Website: www.anthias.co.uk/ training-courses/applied-ftir

Please send your event and training course information to Lily Atkins LAtkins@mjhlifesciences.com

Event News

29 June-2 July 2021

26th International Symposium on Separation Sciences (ISSS 2020) and the 25th International Symposium for High-Performance Thin-Layer Chromatography (HPTLC 2020)

Grand Hotel Union, Ljubljana, Slovenia

E-mail: info@isss2020.si and info@hptlc2020.si

Website: https://isss2020.si/ and https://hptlc2020.si/

5–7 October 2021

Forum Labo

Paris Expo Porte de Versailles, Paris, France

E-mail: infos@forumlabo.com

Website: https://www.forumlabo.com/paris/en-gb.html

11-14 October 2021

The 18th International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA 2021)

Lisbon, Portugal

E-mail: secretariat@LDOrganisation.lu

Website: https://www.spica2021.org

2–5 November 2021

The 10th International Symposium on Recent Advances in Food Analysis (RAFA 2021)

Clarion Congress Hotel, Prague, Czech Republic

E-mail: RAFA2021@vscht.cz

Website: http://www.rafa2021.eu

Training & Events

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