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The Column

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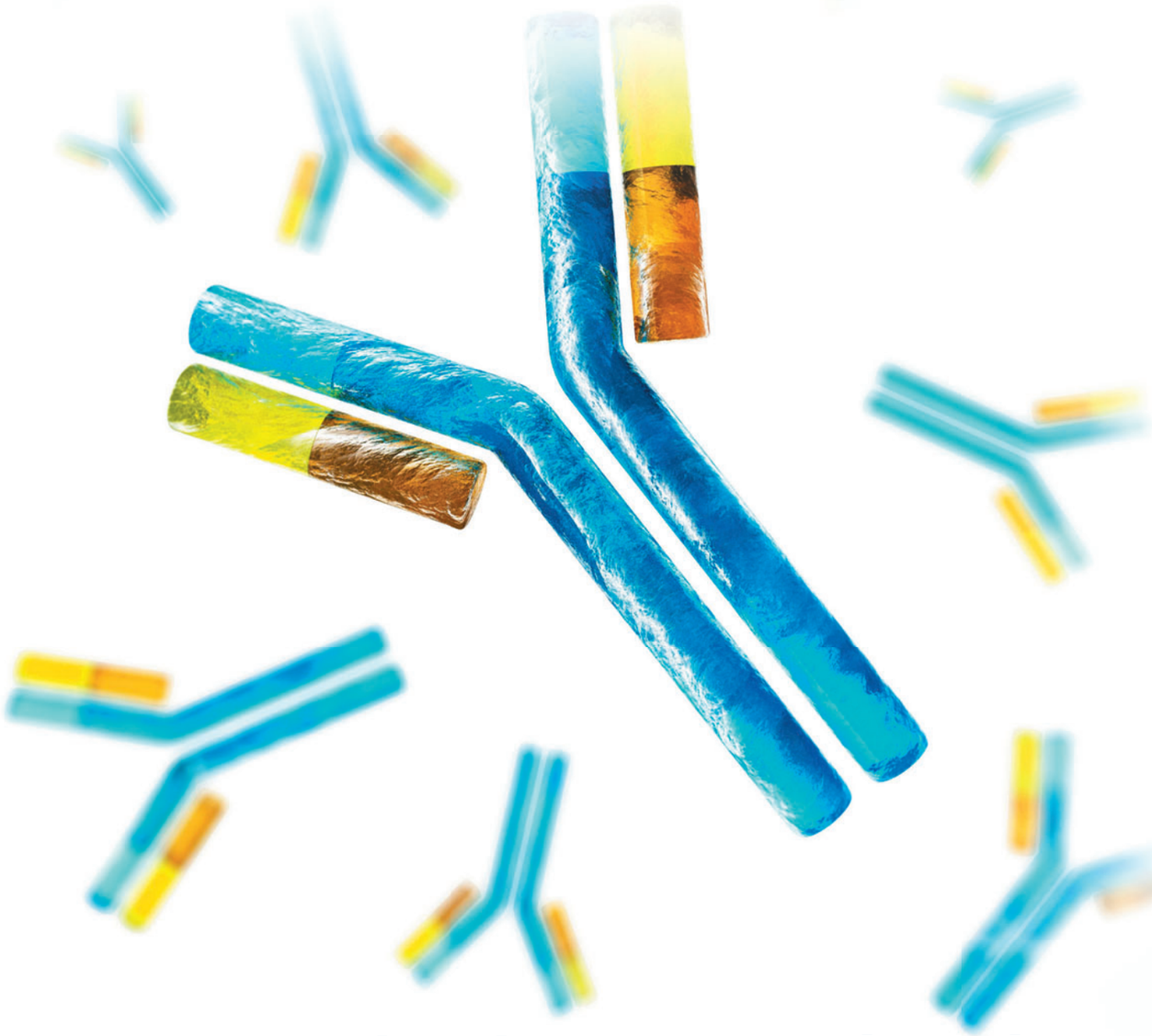

Monitoring Monoclonal Antibodies

The advantages of IEC for analyzing mAbs

Cover Story

Features

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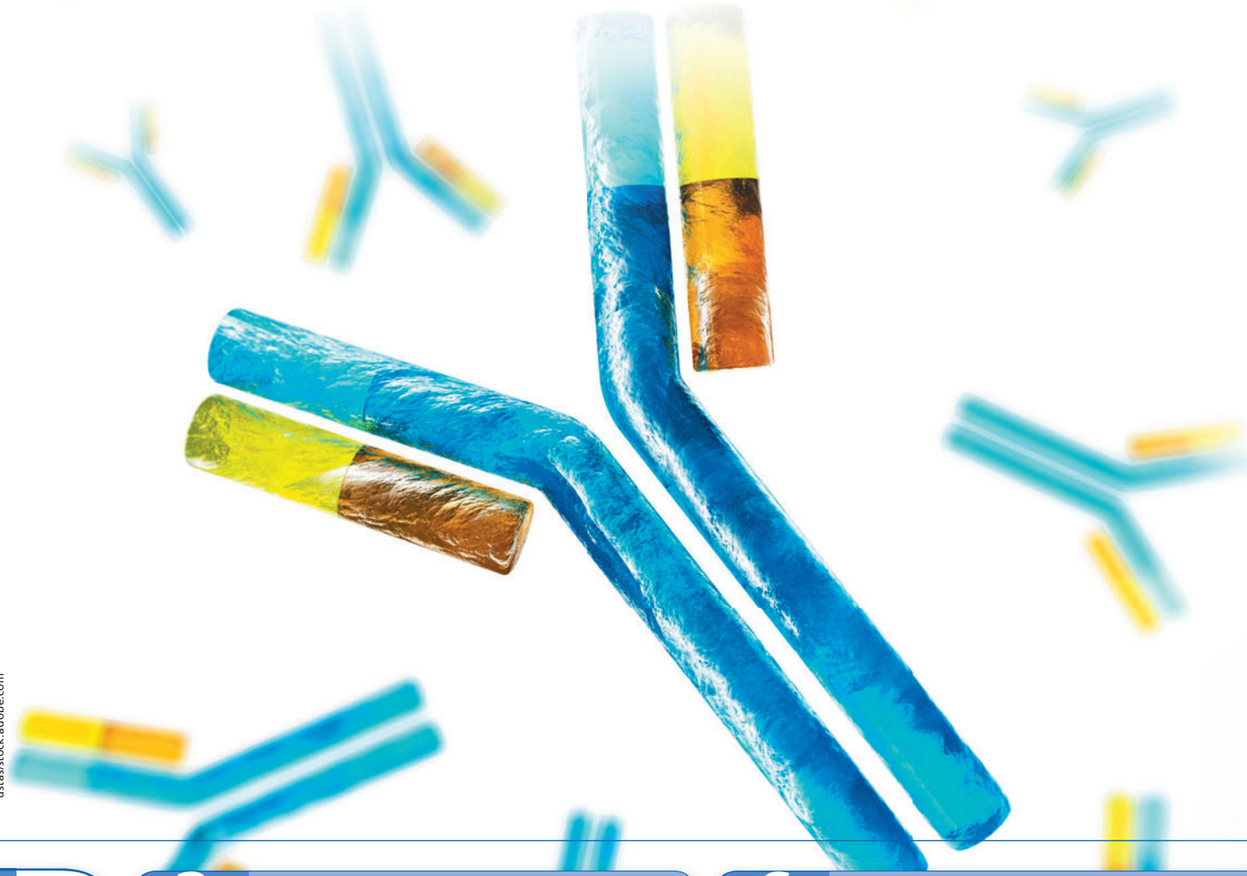
The Benefits of Ion-Exchange Chromatography to Monitor Charge Heterogeneity in Monoclonal Antibodies

The Column **spoke to Richard Shannon from AstraZeneca about his work characterizing monoclonal antibodies (mAbs), why ion-exchange chromatography (IEC) is his technique of choice for analyzing mAbs, and offers his advice for anyone wanting to use the technique.**

—Interview by Kate Jones



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Q. What specific considerations are needed for the characterization of monoclonal antibodies (mAbs)?

A: Monoclonal antibodies (mAbs) are glycosylated multi-subunit proteins containing four subunits covalently joined together by disulphide bonds; there are two identical heavy chains (with one glycosylation site on each) and two identical light chains. Once the mass and sequence of the heavy and light chains have been confirmed using mass spectrometry (MS), the main considerations for characterization of product variants include size variants and charge variants. The size variants can be larger than the monomeric mAb (aggregates) or smaller (fragments). The charge variants, caused by single amino acid modifications or charged glycosylation, can have a pI lower than the parent mAb (acidic species) or higher (basic species). Importantly, for mAbs, it is impossible to characterize and/or quantify every single size and charge variant. A mAb has a molecular weight of approximately 150,000 Daltons with more than 1300 individual amino acid residues. Several amino acids are susceptible to chemical modification during manufacture and storage; for example, deamidation of asparagine, isomerization of aspartic acid, and oxidation of methionine and tryptophan. There may be more than 100 sites of possible chemical modification in the whole molecule, but not all of these will be modified—the reactivity at each site is determined by neighbouring amino acids, solvent accessibility, and flexibility of the peptide backbone. Instead of trying to quantify every modification, we monitor groups of species (aggregates, fragments, acidic species, basic species) and focus our

characterization efforts on modifications that affect the functional activity, safety, or stability of the product.

Q. Why is ion-exchange chromatography your technique of choice to monitor mAbs? Why did you choose this approach and what are the advantages over other techniques?

A: Ion-exchange chromatography (IEC) is one of three common techniques used to monitor the charge variants of mAbs. Capillary isoelectric focusing (cIEF) and capillary zone electrophoresis (CZE) are the other two. In fact, IEC is not our method of choice to monitor mAbs—cIEF is more routinely used to monitor charge variants of mAbs, particularly in early stages of preclinical and clinical development. However, IEC does have some specific advantages over cIEF that make it a particularly useful technique. CZE is less commonly used.

cIEF is usually faster and higher resolution than IEC, and a fit-for-purpose method can be developed more rapidly. These advantages make it the method of choice in early stages of preclinical and clinical development. The key advantage of IEC is that you can collect fractions and analyze the fractions off-line by MS, tryptic peptide mapping, or some other high-resolution method, to find out exactly what modifications are causing the different charge states. Even when cIEF is the preferred method for release testing, IEC with fraction collection is often used to characterize the different charge states present in the cIEF profile.

As a characterization method, IEC is particularly useful because the protein remains in a native state. This enables collected fractions to be tested in a bioactivity assay to establish how a particular modification (or group of modifications) affect the functional activity of the mAb.

In addition, because separation in IEC is due to interactions of the native protein surface with the column stationary phase, changes in surface charge distribution may cause significant changes in retention times. Therefore, IEC may be better than other methods at separating charge (and conformational) variants that alter the protein surface charge distribution. This is important because the functional activity of a mAb is also due to surface charge distribution, but in a much more specific way. Three binding regions known as *complementarity determining regions* (CDR1, CDR2, and CDR3) interact very specifically with the target protein. These regions are fairly flexible and solvent accessible. Modification to amino acids in these regions can seriously impair the binding of the mAb to its target. As separation of charge variants in IEC is

determined by surface charge distributions—including those that influence target binding—you could say that IEC is well suited to separate charge variants that may have altered functional activity.

Even when a chemical modification, such as isomerization of aspartic acid, causes a conformational change, rather than a net charge difference, a change in surface charge distribution may cause a shift in retention time by IEC. IEC may therefore be more able to separate conformational variants that may have reduced functional activity.

I think another advantage of IEC is the flip side to one of its disadvantages. IEC has a lower resolving power than cIEF, but this can contribute to the robustness of IEC as an analytical method. A final charge method is developed to monitor key modifications or attributes that may affect function, safety, or stability of the product. It may not be necessary to monitor all the variants that are separated by a more resolving method, such as cIEF. A less-resolving method may be a more robust method.

Q. Do you have any advice for scientists wanting to use ion-exchange chromatography?

A: One advantage of IEC is that all you need to get started is a high performance liquid chromatography (HPLC) system, an HPLC column, and an HPLC scientist! After that, it's just the usual fun of developing HPLC methods. There is, however, one important factor to determine before you begin: the pI of the molecule of interest. This will then inform your choice of IEC column—anion exchange or cation exchange, weak or strong ion exchanger—and your separation conditions. Separation can be based on a salt gradient, keeping the pH of the mobile phase the same, or a pH gradient. Salt gradients are more common, but pH gradients are gaining in popularity. Whether you use a salt gradient or a pH gradient, the molecule of interest must bind to the column under the starting conditions. For cation exchange chromatography (usually used for mAbs), the pH of the mobile phase must be lower than the pI of the molecule, so that the molecule is positively charged and binds to the negatively charged column. For an anion exchange column, the converse is true.

Q. Please could you talk a little on the development of the antibody–drug conjugate (ADC) platform with inserted cysteine residues (1).

A: Antibody–drug conjugates (ADCs) have the potential to be powerful therapies for the treatment of cancer. A cytotoxic chemical is covalently attached (conjugated) to a mAb via a linker. The mAb directs the cytotoxic drug to the tumour cell, where it is released into the tumour environment, therefore reducing the systemic effects otherwise associated with chemotherapy. The first ADCs were prepared by conjugation of a linker bearing the cytotoxic drug to the mAb, using existing accessible lysine and cysteine amino acid residues in the sequence. This type of conjugation can lead to heterogeneous conjugates, which could have variable numbers of drug molecules per antibody. One approach to reduce this heterogeneity is site specific conjugation, where a reactive cysteine or lysine residue is engineered into the mAb sequence, thus controlling the number and sites of linker conjugation. At AstraZeneca (MedImmune), a cysteine residue is inserted into the sequence in the heavy chain, at a position that does not affect the activity, safety, or stability of the product. A more homogenous ADC product is manufactured, which consistently has two drug molecules per antibody.

Q. Why is an antibody intermediate with inserted cysteine residues more complex than a regular mAb? (2)

A: The inserted cysteine residues are reactive. During manufacture of the mAb intermediate (the mAb before conjugation), the inserted cysteine residues can react with other sulphide-containing compounds present in the bioreactor, such as L-cysteine or glutathione, and become capped. Or they can remain in the uncapped free thiol form. The capped state of inserted cysteines does not impact the manufacture of the final ADC because the initial step of the conjugation reaction involves reduction of the inserted cysteines to their free thiol form. Also, the function of the final ADC is not affected by whether the inserted cysteines in the mAb intermediate were capped or not. However, the capped forms add to the heterogeneity of the mAb intermediate and complicate the charge profile. Glutathione has a negative charge, so glutathione capping can add one or two extra negative charges to the mAb intermediate. This can be observed as two distinct additional acidic peaks in the cIEF and IEC profile. The covalent attachment of L-cysteine to the inserted cysteine does not add a charge in the same way as glutathione, but the difference in pK_a between a free thiol and a disulphide bond is enough of a difference to see additional peaks by cIEF, and possibly IEC. In our laboratory, we have observed additional peaks for the L-cysteine capped species using cIEF, but not for IEC. Therefore, as the capped state was not important for the final ADC product quality, the IEC method was preferred for lot release over the cIEF method because the profiles were less complex and the method more robust.

The peaks from capping of the inserted cysteines may interfere with other peaks that are more important to monitor, such as charge variants from deamidation of asparagine and glutamine residues. In this case, samples can be pretreated with a mild reducing agent that removes the capping species, leaving the rest of the molecule intact. However, an analytical method with sample modification should be employed with caution, partly because some analytical data about process consistency will be lost.

Q. How challenging is the analysis of heterogeneity related to charged variants?

A: The number of possible charge variants present in a mAb is too large to routinely monitor each one. The use of mass spectrometry and tryptic peptide mapping does enable us to take a snapshot of all chemical modifications in the molecule, but these are not routine quality control (QC)-friendly assays. Targeted MS methods using HPLC with simple mass detectors are becoming more common as release methods, but the charge heterogeneity method (IEC or cIEF) is still the primary lot release method used to monitor chemical modifications in mAbs.

Most of the chemical modifications of a mAb will not result in compromised product quality or safety, so the main challenge, already discussed, is to identify the key modifications that affect product quality and design the method to measure these.

Q. What does the future hold?

A: A key advantage of IEC is fraction collection to enable characterization of charge variants. However, this is a laborious task, so how about hooking up a mass spectrometer directly to the IEC column? This has been reported by several groups who have utilized a pH gradient with low concentrations of volatile salt compatible with electrospray mass spectrometry (3). As vendors begin to promote certain columns and mobile phases for this purpose, it may become more routine. However, fraction collection will always be needed because it provides the only means to measure the functional activity of different charge variants.

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Richard Shannon studied chemistry at the University of Sheffield (UK) and stayed on to complete a Ph.D. studying functional supramolecular assemblies. His first industry position was in the analytical department at Pfizer, based in Sandwich (UK), where he worked mainly on HPLC analysis of small molecules. He then took up a position at the MRC Mitochondrial Biology Unit in Cambridge (UK) and worked on the isolation, purification, and analysis of proteins, specifically the Complex I protein from bovine mitochondria. He then trained to be a secondary school science teacher and worked for five years in Cambridgeshire, before returning to research at the University of Cambridge Division of Neurosurgery, where he used HPLC to measure the levels of amino acids and neurotransmitters in brain extracellular fluid from neurotrauma patients at Addenbrookes Hospital. In 2014 he returned to the pharmaceutical industry, taking a position at MedImmune, the biologics unit of AstraZeneca, where he has worked in the Analytical Sciences group on a range of projects including mAbs, peptides, bi-specifics, and ADCs.

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News

UK Launches Community for the Analytical Measurement Science

The Community for the Analytical Measurement Science (CAMS) was officially launched on 27 June at the Royal Society of Chemistry, Burlington House, London, UK.

An industry-led membership network, CAMS represents the first group of its kind and is dedicated to developing research, training, and innovation capabilities in analytical measurement science across the UK and Ireland.

The intention of CAMS is to address the threat of declining skills in analytical measurement science across the UK and Ireland, building on the results of a Royal Society of Chemistry Analytical Division's landscaping exercise and subsequent EPSRC review of analytical science that identified the importance of a healthy analytical measurements science strategy for the UK and Ireland.

To solidify this commitment, CAMS intends to strengthen the existing networks of chemical and bioanalytical measurement excellence throughout the UK and Ireland through an expansion of the regional "centre" for academic training (MSI) and the analytical quality assurance-training institute (BEAM).

"Few things are as important for a modern economy as measurement. Its strategic value is often underappreciated, even though estimates suggest £650 billion of the UK's trade for physical goods alone relies on sound measurement practice," said Julian Braybrook, UK Government Chemist and Director of Measurement Science and Partnerships.

Over £15 million in funding has been committed to date from the Analytical Chemistry Trust Fund (ACTF), the Department for Business, Energy, and Industrial Strategy (BEIS), the Medicines Manufacturing Industry Partnership (MMIP), and over 15 industry and 10 academic members.

For more information, please visit **www.cams-uk.co.uk**

Measuring Chitosan Molecular Weight Using AF4–MALS-RI

A new method for the accurate molecular weight determination of the complex polysaccharides chitosans has been developed using asymmetric flow field-flow fractionation (AF4) coupled with multi-angle light scattering (MALS) and differential refractive index (RI) detectors (1).

A highly sought-after biopolymer, chitosans have multiple functions with well over 200 current and potential applications across a wide range of scientific areas. They can be biologically sourced from the exoskeletons of various crustaceans and insects, as well as the cell walls of certain fungi and fish scales; however, many of its material and biological properties are heavily linked to the molecular weight (MW) of the polymer. Therefore, an accurate MW is crucial to the effective utilization of chitosans in industry.

Unfortunately, the actual task of accurate MW measurement is complicated by their biological source, which can vary greatly and also be affected by parameters such as the season of harvest, or the process of isolation of chitin and the deacetylation into chitosan. The traditional technique used for chitosan MW measurement has been size-exclusion chromatography (SEC); however, for more complex polysaccharides such as chitosans the use of SEC requires prefiltration of samples to remove interfering aggregate fractions, which is a time-consuming process and can lead to considerable sample loss.

The new method described by researchers uses AF4–MALS-RI to separate the polymer from the molecular aggregates found in chitosan solutions. The technique has the added advantage of being able to identify the aggregates present as well as separate them, thereby allowing the determination of MW for a wide range of chitosans while avoiding the sample loss found when using conventional SEC–MALS-RI methods. This was particularly evident for high-molecular-weight chitosans where the required filtration step prior to SEC resulted in a significant sample loss.—L.B.

Reference

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Shimadzu Wins Two Red Dot Design Awards

Shimadzu (Duisburg, Germany) has received two Red Dot Design Awards in the product design category for its new Nexera UHPLC series LC-40 and UV-1900 UV–vis spectrophotometer.

The Red Dot Design Awards are one of the three major design awards in the world with Shimadzu fending off competition to claim this year's awards. Judged based on innovation, functionality, quality, ergonomics, and durability, more than 5500 products were submitted to the product design category by manufacturers and designers from 55 countries.

The award represents the third time in the company's history that they have won a Red Dot Design Award, having previously picked up awards in 2013 and 2018.

The award ceremony was held in Essen, Germany, on 8 July 2019. An actual Nexera series instrument and an explanatory panel showing the UV-1900 will be displayed in the city's Red Dot Museum for the next year.

For more information, please visit: **www.shimadzu.eu/awards-of-shimadzu**

Diagnosing Multiple Sclerosis Using Breath Biomarkers and GC–MS

As a chronic neurological disease, multiple sclerosis affects the brain and spinal cord causing a wide range of symptoms, from problems with vision and movement to sensation or balance. The condition can cause serious disability, but the range and severity of symptoms varies with mild forms also existing.

Diagnosis of the disease is based upon visible symptoms of the disease and confirmed by examination of the cerebrospinal fluids (CSF), an invasive and painful procedure. Magnetic resonance imaging of the brain and spinal cord can also be used for diagnosis, and according to a recent publication, could also predict how their condition will progress, including how disabled they are likely to become (1). However, as routine diagnostic tools, neither technique is adequate.

"There are over 100,000 people with multiple sclerosis in the UK and we often hear that the path to diagnosis is an incredibly stressful time," said Susan Kohlhaas, Multiple Sclerosis Society Director of Research.

As such, there is a drastic need for a noninvasive diagnostic tool. One promising avenue is that of biomarker analysis, with a 2017 (2) preliminary study identifying numerous biomarkers in the breath of multiple sclerosis patients.

That initial study utilized gas chromatography–mass spectrometry (GC–MS) and a nanomaterial-based sensor array to identify significant differences in the volatile organic compounds (VOCs) of multiple sclerosis patients and controls. The new study will confirm the initial results and look to use these results to create a diagnostic tool.

Speaking at the Multiple Sclerosis Society Multiple Sclerosis Frontiers Conference in Bath, UK, Phoebe Tate from the University of Huddersfield's Centre for Biomarker Research said "The new work looked at more subtypes of multiple sclerosis, whereas the 2017 study just looked at relapsing multiple sclerosis. Additionally, the new study also looked at primary multiple sclerosis and secondary multiple sclerosis in to find out if the different subtypes, such as the more inflammatory, actively demyelinating relapsing-remitting multiple sclerosis, and the less inflammatory, progressive primary progressive multiple sclerosis and secondary progressive multiple sclerosis, can be differentiated by a breath test, as well as finding a diagnostic test."

"The techniques used for diagnosis are invasive, expensive, and often laborious, so this exciting development would address a major unmet need. Having a lumbar puncture and even an MRI scan can be an uncomfortable and unsettling experience, which we know people with multiple sclerosis are keen to change," said Kohlhaas.

The researchers are now preparing a larger three-year study with 500 people, which will start later in 2019. This will determine if the test can be rolled out across the NHS, and if it can help to determine how multiple sclerosis progresses.—L.B.

For more information, please visit: www.mssociety.org.uk

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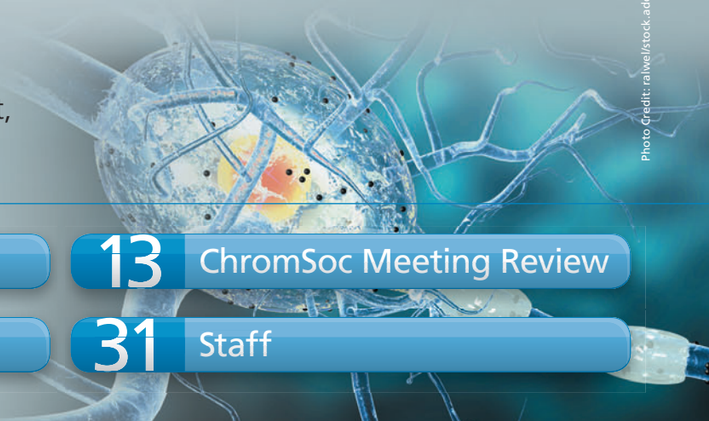

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Peaks of the Month

- **The *LCGC* Blog: Is Hydrogen the Only Viable Gas Chromatography Carrier Gas for the Long-Term?**—As I receive reports from clients in Europe and the United States that helium prices are once again increasing, and warnings are being given regarding yet another laboratory-grade helium shortage, my thoughts turn once again to the use of hydrogen as an alternative carrier-gas for gas chromatography. **Read Here>>**

- **Strategies for the Quantification of Endogenously Present Small Molecules in Biological Samples**—The main objective of this review article is to provide a clear summary of the different methods that can be used to quantify endogenous small molecules. Because of the increased use of mass spectrometry (MS) in the field of bioanalysis, a special focus will be placed on quantification by liquid chromatography (LC)–MS. Practical recommendations to face this bioanalytical challenge, in particular in terms of method validation, will also be provided. **Read Here>>**

- **A Whole Lot of Data Going On: Book Review: Data Integrity and Data Governance by R.D. McDowall**—R.D. McDowall has written an excellent book on data integrity and data governance. Those who need to understand what this is should read the book and follow his advice. He has included both what the regulations and regulators say and what we need to do to be in compliance. This is a scalable approach with enhancements for larger items and shortcuts for smaller items, with numerous examples throughout the book. **Read Here>>**

- **Need a New Instrument to Innovate?**—Calling all innovators (or anyone seeking to justify a new instrument purchase)! **Read Here>>**

- **Cocaine and Illicit Drug Levels Analyzed in Central London**—A group of researchers has investigated the impact of combined sewer overflow (CSO) on pharmaceutical and illicit drug use in central London using liquid chromatography–high-resolution mass spectrometry (LC–HRMS). **Read Here>>**

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News In Brief

InProcess Instruments (IPI) (Bremen, Germany), manufacturer of quadrupole mass spectrometers (QMS) for online gas analysis for research and industrial processes, and Chromatotec Group (Bordeaux, France), specialists in the development, manufacturing, and sales of online gas analyzers, have announced an exclusive distribution partnership for the territories of France, Italy, Benelux, Saudi Arabia, and Taiwan. For more information, please visit: **www.chromatotec.com**

The Pittcon Science Week Committee have announced a collaboration with ChemEd, one of North America's largest conferences for Chemistry Educators, to provide continuing science education for hundreds of teachers. Pittcon have donated $20,000 to fund the cost of equipment for teacher workshops at ChemEd 2019, and teachers who attend the event will have the opportunity to take home materials to recreate the activities in their classrooms. Furthermore, Pittcon are sponsoring six teacher workshops and ten target inquiry workshops at the event. "Pittcon's support will help continue the legacy of ChemEd, teachers sharing with teachers," said ChemEd 2019 Conference Co-Chair Sue Boberor Jill. ChemEd 2019 will take place 21–25 July at North Central College in Naperville, Illinois, USA. For more information, please visit **www.science-week.org**

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How Much Longer Can Chromatography Last?

Incognito is thinking long-term. Is there scope for hope in the future?

As we enter the last six months of the second decade of the 21st century, I want to take the opportunity to briefly reflect on the progress of chromatographic science and look forward to what we might expect in the third and subsequent decades of the century.

However, being Incognito, I'd like to do this in a slightly more challenging way than perhaps would be usual in the popular scientific press.

There is a well-defined model of the technology product development life cycle, which is shown in Figure 1. Most of this figure should be pretty self-explanatory, and whilst it is typically used to define the return on investment of a new technology (usually a single product) from a manufacturing business perspective, I believe it's a reasonable surrogate for the life cycle of an enabling technology, such as chromatography.

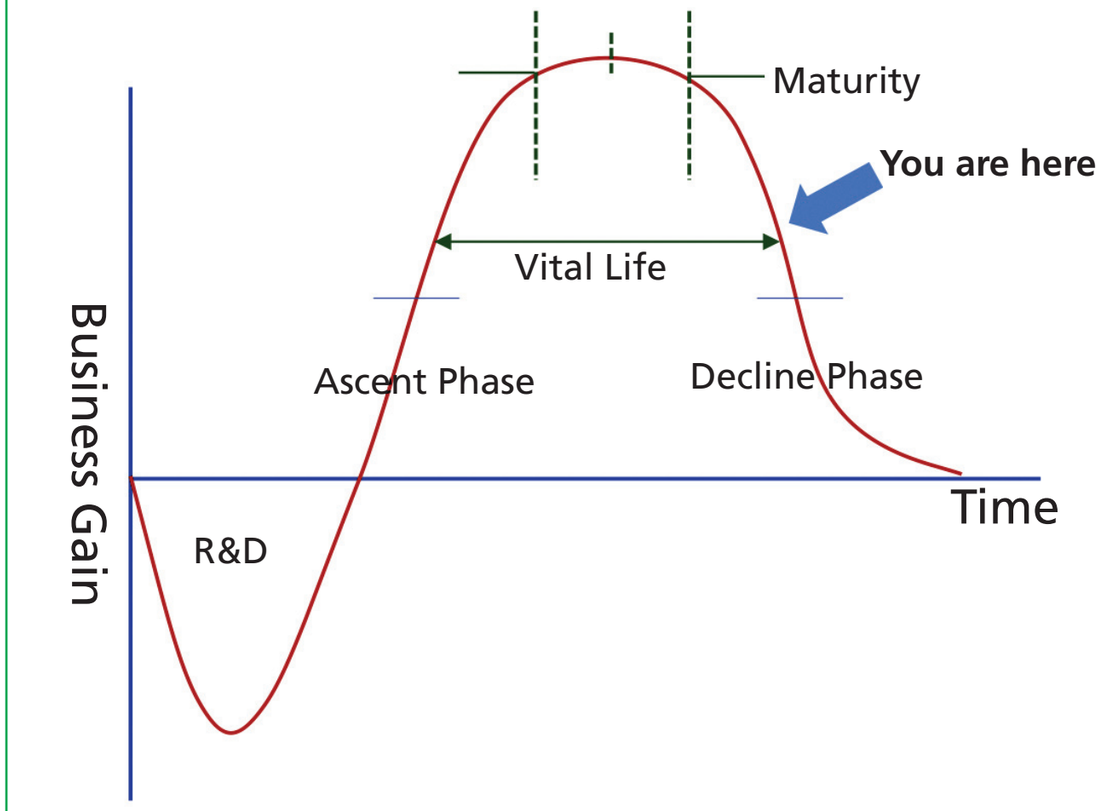
The big question is, where are we currently on the cycle? As you will see in Figure 1, I've given my best guess, although I realize that this perspective may be different depending upon if you are a user within a large, well-funded organization, in a smaller, less well funded laboratory, in academia, or working for an equipment or software manufacturer.

So why do I think we are firmly, as chromatographers, reaching the end of the vital life of our industry and entering the decline phase of the technology product life cycle? What does this mean and what does the future hold for us?

Let me begin by stating that I believe we have never been so well off as chromatographers in terms of the quality and range of our equipment, the number of tools we have within our analytical armoury, and our ability to solve previously unfathomable problems. However, this doesn't mean that we aren't approaching the end of the vital life of our technology. What makes me say this?

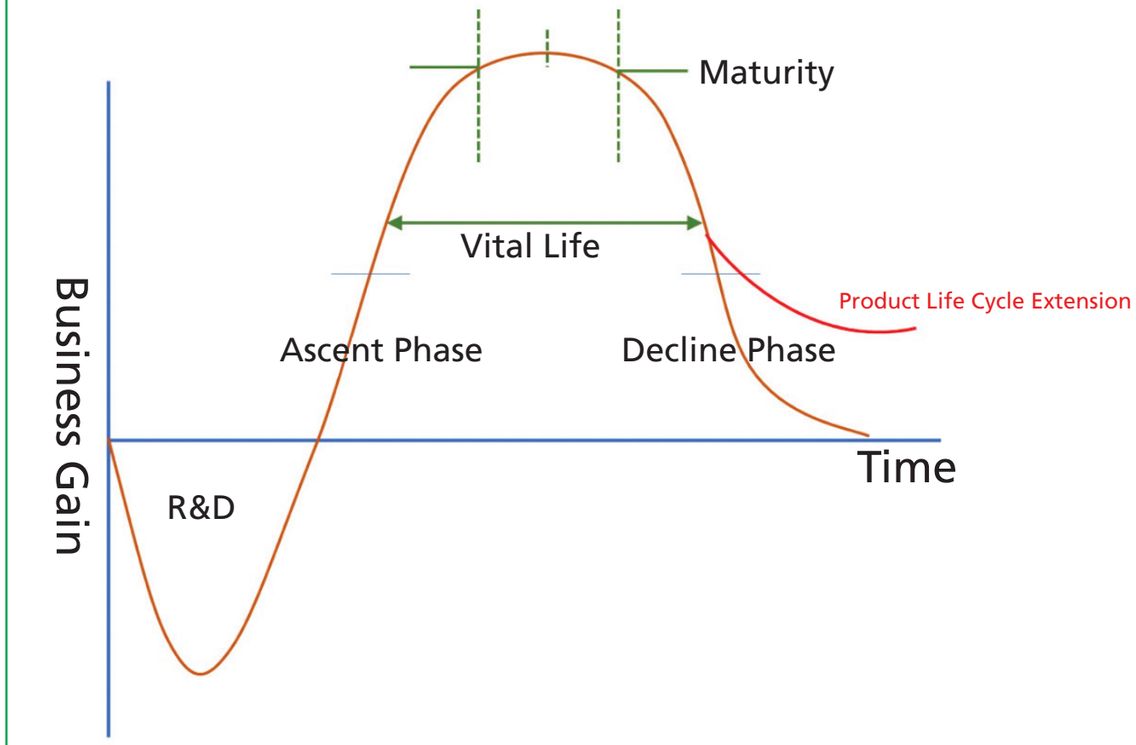
Let's look at the characteristics of technology in the decline phase, from a business perspective (for which the technology life cycle was designed), and see if these fit with anything we see within our industry today:

Figure 1: The technology product life cycle.



- The decline phase is typically characterized by a falling demand for the product or technology. More features, benefits, and capability have been built into each unit and, therefore, more can be done with less.
- In the early part of the decline phase, larger businesses can retain or even grow their profits for several reasons—i: The optimized technology is more reliable and therefore less cost-of-sales need to be attributed to the field maintenance (typically under warranty) of each unit; ii: Whilst research continues into line extensions or minor improvements, the high operating costs of fundamental R&D are largely eliminated; iii: Whilst marketing communications are still necessary to

Figure 2: Product lifestyle extension phase of the technology product life cycle.



maintain sales volumes, the high costs associated with radical new product launches are very much reduced; iv: Smaller competitor sales reduce to break even or even loss making and they exit the market, with their associated sales switching to the bigger brands whose market share increases, and their sales volumes do not contract as quickly as the market; v: The larger suppliers consolidate their product mix, offering fewer, more capable, platforms and manufacturing costs reduce in line with the number of different products and options offered to the market; vi: Smaller retailers and distributors are eliminated in order to optimize profits on each unit sold and on-line sales channels become increasingly important as the pull rather than push sales models are adopted.

So, what can we glean from this? I know that the chromatographic instrumentation industry tends to follow *The Analytical and Life Science Instrumentation Industry* SDi report (1), and from glimpses I've seen of recent reports, there is still growth for most of the major manufacturers in instrument technology, even if this growth is not as aggressive as has been achieved in the past. Obviously, analytical laboratories all over the world still require new instruments, however, I wonder if, like me, you recognize some of the other symptoms of decline that are outlined above?

There are fewer, smaller instrument companies; instrument platforms have become more reliable; and there are fewer options within manufacturer ranges than in the past. The large companies now dominate the marketplace and tend to have their own sales forces, relying less on distributors or "partners", and I believe their support teams are less numerous than they have been previously. I can certainly see that the digital channels for sales are increasing, although this also coincides with the advances in web-enabled selling and the increasing reliance and trust that we as consumers have in on-line information gathering and ordering. It also feels like applications and support teams within the vendor market are shrinking, although I'm sure I will get a response that challenges this thinking. Marry this with the decline in fundamental academic research into chromatographic techniques, which ultimately drives (or at least mirrors) the product innovation from the major manufacturers, and one might begin to worry about the future.

It's useful to consider the truly "enabling" technology and science of recent times, to evaluate the true extent to which our industry is reaching the end of its vital life:

- High performance liquid chromatography–mass spectrometry (HPLC–MS), specifically atmospheric-pressure ionization
- High resolution mass spectrometry (orbital trap and quadrupole time-of-flight [QTOF] being notable examples)
- Triple quadrupole mass spectrometry
- Hydrophilic interaction liquid chromatography (HILIC)
- Ultrahigh pressure (performance) liquid chromatography (UHPLC)
- Core–shell HPLC column packing materials

This is an impressive list and would indicate that my assertion of our position in the technology life cycle is incorrect. Surely, most of these advances are true "game changers" that highlight how our

technologies are not even mature, let along on the verge of decline?

There is a well-known phenomenon called the *product life cycle extension*, the economic model of which is shown in Figure 2.

Here, businesses try to stave off the decline phase of technology by rethinking the positioning of a product or even a whole market by broadening applicability or adding significantly new or improved functionality or utility to the product.

Simple examples include:

- Mobile phones having high-quality camera features to take market share from the digital camera companies
- Breakfast cereal manufacturers repurposing their products into cereal bars to appeal to their increasingly time-poor customers
- Apple Pay and Google Wallet entering the e-payment market to gain market share via customer loyalty and increasing product combination

Are the enabling technologies mentioned previously merely "extensions" to existing technologies or new innovations that will deliver real business benefits to manufacturers?

To my mind there is only one true innovation in the list and this is HPLC–MS, in that until the mid- to late 1990s it simply wasn't possible to successfully interface HPLC techniques with mass spectrometric detection. The other technologies offer real improvements, but fundamentally they existed previously and add performance improvements, rather than being fundamentally enabling. Aside from LC–MS, they all follow the product life cycle extension model rather than the full technology product life cycle. Arguable maybe, and no doubt I'll get some robust challenges to my statements here, but these are my views.

To explain further, let's consider what I would consider "new" technology that might start the life cycle afresh:

- Handheld (or small and portable) spectrometric or spectroscopic devices able to fully elucidate and quantify the contents of liquids, solids, or gases

That's it—short and sweet.

Once we have these, there will be no need to separate individual components in order to introduce them into our detectors. Our detectors will have the ability to structurally elucidate even the most complex analytes (large complex proteins, for example) without the need to simplify them (through digestion, for example) or to remove or separate them from their matrices. Of course, most of us will then be out of work, or be working for the megacorps responsible for developing these techniques.

My guess is that we will be there within 25 years, by which time I'll be "safely" retired. Let's face it, *Star Trek* had the tricorder, a device with similar capabilities in the 1960s, but let's not forget that the series was set in the 23rd century—putting us well ahead of science fiction in this case.

Until then, we will continue to add extensions to our existing technologies, mass spectrometers will become increasingly accurate and sensitive, and chromatography will become ever faster, more efficient, and perhaps even more selective. But we will be extending the technology cycle, not fundamentally reinventing it. So, how safe is your future career and who should you be working for in order to be on the analytical tricorder gravy train? I'll leave you to ponder those fundamental questions.

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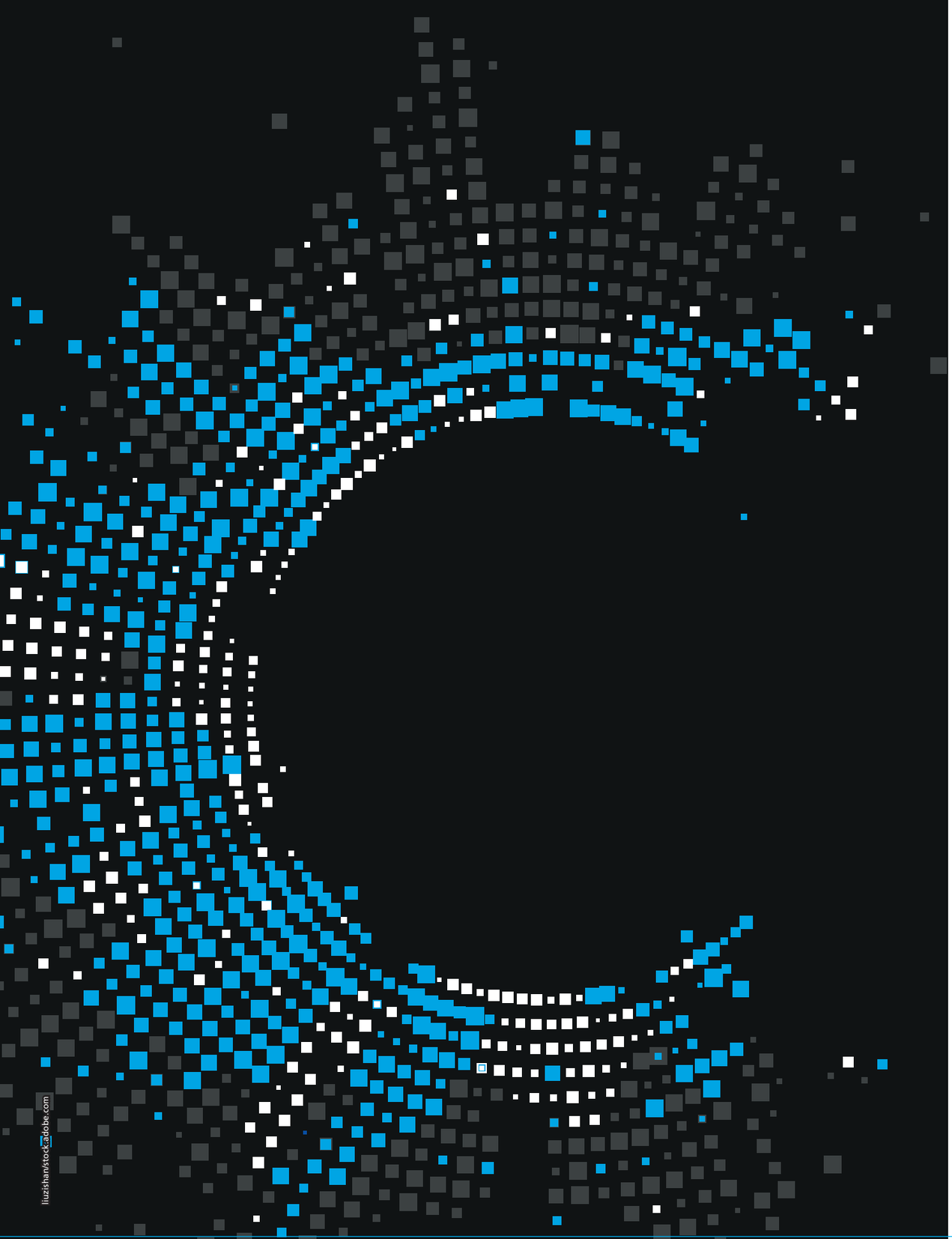


Meeting Review: Emerging Separation Technologies 2019

Paul Ferguson and John Lough, The Chromatographic Society

Organized by The Chromatographic Society and the Separation Science Group of the Royal Society of Chemistry's (RSC) analytical division, the Emerging Separation Technologies meeting was held on the 28 March 2019 at the RSC's central London headquarters in Burlington House.

The third Emerging Separation Technologies meeting organized by The Chromatographic Society and the Royal Society of Chemistry's (RSC) analytical division separation science group was held at the historic Burlington House near Piccadilly, in London, UK. The meeting was opened by one of the principal organizers, Adrian Clarke of The Chromatographic Society, who welcomed the attendees and thanked the vendors for their continued support of the Society's meetings, which enabled international scientists to present. He discussed that the meeting series focuses on "new instrumentation, applications, and analytical approaches" while also "showcasing emerging scientific professionals". The programme (Table 1) was well balanced and, as things transpired, had appropriate timings to allow valuable discussion at the end of each talk. A list of vendors in attendance is listed in Table 2.

Clarke chaired the first session and introduced Peter Schoenmakers of the University of Amsterdam, who discussed the many areas of research in his group focusing on approaches for the separation of complex sample types. His talk "*Current Status and Novel Developments in Multidimensional Chromatography: Separation Challenges Posed by Complex*

Table 1: Presenters and presentation titles from the meeting

Presenter	Affiliation	Presentation Title
Peter Schoenmakers	University of Amsterdam	*Current Status and Novel Developments in Multidimensional Chromatography: Separation Challenges Posed by Complex Samples*
Noor Abdulhussain	University of Amsterdam	*Multidimensional Separations in 3D-Printed Devices*
Eivor Örnskov	AstraZeneca, Gothenburg	*Novel Separation Methods of Polynucleotides*
Adam Taylor	National Physical Laboratory	*Metrology for Mass Spectrometry Imaging in Life Science Research*
Nick Pittman	Waters	*A Novel LC–MS Approach for Enhancing Protein Separations with Improved MS Sensitivity*
Timothy Cross	Thermo Fisher Scientific	*New Paradigms in Liquid Chromatography Separation Efficiency*
Shaun Pritchard	Agilent	*New Developments in SFC and Future Concepts*
Kevin Treacher	AstraZeneca, Macclesfield	*Approaches for the Characterization and Analysis of Advanced Drug Delivery Systems*
Ben Baars	VUV Analytics	*Vacuum Ultraviolet Detection in Gas Chromatography; What are the Perspectives With This New Detector*
Aditya Malkar	Owlstone Medical	*Development of Technology and Workflows for Metabolite Profiling of Human Breath*

Table 2: Vendor products discussed or on display at meeting.

Vendor	Technology Discussed
Agilent Technologies	Ultivo triple quadrupole miniature MS Agilent 8860 (inbuilt software and WiFi software connectivity) and 8690 (automated diagnostic tools) GCs
AGT	Instrument noise minimization and space maximization technologies including MS noise and Ion bench platforms
Anatune/Crawford Scientific	SIFT-MS, Agilent GC-QTof (low energy EI)
HiChrom/ACE	Halo C30, Halo 1000A columns
JayTee	UKAS instrument calibration to ISO17025 standards for LC–UV and GC servicing
Phenomenex	Security Link column/fitting connectors
Shimadzu	New Nexera HPLC and UHPLC ranges
Thermo Fisher Scientific	Vanquish Duo UHPLC
Waters	BioAccord ToF MS, Bioresolve columns and consumables

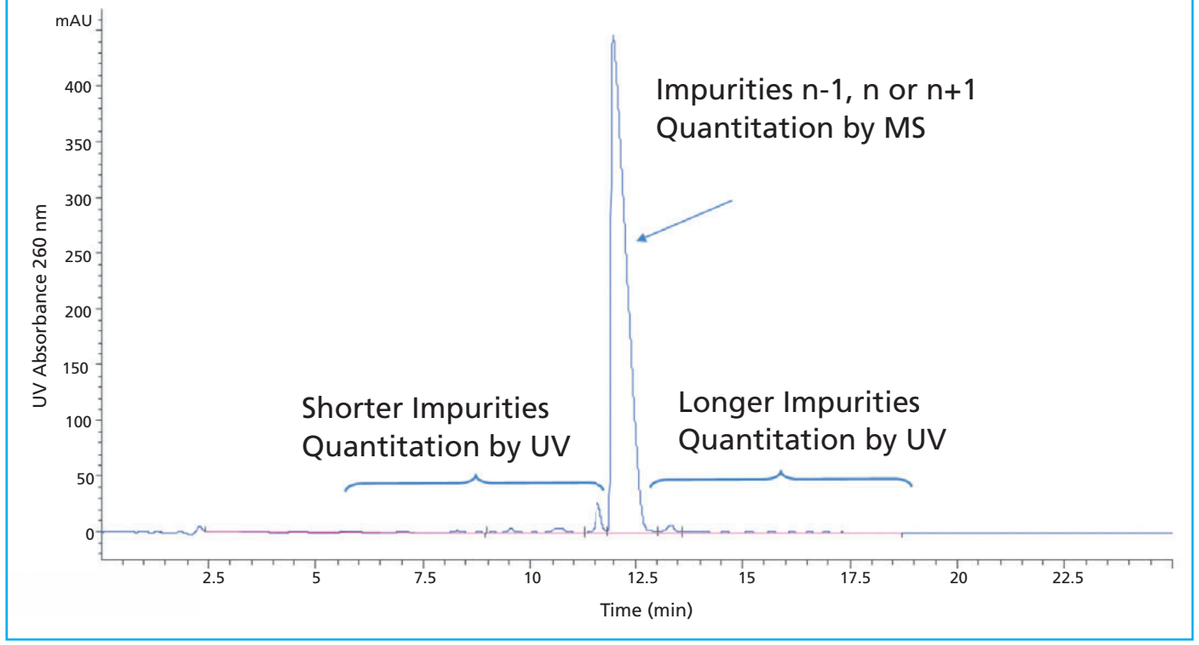
Samples" featured much of his latest work on multidimensional chromatography. His first example illustrated the separation of ethylene oxide-propylene oxide polymers using hydrophilic interaction liquid chromatography (HILIC) × reversed-phase liquid chromatography (LC). HILIC was used to separate different polar head groups in the first dimension while reversed-phase LC separated the analytes by hydrophobicity in the second dimension. He showed a second example of castor oil-derived polyethylene oxide (PEO) where all polar end groups were identified (1).

Schoenmakers then moved on to how chromatography can help characterize higher order polymer structures for both synthetic and biological species. He showed how size-exclusion chromatography (SEC) or hydrodynamic chromatography (HDC) can be used in combination with field-flow fractionation (FFF) to do this. Multiple detectors can be used to help with this, for example, UV, refractive index (RI), viscometry, and multi-angle light scattering (MALS). An interesting observation was that higher salt concentrations in the mobile phase can lead to an increased number of higher order structures. This was exemplified by the identification of β-galactosidase dimers of 200 kDa molecular weight (MW) from *Kluyveromyces* yeast. He also discussed the analysis of polymeric nanoparticles using HDC × SEC, which can provide information on MW and particle size in a single analysis (2).

He then moved back onto multidimensional chromatography of biofluids using this approach to identify steroids in bovine urine (3). He discussed his group's work with Syngenta looking at naturally occurring surfactants using normal-phase LC × reversed-phase LC, which separated ~4800 species and equated to approximately 1 peak per second over the course of the run. He also highlighted his group's work developing *in silico* approaches to two-dimensional (2D) method optimization (the "PIOTR" project), which he said allows methods to be optimized in days rather than months (4).

Figure 1: Noor Abdulhussain of the University of Amsterdam describing her work on the development of 3D separation devices.

Figure 2: IP-reversed-phase LC–UV (MS) separation of a 12-mer oligonucleotide illustrating the approach used to quantify impurities based on both UV and MS detection. The separation conditions are similar to those detailed in reference 12. Data courtesy of Eivor Örnskov, Barbara Adamczyk, and Said Harun, AstraZeneca.



In the next part of Schoenmakers' talk he discussed issues with coupling different types of separation and the importance of the interface; for example, if nonpolar and polar mobile phases are combined they will de-mix leading to poor chromatography in the second dimension. This issue could be alleviated through the use of sample trapping in place of a sample loop in the interface, an action he called *active modulation*. He compared these to a high performance liquid chromatography (HPLC) × HPLC separation and showed many benefits of this approach in terms of analysis time, sample dilution (and therefore method sensitivity), and mobile phase usage (Table 3).

Schoenmakers then discussed how the modulator loops could be replaced with reactors to promote different types of controlled degradation. Samples can be parked and left to react for several minutes before transfer onto the second dimension. He exemplified this in a reversed-phase LC × ion-exchange chromatography (IEC) separation with an immobilized enzyme reactor ("IMER"), which has a zigzag channel for increased surface area. This approach reduced the total analysis time per sample down from 22.5 h to 4 h (5). Another example was the analysis of ethanol-derived sugars in beer where the three-dimensional (3D)-printed reactor included microbes (immobilized microbe reactor—"IMMER"). For the final example he described how the loop was replaced with a light cell for photo-catalyzed degradation in a project called "TOOCOLD". Analytes were parked in the cell and stressed photolytically. This approach has been used to understand synthetic dye degradation in paintings in work sponsored by Amsterdam's Van Gogh museum.

The second talk in the session was from Noor Abdulhussain (Figure 1), who is one of Professor Schoenmakers second year doctoral students at the University

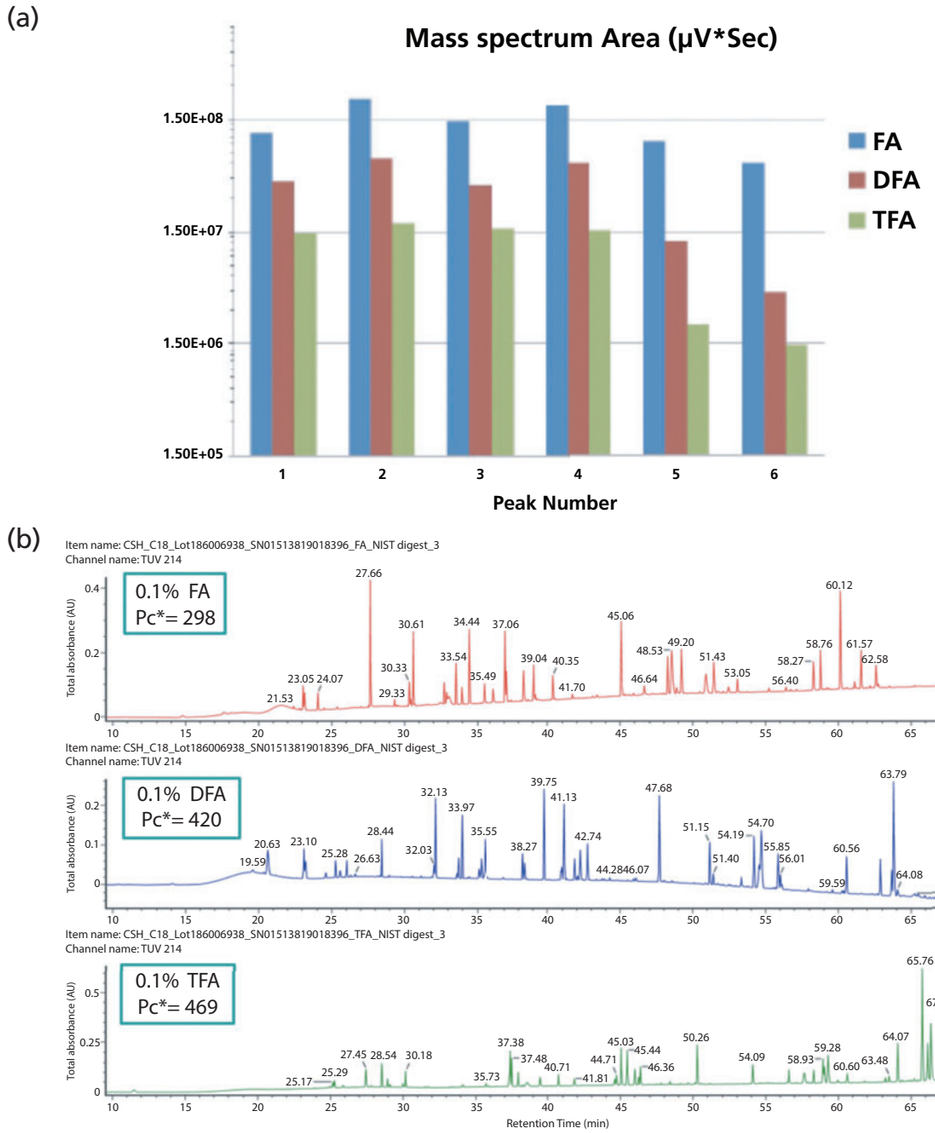
Figure 3: Adam Taylor (NPL) takes questions from the session chair Daniel Meston (The Chromatographic Society) after his talk on "*Metrology for Mass Spectrometry Imaging in Life Science Research*".

of Amsterdam. Her presentation focused on the potential of 3D-printed structures for multidimensional chromatography in a project termed "STAMP" (Separation Technologies For a Million Peaks). She started her talk by stating that resolution in multiple dimensions (spatial) is not a new concept and was first described in 1948 (6). The use of cubic separation approaches to generate high peak capacities was also described by Guiochon in 1983 (7) and more recently by Davydova (8). To generate a peak capacity of 125000, around 10000 channels are required on 3D structure.

She described three approaches to making 3D-printed separation structures:

- Digital light processing (DLP) using materials such as Teflon, which produces 200–400 µm separation channels (9). This approach produces high resolution devices but has limited solvent compatibility.

Figure 4: Comparison of formic acid (FA), difluoroacetic acid (DFA), and trifluoracetic acid (TFA) for MS analysis; (a) comparison of MS signal response for a set of standards and (b) peptide map peak capacity (Pc) comparison. Data courtesy of Nick Pittman and Waters Corporation.



- Selective laser melting (SLM), which uses metal powders such as aluminium, titanium, or steel producing channels of 100–150 µm (10). This produces robust devices but is expensive.
- Fused deposition modelling (FDM), which uses ceramics or polyamide. This approach provides quick prototyping with a variety of materials but has some pressure limitations.

The 3D device she is building utilizes isoelectric focusing (IEF) in the first dimension, which uses a pH gradient and molecules migrate under the influence of an electric field to the pH region where it becomes neutral. At various parts of the fabricated channel, channels to the second dimension are placed. The viability of this device was tested and proved using a pH indicator dye, which separated each coloured component at the correct pH region. She also demonstrated the separation of ribonuclease A from bovine pancreas. She noted that different filament types used for printing the devices had different physical and separation properties.

In the second dimension, a reversed-phase column approach was fabricated. The monoliths were first fabricated using BuMA-EDMA polymers, but liquid leaks were observed as a result of inter-channel voids. When this was replaced with black polypropylene, this issue was prevented. To move the samples between channels, a novel "twisting" mechanism was fabricated to prevent channel leakage (11).

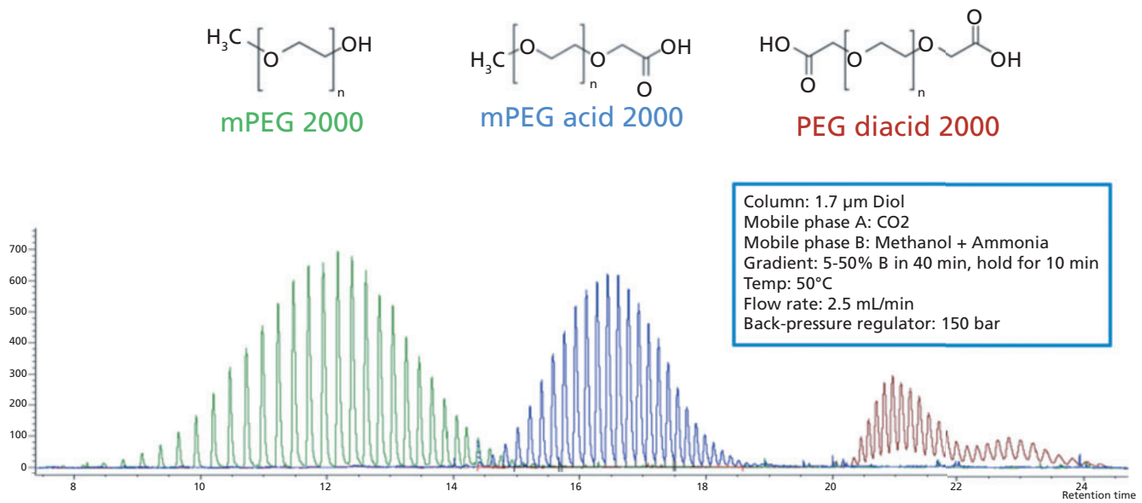
Approaches towards better characterization of biopharmaceuticals are becoming increasingly important as pharmaceutical companies move from smaller to larger molecule development portfolios. After the break, Eivor Örnskov from AstraZeneca discussed approaches to analyze polynucleotides, including antisense oligonucleotides (ASO) and messenger RNA (mRNA), in her presentation "*Novel Separation Methods of Polynucleotides*". Örnskov described how ASOs are fabricated on solid supports and each step adding an additional nucleotide can lead to additional impurities. The longer the oligo, the more impurities, and for standard oligos, these are often removed using preparative chromatography. After purification the number of impurities is in the order of 30 to be analyzed. In addition, these are regulated under small molecule guidelines requiring many of the impurities to be quantified and identified (typically >0.05% area/area). LC methods available today cannot reach those levels, therefore reporting limits 0.2% area/area are applied.

Figure 5: Attendees networking over lunch. Networking is an important feature of these meetings and can be a useful source of scientific information, as well as catching up with friends.

ASO chromatographic methods are largely based on (ion-pairing) IP-reversed-phase LC–mass spectrometry (MS) (12). This methodology separates the oligo and its impurities, the shortmers, the parent oligo, impurities differing n+1 or n-1, and equal length impurities (*n*) and longmers. The shortmers and longmers are quantified by UV while the compounds in the main peak are measured by MS (see Figure 2). Diastereoisomers are formed when the phosphate backbone is thiolated. This increases separation complexity as a 19-mer length oligo will have 2^{19} = 524288 isomers! Separations are controlled by ion-pairing reagent type, stationary phase type, gradient slope, number, and position of sulphur atoms. Methods typically have shallow gradients and low ion-pairing concentrations with tetrabutyl ammonium hydroxide being a common ion-pairing reagent.

mRNA are single strand nucleotides of 800–8000 nucleotides in length and a

Figure 6: SFC separation of PEG and derivatized PEG species used in nanoparticle synthesis. Figure courtesy of Kevin Treacher, AstraZeneca.



ELSD Chromatogram

Table 3: Benefits of using active modulation and UHPLC×UHPLC conditions compared to HPLC×HPLC

	HPLC×HPLC	UHPLC×UHPLC	UHPLC×UHPLC with Active Modulation
Total Analysis Time (min)	200	80	40
Sample Dilution	1529	299	120
Volume of Mobile Phase Solvent Used (mL)	800	120	78

mass in the order of ~3000 kDa. They are synthesized using enzymes that require chromatographic purification to improve quality. From a regulatory perspective, mRNA is classed as either a biologic or gene therapy, but guidelines are vague. However, several critical quality attributes (CQAs) are typically measured including:

- Integrity
- Quantity

Figure 7: Ben Baars delivering his presentation on vacuum ultraviolet detection for GC.

- Purity
- Amount of cap (uncapped form is typically inactive)
- Amount of Poly A tail (tailless form is typically inactive)

For mRNA analysis capillary electrophoretic and IP-reversed-phase LC methods were described. Örnskov said AstraZeneca has developed a charge-based separation with a low viscosity sieving media (capillary gel electrophoresis [CGE]). The gel is based on polyvinylpyrrolidone (1–2% *w/w*), glycerol (10–20% *w/w*), and 112 mM HEPES pH 7.5 (pH has to be between 6 and 9 to prevent degradation). Hydrodynamic injection is used to measure both assay and impurities and UV detection is performed at 260 nm. This methodology was good for measuring longmers, multimers, and foldmers.

The IP-reversed-phase LC method they have developed is based on a 200 Å wide pore column (100 × 2.1 mm i.d., 4-µm C4). The mobile phase is 100 mM TEA acetate–acetonitrile at low flow rate (0.2 mL/min) with a column temperature of 80 °C. It was found to be imperative to include 0.1 mM EDTA in the mobile phase to complex any metal ions in the LC system, column, or mobile phase. This improves peak shape, method robustness, and equilibrates the column more quickly. Örnskov concluded her presentation by illustrating the separation of the 858 nucleotide EPO (mRNA from Trilink) using this methodology.

The next talk was given by Adam Taylor from National Physical Laboratory (NPL) on "*Metrology for Mass Spectrometry Imaging in Life Science Research*" (Figure 3). NPL are working on the grand challenge Rosetta, which investigates the spatial heterogeneity of cancers. To do this, multiple mass spectrometry imaging (MSI) instrumentation is available at NPL including matrix-assisted laser desorption–ionization (MALDI), desorption electrospray ionization (DESI), secondary ion mass spectrometry (SIMS), nanoSIMS, 3D OrbiSIMS, laser ablation-inductively coupled plasma-MS (LA-ICP-MS), and rapid evaporative ionization mass spectrometry (REIMS). The volume of data produced from all these techniques is extremely large and a specialized cloud data storage facility is required for this. All data are stored in a common data format. Much research has focused on data processing, and their automated approach now allows 192 tissue samples (0.5 TB of data) to be processed in two weeks when it historically took 120 days.

The instrumental MS challenges require a balance of spatial resolution, sensitivity, speed, and data file size. NPL has developed high repetition lasers that allow extremely fast "raster" lasering ionization of tissue samples. The company has also developed different MS sources geometries, which lead to increases in ion yields, and it has developed a post-ionization approach, which dramatically increases sensitivity by improving ionization of neutral species.

Significant research is also undertaken on sample preparation to improve tissue sequence coverage. Most samples are formalin-fixed paraffin-embedded (FFPE), but fresh frozen samples offer much better insight into cancer morphology; they do degrade rapidly and are also difficult to obtain during invasive cancer surgery. Liquid extraction surface analysis (LESA) is used to further decrease ion suppression

by salt removal, and is often used in combination with nano-LC–MS for very small surplus.

The final lecture in the session was given by Nick Pittman of Waters, who gave a presentation on "*A Novel LC–MS Approach for Enhancing Protein Separations with Improved MS Sensitivity*". He opened his presentation by discussing the utility of trifluoroacetic acid (TFA) as an ion-pairing reagent for biomolecule analyses because it pairs with protonated nitrogen groups on molecules, decreasing stationary phase silanol interactions and improving peak shape. The use of formic acid (HCOOH) gives poorer retention and poorer peak shapes than TFA but much better MS response (typically 12-fold or higher sensitivity). The use of bridged ethyl hybrid phases gives better peak shape than analogous silica phases, but the charged surface hybrid technology allows a mixture of TFA and HCOOH (0.02% and 0.08% *v/v*, respectively) to be used, which improves MS response over TFA or HCOOH alone.

Waters has recently introduced difluoroacetic acid (DFA) as an alternative to TFA. While retention is lower than TFA, MS response was shown to be at least a twofold improvement in sensitivity (see Figure 4). In addition to this, Waters has also released a new 2.7-µm 450 Å solid core polyphenyl particle that was designed for the analysis of biomolecules. This was exemplified in the analysis of a Pfizer antibody–drug conjugate (ADC). The original method used 0.1% *v/v* TFA, 10% *v/v* IPA at 80 °C (peak capacity of 135), but was translated to 0.15% *v/v* DFA, no IPA at 70 °C (peak capacity of 151 and MS sensitivity increase of 3–4).

The lunch break provided an opportunity to interact with the vendors and discuss their products (Figure 5). The first lecture after lunch was provided by Timothy Cross from Thermo Fisher Scientific, who discussed "*New Paradigms in Liquid Chromatography Separation Efficiency*". He discussed the company's new UHPLC systems and the benefits of dual LC, LC/LCMS, and inverse gradient workflows.

The next vendor presentation was delivered by Shaun Pritchard from Agilent Technologies on "*New Developments in SFC and Future Concepts*". He discussed the ability to perform very large injections of up to 80 microlitres on the company's system, thereby addressing a critical issue with respect to hopes for increasing uptake of supercritical fluid chromatography (SFC). Pritchard also presented work from Agilent collaborators investigating LC × SFC for achiral × chiral analysis (Sabine Heinisch's group at Institut des Sciences Analytiques, France) and LC × SFC utilizing trap columns for comprehensive sample analysis.

The final presentation in the session was delivered by Kevin Treacher of AstraZeneca on "*Approaches for the Characterization and Analysis of Advanced Drug Delivery Systems*". Treacher started his presentation by discussing the enhanced permeation and retention (EPR) effect, which is believed to lead to increased permeation of pharmaceutical drugs at their designated sites of action. This leads to an increase in space between the minimum effective dose and the maximum tolerated dose. This phenomenon has led to the development of pharmaceutical nanoparticle design and their release properties are governed by many factors including size, charge, solubility, cytotoxicity, system clearance, and immune system response.

Treacher said the focus of his talk would be drug conjugates (which include formulations like drug-polymer conjugates, dendrimers, and ADCs) and nanoparticles (which include micelles, liposomes, polymersomes, inorganic, and polymeric nanoparticles). He said that to characterize these formulations he would discuss SEC, SFC, and asymmetric flow field-flow fractionation (AF4).

Nanoparticle size properties are very important. For parenteral administration they are typically sterilized by filtration so must be <200 nm, but also must be large enough to avoid excretion via the kidney (>5–10 nm). Particle characteristics, such as drug loading, particle morphology, and coating type, are also very important; the particles are often coated in polyethylene glycol (PEG) to make them transparent to the immune system. Treacher noted that they are often assembled using poly(L,D-lactide-co-glycolide) or polycaprolactone, which degrade to benign products *in vivo*.

The main instrumentation approach to characterize these particles is via SEC with quaternary array detection (QDA). The detector array may include RI, UV, right-angle light scattering (RALS), low-angle light scattering (LALS), and differential viscometry, which allow determination of important particle properties such as concentration (RI), molecular weight (light scattering), intrinsic viscosity, hydrodynamic radius (viscometer), and solvent interaction (Mark-Houwink coefficient).

Nanoparticle (in this example dendrimers) charge characteristics are very important. Positively charged particles have been found to be cytotoxic and therefore neutral or negatively charged particles are

required (13). A particle size of >80 nm is advantageous and a drug loading of 25–30% *w/w*. Dendrimeric constructs are "grown" based on a poly(L-Lysine) core with either a poly([2-alkyl]-2-oxazoline)s or PEG outer shell controlling the external environment of the particle. Treacher described how the AstraZeneca dendrimer will typically be 20 to 150 kDa and around 30 to 200 kDa with the drug loading. Using a SEC-quaternary detector array (QDA) system, he highlighted its potential for aggregate determination because the physicochemical properties of these systems in solution can be complex.

He also presented an example of particle size distribution determination using AF4 and dynamic light scattering (DLS) detection. He noted that by using this arrangement a shape factor (Rg/Rh) may be determined, which may be used to probe particle-protein interactions.

In a final example, Treacher discussed characterization of PEG and PEG-derivative excipients. He noted that a common excipient is PEG2000, which improves drug solubility. Using SFC he showed impressive separations of mPEG2000, mPEG acid2000, and PEG diacid using a methanol–ammonium hydroxide mobile phase gradient (5–50% *v/v* methanol) and a 1.7-µm diol column (see Figure 6). Using this methodology, it was possible to baseline resolve all the individual oligomers of each of the excipients.

After the break, the first presentation in the final session was delivered by Ben Baars of VUV Analytics on "*Vacuum Ultraviolet Detection in Gas Chromatography; What are the Perspectives With This New Detector*" (Figure 7). He began his presentation by noting that the vacuum ultraviolet name of the technique referred to the wavelength range of the detection (approximately 120–240 nm) rather than the technique being operated under vacuum conditions. In this UV wavelength range, almost all molecules absorb radiation and is therefore a near universal detection (including water and oxygen) (14). The only molecules transparent to the detector are helium and hydrogen, making it an excellent technique to couple to gas chromatography (GC).

In a final note, Baars mentioned that this was his final ever presentation as he is taking a well-earned retirement; The Chromatographic Society and RSC separation science group wish him well.

The last presentation of the meeting was delivered by Aditya Malker of Owlstone Medical Ltd., who discussed "*Development of Technology and Workflows for*

Metabolite Profiling of Human Breath". Malker started the presentation by discussing the formation of endogenous volatile organic compounds (VOCs) produced during body cell function, which typically number over 1000 in any single breath. Exogenous VOCs are also produced and are dependent on factors like food intake, exercise, medication, and smoking. He said that while breath sampling to identify disease markers is well recognized, to date fractional exhaled nitric acid (FeNO) and *Helicobacter pylori* breath tests are in widespread use due to difficulties in "breath bag" sampling, which suffer chemical losses over time, are vulnerable to airborne contaminants, and are difficult to store or transport.

Endogenous VOCs are good markers of bodily functions as the blood sweeps the whole body in one minute and the VOCs are expelled from the lungs during breathing. Owlstone has developed a breath sampler that learns a patient's breathing patterns and can collect samples from different parts of the airways.

Following final questions, Sam Whitmarsh thanked the audience for their attendance and closed a very informative meeting. This edition of the meeting series highlighted the continuing developments within the field of separation science and brought the audience up to date with many emerging analytical trends. It illustrated the novelty and continuing evolution of chromatography and the clear progression of separation science from the previous two instalments of this meeting.

The Chromatographic Society's next meeting will be the Grass Roots IV event "Introduction to Biopharmaceutical Analysis", which will be held in Church Stretton 4–7 October 2019. Further details and registration may be found at https://chromsoc.com/events/. Future RSC separation science group meetings may be found on their website at https://www.rsc.org/Membership/Networking/InterestGroups/separationscience/

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Paul Ferguson is a separation science specialist at a large biopharmaceutical company in the UK. He has worked in the pharmaceutical industry since 1999 following a postdoc at Imperial College London on capillary electrochromatography (CEC) with Norman Smith. Paul has particular interests in UHPLC, SFC, CE, chiral separations, formulated drug sample preparation, green analytical chemistry, and method development. He is a past winner of the Desty Memorial lecture prize (2002), a Fellow and Chartered Chemist in the RSC, and is a visiting lecturer at King's College London. He is currently Honorary Secretary of The Chromatographic Society. He has previously served as Vice President for the Society from 2009 to 2014 and President from 2014 to 2017. He has organized or co-organized several successful symposia for the Society since 2007 including the inaugural Grass Roots educational event held in Grasmere in 2016.

John Lough has taught extensively on separation science, primarily across pharmaceutical sciences courses at his home university, including on the MSc Drug Discovery and Development programme (University of Sunderland) which he leads. He favours the use of industry-orientated, problem-solving laboratory classes on the university's modern analytical instrumentation as a vehicle for giving students the skills and knowledge they need for employment in the pharmaceutical industry. In his research he has covered a wide range of LC themes and LC applications, but he is currently revisiting his longstanding interests in chiral LC and stationary phase selectivity.

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Tips & Tricks GPC/SEC: High Temperature GPC versus Ambient GPC

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Gel permeation chromatography/size-exclusion chromatography (GPC/SEC) is the standard technique to determine the molar mass distribution of synthetic macromolecules. However, some kinds of polymers (for example, polyolefins) are often only soluble in special solvents and require high temperatures to be used during the analysis to keep the sample completely dissolved. Therefore, for the analysis of these polymers, dedicated high temperature GPC systems are used. This article will discuss the pros and cons of both high temperature GPC/SEC and ambient GPC/SEC.

Synthetic macromolecules are a class of products with very different chemistries ranging from water-soluble, charged molecules, such as polyacrylic acid, to very nonpolar, neutral molecules, such as polystyrene. Typical solvents (and thus the mobile phases applied in gel permeation chromatography/size-exclusion chromatography [GPC/SEC]) therefore range from polar, for example, water, to nonpolar, for example, toluene.

Thanks to easily available high-performance materials, liquid chromatography (LC) hardware can be used with (or at least adapted to be used with) many of the mobile phases required in GPC/SEC. With optimized components and suitable seals and wear parts in the system it is possible to use the same equipment for many different applications. This is very attractive, especially for laboratories with a lot of different tasks.

However, there are two important exceptions:

- Applications that require very high salt loads or extreme pH values—specific

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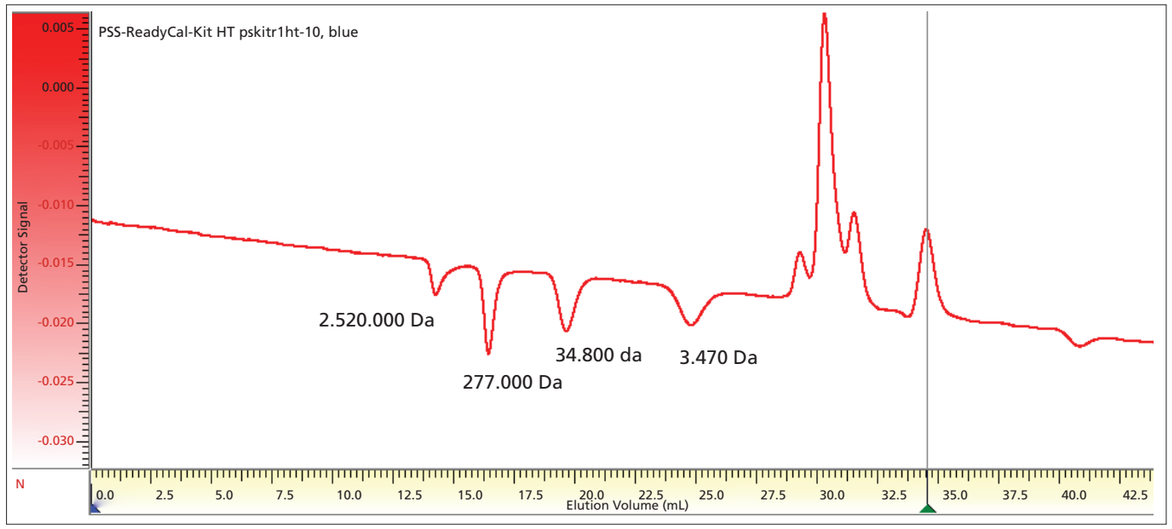
Figure 1: Separation of four different polystyrene reference materials for calibration in TCB detected using a refractive index detector, RI. The signals are negative due to the negative *dn/dc*.



optimized (metal-free/bioinert) hardware might be the better choice.

- Applications that require permanently higher temperatures to keep the dissolved samples in solution. Specialist LC equipment, referred to as *high temperature (HT) GPC*, is used to meet these specific requirements.

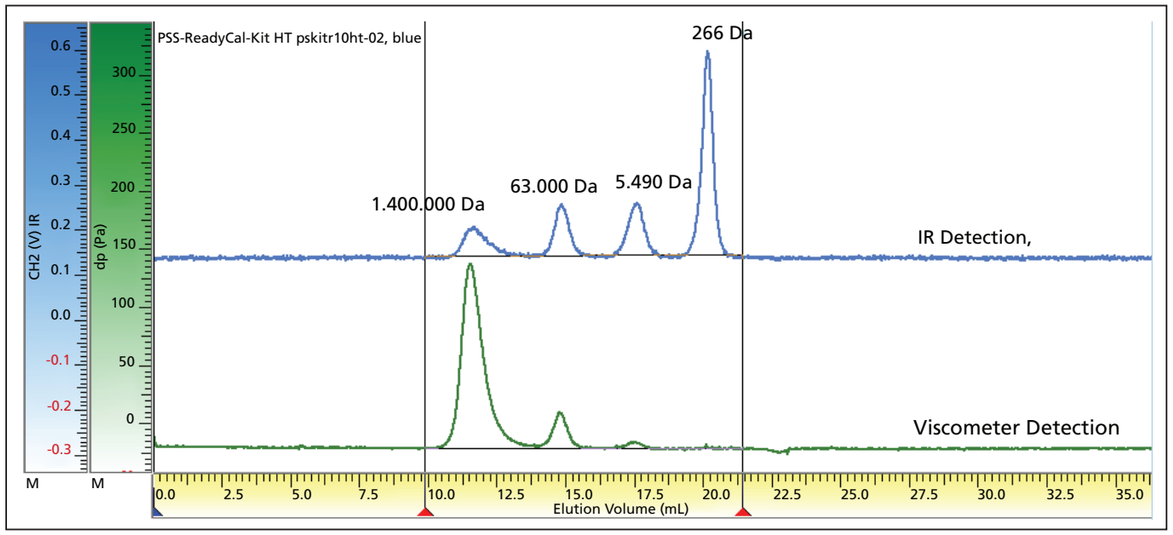
Which Applications Require HT-GPC?

LC requires that the samples be dissolved. This can be particularly difficult for high molar mass samples and crystalline macromolecules.

For crystalline macromolecules the dissolution in an appropriate solvent can be forced when warming up to temperatures slightly below the crystalline melting point. Highly crystalline linear poly(ethylene) (PE) can be dissolved in several solvents (for example, 1,2,4-trichlorobenzene [TCB] or 1,2-dichlorobenzene [o-DCB]) above 160 °C.

Typical HT applications are therefore polyolefins, such as PE, poly(propylene) (PP), and their copolymers. Those

Figure 2: Separation of four different polystyrene reference materials for calibration in TCB detected using an infrared detector, IR(CH_2 vibrations), and an online viscometer (delta pressure).



macromolecules are analyzed applying temperatures in the range of 160–210 °C.

Why is Using a Column Thermostat Not Sufficient?

The influence of temperature in GPC/SEC is an interesting topic to discuss. While the GPC/SEC separation mechanism itself is purely entropy-driven and thus independent of the temperature, there are certain important parameters influenced by temperature to consider (1).

Temperature has an effect on sample solubility. As mentioned earlier, for some macromolecules, warming up to temperatures slightly below the crystalline melting point is favourable. However, while low molar mass poly(ethylene glycol) stays in solution once dissolved in THF, most polyolefins in TCB do not. The samples precipitate when the temperature decreases. Thus the sample solution needs to be kept at an elevated temperature for the whole analysis process. As a consequence the sample injection system, the columns and detectors, and all tubings must be kept at higher temperatures.

Which Detection Options are Available?

In general, the detection options for HT-GPC are the same as for ambient GPC/SEC. Typically, at least one concentration detector is required.

Unfortunately UV detection is not an option because of the missing chromophores in nearly all samples that require HT-GPC. Exceptions are ethylene-vinyl acetate (EVA) or PP-MSA-grafted copolymers, where UV detection could be useful. However, there is no commercial UV detector on the market that can be attached to or used in HT-GPC equipment.

Refractive index (RI) detection is also quite challenging. First of all the refractive index increment, *dn/dc*, of many samples in the applied solvent is small. Therefore the detector response is low, resulting in small signals. In addition, the RI signal is very sensitive to temperature fluctuations. The combination of these two effects is often the reason for poor raw data quality with RI detection in HT-GPC (compare Figure 1).

Very interesting detection options for polyolefins are infrared (IR) detection and the use of high-temperature evaporative light scattering (HT-ELSD) detection (compare Figure 2).

While IR detection with flow-through cells is not possible in ambient GPC/SEC because of the solvents applied, typical HT-GPC solvents are IR-transparent. This offers interesting opportunities: CH_2/CH_3-vibrations can be detected in 1,2,4-trichlorbenzene and 1,2-dichlorobenzene; C=O-vibration can be detected in 1,2-dichlorobenzene. In general, detection of the C-H-vibration in the 3000 cm^{-1} range gives a linear concentration signal with a good signal-to-noise (*S/N*) ratio. Additional information is even available when ratioing two IR-vibrations. This is often used in copolymer characterization to learn more about the comonomer content (2).

HT-ELSD detectors allow for detection of low concentrations, thus reducing the problem of column overloading specifically for high molar mass samples; they can be used for gradient separations of macromolecules (3).

Molar mass sensitive detectors, such as online viscometers and light scattering detectors, are also applied in HT-GPC as additional detectors. As in ambient GPC/SEC, these detectors are used to measure true molar masses and to learn more about branching (4).

Unfortunately the number of narrow distributed polyolefin calibration standards is very limited and only low molar masses are available. Therefore polystyrene is used to calibrate when only sample comparison

is required. Universal calibration with online viscometers or light scattering detection is used if true molar masses are required. However, for polyolefins, light scattering detection suffers from the same problem as RI detection with respect to *dn/dc* (5).

What are the Requirements for Columns?

Typically column stationary phase materials comprise styrene-divinylbenzene, a material that is also quite common in ambient organic GPC/SEC. However, many columns are optimized for the higher temperatures required, so there are specific high temperature columns available.

Of course, heating and cooling processes stress the column stationary phase material. Therefore, to prolong column lifetime, it is recommended to apply low flow rates during heating up and cooling down of columns. The desired flow rate for the analysis itself should be set only when the columns have reached operating temperature. It is also important to never leave the columns at elevated temperatures without flow.

As many polyolefins have a broad molar mass distribution, often column banks are used to ensure that the wide molar mass range is covered with sufficient resolution. This is not unusual when compared to ambient GPC/SEC.

What Else Should be Considered?

Sample preparation for HT-GPC is challenging. A representative sample that can be dissolved without polymer degradation and without initiating reactions during the sample preparation step is required.

As temperature will stress the sample as well as the stationary phase material, it is preferable to limit the sample exposure to higher temperatures to a minimum.

Some samples need to be filtered to remove filling materials like fibreglass or carbon black. Compared to ambient GPC/SEC this step is much more complicated because the filtration needs to be done at the elevated temperatures required to keep the sample in solution. An automated filtering system with backflush cleaning of the filter is a very helpful tool and is a component of some instruments, or alternatively an offline sample preparation device.

As many polyolefins have a broad molar mass distribution and high molar mass, it is recommended to use low concentrations for preparing the sample. To ensure detection with sufficient signal quality the injected mass is increased by adapting the injection volume. An injection volume of 200–300 µL is quite common in HT-GPC, and way above the default 20–100 µL injection volume in ambient GPC/SEC.

It is worth mentioning that solving typical LC problems, which also happen when operating a HT-GPC system, requires much more patience. The system needs to be cooled down to replace frits, columns, or seals. Heating requirements mean that the design of the HT-instruments cannot be as user-friendly as that of ambient GPC/SEC systems, and so checking for leaks and servicing is harder to do. This is also the reason why scientists dedicate one system to HT-applications and use other systems in ambient GPC/SEC.

Summary

- The characterization of polyolefins requires specific HT-GPC hardware; ambient GPC/SEC systems cannot be modified to perform the analysis.
- Autosampler, vials, columns, detectors, and all tubings have to be kept at the high temperature needed to avoid sample precipitation. This high temperature stresses samples, the stationary phase, and the hardware, specifically in the detectors.
- Sample preparation and filtering of the sample is very challenging in HT-GPC because of the high temperatures required to keep the sample in solution.
- Injection conditions for HT-GPC and ambient GPC/SEC are different.
- IR and ELSD are powerful detection options. Other typical ambient GPC/SEC detectors are also used.

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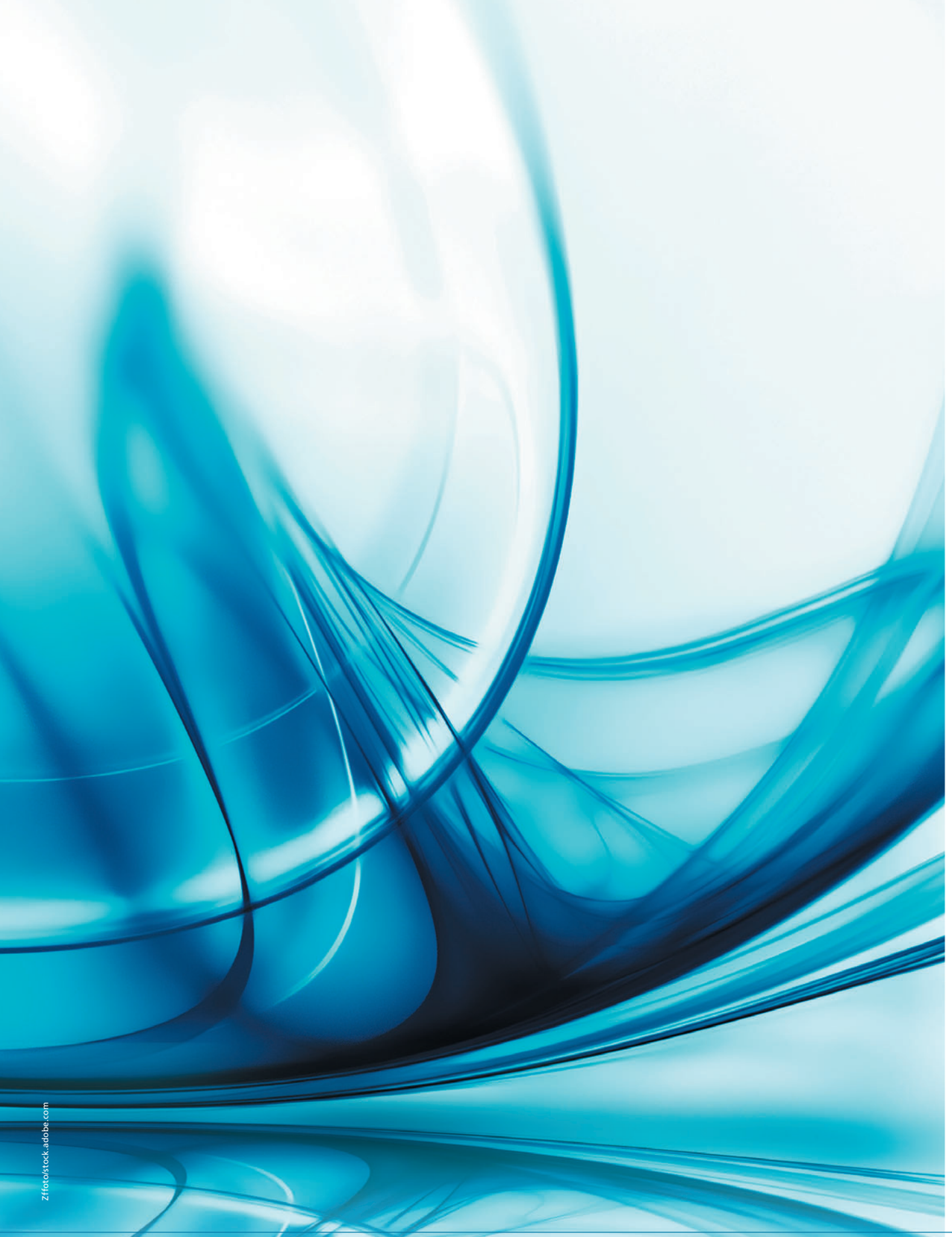

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The Importance of Tuning and Calibration in Liquid Chromatography–Mass Spectrometry (LC–MS)

An excerpt from *LCGC*'s professional development platform, CHROMacademy.com, on the importance of tuning and calibration in liquid chromatography–mass spectrometry (LC–MS).

The importance of tuning and calibration of a mass spectrometer in liquid chromatography–mass spectrometry (LC–MS) cannot be overemphasized. Tuning and calibration is performed to ensure that the mass spectrometer is working correctly, or that the spectra (mass assignment and relative abundance of spectral signals) resemble a previously determined standard. The tuning process will check that spectrometer contamination or degraded electronic components have not changed the assigned mass positions (calibration of the mass axis); that repeatable analysis from instrument to instrument (or laboratory to laboratory) is ensured; and that the spectrometer gives expected relative ratios of ion fragment intensities for a target compound. Tuning acts as a diagnostic tool to indicate the service or cleaning requirements of the spectrometer; it provides a chronicle of system performance, and the matching of fragments from a known calibration compound so as to adjust the mass axis so it agrees with the expected mass assignments. Tuning is essential for all types of mass analyzers, and at high resolution or for trace analysis instrument tuning will help to define attainable resolution and sensitivity.

Tuning

The voltages applied to the various ion source components must be tuned to achieve target ion abundances for various analyte ion masses. This ensures optimal instrument sensitivity and a predictable response across a range of masses. During tuning, the relative and absolute abundances of fragments of a known tuning compound are established, and the mass assignment, resolution, and spectral peak width generated by the mass analyzer are also adjusted and set. The tuning process involves adjusting several mass spectrometer parameters that affect signal processing, as well as voltages and currents associated with ion source components, the mass analyzer, and detector. Because atmospheric pressure ionization-mass spectrometry (API-MS) produces little fragmentation from a single ion, liquid chromatography–mass spectrometry (LC–MS) calibration and tuning is usually carried out using a mixture of compounds. Many substances are available for calibrating the *m/z* scale, including compounds such as bovine ubiquitin, cesium salts, lysozyme, sugar mixtures, and solvent clusters. Proprietary solutions have also been developed by different instrument manufacturers. For many years, polyethylene glycol (PEG) and polypropylene glycol (PPG) were the most widely used calibrants for LC–MS. However, these compounds are very sticky, and tend to remain in the system for long periods, so their use is falling out of favour.

Mass Alignment

All mass spectrometers are calibrated to specify how masses are assigned to peaks. This is done by constructing a calibration curve based upon the mass spectrum of a known reference standard.

Peak Heights

Quadrupole mass spectrometers require calibration to achieve a standardized abundance versus mass response, to correct for any mass discrimination inherent in the mass analyzer. This is done by tuning peak heights for a standard sample. Peak heights can be influenced by other types of analyzers, but the quadrupole case is the best example.

Quantitation

Quantitative methods such as selected ion monitoring (SIM) require the creation of a calibration curve based on the response of varying quantities of a standard sample. SIM or selected reaction monitoring (SRM) methods require some calibration of the mass spectrometer so that the masses or reactions will be accurately monitored.

Peak Shape and Ion Source Tuning

The mass spectrometer parameters must all be adjusted to give both good sensitivity and good peak shapes for a standard reference compound at some specified resolution value.

Mass Axis Calibration

Calibration is done using infused tune compounds that contain spectral lines due to ions whose mass is known to the level of accuracy required, such as unit mass values for quadrupole mass analyzers or accurate mass values for double focusing magnetic-sector mass analyzers. These compounds cover the required operating mass range, with the ions being as evenly spaced as possible. The instrument software tunes the mass axis to a set of pre-programmed tune masses by altering the electronic configuration of the mass analyzer, that is the mass gain and offset for the quadrupole or magnetic field strength for the sector instruments. Mass axis calibration should be independent of the API technique used for ion generation, so long as the calibrant is capable of efficient ionization under a given set of interface conditions. Mass axis calibration is carried out at regular intervals, such as at the beginning of each working day or prior to a campaign of analysis, depending on the laboratory and its applications. When working at high resolution where high mass accuracy is required, a lock mass compound may need to be constantly infused alongside the analyte. This is particularly prevalent with time-of-flight (TOF) mass analyzers, where there may be a tendency for the mass axis to drift, due to the highly sensitive nature of the mass analyzing device.

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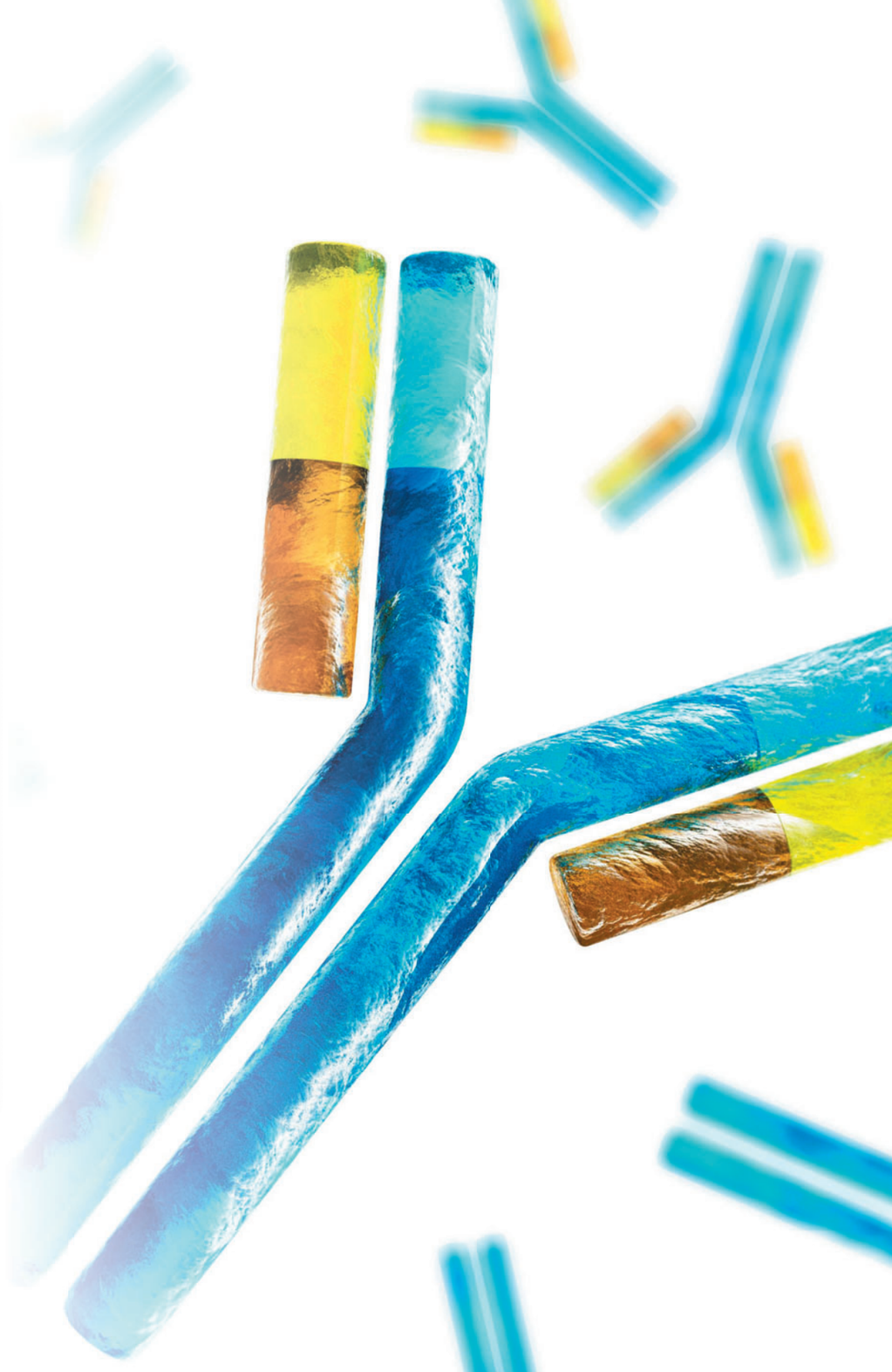

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The Column (ISSN 2050-280X) is the analytical chemist's companion within the dynamic world of chromatography. Interactive and accessible, it provides a broad understanding of technical applications and products while engaging, stimulating, and challenging the global community with thought-provoking commentary that connects its members to each other and the industries they serve.

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