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The Column

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Measuring Microplastic Leaching

A novel automated flow-based platform hyphenated to on-line LC

Cover Story

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Understanding Microplastic Leaching

Microplastic pollution in the environment is a major concern for governments and companies worldwide attempting to understand and reduce the damage they cause. As well as the direct damage these plastic pellets can also act as chemical reservoirs that leach chemical contaminants into the environment and further damage ecosystems. *The Column* spoke to Manuel Miró from the University of the Balearic Islands, Spain, about his research into the pollution caused by chemical leaching from microplastics.

—Interview by Lewis Botcherby

Q. The issue of microplastic pollution in the environment is very well documented but the issue of plastic additives leaching is not as well understood. What is microplastic leaching exactly and what kind of issues does it present?

A: It is true that most of the research efforts in this field have been directed towards the identification and (semi) quantitative analysis of the various types of plastic pellets in aquatic settings and environmental solids. The investigation of toxicity issues in aquatic biota has also attracted interest but the plastics are just seen as physical barriers or contaminants. Microplastic leaching stands for the evaluation of the release of chemical contaminants contained or adsorbed on the solid pellets using a variety of solvents and solutions, and serves to identify the potential role of plastic pellets as vectors of chemical contaminants. Leaching of contaminants from solid substrates is the first step towards deleterious effects onto biota and human health.

Q. In your recent paper you talk specifically about phthalate esters and bisphenol A (BPA) (1). Where do these chemicals originate from, and what dangers do they

represent from an environmental contamination point-of-view?

A: Both types of compounds are added to plastics during the manufacturing process. Phthalate esters are used as plasticizers, that is, endowing flexibility and durability to the polymeric materials. Phthalates are considered endocrine disruptors at the low ng/L and µg/L levels, and thus, their use in toys and childcare products is already regulated. Bisphenol A is another common organic species in polymer manufacturing and is used as an antioxidant or monomer in polycarbonate plastics. The high solubility in water makes it easily leachable into the aquatic environment or into food from polymer packaging. Bisphenol A is known as an estrogen agonist and androgen antagonist thus affects the human reproductive system.

Q. Are there any other notable chemicals which originate from microplastics?

A: Plastic materials launched to the market contain a number of chemicals from distinct classes that might migrate to the environment, including dyes and pigments, UV-filters, anti-oxidants and photoinitiators, just to name a few. Also, emerging contaminants that are replacing banned species, as is the case of Bisphenol S as a substitute of Bisphenol A, can now be found in aquatic settings and biota as a marker of plastic contamination.

Q. You developed an automatic flow-based platform hyphenated to on-line liquid chromatography for investigating the leachability of chemical additives from microplastics? What is novel about this new method (1)?

A: We have proposed for the first time a fully automatic flow injection method that is able to mimic the leachability of plastic additives from microplastics, such as polyethylene and polyvinylchloride, into seawater which is used as a leaching solution under dynamic conditions. This gives us invaluable information relating to the kinetics of release of the target compounds, and also allows the coupling of on-line leachability methods with matrix clean-up, which is the removal of salts by micro-solid phase extraction, and injection of preconcentrated species in high performance liquid chromatography (HPLC).

Q. Can you elaborate on the LC component of this method?

A: We have harnessed to a multi-dimensional separation system by using a short reversed-phase monolithic column for sorptive retention of the leached species in

seawater and removal of salt components under flow-injection conditions followed by switching-valve injection into an analytical column for HPLC separation.

Q. How does this approach benefit the analyst?

A: The on-line hyphenation of sample/extract clean-up with HPLC separation simplifies the analytical workflow and avoids manual treatment steps that might be a source of phthalate contamination.

Q. What challenges did you have to overcome to create this method?

A: The main challenges faced were the difficulty of handling plastic pellets in the flow system, minimizing the contamination issues from the components of the flow setup, and avoiding losses of the leached compounds by sorption onto the tubing of the system.

Q. What steps did you take to overcome these issues?

A: We used a stainless-steel (rather than polymeric) container for packing of the microplastics onto melamine foam that worked as a frit, and included a make-up flow for mixing the seawater eluate with 25% isopropanol for stabilization of the phthalate ester compounds in the way toward the clean-up column.

Q. How did the system perform and what benefits does it offer the analyst?

A: As indicated above, the flow setup operates fully unsupervised because it is controlled by user-friendly software, and integrates dynamic chemical leaching with on-line sample processing of the extracts by sorptive microextraction followed by chromatographic separation. The same system is adaptable to study any type of plastic materials, and plastic-containing solids using leaching solutions to meet the analyst's requirements.

Q. Where else do you believe this method could be useful?

A: The automatic leaching method can be translated to any type of solid sample where bioaccessibility or migration tests of (toxic) organic compounds will be studied and this includes environmental solids, including, sediments, soil, airborne particulate matter, sewage sludge, etc., biological tissues and food commodities, such as mussels, fish, pulse, and other food commodities.

Q. What are you currently working on?

A: We are expanding the applicability of the proposed flow system to evaluate human risks derived from the accidental foodborne ingestion of microplastics that are seen as emerging contaminants. In fact, I am currently coordinating a Spanish Network of Excellence on the impact of emerging contaminants in marine settings, granted by the Spanish State Research Agency (CTM2017-90890-REDT, MICINN/AEI/FEDER/EU).

Manuel Miró received his M.Sc. (1998) and Ph.D. (2002) in Chemistry at the University of the Balearic Islands (UIB), Spain. He is currently Full Professor in Analytical Chemistry at the UIB; Visiting Professor at Charles University (Czech Republic); and member of the International Union of Pure and Applied Chemistry (IUPAC), Chemistry and Environment Division (Subcommittee on Chemical and Biophysical Processes in the Environment). He is the Principal Investigator of the research group FI-TRACE (Flow Injection and Trace Analysis) of the Department of Chemistry at UIB.

Reference

1. K. Fikarová, D.J. Cocovi-Solberg, M. Rosende, B. Horstkotte, H. Sklenářová, and M. Miró, *J. Chroma. A.* **1602**, 160–167 (2019).

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News

ChromSoc Announce ‘Martin’ & ‘Jubilee’ Medal Winners

The Chromatographic Society have announced the winners of their 2020 ‘Martin Medal’ and ‘Jubilee Medal’ as Professor Gert Desmet, Vrije Universiteit Brussel (VUB), Belgium and Professor Deidre Cabooter, University of Leuven, Belgium, respectively.

The Martin Medal is named after Professor A.J.P. Martin who, together with Richard Synge, received the Nobel Prize for Chemistry in 1952 for their seminal work on partition chromatography The award represents the highest honour that the Chromatographic Society confers. The award has been made in recognition of Desmet’s outstanding contributions to the advancement of fundamental chromatographic theory. He is an author of over 300 peer-reviewed scientific papers, 11 patent applications, and a promotor of 35 PhD students. Further to his work on fundamental chromatographic theory, Desmet’s recent research has led to the development of micro-pillar array columns and other miniaturized separation techniques.

The ‘Jubilee Medal’ was created in 1982 to mark the 25th anniversary of the Chromatographic Society, with the intention of recognising up-and-coming separation scientists who have made major use of separation science in their own field or important contributions to a particular area of separations science. Cabooter was awarded the medal in recognition of her contributions to the dvancement of fundamental understanding of band broadening in liquid chromatography. She is the author of over 85 peer-reviewed articles, and is a member of the organizing and scientific committee of the International Symposium on Hyphenated Techniques in Chromatography (HTC). Further to the aforementioned research, she has worked on innovative approaches to improve and streamline the process of method development in liquid chromatography by developing novel hardware. Currently, her research team also develops software strategies based on artificial intelligence to streamline this process in an algorithmic way.

LCGC would like to congratulate both Gert and Deidre on receiving these awards.

2D–LC Characterizes Synthetic Oligonucleotides

A new methodology for the characterization of synthetic oligonucleotides has been developed by researchers from the University of Tübingen, Germany (1).

Synthetic oligonucleotides are a class of synthetic nucle ic acids, which are generally 12–30 nucleotides in length. They have gained considerable popularity as a therapeutic tool and are a promising product for the regulation of gene expression. As they are to be used in humans and in clinical trials, their production must lead to high purity products. However, a number of issues can arise during their synthesis, such as deleted or extended base sequences or base modifications, and therefore the purification methods and assays for their quality control must be robust and thorough.

The dominant method currently uses ion-pair reversed-phase chromatography with triethylammonium acetate as an ion-pairing agent because oligonucleotides are poorly retained by common reverse-phase liquid chromatography (LC). However, as this method is hyphenated to mass spectrometry some drawbacks occur, such as ion-suppression in electrospray ionization. As such a methodology with sufficient selectivity and better compatibility for MS detection is desirable.

Towards this end, researchers developed a multiple heart-cutting (MHC) two-dimensional liquid chromatography (2D-LC) method with ultra-violet (UV) and electrospray ionization (ESI) mass spectrometry (MS) detection. The first dimension features a reversed-phase/weak anion-exchange (RP/WAX) stationary phase which provides the selectivity required to separate structurally similar oligonucleotide sequences. The second dimension reversed-phase column desalts via a diverter valve, and the active solvent modulation enables the oligonucleotide peak to be viewed without interference from non-volatile buffer components and ion-pair agents, which allows oligonucleotides to be detected in MS-compatible conditions.

Reference

1. F. Li *et al., J. Chroma. A.* **1625**, 461338 (2020).

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Shimadzu Announce Alzheimer's Collaboration

Shimadzu and CHU University Hospital of Montpellier, France, have announced a collaboration on a new early detection method for Alzheimer's biomarkers. The focus will be on blood based amyloid-beta analyses which will attempt to screen for amyloid-positive subjects. Abnormal amyloid-beta concentrations can be a marker for Alzheimer's pathology in the brain and could offer a minimally invasive alternative test to the current highly invasive test which utilises positron emission tomography (PET) imaging and cerebrospinal fluid (CSF).

The blood analysis will use a combination of immunoprecipitation (IP) and matrix-assisted laser desorption/ ionization- time of flight–mass spectrometry (MALDI-TOF–MS), and will attempt to assess whether this simple blood analysis method enables early and accurate prediction of amyloid pathology in the brain with an easy-to-acquire blood sample.

For more information please visit: **www.shimadzu.com**

For more scientific background about Shimadzu's blood amyloid-beta analysis:

A. Nakamura *et. al.*, doi: 10.1038/ nature 25456

Determining Microplastic Accumulation Using Pyrolysis GC–MS

Researchers have developed an off-line pyrolysis gas chromatography–mass spectrometry (GC–MS) method to study the accumulation of polystyrene microparticles in filter-feeding organisms (1).

The deadly effects of ingesting microplastic particles on marine organisms have been demonstrated previously with otherwise healthy animals being found to contain an abundance of plastic particles within their digestive tracts leading to an untimely death.

In terms of susceptibility to this issue, filter-feeding organisms are particularly exposed because of their feeding mechanisms. The importance of shellfish as a food resource to many cultures and communities highlights the issue of this susceptibility (2), and potentially constitutes another source of microplastics which could affect human health, especially when combined with other sources such as household fibrous particles.

Mussels have been involved in many laboratory studies on microplastic particles, however, quantitation of microparticles in mussels is a difficult task, often being performed by visual inspection or, in the case of synthetic polymers, with simple physical tools or advanced spectroscopic methods.

Thermal methods and analytical pyrolysis off-line and on-line to GC–MS has been used to identify polymers in numerous environmental matrices, and researchers were keen to use a similar methodology for quantifying plastic polymers within mussels. The study focused on polystyrene because it has been largely used as a reference plastic in bioaccumulation experiments and constitutes one of "the big six" plastics in environmental matrices.

Marine mussels were subjected to short term exposure to polystyrene microspheres (PS-MP), and the accumulation of PS-MPs was assessed in the digestive glands and gills, along with an evaluation of a sensitive biomarker used to determine the general health of mussels.

The method successfully quantified the mass of PS-MPs taken up by the mussels in the laboratory experiment, and provided information on the effect of particle size, exposure level, and tissue type on the bioaccumulation of polystyrene particles in terms of mass concentrations.

While the methodology was a success, researchers also highlighted some experimental factors which require consideration when using an analytical pyrolysis methodology. In particular, the detection of styrene oligomers could potentially be hampered by some experimental factors which the pyrolysis product yields are dependent on, as well as affecting calibration protocols based on styrene monomers, and causing matrix interferences that limit the lower range of detection of polystyrene microplastics. These are common shortcomings in both off-line and on-line pyrolysis but do require future studies.

Reference

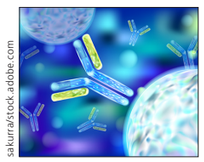
1. D. Fabbri *et al., J. Anal. Appl. Pyrolysis* **149**, 1–7 (2020).
2. Y. Cho *et al., Environ. Pollut.* **245**, 1107–1116 (2019).

Peaks of the Month

- **The *LCGC* Blog: Experiential Learning Interrupted: Reflecting on Teaching Chromatography During a Pandemic**—As our academic year comes to an end, I always take time to reflect on what I have learned in the past year. As an assistant professor in Forensic Sciences and Chemistry at Chaminade University of Honolulu, I take the opportunity to review my courses regularly and implement new tools. Some of them will be successful and others will not. **Read Here>>**

- **How Do You Write User Requirements for Chromatographs and Chromatography Data Systems?**—One of the biggest failures with purchasing chromatograph systems and chromatography data system (CDS) software is either the total lack of or poorly written user requirements. So, how can you write acceptable requirements? Is specifying a chromatograph the same as software? **Read Here>>**

- **New Gas Chromatography Products for 2019–2020**—"GC Connections" presents the column's annual review of new developments in the field of gas chromatography seen at Pittcon and other venues in the past 12 months. **Read Here>>**



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- **Glycosylation Analysis Through Released N-Glycan Workflows**—Post-translational modifications are potential critical quality attributes (pCQAs) routinely assessed in biotherapeutic development. This article demonstrates the method development considerations for sample preparation and chromatographic analysis of N-glycans of therapeutic mAbs. **Read Here>>**

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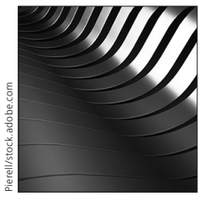
- **Chromatography in the time of COVID-19**—Incognito offers some tips for chromatographers during the current crisis. **Read Here>>**



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- **Investigating Contamination in Biocompatible LC Systems**—Biocompatible LC systems are generally regarded to be chemically more inert than conventional HPLC systems. However, a study into the chromatographic behaviour of some classes of compounds analysed on these iron-free HPLC systems revealed issues typically associated with metal contamination. **Read Here>>**

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News In Brief

Analytik Jena celebrate their 30th anniversary in 2020 and have announced a plan to celebrate customers, partners, and employees who have accompanied and shaped the company over the years. Announcing a motto of "30 Years. 30 Stories.", Analytik Jena will publish stories from this special group throughout the year.

"Many special people have shaped the company over the years. We would like to thank these people and tell their stories in our anniversary year," Said Ulrich Krauss, CEO of Analytik Jena.

You can keep update to date with these stories on their website at:
www.analytik-jena.com/30years

Jen Vanderhoven is to take over as Director of the National Horizons Centre, Darlington, UK, joining Teesside University from Fujifilm Diosynth Biotechnologies (FDB) having spent two years as a Vice-President sitting on the Global Leadership Team. She will be responsible for driving the success of the £22.3 million teaching, research, and training centre of excellence for the bioscience industry, helping the north east of England to meet the challenges of the UK industrial strategy and growing national and international links with industry.

Read more: **www.tees.ac.uk/sections/news/pressreleases_story.cfm?story_id=7445&this_issue_title=June%202020&this_issue=325**

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Molecular Structure of Poly(Lactic-co-Glycolic acid) by SEC with Advanced Detection

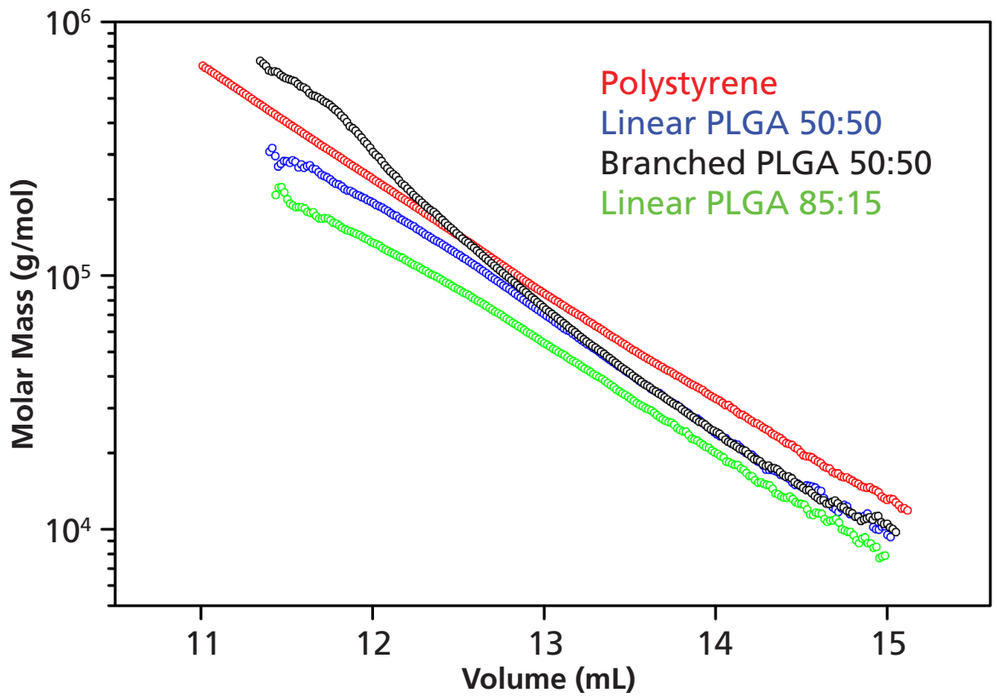
Stepan Podzimek and Daniel Some, Wyatt Technology Corp., Santa Barbara, California, USA

The macroscopic properties of material based on poly(D,L-lactic-co-glycolic acid) (PLGA) polymers are tunable by molar mass distribution and degree of branching, enabling optimization for applications in the pharmaceutical and medical industries. Size-exclusion chromatography followed by online multi-angle light scattering with intrinsic viscosity detection (SEC–MALS–IV) is an advanced analytical method for determining absolute molar mass distributions, identifying polymer conformation and quantifying branching. SEC–MALS–IV overcomes the errors that can be encountered in molar mass determined by conventional SEC, which arise from chemical composition and molecular structure, and provides comprehensive characterization of PLGA to facilitate the targeted development of optimized polymer.

Poly(D,L-lactic-co-glycolic acid) (PLGA) is a copolymer approved by the US Food and Drug Administration (FDA) for a variety of medical devices, tissue engineering and material for drug delivery. The mechanical properties and the rate of biodegradability depend on the ratio of the two monomers, and on their arrangement in a random or block pattern.

In addition to the chemical structure, the properties of PLGA are affected by its molar mass distribution and molecular

Figure 1: Calibration curves of polystyrene and three different samples of PLGA.



structure, that is, linear or branched chains. Molar mass distributions are primarily affected by the synthetization method, usually either ring-opening polymerization of two cyclic diesters of lactic and glycolic acids, that is, lactide and glycolide, (1) or direct polycondensation of lactic and glycolic acid without addition of any catalysts (2). The latter, which avoids the need to purify the final product from the catalyst, typically leads to polymers with lower molar mass.

Control of branching opens further routes to modification of the physical properties and degradability of PLGA. The branched structures are typically induced by the addition of polyfunctional hydroxy components such as glucose

Figure 2: Molar mass and intrinsic viscosity plots of linear PLGA overlaid on signals of MALS detector at 90°, RI detector and viscometer.

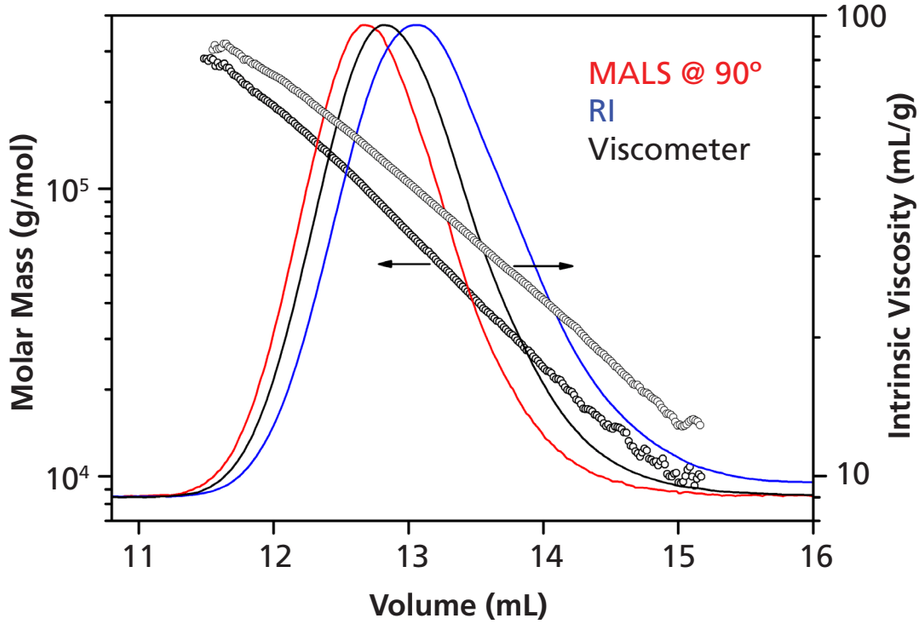
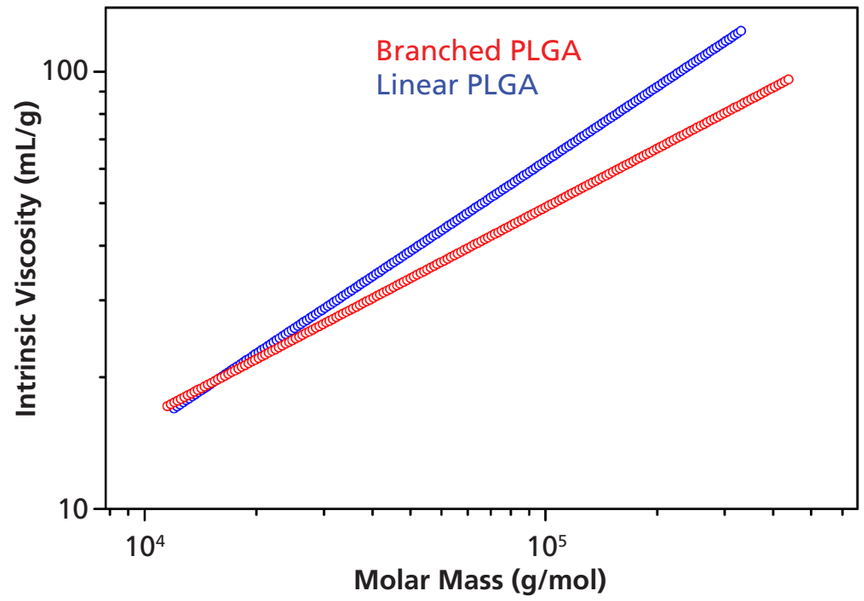


Figure 3: Mark-Houwink plots of linear and branched PLGA.



or pentaerythritol. Comb-like structures may be created with polyacrylic acid or copolymers of acrylic acid.

Polymer Analysis by SEC

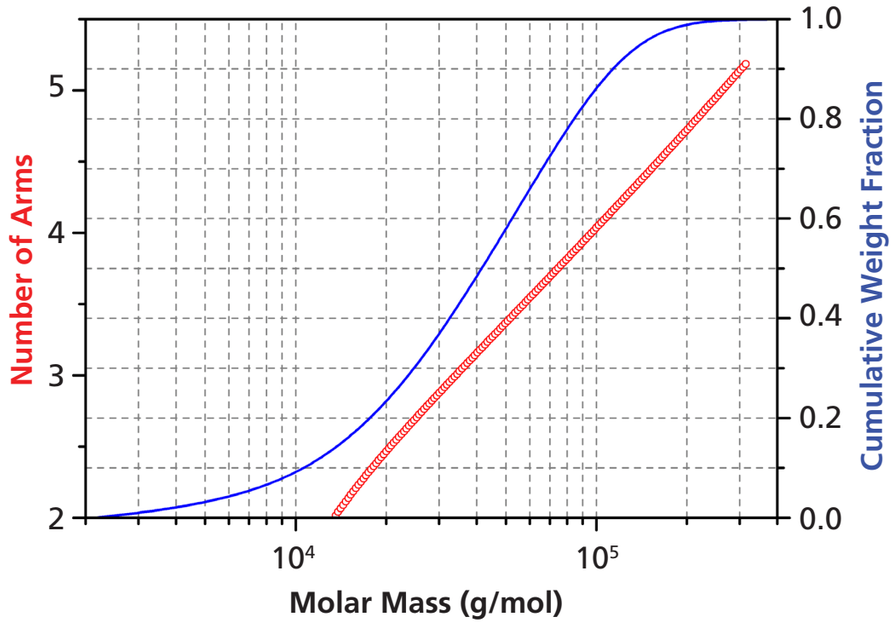
Conventional analytical size-exclusion chromatography (SEC) relies on column calibration to determine molar mass distributions. A calibration curve is constructed, which relates the molar mass of the analyte to elution volume, by means of reference standards of known molar mass. When the mobile phase is an organic solvent, SEC calibration is most commonly performed with narrow polystyrene standards prepared by anionic polymerization.

Conventional SEC is widely used for polymer characterization, but SEC separates polymer molecules according to their hydrodynamic volume, and there is no broadly valid relationship between hydrodynamic volume and molar mass. Consequently, a molar mass distribution obtained with polystyrene calibration is actually that of a hypothetical polystyrene sample that has the same distribution of hydrodynamic volume as the sample under analysis.

The addition of a multi-angle light scattering (MALS) detector upgrades conventional SEC from an unreliable, relative method to an advanced analytical technique. SEC–MALS yields absolute molar mass distributions, as well as an additional important characteristic of macromolecules: the root mean square (RMS) radius *R*. With detailed RMS radius distributions, branching can be detected and quantified as the number of branch units (in randomly branched polymers) or the number of arms (in star polymers).

Whereas molar mass can be measured reliably down to several hundreds of g/mol, the lower limit for *R* analysis by MALS is about 10 nm, roughly corresponding to a linear polymer of about 80 000 g/mol. The majority of material in commercially available PLGA falls below this limit, hence the *R* method of branching analysis is not

Figure 4: Number of arms as a function of molar mass overlaid on cumulative molar mass distribution curve of branched sample depicted in Figure 3.



appropriate. SEC combined with intrinsic viscosity (IV) detection and MALS (SEC–MALS–IV) represents another path to branching analysis which is not subject to a lower size limit, and is even more sensitive to branching than MALS alone.

The Fundamental Theory of MALS and Online Viscometry

The fundamental theory behind MALS and online viscometry can be found in the literature (3). Briefly, the molar mass is determined from the scattering-angle-dependent Rayleigh ratio (intensity of scattered light relative to that of incident light) extrapolated to zero angle:

$$\frac{K^* c}{R_0} = \frac{1}{M} + \cdots \quad [1]$$

where R_0 is the Rayleigh ratio extrapolated to zero scattering angle; K^* is an optical constant including specific refractive index increment, refractive index of the mobile phase, and the operating

wavelength of the MALS detector; *c* is the concentration in g/mL, usually measured by a refractive index (RI) detector; and *M* is the molar mass. The above equation has a second concentration term (the ellipses) that can be neglected without significant error, as concentrations of molecules eluting from SEC are very low.

The IV is measured by an online viscometer connected downstream of the MALS detector. The specific viscosity of the sample eluting from SEC columns is calculated from the pressure difference across the bridge and the pressure drop between the bridge inlet and outlet. The intrinsic viscosity [η] is the specific viscosity divided by concentration; in turn, [η] is used to calculate the viscometric hydrodynamic radius and the Mark-Houwink plot, which is the log–log relation [η]–*M*.

The Theoretical Background of Branching Analysis

The basic principle of detection and quantification of polymer long-chain branching is based on the difference in size between a branched macromolecule and the corresponding linear macromolecule of the same molar mass, as shown theoretically by Zimm and Stockmayer in their famous paper (4). The authors defined branching ratio *g* and related it to the number of arms in star polymers by equations 2 and 3:

$$g = \left(\frac{R^2_{branched}}{R^2_{linear}} \right)_M \quad [2]$$

$$g = \frac{6f}{(f+1)(f+2)} \quad [3]$$

where R^2 is the mean square radius of branched or linear macromolecules with identical molar mass *M*, and *f* is the number of arms in a star macromolecule having arms of random length. Although equations 2 and 3 represent the most fundamentally correct description of branching, their application to PLGA and other smaller polymers is limited by the minimum RMS radius MALS can measure.

An alternative branching ratio *g´* based on the intrinsic viscosity, and hence not subject to the size limit, was derived by Zimm and Kilb in equation 4 (5):

$$g' = \left(\frac{[\eta]_{branched}}{[\eta]_{linear}} \right)_M \quad [4]$$

where [η] is the intrinsic viscosity of linear and branched molecules of identical molar mass. The relationship between the two branching ratios is given by equation 5:

$$g' = g^e \quad [5]$$

where *e* is so-called draining parameter, related to the permeability of polymer coils to the solvent. Although detailed literature data are scarce, it seems that the parameter *e* varies with molar mass and branching. It is expected to fall between 0.5–1.5, so the values ≈ 0.7–1.0 might be good estimates. Other equations relating *g´* with *f* have been published, yielding slightly different results, yet their detailed evaluation is beyond the scope of this paper.

Experimental

The results presented in this paper were acquired with a DAWN MALS photometer, a ViscoStar online viscometer, and an Optilab RI detector and processed with ASTRA 7 software, all from Wyatt Technology (Santa Barbara, CA). The SEC system consisted of an Agilent 1260 HPLC instrument with two PLgel Mixed-C 300 × 7.5 mm columns, also from Agilent. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1 mL/min. Samples were prepared in THF in the concentration of 2.5–5 mg/mL and injected in the volume of 100 µL.

Results and Discussion

The failure of conventional analytical SEC, in terms of column calibration, to properly describe the molar mass distribution is demonstrated in Figure 1, which compares the SEC–MALS plots of molar mass versus elution volume for broad polystyrene and three different PLGA samples. Figure 1 reveals the following: (i) molar mass of PLGA 50:50 measured by conventional SEC with polystyrene calibration is overestimated by 25–30%, (ii) conventional SEC yields markedly larger error for PLGA consisting of 85% lactic acid, and (iii) the plot of commercial branched PLGA overlaps with that of linear PLGA in the region of lower molar masses, and starts to deviate from ≈ 50 000 g/mol. The deviation from the plot of linear PLGA confirms the presence of branched macromolecules in part—but not all—of the sample. It is worth mentioning that while polystyrene calibration would overestimate molar mass of the branched sample in the lower range, it would underestimate values of the most branched fractions with *M* greater than about 150 000 g/mol.

Figure 2 depicts typical data from SEC–MALS–IV, with MALS and specific viscosity measured simultaneously following SEC separation, yielding molar masses and intrinsic viscosities. The data enable calculation of the molar mass distribution, all molar mass averages (number-, weight- and z-averages), and the Mark-Houwink plot.

Figure 3 compares the Mark-Houwink plots of linear PLGA and commercially available branched PLGA. The slope of the plot of branched PLGA is low compared to the linear counterpart, as is typical for branched polymers.

The number of arms f may be calculated using equation 3, and plotted against molar mass. For the sake of simplicity, the draining parameter e was set to unity. Figure 4 depicts an example of such a calculation of f, overlaid with the cumulative molar mass distribution. Note that f, which is a discrete quantity having the values f = 2, 3, 4, …, is plotted here as a continuous quantity. This is because f—at any given molar mass—is averaged over molecules having different number of arms.

The plot in Figure 4 shows increasing number of arms with increasing molar mass, which indicates that not all hydroxyls of the branching compound (likely glucose) created starting points for the PLGA arms. The maximum value of f approaches five, which corresponds to fully reacted glucose. However, the cumulative distribution shows that the weight fraction of molecules with $f > 4$ is less than ≈ 15 %, and that the sample contains a substantial amount, roughly 15%, of linear molecules (since f = 2 corresponds to a linear molecule).

Conclusion

PLGA-based polymers are vital for many pharmaceutical and medical products, and the pharmaceutical industry requires a fast, accurate and highly robust analytical technique to characterize them. This need is fulfilled by SEC with triple detection comprising a MALS photometer, an online viscometer, and an RI detector. This study shows that SEC–MALS–IV can describe the PLGA molecular structure completely, that is, provide the absolute molar mass distribution and the relation between the number of arms and molar mass in star-PLGA. The difference between the correct molar mass by MALS and polystyrene equivalent depends on the monomer ratio, and even more importantly, on the degree of branching. The study also shows that commercially available branched PLGA can contain a significant fraction of linear molecules.

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3. S. Podzimek, *Light Scattering, Size Exclusion Chromatography and Asymmetric Flow Field Flow Fractionation* (Wiley, Hoboken, New Jersey, USA, 2011).
4. B.H. Zimm and W.H. Stockmayer, *J. Chem. Phys.* **17**, 1301 (1949).
5. B.H. Zimm and R.W. Kilb, *J. Polym. Sci.* **37**, 19–42 (1959).

Stepan Podzimek is a scientific consultant for Wyatt Technology with research interests focused on the characterization of molecular structure of synthetic and natural polymers by means of separation techniques and light scattering and viscometric detectors. He also heads the Department of Analytical and Physical Chemistry at SYNPO, a Czech R&D company conducting contract research in synthetic polymers and related materials, and holds a professorial position at the Institute of Chemistry and Technology of Macromolecular Materials at the University of Pardubice, Czech Republic.

Daniel Some is Principal Scientist at Wyatt Technology. He is responsible for scientific and technical marketing content and other projects including instrumentation for macromolecular interactions and real-time process analytics.

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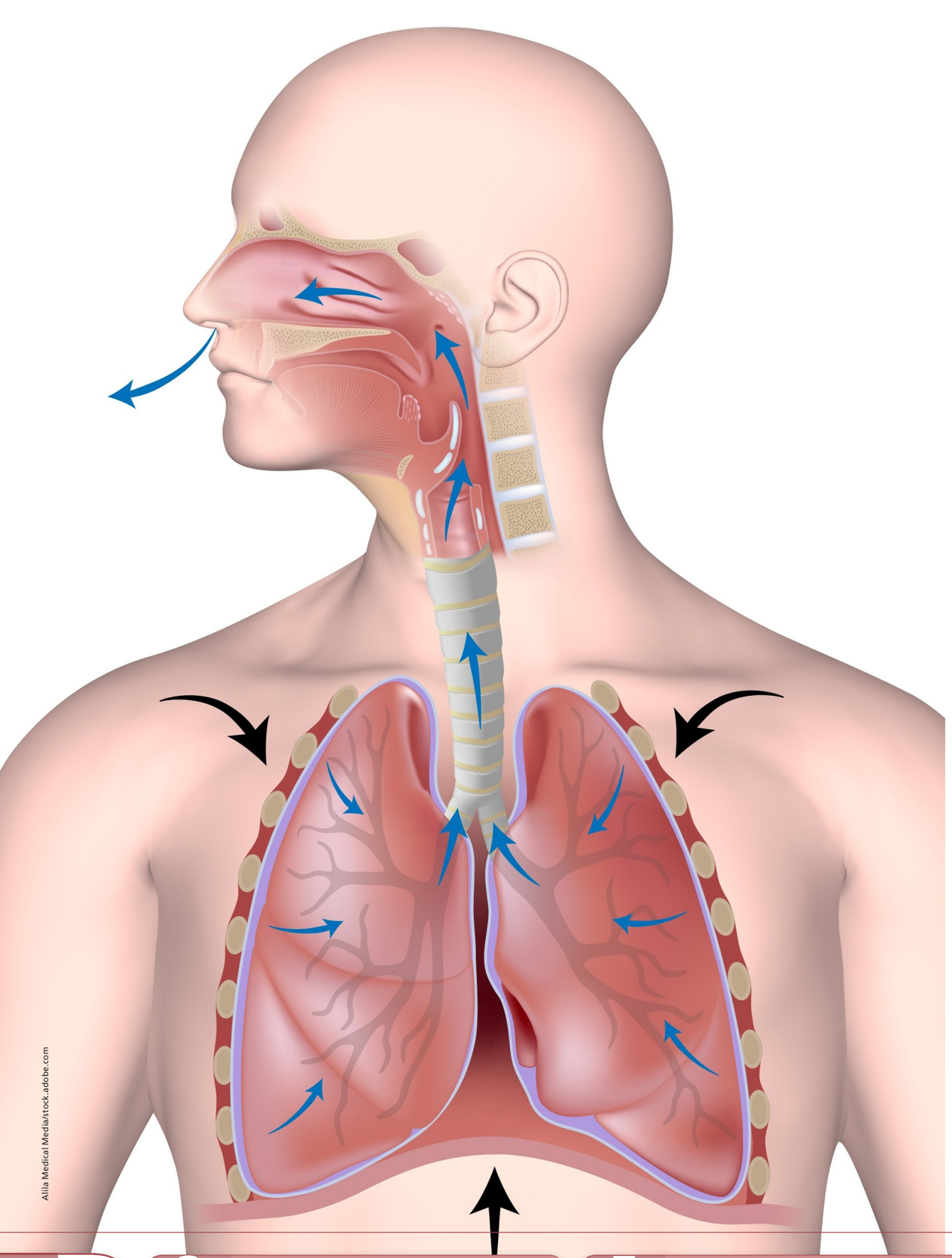

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Detecting Biomarkers in Breath using GC–MS

Max Allsworth, Chief Science Officer, Owlstone Medical, Cambridge, UK

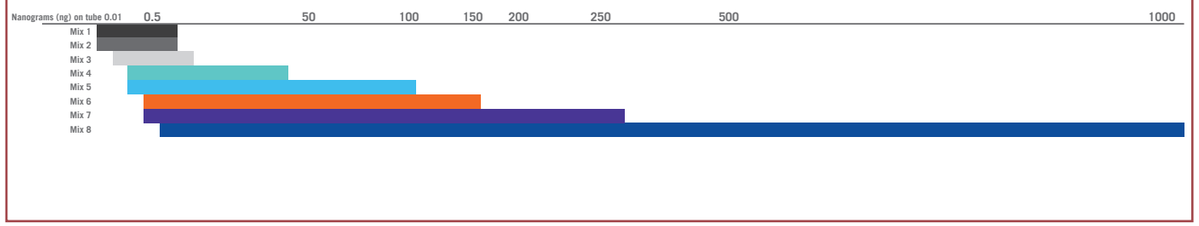
Biomarker studies using exhaled breath are rapidly emerging as a technique for early disease detection and precision medicine. By offering a completely non-invasive experience for patients as an alternative to painful biopsy procedures. A new approach has the potential to enhance patient compliance, while making clinical workflows simpler. Exhaled breath analysis, however, requires a highly sensitive analytical technique capable of accurately measuring the broad range of volatiles present in breath. In this article, we present a proof-of-concept study to demonstrate a reliable and sensitive method to detect analytes in breath samples. Using high-resolution accurate mass (HRAM) mass spectrometry (MS), the method validates how low- and high-abundance biomarkers can be quantified from exhaled breath.

A modern diagnostic technique that involves the analysis of exhaled volatile organic compounds (VOCs), which are products of metabolic activity, has been developed. In certain diseases, such as cancer, where metabolic changes can precede genetic changes, the VOCs in exhaled breath can serve as promising biomarkers for early disease detection.

Traditional biopsy methods involve removing a small amount of tissue using invasive surgical procedures, often requiring anesthesia, a team of healthcare professionals and a post-surgery recovery period. Breath analysis, on the other hand, only requires the collection of breath samples, making it completely non-invasive and pain-free for patients.

Rapidly emerging as a technique in metabolomics studies, the analysis of exhaled breath confronts a very specific analytical challenge: developing a robust method to measure the broad concentration range of different VOCs. With typical

Figure 1: Typical concentrations of VOCs in breath can span across 5 orders of magnitude. Accurate quantitation of breath biomarkers requires sensitive analytical methods that can quantify high- and low-level analytes.



concentrations of chemicals in exhaled breath spanning five orders of magnitude, this type of analysis requires methods that can quantify both high-abundance chemicals, as well as trace-level VOCs at the same time.

Analytical Requirements for Breath Analysis

The analytical pre-requisite for biomarker discovery in exhaled breath involves striking the right balance between three attributes: scanning speed, selectivity (that is, high resolution and accurate mass) and sensitivity. Moreover, the diverse nature of analytes being measured also requires the method to truly represent the comprehensive range of analytes in the samples.

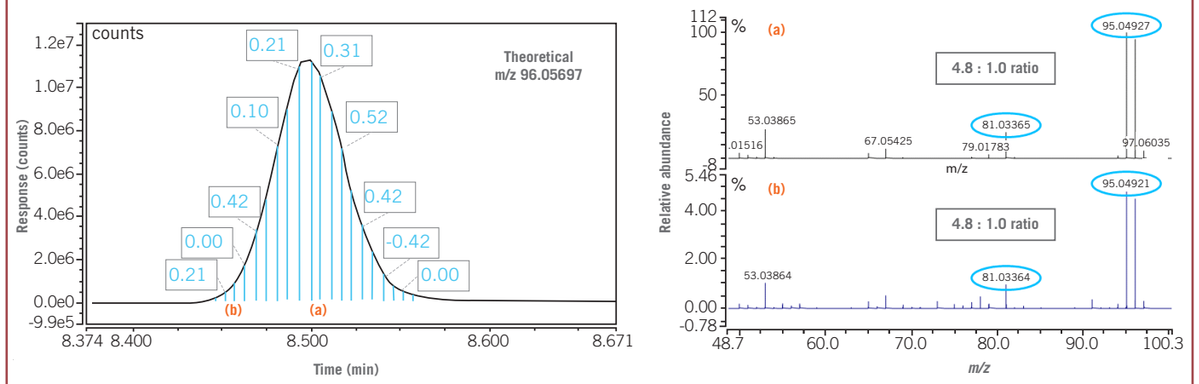
In the past, breath analysis employed time-of-flight (TOF) analyzers. The parallel detection of different ions in this method resulted in good sensitivity over a wide mass range, while the high resolution allowed for accurate compound identification. However, ion saturation in TOF analyzers can sometimes result in inaccurate quantitation of analytes present at higher concentrations, posing a limit on VOC analysis.

Using HRAM analyzers can address this issue because of their characteristic dynamic range. Spanning over five orders of magnitude, HRAM mass spectrometers offer a high resolving power for high- and low-concentration analytes. Additionally, the ability to detect minute differences in mass yields a sub-ppm mass accuracy, making HRAM a good method of choice to quantify trace-level chemicals in breath analysis.

Breath Analysis Workflow

A workflow for non-invasive analysis of breath biomarkers to provide early detection of disease biomarkers in conditions such as cancer, asthma, liver disease and pulmonary hypertension has been developed (1). This workflow involves collecting breath samples from patients and analyzing them using targeted or untargeted methods. In the targeted approach, samples are qualitatively and quantitatively analyzed for a list of known biomarkers, while the untargeted approach involves discovery of unknown biomarkers attributed to a disease. HRAM analyzers are particularly effective for untargeted analysis where the composition and concentration range of analytes are unknown.

Figure 2: Sub-1 ppm mass accuracy can be achieved across the width of chromatographic peaks. Ion ratio stability can be guaranteed at varying concentrations across the width of the chromatographic peaks.

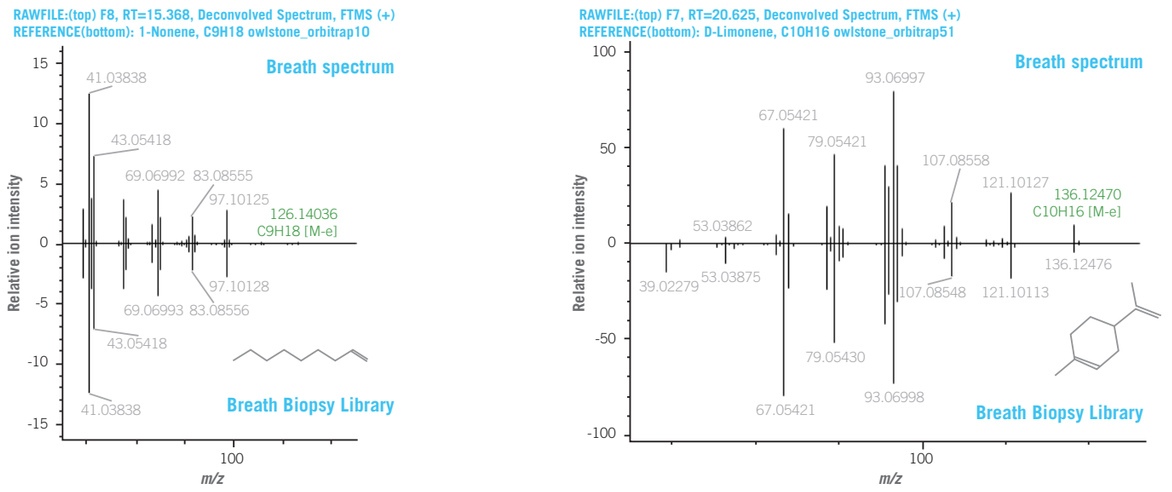


A recent study provided a proof-of-concept workflow of breath sample analysis (1). With the objective of developing a reliable, sensitive and selective method to analyze unknown breath VOCs, the researchersl performed untargeted breath analysis by combining thermal desorption–gas chromatography (TD–GC) with HRAM and validated the method by testing breath samples for smoking-related markers.

Experimental Methods

Breath collection: Breath samples of 1500 mL were collected from twelve individuals, using an ReCIVA Breath

Figure 3: Peak deconvolution can be performed and spectral matching of compounds to a high-resolution breath biopsy library.



1-Nonene
Score 99.7
SI 993, RSI 995
HRF 99.3375, RHRF 99.3441

D-Limonene
Score 99.2
SI 972, RSI 976
HRF 99.17.01, RHRF 99.1701

Sampler (Owlstone Medical). Collected breath samples were captured and pre-concentrated using Breath Biopsy Cartridges (Owlstone Medical) before analysis.

Sample preparation, desorption and MS analysis: Samples were dry-purged to remove excess water, desorbed using the TD100-xr automated thermal desorption system (Markes International) and transferred onto a 30 m × 0.32 mm, 3-µm column (Quadrex) using splitless injection to aid trace analysis. GC separation was achieved using a programmed method on the TRACE 1310 GC oven (Thermo Scientific) prior to MS. Internal standards used for quality control consisted of a 40-compound mixture prepared in methanol with 1 ppm, 100 ppm, and 200 ppm median concentrations. Mass spectral data was acquired on the Scientific Exactive GC Orbitrap MS (Thermo Scientific) using both variable electron ionization (EI) and chemical ionization (CI) capabilities.

Data processing: Data was acquired and processed using Xcalibur and TraceFinder software, both Thermo Fisher Scientific. Automated peak deconvolution features on TraceFinder generated clean mass spectra, while library search capabilities using custom and commercially available spectral libraries identified known and unknown compounds, giving both qualitative and quantitative outputs.

Results

Detecting low- and high-concentration compounds in a single analysis: Sub-1 ppm mass accuracy was achieved over the full chromatographic peak, verifying the detection of low-concentration analytes using this method. The lower mass accuracy improved peak deconvolution, helping to differentiate the analytes of interest in the mixture. Identical relative fragment ion ratios were observed at both low and high concentrations. The high linear dynamic range of HRAM guarantees stable ion ratios across five orders of magnitude, again improving deconvolution and compound identification.

Precise compound identification: To enable precise identification of compounds, the software uses various data filtering scores. Peak deconvolution and spectral matching resulted in more than 1000 entries, which may be "true" peaks associated with the chemicals in breath or false positives. To accurately identify compounds in the breath samples, a mix of hard and soft ionization techniques were used for MS. High fragmentation ionization techniques, such as EI, yielded extensive fragmentation to identify closely related unknown analytes, but with a lower mass range of interpretable data. Soft ionization using CI, generated fragments of higher *m/z* to help differentiate between compounds within a specific class, such as alkanes or terpenes,which further improves compound identification.

Proof-of-concept: Identifying markers of smoking behaviour: The established workflow and settings on the TD–GC–HRAM system were then tested for smoking-related markers using breath samples from smokers and non-smokers as a proof-of-concept demonstration. Collected samples were divided into three groups based on smoking behaviour: never smoked (n = 3), current smokers (n = 4) and ex-smokers (n = 5). A targeted approach was used to detect and quantify smoking-related markers using a customized six-compound library from previously reported data (2). The smoking-related markers in the database comprised of benzene, 2,5-dimethylfuran, toluene, ethylbenzene, m/p-xylene and o-xylene.

Using the above ionization and data filtering methods, fold changes for different smoking markers between the smoking

Figure 4: Breath sample data (n=12) shows high fold changes for 2,5-Dimethylfuran and Toluene in samples from current smokers and suggests correlation of the markers to smoking behaviour.

Compound	Never	Never	Never	Current	Current	Current	Current	Ex	Ex	Ex	Ex	Ex
Benzene	0.96	1.59	0.45	4.21	2.40	1.99	0.32	0.30	0.67	0.76	0.54	0.71
2,5-Dimethylfuran	0.55	1.61	0.84	83.74	59.50	42.66	0.74	0.78	0.50	0.32	0.80	1.31
Toluene	0.35	1.49	1.16	13.70	7.29	6.29	0.74	0.94	0.40	0.46	0.38	2.16
Ethylbenzene	0.53	0.91	1.57	2.17	2.28	2.38	2.07	0.71	1.68	1.46	1.09	1.14
m/p-Xylene	0.49	0.85	1.66	1.90	2.06	1.84	2.12	0.75	1.66	1.52	0.96	0.95
o-Xylene	0.49	0.91	1.60	1.84	2.15	1.98	2.08	0.72	1.69	1.51	0.97	1.01

and non-smoking breath samples were obtained. Low fold changes were observed in most of the smoking-related markers across all three groups, suggesting a low correlation between smoking behaviour and these reported markers. Conversely, high fold changes were observed in "current smokers" for certain markers such as 2,5-dimethylfuran and toluene, indicating a high correlation with smoking behaviour. The fold changes in smoking markers corresponding with the respective smoking behaviour demonstrates the robustness of the breath biopsy method, further corroborating that, with careful experimental design, breath volatiles can offer insights into underlying health conditions.

Conclusion

Given the need for both qualitative and quantitative data from breath samples, the TD–GC with HRAM detection met the three analytical pre-requisites for breath analysis, namely improved sensitivity, greater selectivity, and fast scanning. The broad dynamic range of five orders of magnitude and sub-ppm level sensitivity in the HRAM analyzer enabled detection of both high-abundance and trace-level analytes in a single analysis. Features such as high mass accuracy and strong resolving power, provided improved peak deconvolution, helping to identify and quantify analytes of even trace amounts.

Data filtering algorithms, namely HRF and retention time alignment, contributed to the generation of high-quality datasets by further fine-tuning compound identification. Employing two different ionization techniques, such as EI and CI, each bringing its own area of strength, made it easier to differentiate and gain insights into the chemical structure of closely related compounds.

With untargeted discovery built into its workflow, HRAM acquires and stores the complete profile. This gives researchers the flexibility to return to the dataset at a later stage to re-analyze or even ask a different research question, allowing replication and accomplishment of longitudinal studies. When combined with phenotypic information, these virtual patient profiles can be used to test new hypothesis and augment clinical trials.

This study also demonstrates how the this workflow eliminates time-consuming sample collection and preparation steps involved in conventional, invasive biopsies, thereby saving valuable time in clinical diagnostics, and offering patients a painless experience with precision medicine through non-invasive biomarker discovery using exhaled breath.

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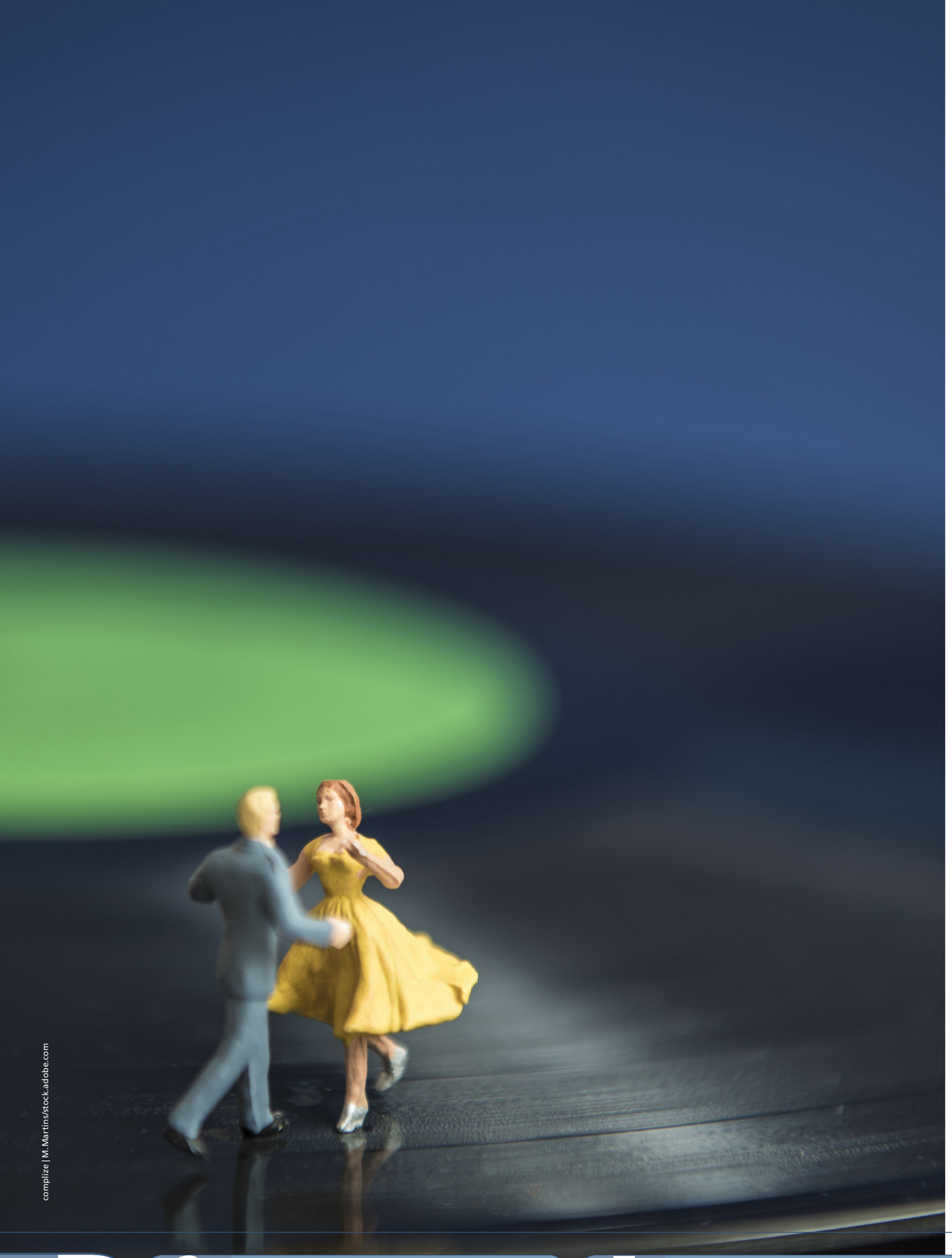

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The *LCGC* Blog: Last Dance, for a While—Time to Share the Wealth

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Kevin Schug takes a break from his popular blog and reveals an exciting new collaboration between *LCGC* and the American Chemical Society Analytical Division, Subdivision on Chromatography and Separations Chemistry, to keep readers abreast of the latest trends and developments in separation science with a new series of blogs from members of this group.

As academic professionals, we seek to have the resources we need to train the next generation of researchers and to have others take notice of our efforts. In my case, as is probably true for many academics, getting started was tough and a lot of hard work. With each successful experiment performed, paper published, new instrument acquired, degree conferred, and grant or contract won, we made strides forward. The failures have far outweighed the successes in numbers, but each success has held far more significance than each failure.

Along the way, a few specific opportunities have provided me some significant advancement and exposure. One of these has been writing the *LCGC* blog. The invitation from *LCGC* to do this came toward the end of 2012. Such a concept was quite foreign to me. I knew that people wrote blogs, but I never really followed them. Even so, this was a pivotal time in my career. We had just finalized the first installation of instrumentation that would ultimately become the Shimadzu Institute for Research Technologies at the University of

Texas Arlington, USA. Part of that agreement included an endowed professorship that would give me some significant discretionary funds to perform research. Further, we had recently embarked on efforts to investigate the potential environmental impact of unconventional oil and gas extraction processes. This effort would lead very quickly to the creation of the Collaborative Laboratories for Environmental Analysis and Remediation (CLEAR), a consortium designed to pull together the needed expertise to handle the multifaceted nature of our environmental research, as well as a new vehicle and brand through which we could raise funds. I was also already working with VUV Analytics, Inc., and they were close to launching their first commercial product (full disclosure: I am a member of the scientific advisory board for VUV). With all of these developments, a new channel into the scientific community through the *LCGC* blog sounded like a nice tool to have in my toolbox.

At first, it seemed quite daunting to come up with a technical topic to discuss each month. After a while, I found that was not the case. I could write about whatever was on my mind and rarely did I need to do any special research to compose the blog. It became a place where I could discuss technical topics of interest, share an overview of recent publications or sets of publications from my group or by others, review conferences I attended, talk about teaching at the university level, or regale everyone with my various experiences and ideas. For example, if you want to consider how working in an analytical laboratory might benefit you in a zombie apocalypse, check out my popular blog entry from October, 2013 (1). Basically, I could write about anything I thought the readers of *LCGC* might like to read.

Very early on, I realized the immense value of the *LCGC* blog as a means to reach a much broader audience with my ideas. Circulation of the newsletters that have featured my blog exceeds 45 000 subscribers worldwide. I am sure not everyone reads my entries, but I have always been heartened by comments I have received (both positive and negative, though the positive have far outweighed the negative) from readers.

More recently, I have been evaluating and weighing the various ways I can and should spend my time. I wrote a recent blog about the various opportunities I have been afforded in the past couple of years, after having risen to the top of academic faculty ranks (2). One thing I did not mention is my role, for the past few years, as an executive committee member in the Subdivision on Chromatography and Separations Chemistry (SCSC), part of the Division of Analytical Chemistry in the American Chemical Society (https://acsanalytical.org/subdivisions/separations/). The current group of officers has charged itself with reinvigorating the subdivision. Efforts have and are being made to increase the visibility of the subdivision, including new initiatives to support and expand dissemination of separation science chemistry research and education. For example, the SCSC is finalizing a partnership with ChromAcademy to promote quality online learning for continued professional development. Stay tuned for more about that.

With my new endeavours taking an increasing amount of my time, and the knowledge that other separation scientists are making their way up the ranks and trying to make a name for themselves, I am pleased to hand over the *LCGC* blog to the SCSC. The *LCGC* blog has been a valuable tool for me, and I think others should have the opportunity to use it to their benefit, as well.

That said, the real benefit will come to us all, as we will be able to view much wider perspectives from leading scientists throughout the world. For example, your next instalment of the blog will come from Assistant Professor Katelynn Perrault at Chaminade University of Honolulu in Hawaii. She is an innovator in the use of comprehensive two-dimensional gas chromatography (GC×GC) for forensics applications, among others. She is also a recent recipient of the 2020 Satinder Ahuja Award for Young Investigators in Separation Science, an award the SCSC has a significant stake in conferring to rising stars. Month by month, new perspectives and topics will be discussed by a host of fantastic scientists. And perhaps every now and then, I will jump back in and provide new perspectives of my own.

Thank you to those who have been casual or faithful readers of my blog. I consider it a privilege to have been able to take up a little of your time each month, and I hope that my insights have been useful and interesting to you. Thanks also to *LCGC* for affording me this exceptional opportunity, and to my students and colleagues for providing me plenty of excellent material to cover. I am confident that the future brings many new and exciting advances to come, and the *LCGC* blog will always be one good place to read about them.

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Chromatographic Innovations for Cell Therapies

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The complexity and challenges of developing, manufacturing, and controlling cell therapies offer today's chromatographic scientists new opportunities to join the journey of discovery and innovation needed to develop and commercialize this new drug modality. This article explores the latest developments and highlights the importance of new capillary electrophoresis–mass spectrometry (CE–MS)-based approaches to understand the efficiency of cell therapy production.

In the opening address of the 8th International HPLC Symposium in 1984, Professor Csaba Horvath emphasized that advances in high performance liquid chromatography (HPLC) were contributing to great gains in life sciences and would play a key role in the future (1). His vision was remarkably prescient because at that time the biopharmaceutical industry was just beginning to manufacture small protein therapeutics such as insulin and growth hormones using genetically engineered microbial expression systems. Over the last three decades, we have witnessed tremendous growth in biopharmaceutical therapeutics, extending from small proteins to large antibodies that have greater power to modulate their biological targets. Innovations from the chromatographic sciences field, such as in the form of very efficient large-scale purification or high-resolution and high-throughput analytical tools, have been instrumental to this revolution.

Today, cell therapies are at the forefront of late-line haematological cancer treatments, and they may offer promise against many

other diseases as well. In immuno-oncology, biopharmaceutical companies are using different types of cells and genetic editing techniques to produce cellular "living drugs" that are designed to enhance the immune system, such as chimeric antigen receptor T (CAR-T) cells designed to eradicate specific cancers. The development of these new products has been spurred by advances in multiple scientific and biomedical disciplines. The complexity and challenges of developing, manufacturing, and controlling these cell therapies offer today's chromatographic scientists new opportunities to join the journey of discovery and innovation needed in the development and commercialization of this new drug modality.

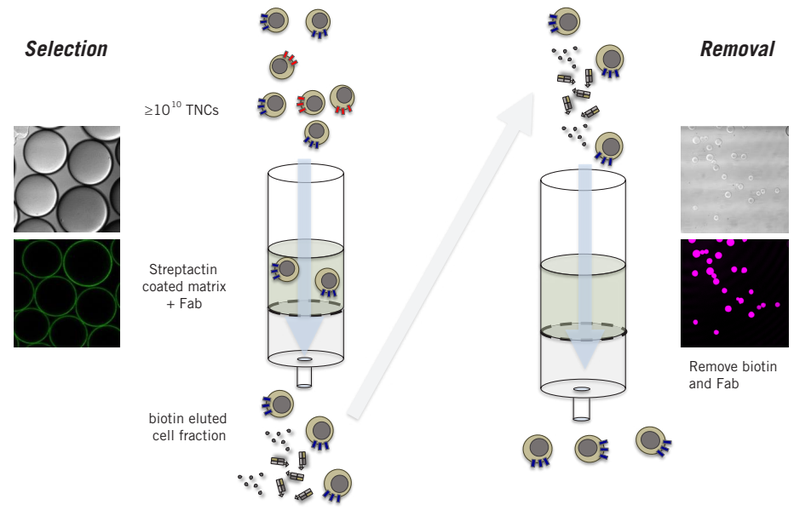
Chromatographic Innovations for Cell Separation

The manufacture of CAR-T drug products involves, in some cases, selection of T cells from the patient's complex starting apheresis materials, activation and transduction with vectors for genetically programming those cells to recognize and attack the tumour cells, followed by cell expansion, formulation, and cryopreservation before re-infusion into the same patient (2). To enable initial selection of the desired cells as biologically optimal starting cells, affinity chromatographic separation methods are being developed (Figure 1) (3). These chromatography technologies offer the possibility to achieve high throughput enrichment of the desired cells, and seamless integration of the upstream and downstream cell manufacturing processes with a high degree of automation.

The ability to select and enrich T cells with certain phenotypic and functional attributes is important at several stages in the multistep manufacturing of complex cell products. Up-front selection of target cells reduces the complexity of patient starting materials and contributes toward a robust and consistent material for further downstream processing with fewer cell contaminants. Additionally, purification of desired individual cell populations from among the modified cells prior to final formulation, especially in the context of current sophisticated gene editing and engineering approaches, has the potential to refine the final drug product. The original high functionality of purified cells is further preserved by design of appropriate chromatographic selection technologies that allow quantitative removal of residual reagents or byproducts from cells (Figure 1).

Cell chromatographic purification technology and instrumentation, in combination with next-generation bioengineering approaches, provide an attractive integrated platform with many benefits. Such platforms enable a fully closed system with automation that could improve the turnaround time and reduce manufacturing costs.

Figure 1: Schematic overview of an automated cell-affinity chromatographic selection workflow. Target cells with specific markers are selected from heterogenous blood products by cell-affinity chromatography. Cells are bound reversibly by interaction between multimerized low-affinity Fab-fragments off the column matrix. Residuals (D-biotin and Fab-fragments) are removed from target cells by single passage over an absorber column. Microscopic images illustrate specific properties of selection and removal matrices, which confer resin functions: binding properties on matrix surface for selection versus inside the matrix void for removal.



Chromatographic Innovations for Analytical Methods

Analytical techniques that are typically used for product characterization and release testing of cellular therapies include flow cytometry to monitor the phenotypes of cells, and molecular analytics to confirm their genetic modification. Sample digestion and treatments that reduce sample complexity could allow more specific analysis of this new product modality by traditional chromatography. Further improved high-resolution methods may enhance the robustness and consistency of ancillary materials, such as complex culture media and various viral vectors that comprise the nascent manufacturing

supply chains and are often considered critical components. For example, analytical methods using monolith columns have been developed to resolve empty from full viral capsids of adeno-associated virus (AAV) (4,5). Such a chromatographic approach may offer increased speed and improved quality control.

Gene editing is emerging as a potentially important tool to achieve desired DNA rearrangements in cellular products. Nearly 20 years ago, microscale separation for DNA such as capillary electrophoresis (CE) was instrumental in decoding the human genome. Today, CE remains a versatile genetic and molecular analysis tool with diverse applications. CE methods have been developed for profiling indels (insertions or deletions) that arise from clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated genetic editing (6). The single base pair resolution of polymerase chain reactions (PCR) amplicons by CE enables discrimination between different types of genetic modifications, and throughput can be improved in the capillary array format. Improved CE-based approaches for monitoring critical genetic modifications and other raw materials could further facilitate the development of gene-edited cellular products.

Cell expansion requires an optimal growth environment where the composition of metabolites, media components, and growth factors can significantly impact cellular phenotypes, proliferation, and differentiation. The introduction of microfluidic based CE–mass spectrometry (CE–MS) devices has allowed rapid quantification of amino acids and potentially may quantify other media components much faster than conventional HPLC-based analytics (7). The speed and throughput of new CE–MS based approaches may improve the process understanding and efficiency of cell therapy production.

Conclusions

Improving the speed and power of separations through innovation in chromatography contributes to the development of cell therapy products. In general, high resolution and high throughput preparative and analytical technologies tailored to the unique chemical and molecular properties of genetically modified cells, vectors, and their process matrices are highly desirable. Multidisciplinary, cross-functional collaborations between scientists in biopharmaceutical companies, analytical instrument companies, and academia may help to bring new and elegant separation solutions to address the challenges in the manufacture and control of cellular products. Although much has changed since 1984, the relative importance of new innovations remains constant. Along with the continuing advances in biology and engineering sciences, chromatographic innovations will help deliver the promise of new cell therapies to patients.

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