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SPECIAL ANNIVERSARY FEATURE

THE STATE OF THE ART

Separation Science: The State of the Art
In this extended special feature to celebrate the 35th anniversary edition of LCGC Europe, key opinion leaders from the separation science community explore contemporary trends in separation science and identify possible future developments.

PEER REVIEW

Method Development for Reversed-Phase Separations of Peptides: A Rational Screening Strategy for Column and Mobile Phase Combinations with Complementary Selectivity
Jennifer K. Field, James Bruce, Stephan Buckenmaier, Ming Yui Cheung, Melvin R. Euerby, Kim F. Haselmann, Jesper F. Lau, Dwight Stoll, Maria Sylvester, Henning Thøgersen, and Patrik Petersson
This review article summarizes the results obtained from the combined efforts of a joint academic and industrial initiative to solve the real-life challenge of determining low levels of peptide-related impurities in the presence of the related biologically-active peptide at a high concentration.

COLUMNS

LIQUID CHROMATOGRAPHY

Pitfalls in Proteomics: Avoiding Problems That Can Occur Before Data Acquisition Begins
Daniel Meston and Dwight R. Stoll
Knowing the tips and tricks of producing quality LC-MS data for peptide analysis can help streamline troubleshooting when problems occur.
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**Constantly Evolving**

*LCGC Europe* is celebrating a rich heritage of innovation in its 35th year and is looking forward to a glorious future supporting the chromatography community with content that matters.

*LCGC Europe* was launched as a humble print magazine in 1987 and has evolved into the leading multimedia platform to support, inform, and educate the separation science community in 2022—and beyond.

The science publishing landscape has changed beyond belief in this time frame, but our audience can rely on *LCGC Europe* to constantly evolve and innovate in multimedia publishing to provide the audience with unbiased educational content, trusted troubleshooting advice, insights from our world-renowned columnists, and best-practice application solutions. The ultimate aim is to keep the chromatography community informed of the latest trends in separation science, advances in the technologies used by chromatographers, innovative analytical approaches, and practical information that can help users perform their jobs more effectively.

Virtual symposiums will never totally replace the experience and value of face-to-face contact at live events, but digital events can provide valuable information without too much time away from the office. This year we organized a series of successful virtual symposiums on *Advances in Gas Chromatography; Advances in Food Analysis; Advances in Biopharmaceutical Analysis* in collaboration with the Research Institute for Chromatography (RIC), Belgium, and the University of Geneva, Switzerland; and *Challenges in Small Molecule Analysis* in collaboration with The Chromatographic Society (ChromSoc).

The ChromSoc collaboration has been fruitful in terms of valuable virtual symposium content, and allows these top-notch, high-profile UK-based live events to be broadcast to a wider audience globally. Next year we will be collaborating with ChromSoc on virtual symposiums covering the hot topics of *Sustainability in Pharmaceutical Analysis, Advances in Gas Chromatography, and Emerging Trends in Liquid Chromatography*. Join our mailing list to keep track of the symposiums *LCGC Europe* and *LCGC North America* are organizing globally in 2023.

*LCGC's* global digital publication, *The Column*, is published monthly and provides readers with content not available in the *LCGC Europe* magazine. Subscription is quick and easy at: [bit.ly/3tbLnOh](https://bit.ly/3tbLnOh). This year we launched the popular interview series *Rising Stars of Separation Science* to celebrate the work of early career researchers. We are looking for fresh nominations for 2023. The entry requirements and nomination forms can be found here: [bit.ly/3UFaS61](https://bit.ly/3UFaS61)

CHROMacademy, produced by *LCGC* and Element, is one of the world’s largest e-learning platforms for analytical chemists, with a mission to elevate practical and theoretical expertise in chromatography amongst younger scientists embarking on their careers and older scientists who could do with brushing up their skills. Find out more at [www.chromacademy.com](http://www.chromacademy.com)

*LCGC* was also a pioneer in promoting and producing educational and sponsored webinars presented by the cream of the crop from the chromatography community and next year will be no different. Find out what is on the horizon from our website [www.chromatographyonline.com](http://www.chromatographyonline.com)

Our passion for innovation led to the launch of the *LCGC Europe-HTC Innovation Award*. This year the award went to Szabolcs Fekete from Waters for his innovative research to develop a complete package of novel approaches to push intact protein and protein subunit chromatography to the next level, including the so-called “ultra-short column”, “multi-isocratic”, “negative gradient segment”, and “on-column fractioning” approaches. Look out for the interview next year with Szabolcs to learn how this technology has been used in the fight against Covid-19.

The sales and editorial team on *LCGC Europe* and *LCGC North America* are dedicated to keeping abreast of novel trends in chromatography and multimedia publishing, and we believe we are leading the way for editorial quality as well as marketing opportunities for leading vendors. We would like to thank all the companies who have supported our print and digital portfolio, our readers, and the esteemed *LCGC* editorial advisory board (EAB), for their continued advice and support.

There is a lot going on in chromatography in 2022, and *LCGC Europe* aims to be the first port of call and a “one-stop shop” information resource for practising chromatographers. We are proud of our past, but we have our eyes firmly fixed on the future.
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November/December Update

Welcome to our special 35th anniversary issue of LCGC Europe! To celebrate 35 years providing top-quality and industry-leading print and digital content to chromatographers, we asked leading luminaries from the world of separation science to reveal the state-of-the-art trends in their areas of expertise, and identify possible future developments. Read on for some valuable insights into the cutting-edge of chromatography.

In our peer-review article this month, the combined efforts of a joint academic and industrial initiative to solve the real-life challenge of determining low levels of peptide-related impurities in the presence of the related biologically-active peptide at a high concentration is revealed.

In LC Troubleshooting, Dwight Stoll and guest author Daniel Meston explain how to avoid common pitfalls that can lead to poor quality data when performing proteomics measurements by liquid chromatography–mass spectrometry (LC–MS).

GC Connections looks back on the history of the electron capture detector (ECD) for gas chromatography (GC). We also look at the life and work of the inventor of the ECD, James Lovelock, who died very recently. He was one of the earliest scientists to bring attention to climate change through Gaia, which explained his theory that the Earth acts as a massive living organism.

Here’s to the next 35 years of innovation!

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

LCGC is a multimedia platform that helps chromatographers keep up to date with the latest trends and developments in separation science, and supports them to perform more effectively in the workplace. Keep updated with our multimedia content by visiting the global website (www.chromatographyonline.com), subscribing to our newsletters, and attending our wide range of educational virtual symposiums and webinars.

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Selected highlights of digital content from LCGC Europe and The Column.

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VIRTUAL SYMPOSIUM
Biopharmaceutical Analysis: The State Of The Art—A Virtual Symposium
Learn about the latest cutting-edge advances in biopharmaceutical analysis in “the real world”.

INTERVIEW
Building Small But Thinking Big
The Column spoke to Brett Paul from the University of Tasmania about the development of truly portable LC systems for use in the pharmaceutical industry and in environmental analysis. Why are they necessary?

E-BOOK
Cannabinoids in Everyday Products: Keys to Successful Laboratory Testing
Tips on how to ensure laboratory compliance in your cannabis testing lab

RISING STAR
Rising Stars of Separation Science: Jessica Pandohee
This month we interviewed Jessica Pandohee about her work in foodomics in prebiotics research for gut health, and the role of GC–O in aroma analysis for coffee-flavoured milk.
Samples containing ionizable compounds are analyzed using buffers, and this can be one of the most important variables controlling retention in a high performance liquid chromatography (HPLC) separation. The pH of the mobile phase determines the presence of ionizable substances, such as analytes and matrix compounds, to be in either an ionized or non-ionized state. In reversed-phase chromatography, ionized species always elute from the column earlier than the non-ionized form. Changing the pH can also improve the selectivity for effective separation of closely eluting or overlapping peaks.

Buffers prevent pH variations and minimize run-to-run variability. **Buffer Selection:** The choice of the appropriate buffer for an application is governed by the buffer characteristics such as $pK_a$, pH range, and UV transparency. As a rule, the buffers should be used for a pH within $\pm 1$ unit of their $pK_a$ value. Within this range, the buffers resist any change in pH. The buffer’s capacity is at its maximum when the pH of the buffer is equal to its $pK_a$. For best results with an ionizable analyte of interest, use a buffer with a pH at least 2 units away from the $pK_a$ of the analyte. For several ionizable analytes of interest, it is preferable to choose a pH value wherein all the analytes exist in the same form—either ionized or non-ionized.

**Measuring Buffer pH:** The pH of the buffer is the pH of the aqueous portion before the organic mobile phase part is added. The addition of an organic solvent normally shifts the pH either up or down and this is consistent for the same buffer. It is not so important to know the exact pH value of the buffer in an organic medium, but it is important to have a consistent pH value because the $pK_a$ of your analyte is also determined in aqueous phase, and the individual $pK_a$ shifts in the final mobile phase are not known.

**Chemical Purity:** The quality/purity of mobile phase additives (buffers, salts, acids, and bases) along with organic solvents utilized in a HPLC experiment must be adapted to the detector sensitivity and elution protocol. In general, compromising purity results in increased baseline noise, ghost peaks, and diminished column lifetime.

**Chemical Compatibility:** Buffer composition, along with mobile phase pH, must be chosen in agreement with instrument, column housing material, and the nature of the stationary phase to prevent corrosion.

**MS Compatibility:** Introducing mineral salts into a mass spectrometer is not advisable and leads to signal suppression, adduct formation, and instrument pollution. Examples of volatile buffers are ammonium acetate, ammonium formate, and ammonium citrate. pH modifiers such as difluoroacetic acid, formic acid, and acetic acid should be used to control pH and help ionization for LC–mass spectrometry (MS).

**Buffer Solubility:** Ideally, the buffer should be completely water-soluble (reversed-phase methods) and should not precipitate during the analysis when mixed with a chosen organic solvent. Buffer concentration must be carefully chosen to avoid precipitation at higher concentrations in the organic solvent; this is particularly the case when using acetonitrile or tetrahydrofuran, as both have limited buffer solubility. If neglected, this can create operational problems with the pumps and instigate HPLC column blockage or back pressure rise.

**Buffer Concentration:** Ideally, the lowest concentration that gives reproducible results should be chosen. Higher concentrations lead to a faster elution of polar molecules. Generally, the buffer concentration should not be lower than 5 mM. Below this concentration, it may not perform as a buffer (this depends on analyte concentration and its buffering capability). Raising the buffer concentration can increase viscosity, which in turn can increase column back pressure. Commonly, the concentration should be kept in the 5 to 100 mM range. A concentration higher than 100 mM of mineral salt buffers wears out the pump’s movable parts faster; therefore, a back-seal wash is recommended to be installed.

Buffers play a crucial role in the majority of HPLC separations. Method development often requires careful selection of buffers and adequate care in their preparation. In conclusion, buffer solutions must be homogeneous, clear, high purity, and free from any particles. If stored, keep in mind that buffers have a limited lifetime, so consider their preparation daily.
Graph Neural Networks for Improved Retention Time Predictions

Alexander Kensert
Gert Desmet
Deirdre Cabooter

To speed up the optimization of chromatographic separations—a process often referred to as method development—models that aim to predict the retention time or retention factor of a compound are frequently used. These models can be empirical or mechanistic in nature, and predict the retention of a compound based on one or more chromatographic parameters, such as the fraction of organic modifier in the mobile phase, is varied a number of times—a number that is typically equal to or higher than the number of parameters in the retention model. Thereafter, a retention model is built by relating the experimentally obtained retention factors to the chromatographic parameter. Well-known examples of such retention models are the linear solvent strength model (2) and the Neue-Kuss model (3). An advantage of this approach is that (structural) information about the compound under consideration is not required. A disadvantage is that multiple informative scouting runs for each compound need to be executed before the model can be built and used, which takes time.

Alternatively, when structural information about the compound under consideration is available, quantitative structure retention relations (QSSR) can be used for retention time or retention factor predictions (4). QSSR are mathematical relations between the retention time of a compound and its structural features. Typically, QSSR are built for a specific chromatographic setup, that is, a fixed combination of stationary phase and mobile phase conditions for a large number of compounds with known structures. The obtained retention times are then correlated with the compounds’ structural features or descriptors to build a retention model. Once the model is available, the retention times of new compounds can be predicted without having to run any new experiments, significantly decreasing the time required to optimize separations. However, before the model becomes available, the retention times of typically at least 50–100 compounds on the particular chromatographic setup are required, while another prerequisite is that the structure of the compound should be known.

To build adequate QSSR, structural features or descriptors that adequately represent the interactions between the compounds under consideration and the chromatographic conditions need to be selected. These descriptors can be physicochemical, quantum mechanical, or topological in nature. Examples include molecular mass, carbon number, polarizability, and (calculated) partition coefficient, although thousands of possible candidates exist (5). Once suitable features have been selected, they are mapped against the experimental retention times or retention factors using regression models or machine learning algorithms, such as multi-layer perceptrons (MLP), random
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complex relations between atoms or short and longer distances, leading atoms via a set of learnable weight and aggregation step to incorporate subsequently undergo a transformation adjacent atoms, to obtain optimized and atoms themselves and their bonds to information about their neighbouring approaches are graph neural networks (radii). This allows the GNN to learn GNNs use information about the numerical. These atom encodings can be obtained by computing the partial derivative of the retention time with respect to the feature matrices. As each row in the feature matrix corresponds to an atom, these gradient activation maps can trivially be superimposed on the molecule. The more intense the green colouring, the more important a substructure is for retention time prediction.

More recent additions to these approaches are graph neural networks (GNNs). As opposed to the more traditional machine learning algorithms, GNNs use information about the atoms themselves and their bonds to adjacent atoms, to obtain optimized and meaningful molecular representations for retention time predictions. This is done by first encoding the atoms numerically. These atom encodings subsequently undergo a transformation and aggregation step to incorporate information about their neighbouring atoms via a set of learnable weight matrices. This is repeated a number of times (layers) for increasing distances (radii). This allows the GNN to learn complex relations between atoms or molecular sub-structures over both short and longer distances, leading to powerful and meaningful molecular representations. The obtained atom encodings are finally reduced into one-dimensional vector representations of the molecules, which are then inputted to a regression model (such as MLP) to produce retention time predictions. A simplified illustration of a GNN is shown in Figure 1(a).

We recently demonstrated the potential of GNNs for retention time predictions in different separation modes (reversed-phase and hydrophilic interaction liquid chromatography) (7). When compared with more traditional approaches, such as MLP, RF, and SVM, GNNs generally performed better than the traditional approaches, with mean absolute errors (MAEs) between the experimental and predicted retention times that were typically 5–25% lower than those obtained with the traditional models, depending on the dataset. This was attributed to the fact that GNNs result in a more expressive and discriminative molecular representation for retention time predictions, due to the learnable molecular representation based on the large number of atom and bond features. It was, however, also observed that GNNs in some cases suffer from some unusually high prediction errors. This was attributed to a possible overfitting of the GNNs to (some of) the training data, which could be explained by the large number of weights that need to be trained at each layer of the GNN. In future research, this could be tackled by adjusting the number of learnable weights or increasing the size of the datasets.

To better understand what molecular substructures contribute to the retention time predictions, so-called gradient activation maps (GAMs) can be computed from the GNNs (8). Visualizing these GAMs can help understand to what part of the molecule the model is “looking” when it makes its retention time prediction and as such help understand what substructures contribute to retention under particular chromatographic conditions. For an example, see Figure 1(b).

In conclusion, GNNs show great promise as new machine learning tools for retention time predictions using QSRR and certainly deserve further exploration. Researchers who are interested in creating their own GNNs for retention time predictions of small molecules are welcomed to use the recently developed open source Python package MolGraph, which can be installed from: https://github.com/akensert/molgraph

References

**The Paradox of Sustainability in Separation Science**

Paul Ferguson

It has become increasingly clear in recent years that climate change has accelerated rapidly and poses an imminent threat to the global ecosystem. At the heart of this issue is the dramatic increases in greenhouse gases—carbon dioxide being the most prominent of these. While it may feel like a long stretch to suggest separation science is a significant contributor to this issue, some simple modifications to the way these measurements are approached can enable significant reductions in the waste generated and carbon dioxide produced during analyses.

As a starting point in discussing sustainability, Anastas and Warner’s seminal 12 rules for green chemistry can be considered as a substrate for analytical chemistry (1). Written in 1998, these rules provide a framework on how to improve the sustainability of various aspects of synthetic chemistry. While this cannot be discussed at length in this article, the concepts detailed in the chapter on reducing volumes (both organic solvents and water), using safer and more sustainable solvents, and increasing instrument efficiency are all within the scope of this discussion (see also Figure 1).

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Let us start by considering how we know if our separation science method is “green”. A key focus area for chromatographic methods and sustainability is the organic solvents used in the mobile phases, as these can be a source of carbon dioxide in their synthesis and also in their destruction (typically incineration) after use as waste. There have been several approaches proposed for objective assessment of method greenness, for example, references 2 and 3. A recent example of this is the “analytical method greenness score” (AMGS) produced by the American Chemical Society Green Chemistry Institute (ACS GCI) (4). The AMGS tool provides a “traffic-light” scoring approach for liquid chromatography (LC) and supercritical fluid chromatography (SFC) methods based on (i) the energy an instrument uses (the analysis time of method and number of samples), (ii) the volume of solvent used (in the chromatographic method and sample preparation), and (iii) safety considerations for the solvent. The tool also highlights the aspects that are detrimentally impacting this score in these three areas. A free to access web interface has been produced to generate these metrics (www.acsgcipr.org/amgs/), and future updates are planned to include gas chromatography (GC) methods.

When discussing organic solvent volumes, four areas should be considered:

1. Reduction: Can a separation be scaled to smaller volumetric flow dimensions without impacting the separation? This will reduce the amount of organic solvent and high purity water used (access to the latter can be limited in some geographic locations).

2. Replacement: Can environmentally hazardous chemicals be replaced with more sustainable or safer options without impacting the measurement? For example, the replacement of acetonitrile with ethanol or acetone.

3. Removal: Can organic solvents be completely removed from the chromatographic method? For example, could techniques such as capillary electrophoresis or ion chromatography (IC) be used instead of reversed-phase chromatography?

4. Recycle: Mobile phases can also be recycled while running isocratic chromatographic methods. While difficult, is it possible to recover certain solvents from the sample diluent or unused/waste mobile phase, for example, ethanol or dimethyl sulfoxide (DMSO)?

In terms of point 1, the move to smaller chromatographic particle sizes in reduced volume column dimensions is an easily accessible and impactful approach. The use of ultrahigh-pressure liquid chromatography (UHPLC) instead of high performance liquid chromatography (HPLC) is a common example of this. Preserving the $L/d_p$ (column length/particle size) ratio to maintain efficiency (ensuring a scalable stationary phase is used with identical ligand and bonding density to preserve relative retention and selectivity) and volumetric flow scaling (many online tools are available to assist with this) can significantly reduce solvent use when moving to smaller column and stationary phase dimensions. It is not uncommon when moving from HPLC- to UHPLC-scale conditions to realize mobile phase savings in excess of 60%. Detrimentally, it is energetically easier to incinerate mobile phases rich in organic solvents than it is to incinerate those rich in water, but it is difficult to quantify this aspect of the mobile phase life cycle.

From a sustainability perspective, it is also important to minimize the number of experiments conducted during the development of a method. The use of in-silico modelling and prediction of analyte retention is very important to enable this. Once the column and mobile phase conditions are established (often through screening, or more recently, prediction of initial conditions based on analyte structure [5]), methods can often be optimized quickly in as few as two to four experiments (6).

The replacement of mobile phase solvents (point 2) can be challenging if a chromatographic method is already established and selectivity needs to be maintained. However, if developing a new method, then the use of methanol, ethanol, or even acetone (not all methods require UV detection!) could be utilized rather than acetonitrile (with ethanol, slightly increased column temperatures may be required to reduce mobile phase viscosity, which will impact system pressure and the usable flow rates). More sustainable and/or safer organic solvents can be chosen from the literature published by process chemists, for example, references 7 and 8.

The use of normal phase chromatography should be avoided whenever possible due to the high volumes of toxic or...
environmentally impactful organic solvents used. Normal phase chromatography was historically used for preparative-scale isolations, chiral separations, or retention of polar molecules. For the latter, hydrophilic interaction liquid chromatography (HILIC) has established itself as a key technique for the analysis of small and biomolecule (for example, glycan) separations. However, the main component of mobile phases used in this technique is typically acetonitrile, often in the order of 95% v/v, which produces significant volumes of organic waste. A greener technique that addresses these three areas is SFC. This technique uses carbon dioxide as the primary component in the mobile phase and typically methanol as co-solvent (in volumes <50% v/v). While carbon dioxide is environmentally detrimental, if the gas is produced from a sustainable source, for example, recycled from the atmosphere, or possibly as a by-product from chemical reactions, then the technique is unquestionably “greener” than many other chromatographic approaches.

While not fully addressing point 3, SFC provides reduced volumes of liquid waste. Alternatively, the use of capillary zone electrophoresis (CZE), or one of its numerous modes, all produce dramatically less waste, while providing a potentially beneficial and complementary separation methodology to chromatographic approaches. However, CE does have limitations, most notably detection sensitivity. Alongside this, the use of IC, which utilizes predominantly aqueous mobile phases, could provide a greener approach to analyzing certain classes of compounds.

Another area for consideration is sample preparation. As more efforts are made to reduce solvent volumes in liquid separations, then sample diluent volumes move further into focus. It is important that analysts have a fundamental understanding of key physicochemical parameters such as analyte pK_a and Log P/D to optimize analyte solubility, alongside understanding of efficient instrumental processes to minimize the proportions of organic solvent used and the absolute volumes of the samples prepared (9). The use of automation to provide reproducible small volume sample preparation will be a key focus area in the future. Equally, the ability to analyze samples without any sample preparation using online analysis approaches (for example, online LC or vibrational spectroscopy such as near infra-red) is another important future direction.

Ultimately, the use of LC as a measurement approach is broadly not green, and, arguably, spectroscopic or spectrometric approaches can provide more sustainable measurements. However, as chromatography often provides the optimal approach to generate measurement data—for example, due to reduced sample matrix interferences—the approaches outlined above can all be considered to reduce the environmental impact of these techniques. While the impact of reducing the environmental burden of separation science is modest compared to the aviation industry for example, I am reminded of the concept of “aggregation of marginal gains” (10). If multiple industries using separation science in any form can reduce their environmental footprint, then these small numbers can become significant and create a positive and sustainable impact.

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How Mature is Gas Chromatography?
An Industry Perspective

Frank David
Pat Sandra

From Theoretical Concepts to an Industry Standard
Capillary gas chromatography (CGC) was introduced about 60 years ago and has evolved through many developmental milestones into an indispensable tool in many analytical laboratories. The authors had the privilege to witness and to actively participate in the growth of CGC technology up to its current state-of-the-art. It has been an exciting and very rewarding journey to see how CGC-based analytical methods have contributed to the better understanding of industrial processes and products, to quality control in foods, pharmaceuticals, and consumer products, to environmental and health protection, and to the discovery of chemicals that are key
markers for aroma or diseases, to name just a few applications of CGC.

Although the fundamentals of CGC have been described by Marcel Golay in the late 1950s, GC using initially metal and later glass capillary columns was not sufficiently robust to be applied in an industrial environment, and GC using packed columns remained the standard until the introduction of fused silica columns at the end of the 1970s. At the University of Ghent in Belgium, we have contributed to capillary GC column technology by the development and optimization of stationary phase coatings, of stationary phase cross-linking and immobilization, through the synthesis of new stationary phases and to the development and evaluation of GC instrumentation, including dedicated CGC inlets (split/splitless, cool on-column and programmed temperature vaporization) and CGC detectors. Together with many academic and industrial research groups, this has led to high performance GC instrumentation and columns. In our opinion, important milestones were the development and introduction of dedicated instrumentation, for example, by Carlo Erba in Europe and by Hewlett Packard in the US. In the mid-1990s, CGC instrumentation became available with state-of-the-art inlets and detectors, with electronic pressure/flow control, on-board diagnostics, and options such as retention time locking and method translation calculators, as could be found in the HP 6890. At RIC, we immediately could benefit from such outstanding performance, as illustrated by detailed hydrocarbon analysis that could be used for fuel control in Formula 1 racing (“the 1995 Schumacher case”), and by high-throughput polychlorinated biphenyl (PCB) analysis, applied at RIC during the Belgian dioxin (PCB) crisis in 1999.

**Into the New Millennium**

Since the new millennium, the revolution in GC technology has changed into an evolution. New GC instruments have further improved in terms of user-friendliness and robustness. Tools are incorporated that increase productivity and reduce downtime. For instance, capillary flow technology facilitates the coupling of retention gaps and columns, allows column back-flushing, enabling effluent splitting to multiple detectors and making multidimensional GC applicable in industry and routine laboratories.

Today, CGC is often considered a “mature” technique. In our opinion, this is correct, but it does not mean that the technique is in decline or that no further progress can be made. Mature stands for high performance, robustness, and wide applicability in a routine and industrial environment.

Parallel with the evolution of CGC equipment, we have encountered a spectacular revolution in mass spectrometers that can be hyphenated to CGC. Note that due to the nature of the gaseous mobile phase, the commercialization of CGC–mass spectrometry (MS) systems was much faster compared with other chromatographic techniques! Next to extremely robust and sensitive single quadrupole systems, laboratories have access to triple quadrupole MS, time-of-flight (TOF-) MS, quadrupole TOF-MS, and orbital trap MS instrumentation, depending on their applications. This has resulted in a trend away from mostly target compound analysis, as known from classical regulatory methods, for example, EPA, towards untargeted screening, as applied in the various “omics” approaches (metabolomics, foodomics, volatolomics).

An important consequence of this revolution is that one might put all trust in the mass spectrometer and “downgrade” the GC system to some kind of “MS-inlet”. The power of high-end mass spectrometers can, in our opinion, only be fully exploited when combined with a high-performance CGC separation (inert flow path, correct sample introduction, correct CGC parameters). Although not the topic of this article, we would like to mention that this is also valid for the selection of the sample preparation procedure: the better the sample preparation is, the better the MS data will be. A great mass spectrometer will not work properly if the ion source is overloaded with dirt!

**The Heart of the GC: The GC Column**

The (r)evolution in sensitivities that can be obtained with state-of-the art CGC–MS equipment has also had an important influence on the development of capillary columns. In the 1980–90s, a wide range of stationary phases was developed for CGC, with the focus on selectivity. Dedicated columns became available for specific applications, such as volatile organic compounds (“624”, “VRX”-type columns), PCBs, and polycyclic aromatic hydrocarbons (PAHs). In combination with the increasing sensitivity of the detector, other column criteria become more important. New developments in CGC column technology should therefore keep this as the focus. If the MS detector is capable of “deconvoluting” solutes, chromatographic resolution becomes less important, while inertness and column bleeding are essential for trace analysis. This also means that column testing should be done in a more critical way—not only by injecting a classical test mixture at 1000 ng/µL using a 1:100 split injection. Remember that the most critical test is your sample.

Obviously, in case of isomer speciation, MS cannot provide a solution and the CGC separation is crucial. To this, there is still room for development of “special” columns that have complementary selectivities, for instance, based on shape...
selectivity, chirality, or specific interaction with functional groups. Research on new stationary phases such as (polymeric) ionic liquids or calix-arenes looks very promising in this respect.

**The GC×GC Case**
Comprehensive GC (or GC×GC) is often considered as the most important milestone in the evolution of gas chromatography since the 1990s. The technique was developed and the first concepts were demonstrated more than 25 years ago. Despite hundreds of publications, books, and dedicated symposia, the implementation of GC×GC instrumentation and methods in industrial laboratories is rather limited, with the exception of the petrochemical industry. We see two reasons here. First, further improvements in hardware and (mainly) software are required to bring the equipment to a more user-friendly level, to offer a reliable and transparent workflow from injection to report. In addition, the technique should be correctly positioned by protagonists of the technique and not applied to analyses that qualitatively and quantitatively can be performed perfectly well using classical one-dimensional GC. GC×GC is not at all a replacement for 1D-GC or GC–MS; it should complement it and be applied to those applications that require sample imaging, group type separation, and significantly higher peak capacity. We encountered a similar situation when (capillary) SFC was introduced. Once it was believed that cSFC would replace both GC and liquid chromatography (LC). Research in GC×GC should therefore not make the same mistake. The technique has a clear potential, can deliver great results in several applications, but it should only be applied when needed.

**The Future of GC**
We are convinced that CGC technology will further evolve. This evolution should focus on further improvement of productivity, usability, energy, gas selection, and consumption. New column heating technologies have been applied in low thermal mass (LTM, resistive heated column) and the recent CGC platforms. These approaches drastically reduce energy consumption, but users need to adapt to the limitations, such as the fact that column trimming is no longer possible and columns are more expensive.

In the past decades, academic research has explored many ways to miniaturize GC instruments, ultimately to chip-GC formats. Micro-GC systems are commercially available and successfully applied in gas analysis (separation of permanent gases), but for applications in other fields major problems are encountered. These often relate to underestimation of the complexity of sample introduction in GC (called the Achilles’ heel of GC), the role of solute capacity, and the required peak capacity. Demonstration of a micro-GC separation using a...
The first use of the term “lipidomic(s)” was for higher productivity, lower costs, miniaturization, and trends towards greener technologies. CGC is currently being applied in more and more industries, beyond classical “home-grounds” such as petrochemical, environmental, and food safety application areas.

A Mature Technique for a Broader Range of Application

To us, CGC, CGC–MS, and GC×GC all have a bright future. Technology will evolve, catalyzed by the need for higher productivity, lower costs, miniaturization, and trends towards greener technologies. CGC is currently being applied in more and more industries, beyond classical “home-grounds” such as petrochemical, environmental, and food safety application areas.

There is still a wide range of potential applications to be discovered in life sciences, product recycling, new energy supplies (characterization of batteries), and more.

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New Frontiers in Lipidomics

Michal Holcapek

The first use of the term “lipidomic(s)” was in 2002, and up until now, almost 10,000 papers on lipidomics have been published (Figure 1), which clearly illustrates that lipidomics is probably the fastest growing omics discipline at present. However, this fast growth is also accompanied by some negative issues. Many newcomers and beginners are entering the lipidomic field, often without adequate knowledge of basic analytical methodology, nomenclature, and data reporting, which can cause confusion in data interpretation and reporting (1).

Recently, the International Lipidomics Society (ILS) has been established with the goal of harmonizing the field, unifying the nomenclature by updating shorthand annotations in cooperation with the LIPID MAPS consortium (2), and introducing a checklist system for transparent reporting of lipidomic data (3). The first Gordon Research Conference on Lipidomics was organized in August 2022 by John Bowden and Kim Ekroos. A smart example of the recognition of lipidomic research is the fact that three distinguished lipidomic researchers (Erin Baker, Shane Ellis, and Livia Eberlin) were awarded prestigious medals at the last IMSC 2022 conference in Maastricht, Netherlands. This is just a brief illustration—and certainly not complete—that the lipidomic community has started to be active in the organization of various events and activities to promote further growth of the field.

The driving force of any research field must be scientific progress in the methodology, which may bring new applications. The lipidomic community pays considerable attention to proper quantitation using appropriate exogenous internal standards (4), including the validation of analytical methods and the use of quality controls (5). The broad consensus is the use of at least one internal standard for each lipid class to be quantified in case of lipid class separation (hydrophilic interaction liquid chromatography [HILIC] and ultrahigh-performance supercritical fluid chromatography [UHPSFC]) or shotgun approaches, but the lipid species separation techniques (reversed-phase ultrahigh-pressure liquid chromatography [UHPLC]) require more. The second important aspect is the lipidomic coverage. Biologists estimate the presence of about 100,000 lipid species in Nature, but the best analytical methods typically report less than 1000, so there is considerable room for improvement in the analytical methodology. However, one method cannot cover this complexity, therefore, we need several complementary analytical methods and sample preparation protocols. Lipidomic analysis is strongly dependent on mass spectrometry (MS) and chromatographic techniques. The main advantage of chromatography is the separation of various isomeric and isobaric lipids, which is sometimes easier than with MS-based approaches. The prevailing technique is UHPLC, but more groups realize the potential of UHPSFC (6), which is perfectly suited for molecules with large hydrophobic parts, such as lipids. From an MS point of view, many researchers appreciate the potential of ultrahigh-resolving power mass spectrometers, typically orbital trap analyzers, and MS imaging for the visualization of tissues. The increasing resolving power in ion mobility has shown its full potential, which may be easily coupled with liquid separation techniques and MS (7). One trend that cannot be overlooked in lipidomic analysis is the shift towards more structural details, such as data on the fatty acyl level or even the double bond level, which could reveal new biological information previously unknown (8).
Multiomics integration of lipidomics with other omics approaches, such as metabolomics, proteomics, transcriptomics, and genomics, is the research direction of the future. Concentrations of lipid species are influenced by the activity of the corresponding enzymes responsible for the particular metabolic pathways involved in the biosynthesis and subsequent degradation of lipids. RNA—both coding and noncoding—shows a correlation with lipidome. The typical example is an alteration of miRNA in cancer. Known genetic mutations have connections to serious human diseases, which is also reflected in the dysregulation of particular metabolic pathways. The integration of multiple omics data is needed for the full understanding of the biological mechanisms of the diseases studied, but such research requires experts for each omics technique and a strong bioinformatic background, which is still in its infancy. I believe that the substantial effort invested in this area will result in fast progress and a better understanding of the biological roles of lipids in disease and health, with possible consequences in biomarker discovery and drug development.

The driving force for progress in lipidomic analysis is biomedical applications and clinical analyses because the translation of key discoveries from academic research laboratories into the real clinical environment could bring substantial benefits to clinicians. Many research groups around the world are working hard to investigate the potential of lipidomics in biomarker discovery applicable to early disease detection, the monitoring of therapeutic response, and helping to select the best possible therapy for a given phenotype, which is known as personalized medicine. The expectations are enormous, but unfortunately, successful clinical translation is rather rare. First, we must harmonize the molar concentrations reported by individual laboratories with their individual workflows, which is the main goal of a running ring trial on human plasma lipidome organized by the Clinical lipidomics Interest Group (CLIG) within ILS. I know of only one example of completed translation from the research laboratory into real clinical use, which is the ceramide test for cardiovascular risk (9) implemented by the Mayo Clinic. At present, the spin-off company created by my university and the FONS company is working on the clinical validation of our lipidomic test for early detection of pancreatic cancer based on blood analysis (10). The methodology is ready to use, with a sample throughput of 20,000 samples/year, but it will still take several years to collect a sufficient number of samples from high-risk subjects to prove the real benefit for the clinical management of cancer patients. It is interesting to note that we observe similar dysregulation patterns for other types of cancer (11). However, the biological mechanism of the observed lipidomic dysregulation has not yet been discovered and will require suitable biological models and a multiomics approach. Another exciting discovery was published by the Fendt group (12) that demonstrated that cancer cells exhibit enormous flexibility, which may result in the desaturation of fatty acyls in unusual positions not common for normal cells. I list here only examples of lipidomic research for cancer and cardiovascular diseases, but there are many examples in the literature about changed metabolic pathways for various lipids in other diseases, such as liver diseases and brain disorders.

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In recent years, multidimensional liquid chromatography (MD–LC) has become a very powerful analytical method for the successful analysis of nonvolatile analytes in complex real-world samples. MD–LC is a powerful alternative technique to conventional one-dimensional (1D)-LC and involves the use of two “orthogonal” liquid separation systems (1). The main advantage of such a technique is linked to the augmented separation power, as a consequence of the increased selectivity and sensitivity of the two systems. In MD–LC, the fractions eluting from the first dimension (1D) are fractionated and re-injected into the second dimension (2D). When only a few selected parts eluting from the 1D are analyzed in the 2D, the approach is regarded as “single” heart-cutting (LC–LC; Figure 1[a]); when two or more selected fractions are diverted into the 2D, the technique is called “multiple heart-cutting” (mLC–LC; Figure 1[b]). In addition, if the whole sample is transferred to the 2D, a full “comprehensive” (LC×LC) approach is accomplished (Figure 1[d]).

Last but not least, if a specific region of the 1D separation is considered for the analysis, a selective LC×LC (sLC×LC) is carried out (Figure 1[c]) (2). Clear advantages of the LC–LC and the LC×LC mode over the 1D-LC counterpart are increased orthogonality (A₀) and peak capacity (nₖ), the possibility to test different retention mechanisms, and better compound organization due to the formation of structured 2D plots; the main disadvantages can be traced back to the instrumental complexity, reduced sensitivity, and need for fast and high flow to employ in 1D separations. Fraction transfer from the 1D to the 2D can be performed either in offline or online mode, in the latter case by the use of a modulator or an interface, which is considered the “heart” of the multidimensional approach. In offline mode, the fractions are manually collected, evaporated, and re-dissolved in a solvent compatible with the 2D mobile phase prior to being re-injected into the 2D. As a consequence, a series of 2D separations, related to the number of fractions from the 1D, are obtained. The simplicity of such an approach avoids the use of any sophisticated modulators or dedicated interfaces; alternatively, this method can bring sample loss or contamination and certainly tedious, time-consuming procedures (3–6).

The capability of the MD–LC technique may be augmented if an MS system is connected to the 2D separations. In fact, this in turn permits unambiguous identification of the compounds, avoiding the need for time-consuming and labor-intensive data interpretation steps.

FIGURE 1: Design of 2D-LC separations. Adapted and reprinted with permission from reference 5.
compound identification in the investigated sample and beneficially allows matrix effects that hamper both quasi-quantitative results to be reduced. Enhanced separations and a decrease of sample complexity prior to MS detection has the advantage of minimizing ion suppression and improving ionization efficiency (8). However, MD–LC does present some pitfalls, for example, length of analysis time, which is normally higher than 1D-LC, and complex equipment with specific data treatment software. Notably, potential immiscibility or incompatibility of the mobile phases in both dimensions have to be considered; in this context, newly developed approaches, for example, fixed solvent modulation (FSM) or active solvent modulation (ASM) have been proposed (9). In this configuration, the implementation of any valve, for example, a 10-port/2-position or 8-port/2-position, normally used for LC×LC, is augmented with a “bypass connector” that establishes a fluidic connection between the 1D pump and 2D column in parallel with the path through the valve. One significant limitation of the FSM approach is that the bypass connection is fixed, and flow will go through both the valve and the bypass connector throughout the analysis. Since the total flow from the 1D pump is split into two paths, the “actual” flow rate through the sample loop is lower than the total flow and dependent on the split ratio. The ASM approach focuses on a new valve-based approach to apply fraction transfer in LC×LC that enables effective focusing of analyte bands at the inlet of the 2D column without the need for any additional instrument hardware. Such an approach offers important advantages over FSM for effective analyte focusing at the 2D column inlet and can be summarized as:

1) More rapid flushing of high solvent strength eluent from the sampling loops at the end of each 1D separation cycle.
2) Less complex mobile phase profiles delivered to the 2D column during gradient elution, which results in less baseline disturbances and a larger practical elution window in each 2D separation.

On the other hand, a practical limitation to the ASM approach is the time it takes to displace the fractions of effluent collected from the 1D column from the sampling loop into the 2D column. Apart from this, it is reasonable to believe the development of ASM represents a significant step forwards in terms of improving the ease-of-use of LC×LC and the overall detection sensitivity of the technique.

On the basis of the above considerations, it is predictable that MD–LC techniques unquestionably present fundamental advancements in terms of increased

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In all areas of analytical chemistry there are obvious advantages to be gained in being able to undertake the analysis at the point of interest or need, and this is particularly so for environmental analysis. Field-portable and long-term “deployable” chromatography systems can provide for robust at-site analysis (monitoring), allow for rapid on-site decision making, and reduce issues arising from sample degradation and/or contamination during transportation back to the laboratory, or during long-term sample storage. Further to this, there are significant financial savings to be had, as laboratory analyses are for the most part clearly more expensive.

The significance of liquid chromatography (LC) as a gold standard analytical technique within the analytical laboratory cannot be underestimated, with this advanced and well-established analytical technology being the go-to technique for all sorts of investigative and confirmatory analytical science, across a hugely diverse range of disciplines and applications. However, translation of the standard liquid chromatograph to a mobile, portable, and even deployable instrument has been curiously slow, that is until now.

Today, there are a number of small-footprint LC-based systems commercially available, which are designed for portability, and interest in these systems has been growing rapidly (1–7). Milton Lee and co-workers led this most recent charge in and around 2014, reporting on portable nano-flow LC systems, which were shown to deliver impressive performance (8–10). Their first prototype was based on an isocratic piston pump, with stop-flow injection, capillary column, and on-capillary UV absorbance detector. In 2015, Sharma et al. reported upon a more advanced version of their previous system, which now included gradient-nanoflow dual-pumps and an on-capillary deep-UV-LED-based detector (9). The pumps were able to operate up to an impressive 550 bar, covering flow rates of 0.5 to 10 µL/min. The dimensions of this system were 31 × 18 × 14 cm and it weighed a mere 8.0 kg—it could also be battery operated. It was later applied to several “in-field” applications, including illicit drug analysis (10).

Running parallel to the above developments is an ongoing project focused on a more modular approach to portable liquid chromatography, initiating orthogonal, separation power, and sensitivity—even more enhanced if powerful MS detectors are hyphenated—thus leading to an ever increasing number of applications in several research fields.
from early work by Li et al., and involving a prototype open-platform LC with micro-syringe pumps capable of simple gradient programs (11). The first micro-LC was built using micro-syringe pumps (20 µL volume), mini-switching valves, and a manual injector all assembled on a breadboard, and used a commercial reversed-phase monolithic capillary column, with on-capillary deep-UV-LED absorbance detection. The dimensions were just 25 × 25 cm, weighing in at just 1.3 kg. This system was further developed by Lam et al., with modifications including a Z-cell-based LED detector, column heater, and an automated injector. This chromatograph weighed ~ 2.7 kg, and in this version all components were contained within a compact 3D-printed portable housing. This upgraded version was lightweight, small-size, and was demonstrated for the in-field analysis of plant extracts (12). A fully modular and re-engineered descendant of these earlier platforms was produced in 2020 (13). This full gradient capillary system (dimensions now of 30 × 45 × 12 cm and 7.2 kg weight) consisted of two syringe solvent pumps capable of handling up to 330 bar across flow rates of 1 to 100 µL/min, with retention time precision of < 1% (RSD). This instrument now included custom-built software and came with an open architecture design and a flexible configuration to fit a variety of intended on-site applications.

In the area of inorganic ion analysis, the standard laboratory (chromatographic) approach would be to use ion chromatography (IC), most commonly with suppressed conductivity detection. This now long-established methodology is the gold standard analytical technique for inorganic anions and cations, and is likely to remain so for the considerable future—particularly for the analysis of natural and potable waters. Interestingly, so-called portable ion chromatographs were commercially available as far back as the early 1980s, although they did not generate a great deal of attention or application at that time. However, following a hiatus of three or so decades, there are today at least three commercial suppliers of truly field-portable ion chromatographs, engineered from the ground up to be applied out of the laboratory and operate effectively at the point of need (14–16).

One area where field-deployable ion chromatography has a great deal of potential is in the field of nutrient monitoring. Inorganic nutrients—namely ammonia, nitrite/nitrate, and phosphate—are of course the lead culprits in freshwater eutrophication. Today, almost every environmental monitoring organization around the globe routinely check for these species, predominantly via costly and slow grab-sampling and lab-based analysis. For us, portable, low-cost, and low-pressure ion chromatography, including the push for “at-site” and long-term deployable systems, has been a focus of our research efforts dating back over two decades (17), but the last few years has seen this idea really take-off, and nowadays affordable commercial systems are available for precisely these applications (16). Recently, to test the robustness and reliability of one such field-deployable ion chromatograph under extreme environmental conditions, we set up a simple monitoring experiment within a greenhouse, in the Australian (Tasmanian) springtime, where ambient conditions can vary from between 4 to 40 °C. The experiment involved a field-portable ion chromatography-based system, which employs direct UV absorbance detection for nitrite and nitrate quantification, and which is a fully automated and wirelessly monitored system, capable of unmanned deployment for several months at a time (16). With a model sample system in place consisting of a large, filled water tank, containing a bottom layer of sheep-manure (ammonia source), covered by a layer of soil and a top layer of sand, the system was set to monitor the production and equilibrium of nitrite and nitrate concentrations over a period of several weeks. Figure 1(a–b) shows the system in place within the greenhouse and the analytical data generated from this controlled field demonstration. The temperature plot demonstrates the daily extremes of temperature, whilst the rapid growth of nitrate and slower continual rise of nitrite levels in the water tank can be continually monitored. Given the number of sample chromatograms run during this deployment, it’s not difficult to see the savings in lab time and costs, when compared with taking grab-samples run on an ion chromatography system within a centralized laboratory.

Of course, the ultimate test of an analytical system’s portability is taking it on a sampling journey! Figure 1(c)
really demonstrates this new philosophy and capability. With a fully portable and battery-powered ion chromatograph, we are able to undertake river cruises and map nutrient levels as we go (and simultaneously enjoy some of our state’s most scenic and evidently unpolluted waterways), which for a chromatography-based analyzer is really quite something. The benefit here is improved spatial coverage and mapping of dynamic systems, particularly inputs and temporally variable point sources, which can lead to better understanding and management of river catchments in the future.

In summary, it would seem clear that a number of instrument manufacturers and innovators have begun to see liquid chromatography as a platform technology that is indeed amenable to portable and “at-site” or deployable applications. This has awoken end-users to new possibilities and potential cost savings and process improvements. We certainly foresee a continuation in this direction and more systems coming to the market—hopefully with inherent flexibility and modularity to allow greater application to really interesting “out-of-lab” challenges.

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References

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On the Contemporary Analysis of Protein Biopharmaceuticals
Koen Sandra
Pat Sandra

The publication of the first LCGC Europe issue back in 1987 followed a major milestone in biology, the FDA approval of the first therapeutic monoclonal antibody (mAb). This event, together with the commercialization of recombinant insulin a few years earlier, marked the birth of a new class of medicines that have drastically reshaped the pharmaceutical landscape. Protein biopharmaceuticals are currently being developed and approved at an explosive rate and have attracted great interest from both biotech and big pharma. Their success is driven by their efficacy in disease areas with a high unmet medical need, such as oncology, autoimmunity, and infectious diseases. Antibodies reign supreme but protein biopharmaceuticals come in many flavours, all sharing one common denominator, an enormous therapeutic or prophylactic potential with a layer of immense structural complexity highly demanding towards analytics.
Privileged and from the first row, *LCGC Europe* witnessed how the analytical community rose to the challenge and sharpened the analytical toolbox. An inspiring creativity amongst peers throughout these three decades resulted in many innovative chromatographic and mass spectrometric tools for the in-depth study of these ever-more complex molecules.

Today these fascinating study objects are chromatographically resolved from top to bottom (protein, peptide, glycan, amino acid, monosaccharide) using a diverse set of separation principles, stand-alone or in conjunction, tackling different physicochemical properties (size, charge, hydrophobicity, hydrophilicity, affinity). To cope with the slow chromatographic diffusion, sub-2-µm fully porous or sub-3-µm superficially porous particles are widely employed. Ultrahigh-performance liquid chromatography (UHPLC) instrumentation has been made available to successfully operate the latter particles. Biocompatible or bioinert systems have been developed preventing corrosion at high salt concentrations and analyte loss. Stationary phases have been coated with hydrophilic layers to block nonspecific interactions with hydrophobic proteins, such as antibody–drug conjugates, in size-exclusion chromatography (SEC), or they have embedded charged functionalities to boost resolution in reversed-phase LC-based peptide mapping while using mass spectrometry (MS)-friendly formic acid (FA) instead of the ion-suppressant trifluoroacetic acid (TFA). Difluoroacetic acid (DFA) has been proposed as a hybrid between FA and TFA, offering decent chromatographic resolution and MS sensitivity. Wide-pore reversed-phase LC particles capable of withstanding temperatures up to 90 °C allow separations of even the most challenging proteins, and the on/off elution mechanism enables fast measurements, with ultra-short columns largely circumventing the negative impact of temperature on protein integrity. The introduction of wide-pore particles in hydrophilic interaction liquid chromatography (HILIC) has extended the use of this technology beyond mapping of fluorescently labelled glycans and allows glycoforms to be resolved at the protein level. Novel antibody degrading enzymes, such as IdeS, have been released to facilitate the latter. Glycans themselves are nowadays enzymatically liberated and tagged in record times with fluorophores with high proton affinity that boost MS sensitivity by at least 50-fold over the golden standard 2-aminobenzamide (2-AB). Column hardware (frits, inner wall) has been prepared stainless-steel-free, allowing recovery of challenging analytes, such as phosphorylated glycans in HILIC and antibody dimers in SEC, and preventing on-column oxidation during peptide mapping. Multidimensional liquid chromatography has successfully been adopted by the biopharma industry to drastically increase resolution, to make first dimension separations compatible with MS and to obtain orthogonal information. The technology has even
been stretched from two to five dimensions by incorporating (electro)chemical and enzymatic reactors and thereby maximizing information gathering in a fully automated manner. The quest for ever more dimensions has recently reopened a discussion within the chromatographic community on terminology.

Electrospray ionization (ESI)—invented a couple of months after the inauguration of *LCGC Europe*—has given wings to these molecular elephants, enabling successful mass analysis using high resolution time-of-flight (TOF) or orbital trap MS instruments equipped with various complementary fragmentation modes employed at top, middle, and bottom level. The coupling of historically incompatible chromatographic methods, such as SEC, ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), or affinity chromatography, with MS has been made possible by two-dimensional liquid chromatography (2D-LC), in which the second dimension enables desalting, or by direct MS hyphenation using volatile mobile phases. The latter sprays proteins under native conditions, contrasting with reversed-phase LC or HILIC–MS that operate under denaturing conditions, opening up perspectives towards studying protein-protein interactions and measuring previously unmeasurable proteins, as charged envelopes are shifted to higher m/z values where spatial resolution is at its maximum. Beyond primary structural characterization, native MS, ion mobility (IM), hydrogen-deuterium exchange (HDX), and cross-linking (XL) MS have been shown appealing for higher order structural analysis, epitope mapping, and to assess structure/function relationships.

LC–MS has furthermore been applied to study protein biopharmaceuticals in complex matrices in support of pharmacokinetic/pharmacodynamic (PK/PD) programs and to identify and quantify host cell proteins (HCPs) at low ppm levels—measurements historically performed by ligand binding assays. Throughout the years we have seen products become increasingly complex and witnessed analytics become more and more performant and robust. With that impressive analytical arsenal on the battlefield, we can rest assured that well-characterized and safe innovator and biosimilar products reach the patient. Yet there remains so much to be explored, and with all these opportunities on the shelf, it is highly rewarding as a scientist to be involved in biopharmaceutical analysis today. What will the next 35 years bring? Will nucleic acid-based medicines deliver on their promises? Will we separate our solutes on pillars instead of particles? Will we assess all structural attributes at once or will we keep on using dozens of methods and the most expensive instrumentation out there? Will we continue to handle these enormous amounts of data in a semi-automated manner or will we finally get all the information through one push of the– button? Curious to see how the field will be moulded in the next 35 years. Stay tuned…for a little longer.

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Nontargeted Screening in Environmental Analysis: An Example Showing Migration of Chemicals from Reusable Sports Plastic Bottles into Drinking Water

Selina Tisler
Jan H. Christensen

Reusable plastic water bottles are popular, especially during sport, due to their lightweight and impact-resistance properties. However, little is known about the migration of compounds from plastic into the drinking water, as a wide variety of intentionally added substances (IAS) are used in the production of plastic. Furthermore, an unknown number of nonintentionally added substances (NIAS) contribute to the chemical complexity. NIAS are often unknown themselves (1), and can be introduced during the production as impurities, as well as breakdown or transformation products (TPs) caused by multiple stressors such as sunlight, physical use, or elevated temperatures (2).

Bisphenol A (BPA) is one of the chemicals of concern in plastics, and products are nowadays sold as BPA-free. However, BPA has been replaced by bisphenol S and F, which have been shown to be carcinogenic as well (3). The new prioritization and identification of the regrettable substitutions will take additional time for risk evaluation before they can be phased out. Thus, a more broad screening of known and unknown compounds such as nontargeted screening (NTS), not yet included in regulations or databases, is needed.

In this study, we investigated the chemical migration into drinking water stored for 24 h in new bottles, used bottles, and bottles washed in the dishwasher. NTS by liquid
chromatography–high-resolution mass spectrometry (LC–HRMS) was performed to identify these compounds. The experiment was designed to be as close as possible to typical consumer use. The chemical composition of the stored tap water was investigated after each migration step and compounds were prioritized based on systematic changes in peak intensities across the treatments. The entire study is published in the Journal of Hazardous Materials (4).

**Nontarget Screening for Prioritization of Chemicals**

Using the NTS approach, hundreds of chemicals were analyzed migrating out of the plastic from new and used sports plastic bottles. After a dishwasher cycle of the same bottles, thousands of additional, dishwasher soap-related, compounds migrated into drinking water stored for 24 h. In Figure 1, the 2000 compounds detected in the new bottles are shown in a heat map that shows the origin of the chemicals. Around 100 plastic-related compounds from the new purchased bottles were flushed away after washing the bottles in the dishwasher (a). However, another 50 compounds (b) were still in the drinking water after the dishwasher but were removed after additional flushing. One hundred and seventy-five compounds were observed to migrate out of the bottles consistently (c), even after flushing the bottles with drinking water.

It is of special concern that the concentration of some of these chemicals was even higher in the drinking water after the dishwasher cycle—indicated by vertical lines with more red colour in the row "after the dishwasher". Most of the detected compounds were derived from the dishwasher soap, and so were detected after the dishwasher cycle (d) but removed after additional flushing of the bottles. Some of the dishwasher soap adsorbed to the material and could not be flushed away by additional water flushing (e).

**Dishwasher Soap Adsorbs More to Plastic than to Glass**

Surfactants that originated from the dishwasher soap were removed to more than 90% after additional flushing. However, there were several compounds still adsorbed to the plastic surface. The main detergents, which were responsible for the 60 most intense peaks, were identified as homologous series from polyoxyethylene lauryl (C12) ether and polyoxyethylene (C14) ether. These homologue series contained an increasing number of EO groups, with decreasing retention time as a function of polarity. The homologue series showed a removal trend that correlated with the number of EO groups, therefore the higher the number of the EO homologues in the series, the better the removal after additional flushing. The very polar homologue with the highest molecular mass and most EO groups \( \text{C}_{12}\text{H}_{26}\text{O}_{13} \) was almost...
completely removed (99% removal of the original intensity left), whereas the most nonpolar homologues \((C_{12}H_{23}O)\) were on average removed less than 30% after flushing.

**DEET—Insect Repellent in Plastic?**
The identification of the insect repellent DEET in the plastic bottles was surprising at first glance and shows the strength of NTS. DEET was detected in the water from all plastic bottles, with the highest peak intensity in the older, previously used, bottles. DEET was also detected at low peak intensities in the new bottles but not in the glass bottles nor in the solvent blanks. Therefore, the dishwasher and laboratory blanks could be excluded as a source for DEET. We suspect that DEET, which is a ubiquitous water contaminant (5), has another origin other than its use as an insect repellent. DEET demonstrates the properties of a plasticizer (6), and for that reason it could be introduced in the plastic as a plasticizer itself or it could be formed during the use of the bottles by the degradation of other chemicals. We detected a good correlation between DEET and the plasticizer laurolactam, which has the chemical formula \(C_{10}H_{19}NO\) and only 6 H less than DEET. It is possible that some impurities could remain during the laurolactam production process, where there is incomplete desaturation during workup, and therefore a similar compound to DEET could be formed. However, DEET could also have been formed from laurolactam itself during the dishwasher process, with the aid of catalyzing compounds in the plastic (for example, quinones such as anthraquinone).

*Other Detected Compounds and Toxicity*
We detected >400 plastic-related compounds as well as >3500 dishwasher-related compounds by LC–MS NTS. The chemical structure was identified for around 50 prioritized compounds. Most plastic-related compounds were plasticizers, antioxidants, and photoinitiators. The highest peaks were detected for oligomers suspected to originate from the biodegradable polyester polycaprolactone. The most concerning compounds were photoinitiators because of their high potential adverse effects. Irgacure 369 is an amine co-initiator, a group well-known for their endocrine-disrupting effects, and 4-methylbenzophenone has shown carcinogenicity, reproductive toxicity, and skin contact toxicity in animal testing. Anthraquinone is of concern because of its breakdown products that may be toxic and carcinogenic. The dishwashing process enhanced the migration of plasticizers, antioxidants, and photoinitiators into the drinking water. Therefore, the highest predicted toxic hazard was calculated (by Cramer’s rule) for the used plastic bottles that had been filled with drinking water after the dishwasher and without further flushing. The toxicity decreased after additional flushing.

Due to the lack of analytical standards in NTS, no concentration of the identified compounds could be determined. Therefore, the risk of the stored drinking water in these reusable bottles currently remains unknown. More research is needed to identify and quantify more of the unknown unknowns detected by NTS, as well as toxicological tests to assess the relevance and risk of the newly identified compounds. The study raises the question as to whether plastic bottles are suitable for re-use, especially when they are labelled as biodegradable plastic. The study emphasizes that the production of biodegradable plastic bottles does not mean that the bottles are necessarily made of naturally occurring compounds. Instead, it can be speculated that plasticizers will migrate more easily into consumer drinking water when the biodegradable plastic bottles slowly degrade during use.

*Other Uses of NTS in Environmental Analysis*
NTS have been and are currently used for the analysis of many different environmental and human matrices. One area of research where the use of NTS is in its early phase is for human biomonitoring. Target analysis of endocrine-disrupting chemicals (EDCs) in urine and serum has been applied since the early 2000s. Some of the prioritized EDCs have been phthalates and their metabolites, bisphenols, and parabens, while wide scope NTS analysis has been rare (7,8). In the study by Tkalec (8), an extraction method for human biomarkers in urine was developed and combined with LC–HRMS analysis and a suspect screening and NTS approach, and the workflow was validated by spiking known EDCs.

Another area of environmental NTS that is more developed is wastewater-based epidemiology. It has been used to screen and prioritize potentially persistent, bioaccumulating, and toxic wastewater contaminants (9) and to investigate removal processes (9–12). The same has been done for advanced oxidation processes (AOP) of groundwater.
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The Future of Column Packing Technology

Columns packed with chemically optimized stationary phase particles are the workhorse of liquid chromatography (LC) analysis. Despite the decades of work spent in optimizing particle manufacturing and column packing procedures, we still lose about 50–60% of their separation potential to the random nature with which these particles are being packed. What if we could three-dimensionally (3D)-print these particles in ordered packing layers?

It is by now very well-known that the infamous eddy dispersion wastes more than half of the number of theoretical plates $N$ that state-of-the-art high performance liquid chromatography (HPLC) columns would be able to produce. This has been demonstrated in a number of computational studies studying the band broadening in ordered sphere packings, as these represent the ultimate degree of eddy dispersion elimination (1,2). Roughly speaking, reduced plate heights can be reduced from around $h = 1.9–2$ (fully particles) to around 0.8–1.0 near the optimum, while at $4 \times$ the optimal flow rate of the random packed bed gains already amount up to a factor of 3 to 4.

A first solution to produce chromatographic beds with a perfectly ordered structure are pillar-array columns (3). These are produced using state-of-the-art micromachining to produce silicon pillar arrays that are subsequently electrochemically anodized to produce a mesoporous outer shell. Because of their high efficiency, pillar-array columns have rapidly}

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The joint efforts by environmental scientists worldwide have accelerated the development of a first guideline towards standardization of NTS in water analysis, which was published by the German Water Chemistry Society, “Non-Target Screening” Expert Committee (14).

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to prioritize compounds persistent to AOP processes, as well as TPs created by UV and hydrogen peroxide treatment that cannot be removed by active carbon filters (13). Many more applications within environmental analysis have been proposed and tested, such as soil, sediment, and biota, showing the wide applicability of NTS for environmental analysis. Key developments are currently within analytical methods alternative to reversed-phase LC–HRMS for more water-soluble compounds (persistent mobile and toxic compounds [PMTs]) such as hydrophilic interaction liquid chromatography (HILIC), supercritical fluid chromatography (SFC), and ion and ion-pair chromatography (IC); workflows for more reliable peak detection, grouping of features, drift correction, and quantification without standards; and compound databases for precursors and TPs. Because of their high efficiency, pillar-array columns have rapidly...
grown into the column of preference for many key players in the proteomics area (4,5). Limitations of the production method are the small scale of the produced micro-arrays, typically only suitable for the nano-flow format, although recently some new designs targeting the micro- and capillary-flow regimes have been proposed. Because the etching process is unidirectional, only 2.5D-beds (pillar arrays) can be produced. This implies the top and bottom surfaces of the channel inevitably induce an extra velocity gradient, extending its effect across the entire channel depth. This in turn generates an additional degree of dispersion not present in 3D beds. In addition, and more importantly, the number of available stationary phases is limited, since pillar-array columns are currently a single-vendor product. This poses a considerable hurdle to their widespread use, as analysts—especially those in the pharmaceutical industry—have many good reasons to stick to the stationary phase and even the specific brand their original methods have been developed with.

The above implies there remains a strong impetus to produce ordered 3D beds, preferably making up a monolithic body of a suitable mesoporous material (silica, some polymer types). When contemplating the possibility of producing ordered 3D packings, the first technology that comes to mind is 3D-printing. However, most of the available 3D-printing methods (filament printing, stereolithography, digital light processing, inkjet/polyjet [3DP] printers) do not offer the required ultra-small printing resolution (which is needed to produce a packing structure suitable for analytical chemistry, see below). Up to this point, 3D-printing in liquid chromatography has therefore been limited to printed devices targeting, at best, the preparative-scale (6), where packing structure sizes in the 20 µm range are on the verge of being competitive. Other 3D-printing projects have been even more modest and only aimed at printing the exterior column housing (7). The only 3D-printing approach enabling the sub-micrometre printing resolution required for analytical liquid chromatography is two-photon polymerization (2PP) (8). As this approach requires the simultaneous
absorption of two instead of only one photon to activate the photo-initiator molecules, and, as the probability for such an event is only high enough in the central portion of the focal spot, resolutions below the wavelength of the light can be achieved. Using tailored monomer mixtures, voxel (= “volumetric pixel”) sizes as small as 200 nm can be printed using 2PP, which is small enough to print structures with 0.6 µm through-pores without suffering from excessive pore-to-pore shape and size differences. This pore size is comparable to the through-pore in a 2 µm particle-packed column, as is needed to be competitive with these columns. However, these very small voxel sizes inevitably also come at the expense of very low volumetric printing speeds. In a recent study (8), we showed the main drawback of this approach is that the printing process is inherently slow, requiring several 100’s of hours (!) to just print a structure with the dimensions of a typical nano-LC column (75 µm internal diameter [i.d.] cylindrical tube with length L = 15 cm). Another limitation is the available 2PP-optimized printing resins. These are mostly of the acrylic type, and are hence difficult to derivatize, let alone amenable to forming a mesoporous structure. The ideal 3D-printed bed for liquid chromatography should be made of mesoporous silica, but the direct printing of ceramic structures is up to now limited to filament printers, which come nowhere close to the required resolution.

An ideal 3D-packed bed could be realized by developing a technology that can deposit entire layers of (chromatographic) spherical particles at the same time. This would speed up the printing process tremendously and produce structures made with suitable material. An approach to realize this (Figure 1) is under investigation in PrintPack, a research project funded via an Advanced ERC Grant. In its first realization (9), we managed to produce uniform monolayers using a two-step process. First, we assembled two-dimensional (2D)-sphere arrays by suspending the particles in an electrostatic cell and simultaneously attracting them using a vacuum-driven flow onto a silicon membrane perforated by an ordered array of through-pores and subsequently brushing away the inevitable excess particles in a single or double sweep with a dense brush while maintaining the vacuum to keep the bottom layer of particles in place (Figure 2). As can be noted from Figure 2, this approach allows error-less ordered sphere particle monolayers to be produced. In the next step, we could demonstrate that the monolayers can be flawlessly transferred onto a second substrate, provided this is made of an elastomeric material, for example, carbon tape or polydimethylsiloxane (PDMS). The project’s next phase will target contacting the layer of particles with a (sub-micrometre) thin layer of photocurable glue, and then deposit the particle layer onto a stack of previously deposited layers with the glue layer facing downwards (Figure 1[b–c]). Subsequent illumination with UV-light would then fix the particles in their position, followed by the release of the vacuum-assembly tool holding the particles during the assembly process. As already demonstrated, this can be realized by making use of the reversibility of the vacuum-driven holding mechanism, that is, by applying an overpressure instead of an underpressure.

Although many hurdles are still to be overcome, for example, putting the produced arrays in perfect-fitting and high-pressure-resistant housing and finding ways to handle the polydispersity of some commercial HPLC particle types, it is believed this particle assembly and deposition process holds the promise that in the future one would be able to build perfectly ordered monolithic particle beds, offering twice the efficiency of our current randomly packed columns.

Acknowledgements
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The development of multidetection field-flow fractionation (FFF) has made a global impact on the research and development of advanced separation and analysis of novel, supramolecular, and complex polymers. Owing to their multiple functionalities and applications in advanced nanotechnology, the accurate and reliable characterization of advanced materials is essential for both the material design and development processes. As highlighted in Figure 1, the utility of FFF, namely asymmetrical-flow FFF (AF4), hollow-fibre-flow FFF (HF5), and thermal FFF (ThFFF), has been instrumental in extending the frontiers for the separation and analysis of supramolecular and complex polymers (1). These FFF subtypes have the ability to separate at low-shear conditions as a function of distinct separation force-fields across a flat ribbon-like channel with a laminar and parabolic solvent flow-profile. Examples of force-fields are a thermal gradient and a cross-flow stream, applicable for ThFFF and AF4/ HF5, respectively.

Essential AF4/HF5 research avenues focus on the deep understanding and elucidation of structural transformations within complex macromolecular samples depending on different parameters, such as molar mass, cross-linking, pH, and concentration. Here, we are using the power of multidetection to provide a large amount of information within a single measurement. We consider a variety of parameters that describe the molecular shape in dilute solutions, such as the scaling or Mark-Houwink exponent, as well as the ratio of the hydrodynamic radius ($R_h$) to the radius of gyration ($R_g$) (shape parameter). Additionally, apparent density, which is molar mass-dependent, aids in validating the conformational data. Particularly noteworthy is the AF4-5D (quintuple detection consisting of multi-angle light scattering [MALS], dynamic light scattering [DLS], refractive index [RI], UV, and viscometry) applicable for evaluating the internal folding process of single-chain nanoparticles (2). Here we demonstrate that increased internal cross-linking density results in higher compactness.

Continuously improving AF4 protocols for in-depth vesicle characterization (polymersomes [3] and exosomes [4]) are of the utmost importance to understanding structural transformations dependent on loading or modification processes. Here, it is possible to get quantitative and qualitative information, as well as the locus of the encapsulated drug or protein, which helps in optimizing the loading or modification processes. AF4 elucidates the intra- and intermolecular interactions between various types of fragile nanostructures (the combination of polymers, proteins, nanoparticles, and/or enzymes) and their conformational effects (5).

A single measurement can provide even more information when coupling state-of-the-art quintuple-detection to ThFFF. In addition to molar mass, size, shape, apparent density, and intrinsic viscosity, we can also separate according to microstructure, topology, and composition owing to the thermophoretic properties of the analytes.

Regarding ThFFF’s capability in the characterization of polymers, great advances have been made to transform

![FIGURE 1: The latest advances from AF4 (a) used for the characterization of complex nanoparticles, for example, (b) exosome, as well as from (c and d) ThFFF used as a branching characterization tool. Adapted and reprinted with permission from references 1, 4, 6, 7, and 8.](image)
from an empirical trial-and-error procedure to a targeted theory-based method, including retention predictions (6). Furthermore, ThFFF has evolved its potential to act as a complementary tool for branching or topology characterization, as shown recently for aromatic-aliphatic polyester (7) and chain-walking polyolefins (8). This sets the basis for ThFFF to be a promising branching characterization tool for polyolefins.

ThFFF has also shown its potential in the characterization of polymeric nanoreactors (9). A new trend in the development of ThFFF is the characterization of metal-polymer hybrids for subsequent exploitation of their plasmonics as highly sensitive electromagnetic probes for investigating size, shape, and composition dynamics with higher resolution. When exposed to electromagnetic irradiation, metal nanoparticles exhibit highly sensitive and intensive plasmonic behaviour as a function of shape, size, and composition, and this is analyzable with higher reliability using advanced UV–vis spectroscopy coupled to ThFFF.

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were rewarded. Out of this induction period, we are experiencing an "exponential" evolution in hardware and software development. Many of the criticisms of the technique in the past have been overcome or solved, and workflows have been developed and automated to solve specific problems. These solutions have been possible because of the synergies between all the key players: academia, private companies, and instrument manufacturers. Application problems have been turned into drivers for new hardware development and more intuitive software solutions.

Instrument manufacturers, directly or with their solution partners, offer a full portfolio of hardware tools. Thermal modulation, with or without cryogenic fluids (CO$_2$ or liquid N$_2$), represents the gold standard in terms of efficiency and resolution (3). Available solutions cover "fully equipped" instruments or "add-on" modulators for the upgrading of existing one-dimensional (1D)-GC to GC×GC platforms. Moreover, for high-throughput analyses in control or industrial laboratories, flow modulation with different principles of operation (differential-flow modulation [4] and multi-mode modulation [5]) offers suitable performance, robustness, reliability, and cost-effectiveness. With software-assisted procedures, even the translation of methods from thermal to flow modulation is possible (6), facilitating the application transfer from R&D to control laboratories.

Mass spectrometry (MS)—high or low-resolution—is the most common detector in combination with GC×GC. Providing orthogonal information about analyte identity and amount present, it is fundamental for the accurate interpretation of the chemistry behind many phenomena. In those applications where the confirmatory role of MS is not mandatory, single-channel detectors such as the flame ionization detector (FID) are optioned, providing a wider linearity range of responses and quantification options without the need for external calibration, for example, by response factors (7).

However, hardware advances and market consolidation of the GC technique should be accompanied by equivalent efforts in the data processing. If the advantages of comprehensive two-dimensional (2D) chromatography are clear to most GC users—improved efficiency, resolution, separation power, ordered retention logic—the change of mind required for data processing and interpretation—from profiles to fingerprints—has been the barrier some could not overcome.

As a passionate user and convinced supporter of GC×GC, I must say that the key to unlocking the deserved success of the technique is to help analysts access the data with intuitive and efficient tools.

In my interaction with applied research units in food manufacturing companies, they are often highly resistant to analytical innovation. They are open to new tools if they are related to food safety risks, if a complex measurement is establishing the safety compliance. Food analysis should be fast, simple, informative, and reliable enough to provide answers to multifaceted questions, for example, is my product compliant with the reference? Is my primary material of suitable quality? Is the new processing technology improving sensory acceptability? Is climate change impacting crop quality? Each of these questions refers to complex (biological) phenomena where informative markers and decision-making tools are not just single but complex patterns of analytes. In this context, which shares common features with many other application areas—such as petrolemomics, metabolomics, environmental analysis, and flavour and fragrance—GC×GC represents the innovation we all need(ed) to take a step ahead supporting innovation, safety, and quality of food.

I see great potential in the application of artificial intelligence (AI) concepts and tools at the data processing level, for data mining, and even applied to the global method workflow.

Computer vision, for example, is a field of AI where comprehensive two-dimensional chromatography can take tremendous benefits. It is defined as "a field of artificial intelligence that enables computers and systems to derive meaningful information from digital images, videos, and other visual inputs—and take actions or make recommendations based on that information. If AI enables computers to think, computer vision enables them to see, observe, and understand" (8). Luckily we have software tools that have been developed in full alignment with this definition (9).

Pixel-based approaches for prompt visual comparisons, peak-region features, and tile-based Fisher-ratio analysis for fingerprinting are all new data processing tools capable of “seeing” compositional differences and support the “understanding” of discriminant patterns of chemicals between samples (9). As for biometric fingerprinting, pattern recognition algorithms give access to a higher level of information; in forensics, we identify individuals based on a fingerprint identification, in analytical chemistry we capture chemical diversity and interpret phenomena.

Another intriguing application of AI is in the sensomic area. The
concept of sensomic-based expert system (SEBES) (10) has recently been introduced and this predicts key aroma signatures of food without using human olfaction—an AI-smelling machine. In practice, SEBES is a workflow capable of extracting, isolating, and quantifying key food odorants (KFO), those responsible for the sensory features that shape food sensory “identity” (11). By capturing KFO signatures, we enable our systems to smell like a human nose and our analytical workflows to screen for hundreds of samples without any fatigue. Not surprisingly—at least for us—GC×GC is the core platform of SEBES, and it can be fully automated for many food products, for example, extra-virgin olive oil aroma blueprint (12), raw hazelnuts spoilage (13), and cocoa and chocolate aroma quality (14).

In conclusion, GC×GC offers the chance to approach Nature’s (chemical) complexity by more accurately delineating piece by piece all of its parts; we do not need to simplify measurements because we make the way we look at it easier. For this reason “the full potential of GC×GC was probably not clear at its introduction, but its widespread adoption in different areas and the infusion of strategies and concepts from other disciplines, have definitely highlighted its central role of missing technique: from a technique that did not exist…to a technique that was missing” (15).

Finally, I would like to thank my mentor, Carlo Bicchi, for his inspiration and scientific contribution to multidimensional GC.

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Analytical chemistry is an important tier of environmental protection and has been traditionally linked to the assessment of the environmental quality status of systems. Although essential, analytical chemistry may also contribute to further environmental problems mainly due to the high energy demands and large quantities of hazardous substances that may be used or generated throughout an analytical procedure. These two distinct and contradictory roles of analytical chemistry were highlighted by Paul Anastas a year after the introduction of green chemistry (1), when the concept of green analytical chemistry (GAC) was defined as an emerging area, relevant to the research arena and commercial sector. At that time, traditional sample preparation methods were identified as a major source of the total negative impact of analytical methodologies on the environment and their replacement with contemporary ones was considered central in settling greener analytical methods. Indeed, early sample preparation methodologies were tedious, time-consuming, and, more importantly, expended large quantities of resources that resulted in the generation of hazardous laboratory waste.

In 2013, the concept of GAC was formulated in the form of 12 principles that expressed the willingness to care for the environment and human safety as part of the development and application of analytical procedures (2). The introduction of GAC aimed to redefine and reevaluate analytical methods by addressing safety of solvents/reagents, toxic laboratory waste generation, workers’ safety, and energy efficiency. In the formulation of GAC, the first principle suggested applying direct analytical techniques to avoid sample preparation and also concluded that any “green” action taken during the sample preparation step (for example, minimal use of energy, safety for operator, use of non-toxic reagents or reagents from renewable source) would have a negative impact on accuracy, precision, selectivity, sensitivity, and detectability of the analytical process. This was not a well-reflected assumption, especially when considering the analytical performance of mature and green sample preparation technologies available at that time (for example, the solventless and reagentless solid-phase microextraction [SPME]). The use of direct analysis may be a straightforward approach to address problems related to sample preparation. However, to meet sensitivity needs and overcome matrix-related problems, the use of sophisticated, expensive, and energy-consuming instrumentation is required that generally shifts the environmental impact from sample preparation to the determination step. More importantly, direct analysis is not always an option and a step of sample cleanup, analyte enrichment, or analyte conversion into a form suitable for analysis is commonly needed. In other words, sample preparation remains a key step in analytical procedures.

The first principle of GAC was commonly misinterpreted and created the false impression that omitting the sample preparation step is a green approach, fully neglecting the “green” technological advances in the field. The “exclusion” of sample preparation from GAC also created a gap by not considering cases where direct analysis was not an option. Instead of neglecting this step, efforts should have been devoted to fully defining sample preparation within the context of green chemistry and GAC. Over the years, redefining sample preparation to address sustainability issues and promote the practice of green sample preparation (GSP) has become a necessity. After all, green chemistry was never about what to stop
doing, but was always about invention and the things one can do better.

Earlier this year, the concept of GSP was proposed (3). It was formulated in the form of 10 principles that represented a road map towards the development of overall greener analytical methodologies (Figure 1). This set of principles represented the optimum number of characteristics needed to describe the inner structure of the GSP concept, its properties, and function mechanism. The 10 principles were not isolated but formed an integrated system of design (Figure 1), where improvements achieved by aligning to the fulfilment of a given principle could synergistically help to reduce the deficiencies associated with other interconnected principles. GSP set goals that were common to GAC but also had several distinctive and innovative features. In every case, the GSP approach put sample preparation at centre stage and translated greenness based on the needs and requirements of sample preparation. The aspects considered by GSP included the use of safe solvents/reagents; materials being reusable and from renewable, recycled sources, minimizing waste generation and energy demand; minimization of samples, chemicals, and materials; procedure simplification and automation; operator’s safety; and preparing a high number of samples per unit time. Based on the advances in sample preparation, several contemporary and mature sample preparation technologies align with the principles of GSP and fulfil the requirements for greening this key step in analysis. It is equally important to note that the adoption of these methods in the laboratory not only aligns with the principles of GSP but also assists in improving the analytical characteristics of the overall method. The latter contrasts with the GAC approach that faces the challenge of reducing the environmental impact of methods without negatively impacting the analytical efficiency of the method, that is, sensitivity, selectivity, accuracy, precision, robustness, and, in turn, the quality of the analytical information obtained.

Current environmental challenges are of a global scale, highlighting the urgent need to align with pollution abatement and the principles of sustainable development. Analytical chemists face increasingly complex interrelated problems both on-site and at the laboratory and the application of environmentally benign analytical practices has become a critical factor to consider. The adoption of contemporary sample preparation practices with a low environmental impact should therefore be a priority for all researchers, practitioners, and routine analysts. Greening analytical methods is directly connected to greening the sample preparation step. In other words, there is no green like more green.

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Method Development for Reversed-Phase Separations of Peptides: A Rational Screening Strategy for Column and Mobile Phase Combinations with Complementary Selectivity

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This review article summarizes the results obtained from the combined efforts of a joint academic and industrial initiative to solve the real-life challenge of determining low levels of peptide-related impurities (typically 0.05–1% of the drug substance) in the presence of the related biologically active peptide at a high concentration. A rational screening strategy for pharmaceutically important peptides has been developed that uses combinations of reversed-phase ultrahigh-pressure liquid chromatography (UHPLC) columns and mobile phases that exhibit complementary reversed-phase chromatographic selectivity using either UV- or mass spectrometry (MS)-compatible conditions. Numerous stationary and mobile phases were categorized using the chemometric tool of principal component analysis (PCA), employing a novel characterization protocol utilizing specifically designed peptide probes. This was successfully applied to the development of a strategy for the detection of impurities (especially isomers) in peptide drug substances using two-dimensional liquid chromatography coupled with MS detection (2D-LC–MS).

KEY POINTS

- A column and mobile phase reversed-phase chromatography characterization protocol employing relevant peptide probes is described.
- Principal component analysis of the characterization database allowed the identification of column and mobile phase combinations with differing or similar chromatographic selectivity.
- The movement from small molecule-based new drug entities to larger and more complex biomolecules, such as peptides and proteins, has increased in recent years (1,2). This has led to a re-evaluation of how chromatographers perform efficient, rapid, and rational development of purity methods for peptide-based pharmaceuticals—that is, samples that consist of a biologically active peptide present at a high level and a number of related peptide impurities present at a low level (typically 0.05–1% of the drug substance). Peptides present a different analytical challenge compared with small molecules because of their size and unique physical-chemical properties. Reversed-phase chromatographic separations with either UV absorbance (UV) or mass spectrometric (reversed-phase chromatography–MS) detection are the most important analytical techniques for the analysis of intact peptides and their related impurities (oxidation, deamidation, racemization, isoAsp formation). There is even a...
current drive to replace several traditional analytical methods (reversed-phase chromatography, ion-exchange chromatography [IEC], imaged capillary isoelectric focusing [icIEF], CE-SDS [capillary electrophoresis in which proteins are denatured with sodium dodecyl sulphate and then separated based on size in a gel (for example, dextran, polyvinyl alcohol, polyethylene oxide, polyethylene glycol]), hydrophilic interaction liquid chromatography [HILIC], enzyme-linked immunosorbent assay [ELISA]) with one reversed-phase chromatography–MS method that is fit for purpose for the characterization of larger proteins, antibodies, and their related impurities as digests—this is referred to as multiple attribute monitoring (MAM) (3). There are several challenges related to peptide analysis; however, the “Achilles’ heel” of liquid chromatography (LC)–MS lies in its inability to accurately determine isobaric compounds such as isomers—which are difficult to separate chromatographically and have identical mass-to-charge ratios (m/z). It is therefore vital to have a better understanding of the separation of peptide impurities—especially isomers—as a function of column and mobile phase conditions (both UV- and MS-compatible).

This article summarizes the findings of our recent research to:
1) Develop a column and mobile phase reversed-phase chromatography characterization protocol employing relevant peptide probes.
2) Utilize the characterization results to identify column and mobile phase combinations that provide complementary selectivities for method development purposes or similar selectivities for the identification of “equivalent” columns.
3) Define and demonstrate a strategy for detection of impurities (especially isomers) in therapeutic peptides using two-dimensional (2D)-LC–MS.

The work described in this review article is an example of combining the expertise within both the pharmaceutical industry and academia to solve a real-life challenge (4–10). The reader is directed to the original papers for more details relating to the studies.

**Experimental**
For complete details of the experimental conditions, readers are referred to references 4–10.

**Development of a Peptide Reversed-Phase Chromatography Characterization Protocol (4,5)**
Column characterization protocols employing small molecules have been used successfully for decades to classify reversed-phase chromatography stationary phases and provide a better understanding of potential mechanisms of interaction between the stationary phase and analytes. The most common are the hydrophobic subtraction model, developed by Snyder et al. (11), and the extended Tanaka protocols, enhanced by Euerby and Petersson (12,13). However, for the reversed-phase chromatography of peptides, it was deemed imperative to use relevant peptide probes to categorize the stationary phases classed as suitable for peptide analysis. This was proven by the fact that the small molecule databases failed to correlate with the Peptide RPC Column Characterization Protocol (6).

[Ile27]-Bovine GLP-2, which contains a 33 amino acid sequence (see reference 4, table 2), was selected as the base sequence to develop the peptide probes. The peptide was synthesized in two parts: a more hydrophilic portion (1‑15) and a more hydrophobic segment (16‑33) were modified systematically at one position along the peptide chain. These modifications were made to investigate the effects of hydrophobicity, aromaticity, and changes in charge, as well as

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changes that reflect common degradative pathways (isomerization, racemization, and deamidation products that can prove difficult to resolve) (see reference 5, table 1 for the peptides used in the final protocol). For a full rationale for the selection of [Ile27]-Bovine GLP-2 and the design of the probes, see reference 4.

The characterization protocol was performed at both low and intermediate pH conditions to alter the ionization state of the stationary phase and peptide probes. Solutions of 0.1% v/v formic acid and ammonium formate (native pH values of 2.7 and 6.5, respectively) were employed. The use of the ion-pairing reagent trifluoroacetic acid (TFA) as a mobile phase additive was not considered for use in the characterization protocol because it suppresses any potential selectivity differences between the stationary phases (4,7). Selectivity between two probes of interest was measured as the difference in normalized retention times (that is, delta values). In this way, any contribution from instrument dwell or column volumes could be eliminated, and variations caused by analyst, mobile phase, or laboratory differences reduced. In total, 33 delta values were obtained for each mobile phase. The Peptide RPC Characterization Protocol was then applied to 14 stationary phases. The results were evaluated using the chemometric tool of principal component analysis (PCA), which condenses the data into a selected number of principal components (PCs), where the number of PCs is less than the number of experimental variables. In our case, it was found that the first two PCs carried 56% of the measured variance in the delta values. Based on the prior knowledge of the stationary phases, the manufacturer’s information, the Tanaka column characterization data, and their position within the score plot, the stationary phases could be classified into three groupings, as described in the next section. From the PCA loading plot, numerous duplications of data from the variables (delta retention time values) were revealed, and so many measurements could be removed without any loss of information. The characterization protocol could therefore be streamlined by reducing the number of delta values determined from 66 to eight measurements and using nine instead of 26 peptides (see reference 6, table 2).
The retentivity and selectivity of the peptide probes were observed to be dominated by hydrophobicity, and then electrostatic/polar interactions. Despite the random coil nature of the peptides in mobile phases, it was observed that they changed conformation within the stationary phase (4). The second order structure of the peptides contributed significantly to the elution of the peptide, which was highlighted by large selectivity differences. This further complicated an interpretation of the retention mechanisms between the stationary phase and peptides.

Based on a statistical analysis of data from a reduced factorial design, the Peptide RPC Column Characterization Protocol was shown to be robust. This allowed specifications for the preparation of the mobile phases to be set, as well as critical appraisal of the impact of small fluctuations in operating conditions, batch-to-batch variability in columns, and instrument characteristics. The results indicated that the spatial differences observed in the characterization score plots were caused by real selectivity differences between stationary phases, and not variations in experimental conditions. For a full list of the steps employed to improve the reproducibility and reliability of the procedure, as well as the definitive protocol, see reference 5.

**Characterization of Stationary Phases for Peptide Analysis (6)**
The Peptide RPC Characterization Protocol was then applied to a more extensive range of stationary phases (43 differing phases to date) covering a range of ligand functionalities. The results were assessed using PCA, and the first two PCs carried 80% of the variance in the dataset (Figure 1). The stationary phases were divided into the three categories based on manufacturer information, the extended Tanaka small molecule column characterization results, and their position within the PCA score plot. These categories were similar to those that emerged in the preliminary study: 1) neutral phases; 2) phases possessing negative charge/polar character; and 3) those with a positive charge character. The neutral phases (marked in red) were clustered in the top right quadrant of the score plot. These phases possessed alkyl moieties with a high ligand density and endcapping. The negative/polar phases (marked in blue) were located predominantly in the lower right quadrant. These phases typically had lower ligand density, lacked endcapping, or possessed a ligand architecture that promoted access to the silica surface. The third group had some form of positive

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character (marked green) on the stationary phase, either via the ligand functionality, or positively charged sites on the silica surface. These phases were scattered within the upper and lower left-hand quadrants. These results were in line with the position of the delta values in the PCA loading plots. For example, stationary phases with a positive character in the left-hand side are dominated by $\Delta(9,1)$ FA (negative character), which probes the positive character of the stationary phase. Those phases on the right are dominated by the $\Delta(26,13)$ values (positive character), which explores the negative character of the stationary phase. For a full explanation of the PCA results, readers are directed to reference 6.

Stationary phases that are within close spatial proximity to one another in the PCA score plot should provide similar chromatographic profiles, while stationary phases spatially separated in the PCA score plot should provide different chromatographic elution profiles. Both scenarios were demonstrated using tryptic digested peptides, as shown in Figure 2. The selectivity (S) values (14) for the dissimilar (S value = 33) and similar (S value = 4) phases relative to a standard C18 were of a similar magnitude to those previously observed for small molecular weight analytes (15). The Peptide RPC Column Characterization database should be freely available on the ACD/Lab website later in the year (16).

**Characterization of Mobile Phases for Peptide Analysis**

In liquid chromatography, the mobile phase is known to have a major impact on selectivity. In our work, 51 novel and routinely used mobile phases, suitable for both MS- and non-MS-compatible conditions, were evaluated. Mobile phases with pHs ranging between 2.3–7.5 were adjusted with NaCl, kosmotropic (Na$_2$SO$_4$ and [NH$_4$]$_2$SO$_4$) or chaotropic (sodium perchlorate [NaClO$_4$] and ammonium hexafluorophosphate [NH$_4$PF$_6$]) salts, or ion-pair reagents (triethylamine [TEA], TFA, heptafluorobutyric acid [HFBA], and sodium 1-butanesulfonate [BuSO$_3$Na]). Other mobile phases covering a pH range of 1.8–7.8 were also assessed and included typical acids such as TFA, as well as less commonly used additives such as difluoroacetic acid (DFA) and methanesulfonic acid (MSA). Higher pH mobile phases were not investigated due to their potential to cause deamidation and isomerization/racemization of peptides.
In addition, low retention of the peptide probes was observed at high pH due to mutual repulsion of the negatively charged peptide and stationary phase. For the full list, rationale, and preparation of the mobile phases, see reference 7. The study was performed using a general gradient elution profile with a new generation “low acidity” C18 column, which was representative of a neutral, reversed-phase column.

The first two PCs carried 91% of the measured variance in the dataset (Figure 3), with a clear link to electrostatic interactions represented by the first principal component, based on the positions of Δ(9,1) and Δ(26,13) values. The first principal component was also dominated by aromatic, phenolic, and hydrogen bonding interactions due to the location of Δ(16,13), Δ(24,13), and Δ(8a,1) values, respectively. In Figure 3, the left-hand side of the score plot is governed by the low pH conditions (pH ≤ 2.7), with a clear increase in pH across to the right-hand side of the score plot (pH ≥ 6.5). There is also a convergence as the pH increases, indicating that as the pH increases, the selectivity differences due to other factors are not as large as those observed at low pH conditions. The second principal component reflected steric interactions (Δ(14,13) and Δ(15,13)). The results indicated that pH is a major contributor to selectivity differences and should be evaluated in any method development screening approach.

Kosmotropic and chaotropic salts are a particularly interesting range of additives due to their ability to make or break water structure, which can affect the solvation shell around peptides and proteins. The chaotropic salts appeared to provide greater selectivity differences than the kosmotropic salts. Despite the potential selectivity differences provided by the perchlorate (ClO₄⁻) additive, there are some disadvantages that could limit its widespread use—namely, health, safety, and environmental concerns. The nonvolatile ammonium hexafluorophosphate additive is a viable alternative that offers even larger selectivity differences under low pH conditions, without the explosive properties of perchlorates.

The ion-pairing reagents are an interesting subset of additives that can vastly alter the elution profiles based on the various interactions of the ion-pairing reagent with analyte and stationary phase. The hydrophobicity of the ion-pair can also be advantageous for peptides with different polarities, highlighting the importance of evaluating several ion-pair reagents. The ion-pair reagents TFA, TEA, HFBA, and butanesulfonate (BuSO₃⁻) were compared against no ion-pair reagent, at the four different pH conditions (pH 2, 3.6, 5.1, and 7.5). As hexafluorophosphate and perchlorate possess ion-pairing properties, these additives were also included in the comparison; however, the ionic strength was significantly higher, therefore the value of this comparison is somewhat limited. The increase in retention of the peptide probes due to the addition of a given ion-pair reagent correlated with the hydrophobicity of the ion-pair reagent: none < TFA < BuSO₃⁻ < HFBA. At pH 2.3, the BuSO₃⁻ and HFBA ion-pair reagents had the largest spatial difference in the score plot. However, with the inclusion of the chaotropic salts at pH 2.3, this distance was extended substantially, suggesting considerable selectivity differences. The TFA-containing mobile phase was located closest to the non-ion-pair reagent mobile phase, suggesting minimal selectivity differences between the two conditions. As the pH was increased, the selectivity differences for the peptide probes diminished—particularly when using the anionic ion-pair reagents TFA, HFBA, ClO₄⁻, and BuSO₃⁻. At pH 2.3, the peptide probes possessed more positive character than negative charge, which facilitated ion-pair formation, whereas under pH 7.5 conditions, the peptides possessed a mixture of negative and positive residues in the chain. As such, the peptides possessed an overall neutral or slightly negative surface charge, which would not interact sufficiently with the anionic ion-pair reagents.

Although the mobile phase characterization was based on a C18 column, there was a good indication that the results may be applicable to a wide range of commercially available stationary phases that includes those possessing a neutral, polar/negative, or positive character (see reference 7, supplementary material figure 2).

Establishment of a Screening Strategy for Development of Methods for Assessment of Pharmaceutical Peptide Purity (8)
The findings from the column and mobile phase characterization were combined into a reversed-phase ultrahigh-pressure liquid chromatography (UHPLC) method development screening strategy for the rapid assessment of peptide-based pharmaceutical purity (8) using automated column and mobile phase screening technologies (see next section). The protocol recommends the use of five disparate columns with varying degrees of charge—positive and negative/ hydrophobic character—and six volatile/ nonvolatile mobile phases within the pH range of 2.3 to 5.1 (see Table 1). The ion-pair/chaotropic reagent ammonium hexafluorophosphate at pH 2.3 was shown to be an extremely useful mobile phase additive, as it produced highly complementary selectivity and good peak shape. Methanesulfonic acid was found to be a good alternative to the ubiquitously employed TFA, which failed to generate acceptable separation for the many peptides investigated, highlighting the importance of screening disparate mobile
phase additives. However, TFA was found to be vital for acceptable chromatography of highly basic peptides such as melittin. The success of the method development strategy was demonstrated using a range of degraded peptides and peptide diastereomers corresponding to a racemization of a single amino acid in the target sequence. The protocol was found to be highly effective for identifying UHPLC conditions that generate complementary selectivity, good peak shape, and a high probability of separating the peptide impurities (see Figure 4). For this type of sample, it is important to use mobile phases with high ionic strength to mitigate mass overload, which can lead to a very broad/asymmetric main peak; this in turn can result in the possible masking of closely eluting impurities (7). The screening also identified partial separation of all the diastereomeric products (see Figure 5), which could form the basis of a good starting point for further optimization.

**Recommended Method Development Strategy (Figure 6)**

From the initial screening of five columns and six mobile phases (Step 1, 30 total combinations), the best column/mobile phase combination that satisfied the pre-set separation criteria (Step 2) was used to construct a 3 × 3 (Step 3, nine input experiments) gradient time vs. temperature (t<sub>G</sub> vs T) retention model (17). If adequate resolution is not achieved, different organic modifiers, such as isopropanol (2-PrOH) or methanol (MeOH) in place of or in combination with acetonitrile (MeCN) as an optional step (Step 2.1), can be investigated. Due to the high viscosity of 2-PrOH, it is typically used in combination with MeCN, for example, 20:60:20 (v/v/v) 2-PrOH–MeCN–H<sub>2</sub>O.

There were relatively large differences in retention of GLP-2 (1-15), insulin, angiotensin I, and melittin when using the different column and mobile phase combinations (see reference 8 and 9 and Figure 5). Most column and mobile phase combinations have a retention relative to the C8 column, with TFA within ±20%. The largest differences in relative retention were observed for acetic acid–ammonium bicarbonate buffer (AA–NH<sub>4</sub>HCO<sub>3</sub> pH 7.0) in combination with the fluorophenyl columns (56% decrease in retention compared with the C8 column). This information is important for the design of column and mobile phase screens. Based on the retention for a scouting gradient employing the C8 column with TFA, it is possible to either extend or shift the screening gradient to achieve sufficient retention with the fluorophenyl column. Our data from the use of ammonium hexafluorophosphate and methanesulfonic acid have shown that these additives do not adversely affect the stability of C18 columns, nor cause any irreversible adsorption/memory effects. No pH hysteresis effects were observed with any of the stationary phases on mobile phase pH cycling. Although no major problems have been observed with the use of the novel mobile phase additives ammonium hexafluorophosphate and methanesulfonic acid, the authors would recommend that these mobile phases be used with caution until their appropriateness for long-term, routine use has been established.
From the two-dimensional model of the dependence of retention on gradient time and temperature, the elution conditions (temperature, initial and final %B in the gradient, and the gradient slope) can be adjusted to produce a candidate separation (Step 3). This separation can then be further fine-tuned to increase the resolution or shorten the analysis by investigating the resolution as a function of particle size ($d_p$), column length, and flow rate while maintaining a constant optimal selectivity by keeping $(t_G \times F)/V_m$ constant (18) (Step 3.1). Once the optimum method has been located, the robustness of the proposed methodology can be assessed from the retention model (Step 4). The effect of small permittable changes to the gradient slope and temperature on the separation criteria can be rapidly performed in silico to provide both graphical (calculated chromatograms) and numerical ($\delta t_r/\delta%$MeCN and $\delta t_r/\delta T$) guidance for system suitability adjustments. If the methodology appears to be robust, it can be validated experimentally using a reduced factorial design (19) and ICH guidelines (20) (Step 5). If the desired separation criteria have not been met (Step 3.2), then another set of column and mobile phases with different selectivity should be chosen, and the process restarted (Step 1).

**2D-LC–MS-Based Determination of Peak Purity (9,10)**

The final two papers in the series (9,10) describe a strategy for determination of peak purity using 2D-LC–MS, based on the findings from papers (4–8), where the focus was on isomer selectivity using volatile mobile phases to allow MS detection. The objective was to identify column and mobile phase combinations...
that can be coupled in the first (1D) and second dimension (2D) separation to maximize the probability of determining isomers that may coelute in the 1D separation. One typical application for peak purity analysis using 2D-LC–MS would be during the validation of reversed-phase chromatography methods used for the analysis of peptide-based pharmaceuticals, to investigate whether an isomeric impurity coelutes with the main peak.

Furthermore, in addition to the need to identify suitable column and mobile phase combinations, an additional challenge involves defining the very shallow gradient required for the 2D separation. To achieve this, retention modelling (18) was investigated to assess if this could be employed to define such gradients in a rational way. Reference 9 focuses on the selection of suitable columns and mobile phases, whereas reference 10 aims to define and illustrate proof-of-concept for a workflow that yields appropriate gradient conditions for the second dimension.

Our findings suggest that a logical peak purity strategy based on 2D-LC–MS would consist of coupling the existing 1D peak purity methodology (as currently used in the laboratory) with a 2D separation consisting of a neutral character C8/C18 column and acetic acid–ammonium acetate (AA–NH₄AA pH 5.1), which has been shown to be highly effective for the separation of isomers (for example, see Figure 7). If poor peak shape is obtained with AA–NH₄AA pH 5.1, an alternative mobile phase that could be employed would be TFA. Formic acid–ammonium formate (FA–NH₄FA pH 3.6) is also an alternative, but TFA is more likely to give improved peak shape for basic peptides due to its ion-pairing properties. If the 1D method that is being evaluated for peak purity involves a mobile phase consisting of AA–NH₄AA pH 5.1, then it is recommended that the 2D separation utilizes TFA or FA–NH₄FA pH 3.6 mobile phases.

Previous investigations concluded that the MS response for peptides when using mobile phases buffered with FA–NH₄FA pH 3.6, TFA, or AA–NH₄AA pH 5.1 is approximately 20, 20, and 5% of the MS signal obtained with 0.1% formic acid (see reference 7, supplementary material table S1). With a typical sample load of approximately 3 µg of peptide on column, these responses should allow determination of isomers levels at <0.1% of the main component when using 2D-LC–MS. An example of results obtained from this workflow for the development of a 2D-LC–MS method is shown in Figure 7.

**Conclusion**

A peptide reversed-phase chromatographic column and mobile phase characterization protocol was developed using peptide probes synthesized for the purpose of interrogating the effects of mobile and stationary phase chemistry on separation selectivity. These probe molecules reflect chemistries encountered in common degradation pathways of peptides that include isomerization, racemization, and deamidation products that are often challenging to separate. Forty-four differing columns, all described by their manufacturers to be suitable for peptide analysis, and 51 different UV- and MS-compatible mobile phases, covering a pH range of 1.8 to 7.8 (including ion-pair reagents), have been characterized using the Peptide RPC Characterization Protocol. PCA was used to successfully categorize the columns into three sub-classes. This procedure was validated in that columns shown to be similar in the PCA score plots did, in fact, yield similar impurity profiles for several peptides. At the same time, columns judged to be dissimilar in the PCA score plots produced different selectivity profiles with several peptide samples. This allowed the selection of columns with complementary chromatographic selectivity for method development strategies, or similar ones in the case where “equivalent” columns were needed.
PCA of the data resulting from the study of different mobile phases highlighted that pH was a major contributor to selectivity. The chaotropic/ion-pair reagent ammonium hexafluorophosphate was found to generate interesting selectivity differences and merits further investigation. TFA, which has been ubiquitously employed in the analysis of peptides, was demonstrated to suppress selectivity differences between the stationary phases that are otherwise evident when using other mobile phases.

A reversed-phase chromatography method development strategy (30 total mobile/stationary phase combinations using screening technologies) based on the PCA findings was proposed and employed to separate a number of peptides and their impurities (including their diastereomers).

Finally, the work has identified columns and mobile phases that are sufficiently complementary and can be used in the second dimension of 2D-LC–MS methods for the purpose of assessing main peak purity in candidate reversed-phase chromatographic methods.

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Pitfalls in Proteomics: Avoiding Problems That Can Occur Before Data Acquisition Begins

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The analysis of peptides using liquid chromatography–mass spectrometry (LC–MS) for proteomics applications is powerful and can yield tremendously rich data sets. However, the exquisite sensitivity and global applicability of MS detection also makes it prone to contaminants that can easily and seriously compromise the quality of a data set. A short list of tips and tricks can increase the likelihood of consistently producing good LC–MS data in this context and streamline the troubleshooting process when problems do eventually occur.

Some applications of liquid chromatography (LC) involve highly specific “tips and tricks” that are acquired by analysts over time, and they can make the difference between producing data that is either terrible or great. The analysis of peptides by LC coupled with mass spectrometry (LC–MS) is one such area where it can appear that highly skilled analysts possess superpowers or “golden hands” that enable them to acquire better data than anyone else in a laboratory. In the end though, careful attention to detail and a thorough understanding of the conditions and chemistry involved in these analyses can help avoid problems before they occur or accelerate troubleshooting problems when they do appear. For this month’s instalment of LC Troubleshooting, I invited Daniel Meston to share his short list of knowledge, tips, tricks, and advice related to analyzing peptides by LC–MS. There are many details that are important to consider in the preparation of samples and in the instrument prior to LC–MS data acquisition that can increase the likelihood of acquiring high quality data.

- Dwight Stoll

LC–MS has become the gold standard methodology used for proteomics, which is the identification and quantification of protein abundances in biological samples. This is because of the ability of LC–MS to provide sensitive detection for untargeted peptides and identify unknown peptides through tandem MS (MS/MS) fragmentation and comparison of the experimental spectra to theoretical spectra prepared in silico. Subsequently, LC–MS-based proteomics tools have become invaluable in diverse disciplines ranging from immunology to microbiology and food chemistry. However, analysis of biomolecules is generally susceptible to a number of unique and perilous sample preparation pitfalls that can severely degrade the quality of data acquired, even when the most sophisticated LC–MS systems are used, which is because of the unique chemistry and size of large biological macromolecules, such as proteins, peptides, and oligonucleotides to name a few. In this instalment, we provide our views on some of the most common pitfalls in sample and instrument preparation relevant to proteomic analyses by LC–MS. We hope that increasing awareness of these potential problems can help users mitigate them and produce higher quality data in general.

Adding Contamination

All mass spectrometric methods require the ionization of the analytes of interest before they can be separated and ultimately detected. For the peptides encountered in proteomic workflows, the ionization step is generally straightforward because conventional tryptic digestion of proteins often results in amino acid chains with terminal amino groups. Indeed, the protonation of peptides is so favourable that they are often observed as multiply charged positive ions. Given the ease of ionization of peptides, MS can almost be regarded as a “universal detector” for peptide analysis. However, this sensitivity can also be a double-edged sword in the sense that the detector will also respond to very low concentrations of easy-to-ionize contaminants in
our samples, thus degrading the overall quality of the acquired data. **Polymers:** Polymers are perhaps the most frequently encountered type of contamination in proteomic samples, probably because they are present in most laboratories at levels high enough to matter to LC–MS work. A few examples of polymer sources include skin creams and moisturizing products, pipette tips, chemical wipes that contain an abundance of different polyethylene glycols (PEGs), and siliconized surfaces that contain polysiloxanes (PSs). The presence of these contaminants can be readily recognized in MS spectra by their characteristically large numbers of regularly spaced peaks in the spectra (44 Da spacing for PEG, 77 Da spacing for PS).

Another source of PEG contaminants is the use of surfactant-based cell lysis methods that are routinely employed in most molecular biology laboratories and involve surfactants such as Tween, Nonident P-40, and Triton X-100. Residual surfactant in samples that are produced using these chemical lysis methods have the potential to produce MS signals that can largely obscure the MS signal of the target peptides, thus rendering the data useless. A dramatic example of such a result is shown in Figure 1. When such protocols are used, extreme care should be taken to selectively remove the surfactants from the sample prior to analysis. Although it is certainly possible to remove PEG contaminants using solid-phase extraction (SPE), it is far easier in the long run to avoid the problem altogether and not use the surfactants for cell lysis in the first place. **Urea:** In addition to problems with surfactants, urea used in cell lysis buffers can also cause problems. It is known that urea can decompose to isocyanic acid, which can in turn covalently modify free amine groups in peptides through carbamylation reactions (1). This chemical modification of the peptide can be accounted for in peptide identification software, but only if the software is instructed to look for this. **Residual Salts:** In general, residual salts can negatively impact chromatographic performance as well, and they can cause physical damage to fluidics and the MS-interface instrumentation by scratching the surfaces and clogging the emitter. As a result, residual salts should be removed from the sample prior to injection. Both urea and salts are most commonly removed using a reversed-phase clean-up step, such as SPE. **Water Quality:** Not all high quality laboratory water is created equally, and there are many ways problems can arise with the water used for sample and mobile phase preparation. For example, in-line filters that are used to filter out deoxyribonucleic acid (DNA) can inadvertently lead to contamination of the water with PEG. Even the highest quality water produced on-site in the laboratory can begin to accumulate contaminants within a few days of production. Generally, we should avoid using water that has been sitting around for more than a few days after opening the bottle (if purchased) or production by a polishing system on-site. It is good practice to dedicate a specific subset of mobile phase bottles in the laboratory for LC–MS use only and to avoid washing them with any kind of detergent (2,3). **Keratins:** The most abundant protein contamination found in proteomic samples are the keratin proteins that make up our skin, hair, and fingernails. It is not uncommon to observe more than 25% of the peptide content of a proteomics sample to originate from keratin-derived peptides. Any steps that can be taken to reduce this type of contamination will improve the ability to detect low-abundant proteins of interest. In general, natural fibre clothing, such as wool, should not be worn in proteomics laboratories. Ideally, all sample preparation steps should be performed in a laminar flow hood to prevent dust and skin from the analyst and laboratory air from entering samples. Additionally, gloves should be worn at all times and replaced after touching a contaminated surface, such as stopwatches, pens, and

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**FIGURE 1:** Comparison of LC–MS total ion chromatograms for the same peptide sample before (red) and after (black) cleanup by SPE to remove PEG contaminants. If the PEG is not removed, those species completely dominate the MS signal, making extraction of useful information about the target peptides extremely difficult.
A number of strategies to minimize concentration of low-abundant peptides. A lesser appreciated pitfall in proteomics sample preparation is adsorption of the peptides of interest to the vessel used for sample pretreatment (for example, digestion with trypsin), as well as LC sample vials. Peptides are multifunctional molecules with tremendous diversity in properties, including hydrophobicity and the number of acidic and basic functional groups. These properties make them prone to adsorption onto materials with very different properties, such as glass and plastics. This observation is also true for proteins but to an even greater degree (5). Adsorption of peptides from analytical samples onto such surfaces has been observed within timeframes as low as an hour after being placed in the LC vial, and they can result in a significant decrease in the apparent concentration of low-abundant peptides. A number of strategies to minimize sample adsorption to glass LC vials have been demonstrated, including the use of surfactants (which is not recommended for LC–MS, as discussed above). “Priming” vessels with a sacrificial protein, such as bovine serum albumin (BSA), is also done, similar to what is done with LC columns (4). The idea here is that the vessel is rinsed with a solution of a protein that is unrelated to the target proteins being analyzed (or synthetic peptides not found in biological samples); adsorption sites on the material are subsequently saturated and therefore unable to further adsorb peptides or proteins of interest from the analytical sample. Many vendors also offer “high-recovery” LC vials and other products that are engineered to minimize such undesirable analyte adsorption. An additional point to be aware of in this area is related to the removal of the sample solvent during the sample preparation process (for example, vacuum centrifugation to remove organic solvents). Completely removing all of the solvent promotes strong analyte adsorption onto surfaces; thus, it is helpful to avoid complete drying and to leave a small amount of liquid in the vial to increase analyte recovery.

Plastic micropipette tips present another opportunity to lose peptides to adsorption. With this in mind, it is important to limit the number of sample transfers during sample preparation, which has spurred the development of a number of so-called “one-pot” sample preparation methods that minimize contact between the sample and vessel, including nanoPOTS (6), SP3 (7), and FASP (8). From this research, it is becoming increasingly clear that single reactor vessel sample preparation protocols are superior to conventional protocols in the proteomics area.

Finally, when transferring peptide samples, it is best to avoid contact with metals. For example, most glass syringes are fitted with stainless steel needles by default. If a peptide sample is drawn up through a metal needle, peptides can be lost to adsorption on the metal surface. One way to avoid this scenario when using glass syringes is to remove the plunger, fill the sample into the glass syringe barrel using a glass pipette, and then push the sample out of the syringe using the plunger, but through a PEEK capillary fitted to the syringe via a Luer lock fitting, rather than pushing it through the metal needle. Doing so is particularly important in the case of MS calibrations where many vendors utilize a peptide calibrant that can be depleted by metal syringe needles.

**Protein and Peptide Adsorption**

Peptide adsorption to the LC columns has been addressed in previous installments of “LC Troubleshooting” (4). One way to avoid this scenario when using trifluoroacetic acid (TFA) as a mobile-phase additive to improve chromatographic performance for peptides (that is, better peak shape, and increased retention, especially for hydrophilic peptides), this practice is generally frowned upon in the proteomics community because TFA can dramatically suppress peptide ionization, leading to lower overall detection sensitivity compared with using formic acid. An alternative to using TFA in the mobile phase is to add it to the sample and use formic acid to acidify the mobile phase. In this case, the TFA can enhance the retention of hydrophilic peptides on the precolumn, preventing them from eluting into the waste line. Meanwhile, the TFA exiting the precolumn is diverted to waste rather than entering the analytical column and MS interface.

Proteomics applications, which are outside conventional peptide sequencing, may also require specific buffers that preserve the structure or stability of proteins during analysis. It is not uncommon for these buffers to lead to salt precipitation on the electrospray emitter and the source cone of a MS system. In this way, one must optimize the conditions to use volatile buffers, such as ammonium acetate, or to physically clean the surfaces with water to prevent significant sensitivity loss. Recently, it has been demonstrated that postcolumn suppressor technology provides more flexibility in terms of the types of buffers that can be used, some of which are normally considered MS-incompatible (9).

Finally, the age of prepared mobile phases can be particularly problematic in proteomics applications where nano-flow systems are prevalent and mobile phase

**Sample Matrix Incompatibilities**

Other aspects of the sample matrix are worthy of careful consideration. Although it is attractive to consider using trifluoroacetic acid (TFA) as a mobile-phase additive to improve chromatographic performance for peptides
Consumption is so low that a 100-mL batch of the mobile phase can last several weeks. Specifically, when buffer components with differing volatilities are mixed together, there can be selective evaporation of the more volatile component that will change the elution or ionic strength of the mobile phase, which can lead to changes in both absolute and relative peptide retention times. Primarily, this occurrence comes from incorrect viscosity values that change the mixing behaviour of modern nanoLC instruments. Additionally, changes in ionic strength will have a direct effect on the retention factor of the analytes, which will lead to variation in retention time. As a rule of thumb, mobile phases should be replaced every 1–2 weeks to ensure reproducible results. Additionally, in the case of pumping systems that rely on known mobile phase viscosities for accurate flow rates, the mobile phase viscosity should be determined when changing solvents. Instruments that require this feature build in software routines to facilitate these measurements. An additional point is to consider not using conventional membrane degassers when using nano-flow rate because the flow rate is so slow during this process. It has been noted in our laboratory that volatile buffer components, such as formic acid, are preferentially removed from the mobile phase during degassing. In these situations, it is better to utilize alternative degassing measures, such as sonication, that have a low risk for additional contamination of the mobile phase.

**Optimizing the Electrospray Source Voltages in nanoLC–ESI–MS**

Finally, as we reach the last step prior to the actual acquisition of LC–MS data, we must think about settings for the MS detector itself. Arguably, one of the most consequential missed opportunities in data acquisition for proteomics workflows is related to the optimization of electrospray source voltages. Once analytes are ionized, they are steered through the MS instrument in the direction of the detector using carefully manipulated electric fields. Therefore, analyte detection is highly dependent on the voltage settings for the ion optics that guide the motion of the ionized analytes. Failure to optimize these voltages can result in peptides over large regions of the mass range not making it to the detector, or significantly reduced numbers of detected ions.

Before analyzing any experimental samples, it is worth taking the time to incrementally change the ion source parameters step-wise (for a representative ion infused at the flow rate that will be used in the LC–MS method, we recommend the doubly charged ion of Glu-Fib or angiotensin II), while monitoring the signal change and the spectrum quality. For most modern MS instruments, this tuning step can be done automatically within the MS data acquisition software, but it can be beneficial to further manually fine-tune parameters to maximize signal for the ions of interest. Finally, once the detector settings have been adjusted, it is a good idea to analyze a well-characterized quality control (QC) sample (for example, a cytochrome C digest or HeLa cell digest) to enable a final check on the data quality before analyzing any novel samples.

**Summary**

Because of the size and varied chemistry of peptides found in proteomics samples, it is important to pay attention to details of each step leading to the acquisition of LC–MS data. This includes sample preparation chemistries, sample handling, optimization of instrument parameters, and control of mobile phases. Paying attention to these details from the start can help avoid data quality problems entirely and simplify troubleshooting when problems do arise. We hope that the tips and tricks discussed here are food for thought for analysts of all levels of experience, and ultimately improve data quality and minimize loss of precious samples and instrument time because of unanticipated problems.

**References**


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Selectivity and Sensitivity: The Electron Capture Detector (ECD), Its Unique Inventor James Lovelock (1919–2022), and GAIA

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The electron capture detector (ECD) is among the original classical detectors for gas chromatography (GC). It is highly selective and sensitive for electron-absorbing compounds, especially those containing halogens. The ECD was developed in the 1960s by James Lovelock (1919–2022), who passed away earlier this year. The ECD is among the early detectors that is used in a relatively unmodified form today. The ECD was crucial in the discovery of chlorofluorocarbons (CFCs) in the upper atmosphere, ultimately leading to the international agreements limiting their use and reducing the ozone hole. In this instalment, we review the ECD and its principles of operation and discuss the general trade-offs in detection between selectivity, ease of use, and sensitivity. We also look at the unique life and work of Lovelock, both inventor of the ECD and one of the earliest scientists to bring attention to climate change through GAIA, which explained his theory that the Earth acts as a massive living organism.

This summer, I was watching the news show “Sunday Today”, and heard a name familiar to many gas chromatographers: James Lovelock. To chromatographers, Lovelock invented the electron capture detector (ECD) for gas chromatography (GC). He was featured in the weekly “Life Well Lived” segment, where the show features people who are not widely known to the public but made contributions to public life (1). The segment describes how the ECD and Lovelock’s own work provided major influences on climate science today, nearly 60 years later.

Detection in GC remains unique in analytical chemistry because of the large number of customized detectors. For early workers, detection was especially challenging because the detector was required to be sensitive, selective, and able to detect a low concentration or mass of analytes in a rapidly moving stream of carrier gas. Interestingly, many of these early detectors, including flame ionization detectors (FIDs), thermal conductivity detectors (TCDs), and ECDs remain widely used today, along with myriad additional ionization, physical property, and spectrometric detectors. Schug and McNair provided a summary and “grade sheet” for commonly used GC detectors (2). The ECD was graded A+ for limit of detection (LOD) and specificity, but it was given a C grade for qualitative speciation, linear range, and robustness, and a D grade for universal response.

A summary of the basic principles and operation for the ECD is provided in textbooks on GC and available in ChromAcademy, LCGC Europe’s online learning platform (3–5). Overall, the ECD provides a unique combination of selectivity and sensitivity. Implied by its name, the ECD offers selectivity for compounds that include atoms that are favourable for attracting or capturing an electron. Recalling general chemistry, note that elements on the right and up in the Periodic Table tend to attract electrons. These include the most common heteroatoms, especially halogens. The ECD is highly selective for compounds containing these elements, and by contrast, generates little or no signal for common hydrocarbons. The ECD presents a useful complement and contrast to the FID, which gives strong signals for hydrocarbons.

Highly selective detectors, in general, offer greater sensitivity for those analytes for which they are selective. The ECD is noted to be the most sensitive of the classical detectors, with detection limits easily in the part-per-billion (ppb)
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The operating principle, while not fully understood, first involves the production of electrons from a radioactive source, typically $^{63}\text{Ni}$. For safety, a sealed source is used and usually the detector manufacturer will have a blanket radioactive isotope license in case the end user does not. Radioisotope licensing was an early barrier for ECD users. If you ever need to dispose of an ECD, you should consult with the manufacturer, a licensed health physicist, or radiation safety officer for proper disposal procedures.

Figure 1 shows a schematic diagram of a typical ECD. The operating principle, while not fully understood, first involves the production of electrons from a radioactive source, typically $^{63}\text{Ni}$. For safety, a sealed source is used and usually the detector manufacturer will have a blanket radioactive isotope license in case the end user does not. Radioisotope licensing was an early barrier for ECD users. If you ever need to dispose of an ECD, you should consult with the manufacturer, a licensed health physicist, or radiation safety officer for proper disposal procedures.

Figure 1 shows a schematic diagram of a typical ECD. Note the stronger argon detector signals for the hydrocarbon and fluorinated compound, for the singly-chlorinated benzene, and the strong ECD signals for the multiply chlorinated compounds.

In 2007, Ettre and Morris provided a thorough discussion “The Saga of the Electron Capture Detector” in *LCGC Europe* (7). They traced the developments that led to the ECD beginning in 1948, showing a fascinating scientific story that began with studying a different problem (whether drafts of cold air in a room really do cause illnesses) for which Lovelock developed an anemometer that worked by disturbing the slow flow of positive ions from a radon source. This anemometer was so sensitive that the signal was disturbed by small amounts of cigarette smoke in the atmosphere, capturing electrons, although at the time this was seen as a problem and not of any interest. Recall that smoking was much more prevalent in the 1950s than it is today. In my own career working with modern ECDs, I have observed deflections in ECD signals because of the presence of cigarette smoke odours. The full story, presented by Ettre and Morris, of how this observation ultimately led to today’s ECD is well worth the read.

The Science Museum of London also has an early ECD and other equipment, including Lovelock’s own home-made gas chromatograph on display and an extensive treatment of Lovelock’s life and career, along with Lovelock’s entire personal archive of documents and letters (8).

**Lovelock, Atmospheric Chemistry, and GAIA**

Lovelock’s prolific scientific career, spanning seven decades, included many more contributions than inventing the ECD for use in GC. As with many of the early gas chromatographers, his career started in other areas. His first publication in 1941 related to the use of hypochlorite spraying for disinfectants (9). Then, he had a prolific publication record in the general area of disinfectants, sports training, prevention of the common cold, and other medical topics through the remainder of the 1940s and early 1950s. After earning his Ph.D. in medicine in 1948, a shift towards instrumentation and measuring devices was seen throughout the 1950s. Through the 1950s, his medical research shifted to many aspects of preservation and chemical effects on red blood cells and some interestingly titled articles related to the re-animation of rats and mice frozen to 0 °C.

As Ettre and Martin describe, in the 1950s, Lovelock’s laboratory was in close proximity to A.J.P Martin and A.T. James, who invented GC, and they became collaborators, with Martin suggesting to Lovelock that he develop a highly sensitive detector that was operable by average chemists. They noted that the most sensitive detectors of the day were not easily operated by most chemists. The first mention of GC in the title of an article written by Lovelock occurred in 1958. In the article, he discussed an early
detector, not the ECD but the argon ionization detector, which like the ECD used a radioactive source to produce electrons that ultimately ionized analytes. It was a nearly universal detector, used for many applications that were ultimately supplanted by the FID, which did not require a radioactive source (10). Using the argon ionization detector, Lovelock then published numerous papers on the ionization mechanisms of organic compounds in the vapour phase, most often using GC with the argon ionization detector.

Following the initial invention and publication of the ECD (6), Lovelock worked with Zlatkis to commercialize the detector, founding a company called Ionics Research, which provided ECDs to PerkinElmer and the Wilkens Instrument and Research Company, which later became the Chromatography Division of Varian bought in 2010 by Agilent Technologies. In 1962, coincidentally with the publication of the classic book Silent Spring by Rachel Carson, the first papers demonstrating ECD of pesticides at the picogram level were being published; although, as pointed out by Ettre and Morris, the ECD is often incorrectly cited, including in the “Sunday Today” segment, as one of the techniques on which Carson’s findings were based (11). However, there is clear connection between Lovelock’s work, the ECD, the early environmental movement, and today’s challenges with climate change.

Using the ECD, Lovelock published the first work demonstrating the presence of chlorofluorocarbons (CFCs) in the atmosphere over the Atlantic Ocean between England and Antarctica (12). This work demonstrated the presence of Freon-11 and other compounds resulting from the use of aerosol cans and refrigerants. Roland and Molina then used this work in developing the theory that halocarbons in the atmosphere were reacting with stratospheric ozone in a chain reaction, causing the depletion of ozone and the famous “ozone hole” (13). Roland, Molina, and Crutzen shared the Nobel Prize in 1995, and this work resulted in international treaties limiting the use of halocarbons.

We close with GAIA, a fundamental aspect of climate science today, which was mocked by most when introduced by Lovelock in the 1970s. Much like the seeming lack of interest in the 1950s in a detector that was affected by cigarette smoke in the room, GAIA, named for the Greek goddess of the personification of Earth, proposed that the Earth is a single self-sustaining organism, and this idea was universally rejected by the scientific community of the day (14). The basic principles and problems described by Lovelock in his original work remain debated in climate discussions today. With the invention of the ECD, its use in discovering CFCs in the atmosphere, work in planetary, medical, space, and climate science, James Lovelock was
References

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VENDOR VIEWPOINTS

KNAUER

Q. What do you consider the most important development in separation science in the past 35 years?
In the field of LC, I can name many developments. We have seen low dispersion systems, sub-3-µm stationary phases, all coming onto the market—without counting the number of different detectors and electrospray ionization sources to interface LC and MS. In my opinion, if we need to name just one, the most important development is the synergistic effect of all these contributions. The result? Liquid separations are now versatile, robust, and widespread techniques for the efficient analysis and purification of samples.

Q. What do you think is currently the most pressing problem or need in separation science?
Beside numerous open questions, many readers will agree with me that the main pressing problem is the lack of expertise in the field of chromatography. We can see it as a positive phenomenon because it tells us that chromatography is popular, reaching laboratories and users outside the “expert zone”. Our role as chromatography experts is to help new users become familiar with these techniques and support them in method development. I strongly believe that a conscious approach to the technique creates more environmentally friendly separations. Why? Because you need less trials for developing a method and you are empowered to optimize the analysis and obtain results in the shortest time possible. This can translate into a big solvent saving! The lack of expertise together with the popularization of LC will fuel innovation. In the last 35 years, we have focused on improving the performance of LC systems. For the next 35 years, I see more expertise being added to the system and more attention being paid to system design, user interface, software, sample preparation, and energy and solvent consumption. Removing challenges for the users will lead to a wider and greener dissemination of LC.

TOSOH BIOSCIENCE

Q. What do you consider the most important development in separation science in the past 35 years?
Since its introduction, HPLC has been constantly evolving, and Tosoh has been part of it: Our column brand TSKgel turned 50 last year. A lot has been done to improve analysis speed and resolution. There has been tremendous development in multidimensional and comprehensive methods: In the 1990s, 2D approaches were still quite exotic and performed on self-configured systems, not to mention the lack of appropriate software. Today, this technology is commercially available. In the last two decades, MS detection has given HPLC a major boost. Biomolecules are becoming increasingly important therapeutics. As the separation of biomolecules has traditionally been our focus, we are accompanying the increasing demand for dedicated columns for the characterization of biotherapeutics. Increasing selectivity is currently a hot topic, for example, through the use of affinity ligands.

Q. What do you think is currently the most pressing problem or need in separation science?
Similar to analytical science in general, separation science is often considered an enabling technology rather than a science in its own right. The number of research groups dedicated to fundamental research in separation science has declined, resulting in a limited number of qualified young chromatographers leaving academia. At the same time, the industry is desperately seeking professionals in analytical science and especially in chromatography because it is essential, from R&D to quality control. Technologically, the excellent columns and instruments/detectors available today need to be integrated into platforms that enable rapid and efficient analysis of multiple COAs at once. For stationary phases, there is a need for alternate selectivities, for column formats ready for integration in automated workflows, and (U)HPLC columns optimized to take full advantage of the sensitivity gain offered by MS and LS.
Thermal Desorption

Unity-xr is an analytical thermal desorption instrument that provides exceptional performance for VOCs/SVOCs collected onto 3½-inch sorbent tubes, while offering unmatched upgrade flexibility for tube, canister, and online automation, according to the company. The instrument is reportedly perfect for laboratories with low throughput requirements, or for analysts needing a versatile thermal desorber.

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Postnova Analytics GmbH, Landberg, Germany.

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www.vici-dbs.com
VICI AG International, Schenkon, Switzerland.

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www.lni-swissgas.eu
LNI Swissgas, Milan, Italy.

Bioinert (U)HPLC Columns

YMC-Accura Triart (U)HPLC columns are characterized by a bioinert surface coating for challenging substances (oligonucleotides or peptides/proteins). According to the company, they provide excellent peak shapes, prevent carryover without any preconditioning, offer secure recovery, and are ideal for use in highly sensitive LC–MS analyses.

https://ymc.eu/d/brDoj
YMC Europe GmbH, Dinslaken, Germany.

Headspace Sampler

The 2t sampler is the first manual system for static headspace that allows the quantitative application of this technique in a simple and economical manner, according to the company. The product meets all CE requirements. The sampler is suitable for applications such as volatiles in pharmaceuticals, flavour analysis in food and cosmetic products, alcohol and other toxic compounds in blood, and many more.

https://www.teknokroma.es/head-space/injector-manual/
Teknokroma Analítica S.A., Barcelona, Spain.
The Applications Book

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Rugged, Reliable Semivolatiles Analysis on Rxi-SVOCms Columns

Chris Rattray, Restek Corporation

Semivolatiles analysis is an essential part of environmental testing programmes, yet it can be difficult for laboratories to run methods efficiently because target analyte lists are extensive and contain a diverse array of active compounds. To accurately report semivolatiles, particularly reactive ones, highly selective and inert columns must be used to ensure good chromatography and stable calibrations. In this report, Rxi-SVOCms columns were evaluated for both chromatographic performance and ruggedness. These columns are designed specifically for semivolatiles analysis using new polymer and deactivation chemistries that produce highly inert columns with tightly controlled selectivity for a wide range of analytes (acidic, basic, and neutral). Our testing of 94 semivolatiles from EPA Method 8270 specifically assessed peak shape and resolution for critical compounds, calibration stability, and ruggedness.

Chromatographic Performance

As shown in Figure 1, problematic reactive semivolatiles show highly symmetrical peak shapes and good responses on Rxi-SVOCms columns. In addition, excellent resolution (≥85% valley) is obtained for benzo[b]fluoranthene and benzo[k]fluoranthene (peaks 88 and 89), which are isobaric PAHs that must be separated chromatographically, as well as for indeno[1,2,3-cd]pyrene and dibenz[a,h]anthracene (peaks 92 and 93). Good chromatographic separation allows for positive identification, easy integration, and accurate reporting.

Calibration Stability

To evaluate calibration performance, calibration standards were analyzed on six different columns. The passing calibration range for most compounds was 1–120 µg/mL. Only benzoic acid, 2,4-dinitrophenol, and 4,6-dinitro-2-methylphenol fell outside this range, and these three compounds all had a range of 2.5–120 µg/mL. In addition, the overall average %RSD for response factors across all compounds and columns was just 6.00%, and all individual compounds, except benzoic acid, had very low %RSD values indicating that calibration performance was very consistent. Individual results for all compounds are reported at www.restek.com/Rxi-SVOCms. Extremely low and consistent results ensure that calibrations will last longer, allowing more samples to be run before recalibration is required.

Figure 1: Rxi-SVOCms columns provide excellent chromatographic results, reliably producing good peak shape and resolution even for problematic compounds. (Analysis of 94 semivolatiles from EPA Method 8270; peak identifications and conditions are available at www.restek.com/Rxi-SVOCms.)
Ruggedness
Improved column chemistry ensures that the chromatographic performance of Rxi-SVOcms columns is maintained even under very aggressive conditions. We tested this using a 300-sample ruggedness test, which was conducted as follows. Each day, 30 injections of a diesel particulate extract (NIST SRM 1975) were made, and a continuing calibration verification (CCV) standard was run after every 10 sample injections. After the 3rd daily CCV, the column was trimmed, and the liner, septum, and inlet seal were replaced. This sequence was repeated for 10 days, and the entire experiment was repeated on a second column. As shown in Figure 2, even after 300 injections, performance was easily restored with a quick column trim as evidenced by fewer than 10% of compounds failing the post-trim calibration check. Bringing back performance with simple routine maintenance means more samples can be analyzed with less downtime and fewer column replacements.

Conclusion
Designed specifically for semivolatiles analysis, Restek’s new Rxi-SVOcms columns ensure consistent performance that will keep calibrations passing longer, so laboratories can run more samples before needing to recalibrate the instrument or replace the column. For chemists in the environmental industry who are slowed down by variable column performance, frequent calibration failures, and poor column lifetimes, using a rugged Rxi-SVOcms column can ensure data requirements are met longer and downtime is minimized.

Figure 2: Column performance is completely restored by trimming following repeated exposure to a highly complex sample (diesel particulate extract). The blue line includes all CCV injections and demonstrates that performance was first lost, as expected due to contamination from the sample matrix, and then fully restored following maintenance. The green line plots only post-maintenance CCV injections and demonstrates calibration performance stability.
Healthy Chocolate?—UHPLC Determination of Ingredients in Dark Chocolate

Juliane Böttcher, Kristin Folmert, and Kate Monks, KNAUER Wissenschaftliche Geräte GmbH

Chocolate...a healthy food? Recently, polyphenols have gained in attention due to their antioxidant capacity and their possible beneficial implications in human health (1). As more studies associate an increasing number of health benefits to its high antioxidant content, dark chocolate has been labelled as a “superfood”. But not all ingredients are considered to have health-beneficial properties. In this application note, 14 chocolate-relevant compounds were separated in under 1.5 min.

Cocoa products contain many physiologically active compounds. The high level of fat contributes to the high energy content of the cocoa bean. Despite its high nutritional value, however, the presence of caffeine and theobromine alkaloids may limit its potential as a nourishing food. The determination of the levels of methylxanthines and polyphenols in cocoa products is becoming increasingly important in the light of recent concern about the health effects of these compounds and their widespread consumption by the public. Methylxanthines such as theobromine and caffeine are typical compounds present in coffee, tea, chocolate, and products made of them (2,3). Beside polyphenols and methylxanthines, this application allows the simultaneous determination of common preservatives, sweeteners, and flavouring substances that can also be present in dark chocolate.

Sample Preparation
The sample preparation of chocolate included the following steps:

1. Defatting: 1 g of a chopped chocolate sample was defatted with 3 x 10 mL hexane. For this, 10 mL of hexane was added to the sample, sonicated, and centrifuged. The hexane supernatant was decanted and discarded each time.

2. Drying: The chocolate residue was dried overnight. Faster drying is possible using a slight nitrogen gas stream.

3. Extraction: The dried sample was then extracted with a 80:20 (v/v) mixture of methanol and water. A 5-mL measure of the extractant was added to the dried sample, sonicated, and finally centrifuged. The supernatant was collected. The extraction procedure was repeated two more times, to receive in total 15 mL of extracted supernatant.

4. Concentration/filtration: The extract was concentrated to a volume of 1–2 mL. The concentrated extract was then diluted with water to a volume of 10 mL. Last, the sample was filtered through a syringe filter with a pore size of 0.45 µm.

5. Injection: 2 µL of the prepared sample were injected to the HPLC system.

Results
In Figure 1, a mixed standard containing acesulfame K, theobromine, saccharin, theophylline, caffeine, chlorogenic acid, catechin, epicatechin, 4-hydroxybenzoic acid, vanillin, guaiacol, sorbic acid, methyl paraben, and propyl paraben was separated in 1.5 min. The presence of all components in one sample matrix is not common to clearly identify and quantify all peaks, but the separation is technically feasible. As seen in Figure 1, the peaks are all baseline separated except guaiacol and sorbic acid. However, resolution was sufficient to clearly identify and quantify all peaks. Furthermore, two different

Figure 1: Chromatogram of mix standard: 1) acesulfame K, 2) theobromine, 3) saccharin, 4) theophylline, 5) caffeine, 6) chlorogenic acid, 7) catechin, 8) epicatechin, 9) 4-hydroxybenzoic acid, 10) vanillin, 11) guaiacol, 12) sorbic acid, 13) methylparaben, 14) propylparaben.

Figure 2: Overlay of extracted chocolate samples (85% cocoa – red, 75% cocoa – blue); 2) theobromine, 5) caffeine, 8) epicatechin.
chocolate samples were prepared. Both samples were dark chocolates with 75% cocoa solids and 85% cocoa solids, respectively. Figure 2 shows the overlaid chromatogram traces of the extracted samples with 75% cocoa (blue) and 85% cocoa (red). As expected because of their natural occurrence in cocoa plants, a high level of theobromine and caffeine was measured; epicatechin was also detected. Referring to the higher cocoa amount, the values for caffeine and theobromine in the sample with 85% cocoa were higher. Figure 3 displays the overlay of the mixed standard and the chocolate sample containing 85% cocoa.

**Conclusion**

This method allows a robust, sensitive, and fast determination of several ingredients present in dark chocolates. Because of the variety of determined compounds, from sweeteners up to polyphenols and methylxanthines, the method is suitable for quality control even in a complex matrix. The relatively time-consuming sample preparation is mandatory but tolerable due to the fast analysis time.

**Materials and Methods**

**Table 1: System configuration**

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Description</th>
<th>Article No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pump</td>
<td>AZURA P 8.1L</td>
<td>APF45PA</td>
</tr>
<tr>
<td>Autosampler</td>
<td>AZURA AS 6.1L, 1240 bar</td>
<td>AAA10AA</td>
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<tr>
<td>Detector</td>
<td>AZURA DAD 6.1L</td>
<td>ADC11</td>
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<tr>
<td>Flow Cell</td>
<td>Standard KNAUER LightGuide UV</td>
<td>AMC19XA</td>
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<td>Thermostat</td>
<td>AZURA CT 2.1L</td>
<td>ATC00</td>
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<tr>
<td>Column</td>
<td>ACE Excel C18-Amide, 100 x 2.1 mm</td>
<td>–</td>
</tr>
<tr>
<td>Software</td>
<td>OpenLAB CDS EZChrom Edition - Workstation</td>
<td>A2600-1</td>
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<tr>
<td>Software</td>
<td>OpenLAB CDS EZChrom Edition - PDA/3D UV</td>
<td>A2611-1</td>
</tr>
</tbody>
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**Table 2: Method parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent A</td>
<td>10 mM ammonium formate pH 2.8 in water</td>
</tr>
<tr>
<td>Eluent B</td>
<td>10 mM ammonium formate in acetonitrile–water</td>
</tr>
<tr>
<td>Gradient</td>
<td>Time [min] % A % B</td>
</tr>
<tr>
<td></td>
<td>0 100 0</td>
</tr>
<tr>
<td></td>
<td>1.50 100 0</td>
</tr>
<tr>
<td></td>
<td>2.00 0 100</td>
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<td></td>
<td>2.02 100 0</td>
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<tr>
<td></td>
<td>5.00 100 0</td>
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<tr>
<td>Flow Rate</td>
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<tr>
<td>Detection</td>
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<tr>
<td>Data Rate</td>
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<tr>
<td>Time Constant</td>
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</tr>
<tr>
<td>Injection Volume</td>
<td>2 μL</td>
</tr>
</tbody>
</table>

**References**

ANAKON 2023

After a hiatus of four years due to the Covid pandemic, the ANAKON conference series is back in 2023! The ANAKON conferences are the flagship events of the German, Swiss, and Austrian analytical chemistry communities and jointly organized every other year by the Fachgruppe Analytische Chemie of the German Chemical Society (GDCh), the Division Analytical Sciences of the Swiss Chemical Society, and the Austrian Society of Analytical Chemistry (ASAC) within the Austrian Chemical Society (GÖCh). ANAKON 2023 will be organized in Vienna, Austria, from 11–14 April 2023 by the Conference Chairs Martina Marchetti-Deschmann, Erwin Rosenberg, and Victor U. Weiss at TU Wien. The conference venue is located right in the centre of Vienna, next to many of the city’s landmark historical sights. Spacious and well-equipped lecture theatres and ample space for a company exhibition and poster displays guarantee vivid interaction of the participants during breaks and social events.

ANAKON 2023 is a conference open to all fields of analytical chemistry. It is intended to showcase new developments in instrumentation and analytical methodology, as well as important novel applications. A great variety of topics will be addressed at this conference, including—but not limited to—separation sciences, mass spectrometry (including ion mobility separation), miniaturization, microfluidics, imaging and lab-on-a-chip technologies, sample preparation, sensors, surface analysis, spectroscopy from the macro- to the nanoscale, and hyphenated techniques for more comprehensive analysis. The application of the above-mentioned methods to the fields of bio- and clinical analysis, environmental analysis, and food analysis, and the application of analytical techniques in the determination of micro- and nanoplastics or in the field of cultural heritage science will be highlighted. In addition, ANAKON 2023 will also host the Austrian MassSpec Forum. Besides fundamental studies, many presentations at this embedded event will cover the omics field, showing the importance of combining separation techniques with mass spectrometry to dig deep into very complex samples.

The scientific programme will boast plenary as well as keynote lectures in which the state-of-the-art and innovative applications of analytical techniques will be presented by prominent speakers. Each keynote lecture opens a session in which contributed lectures will present further facets of the topic addressed by the headline speaker. The scientific programme of the conference will be entirely in English.

A particular highlight of the conference programme will be the Pregl-Award Session, during which the Fritz-Pregl Medal will be awarded, one of the most prestigious awards presented by the Austrian Society of Analytical Chemistry. This Pregl Session will also be organized to honour the 80th birthday of Wolfgang Lindner (University of Vienna)—an earlier recipient of the Fritz Pregl Medal. In addition to the designated Pregl Medal awardee of 2023, several other earlier recipients of the Pregl Medal will give keynote presentations at this session.

Selected presenters of poster contributions will have the chance to participate in the “science slam” in which they can present their poster in the form of a flash presentation. The best presentations will be awarded with poster prizes.

The scientific programme will be complemented by a delightful social programme throughout the conference and by a company exhibition that is located within the same space as the poster sessions and the coffee breaks.

Submission of abstracts for scientific contributions is possible until 31 December 2022. Registration at early bird rates is possible until 31 December 2022.

Email: office@anakon2023.at Website: www.anakon2023.at

1–3 FEBRUARY 2023
The 10th International Symposium on the Separation and Characterization of Natural and Synthetic Macromolecules (SCM-10)
Amsterdam, Netherlands
E: info@scm-10.nl
W: https://scm-10.nl

15–17 FEBRUARY 2023
The 3rd Australian Symposium on Advances in Separation Science (ASASS 2023)
Hobart, Tasmania
E: vipul.gupta@3dmade.com.au
W: www.asass2023.org

18–22 MARCH 2023
Pittcon 2023
Philadelphia, Pennsylvania, USA
E: info@pittcon.org
W: http://pittcon.org

19–21 APRIL 2023
Analytica Vietnam
Ho Chi Minh City, Vietnam
E: analyticanvietnam@messe-muenchen.de
W: www.analyticanvietnam.com/en/

24–26 APRIL 2023
34th International Symposium on Polymer Analysis and Characterization (ISPAC 2023)
Stellenbosch, South Africa
E: contact@ispac-conferences.org
W: https://ispac-conferences.org

18–22 JUNE 2023
HPLC 2023
Düsseldorf, Germany
E: oliver.schmitz@uni-due.de/michael.laemmerhofer@uni-tuebingen.de
W: www.hplc2023-duesseldorf.com

17–22 SEPTEMBER 2023
6th International Mass Spectrometry School
Cagliaria, Sardinia (Italy)
E: gianluca.giorgi@unisi.it
W: www.spettrometriadimassa.it/imss2023/#hero

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