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Tips & Tricks: Influence of Sample Dispersity on the GPC/SEC Separation of Multi-Component Systems

Wolfgang Radke, Miriam Lossa, and Jasmin Preis, PSS Polymer Standards Service GmbH, Mainz, Germany

The main task of gel permeation chromatography/size-exclusion chromatography (GPC/SEC) is the determination of molar mass distributions and molar mass averages. For this purpose, broad peaks, mostly without fine structures, suffice. However, sometimes two- or multi-component systems must be analyzed, and the different components need to be separated for quantitation. While chromatographic conditions can be adjusted to achieve fully separated peaks in high performance liquid chromatography (HPLC) of low molar masses, this is often not the case in GPC/SEC. This instalment of Tips & Tricks focuses on whether a complete separation can be achieved using GPC/SEC and, if so, under what conditions.

Gel permeation chromatography/size-exclusion chromatography (GPC/SEC) is usually applied to determine molar mass distributions and average molar masses. However, to adjust application properties, the use of polymer mixtures has increased...
over the years, which has led to a demand to quantify the amounts of the different polymeric components within a mixture. For this purpose, customers often attempt to apply GPC/SEC.

Ideally, GPC/SEC separates according to the size of macromolecules in solution. The size of a macromolecule is related to its molar mass. Thus, two polymers differing in molar mass can be expected to exhibit different hydrodynamic volumes, allowing for a separation by GPC/SEC.

The resolution for a particular sample analyzed in GPC/SEC can be enhanced using a column bank or column combination instead of a single column, as discussed in a previous issue of Tips & Tricks in GPC/SEC (1).

The effect of a prolonged separation path on the resolution is exemplarily shown in Figure 1, which compares chromatograms of bovine serum albumin (BSA) separated with a single column as well as with a set of two columns. The x-axis was adjusted to allow for direct comparison of the chromatograms, despite a different number of columns. In both chromatograms the peaks of monomer, dimer, and trimer are observed. However, the peak resolution of the blue curve is superior to the black.
chromatogram due to the increased column length. This is particularly visible when the heights of the peak minima between the peaks are compared.

Thus, when it is necessary to separate a mixture of two polymer samples differing in molar mass, and to quantify the amount of each component, it appears as if the task to be solved lies in finding a suitable column combination.

Figure 2 shows chromatograms of three narrowly distributed poly(ethylene glycol) standards run on a single column and on a column bank consisting of three identical columns. Again, the x-axis was adjusted to allow for direct comparison of the chromatograms, despite a different number of columns. The average molar masses of two adjacent peaks differ approximately by factor 2–3. The dispersities \( D = M_w/M_n \) lie below \( D = 1.1 \), and, under these conditions, a full baseline separation is almost achieved.

However, in contrast to the example shown in Figure 1, there is no substantial gain in resolution of two adjacent peaks despite increasing the column length by a factor of three.

Why can a distinct enhancement of resolution be observed in Figure 1 but not in Figure 2—although in both cases the column length was increased?

The answer lies in sample dispersity. While in Figure 1 the individual peaks of monomer, dimer, and trimer correspond to monodisperse species, for which the peak width is dominated by band broadening effects, each peak in Figure 2 represents a sample exhibiting a—although narrow—molar mass distribution. This means that each peak does not contain molecules of only one specific molar mass and thus one specific hydrodynamic size, but is composed instead of a large number of very similar molecules differing in molar mass. Consequently, each peak of a given average molar mass consists of a large number of molecules of different sizes, resulting in a broadened chromatographic peak if separated by GPC/SEC.

Depending on the widths of the molar mass distributions, two samples differing in average molar masses may contain species of identical molar mass and thus identical size, which will elute at the same
GPC/SEC elution volume. Such overlapping size distributions do not allow for a proper separation of each component by GPC/SEC, which makes quantitation difficult or even impossible.

This becomes clear in Figure 3, which shows the molar mass distribution of the same standards mixture as depicted in Figure 2. Indeed, both low molar mass standards contain molecules in the range of approximately 800–1100 g/mol, while both of the two higher molar mass peaks contain molecules within approximately 1500–2500 g/mol.

However, we can conclude from Figure 2 and Figure 3 that narrowly distributed samples can almost be baseline separated using suitable GPC/SEC columns, if their average molar masses differ by a factor of approximately 2–3. Therefore, the separation of narrowly distributed samples differing in average molar mass by factor 2–3 is possible in principle, as the molar mass distributions and therefore the size distributions do not severely overlap (Figure 3).

This instalment of Tips and Tricks in GPC/SEC will elucidate the effect of molar mass (or more precisely, size) dispersity on the separation of polymer mixtures.

To get a better understanding of how dispersity affects separation in GPC/SEC, we will assume we have mixtures of two polymer samples that differ in their average molar mass. We will also assume that the same relationship between molar mass and hydrodynamic size exists for both polymer samples. Thus, identical molar masses in both distributions will correspond to identical hydrodynamic sizes and consequently to identical elution volumes in GPC/SEC.

We have seen that for narrowly distributed samples differing by a factor of 2–3 in molar mass, a good separation is possible. In industrial polymerization processes, narrowly distributed samples are, however, rarely produced. Samples prepared by free radical polymerization or polycondensation reactions feature typical dispersities of \( D > 1.5–2 \).

To investigate whether or to what extent separations of mixtures of such industrial samples can be achieved, we assume the samples to feature Schulz-Flory distributions (2), with dispersities of \( D = 2 \).

Figure 4 shows calculated molar mass distributions for 50/50 (w/w) mixtures of the first sample with a weight-average molar mass of \( 2 \times 10^4 \) g/mol and the second with a weight molar mass of \( 4 \times 10^4 \) g/mol (red), \( 6.7 \times 10^4 \) g/mol (blue), and \( 9.1 \times 10^4 \) g/mol (black), each with a dispersity of \( D = 2 \).
of a sample with a weight-average molar mass of $2 \times 10^4$ g/mol, and samples with weight-average molar masses of $4 \times 10^4$, $6.7 \times 10^4$, and $9.1 \times 10^4$ g/mol.

While for the narrowly distributed samples in Figure 2 a factor of 2 between the molar masses resulted in an almost baseline separation, the molar mass distribution of the 50/50 mixture of the two broadly distributed samples does not reveal any indication of the existence of two components mixed together, although the average molar masses of the components differ by the same factor of 2. Therefore, even on a well-separating GPC/SEC column, the resulting chromatogram is expected to show only one broad monomodal uniform peak.

When the molar mass difference between the components corresponds to a factor of 3.3, a weak shoulder can be observed in the chromatogram of the two-component mixture. However, this weak shoulder does not allow for quantitation of both components.

Only when the ratio of weight-average molar masses exceeds approximately a factor of 4.5 do two minima become visible in the molar mass distribution of the mixture.

Even if the average molar masses of two polymer samples, each featuring a dispersity of $D = 2$, differ by a factor of 10, a substantial overlap still exists in the molar mass distributions (Figure 5). Consequently, to separate the broadly distributed components for quantitation by GPC/SEC, the differences in their average molar masses must exceed approximately a factor of 10.

The problem of insufficient separation is related to the width of the molar mass distributions. In Figure 5, we see that the molar mass distribution of the sample with a molar mass of $M_w = 2 \times 10^5$ g/mol and $D = 2$ ranges from below $10^4$ g/mol to above $10^6$ g/mol. Likewise, the molar mass distribution of the sample exhibiting a molar mass of $2 \times 10^4$ g/mol and $D = 2$ also covers approximately two orders of magnitude in molar mass.

Besides the differences in molar mass, the relative ratios of both components also affect whether separated peaks can be observed for each component. In Figure 6, a sample with a weight-average molar mass of $2 \times 10^4$ g/mol is mixed with a sample with a molar mass of...
6.7 × 10^4 g/mol, each having a dispersity of D = 2. The weight ratio of both components was varied to investigate the effect of the weight ratio on peak overlap.

Adding a small amount (10%) of the high molar mass component to the lower molar mass component resulted in a weak shoulder at the high molar mass side of the distribution, while the addition of 10% of the low molar mass to the high molar mass component did not reveal any indication of the existence of a mixture. Again, quantitation is not possible without further information on the components.

It should be stressed that the insufficient separation of both components does not result from insufficient resolution of the GPC/SEC column or column combination but instead results from identical molar masses being present in both components of the two-component mixture. Therefore, enhancing the resolution by changing the GPC/SEC column or column combination will not overcome the general problem of overlapping molar mass (size) distributions.

In the calculations shown before, the problem of insufficient separations was discussed based on molar mass distributions. However, GPC/SEC separation relies on the molecular size (hydrodynamic volume) in solution and is not based on molar mass. Therefore, two polymers of identical molar mass might differ in their hydrodynamic size and might therefore elute at different elution volumes. However, this shift is usually not sufficient to overcome the effect of the peak width of broadly distributed samples.

If the molar mass distribution for each individual component of the mixture is known, quantitation might be possible using more sophisticated computational approaches, even for mixtures of chemically identical components.

If the sample components differ in their chemical composition, selective detection can be helpful to overcome the separation issue. Alternatively, separations by interaction chromatography might be an option.

**Summary**

- The success of GPC/SEC to separate mixtures of polymers strongly depends on sample dispersity and difference in molar mass (hydrodynamic size).
- Almost baseline resolution can be achieved for narrowly distributed samples, provided their weight-average molar masses differ by a factor of approximately 2.
- For 50/50 mixtures of typical technical samples, each featuring a dispersity of D = 2, the molar mass distributions do not show any indications for a two-component mixture as long as the molar mass averages differ by a factor less than approximately 3.5.
- The observation of a second maximum in 50/50 mixtures of broadly distributed samples requires the average molar masses to differ by a factor of at least 5.
- Quantifying the amounts of broadly distributed samples in a mixture requires the average molar masses to differ by a factor of more than 10.
- A minority amount of a broadly distributed sample of high molar mass in a majority of a broadly distributed polymer of low molar mass is easier to detect than a minority of low molar mass polymer in a high molar mass sample.
- The limited capability to separate mixtures of broadly distributed samples is not related to insufficient separation capabilities of the GPC/SEC column or column combination, but it is a consequence of the width of the molar mass distributions.

**References**


Miriam Lossa studied applied chemistry (B.Sc.) and completed her masters in bioanalytical chemistry and pharmaceutical analysis (M.Sc.) at Hochschule Fresenius in Idstein (Germany). She works at PSS in the R&D department, with a focus on the analysis of biopolymers.

Jasmin Preis studied polymer chemistry in Mainz (Germany) and is senior director of the chemical production department at PSS. She works on porous particles for chromatographic applications, as well as on polymer characterization using SEC coupling techniques.

Wolfgang Radke studied polymer chemistry in Mainz (Germany) and Amherst (Massachusetts, USA) and is head of the PSS application development department. He is also responsible for instrument evaluation and for customized trainings.
Markes Official Opening of New Site

Markes International moved its entire Welsh operations in March this year to relocate from Llantrisant to a newly refurbished site in Bridgend, which is twice as large as its former premises.

Markes’ Managing Director, Tim Hawkins, commented that the pandemic delayed any official opening, but with restrictions lifting, September was now a great opportunity for an official opening. Before cutting the ribbon, Hawkins and Markes’ Head of Business Operations, Pamela Simpson, took Economy Minister Mr Gething on a tour of the site, starting with the company’s demonstration laboratory, before moving on to its 52,800-square-foot factory and bespoke-fitted research and development facilities.

Markes develops and manufactures all of its specialist laboratory instruments at the site, which is its largest in the UK. Markes also has sites in the US, Germany, Canada, China, and Asia Pacific. The company’s instruments are used to detect chemicals in solids, liquids, and gases in much smaller quantities than have been previously possible. The instruments are used in numerous sectors such as environmental monitoring, defence and forensics, food and drink—and often in ground-breaking ways, such as detecting COVID-19 and cancer in breath and biological samples.

Explaining why Markes had chosen Bridgend to relocate to, Hawkins said: “Location is everything for a manufacturing business like ours. This is an established industrial area, so we benefit from a readily available skill set.”

For more information, please visit: https://markes.com/content-hub/news/vaughan-gething-ms-officially-opens-markes-international-s-new-bridgend-hq

Analyzing PFAS in Human Breast Milk Using Lipidomics

Lipidomics researchers from Örebro University have developed a method to analyze levels of per- and polyfluoroalkyl substances (PFAS) in human breast milk using ultrahigh-pressure liquid chromatography combined with quadrupole time-of-flight mass spectrometry (UHPLC–QTOF-MS) (1). The study aimed to assess the impact of PFAS on the composition of breast milk and the potential effect on nutrition and infant health. The analysis of breast milk is complicated because the composition varies highly between individuals. PFAS are a growing concern because they are, or can degrade to, persistent chemicals that accumulate in humans, animals, and the environment (2). This bioaccumulation effect increases the levels of PFAS and potential detrimental effects on human health. This is the first study that indicates PFAS can change the composition of breast milk, according to a press release on the University’s website (3).

In this study involving 44 participants, the researchers measured the levels of PFAS and lipids in a mother’s serum and performed lipidomics analysis of breast milk collected shortly after the birth and then three months later. The published research concluded that PFAS levels were inversely proportional to total lipid levels in the breast milk collected after the delivery. In the group with highest exposure, the ratio of acylated saturated and polyunsaturated fatty acids in triacylglycerols was higher. High exposure to PFAS was linked to alterations in phospholipid composition, which could increase the size of milk fat globules and could possibly lead to slower infant growth and higher intestinal inflammatory markers. The researchers concluded that PFAS present in human breast milk could possibly have a detrimental effect on the health and growth of the children with higher levels of PFAS.

Professor Tuulia Hyötyläinen, who performed the study in collaboration with Matej Orešič, Professor of medical sciences at Örebro University, and a team of clinical researchers from the University of Helsinki, commented that breast milk is becoming less nutritious because of chemical exposure that causes changes in the composition of breast milk, with less fat content in the milk. There is also an increase in saturated fats at the expense of the healthier, unsaturated ones, according to Hyötyläinen.

Researchers at Örebro University will proceed with an expanded study of 380 women and their children.—A.M.

References
**Peaks of the Month**

- **The LCGC Blog: Don’t Fear the Automation**—With modern chromatography modelling software, is it time to hang up our laboratory coats? Is resistance to software solving separations futile? [Read Here>>]

- **Sweating the Small Stuff—Are You Sure You Are Using Your Pipette Properly?**—Most analytical chemists believe their pipetting technique is infallible, but few of us are actually following all of the recommendations within the relevant guidance. Incognito investigates good pipetting practice and busts some of the urban myths behind what is probably the most widely used analytical tool. [Read Here>>]

- **Gas Chromatography Tandem Mass Spectrometry Analysis of Ethylene Oxide: An Emerged Contaminant in Seeds and Spices**—Two alternative approaches based on gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) were developed to control the maximum residue limit of the banned insecticide, ethylene oxide (EtO), and its transformation product, 2-chloroethanol (2-CE). [Read Here>>]

- **Determination of Organophosphate Esters in Water Samples Using Gas Chromatography–Mass Spectrometry and Magnetic Solid-Phase Extraction Based on Multi-Walled Carbon Nanotubes**—A method based on GC–MS coupled with magnetic SPE with multi-walled carbon-nanotube-coated Fe₃O₄ as adsorbent was developed for the analysis of four organophosphate esters (OPEs) in ambient water samples. The method was validated for its comparable sensitivity and potential application prospect. [Read Here>>]

- **Analyzing Glycerol in Biodiesel Using SPE and HPLC-RI**—Researchers have developed a viable alternative to existing GC methods for glycerol determination in biodiesel using SPE–HPLC-RI. [Read Here>>]

**News In Brief**

**Thermo Expands Biologics Capacity**
Thermo Fisher Scientific (Waltham, Massachusetts, USA) has taken over operational responsibility for a new biologics manufacturing site in Lengnau, Switzerland, as part of its strategic partnership with CSL Limited announced last year. The Lengnau site will become part of Thermo Fisher’s global biologics manufacturing network, and approximately 200 employees will join Thermo Fisher. The Lengnau site is a 1.5 million-square-foot facility that will leverage highly flexible bioproduction technologies, including both single-use and stainless steel, with up to 12,500 L bioreactor capacity. This will provide biopharma companies with a pathway from development to large-scale production as manufacturing needs evolve. [https://thermofisher.mediaroom.com/2021-10-01-Thermo-Fisher-Scientific-Opens-Biologics-Manufacturing-Site-in-Lengnau,-Switzerland](https://thermofisher.mediaroom.com/2021-10-01-Thermo-Fisher-Scientific-Opens-Biologics-Manufacturing-Site-in-Lengnau,-Switzerland)

**YMC Expands Laboratory Capabilities**
YMC (Kyoto, Japan) is significantly expanding its laboratory capabilities in the USA by opening labs in Devens, Massachusetts, and Thousand Oaks, California (USA) to complement their long-standing partner lab in Maryland. These new laboratories are fully equipped and staffed with industry-experienced scientists. Much more than testing or demo labs, YMC will be using these facilities to conduct relevant separation studies and other research-based activities. [https://www.ymcpt.com/sites/default/files/YMC%20Insight%20Centers%20Labs%20PR%20EXTOK%20270821.pdf](https://www.ymcpt.com/sites/default/files/YMC%20Insight%20Centers%20Labs%20PR%20EXTOK%20270821.pdf)
Rising Stars of Separation Science: Jelle De Vos

This month we interview Jelle De Vos, Senior Postdoctoral Researcher at the Department of Chemical Engineering, Vrije Universiteit Brussel, Belgium, about his recent work to develop a chromatographic method to characterize nucleocapsid proteins from SARS-CoV-2, and the development of spatial three-dimensional liquid chromatography (3D-LC) as a separation concept.

—Interview by Kate Jones

Q. When did you first encounter chromatography and what attracted you to the subject?
A: Analytical chemistry and chemical analysis were very important subjects when studying chemistry at Ghent University. It was Pat Sandra who introduced me to the world of chromatography. In his own style, he told us about “where chromatography made the difference”, and his recollection of how he became “the analytical scientist that saved Belgium” during the Belgian dioxin crisis in 1999. What I found most fascinating is how this technique and these “complicated” machines were able to quantify minute amounts of analyte molecules in a sample. This persuaded me to specialize in analytical chemistry during my M.Sc. studies, which is where I took the course “Advanced Chromatography”. Frederic Lynen, along with guest speakers Roman Szucs and Gert Desmet, showed me the current state-of-the-art in separation sciences. Intrigued by the challenges of new technology and the fundamentals of liquid chromatography (LC), I finished my M.Sc. thesis at the Vrije Universiteit Brussel (VUB) in the group of Sebastiaan Eeltink.
Back then, 2.6-µm core–shell particles found their reintroduction into the field, which was the perfect opportunity for me to learn more about LC.

Q. Can you tell us more about your Ph.D. thesis?
A: Making scientific discoveries and trying to make the difference by pushing the field forwards have always been my main motivators to stay in academia and pursue a Ph.D. I was fortunate to have the opportunity to work on many diverse projects. One part of the thesis focused on advances in ultrahigh-pressure liquid chromatography (UHPLC) technology, including system design and pushing the performance limits of LC by working at 1500 bar using 1.5-µm core–shell particles. The intrinsic time and performance gains at these conditions were demonstrated for a complex mixture of wastewater pollutants. In addition, the possibilities and limitations of method speed up for the separation of native proteins using aqueous size-exclusion chromatography (SEC) were explored.

Jelle De Vos is a Senior Postdoctoral Researcher at the Department of Chemical Engineering of the Vrije Universiteit Brussel, in Belgium. He studied chemistry at Ghent University and obtained his M.Sc. degree in 2012. In the same year, he started to work as a doctoral researcher in the Bio-Analytical Separation Science Group at VUB. Jelle conducted research on advancing the performance limits of ultrahigh-pressure liquid chromatography (UHPLC) technology and developing a novel solvent-assisted modulation strategy for multidimensional separations. His Ph.D. thesis was supervised by Prof. Dr. Sebastiaan Eeltink and Prof. Dr. ir. Gert Desmet. He obtained his Ph.D. in engineering sciences in 2016. Following his Ph.D. studies, he was selected for an FWO Postdoctoral Fellowship, which was extended in 2019. He performed an international research stay at the Institute of Bioprocess Science and Engineering at the University of Natural Resources and Life Sciences in Vienna (Austria) in 2019–2020, under the supervision of Prof. Dr. Alois Jungbauer. Whilst there, his research focused on mass transfer in microfluidic devices, applied biopharmaceutical processing (downstream processing), and analytics of antibodies and proteins from SARS-CoV-2.
The other part of the thesis described the fundamental and applied work that I’d done on a generic postcolumn refocusing strategy that mediated the effects of chromatographic dilution. This approach involved trapping target analytes on a trap column, after an analytical separation, and focusing them using a strong solvent before detection, which resulted in signal enhancement. Lately, this approach has gained more interest in two-dimensional LC (2D-LC).

**Q. What chromatographic techniques have you worked with?**
**A:** All liquid chromatography modes for both small-molecule analysis and bioanalysis. Recently, I have been working more on multidimensional LC and LC–mass spectrometry (MS) hyphenation.

**Q. You recently developed an analytical hydrophobic interaction chromatography (HIC) multi-angle light scattering (MALS) detection method to characterize SARS-CoV-2 nucleocapsid protein (1). What is novel about this approach and what does it offer over other methods?**
**A:** In this study we produced for the first time high titers of nucleocapsid protein (NP) from SARS-CoV-2 expressed in *E. coli* with a CASPON tag, and established a fully optimized downstream processing (DSP) approach. The HIC–MALS analytical step allowed for fast and reliable screening of product quality from the different iterations when optimizing DSP. The molecular weight information from MALS showed us that the main NP fraction is consistent with a dimerized protein, which was exciting to discover since not much was known about SARS-CoV-2 back then. The main advantage of this HIC–MALS method is that it allows for a screening method of biomolecules under native conditions, providing equivalent information to traditional reversed-phase LC–MS methods.

**Q. What challenges did you encounter when developing this method and how did you overcome them?**
**A:** The most challenging part of completing the research was optimizing and completing the method during the pandemic, when many travel and work restrictions were imposed. The HIC–MALS method itself produced the data we required after some optimization work. Getting the required mass tuning and accuracy in order was a bit more challenging, but fortunately I could rely on very skilled colleagues.
Q. How will this research benefit further SARS-CoV-2 analysis?
A: This research was part of a unique multi-department effort at the University of Natural Resources and Life Sciences (BOKU) to study SARS-CoV-2 antigens. A comprehensive evaluation of antigens from several biotechnological platforms was performed to identify reliable candidates for serodiagnostic tests. Two easy-to-implement SARS-CoV-2 ELISA antibody tests, based on the spike receptor binding domain and the nucleocapsid protein, were developed and evaluated using time-resolved acute and early convalescent samples from hospitalized COVID-19 patients.

Q. Your main area of research is focused on establishing spatial three-dimensional liquid chromatography (3D-LC) as a separation concept (2). Can you talk a little about the theory behind this concept?
A: It was the late Georges Guiochon who advocated to revisit planar chromatography and showed the potential of spatial chromatography in the 1980s. In spatial 3D-LC, components are separated in the spatial domain of a separation device, with each compound band being characterized by X, Y, and Z coordinates in a three-dimensional separation body. The sample compounds are simultaneously introduced from one dimension to the following, and their development occurs in parallel, leading to a substantial gain in analysis time compared to conventional multidimensional LC. Our group at the VUB realized the first prototype devices for spatial LC and has been developing the technology from the ground up. Many challenges lay in getting the devices leak-tight at high operating pressures of several hundreds of bar, introducing the stationary phase in microfluidic channels that were sub-500-µm internal diameter (i.d.), and achieving a parallel flow control and injection mechanism. Luckily, the toolbox of the engineer working with microfluidic devices has been expanded lately with the advent of novel thermoplastic materials and 3D-printing technology.

Q. What projects are you working on next?
A: I am confident that microfluidic technologies will overtake the analytical laboratory simply because they offer much more versatility, with integrated components on one module and user-friendly modular devices at lower production costs over conventional technology. My recent projects focus on new microfluidic devices and technology for multidimensional liquid chromatography and hyphenation to mass spectrometry.

Q. What advice would you give to someone who wants to focus their research on spatial 3D-LC?
A: This project requires insights from both separation science and engineering science, which increases the challenge significantly to create a viable prototype device. My main advice would be to always keep the target application in mind and, most importantly, the operator. Many technologies have failed because they were too difficult to handle or showed too little advantage over conventional technology. With spatial 3D-LC being a new technology in separation science, it is important to have all the building blocks right from the start.

References

Rising Stars of Separation Science
The Column will be running a series of interviews in 2021, featuring the next generation of separation scientists. If you would like to nominate a “rising star” for consideration, please send the name of the candidate and why they deserve recognition to Alasdair Matheson, Editor-in-Chief, LCGC Europe and The Column at amatheson@mjhlifesciences.com
The 17th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-17) will be held in person from 26 to 28 January 2022, at Conference Center Het Pand in Ghent, Belgium. HTC-17 will be organized under the auspices of the Royal Flemish Chemical Society (KVCV) and the Separation Science Group of the Royal Society of Chemistry (SSG RSC).

Deirdre Cabooter, University of Leuven, Leuven, Belgium

The HTC conference has been the premier platform for state-of-the-art developments in separation technologies and hyphenated techniques for more than 30 years. The conference typically encompasses three parallel sessions consisting of plenary lectures, keynote lectures, tutorials, oral, and poster-flash presentations. One of the parallel sessions will be dedicated to young emerging scientists (YES!). The symposium will also host an attractive technical exhibition where vendors will present their newest instruments and developments, topped with technical seminars.

We will cover fundamental and practical aspects of liquid phase (LC) and gas chromatography (GC), including multidimensional LC, multidimensional GC, supercritical fluid chromatography (SFC), LC–mass spectrometry (MS), and GC–MS. The programme will include topics such as three-dimensional separations, automated sample preparation, online analyzers and sensors, ion-mobility and native mass spectrometry, emerging detectors and separation modes, method development and artificial intelligence, column technology and stationary-phase developments, system design and optimization, miniaturization and chip technology, and data mining and curation. We will also address bottlenecks and describe trends and new technologies for a wide range of applications, including (bio-) pharmaceuticals, macromolecules, medical diagnostics and clinical applications, forensic analysis and doping control, food analysis and safety, environmental analysis, energy, green approaches, high-throughput analysis, -omics (lipidomics, metabolomics,
proteomics), and biomarker discovery, as well as modern industrial applications and natural products.

As always, we will ensure abundant networking opportunities during the conference, with an informal beer tasting event organized during a nocturnal poster event, a conference dinner in the beautiful PoortAckere monastery, and lots of dissemination activities.

A number of awards will be presented at HTC-17. The HTC/LCGC Innovation Award, sponsored by *LCGC Europe*, will be offered to a researcher with less than 15 years’ experience after obtaining his or her Ph.D. for “a pioneering contribution to the field of separation sciences by introducing new methodologies, new instrumentation, or new techniques in the field, with a strong focus on applicability”. The most innovative oral contribution presented during the conference will receive the HTC-Award. The most innovative poster contributions will receive the HTC-Poster Award. We will also organize a YouTube contest for young scientists that will be sponsored by Elsevier’s *Journal of Chromatography A* and *Journal of Chromatography Open*, a new open access journal.


After a challenging time where meeting and networking have been severely restricted, we very much look forward to seeing you in Ghent!

For more information and registration, visit the HTC-17 webpage at: https://htc-17.com/
E-mail: htc17@kuleuven.be

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The Importance of Analyzing Sulphur Compounds in Food

Rebecca Kelting* and Erich Leitner†, *Shimadzu Europa, Duisburg, Germany, †Graz University of Technology, Institute of Analytical Chemistry and Food Chemistry, Graz, Austria

Volatile sulphur compounds can significantly contribute to the flavour or the off-flavour of different foods and beverages. Some of them belong to the most potent aroma substances that are known, and very often the concentration level determines a positive or negative perception. For example, the compound 4-methyl-4-sulfanylpentan-2-one has a reported sensory threshold in water of 100 picogram per litre with a pleasant aroma of tropical fruits (maracuja), which turns into an annoying cat urine smell at elevated concentrations. The identification and quantification are real analytical challenges, demanding selective and sensitive methods. The analytical approaches are demonstrated on products such as beer, wine, coffee, and vegetables.

Considering the human senses, smell is the most important driver for acceptance or rejection. Only volatile substances with a maximum molecular weight of 400 Dalton trigger odour perception.

Odours and odour impressions have fascinated humankind since ancient times and are a vital part of its survival. It is not only the positive changes during food preparation and conservation of food and the fragrance of spices, plants, and essential oils that are important signals for people but also the alarming smell of spoilt and rotten products.
is normally the tiniest part of a food or a beverage. To pick out the relevant compound that contributes to the product is a tricky task. In addition, the human smell system perceives odorants over a range of 10 to 12 orders of magnitude, ranging from the upper milligram down to the picogram per kilogram level.

The Roles of Organosulphur Components
Of the various organic compounds, sulphur-containing molecules belong to a very potent class that influence the smell of various products such as fruits (durian, maracuja) and vegetables (garlic, onion, horse radish, cauliflower), alcoholic beverages (beer, wine), meat and seafood, and coffee. Unfortunately, organosulphur components can also be responsible for off-flavours. Table 1 lists some compounds identified in various food products with their sensory descriptors and the lowest reported sensory threshold.

The sensory threshold is not a fixed value but instead depends heavily on the matrix and the sensitivity of different persons. There are huge variations over several orders of magnitude between humans. Also, the descriptors are very often dependent on concentration and can change their odour quality with increasing concentration. Further, it should be stressed that only compounds in a concentration above the sensory threshold can contribute to the overall product smell.

The Maillard Reaction
The sources and the formation pathway of sulphur compounds are multifaceted, ranging from enzymatic reactions to microbial activities and thermal generation, mainly via the Maillard reaction (1).

For example, the β-thioglucosidase enzyme myrosinase hydrolysis glucosinolates can be found in horse radish. By the enzymatic reaction, isothiocyanates, mainly allyl isothiocyanate, are formed. Also well documented is the enzymatic reaction in Allium species (garlic, onion), where the enzyme allinase triggers the formation of different volatile sulphur species.

In the case of Maillard reaction-induced sulphur components, the amino acids methionine and cysteine are important precursors for key components in heat-treated products. Various microbiological pathways can generate volatile and odour-active compounds during fermentation and can contribute either in a positive or a negative way to the quality. Very volatile sulphur compounds (VVSC) are particularly responsible for reductive off-flavours in wine. In addition to hydrogen sulphide, other mercaptanes taint the wine with notes of rotten egg, sulphur, and onion.

Coffee
The volatile fraction and the aroma display the most complex composition of all known food products. One of the reasons is the high roasting temperature, which can be up to 400 °C for short-time roasting. Aside from other Maillard products, such as pyrazine, which contributes to nutty, roasted flavour notes, degradation products from trigonelline, chlorogenic acids, lipids, and carotenoids, it is mainly sulphur compounds that have an impact on the aroma of roasted coffee (2). The very volatile compound methanethiol is a good marker substance for the freshness of coffee due to its high volatility and reactivity, respectively (3).

Figure 1 shows the increase of sulphur compounds from green beans to the final product. So far, over 100 sulphur species have been reported in the literature; in the chromatogram shown 150 sulphur species were detected. The only compound found in the raw coffee was dimethylsulphide.

Identification of all the sulphur compounds is a laborious and tricky task.

Figure 1: Sulphur formation coffee SCD: the increase of sulphur compounds from green beans to the final products.
Due to the low sensory threshold of some of the compounds and heavy interferences from other compounds, detection with gas chromatography–mass spectrometry (GC–MS) is tough or even impossible in some instances. Two-dimensional comprehensive GC×GC coupled with mass selective detection (MSD) can be a powerful tool to increase selectivity and sensitivity of the separation and identification of coffee volatiles.

Figure 2 displays a two-dimensional chromatogram of 100 mg of freshly roasted ground coffee powder. Sample preparation was done via solid-phase microextraction (SPME) at 40 °C for 5 min. Even with this powerful separation technique, interferences occur but even high impact sulphur compounds are identified. Some of them are marked in the chromatogram.

**Beer Brewing**

The presence of volatile organosulphur compounds that potentially contribute to the flavour and odour impression of food on consumers, and their ambivalence, is also important in the world of beer brewing. This starts with the ingredients—malt, yeast, and hops (4,5)—and ends with the final product—the canned, bottled, or tapped beer.

Even though hops oil contains less than 1% sulphur compounds (4,5), they still influence its aroma, both positively and negatively. They can be divided into four types: alkyl- and polysulphides, thioesters, polyfunctional thiols, and sulphur-containing terpenoides (4).

Alkyl- and polysulphides display a cabbage-like, onion-like smell, reminiscent of boiled vegetables, and should therefore be present only in small amounts. The rather negative judgement of sulphides also holds true for thioesters (4). Polyfunctional thiols only occur in ppb amounts but have extremely low odour thresholds and still contribute to the aroma of hops. The latter group includes 4-mercapto-4-methyl-2-pentanone (4-MMP), identified as a key compound for hops aroma, having a blackcurrant odour impression at low concentrations and an odour threshold of only 0.8 ppt (4).
The sulphur compounds present in hops do not contribute to the aroma of beer by default, since the volatile sulphur compounds added at the beginning of the beer production are mostly lost during the process. Yet, a second portion of hops can be added shortly before the end of the boil or in the whirlpool (late hopping) to give the product a hoppy, floral note. Adding the second portion of hops even later to the green beer after fermentation, also called dry hopping, is an important factor in the production of craft beers (6). Since volatile organic compounds from the addition of hops remain in the beer and are used for “fine-tuning” the taste and odour impression, the sulphur compounds that contribute to hops aroma are important, both for hops and beer producers. Factors such as hops variety, growing conditions, and harvesting time contribute to the organosulphur pattern and content seen (4,5).

**Hops**

In combination with the low odour thresholds, sulphur compound monitoring in hops is analytically challenging. Figure 3 shows the chromatograms of a hops extract investigated using GC coupled with sulphur chemiluminescence detection (SCD) (top) and mass spectrometric detection (bottom), respectively.

Sample introduction can either be performed via headspace (HS) sampling of hops powder and pellets, or as liquid injection of a hops extract. The first approach simplifies sample preparation and reduces matrix contributions, the latter relates to the alcoholic extraction process during hopping of a beer. The sulphur content analysis of beer as the final product can also be done using GC–SCD with headspace sampling. The beer is filled into headspace vials and quantitation on target species is performed using standard addition. Beer samples may show various sulphides: dimethylsulphide (DMS), dimethyl disulphide (DMDS), and dimethyl trisulphide (DMTS), of which DMS is the most pronounced one (7), with a sweetcorn character and a threshold of 0.03 mg/L (8). In addition, thiols and thioesters can be seen, specifically methanethiol and S-methyl thioacetate.

**Table 1: Important sulphur compounds and their odour impressions**

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Flavour Description</th>
<th>Odour Treshold (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methyl-2-butene-1-thiol</td>
<td>C₅H₆S</td>
<td>skunky, foxy, meaty, coffee</td>
<td>0.0002</td>
</tr>
<tr>
<td>4-Mercapto-4-methylpentan-2-one</td>
<td>C₆H₁₀OS</td>
<td>maracuja, box tree, cat urine</td>
<td>0.0008</td>
</tr>
<tr>
<td>3-Mercapto-3-methylbutylformate</td>
<td>C₇H₁₀O₂S</td>
<td>fruity odour, blackcurrant and catty</td>
<td>0.0035</td>
</tr>
<tr>
<td>2-Methyl-3-furanthiol</td>
<td>C₅H₁₀OS</td>
<td>meat-like, roasted coffee</td>
<td>0.005</td>
</tr>
<tr>
<td>Dimethyltrisulphide</td>
<td>C₅H₁₄S₃</td>
<td>penetrating odour of fresh onion; cabbage, burnt, cooked</td>
<td>0.01</td>
</tr>
<tr>
<td>Methanethiol</td>
<td>CH₄S</td>
<td>rotten cabbage, coffee</td>
<td>0.02</td>
</tr>
<tr>
<td>2-Furfurylthiol</td>
<td>C₅H₁₀OS</td>
<td>classic coffee odour, coffee-like, caramellic-burnt, sweet</td>
<td>0.04</td>
</tr>
<tr>
<td>Methional</td>
<td>C₅H₁₀OS</td>
<td>onion- and meat-like</td>
<td>0.2</td>
</tr>
<tr>
<td>Dimethylsulphide</td>
<td>C₅H₁₀S</td>
<td>wild radish, sharp, green, cabbage, vegetable-like</td>
<td>0.3</td>
</tr>
<tr>
<td>Dimethyl disulphide</td>
<td>C₅H₁₀S₂</td>
<td>intensive, onion-like character</td>
<td>3</td>
</tr>
<tr>
<td>2-Mercaptoacetate</td>
<td>C₅H₁₂O₂S</td>
<td>rotten vegetables</td>
<td>40</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>C₅H₁₀OS</td>
<td>barnyard</td>
<td>130</td>
</tr>
<tr>
<td>Allylisothiocyanate</td>
<td>C₅H₁₄NS</td>
<td>pungent, horse radish</td>
<td>1300</td>
</tr>
</tbody>
</table>

The sulphur compounds present in hops do not contribute to the aroma of beer by default, since the volatile sulphur compounds added at the beginning of the beer production are mostly lost during the process. Yet, a second portion of hops can be added shortly before the end of the boil or in the whirlpool (late hopping) to give the product a hoppy, floral note. Adding the second portion of hops even later to the green beer after fermentation, also called dry hopping, is an important factor in the production of craft beers (6). Since volatile organic compounds from the addition of hops remain in the beer and are used for “fine-tuning” the taste and odour impression, the sulphur compounds that contribute to hops aroma are important, both for hops and beer producers. Factors such as hops variety, growing conditions, and harvesting time contribute to the organosulphur pattern and content seen (4,5).
odour of lager-style non-alcoholic beers (9). Consequently, it was found to be present in the non-alcoholic beer samples analyzed, but in lower concentrations when comparing the beers with their non-alcoholic counterparts (7).

Aside from the control of the amount of “typical” sulphur compounds in beer, typical off-flavours could also be monitored analytically, such as 3-methyl-2-buten-1-thiol (3M2B1T or MBT, also known as “light struck flavour” or “skunk thiol”), which was discovered in 1952 and is formed by the reaction of iso-alpha-acids with sulphur compounds under the influence of UV or visible light (10).

References
2. I. Flament, Coffee Flavor Chemistry (Wiley, 2001)

Erich Leitner is the head of the Institute of Analytical Chemistry and Food Chemistry at Graz University of Technology. His research focus is on the identification of volatile and odour-active substances in food and food contact materials. He employs a number of human sensory techniques and ultra-trace analytical methods, mainly based on gas chromatography, in his work.

Rebecca Kelting holds a doctorate degree in chemistry from the Karlsruhe Institute of Technology (KIT), Germany. She works as a GC and GC–MS product specialist for Shimadzu Europa in Duisburg, Germany, currently focusing on gas chromatographic application and product support.

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Detecting Prohibited Pharmaceutical Active Substances in Milk

*The Column* spoke to Irma Bongers from Wageningen Food Safety Research (WFSR) in The Netherlands, about a recent research project to analyze prohibited pharmaceutical active substances (PPAS) in milk.

—*Interview by Alasdair Matheson*

Q. Why is the analysis of residues of prohibited pharmacologically active substances (PPAS) in milk important? Can you provide some background information on this research project?

A: Wageningen Food Safety Research (WFSR) is responsible for the monitoring and control of food production within The Netherlands. We analyze about 135,000 food samples yearly using multiple test methods. An important topic within food safety is testing for residues of pharmacologically active substances that may be present in foods of animal origin, such as muscle, milk, and eggs. Within the EU there is strict legislation regarding the use of pharmaceutically active substances in food production, and some substances are even marked as prohibited by Commission regulation (EU) No 37/2010.

Q. How do these analytes enter the food chain? What detrimental effects can occur when they do?

A: PPAS are rarely used and found in food. However, many substances that have a pharmaceutical effect can be used for the treatment of (sick) animals. For example, nitroimidazoles are an important antiparasitic agent with a high biological activity and are therefore very effective. However, nitroimidazoles are prohibited because concerns have been raised about toxicity, mutagenicity, and genotoxicity.
In general, some substances are marked as prohibited by the EU due to potential public health risks as a result of exposure to toxic residues or related metabolites and the potential for development of antimicrobial resistance. Antimicrobial resistance could lead to ineffective antibiotics and reduce treatment options in human and animal health.

Q. You worked on a method to analyze residues from five different classes of these substances in a single method (1). What analytical techniques were used previously?
A: Before this new multi-class method was developed and implemented in routine analyses, five different single-class methods were used. This means a method, including a complete sample cleanup and liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis, (i) for the group of nitrofurans, (ii) for the group of nitroimidazoles, (iii) for dapsone, (iv) for chloramphenicol, and (v) for chlorpromazine.

The sample preparation for 20–50 samples using five different methods takes an analyst five times one and a half workdays. This is followed by five times 8–20 min analysis time on the LC–MS/MS instrument.

Q. What were the main challenges you encountered to develop a single method to analyze this wide combination of analytes and how did you overcome them?
A: The wide combination of PPAS makes the sample preparation challenging, because the different physicochemical properties limit the possibilities for single method cleanup. On the other hand, sufficient recovery of all PPAS is required, and on the other hand, you want to remove as much unwanted matrix compounds as possible. With a liquid–liquid extraction (LLE) using ethyl acetate we were able to create sufficient recovery for all PPAS. However, the matrix compounds in the milk caused an unwanted gel formation during this extraction step. We found that this gel formation could be prevented by using the AOAC dispersive SPE kit while maintaining sufficient recovery.

Developing the LC–MS/MS analysis was also challenging because all PPAS are prohibited and therefore zero tolerance for the presence of these substances is applicable. A zero-tolerance policy requires high sensitivity. For a sensitive detection, two of the 16 PPAS included have to be measured in the negative ionization mode of the mass spectrometer, while the highest sensitivity is achieved in the positive mode for two other compounds. Therefore, an instrument was needed that was fast enough to quickly switch between both ionization modes. Additionally, an optimized chromatographic method ensured separation of most PPAS. In combination with the scheduled multiple reaction monitoring (MRM) detection, this method resulted in a more sensitive detection.

Q. What is novel about your approach?
A: Within the field of food safety a lot of research has been done. Many papers have been published on the analysis of a single class of residues of regulated and/or prohibited pharmaceutical active substances. Several methods are even published about the combination of PPAS in a single method. However, to our knowledge, no fully validated method in milk has previously been reported that includes 16 compounds belonging to five different classes. This new approach results in a far more cost-effective surveillance of the PPAS in milk, using one multi-class method instead of multiple single-class methods.

Q. Are you planning to develop this research further?
A: The published method is successfully implemented in the routine control of the National Residue Monitoring Programme. However, we continue method improvement and we are currently working on an expanded version of this method to broaden the number of substances. In addition, we will add nitroimidazole, colchicine, and some extra metabolites of the substances already included.

Another development area is the efficiency of the sample cleanup. The LLE in the sample cleanup is a time-consuming step, particularly if the sample numbers expand. Therefore, we will try to improve this step with a new approach.

Q. What other projects are you working on at the moment?
A: Another project I am working on is an automatized cleanup and detection of nitroimidazoles using the dried fluid spot technique. A small fluid sample (around ten microlitres) is spotted onto a paper card. This card is placed in an autosampler and the sample is extracted from the card using
a flow-through technique. The flow goes directly to an inline solid-phase extraction (SPE) unit for the clean-up and is separated and detected by LC–MS/MS.

Reference

Irma Bongers currently works at Wageningen Food Safety Research (WFSR), Wageningen University and Research, The Netherlands. After she completed her master’s degree in analytical chemistry at the University of Amsterdam five years ago, she started working at WFSR, focusing on veterinary drug residues in food and environmental matrices.

For a special supplement on food analysis organized by the RAFA 2021 organizers in collaboration with LCGC Europe called “Advances in Food and Beverage Analysis” go to https://bit.ly/2Xcfc4S. For details on the RAFA 2021 Virtual Symposium go to www.rafa2021.eu
In this instalment of the LCGC Blog, it’s time to take the lid off the black box and take a closer look inside electronic pneumatic controller (EPC) devices.

Electronic pneumatic controllers (EPC) were introduced into gas chromatography (GC) systems from the mid-1990s onwards and do an excellent job of regulating gas flow and pressure for GC inlets, columns, and detectors. They take the hard work out of manual gas flow, pressure adjustment, and manual-flow measurement, and they typically provide accurate and reproducible results, time after time. One simply selects the required pressure or flow from the instrument or data system and then checks the instrument readback to see if the set point has been successfully achieved. Truly black box technology, what can possibly go wrong? Well, regular readers will know that, while I support innovation and improvements in engineering capability, I
am inherently sceptical of anything with the “black box” tag and believe that unless we inherently understand how something works, we can neither fully harness the potential benefits, nor properly troubleshoot when something goes wrong. Therefore, read on as we open the lid and take a good look inside these particular black boxes.

So, how do EPC devices work? Typically, a forward-regulated gas supply from the source is provided to the EPC unit that contains a variable metering valve that can be varied according to the set point for the flow or pressure required. The required gas flow or pressure value is sent to the EPC controller from the GC system and a flow or pressure transducer is used to monitor the actual flow or pressure and the metering valve adjusted to obtain and maintain the desired set point value. This is known as a “closed-loop” control system. As can be seen in Figure 1, frits of fixed diameter may be included to provide adequate flow resistance downstream of the EPC pressure sensor, or upstream of a flow sensor. Most EPC systems will calculate required pressure drops for the column dimensions and the gas type to account for the different compressibility of our common carrier and detector supply gases. Other factors may also be compensated for, such as controller temperature, gas supply pressure, and atmospheric pressure, which can reduce instability and drift in the gas flows delivered to the GC inlet, detectors, or auxiliary equipment.

Figure 1 shows a typical EPC-controlled split/splitless inlet operating in split mode. The total flow into the inlet is set and monitored by valve A and flow sensor A. The flow through the split line is controlled by valve B and pressure sensor B. Valve C and pressure sensor C control the flow through the septum purge line. The flow through the column is not directly sensed but is the balancing flow between that which enters the inlet via valve A and the flows through valves B and C. So, let’s say we need a column flow of 1 mL/min and a split ratio of 100:1. Approximately 104 mL/min will be supplied via valve A, typically around 3 mL/min will leave the inlet via valve C and the septum purge line (which is physically separated from the inlet region occupied by the sample when injected), and 100 mL/min will leave via valve B and the split line. The remaining and balancing flow of 1 mL/min will be transferred to the column.

Figure 1 illustrates the EPC control of a split/splitless inlet; the configuration for gas control to detectors and auxiliary and sampling equipment may be different, but all work on similar principles.

The astute reader will have noted that in our inlet example, only the carrier supply flow into the inlet is measured as a flow. The actual flow of the carrier through the column is not directly measured by the instrument, rather it is calculated from a differential of flow into the inlet (flow sensor A) and pressures out of the inlet (pressure sensors B and C). Most GC vendors will have a calculator that highlights the principles on which this principle works and using such a calculator I was easily able to compute that to obtain a carrier gas linear velocity of 35 cm/sec of helium through my notional

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**Figure 1:** Schematic diagram of an EPC module used to control gas flows in a split/splitless inlet (Figure courtesy of Agilent Technologies, Santa Clara, California, USA).
30 m × 0.32 mm, 0.1-mm GC column at 50 °C, the carrier gas pressure required at the inlet is 10.01 psig, which will result in a column (outlet) flow of 2.13 mL/min. For a split ratio of 50:1, the required split flow can be calculated as 106.5 mL/min. Of course, I could have calculated the required pressure to obtain a particular column flow, rather than a linear velocity, in exactly the same manner. These same calculations are carried out “on-board” the GC system and the required pressures for the inlet set by the EPC control units. This leads me to highlight the first potential “gotcha” when using EPC systems for gas control. Unless the end user specifies the correct column dimensions in the chromatograph (or data system), the actual flow through the GC column may be different than intended, even though the GC system will report the correct flow value. Let’s look at a quick example of this. Provided the GC system can attain and maintain the pressure set point required for what it has calculated as the correct flow rate or linear velocity of the carrier through the column, it will carry on regardless. If we suppose that an error was made in the previously cited example and the internal diameter of the column was entered as 0.25 mm rather than 0.32 mm, then the instrument would believe it needed to set an inlet pressure of 16.76 psig to achieve the desired 35 cm/sec carrier gas linear velocity. As the actual internal diameter of the column was 0.32 mm, the actual column flow would now be 4.18 mL/min and the average linear velocity 57.4 cm per sec, a difference in flow rate of 196% and one would notice very much shorter retention times and perhaps compromised analyte resolution. Further, because the split flow rate would remain unchanged, the split ratio would be in the order of 25:1, risking column overload and perhaps further compounding any issues with the resolution of critical peak pairs. One also needs to remember that as the column length is “trimmed” to maintain good chromatography, this will also influence the pressure required to obtain the necessary flow or linear velocity. If significant lengths of the column are trimmed, one should note on the column hangar tag or column log the “new” length of the column. The lesson here is to be very careful to properly specify and check the column dimensions (including stationary-phase film thickness) when prospering for GC analysis. While EPC control is highly advantageous, it is by no means infallible. Of course, one might choose to manually check the column flow prior to analysis to ensure it is correct, using an electronic flow meter. Here is the second issue that I regularly get questions on: “Why does my flow meter read a different flow to the value reported by my GC system?” Of course, one answer could be that there has been an error in setting the GC column dimensions in the systems as discussed above, but even when the dimensions are correct, there can be differences between the instrument reported value and the measured value. Some of these differences can be caused by the type of flow meter used and its calibration. These topics are beyond the scope of the current discussion, but I would point readers to an excellent article in LCGC magazine by John Hinshaw on measuring gas flow for gas chromatography (1). In accounting for differences between instrument flow values and those measured by modern electronic flow meters used to manually check the flow rates produced, we must consider the concept of standard temperature and pressure (STP). In most cases, the standard pressure will be assumed to be 101.325 kPa but standard temperature can vary depending upon the industry we work in, geographical location, and the manufacturers of the GC equipment and metering devices. The International Union for Pure and Applied Chemistry (IUPAC) specify the standard temperature for gas measurement to be 273.15 K (0 °C), but temperatures of 20 and 25 °C are also used for “standard” gas measurement by various bodies and indeed by equipment manufacturers. While some EPC units and flow meters can adjust for differences between standard and local conditions, clearly those that cannot need to have some correction factor applied to the measurement in order to account for the variations between the conditions under which the equipment was calibrated and local conditions. Fortunately, there is a fairly simple equation that can be used for these purposes:

\[ F^o = F_a \cdot \frac{T^o}{T_a} \cdot \frac{P_a}{P^o} \]  

[1]  

In this equation, the superscript “o” designates standard or reference conditions under which the equipment was calibrated, and the subscript “a” designates ambient (local) conditions under which the measurements are being made. If we assume an STP of 0 °C and 101.325 as the standard conditions and an instrument flow reading of 2.1 mL/min, let’s see what differences there would be between flow meter measurements using a made-in-a-cooler laboratory under lower atmospheric pressure conditions.
they can highlight that, where methods are to be transferred between laboratories in different parts of the world with varying ambient conditions, some account must be taken to standardize the measurement of flow. It should also be noted that some flow meters impose a back pressure on the gas flow, which may change the response of the EPC system and then cause spurious flow reading. Again, I would urge readers to consult reference 1 for further information.

A further contributing factor to the differences between flow meter and instrument readings involves the “drift” that can be encountered by EPC devices over time. While the “slope” (sensitivity) of EPC metering valve calibration tends to be steady, the “offset” values in terms of valve position versus metered gas flow can from time to time need to be reset. There is no pre-defined time interval for this operation, but some manufacturers will include an EPC reset or zero function that will allow the user to reset the EPC valve position and “calibration,” and you should consult your manufacturer’s literature for guidance. Care should be taken as flow zeroing tends to be carried out with gas flowing, whereas pressure zeroing requires no gas flow.

The final EPC topic worthy of consideration is that of carrier gas operation in constant pressure or constant flow mode during GC oven temperature programming. As the oven or column temperature increases, the gas viscosity also increases, and under constant inlet pressure conditions, the carrier gas flow and linear velocity will reduce. This is very often not the best situation for chromatography (long analysis times and broadening peaks) and several detectors also work more optimally with a constant flow situation. The ionization efficiency and fragmentation pathway analytes with the ion source of mass spectrometric detectors can be dependent upon ionization source pressure and therefore the flow into the ion source. Flame ionization detectors using hydrogen as the carrier can show changes in response if the volume of hydrogen to air and makeup gases changes. Therefore, it is better to keep a constant flow of hydrogen from the column during the temperature program.

EPC units are calibrated to calculate the required pressure drop across a column (provided we input the correct column dimensions!) versus temperature in order to maintain a flow rate set point. In this way, as the oven temperature rises, the EPC module can increase the inlet pressure so that constant flow is maintained as the column temperature and carrier gas viscosity increases. In our previous example, the 10.01 psig required to obtain 35 cm/

\[ F^o = \frac{2.1 \times 275.15 \times 99.200}{303.15 \times 101.325} = 1.87 \text{ mL/min}[3] \]

While these differences are not vast, they do go some way towards explaining why meter readings can be different from those displayed on the instrument, and
sec helium flow at 50 °C will rise to 15.02 psig to maintain this linear velocity at 300 °C. This helps to overcome some of the disadvantages of the constant pressure operation described above and illustrated in Figure 2.

It is often advantageous to maintain a constant flow of carrier gas into a detector, and therefore, even if one is operating in constant pressure mode for the GC carrier gas, detector EPC units are often capable of ramping the flow of a fuel or make-up gas during the temperature program, such that, as the carrier gas flow decreases, total flow experienced by the detector remains constant.

While these EPC operations are highly beneficial, the operator needs to be aware of how to implement them within the GC hardware or controlling software and of everything that has been said above regarding EPC operation under non-standard conditions. Recalibration or zeroing needs should also be kept in mind for trouble-free operation.

I hope that this deeper dive into the working principles and potential issues of EPC modules in GC has helped to broaden your understanding. Certainly, these advances in gas control have brought many advantages to practical GC usage, but, as always, without a deeper understanding of the working principles and limitations of such systems, one cannot ever truly exploit these advantages or properly investigate issues when trouble occurs!

Reference

Tony Taylor is the Chief Scientific Officer of Arch Sciences Group and the Technical Director of CHROMacademy. His background is in pharmaceutical R&D and polymer chemistry, but he has spent the past 20 years in training and consulting, working with Arch Sciences Group clients to ensure they attain the very best analytical science possible. He has trained and consulted with thousands of analytical chemists globally and is passionate about professional development in separation science, developing CHROMacademy as a means to provide high-quality online education to analytical chemists. His current research interests include HPLC column selectivity codification, advanced automated sample preparation, and LC–MS and GC–MS for materials characterization, especially in the field of extractables and leachables analysis.

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**Website:** www.chromacademy.com/channels/infrared/principles/introduction-to-infrared-spectroscopy

### MISCELLANEOUS

**Coping With COVID-19: Remaining Productive and Safe in the Analytical Laboratory**  
**Online webcast from CHROMacademy**  
**Website:** www.chromacademy.com/channels/basic-lab-skills/technique/coping-with-covid-19-remaining-productive-and-safe-in-the-analytical-laboratory

**Absolute Basics of Metabolomics**  
**6–7 December 2021**  
**Online—virtual classroom**  
**Website:** www.anthias.co.uk/training-courses/basics-metabolomics

**Practical Essentials of Pyrolysis**  
**3 December 2021**  
**Online—virtual classroom**  
**Website:** www.anthias.co.uk/training-courses/practical-essentials-of-pyrolysis

**Introduction to Infrared (IR) Spectroscopy**  
**Online webcast from CHROMacademy**  
**Website:** www.chromacademy.com/channels/infrared/principles/introduction-to-infrared-spectroscopy

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**Please send your event and training course information to Kate Jones kjones@mjh lifesciences.com**
Event News

26–28 January 2022
The 17th International Symposium on Hyphenated Techniques in Chromatography and Separation Technology (HTC-17)
Het Pand Conference Center, Ghent, Belgium
E-mail: htc17@kuleuven.be
Website: https://htc-17.com/

5–9 March 2022
Pittcon 2022
Georgia World Congress Center, Atlanta, Georgia, USA
E-mail: info@pittcon.org
Website: https://pittcon.org

21–24 June 2022
Analytica 2022
Messe München, Munich, Germany
E-mail: info@analytica.de
Website: www.analytica.de/en/

11–14 October 2022
The 18th International Symposium on Preparative and Industrial Chromatography and Allied Techniques (SPICA 2022)
Lisbon, Portugal
E-mail: secretariat@LDOrganisation.lu
Website: www.spica2022.org/
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The Column (ISSN 2050-280X) is the analytical chemist’s companion within the dynamic world of chromatography. Interactive and accessible, it provides a broad understanding of technical applications and products while engaging, stimulating, and challenging the global community with thought-provoking commentary that connects its members to each other and the industries they serve.

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