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In Memoriam: Michael J. Hennessy, Sr.

Michael J. Hennessy, Sr., was the beloved chairman and CEO of MJH Life Sciences, parent company of LCGC Europe.

Hennessy spent his career turning his passion for building businesses and creating jobs into a run of successful ventures and brands. Following his graduation from Rider University in 1982, he started his career in medical publishing as a sales trainee. In 1986, Hennessy became chief operating officer of Medical World Business Press, which was part of the launch of medical newspapers and other media products. The company prospered and was eventually sold to a Boston-based venture capital firm.

Hennessy launched Multimedia Healthcare, LLC, in 1993 and built a portfolio of award-winning clinical journals. In 2001, Freedom Communications, Inc., acquired Multimedia Healthcare, about the time that Hennessy was pioneering a new approach to print and digital publishing with Intellisphere, LLC (now part of MJH Life Sciences). Guided by the principles of innovation and entrepreneurial spirit, and reflecting its founder’s dedication to improving quality of life through health care research and education, Intellisphere publishes a variety of integrated print and digital products focusing on a range of topics in research and clinical medicine.

To build a comprehensive multimedia and education platform, Hennessy added additional companies and capabilities to the MJH Life Sciences portfolio. In 2004, he acquired Healthcare Research Analytics, which has been the leader in health care market research for over 30 years. In 2005, Hennessy acquired ArcMesa Educators, LLC, leaders in online certification for physicians, pharmacists, nurses, and other health care professionals.

In February 2008, Hennessy acquired the rights to the journals Pharmacy Times and The American Journal of Managed Care, both recognized in their respective markets as authoritative, trusted media platforms that provide essential information to a large audience of health care professionals. In April 2011, MJH Life Sciences acquired Physicians’ Education Resource, LLC (PER), an accredited continuing medical education company that is an industry leader in producing high-quality, first-rate oncology and haematology meetings and conferences.

In 2019, MJH Life Sciences made its largest acquisition to date when it acquired the Healthcare and Industry Sciences divisions of UBM Medica, nearly doubling the size of the organization and adding European legacy titles such as LCGC Europe, Pharmaceutical Technology Europe, and Ophthalmology Times Europe to this already impressive portfolio. This acquisition made the organization the largest independently owned medical communications company in North America. In addition to acquisitions, Hennessy organically developed ancillary in-house agency divisions with Proximyl Health, Truth Serum NTWK, and MJH Global Medical Affairs.

Later in 2019, Hennessy elevated his own role to Chairman while naming his son, Mike Hennessy Jr, to assume the leadership role of the organization and carry on the family legacy. Under Mike Jr’s leadership, the company enhanced its global potential by entering into a long-term partnership with BDT Capital Partners, LLC in November 2021.

Due to his broad business and educational experience and his understanding of the challenges facing New Jersey, Hennessy’s counsel and insight had been sought by several organizations, including his alma mater Rider University, where he served on the Board of Trustees and was elected to the Executive Committee. In addition to being active in state and national politics, Hennessy also had a long record of service at the local level, where he was a strong advocate for veterans and environmental issues.

Hennessy was preceded in death by his wife, Patrice Hennessy, who bravely battled cancer for almost 10 years until her death in January 2020. Hennessy donated $4 million to Rider University to expand the Science and Technology Center at their alma mater. The Mike & Patti Hennessy Science and Technology Center is set to be completed in 2022.

Mike Hennessy Jr,
President and CEO, MJH Life Sciences™
January Update

Happy New Year! We kick off 2022 with our cover story on the analysis of complex biological fluids using asymmetrical flow field-flow fractionation (AF4). Biopharmaceuticals are increasingly being used in medical treatments and therefore the need to characterize these components is growing. This peer-review article looks at how AF4 has shown increasing applicability for the characterization of components in blood plasma and serum.

This month’s LC Troubleshooting is focused on those topics that are the “bread and butter” of liquid chromatography (LC) troubleshooting—those elements that come up time after time. This instalment focuses on problems related to pressure—too low, too high, and fluctuating. Developing a list of the possible causes can help with troubleshooting.

GC Connections takes a look at Golay’s famous equation that most people see as relating HETP (height equivalent to a theoretical plate) to the carrier gas flow rate or average linear gas velocity in a capillary column. Developed in the 1950s, what is its relevance today?

Introduced in 2003, the “quick, easy, cheap, effective, rugged, and safe” (QuEChERS) sample preparation approach has become widely adopted in many applications. Sample Preparation Perspectives reviews the update of QuEChERS to QuEChERSER (“more than QuEChERS” or also “efficient and robust”) to better take advantage of the features provided by modern MS-based detection tools.

As one of the most popular drinks in the world, the interest in beer styles and flavours continues to grow. In this 7-min method, easy analysis of bitter flavours in beer are achieved with a minimum of sample preparation.

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.

Mike Hennessy Jr,
President and CEO, MJH Life Sciences™
Biopharmaceuticals such as proteins, antibodies, peptides, and nucleic acids are increasingly being used in practically all branches of medicine. The market for these and other biologics has been developing at a faster rate than the market for all drugs and it will continue to grow (1). Yet, the complexity of these structures poses a significant challenge for biopharmaceutical developers and often requires an in-depth characterization to gain understanding and to ensure safety and efficacy (2). A major concern with pharmaceutical proteins is their propensity to form aggregates. These aggregates can exist as small oligomers up to large sub-visible or even visible precipitates, and are of concern because they can reduce drug efficacy and give rise to adverse toxicological and immunological responses (3,4).

While characterizing biopharmaceuticals in formulation is certainly important, it is also of interest to perform the characterization of the drug product after its administration (5). This implies the analysis of blood samples after drug administration. However, blood samples are difficult to
analyze due to the complex nature of their components and their wide size range, which requires extensive sample preparation prior to analysis. Similar to blood plasma, the analysis of proteins in biological fluids such as cerebrospinal fluid (CSF) and amniotic fluid is challenging, and until recently only possible to achieve either in vivo or ex vivo.

The most common method for determining the size and amount of proteins and sub-visible aggregates is size-exclusion chromatography (SEC) in combination with suitable detectors. However, some complications with SEC are that it has high surface area and shear forces, which may be a problem for analysis of large-sized aggregates with the potential to adsorb to surfaces. To achieve high recovery and proper elution of analytes in SEC, the separation often uses a mobile phase that does not agree with physiological conditions with respect to ionic strength, pH, and composition. It is therefore recommended to verify SEC data with complementary orthogonal methods because SEC can give erroneous estimates of the aggregate levels (6,7,8). Analytical SEC is rarely reported to be used for the analysis of biological fluids (without extraction protocols to remove matrix components). This is presumably because samples such as blood plasma could clog the column or rapidly deteriorate its performance due to adsorption of matrix components to the column.

Asymmetrical flow field-flow fractionation (AF4), an orthogonal technique to SEC, has been used to characterize biomacromolecules and pharmaceutical molecules (9,10,11), and it has been proven to be a powerful tool to characterize

![FIGURE 1: AF4-UV-fractogram from the analyses of blood serum, amniotic fluid, and cerebrospinal fluid (serum and amniotic fluid diluted 100× with PBS prior to injection, CSF diluted 10×). Injection volume = 10 µL and flow rate = 0.5 mL/min. The fractograms of reference proteins (grey lines) were added for comparison. Shaded areas (yellow and green) indicate the elution of serum albumin and immunoglobulin G reference proteins and the corresponding peaks in the sample matrices.](image1)

![FIGURE 2: AF4-UV-fractogram from the analysis of blood plasma from mouse, rat, monkey, and human as indicated in the respective graph. All samples were diluted 100× with PBS prior to analysis. For reference, the fractograms from a range of proteins (myoglobin, ovalbumin, bovine serum albumin, immunoglobulin G, ferritin, and thyroglobulin) in the molecular weight range 17–662 kDa are overlaid (grey dashed lines). Injection volume = 10 µL and flow rate = 0.5 mL/min.](image2)
**FIGURE 3:** AF4-UV-fractogram from the analysis of milk processed in various ways. The secondary x-axis shows the hydrodynamic size of the eluted components, and the right-side y-axis the r.m.s. radius as determined by online MALS. Adapted and reproduced from reference 20: https://creativecommons.org/licenses/by-nc-nd/4.0/

**FIGURE 4:** AF4-UV- and FL-fractograms from the analyses of a FITC-labelled antibody in phosphate-buffered saline (PBS 1X, pH 7.4, blue traces) and in cerebrospinal fluid (CSF, red traces). For reference, protein standards (grey traces) are overlaid together with blank CSF (green traces).

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proteins, antigens, and antibodies (12,13). One critical step when applying AF4 is to choose the proper detection technique and factors such as selectivity and sensitivity should be considered. The combination of AF4 with fluorescence (FL), enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), or mass spectrometry (MS) offers the possibility of obtaining information about the size distribution of the biopharmaceutical in blood, the aggregation potential after administration, the interaction of the protein with different matrix components, and potentially applying the methodology to the study of other biological fluids.

Discussion
Several methodologies exist that have the ability to selectively detect and quantify the amount of a component in a biological fluid. Commonly used techniques are ELISA, SPR, and MS. A limitation of all these techniques is that it is difficult or not possible to determine if the analyte response originates from the analyte in monomeric form or if it is from an aggregated (or conjugated) species. In some cases, there is a significant loss of signal when the analyte is present in a biological matrix. It may then be suspected that the analyte is aggregated or associated with matrix components, thereby giving a loss of signal. While such information may give valuable indications, it is often inconclusive and still does not give any information on the size of the aggregates. Furthermore, quantification is often difficult or not possible.

If the sample in the biological matrix could be size-separated and then detected it would enable more detailed information on the analyte size distribution, potentially offering answers to if the analyte is aggregated or associated with matrix components, and to what extent. An alternative methodology is AF4. In contrast to SEC, a sample passing an AF4 channel is subjected to significantly lower shear forces and exposed to much smaller surface areas. Also, clogging of the channel is no longer an issue, and AF4 generally works very well with mobile phases of physiological ionic strength and pH.
Different Sample Matrices
As reported elsewhere, AF4 can be applied to a variety of different biological fluids (14,15,16,17,18). Blood serum and plasma are perhaps the most obvious and interesting from a pharmaceutical point-of-view, but the methodology is equally applicable to other interesting biological matrices such as CSF and amniotic fluid (Figure 1).

In AF4 (Brownian mode), the sample components are eluted in order of increasing size. The eluted fractions can be collected and measured offline, or monitored online by an UV detector, as in the Figure 1 example.

As can be seen in Figure 1, the different fluids share some characteristics: the most intense peak (at ~4 min) corresponds well with the elution time of serum albumin reference (67 kDa), which should be the most abundant component, with the second most intense peak being dominated by IgG (150 kDa). A significant difference between the different matrices lies in the total amount of proteins, so that, for example, the lower protein content CSF gives considerably lower signals than the serum.

It is equally possible to apply the same methodology to serum and plasma from different animals (Figure 2), which can be helpful when investigating if a component behaves differently in different animal types. In the example (Figure 2), the different plasma samples all contained an active pharmaceutical ingredient (API). Fractions were collected after the AF4 size separation and analyzed by ELISA for quantification of the API. As can be noted from the four plasma fractograms in Figure 2, the general profile is similar irrespective of the source, with the difference being that protein levels, and especially antibody levels, show some variability. The different antibody levels may be related to pattern-variations of age, health, and infection history between individuals.

Biological samples and matrices can also contain very large colloidal structures such as lipoproteins, vesicles, and viruses. One example of a biological fluid with an abundance of large colloidal structures is milk (Figure 3). Due to the large size range over which AF4 is applicable, it can
also be applied for the analysis and characterization of casein micelles present in milk (19,20,21).

In Figure 3, AF4 was used to investigate the impact of pasteurization of milk and reconstitution of milk powder on the size and structure of casein micelles. The most abundant component of the milk samples was confirmed to be casein micelles (elution 16–45 min, radius size range of 30–300 nm), with the difference being that the most abundant size (peak apex) depends on the milk type. In the same analysis, the first population of the fractogram consists mainly of serum (whey) proteins and other relatively small and low molar mass components of the milk (<6 nm in hydrodynamic radius and peak maximum at 2.8 min). The second population (between 6–15 min)—of which there is substantially less in the raw and pasteurized milk samples—consists partially of serum protein aggregates.

Separation Considerations

As has been shown, it is possible to size-fractionate biological fluids containing a wide variety of components, with sizes ranging from small proteins up to ~0.5 µm colloidal structures. However, some aspects of the sample and the aim of the study must be considered before starting the experiments. One must primarily consider the size range of interest—is it peptides and small proteins, or large colloids? (AF4 can, in Brownian mode, cover from ~2 nm to approximately 1000 nm hydrodynamic diameter.) Depending on the size range to be investigated, different separation conditions should be used for optimal resolution. One should also consider the sample load as many biological fluids have a very high protein content. For example, blood serum and plasma are often in the 50–100 mg/mL protein range. To avoid overloading the AF4 channel (otherwise detrimental effects on resolution can be experienced), only small volumes of sample need to be injected or the sample needs to be diluted prior to analysis. Another consideration is that in many cases it is preferable to investigate the sample under conditions as close as possible to physiological conditions. Thus, it is suitable to use a carrier liquid that has a physiological pH and ionic strength. Some biologically active components, for example, enzymes or milk as in the earlier example, may require certain ions (Ca²⁺ or Mg²⁺) to maintain their structure or function. Such considerations may impact the choice of buffers used for both dilutions and as the carrier liquid.

Detection Considerations

Biological fluids are complex and contain millions to trillions of different components. While AF4 is able to separate a wide range of macromolecular and colloidal components, the resolution is not sufficient to resolve all of them. Thus, one of the critical steps when applying the methodology is to choose the detection technique. Due to the complexity of the sample matrix and the often low concentration of the analyte, two factors are particularly important: selectivity and sensitivity. As the analyte will be coeluting with other substances, selectivity is necessary for accurate concentration determinations. As the analyte is often present at low concentrations in body fluids, and the sample becomes further diluted during the AF4 separation, there is also demand on the detection sensitivity. The most straightforward selective detection technique is via fluorescently labelling the component of interest. This approach has the added benefit of being easily performed online. An example is presented in Figure 4: a fluorescently labelled antibody is present in CSF, making it straightforward to monitor by AF4 equipped with an online fluorescence detector.

A potential drawback with FL-labelling is that the introduction of the chemical groups with fluorescence activity might affect how the component interacts with matrix components. From that perspective it would therefore be better to use a detection technique that is able to selectively measure an unlabelled component. Examples are immunoassays such as ELISA or SPR (Figure 5) (15). Besides the obvious benefit of not needing to go through a labelling procedure, any concerns that the methodology incurs changes in the physicochemical properties of the analyte are eliminated. However, immunoassays are not without caveats either. First, there must be an antibody against the target analyte. For late-stage pharmaceutical candidates this may be the case, but in the early stage it is not always available. Another more fundamental consideration that should be taken into account is the detectability of the analyte if present in an aggregated state or associated with matrix components. It is not guaranteed that an immunoassay technique (such as ELISA or SPR) has the ability to give a response for an oligomer or aggregate (at least it is not certain
that the detection will have the same sensitivity or response factor for an aggregate). In the example of SPR on a stressed trastuzumab, the large aggregates were detected when the stressed trastuzumab was in a buffer system. However, when the stressed trastuzumab was in a blood serum matrix, the largest aggregates were not detected (15). This may be caused by matrix components associating with the large aggregates, making them less accessible to the SPR-sensor surface.

**Limitations**

During the separation, the sample is diluted, and the run-time of the complete process (from injection to detection or fraction collection) is at least several minutes up to an hour. For example, if a protein aggregate dissociates upon dilution and does so within seconds or minutes, such protein aggregates would not be detected or accurately quantified by the methodology. In summary, for a system where the analyte aggregates or associates rapidly and is dependent on concentration, results may be inaccurate.

AF4 fractionates by hydrodynamic size. Thus, if there are changes in an analyte that occur but that do not significantly change the hydrodynamic size of the analyte, they will not be noticed by any shift in elution time. An example would be the association of a peptide internally within a large protein structure may go undetected (although FL-labelling of the peptide or MS against the peptide would be able to detect this).

**Summary**

There is a renewed interest in AF4 as it may be helpful with regards to analyzing complex biological fluids. The methodology becomes especially interesting when combined with selective detection techniques, such as FL, ELISA, SPR or MS, as the combination then offers the potential to provide information on analyte behaviour in biological systems that is difficult to obtain by any other technique.

**References**


**Mats Leeman** is a senior scientist at Solve Research and Consultancy in Lund, Sweden, where he works with size characterization of proteins, polymers, and nanoparticles. Mats holds a Ph.D. in technical analytical chemistry from Lund University. He has been using AF4 for more than 15 years for various applications within the pharmaceutical, specialty chemicals, and biotech industries.

**Alejandra Castro Nilsson** is a senior scientist and CEO at Solve Research and Consultancy in Lund, where she works with the characterization of biopolymers, proteins, and nanoparticles. Alejandra holds a Ph.D. in food engineering from Lund University. She has worked with AF4, light scattering, laser diffraction, and chromatographic techniques for more than 10 years for various applications, including polysaccharides, proteins, nanoparticles, and biopolymers.

**Lars Nilsson** is Professor of Formulation Technology at Lund University. His research focus is on applied colloid chemistry and formulation technology as well as development and application of AF4 methodology for the separation and characterization of biomacromolecules and nanoparticles within the life sciences. His AF4 work currently focuses on the analysis and characterization of therapeutic proteins and peptides in relation to formulation aspects and biological fluids.
In the fourth episode of this six-part podcast, David Perlman, senior principal scientist and director of ultrasensitive proteomics at the Merck Exploratory Sciences Center, discusses where LC-MS proteomics has the largest impact, the need for single-cell proteomics, the promise single cells have on our day-to-day lives, and more.

**PERLMAN:** What did you decide to work in the field of proteomics, particularly using LC-MS technologies?

**Perlman:** I have a diverse background across multiple fields in biological, physical, and chemical sciences, but I find these areas are all mutually beneficial and mutually inform and enhance one another. They come together to help me better understand complex biological systems and build better working models.

LC-MS-based proteomics is the way to physically interrogate the chemical makeup of cells and tissues at a meaningful resolution. There’s no better way to get at the protein composition of biological systems and examine their dynamics in normal, healthy states as well as disease states. Other analytical techniques measure proteins or other components within the cell, but most of these involve antibodies, which themselves have huge caveats and provide a very narrow tunnel vision of the epitopes they were raised to detect, or the techniques involved in direct measurements or measurements of species within the cells like RNA transcripts are often widely divergent from cellular phenotype. These other techniques can provide a lot of data, but these data can be biologically muddled or misleading. So, there is no other way for an unbiased quantitative measure of protein levels within cells and no better way to follow their dynamics across space, time, or stimuli than LC-MS-based proteomics. For me, it’s the means to understand the mechanisms of life and roots of disease—that’s why I’m drawn to it.

**LCGC:** Considering that LC-MS proteomics is just a tool, where is its largest impact?

**PERLMAN:** First, I disagree with the assertion that LC-MS-based proteomics is just a tool. I describe it instead as a rapidly developing transformative-enabling technology. It’s a field or several fields, and it’s improving rapidly year after year, which ultimately provides greater delivery of quantitative information about more species within cells but also greater impact on the way we develop drugs, understand disease, identify and vet new smarter drug targets, etc. It’s an ever-increasingly powerful, fundamental technique with no less of an impact on biology and medicine than microscopy. It has already become a central component of biomedical research, and its use is only going to intensify over the coming years.

**LCGC:** What are the main technological challenges with moving cutting-edge proteomics research into real-life applications?

**PERLMAN:** This is a great question because the technology could be more widely used than it is. A huge barrier historically has been the difficulty in setting up and maintaining a state-of-the-art LC-MS-based proteomics platform, especially the finickiness and lack of robustness of the highest end LC-MS technologies.
However, this has been changing rapidly; the more the technologies are made user-friendly and robust, the better. This gets them into more hands of creative experimentalists and biologists and not solely operated by specialized instrumentalists. Other than that, the impact of current proteomics on real-life applications is limited mainly by the inspiration and educated engagement of the researchers themselves, which I hope is growing year by year.

**LCGC:** You are one of the first who started to work on single-cell proteomics. Can you briefly explain the need for this type of application?

**PERLMAN:** Cellular heterogeneity is a feature of biology, a feature of disease, and a feature of the response to therapeutics, so it really matters. With single-cell proteomics, we aim to characterize cellular heterogeneity, so we can understand how real biological multicellular systems work. More specifically, single-cell proteomics can be used to help better understand the mechanisms of resistance to chemotherapy drugs so that smarter treatments or combinations can be developed. It can also be used to help define antigens that are co-expressed on the surface of cells, such as cancerous or other disease cells, which could be exploited for targeting these cells with something like a therapeutic antibody or drug antibody conjugate. Single-cell proteomics can be also used to understand how certain types of immune cells like macrophages change their phenotype from pro-inflammatory to anti-inflammatory states or vice versa during the course of disease and how this change could be reversed by therapeutics.

**LCGC:** Nano-LC coupled with MS is established as the gold standard in bulk proteomics. What are specific requirements for ultra-sensitive analysis specific to LC instruments?

**PERLMAN:** For ultrasensitive proteomics, we need ultra-low flow rates, so we need LC systems that reliably deliver run-after-run at these flow rates with smooth, uniform gradients. We need the highest performance columns as well for low-abundant samples that concentrate the analytes into the sharpest possible peaks. We also need autosampler features such as reliable pickup of small volumes and vial bottom-sensing so that the entirety of samples can be injected. It’s also critical that the system fittings and liquid junctions are configured to produce true zero dead volumes and that the internal fluid paths are minimized. All this is necessary to reduce losses that otherwise bring down the experiment in a proverbial death by a thousand cuts.

**LCGC:** What can make LC-MS proteomics of single-cells a widely adopted technique? What are the requirements for analytical throughput and proteome depth?

**PERLMAN:** So far, it’s been the domain of a few brave souls, and this is largely because of the artisanal nature of some of the initial sample preparation techniques and the extreme difficulty of setting up and maintaining an ultrasensitive proteomics platform at the utmost levels of performance. This has been a big barrier, even a psychological barrier for some, but this is changing. As the sample-prep techniques become well adapted for miniaturization and automation, and as the technologies for LC-MS become more robust, easier to use, and faster, this field will open up to more practitioners. I expect explosive growth in the next few years.

Regarding throughput and proteomic depth, it depends on the type of experiment. As a rule of thumb, we shoot for 2,000 to 5,000 proteins quantified per single cell and do it at a rate of 1,000 or more cells a day to make it through meaningful experiments in a reasonable amount of time. We’re currently approaching this depth but not yet at this throughput. We’d love to see numbers in the tens to hundreds of thousands of cells per day, but a thousand cells per day is likely attainable with existing or slightly improved technologies.

**LCGC:** LC-MS sensitivity is one of the key requirements for single-cell and limited sample amounts analysis. What are the other technical requirements?

**PERLMAN:** Injection carryover must be minimized otherwise every cell looks the same. Sample injections, LC gradients, and column performance have to be optimized and entirely uniform run-to-run so that the data align across large data sets. One key feature of single-cell proteomics is you have to have all your eggs in one basket. Every bit of every sample must be injected—you only have one shot to make the most of that sample. All your instrument parameters must be optimized for low-abundance materials to be able to extract as much data as possible out of this fleeting sample as it rushes by. For the same reason, the more onboard and on-the-fly instrument intelligence—which will greatly improve the quality of both shotgun and targeted single-cell LC-MS experiments in the future—that can be leveraged, the better to enhance the overall quality and depth of the data in the end.

**LCGC:** Analysis of single cells has a lot of promise that might directly affect each of us. How long do you think it will take to see this happen?

**PERLMAN:** Other single-cell analytical methods such as microscopy or flow cytometry have been a central component of biomedicine for decades and are impossible to divorce from the development of the medicines and vaccines that are currently on the market. My assertion is that because of its additional power and depth of molecular insight that is so closely tied with cellular phenotype, single-cell proteomics will be integrated rapidly into discovery and developmental-phase research for the next generation of medicines. I expect this transformation to occur within the next three to five years.
Some “LC Troubleshooting” topics never get old because there are some problems that persist in the practice of liquid chromatography (LC), even as instrument technology improves over time. There are many ways for things to go wrong in an LC system that ultimately manifest as deviations from the expected pressure. Developing a short list of the likely causes of these deviations can help streamline our troubleshooting experience when pressure-related problems occur.

Writing this “LC Troubleshooting” column and thinking about topics each month is interesting in the sense that there are some topics that just never get old. Whereas, in the chromatography research world, certain topics or ideas become obsolete as they are displaced by newer and better ideas, in the troubleshooting world there are certain topics that have remained relevant since the very first troubleshooting article appeared in this magazine (LC Magazine at that time) in 1983 (1). Over the last few years, I’ve focused several “LC Troubleshooting” instalments on contemporary trends (such as the relatively recent advances in our understanding of the effects of pressure on retention [2]) in liquid chromatography (LC) that are affecting the way we approach our interpretation of LC results, and approach troubleshooting with modern LC instruments. With this month’s instalment, I am starting a series focused on some of the “bread and butter” topics of LC troubleshooting—those elements that are essential for any troubleshooter, no matter the vintage of the system we are working with. The topics at the heart of this series will be highly related to the well-known “LC Troubleshooting” wallchart (3) that hangs in many laboratories. For the first instalment in this series, I’ve chosen to focus on problems related to pressure (too low, too high, or fluctuating). I hope LC users young and old will find some useful tips and reminders related to this important topic.

Everything is Possible
In the area of pressure problems, everything is possible. Sometimes pressure is unexpectedly low but stable. Other times the pressure is too low, and appears to steadily decrease. The same is true for pressures that are higher than expected. In other cases, the observed pressure may seem to be about right, but it is fluctuating more than usual. Figure 1 illustrates the idea that pressure problems appear in all kinds of different ways, and lists the specific situations that are discussed in this article. The list of pressure-related problems shown in Figure 1 is not exhaustive; in this instalment, I focus on those problems that I see most frequently in practice.

What Is To Be Expected?
A critical step in any troubleshooting exercise—but one that I think is underappreciated—is recognizing that there is a problem to be solved. Recognizing that there is a problem usually amounts to recognizing that what is happening with the instrument is different from what is expected to happen, and our expectations are formed from theories, empirical knowledge, and experience (4).

Before getting into details about what we can expect about pressure, a few words to clarify what it is and how it is measured in LC instruments are warranted. In LC, when we say “pressure”, we are really talking about a “pressure drop” or a “pressure difference”. These more precise terms are indicated in various equations that relate pressure drop to other variables, such as flow using the symbol $\Delta P$. Most commercially available LC systems have a single pressure readout associated with the pump that reports the pressure drop between the pump and the outlet of the system (the outlet side of a detector flow cell) that, for all practical purposes, is zero, because the atmospheric pressure of about 1 bar is usually small compared to LC operating pressures. This single pressure readout quantifies the total pressure drop across the entire flow path, but does not tell
us anything about the pressure drops across individual elements of the flow path (for example, filters, different pieces of connecting tubing, and the column).

**Pressure Drop Across Connecting Tubing:** Most practical high performance liquid chromatography (HPLC) is done under conditions where flow through connecting tubing in the system is laminar. Under these conditions, the pressure drops across the different pieces of tubing can be calculated with the accuracy needed for troubleshooting purposes using Poiseulle’s Law:

\[
\Delta P_{\text{tub}} = 128 \cdot \frac{\eta \cdot L_{\text{tub}} \cdot F}{\pi \cdot d_{\text{tub}}^4}
\]  

where \(\eta\) is the dynamic viscosity of the mobile phase, \(F\) is the flow rate, and \(L_{\text{tub}}\) and \(d_{\text{tub}}\) are the length and diameter of the tubing, respectively. Calculating the pressure drop using equation 1 is straightforward when all of the values needed are available; however, the dependence of viscosity on mobile-phase composition and temperature is a bit complex. Fortunately, there are some freely available web-based tools that take these factors into account (for example, see reference 5, and https://www.multidlc.org/ dispersion_calculator), and provide users with quick estimates of the expected pressure drops for the tubes in their systems.

**Pressure Drop Across Columns:** The pressure drop across the LC column itself (assuming the column is packed with particles) can be calculated using equation 2 (or similar). Like equation 1 for open tubes, the pressure drop depends on the column length, mobile-phase viscosity, and the flow rate (through the interstitial mobile phase velocity, \(u_i\)). Different in equation 2 are the \(\Phi\) term that quantifies the permeability of the packed particle bed and the particle size term, \(d_p^2\):

\[
\Delta P_{\text{col}} = \frac{\Phi \eta u_i L_{\text{col}}}{d_p^2}
\]  

As with equation 1, calculating the pressure drop across the column is straightforward once all of the values for length, viscosity, and other variables are in hand, but they are not all easy to come by. Once again, there are freely available simulators that can calculate the pressure drop for conditions of interest. Two such simulators that I am familiar with are the web-based LC simulator maintained by my group (https://www.multidlc.org/hpclsim), and the spreadsheet-based simulator developed more recently.
by Professor Davy Guillarme’s group (see [6]; https://ispso.unige.ch/labs/fanal/practical_hplc_simulator.en).

**Other Elements of the Flowpath:** The other elements of an LC flow path that can contribute substantially to the pressure drop measured at the pump are inline filters (and guard columns, though these can be treated like columns as above). Most inline filters sold for use in analytical LC systems are designed in a way that they will not contribute more than a few bar to the total pressure drop under typical conditions (that is, less than 5 mL/min). When debris begins to accumulate on the filter, the pressure drop across the filter will increase, and become higher, and sometimes nonlinearly, dependent on operating conditions (for example, flow rate and mobile-phase composition).

Because it is difficult to cope with this hard-to-predict behaviour, in my laboratory we simply change the filter if the pressure drop across it exceeds about 10 bar.

**Using These Numbers in Troubleshooting Practice:** Throughout the hundreds of “LC Troubleshooting” articles John Dolan wrote, he emphasized the value of “rules of thumb” in effective troubleshooting (7). I completely agree, and think the value of these ideas—which are informed both by theory and experience—cannot be overstated. As an example of a rule of thumb that is useful in the context of troubleshooting pressure problems, the one that we use in my group is that the pressure drop across all of the tubing (no column) in a “typical” LC system is about 30 bar under the following conditions (if the observed pressure is much lower or higher than that, this should trigger a thought that something is not right):

- All tubing from injector to detector is 0.005” i.d. (120 µm); total length is about 60 cm. The capillary from pump to injector is usually larger in diameter, and does not contribute much to the total pressure drop.
- Flow rate, 1 mL/min.; Temperature, ambient; mobile phase, 100% aqueous.

**Situations Involving Pressure That Is Lower Than Expected**

There are two main problems that lead to pressures that are lower than expected.

- **Leaks:** There are many different ways that a leak can occur in a LC system. The most common ones I see are related to connections (for example, tubing to valve, or tubing to column). Usually, these leaks are relatively small (that is, a few microlitres per minute), and can be corrected by tightening the fitting slightly. However, be careful—forcing a metal ferrule into a connection too much can deform the port (such as a column endfitting or valve port). If you feel like the connection is already very tight, then it is better to throw the capillary away and start with a fresh one. I always tell my students that “too loose is much better than too tight”. Another way of saying this is that it is much cheaper to replace several capillaries than it is to replace a valve stator because a capillary was overtightened and deformed or stripped the threads on a valve port. Significant leaks can also occur between the pump pistons and seals. We should never be able to see liquid emerging from the pump head in the area of the piston/seal. If liquid is observed, the seals are probably leaking and should be replaced. In my experience this problem does not occur nearly as frequently as it did with pumps 20 years ago. Finally, it is possible for polyetheretherketone (PEEK) tubing to burst, even when working below the advertised pressure limit of the tubing. When this happens it usually leads to a major, obvious leak, whereas leaks with connections and pump seals tend to be more subtle.
- **A partially obstructed solvent inlet filter:** LC pumps rely on a free, steady flow of solvent from the solvent bottle to the inlet check valve to work properly. If the inlet filter on the end of the line in the solvent bottle becomes partially obstructed by particulates or bacterial growth, this can slow the flow to the point where the pump is “starved” of solvent, and unable to deliver flow to the column at the specified flow rate, leading to lower than expected pressure. If one suspects that this might be the problem, a quick check involves simply removing the inlet filter from the solvent line. If the pressure returns to normal after the filter is removed, then the filter needs to be cleaned at a minimum, and it is usually best to just replace it altogether.

**Situations Involving Pressure That Is Higher Than Expected**

Most problems involving higher than expected pressure are somehow related to accumulation of debris somewhere in the system. The origins of this debris vary; it can come from particulate matter in the injected sample, molecules that
are soluble in the sample solvent but precipitate in the mobile-phase stream, polymeric material shed by pump and injector seals, and so on. The specific nature of the problem that results from this debris depends strongly on how the system is configured. Determining where the obstruction is in the system can be tricky. A systematic approach to finding out where the problem lies involves removing components from the flow path one at a time, starting from the downstream end. For example, suppose we are running at 1 mL/min, and we observe a pressure at the pump of 600 bar, which is high compared to a normal operating pressure of 250 bar. With the flow off, remove the detector from the flow path. Turn the pump back on and record the pressure. If it has only decreased by 5 bar to 595 bar, then we know that the obstruction does not lie in the detector flow path. Again, with the flow off, remove the tubing between the detector and the column outlet. Turn the flow back on and record the pressure. If it has decreased another 10 bar to 585 bar, then we know that the tubing between the column outlet and the detector is not the source of the problem. Next, remove the column, turn the flow back on, and record the pressure. There should be a significant difference between the pressure recorded with and without the column connected. Suppose in this case that the pressure is still 365 bar even without the column connected, which would be abnormal in any typical analytical LC system. Next, suppose that upon removing the inline filter installed immediately upstream from the column the pressure drops to 20 bar. This would tell us that the pressure drop over the filter itself was 345 bar (far higher than expected), indicating that the filter should be thrown away and replaced. This “one-piece-at-a-time” approach can feel tedious when trying to get an instrument back on track, but it is the most reliable way to isolate the problem. The three most commonly encountered scenarios are:

- An obstruction in an inline filter: In my laboratory, partially plugged filters account for 95% of our high pressure problems. Once it is clear that a filter is obstructed, one could try backflushing them, but this solution is usually short-lived. It is far better in the long run to just replace the filter.
- An obstruction in a piece of capillary tubing: This does not happen very often if inline filters (0.2–0.5 µm porosity) are used immediately after the sample injector. If inline filters are not used, then the capillaries of the smallest diameters

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and the ones furthest upstream (that is, closest to the injector) are the most likely to become blocked. Reversing the flow through an obstructed capillary will occasionally be sufficient to remove the debris, but the most reliable solution is to just replace the capillary.

- **An obstruction at the inlet of a column:** This can also be largely prevented through routine use of an inline filter upstream from the column. If an increase in pressure drop across the column is observed over time, reversing the flow through the column can flush debris out of the inlet frit, but, in my experience, the reduction in pressure is usually short-lived. It is also important to note that some column manufacturers use frits with larger porosities at the column inlet; reversing the flow is then a bit risky, because some particles could go through the frit and be lost from the column (thank you to Professor Chuck Lucy for this reminder). The bottom line is that it is best to avoid problems like this with the column by protecting it, through routine use of inline filters and/or guard columns.

### Situations Involving Pressure That Appears To Be Fluctuating

Most modern LC pumps are based on some variation of a reciprocating dual piston design, where the pressure variation that occurs at the end of each piston stroke can be minimized, but is difficult to eliminate entirely. The specification for modern pumps is that the pressure variation should not exceed about 1%. If the observed variation is much larger than 1%, then it is most likely because of one of two reasons.

- **Malfunctioning check valves:** A typical reciprocating dual piston pump design relies on two check valves to keep the mobile-phase flow moving in the direction of the column. When solvent is being pushed out of the pump head toward the column, the inlet check valve closes so that solvent cannot travel back towards the solvent bottle. When solvent is being drawn from the solvent bottle into the pump, an outlet check valve closes to prevent solvent from flowing backwards into the pump head. When working properly, these check valves open and close multiple times per minute. If one or both of them does not open or close properly, there will be a significant interruption in the flow to the column, which in turn manifests as a change in pressure. Determining which of the two check valves is faulty can be tricky. One approach is to first replace the inlet check valve with one that is known to be functional. If this does not solve the problem, then re-install the original inlet valve, and change out the outlet check valve with one that is known to be functional. If this does not reduce the pressure fluctuation, then the problem does not lie with the check valves.

- **Gas bubbles in the pump head:** Even a pump with properly functioning check valves can get tripped up by an air bubble. If air becomes trapped in a pump head (for example, after the pump has accidentally run dry, because an inline degasser is not working properly, or after a prolonged period without use), it can lead to severe flow or pressure fluctuations. Purging the pump at high flow rate (with the column disconnected) is often effective for dislodging the bubble and returning to normal operation. If this does not resolve the problem, purging the pump head with isopropanol is another approach that often works well.

### Summary

In this first installment on essential topics in LC troubleshooting, I have discussed situations where the observed system pressure is somehow different from what is expected or normal. Effective troubleshooting for this type of problem begins with a sense for what the expected system behaviour is, so that a deviation from those expectations is noticeable. While there are many different potential causes of pressure-related problems (too low, too high, or fluctuation), most problems can be connected to five or six specific causes. Understanding this short list of likely causes provides a good place to start troubleshooting, but it does not capture all possibilities. Readers interested in learning about a deeper list of causes and solutions are referred to the “LCGC Troubleshooting” wallchart (3).

### References


### ABOUT THE COLUMN EDITOR

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In this instalment of “GC Connections,” we take a close look at Golay’s famous equation that most people view as relating height equivalent to a theoretical plate (HETP) to the carrier gas flow rate or average linear gas velocity in a capillary column. We explore the original works of Golay and Keulemans. We also look closely at the original equation, the assumptions involved with its development, and the assumptions (both good and bad) that have been applied to HETP over the years. In part two, we will explore the consequences and relevance of this theory today, with temperature programming, very long (and very short) columns, high pressure drops, and vacuum outlet detectors.

The height equivalent to a theoretical plate (HETP) is one of the most important parameters used to measure column performance. HETP is the length of column required to generate one theoretical plate, with a theoretical plate being one transfer process of an analyte molecule between the mobile phase and the stationary phase. We are all familiar with considering the number of theoretical plates in a column or the number of theoretical plates in a length of column, as these are often stated by column vendors as a measure of column quality. More theoretical plates and more theoretical plates per metre are often considered good, leading to better resolution. A 30 m column with 100,000 theoretical plates is said to have 3333 theoretical plates per metre.

We use a chromatogram to measure theoretical plates (as shown in Figure 1), using equation 1, where \( N \) is the number of theoretical plates, \( t_R \) is the retention time of the peak and \( W_b \) is the peak width at the baseline. \( N \) and HETP (as discussed later) are dependent on many parameters, including temperature, carrier gas flow rate, inlet and outlet pressures, column dimensions, and the choice of carrier gas. One challenge in comparing plate numbers between different columns is that the experiments will almost always have been performed under different conditions, making true comparisons difficult.

\[
N = 16\left(\frac{t_R}{W_b}\right)^2 \tag{1}
\]

HETP is then calculated using equation 2, where \( L \) is the column length and \( N \) is the number of theoretical plates calculated using equation 1:

\[
H = \frac{L}{N} \tag{2}
\]

As we will discuss in detail shortly, \( H \), or HETP, is also defined as the rate of band broadening. As we know, the peaks or bands get wider in all chromatographic techniques as the peaks and bands traverse the length of the column. HETP provides a measure of the rate of the broadening, with a smaller HETP indicating slower band broadening, leading to sharper peaks.

Origins of Golay’s Famous Equation

How was HETP as a measure of the rate of band broadening originally defined? This definition goes back to the earliest days of gas chromatography (GC) in the 1950s and the work of A.J.P. Martin and A.T. James, the inventors of GC; J. Van Deemter, who developed the original theory of band broadening for packed columns; Golay, who developed the theory for capillary columns; and Keulemans, who provided a fundamental conceptual definition for a theoretical plate.
Keulemans presented a picture of the separation process in chromatography in a classic text, *Gas Chromatography*, published in 1959 (1). In the foreword to this short text, A.J.P. Martin described it as “an admirably clear account of all the practical and theoretical aspects of this rapidly growing method”. The clear influence of the text is seen in the highly recognized 1965 text, *Basic Gas Chromatography*, by McNair and Bonelli (2).

As illustrated in Table 1, Keulemans described each theoretical plate as a liquid–liquid extraction between immiscible solvents. Starting with a flask containing a solution of an analyte dissolved in a solvent, say, water. The solution is mixed with an equal volume of hexane. If the partition coefficient is 1, then equal masses of solute are present in each phase. The hexane layer is then removed and mixed with an equal volume of water in a second flask and fresh hexane is added to the first flask. Each flask now contains half of the original analyte, equally distributed between the two phases. This process can be repeated with a third, fourth, fifth, and up to any number of flasks, ultimately resulting in a distribution of analyte among all the flasks (seen in Table 1), calculated for 10 flask theoretical plates, 10,000 molecules, and a distribution constant between the two phases of one.

The numbers in Table 1 represent the number of molecules out of the 10,000 in each flask or theoretical plate. We can note immediately that as the number of flasks increases, the number of molecules in each flask decreases, analogous to the chromatographic peak becoming broader and shorter. Also note that there are still some molecules in the initial flask, even after 10 extractions, an often-unappreciated consequence of chemical equilibrium: There are always some molecules left behind.

The description provided above makes several assumptions that, although illustrative of the chromatographic process, do not account for all processes that occur in real columns. First, it assumes that each theoretical plate occurs independently and has no direct contact with the other theoretical plates. Obviously in a column with a mobile phase, this assumption cannot be true as the mobile phase is continuously transporting solutes along the column. Second, it assumes that there are no

---

**TABLE 1: Illustration of a 10 theoretical plate distribution of 10,000 molecules with a partition coefficient of 1**

<table>
<thead>
<tr>
<th>Flasks Representing Theoretical Plates</th>
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<tbody>
<tr>
<td>Step</td>
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<tr>
<td>10</td>
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</tbody>
</table>

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**FIGURE 1: Simple chromatogram showing the classical calculation of $N$, the number of theoretical plates.**
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barriers to mass transfer at the phase interfaces. In a real system, of course, the kinetics of mass transfer across the phase interfaces must be considered. Finally, there is no consideration of diffusion both within and between the two phases. In a column, the solute molecules distribute throughout the mobile and stationary phases. Golay’s equation, described below, uses a description of the chromatographic process like Keulemans, and accounts for these assumptions, plus the dimensions and physical conditions observed within columns.

The “Golay Equation”
Golay provided a thorough description of an equation describing the rate of band broadening in the proceedings of the “Gas Chromatography 1958” symposium organized by the Hydrocarbon Research Group of the Institute of Petroleum and the Koninklijke Nederlandse Chemische Vreniging in Amsterdam, The Netherlands, in May 1958. The symposium volume was published in 1959, edited by Desty (3). The equation for circular capillary columns is presented as equation 3:

\[ du = \left( \frac{2D}{v_0} \left( \frac{1}{2} \frac{k^2 L}{6(1+l^2)C} \right) + \frac{k v_0 L}{6(1+l^2)C} \right) dx \]  

Note that Golay also described a similar equation for rectangular capillaries. At the time, there was considerable discussion about which would be better, and clearly circular capillaries have become far more popular. Interestingly, with recent interest in columns etched into silicon wafers for “laboratory on a chip” applications, rectangular capillaries are increasing in usage and importance. This equation is popularly called the “Golay Equation”, but it combines the work of many early researchers in chromatography.

Definitions of the many variables are discussed in the “deeper dive” below. Interestingly, the proceedings volume includes commentary following the formal text of the talk, in which substantive discussion among the symposium participants was captured. The names of those participating in that discussion read like an all-star lineup of modern chromatography inventors, including Golay, A.J.P. Martin, R.P.W. Scott, I.G. McWilliam, and others. It was traditional in the past to assign scribes to record the discussions. In one especially important exchange between Golay and Martin, they appear to agree, possibly begrudgingly, to use the definition of HETP presented by Keulemans and illustrated above (4).

In the original work, several related forms of the equation are presented, relating to specific situations that may occur in the making of capillary columns; the form used here is equation 31a from that work, chosen for simplicity in discussing the basic principles and consequences. The first thing to note is that this is a differential equation relating to \( du \), a differential of peak width to \( dx \), a differential of length or distance. The terms enclosed in the parentheses provide what we term as HETP or \( H \), the height equivalent to a theoretical plate, so the equation could be conceptually simplified to equation 4. Interestingly, similar to the Keulemans description, the independent variable is the length of the column (\( dx \)), as the differential is distance and the dependent variable is the peak width, represented by the differential of the second statistical moment (\( du \)).

\[ du = (HETP)dx \text{ or } \frac{du}{dx} = HETP \]  

Equation 4 shows us that the rate of band broadening as the band traverses the column is equal to HETP.

As we have learned in short courses and textbooks, smaller HETP means a slower rate of band broadening and sharper peaks. In many textbooks and courses, the terms within the parentheses in equation 3 are simplified to the form shown in equation 5. As we will see in Part 2 next month, equation 5 is an oversimplification that can generate confusion and possibly lead to false directions in method development and optimization.

\[ HETP = \frac{B}{v_0} + C_m v_0 + C_s v_0 \]  

Note the average velocity of carrier gas (\( v_0 \)) is usually chosen as the independent variable in equations 4 and 5, leading to the “van Deemter” or “Golay” plots often seen in discussions of column performance in the scientific literature. The three terms are often described as representing analyte diffusion in the mobile phase (the “\( B \)” term), analyte diffusion in the stationary phase (the “\( C_m \)” term), and analyte diffusion in the mobile phase (the “\( C_s \)” term). In developing equation 3, Golay noted the similarity in form to the equation of van Deemter, developed a few years earlier.

A Deeper Dive into Golay’s Equation
Whether we perform detailed calculations based on it or not, equation 3 provides a very useful description of the many variables and processes relating to band broadening and, ultimately, to peak widths and resolution. Some important general principles are discussed here; for a detailed description of all variables and how they are used to evaluate column performance, see these book chapters or the discussion in ChromAcademy, LCGC’s online chromatography learning platform (5–7). In short, to minimize HETP, the quantities in parentheses in equation 3 should be minimized.
In the first term within the parentheses, often called the “B” term using the terminology in equation 5, diffusion in the mobile phase is considered. The numerator, \(D\), is the diffusion coefficient of an analyte molecule in the gas phase, which for simplicity can be considered a constant for that analyte at a given temperature in a given carrier gas. Diffusion coefficients in the gas phase are mainly dependent on the identity of the analyte, the solvent gas, and the temperature. The denominator is the average carrier gas velocity in the column. Not surprisingly, this suggests an inverse relationship between HETP and average carrier gas velocity.

The second term, often abbreviated as \(C_m\), relates to mass transfer, also in the mobile phase, and is the major difference between Golay’s equation for capillary columns and the original van Deemter equation for packed columns. Capillary columns usually have much larger phase ratios (volume of mobile phase divided by volume of stationary phase) than packed columns, so mass transfer in the mobile phase becomes important. Note that in this term, the same diffusion coefficient described above is now in the denominator and the average carrier gas velocity \(v_c\) is in the numerator, suggesting a proportional (linear) relationship between HETP and carrier gas velocity.

Additional variables in the \(C_m\) term include the square of the column radius, \(r_0^2\), and a complex polynomial of the retention factor \((k)\). Note that all of the variables, except for the column radius are temperature dependent and that the retention factor is also related to the column dimensions and average linear gas velocity.

The third term, \(C_s\), relates to mass transfer in the stationary phase. Similar to the \(C_m\) term, the \(C_s\) term is linear with the average carrier gas velocity and the square of the column radius \((v_c, r_0^2)\). Also, similar to the \(C_m\) term, the denominator includes a diffusion coefficient, this time in the liquid phase, which depends on the specific analyte, liquid phase, and temperature. Additionally, the analyte partition coefficient \((c)\) into the liquid phase is part of the denominator. The term also includes a polynomial function of the retention factor \((k)\).

Taken together, the three terms of the expression describing HETP, as presented by Golay, have numerous dependencies, and many of the variables that make up the equation have their own dependencies. The column radius is fixed and appears in two of the three terms. All the other variables are dependent on temperature. The average carrier gas velocity is dependent on the choice of carrier gas, the column dimensions (length and inside diameter), and temperature. The partition coefficient depends on the chemical nature of the analyte and stationary phase, and the retention factor depends on the partition coefficient, column phase ratio, and is further related to the average carrier gas velocity.

In our next installment, we will examine these dependencies and the assumptions upon which our traditional view of HETP, based on the “Golay Equation”, are founded. We will also discuss their relevance in today’s capillary GC. The major changes between thinking about GC from the 1950s, when these ideas were first developed, and today include: the nearly universal use of temperature programming, smaller diameter capillary columns, thinner stationary phase films, and high vacuum column outlets, as seen in GC–mass spectrometry (MS). We will see that many of the traditional views that we were all taught (and still teach) about HETP, peak broadening, and peak widths in gas chromatography deserve some new thinking.

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The QuEChERSER Mega-Method

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Introduced in 2003, the “quick, easy, cheap, effective, rugged, and safe” (QuEChERS) sample preparation approach has been widely adopted in many applications, particularly in chemical residue analysis of foods. Prior to QuEChERS, sample preparation generally entailed several time-consuming, labour-intensive, and reagent-excessive steps, but the commercialization at the time of powerful, cost-effective, benchtop gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem MS (LC–MS/MS) instruments enabled the implementation of the QuEChERS procedure. Despite analytical technologies continuing to improve over the last two decades, many laboratories are still using QuEChERS protocols developed for outdated instrumentation. Recently, QuEChERS has been updated into QuEChERSER (with “efficient and robust” being added to the portmanteau) to better take advantage of the features provided by modern sample preparation and analytical techniques. Most notably, QuEChERSER is a “mega-method” that covers a broader scope of polar and nonpolar analytes in diverse sample types. In this article, the new QuEChERSER approach and its advantages over QuEChERS are discussed.

Twenty years ago, the “quick, easy, cheap, effective, rugged, and safe” (QuEChERS) approach for sample preparation was developed by Anastassiades and Lehotay at the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) Eastern Regional Research Laboratory (1,2). The goal was to develop the most efficient method for the multiclass, multiresidue analysis of pesticides in foods, which had been a long-standing wish for regulatory and industry laboratories for decades. However, increasing concerns, such as excessive glassware, use of chlorinated solvents, high labour needs, and low sample throughput, were putting pressure on laboratories to adopt more efficient practices. Simultaneously, more sensitive, smaller, and less expensive gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem MS (LC–MS/MS) instruments were also being introduced, which allowed for a broader scope of analysis while still maintaining high selectivity in detection. The introduction of these new GC–MS and LC–MS/MS instruments meant that sample preparation needed to recover a wide range of polar and nonpolar pesticides in the same method. Because chemical interferences were less likely to occur, cleanup could be “just enough” to reduce indirect matrix effects and avoid instrument contamination.

We decided to start from scratch, and QuEChERS essentially grew out of brute force experimentation of each aspect in the extraction and cleanup process (2). We eventually achieved the most streamlined protocol to yield high recoveries with sufficiently clean final extracts for moistened food samples. Acetonitrile was shown to work best for extraction. MgSO₄/NaCl induced the most effectively tailored phase separation to avoid proteins and sugars, and dispersive solid-phase extraction (d-SPE) cleanup with a combination of anh. MgSO₄, primary secondary amine (PSA), C18, and graphitized carbon black (GCB) sorbents worked well to retain lipids (including fatty acids) and chlorophyll. We realized that traditional SPE cartridges were expensive, inflexible, and inefficient in “chemical filtration” applications in which the extract also serves as the eluting solvent, so we decided to just combine the extract with the sorbents.

Two versions of QuEChERS were independently validated in successful interlaboratory trials for analysis of pesticide residues in foods (3,4). In 2010, Majors interviewed Lehotay and Anastassiades for LCGC to discuss the past, present, and future of QuEChERS (5). Since then, many reviews have been published about QuEChERS, including descriptions of how it has expanded beyond the monitoring of pesticides in foods (6–8). Lehotay and Chen (9) plotted the trend in scientific publications involving QuEChERS, tracking a total of 2565 papers on the topic through 2017. In a continuation
of the same search in Web of Science, QuEChERS has grown from nearly 450 to >600 papers per year, totaling 4499 publications through 2020. More than 30 vendors worldwide market QuEChERS products, which has helped it become a staple approach in sample preparation. The chemistry of QuEChERS possesses a flexibility that enables it to be a template for modifications, while still maintaining streamlined protocols for a wide range of applications.

Despite the popularity of QuEChERS, analytical instrumentation has continued to improve in the past two decades, which enables even more streamlined sample preparation with broader applicability. For example, extract and inject methods are now feasible in many cases to overcome matrix effects using highly sensitive and selective MS-based analyses (10,11). However, robust cleanup is still often needed to provide long-term ruggedness in high-throughput applications involving complex matrices.

Recently, an updated approach in streamlined sample preparation, dubbed QuEChERSER, has been introduced (12–16). QuEChERSER adds “efficient and robust” to the QuEChERS portmanteau, but more importantly, QuEChERSER is a “mega-method” that covers a wider polarity range than QuEChERS. The most effective way to gain laboratory efficiency is by reducing the number of methods needed to analyze the same scope of analytes. For example, four individual methods are typically used to monitor pesticides, environmental contaminants, veterinary drugs, and mycotoxins in pertinent foods, but with QuEChERSER, the same sample can be prepared by the same method to cover the contaminants in all kinds of foods.

This article describes and explains the changes made in QuEChERS leading to the development of the QuEChERSER method.

**Table 1: Differences between QuEChERS and QuEChERSER (highlighted in bold)**

<table>
<thead>
<tr>
<th>Step</th>
<th>QuEChERS</th>
<th>QuEChERSER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk Sample Communication</td>
<td>1-Step room temp. (or dry ice)</td>
<td>1-Step liquid nitrogen or dry ice, 2-Step room temp.</td>
</tr>
<tr>
<td>Test Portion</td>
<td>10–15 g (2–5 g) add water to dried or oily samples</td>
<td>&gt;0.25 g (2–5 g) may add water to complex dried samples</td>
</tr>
<tr>
<td>Extraction</td>
<td>1 mL/g acetonitrile (optional buffer)</td>
<td>5 mL/g 4:1 (v/v) acetonitrile–water (buffer probably not needed)</td>
</tr>
<tr>
<td>Shaking Time</td>
<td>1–10 min (up to 60)</td>
<td>1–10 min (up to 60?)</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>3 min at &gt;3000 ref</td>
<td>3 min at &gt;3000 ref</td>
</tr>
<tr>
<td>Salt-Out (Shake 1 min and centrifuge 3 min)</td>
<td>4 g 4:1 (w/w) MgSO₄–NaCl per 10 g sample (can be added in 1-step)</td>
<td>A) Non for LC (take 200 µL) B) For GC, decant = 10 mL into 2 g 4:1 (w/w) MgSO₄–NaCl (1 g per g sample)</td>
</tr>
<tr>
<td>Cleanup</td>
<td>(d-) SPE with 0.25 g 3:1:1 (w/w/w) anh. MgSO₄–PSA–C18 per g equiv. sample Many options (GCB, Z-Sep, EMR L, ChloroFiltr, etc.)</td>
<td>A) For LC, evaporate MeCN, add initial mobile phase, ultracentrifuge for 5 min B) For GC, (μ)SPE with 45 mg 20:12:12:1 anh. MgSO₄–PSA–C18–CarbonX per 0.3 mL extract</td>
</tr>
<tr>
<td>Final Extracts</td>
<td>1 g/mL (adjustable)</td>
<td>LC) adjustable GC 0.25 g/mL</td>
</tr>
<tr>
<td>Scope</td>
<td>LC- and GC-amenable pesticides, environmental contaminants, mycotoxins, et al. (depending on cleanup)</td>
<td>LC- and GC-amenable pesticides, environmental contaminants, mycotoxins, et al., vet. drugs, and more relatively polar analytes</td>
</tr>
</tbody>
</table>

**QuEChERSER vs. QuEChERS**

Table 1 outlines each step in the QuEChERS and QuEChERSER templates for comparison, and the following discussion is sectioned by the individual components. Only the differences in bold text (Table 1) are discussed. Otherwise, both methods are streamlined to employ liquid extraction of sample test portions in tubes by shaking for 10 min followed by centrifugation for 3 min. **Sample Comminution:** An easy way to increase practical efficiency in sample preparation is by reducing test portion sizes. However, sample processing (comminution) is rarely included by most chemists as part of the analytical method even though it is as equally (or more) important as any other step. As discussed in previous papers (9,12,17–20), comminution must not be ignored! Fundamentally, all analytical chemists must understand that ineffective sample comminution relative to the test portion size for analysis leads to a “garbage in, garbage out” situation. No matter how accurate the sample preparation and analytical methods are demonstrated to be based on the validation and quality control (QC) results, the expenses and effort put into the analyses are rendered meaningless if the analyzed test portion fails to represent the original sample.
Prior to QuEChERS, most methods called for 50–100 g test portions of bulk processed food samples in pesticide residue analysis, but studies showed that 10–15 g subsamples could yield similar results with better comminution practices, such as using two steps, high-quality devices, or cryogenics (18). In QuEChERSER, test portions can be further reduced to 1–5 g by the same concepts, but it is better to use liquid nitrogen rather than dry ice for reasons described in the literature (12,19). For QuEChERSER, a safe, facile, and high-throughput protocol was developed using conventional sample processors for (unfrozen) raw bulk food commodities applying liquid nitrogen in a single step. With practice, two technicians alternating two containers for a single device can comminute a batch of 40 bulk samples and weigh out the test portions in centrifuge tubes ready for extraction in approximately 4 h.

This type of efficient comminution using liquid nitrogen can be done for any sample preparation method to gain the benefits of reduced test portion sizes. Independent of the subsequent steps, the degree of subsample homogeneity can be empirically measured by adding a QC spike to the bulk samples prior to comminution. The repeatability and recovery of the QC spike is then determined for each batch of samples to isolate and assess the sample processing step, as well as similar QC spikes added just prior to the sample preparation and analysis steps to follow (12,20). The measurement uncertainty and contributions of each step in the overall method are then calculated using summation of squares to determine the limiting source of error for control and improvement in routine monitoring. Despite the smaller test portion, QuEChERSER has been demonstrated to yield about 5% lower overall relative standard deviation (RSD) (better reproducibility) than QuEChERS (12,20).

**Extraction:** Prior to QuEChERS, extraction typically called for 2 mL or more of the solvent. QuEChERS reduced this ratio to 1 mL/g sample for three reasons: 1) a greater volume could not fit well within the convenient 50 mL extraction tubes; 2) use of the smaller solvent-to-sample ratio led to more concentrated extracts for potentially lower detection limits (without solvent evaporation); and 3) a 1 mL/g ratio was determined to be acceptable for complete extraction of the tested incurred analytes in real samples (2–4). In the latter case, using more solvent per test would have been desirable to provide thorough extraction for all types of analytes and matrices, but the former two reasons precluded a change to the method, which is one of the reasons why QuEChERS is not ideal for extracting lipophilic analytes in relatively fatty matrices. Furthermore, analytical tools are capable of orders of magnitude lower detection limits nowadays than when QuEChERS was developed, thus more dilute extracts are no longer a problem, and in fact, dilution is often used to overcome matrix effects in MS analyses.

The reduction of the test portion size in QuEChERSER allowed for the reconsideration of the 1 mL/g ratio used in QuEChERS. Samples of 2 g in 15 mL centrifuge tubes can be readily extracted with approximately 10 mL of solvent (or 30 mL for 6 g test portions in 50 mL tubes). As it turned out, the multiclass, multiresidue method developed in my laboratory for veterinary drug residues employed 10 mL of a acetonitrile–water solution in a 4:1 (v/v) ratio for extraction of 2 g test portions of animal tissues (10,13,21). This 5 mL/g solvent-to-sample ratio has been shown to fully extract many incurred drugs in different matrices, and the USDA Food Safety and Inspection Service has been using this approach routinely in the National Residue Program for nearly a decade (22). In QuEChERSER, this proven extraction protocol was simply extended to also include pesticides, environmental contaminants, and other types of analytes in the same sample.

Another major factor for extraction in nearly all methods is the amount of water in the sample or extraction solvent. For dry commodities, such as grains, QuEChERS and other nonaqueous extraction methods require the addition of water to the test portions prior to extraction with the organic solvent. However, the extraction solvent already contains 20% water in QuEChERSER, thus the water addition step is avoided. QuEChERS for dry samples typically entails 2 g samples and 5 mL of water, followed by extraction with 10–15 mL of acetonitrile, which is not too different from the aqueous acetonitrile extraction conditions in QuEChERSER.

The limitation in QuEChERS with nonpolar analytes in fatty food matrices and soils was also addressed in QuEChERSER by an increased solvent-to-sample ratio. Even though isotopically labelled surrogate standards are used in both methods to compensate for <100% recovery of legacy chlorinated pesticides and environmental contaminants in fatty foods, the accuracy of the QuEChERSER method is substantially better than with QuEChERS (13,16). Better cleanup using mini-cartridge SPE rather than d-SPE prior to GC analysis is another major reason, and is discussed later in this article.

**Precipitation Cleanup and Ultracentrifugation for LC Analysis:**
An advantage and disadvantage of QuEChERS is that the same final extract is usually used for both GC and LC analyses, which is a convenient
and efficient benefit, but it also limits the performance and scope of the analyses, particularly for LC-only analytes. Hydrophilic analytes do not partition into the acetonitrile layer during the salting-out partitioning step, and in (d-)SPE, PSA and its alternatives retain many LC-amenable carboxylic acid analytes. Furthermore, the final extract in QuEChERS consists almost entirely of acetonitrile, which is not ideal for injection in reversed-phase LC. The first-to-elute analytes tend to have poor peak shapes even when the final extracts are partially diluted with water.

In QuEChERSER, a small aliquot (200 µL) of the initial extract before phase separation is taken for LC analysis, which expands the analytical scope to many polar compounds (such as many drugs) that do not partition into acetonitrile from water. This extract can be taken directly for LC analysis as done in extract and inject methods (10,13–14), but this runs into the same problem as in QuEChERS with poor peak shapes of the most polar analytes.

In the QuEChERSER method, a solution that also provides cleanup is to perform a solvent-exchange step that matches the final extracts with the initial mobile phase. A common evaporation device using nitrogen flow evaporates a batch of 200 µL extracts at 40 °C in approximately 5 min. Then, the initial mobile phase solvent is added (750 µL), which does not dissolve the hydrophobic matrix components that had precipitated from the extracts in the micro-centrifuge tubes. Nonpolar analytes also precipitate, but those are analyzed by GC anyway, which minimally affects the overall scope of analysis.

Otherwise, sorbent-based cleanup tends to lose certain analytes, which was shown in a recent comparison study involving four veterinary drug methods (23). Similarly, in the case of ultrafiltration, adsorption of some analytes always occurs in mega-methods for any one type of membrane material, especially in a highly aqueous solvent as in this application (24). Furthermore, exposure of final extracts to filters and sorbents sometimes adds more potential interferants in the analysis than they remove. Thus, QuEChERSER employs a 5 min ultracentrifugation (13,000 relative centrifugal force) step after precipitation cleanup to maintain the exceptionally broad scope of analysis. Although vendors who sell sorbents and filters do not promote precipitation cleanup and ultracentrifugation for sample preparation, these are excellent tools that analytical chemists should not neglect.

Another key advantage of removal of the nonpolars by precipitation is that these components tend to be highly retentive in reversed-phase LC, which can pose a serious problem with matrix effects and ghost peaks. Michlig and others studied this aspect in the analysis of highly complex hemp matrices (15). The precipitation cleanup greatly decreased the extent of matrix components coeluting with analytes as the mobile phase became less aqueous. Ghost peaks were also reduced by this type of cleanup, and the elimination of the possibility of ghost peaks was achieved by backflushing the columns with an organic solvent phase between injections (15). In long sequences, ghost peaks are often the cause of matrix effects in subsequent injections, despite the fact that cleanup may appear to be sufficient for individual samples. We have recently implemented alternating dual-column switching with backflushing in our QuEChERSER studies, which will be published in the future.

**Salt-Out Partitioning and Automated µ-SPE Cleanup for GC Analysis:** One of the elegant aspects of QuEChERSER is that the salt-partitioning and cleanup steps are tailored for GC-amenable analytes without compromise to accommodate LC analysis. After the small aliquot of initial extract is taken for LC, the remaining supernatant extract (10–11.7 mL) is decanted into 2 g of the same salt combination as used in QuEChERS. As demonstrated in the seminal publication (2), the use of MgSO₄ combined (or not) with other salts for phase separation in QuEChERS is very robust in that different amounts of the salts per extract volume yield 100% recoveries for a wide range of GC-amenable pesticides. Thus, 2 g of the commercially available QuEChERS salts conveniently packaged in 15 mL centrifuge tubes were chosen for use in QuEChERSER. The protocol simply calls for pouring the supernatant (extract) into the tube containing the salts followed by 1 min shaking and 3 min centrifugation.

In the final step, automated instrument-top sample preparation (ITSP or µ-SPE) is used for extensive cleanup (12–16,20,25–27). The approach is commercially available from several vendors for installation on any brand of GC. The analyst merely needs to transfer 1–1.5 mL of the acetonitrile extracts (upper layers) into autosampler vials and load the trays as normally done for instrumental sequences. The robotic liquid handler that also serves as an autosampler conducts the mini-column SPE cleanup step for each extract just before GC injection. The cleanup is done in parallel with analysis of the previous sample, which is also done in parallel with LC analysis, to maximize sample throughput.

The same ITSP cleanup sorbent combination of 45 mg 20:12:12:1 and MgSO₄–PSA–C18–CarbonX per 300 µL extract can be used in QuEChERS or QuEChERSER, but the fourfold less equivalent sample concentration in the latter method is much less
likely to overload the mini-cartridges. Modern instruments are still capable of achieving the needed detection limits in QuEChERSER, and injection volume can be increased if needed. The same sample–sorbet ratios could be conducted in batch-wise fashion using traditional cartridges loaded in a manifold or in a centrifugal SPE format, but this has at least four drawbacks compared to the automated approach: 1) traditional SPE costs more and involves attentive labour; 2) ITSP in parallel with analysis has higher sample throughput and permits larger batch sizes; 3) ITSP produces highly consistent, controlled, and slow (2 µL/s) flow rate for better precision; and 4) operation in batches loses the benefits of “just-in-time” cleanup prior to injection. The capital expense and maintenance of the robotic liquid handler are drawbacks with any automated system, but they should pay off in the long run.

In QuEChERS, d-SPE has several beneficial features (fast, flexible, easy, and inexpensive), but it sacrifices a degree of cleanup compared with column SPE. Automated µ-SPE has a similar extent of cleanup as cartridge-SPE, but it also possesses the advantages of speed and ease akin to d-SPE for reasonable cost and robustness. Although d-SPE has practical advantages over traditional SPE, it still takes time and care in pipetting to avoid sorbent particles from being transferred to the autosampler vials. QuEChERSER obviates this problem, and more importantly, it involves plenty of extract volume for easy and accurate transfers for both LC and GC analyses. A limited amount of final extract constitutes a drawback in QuEChERS, especially for reduced test portion size as in QuEChERSER.

Not only does QuEChERSER with ITSP recover a wider scope of analytes than QuEChERS with d-SPE, it also works without modification for a greater range of sample matrices. The effect of the increased solvent–sample ratio has already been discussed, but the more dilute extracts using the combination of cleanup sorbents in ITSP does an excellent job to remove common matrix components in meats (13), fish (14,15), hemp (16), eggs, grains, fruits, and vegetables (12). Although milk and soils have yet to be tested in QuEChERSER, the method is expected to work better than QuEChERS for those and other sample types. If needed, the same cleanup options can be done in QuEChERSER as in QuEChERS, such as hexane partitioning (21), enhanced-matrix removal (28), magnetic-SPE (29), and freezing out (6–9).

Analytical Aspects: Although analysis is distinct from sample preparation, the author would be remiss not to mention that low-pressure (LP) GC–MS (30) and analyte protectants (31) serve as valuable techniques in conjunction with QuEChERS, QuEChERSER, and other methods. LPGC matches the speed of ultrahigh-pressure liquid chromatography (UHPLC) with ≈13 min cycle times (from injection-to-injection), which is approximately threefold faster than conventional GC analyses that would normally limit sample throughput. With respect to QuEChERS and QuEChERSER, LPGC enables large-volume injection of acetonitrile in a standard hot splitless injector (12–16,30), which is a key feature to further lower detection limits. Similarly, QuEChERS and QuEChERSER are well-suited for using analyte protectants, which tend to be relatively polar sugar derivatives, because they are soluble in acetonitrile final extracts. Analyte protectants fill active sites in the GC–MS system, which acts to reduce peak tailing, improve accuracy, enhance signal, decrease matrix effects, and increase ruggedness in high-throughput analyses (31).

Last, targeted analysis in both LPGC and LPLC permits the use of summation function peak integration to greatly reduce analyst time spent on data review and manual re-integrations (32). Summation integration is commonly used when an analyte consists of multiple or misshapen peaks because the default integrator fails in those (and other) situations. Default software integrators do not provide fully trustworthy chromatographic peak integrations in mega-method analyses, especially at low concentrations. Despite that matrix interferences rarely occur when using highly selective MS-based detection, one set of integrator settings for each analyte rarely integrates all the peaks reliably throughout a sequence. However, summation function integration does not fail because it simply entails setting of the start and stop times for each analyte peak before and after the known retention times, accounting for peak widths. If the peaks consistently fall within the start and stop times (and there is a chromatographic problem if they don’t), then the peaks will always be integrated from the baseline, usually better than the conventional integrator or human can do. Then, MS-based identification criteria, including signal or concentration thresholds, indicate if the targeted analyte is present or not (33).

Conclusions
Technology advances in analytical chemistry and real-world laboratory methods need to be updated periodically to incorporate new proven capabilities. The QuEChERS approach was developed to suit the analytical devices and instruments commercially available 20 years ago, and the time has come for an updated generic sample
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References

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Beer is one of the most popular beverages in the world, and consistently ranks at the top of the list for consumers of alcoholic drinks. Growth of craft breweries and consumer interest in beer flavour has led to a wealth of readily available beer styles and flavours. In 1984, there were fewer than 100 breweries in the United States (1). The most predominant beer and perhaps the only style available at many locations in the United States was North American lager. By 2019, there were over 8000 breweries offering hundreds and hundreds of beer styles, as defined by the Brewer’s Association “Beer Style Guidelines” (2). This surprising variety of beers can be obtained from four basic ingredients: water, malted grain, spices (hops, fruits, and other consumables), and yeast. Control of the ingredients, brewing process, fermentation, packaging, and storage has a significant impact on the consistency of the final product, and also presents a challenge for brewing scientists monitoring the components contributing to the flavours in beer (3).

Beer bitterness derives from using hops during brewing, providing an important flavour balance to the sweet flavours of malts and grains (4). The dried hop cones or strobile used in brewing contain significant amounts of polyketides, particularly the humulones, or α-acids (AA), which can represent 2–15% of the total mass of the hops. The major homologues of the AAs are the co-, n-, and ad-humulones, which collectively account for greater than 98% of the α-acid or AA content. (5). During the brewing process, the AAs are isomerized to the more water-soluble isohumulones, or iso-α-acids (IAA), via an acyloin rearrangement.
Beer bitterness is often described in terms of international bittering units (IBU), with a scale ranging from values of 5 to 100 (6). With the advent of craft brewing and very hoppy beers, some extremely high values of IBU have been claimed. The IBU numeric value has been suggested as approximately equivalent to the concentration of isohumulones in beer in parts per million (ppm). However, actual sensory beer bitterness is more complex and depends on the concentrations of a number of potential bittering agents (7–9).

The combination of extraction of humulones AA from hops and conversion to IAA during brewing is described as hops utilization. Predicted values of IBU can be determined by calculating the amount of hops, percent AA content, volume of the wort, sugar concentration, and time of hop addition (10). This is particularly important for design and implementation of new beer recipes. The traditional method of analysis of beer bitterness relies on the spectrophotometric UV determination of IAA content of an organic extract of beer at 275 nm (11,12). A disadvantage of the spectrophotometric method is the inability to separate absorption of IAA from interfering components that absorb in the same region of 275 nm, but do not exhibit the same contribution to beer bitterness. The growth of the craft beer industry has led to a marked increase in strongly hopped and flavoured beers, resulting in significant changes in the absorption profile of organic extracts of beer. Late- or dry-hopped beers, in which hops are added at the end of the boiling process or after primary fermentation, result in higher concentrations of humulone AA and related oxidized products. Their contribution to UV absorption can result in unexpectedly high values of IBU by the spectroscopic method. Complete analytical separation of all the homologues and isomers of IAA has been achieved by high performance liquid chromatography (HPLC) with UV detection (13–16) and by liquid chromatography–mass spectroscopy (LC–MS) (17,18).

We recently introduced a rapid LC–MS method for determining the AA and IAA content in commercial beer samples using selective ion monitoring (SIM) (19). By employing multiple injections in a single experimental run (MISER), we were able to analyze 70 different beer samples in a total of 74 min. The single misergram allows facile comparison of the relative concentrations of AA and IAA in the beer samples in a single chromatographic display (20,21).

MISER analysis has been used in our introductory course at the University of Missouri–St. Louis, “Beer Brewing:
In this report, we take advantage of a set of beers prepared in our beer brewing course to analyze hops utilization determinants of IBU and measure beer bitterness components. Knowledge of the individual brewing recipes allowed direct comparison of predicted IBU from hops additions, measurement of IBU by spectroscopic analysis, and final determination of the concentration of AA and IAA by SIM LC–MS in the finished beer samples. A sample set of commercial beers from a local brewery are also compared for content of AA and IAA.

**Experimental**

**Reagents:** Methanol and acetonitrile (HPLC-grade) were purchased from Fisher Scientific. Ammonium formate (NH₄HCO₃) and formic acid (HCOOH) were purchased from Sigma–Aldrich. Ultrapure water was obtained from a Milli-Q Integral 10 from Millipore. Standards ICS-3 (dicyclohexylamine salts of trans-iso-α-acids) and ICE-3 (hops extract standard) were obtained from the American Society of Brewing Chemists (ASBC Check Sample Service; asbc@scisoc.org).

Unhopped beer, used for preparation of standard solutions, was prepared by addition of 225 g of dry malt extract (DME) to 1 L of boiling water. The mixture held at a boil for 15 min, the hot wort cooled in an ice water bath and subsequently transferred to a 4 L glass fermentation bottle equipped with an air lock. To this mixture, 5 mg of dry yeast (Safale-05) was added. After one week, the beer was transferred to a clean, sanitized 4 L glass fermentation bottle equipped with an air lock. After an additional two weeks, the unhopped beer was transferred to standard beer bottles, capped, and stored in a refrigerator at 4 °C. Humulone and isohumulone standards were prepared by dissolving a suitable amount of AA or IAA in a minimum amount of ethanol and diluted with unhopped beer to provide standard solutions of 5, 10, 25, and 50 ppm.

**Sample Preparation:** Beer samples were collected immediately upon opening the specific bottle of carbonated beer. Several millilitres were transferred via gravity filtration through Whatman qualitative P8 filter paper into a 15 mL plastic centrifuge tube. Samples were capped, frozen, and stored for several days. Aliquots of the thawed samples were transferred into HPLC analytical vials for placement in the LC–MS autosampler. Samples were analyzed directly without dilution. Standard solutions of known concentration of humulone and isohumulone were prepared in unhopped beer.

**Instrument Details:** MISER LC–MS experiments were performed on a Shimadzu LC–MS-2010A equipped with two LC-20AD mobile phase pumps and a SIL-10AD autosampler. The mass spectrometer was operated in electrospray (ESI) negative mode with capillary voltage of 1250 V. SIM was conducted to continuously monitor ions at 347 and 361 m/z, corresponding to the deprotonated molecular ions of the AA and IAA homologues. The source and desolvation temperatures were 275 and 250 °C, respectively. Nitrogen nebulizing and drying gas flow rates were optimized at 1.5 L/min and 0.1 MPa, respectively.

Chromatographic separation of AA and IAA was achieved using isocratic conditions with an Agilent Poroshell 120 EC-C18 HPLC column having dimensions of 30 mm × 2.1 mm i.d. and 2.7 µm particle stationary phase. Mobile phase consisted of an isocratic mixture of 2 mM ammonium formate (HCOONH₄) in 1:1 acetonitrile–water with a flow rate of 0.20 mL/min. MISER experiments were performed using sequential 5 µL injection volumes for each beer sample at intervals of 5–7 min.

**Results and Discussion**

Students in our introductory science course on beer brewing (22) have been preparing small batches of beer using recipes developed in class (Table 1). Over two semesters with 40
students, we brewed 13 different recipes. Students worked in pairs and some teams chose identical recipes. We obtained a wide range of beer styles from very light ales (2A, 5A, 7A, and 12A) to dark stouts and porters (4A, 8A, and 9A).

The hop addition sequence is very dependent on the specific recipe. Timing of hops addition during brewing and the amount added has a significant impact on the sensory taste profile of the final beer. Hops added at the beginning of the boil contribute to beer bitterness and undergo comparatively significant conversion from AA to IAA. Additions of hops near the end of the boil contribute to flavour and aroma, but have less impact on beer bitterness because of low levels of conversion of AA to IAA. The sugar concentration of the wort (boil gravity) also has an impact on extraction of AA, with higher sugar concentration resulting in less incorporation of bittering components. Hops utilization can be modelled as a function of boil time and wort boil gravity and used to calculate IBU for specific recipes. The calculated IBU value was determined using the Tinseth method from published utilization tables and free online homebrew calculators (10,23,24).

Values of IBU were determined using the industry standard spectroscopic method (11,12). Absorption of an isooctane extract of acidified, decarbonated beer at 275 nm can be related directly to IBU. For a number of the beer samples, the spectroscopic IBU showed significant deviation from the calculated value. This is not unexpected—variations can occur because of age and storage of the hops as well as deviations from the brewing process. Aged or improperly stored hops can result in higher concentrations of oxidized products, such as humulinones, that can contribute both to higher UV absorbance of the isooctane extract and higher perceived bitterness in the beer (25). Four of the beer samples, English brown ale, weissbier, imperial IPA, and porter (1A, 2A, 3A, and 9A), exhibited significantly higher spectroscopic IBU measurements compared to the calculated value. The imperial India pale ale (IPA) showed the greatest UV absorption, resulting in an IBU of 120 compared to the calculated value of 48. The solubility of IAA in beer is dependent on the concentrations of sugars and pH but limited to approximately

![FIGURE 3: Analysis of standard solutions of IAA and AA prepared in unhopped beer by SIM LC–MS operated in negative ion mode at m/z 347 and 361 for co- and n-/ad-isomers, respectively. Chromatographic conditions were identical to those used for beer samples (see details in Figure 2 caption).](image)

![FIGURE 4: MISER LC–MS chromatogram of isohumulones IAA (red) and humulones AA (blue) using SIM at 361 m/z of craft beer samples. For chromatographic conditions: see Figure 2 caption.](image)

We look forward to serving you!
FIGURE 5: Scatter plot of signal intensities for compounds IAA and AA from the combined set of student beers and craft beer samples shown in Figures 2 and 4, respectively.

TABLE 1: Student beer characteristics

<table>
<thead>
<tr>
<th>Beer</th>
<th>Recipe Style</th>
<th>Calculated IBU</th>
<th>IBU (275 nm)</th>
<th>IAA (ppm)</th>
<th>AA (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>English Brown Ale</td>
<td>31</td>
<td>52</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>2A</td>
<td>Weissbier</td>
<td>10</td>
<td>24</td>
<td>6</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>3A</td>
<td>Imperial IPA</td>
<td>56</td>
<td>120</td>
<td>25</td>
<td>63</td>
</tr>
<tr>
<td>4A</td>
<td>Dry Irish Stout</td>
<td>18</td>
<td>11</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>5A</td>
<td>Lampo Blanco</td>
<td>15</td>
<td>24</td>
<td>7</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>6A</td>
<td>Blueberry Blonde Ale</td>
<td>18</td>
<td>17</td>
<td>17</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>7A</td>
<td>Cream Ale</td>
<td>14</td>
<td>15</td>
<td>9</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>8A</td>
<td>Coffee Stout</td>
<td>19</td>
<td>23</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>9A</td>
<td>English Porter</td>
<td>20</td>
<td>46</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
<td>10A</td>
<td>American Pale Ale</td>
<td>27</td>
<td>29</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>11A</td>
<td>India Pale Ale</td>
<td>33</td>
<td>28</td>
<td>54</td>
<td>27</td>
</tr>
<tr>
<td>12A</td>
<td>Cherry Ale</td>
<td>8</td>
<td>10</td>
<td>2</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>13A</td>
<td>English Southern Mild</td>
<td>25</td>
<td>18</td>
<td>55</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

*Details concerning brewing recipes are available (22). The IBU is determined by calculating the amount of hops, addition time, % AA content, and Wort boil specific gravity using the Tinsel method (10,23,24). IAA and AA values in ppm were determined by evaluation of combined chromatographic peak areas of co-, ad-, and n-humulones and isohumulones. LOD indicates the limit of detection.

100 ppm (26). In the case of beer 3A, the unusually high absorbance and IBU value indicate the presence of additional UV absorbent compounds.

Analysis of the beer samples by LC–MS was achieved using MISER. This provides visualization of all of the beer samples in one chromatographic output or “misergram” (20,21). The beer samples were filtered, degassed, and added to sample vials for direct injection and analysis by LC–MS (Figure 2). A single 120 min misergram of beer samples 1A–13A along with IAA and AA standards was obtained using SIM in negative ion mode at 361 m/z. It is worth noting that the chromatographic conditions employing a short column and strongly eluting mobile phase result in the coelution of the homologues co-humulone (MW 348), ad-, and n-humulones (MW 362). However, the isomeric AA and IAA are well resolved because of their inherently different physical properties. Therefore, we observe a single chromatographic peak for the homologues of IAA and a second more retained peak for the homologues of AA. The misergrams obtained from either set of negative parent ions (SIM at 347 or 361 m/z) are very similar in profile and useful for qualitative assessment of relative amounts of IAA and AA. The misergram is divided into 7-min intervals, with vertical hash marks differentiating the results for each beer sample. The IPA (11A) contains the greatest amount of IAA, while light ales 5A and 12A contain the least IAA. These chromatograms are also useful for assessing the utilization of hops or conversion of AA to IAA. Imperial IPA (3A) and English Porter (9A) show low levels of utilization, as reflected by greater residual amounts of AA. Both of these recipes (3A and 9A) included late hops additions during the boil, which would be expected to result in higher concentrations of un-isomerized AA. Quantitation of AA and IAA in each beer sample was determined by combined content of the individual homologues of co-, ad-, and n-humulones and isohumulones, respectively. Comparison of each beer sample was made to the standard curves obtained from commercial samples of IAA and AA in unhopped beer, as shown in Figure 3. For instance, the total concentration of AA was obtained by the sum of the ppm concentrations of cohumulone (MW 348) and n-ad-humulone (MW 362) obtained from two separate LC–MS chromatograms. The content of all homologues of AA and IAA was obtained for each beer style using the summation of the concentrations in ppm obtained from two misergram plots using SIM of 347 and 361 m/z (Table 1). A set of beers was also collected from a local brewery for evaluation using MISER LC–MS (Figure 4). The sample set included a range of products from a lemon shandy prepared from a light lager and lemon soda (2B) to the more...
bitter pilsners (6B and 7B), double IPA (8B), and American IPA (9B). From this small set of nine beers, the American IPA (9B) shows the greatest amount of AA, as evidenced by the second peak (blue). A comparison of peak heights of AA and IAA at 361 m/z in the MISER chromatograms (Figure 2 and 4) can also be displayed as a single scatter plot (Figure 5). This analysis is qualitative in nature because we are comparing peak heights rather than integration values and only the co- and n-IAA and AA at 361 m/z are displayed. However, the scatter plot provides useful visualization of two important aspects: 1) relative amounts of AA and IAA in different samples, and 2) ratios of AA/IAA in a single sample. Similar beer styles also appear as clusters that can help identify features of particular recipes. For example, commercial weissbeer (1B) and lemon shandy (2B), known to have low bitterness and very little hops aroma, appear in the lower left-hand corner of the plot. A number of student beers (weissbeer 2A, lamento bianco 5A, cream ale 7A, and cherry ale 12A) were also characterized by low bitterness and hops aroma, appearing in the same general area of the scatter plot. Using this scatter plot from both the student and commercial samples provides a comparison between our classroom recipes and standard beer styles.

Conclusions
The results of this investigation illustrate the utility of using multiple injections in a single chromatogram MISER coupled with single ion monitoring LC–MS to obtain graphical, qualitative analysis of humulones/isohumulones ratios (AA/IAA) in sample sets of beers. By comparison to standard curves obtained at both 347 and 361 m/z, it is possible to determine concentrations of the combined homologues of AA and IAA. The technique is rapid, allowing analysis of large sample sets with a minimum of sample preparation. A simple decarbonation and filtration is all that is required prior to transfer to HPLC sample vials. The method is also useful for monitoring the brewing process and hops utilization during the boiling of the wort. Components that interfere with the traditional UV spectroscopic determination of IBU are largely eliminated by SIM and chromatographic separation. The ability to view both AA and IAA concentrations allows direct comparison of hops utilization and the sequence of hops additions in beer brewing recipes.

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References

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