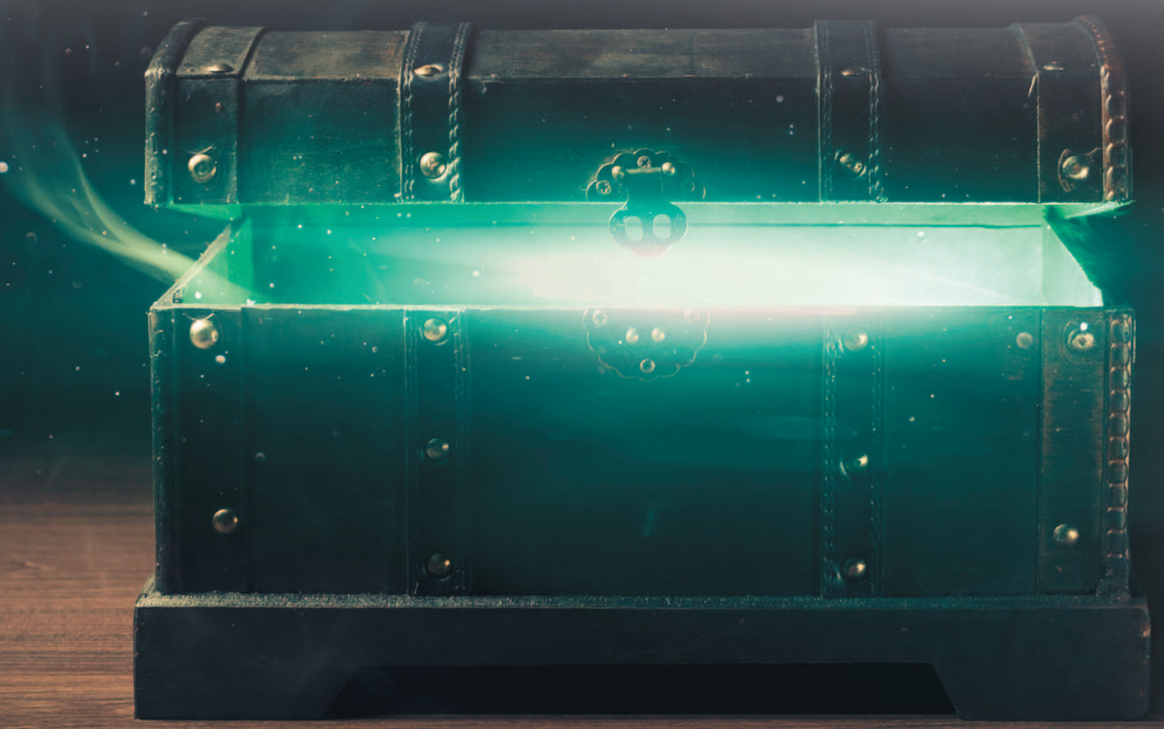
21 September 2018 Volume 14 Issue 9

The Column

the interactive e-publication for the global separation science industry



Opening Pandora's Box

What will multipath LC–MS offer to chromatographers?

UBM

Cover Story

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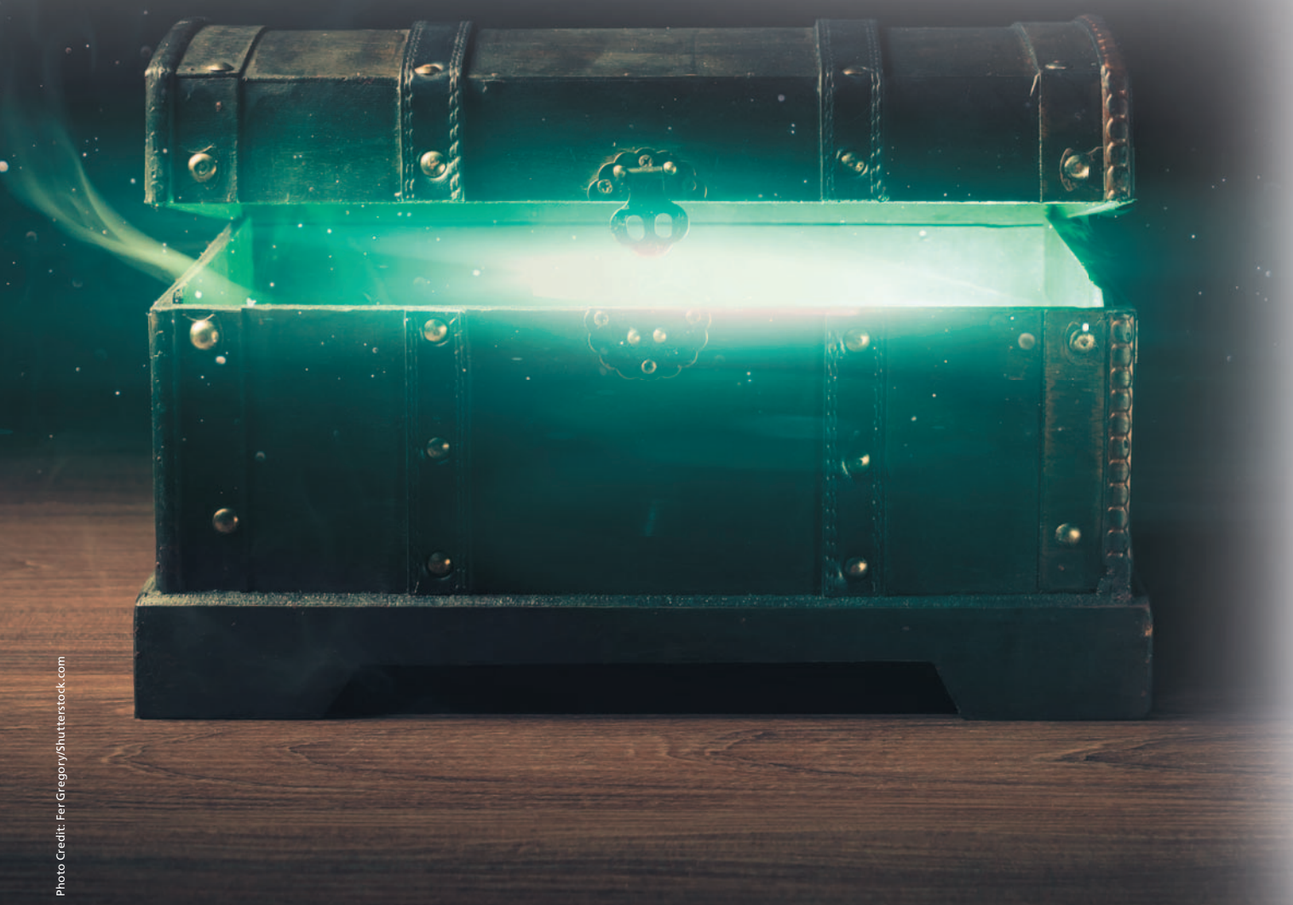
Multipath Liquid Chromatography–Mass Spectrometry: **A Veritable Pandora's Box**

Kevin A. Schug, Department of Chemistry and Biochemistry, The University of Texas (UT) at Arlington, Arlington, USA

The drive behind multipath liquid chromatography (LC), a new concept developed by Kevin Schug and his group, is presented.



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I suppose that Pandora's Box usually has a negative connotation, but in this case it is not so. For several years, our group has been working on a concept that we have termed *multipath liquid chromatography* (LC). The main idea is to target multiple classes of compounds following a single injection of a sample, the components of which are segregated on-line and directed to separate appropriate paths for simultaneous separation; the streams are then recombined for detection. I believe that this approach would be powerful for biomarker quantitation, where it would be more informative to track both metabolite and protein biomarkers to better define a disease state, or in the case of antibody–drug conjugate (ADC) development, where the metabolism of the ADC might involve understanding both the levels of the released drug and the remaining protein. Performing these analyses with one injection would certainly be preferable to having separate methods (and likely, sample preparations) to target each class separately.

The initial embodiment of the multipath system uses restricted-access media (RAM) to separate proteins from small molecules; each class is then directed to its own LC separation and the separations are then joined back together via a tee connection to enter the mass spectrometer detector (1). There is a lot of room to make this concept more complex and more powerful.

The reason this approach has taken so long to develop is that the overarching concept contains many little intricacies, each of which needs to be studied and optimized. We would like to be able to implement automated method scouting to reduce method development time; to target and quantify intact proteins, to avoid extra steps and uncertainties associated with protein digestion; and to incorporate

comprehensive multidimensional LC (LC×LC) in each of the paths. We still have a lot of work to do to be able to reach these goals, but we have made headway in many aspects.

I have written previously about our efforts to use automated method scouting to streamline method development (2). Using a diverse set of small molecules, in two separate studies we screened a series of commercial columns to characterize their performance in reversed-phase mode (3,4). In the second case, we also screened a column set in aqueous normal-phase mode. The take-home message was that automated method scouting, using a generic mobile phase gradient to determine which column provides the best selectivity and peak shapes for separating a set of desired analytes, is a much more efficient route to method development than sticking with one column and trying to find a mobile phase that works. After all, changing the stationary phase is the most effective way to affect selectivity for small-molecule separations.

A bit more challenging was figuring out how to target intact proteins using triple-quadrupole mass spectrometry (MS). I also talked about this before in another *LCGC* Blog instalment (5). It was a good thing that we started working on this approach before we really consulted people. In the literature, there was very little to no precedent for using multiple reaction monitoring in triple-quadrupole MS, like what you would do for small molecules, on intact proteins. Most people claimed it would not work, but we were successful.

We were able to create a reasonably sensitive, but highly specific, means for top-down quantitation of intact proteins (6). The most important part of the experimental design turned out to be modulating the collision energy to a point where not all of the precursor ion was fragmented; under those conditions, a series of highly reproducible and specific product ions could be generated. We have not analyzed a protein for which this approach fails to work.

We also spent considerable effort to better understand ion transmission properties of proteins vs. small molecules in the gas phase, inside the triple-quadrupole analyzer (7,8). Overall, we concluded that there probably is some opportunity to develop a system that is tailored and more amenable to protein analysis, since essentially all triple-quadrupole instruments are dedicated to (and presumably tested for) small-molecule analysis.

Figuring out how to detect the intact proteins allowed us to return back to studying their separation. A dearth of literature exists on the topic, but much less of that previous work considered direct detection by MS. Thus, many reported intact protein separation conditions from the 1980s and 1990s incorporated non-MS-compatible mobile-phase conditions. We were able to study both stationary-phase and mobile-phase effects on the separation of a model set of proteins and to define some generally good conditions for MS-compatible separations (9). The most important aspect of this work was to realize that a compromise needed to be made with mobile-phase additives. Formic acid is good for MS sensitivity to detect the intact proteins, but it generally provides for poor peak shapes. Trifluoroacetic acid is a common additive for generating good peak shapes for proteins, but it can suppress electrospray ionization response. We found that a combination of the two, where just a small amount (0.05%) of trifluoroacetic acid is used along with a standard amount (0.1 or 0.5%) of formic acid, provides excellent performance.

We continue down the path. At the recent HPLC conference in Washington D.C., I presented some of our recent headway towards generating separation conditions orthogonal to those I discussed above.

The impetus for those efforts was to move towards LC×LC of intact proteins, and I was also able to show some good progress on that front. We are currently preparing manuscripts to more comprehensively report on those advancements. Stay tuned for more development on the multipath LC concept.

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Kevin A. Schug is a Full Professor and Shimadzu Distinguished Professor of Analytical Chemistry in the Department of Chemistry & Biochemistry at The University of Texas (UT) at Arlington. He joined the faculty at UT Arlington in 2005 after completing a Ph.D. in chemistry at Virginia Tech under the direction of Prof. Harold M. McNair and a postdoctoral fellowship at the University of Vienna under Prof. Wolfgang Lindner. Research in the Schug group spans fundamental and applied areas of separation science and mass spectrometry. Schug was named the *LCGC* Emerging Leader in Chromatography in 2009 and the 2012 American Chemical Society Division of Analytical Chemistry Young Investigator in Separation Science. He is a fellow of both the U.T. Arlington and U.T. System-Wide Academies of Distinguished Teachers.

E-mail: kschug@uta.edu
Website: www.chromatographyonline.com

News

Waters and Restek Announce Co-Marketing Agreement

Waters (Milford, Massachusetts, USA) and Restek (Bellefonte, Pennsylvania, USA) have entered into a co-marketing agreement aimed at food safety laboratories and promoting the use of Waters gas chromatography–mass spectrometry instruments with Restek GC consumables.

"We are delighted to enter into this agreement with Restek," said Jeff Mazzeo, Vice President, Marketing, Waters Corporation. "Like Waters, they are strongly committed to making customers successful, [their] GC consumables and technical support play an important role in helping our customers monitor for pesticides and other contaminants and meet the sensitivity requirements of global regulated methods," continued Mazzeo.

Under the terms of the agreement, Waters and Restek will work together to provide food safety laboratories with training and applications support of GC–MS methods and workflows for pesticide monitoring and screening.

"Restek is excited to work with Waters on this new venture," said Rick Lake, Vice President, Marketing, Restek Corporation. "By collaborating, we can combine our unique expertise to help analysts around the world perform the vital job of ensuring the safety of a food supply we all share," added Lake.

For more information, please visit: **www.waters.com/tqgc** or **www.restek.com**

Py-GC–MS Analysis of Japanese Jõmon Period Lacquerware

Analysis of red lacquerwares from the Japanese Jõmon Period (approximately 14,000 to 500 BC) by pyrolysis gas chromatography–mass spectrometry (Py-GC–MS) has revealed the materials and fabrication techniques used in their production (1).

The Jõmon period of Japanese prehistory started roughly 14,000–10,000 BC and ended around 500–300 BC. This incredibly long time period saw many developments within the Japanese culture including the production of lacquer craft products such as containers, tableware, and jewellery among many others everyday items. Lacquer tapped from *Rhus vernicifera*, commonly known as lacquer trees, is the oldest natural coating material used in Japan, and research into the lacquer culture forms an important part of many archaeological studies. As lacquer is not native to Japan (2), there remains many questions to be resolved around the lacquer culture including when, where, and how was the lacquer culture established? Further questions remain about the cultural and social implications of the lacquerware. In order to answer these questions researchers have studied lacquerware samples belonging to the period from various sites such as the Niigata Prefecture and the Iwate Prefecture (3,4), as well as the Minamikonuma ruins located in Saitama City (1).

In the most recent study, researchers used attenuated total reflection Fourier-transform infrared spectrometry (ATR-FTIR), energy dispersive X-ray fluorescence (EDXRF), and Py-GC–MS to analyze 16 lacquerwares excavated from the Miniamikonuma ruins, Saitama City, Japan.

The results indicated that lacquerwares from the metaphase of the Jõmon period had a simple one-layer coating of iron (III) oxide (Fe_2O_3), whereas lacquerwares from later stages of the Jõmon period had 2–7 coating layers and added a mercury sulfide (HgS) layer at a later stage as an additional red pigment. Urushiol chemical products were detected using Py-GC–MS suggesting all lacquerware products were coated with lacquer sap collected from a *Toxicodendron vernicifluum* lacquer tree. These results indicate a definite advancement in lacquer craft production technique towards the end of the Jõmon period. — L.B.

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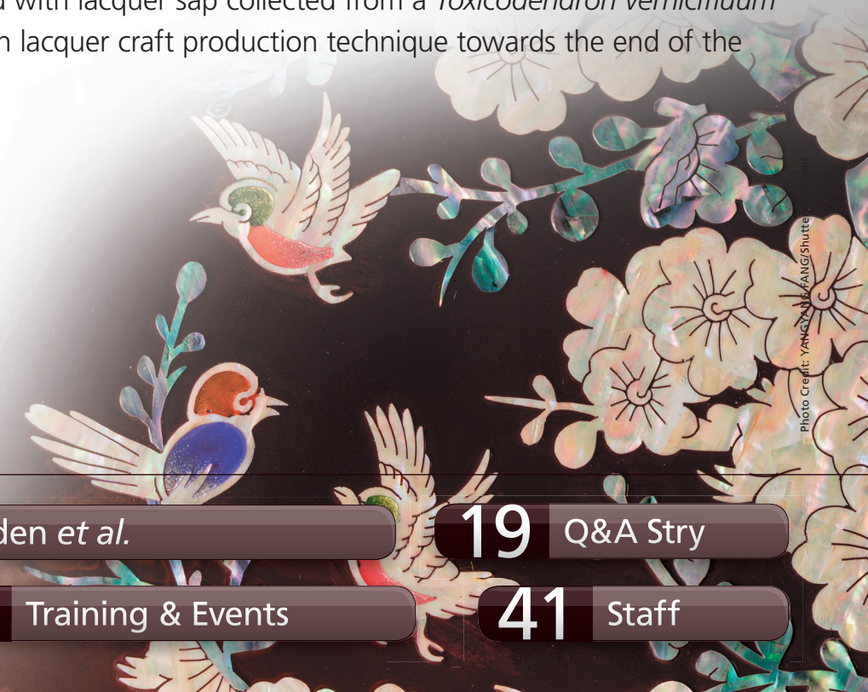

Photo Credit: YANG XIE PANG/Shutterstock.com

Shimadzu Receive NRW.Invest Award

Shimadzu has been awarded the 2018 NRW.Invest Award by the North Rhine-Westphalia (NRW) region of Germany. Following 50 years of presence in Europe, the Japanese company has been rewarded for its commitment to investment within Germany's industrial heartland and most populated federal state. Shimadzu received the award at the end of June from Andreas Pinkwart, the federal state's Minister for Economic and Digitalization, and Petra Weassner, the managing director of the NRW.Invest economic development agency.

Opening a new facility in the city of Duisburg in 2017, Shimadzu has a long-standing commitment to the area, going as far as to place its official European headquarters in the region.

"We are very pleased with the award," said Jürgen Kwass, Managing Director Shimadzu Europe, "and we are very happy to call Duisburg home."

Starting in 1968 with just five employees based in Düsseldorf, the company has grown to a workforce of 230 employees within the region, and more than 700 throughout Europe who are supervised from Duisburg.

"International investors are essential for North Rhine-Westphalia. They are important employers, drivers of innovation and economic engines at the same time," explained State Minister Pinkwart.

For more information, please visit: **www.shimadzu.eu**

Retroactive Toxicology Study on Adolescents Using GC–MS

Toxicological surveillance of illicit prescription and illegal drug abuse has been carried out using post-mortem data from Clark Country, Nevada, USA, with gas chromatography–mass spectrometry (GC–MS), headspace GC–MS, GC×GC–MS, liquid chromatography (LC)–MS, and enzyme-linked immunosorbent assays (ELISA) (1).

A modern-day pestilence, substance abuse knows no class, race, or age. Carving through demographics outside of the socially expected and at numbers which most infectious diseases would struggle to compete with—even if allowed to spread unabated. National surveys in the US have revealed a doubling of Americans aged 12 and older from 2004 to 2014, an astounding 6.5 million individuals, who use prescription drugs for conditions other than medical use (2,3). Particularly alarming was the rate of drug use in adolescents with an estimated 655,000 adolescents aged 12–17 abusing prescription drugs—around 2.6% of adolescents within the US (2). Studying the effects of such abuse has proven difficult for researchers to fully quantify, a situation further complicated by the rapid brain and body development associated with these phases in life. Drug use during these periods may result in neurological changes and behavioural consequences that differ from those recorded in adult populations (4).

The majority of research on opioid abuse and associated factors among adolescents has been taken from the National Survey on Drug Use and Health (NSDUH), a self-reported survey conducted annually with approximately 20,000 adolescents aged 12–17. There are positives and negatives to self-reported data, however, and additional information would be a welcome addition in trying to understand overarching trends. As such, researchers investigated 526 referred cases through autopsy on subjects aged 12–17 over an 11-year period 2005 to 2015 in Clark Country, Nevada, USA. Comprehensive toxicological examination of heart blood, subclavian blood, urine, liver tissue, stomach contents, and vitreous fluid was carried out using GC–MS, headspace GC–MS, GC×GC–MS, LC–MS, and ELISA.

The retrospective toxicology found that 47% of individuals had positive drug toxicology at the time of death with illegal drugs being found in 35%, prescription drugs in 19%, and over the counter in 7%. Prevalence rates exceeded those in nationally self-reported data (2). The most commonly used drug was tetrahydrocannabinol (THC), which was found in 29.7% of subjects. Excluding THC, illegal prescription opioids and benzodiazepines were used approximately 1.7 times as much as all other illegal-drugs.

Prescription drugs were found in 1 out of 5 adolescents with 39% being positive for one and 61% being positive for poly-prescription drug use. Of the adolescents positive for prescription drugs, 50% were also positive for illegal drugs.

While the study did have limitations, it offered a brief insight into a severe problem blighting American culture, and with drug trends in youths evolving at a rapid speed, more data are required to fully comprehend the situation and more effectively target prevention initiatives. — L.B.

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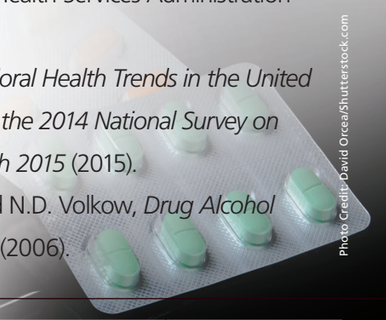

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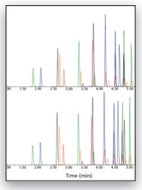
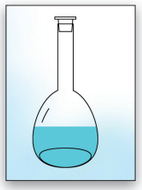
Peaks of the Month

- **The *LCGC* Blog: A Weighty Problem with Calibration:** Chromatographic methods often require that the analyte response is calibrated (and validated) over a wide concentration range when the analyte concentration in the sample is either unknown or is expected to vary widely. Bioanalysis, environmental, and clinical applications are just a few examples of where this may be the case. **Read Here>>**

- **Ten Common-Sense Corollaries in Pharmaceutical Analysis by High Performance Liquid Chromatography** — This instalment describes ten corollaries in high performance liquid chromatography (HPLC) and pharmaceutical analysis that most practitioners are likely aware of but may overlook the rationales behind them. **Read Here>>**



- **When Do We Need Sub-2-µm Superficially Porous Particles for Liquid Chromatography Separations?** — The use of superficially porous particles (SPPs) for modern high performance liquid chromatography (HPLC) is now very common. Initially, SPPs rose as an alternative to sub-2-µm fully porous particles (FPPs). In recent years, many column manufacturers have developed 2-µm and smaller SPP-based products. This article investigates the practical utility of these smaller SPP designs. **Read Here>>**



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- **Solvents: An Overlooked Ally for Liquid Chromatography–Mass Spectrometry** — Choosing an inappropriate solvent can significantly undermine the quality of results, even when using the most advanced technology. This article discusses the importance of selecting the correct grade of solvent for LC–MS analyses and some of the challenges arising from an insufficiently pure mobile phase. **Read Here>>**

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- **Forensic Profiling of Human Odour Using GC×GC–MS** — Researchers from ESPCI Paris and the Institut de Recherche Criminelle de la Gendarmerie Nationale have developed and optimized a comprehensive two-dimensional GC–MS method for the forensic profiling of human hand odour. **Read Here>>**

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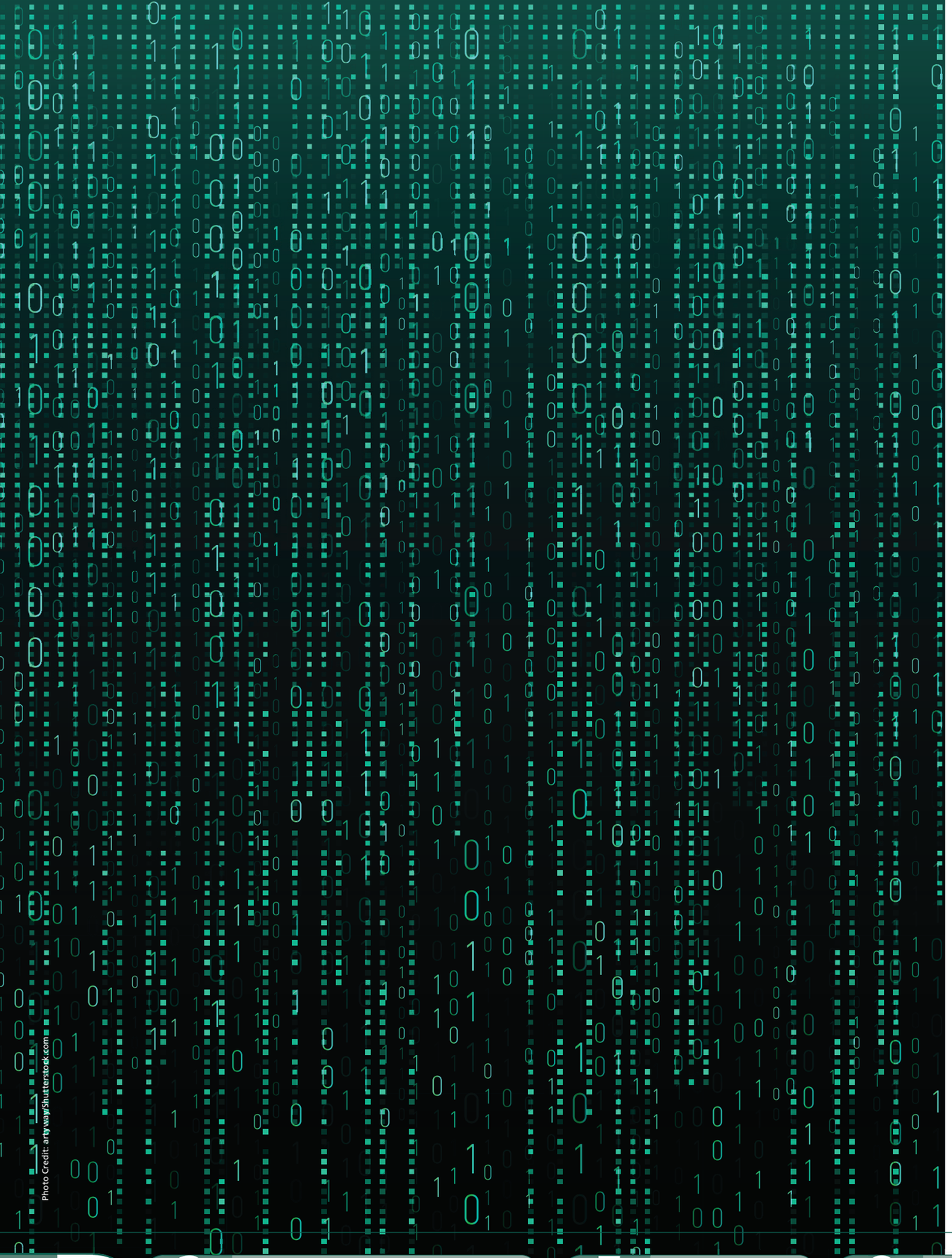
News In Brief

YMC Co., Ltd. (Kyoto, Japan) has announced a formal agreement to acquire the pharmaceutical systems business of Lewa-Nikkiso America Inc. Based in Devens, Massachusetts, USA, the Lewa division of the Nikkiso Corporation is a provider of advanced production-scale chromatography systems for the bioprocessing and the pharmaceutical industry. The transaction includes staff and assets dedicated to the development, manufacture, and sales of batch and continuous HPLC, LPLC, SMB, and buffer and caustic dilution systems for the biopharmaceutical and pharmaceutical market, including the manufacturing site located in Massachusetts, USA.
For more information, please visit: **www.ymc.co.jp/en** or **www.lewa.com**

Agilent Technologies Inc. (Santa Clara, California, USA) has received the 2018 IBO industrial award for their Ultivo Triple Quadrupole LC–MS system from *Instrument Business Outlook (IBO)*. The award recognizes excellence in the industrial design of an analytical instrument and how the design can improve a product's functionality and the end user experience. "We are honoured to receive this award," said Monty Benefiel, Agilent Vice President and General Manager of the company's mass spectrometry division.
For more information, please visit: **www.agilent.com**



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Are You Producing Data or Information?

Can we see the wood for the trees?

We spend most of our working lives producing data, both qualitative and quantitative. It is our primary output and the reason we are employed. My simple question is: Do we focus on the data rather than the information that the data might represent?

Data systems can generate a "result" if we input sample and standard weights, dilution factors, concentrations of standard solutions. We can report the qualitative output without a lot of thought—yes, it's there or no it's not. The mass spectrometry (MS) library search tells us that its compound X, or its accurate mass is Y, or its empirical formula is such and such. But what does this actually mean to our clients (either internal or external)? As we industrialize the science and push for higher efficiencies and greater productivity, do we lose the ability to generate information rather than data? To me these are two very different things.

The ability to produce information that guides a project, aids in troubleshooting a process, or allows properly informed commercial decisions within the business is often much more valuable than a raw number, a list of compounds, or an accurate mass and empirical formula. All too often we work as disparate functions; the analytical laboratory is a satellite (physically or metaphorically) that serves many masters and exists to pass or fail, confirm or deny, and provide the data they have requested. Sometimes in the laboratory we might not even know who "they" are, "they" may never venture into the analytical space. This isn't right, it isn't how it should be.

I wonder how many clients actually know what information we are capable of producing, how many of them really understand our techniques in order to make the right analytical "requests"? Do they know which questions to ask?

I believe there are two aspects that need to be developed to properly function as a key provider of information:

1) To allow users of the service to better understand our capabilities and empower

them to ask the right questions of us and to better understand our outputs;

2) For analytical chemists to strive to understand more of the context of their work, the background to the analysis, the chemistry involved, and the place of the results in this wider context.

In respect of point number 1, I remember a time when a great deal of emphasis was placed on educating analytical service users on the background to our techniques and the capabilities of our instrumentation. There were many training sessions on the use of data from the analytical laboratory and the inferences that could or could not be drawn from data produced from the various different techniques. I see precious little of this happening in the modern analytical laboratory. In fact, the fashion now seems to be that when things have gone wrong or unusual data are produced the laboratory manager or team leader is summoned to the project meeting. I wonder, do they know enough about the analysis to draw proper inference from the data and provide a guiding hand to the project (whatever this may be)? Sometimes I wonder if the presence of the analytical chemist who is the subject matter expert or has carried out the analysis would be a lot more informative!

Often the recipients of the data are unaware of the limitations, unable to properly interpret the numbers or the facts, not aware of the level of error or uncertainty which accompanies the data. I am certain wild decisions are made from data, which are wholly unsupported by the analytical output. There is a worrying disconnect.

I believe we are a little scared of point number 2. In discussions on this topic I hear responses such as "It's not our place to make that decision", "it's a project decision", "that's not something we can comment on", "we aren't in a position to make comments on why this has occurred". My simple counter question to these statements is "why not?" Do we not want to be the ones who take the blame if our advice is incorrect? Do we not have the knowledge to give advice on what might be done to overcome the issues that we may have highlighted? Do we not have time to get involved?

In truth the answer is probably a little of all of the above. We often don't have time to get involved in every project that needs a little more commentary on our data. There will be many cases where we don't have the breadth of knowledge for us to properly interpret our data in the wider context. And yes, why should we stick our necks out and be the ones to take the rap if our judgement is awry? Analytical science used to be much more collaborative; we used to get involved in meetings with our service users and help

them to understand what can and, crucially, cannot be inferred from our data. Why has this changed so much? I believe it is, once again, down to industrialization, where in many cases we are simply producers of data, both qualitative and quantitative. This is in some ways self-inflicted and we have undertaken this role too lightly, after all, it's much easier to be insular, much more straightforward to make sure that, according to our own processes and procedures, our data are of excellent quality. But beware the lack of the wider context, of not seeing the wood for the trees.

If we have a specification or expected range of values for this number, then we may have a feel for the validity of the data, however, if the analysis is investigational or if no expected range exists, then do we ever question the number that is produced?

We are very concerned about the system suitability and quality control (QC) data hitting their specification targets and can often spend hours tabulating the data and ensuring that we are "compliant" and that the system is producing "fit-for-purpose" data. But does this mean that our data are "correct" in a contextual sense?

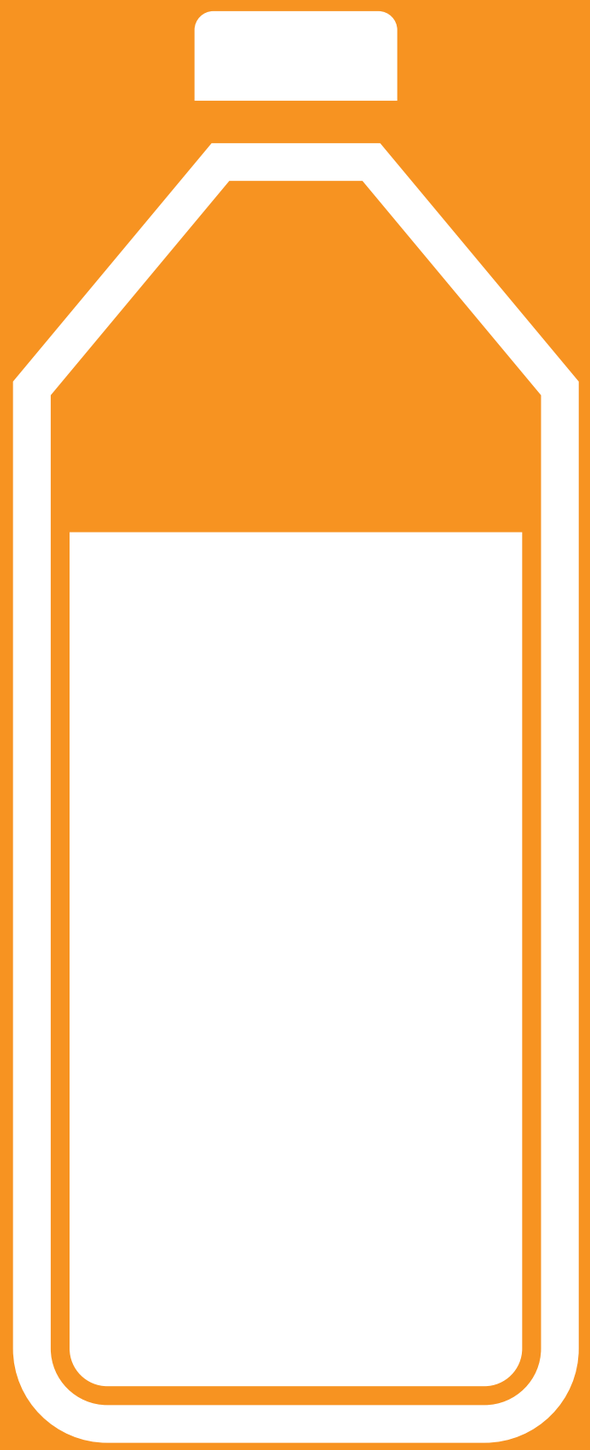
QC laboratory methods are very often undertaken without any idea of the analyte structure or the physical chemical properties of the chemical nature of the matrix. It's perfectly possible to produce "fit-for-purpose" data (from the checks and balances constituted by QC and system suitability), but knowing the context must surely help with troubleshooting or interrogating the data for abnormalities not seen in individual or smaller numbers of data points. A good point to consider here is the out of specification (OOS) result. What happens in your laboratory when an OOS event occurs? Sometimes this will be reported as a failed batch and a re-test will occur, after which a decision will be made, usually according to an investigation protocol. But how much investigation is undertaken outside the confines of the protocol? What can be learned about the manufacturing process, the synthetic route, or the pilot plant production method in light of the failed sample?

And how much is automation to blame in our lack of context? We often use templates to generate written reports and may populate tabulated data using information electronically transferred from the data system. In theory we may place the sample on one end of the system and the results are reported to us at the other. How much does this allow us to understand the nature of the failed results?

Studies with airline pilots show that whilst automation allows them time to think ahead and consider the wider context of the flight, any problems or struggles with automation makes the pilots turn inward and concentrate much more on the tasks at hand and become more obsessed with solving these perhaps minor issues—often to the detriment of the big picture. Given the higher levels of automation in the laboratory—is there anything we can learn from this?

Of course, by no means is every laboratory guilty of being so insular, but I do see a general trend in which we are much more concerned that our data are "correct" without considering what information that may represent to the service user. Denis Waitley, the American author and motivational speaker, wrote, "You must look within for value, but must look beyond for perspective." I think we can benefit greatly from bearing this in mind in our daily work.

Contact author: **Incognito**
E-mail: **kate.mosford@ubm.com**



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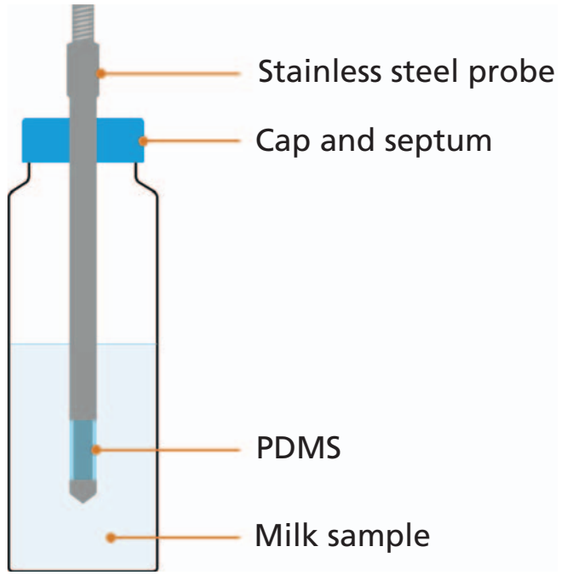
Combining Sorptive Extraction with Two-Dimensional Gas Chromatography for the Flavour Profiling of Milk

Rebecca Preston, Laura McGregor, and David Barden, SepSolve Analytical, Peterborough, UK

This proof-of-principle study shows that polymer-based sorptive extraction probes, coupled with secondary focusing by thermal desorption and analysis by flow-modulated two-dimensional gas chromatography with time-of-flight mass spectrometry or flame ionization detection (GC×GC–TOF-MS/FID), can be used to separate and identify flavour compounds in milk. As well as comparing the profiles of dairy and non-dairy milks, this article highlights the practical benefits of this sampling procedure, the ability of two-dimensional GC to physically separate components that would coelute in one-dimensional GC, and the use of software tools to improve workflow.

Like many other foodstuffs and beverages, the quality of milk as perceived by the consumer depends crucially on the presence of aroma-active compounds. This sensitivity can be attributed in part to the relatively bland flavour of milk, which allows even modest changes in compound profiles to be perceived (1). The flavour of milk is nevertheless influenced by the presence of a large number of compounds from numerous

Figure 1: Sampling setup for immersive sorptive extraction.

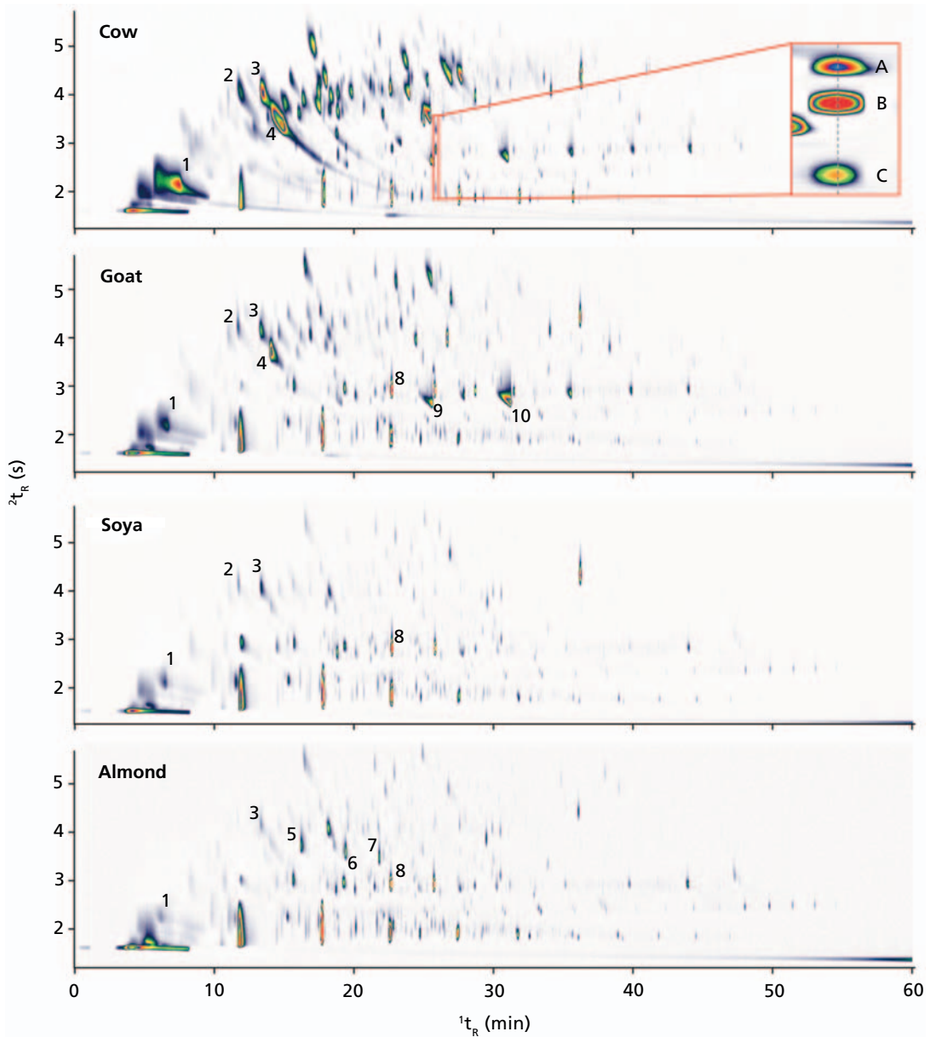


chemical groups, many present at levels below their flavour threshold (2). Reliable analysis of the volatile components of milk is therefore valuable in research to understand the factors affecting the flavour profile, and in routine monitoring to ensure high quality or identify the cause of off-flavours.

Approaches to sampling of milk volatiles are numerous (3). In the past, liquid–liquid extraction (LLE), solid-phase extraction (SPE), and distillation were widely used, often in studies that focused on the fatty-acid components of the volatile organic compound (VOC) profile by derivatizing them to their methyl esters. However, in the last 20 years these methods have largely been supplanted by headspace approaches (or less often, purge-and-trap [4]). Headspace analysis provides profiles that are representative of the volatile emissions from the sample, as well as offering greater operational simplicity and reduced artefact levels (5). However, the low concentrations involved means that a preconcentration stage is essential, and to this end sampling of the headspace onto a sorbent is used—typically either in static headspace mode using a solid-phase microextraction (SPME) fibre (6), or using a dynamic headspace device in conjunction with a sorbent-packed tube and transfer to the gas chromatography (GC) system via trap-based thermal desorption (7,8,9).

Despite the advantages and popularity of these approaches, there remains a place for easy-to-use methods able to extract volatiles from the bulk liquid, both for a deeper understanding of the full volatile content of the liquid sample (as opposed to the subset of compounds released into the vapour phase), and for monitoring less-volatile organic compounds such as contaminants. SPME fibres, although occasionally used for immersive sampling of milk (10), are easily

Figure 2: GC×GC–TOF-MS colour plots of the four milk samples. Peaks contributing >5% of the total response from identified compounds are labelled: 1. Acetic acid; 2. Methyl pyruvate; 3. Furfural; 4. 2-Furanmethanol; 5. 2,5-Dimethylpyrazine; 6. 2-Ethyl-6-methylpyrazine; 7. 3-Ethyl-2,5-dimethylpyrazine; 8. Nonanal; 9. Octanoic acid; 10. Decanoic acid. The inset shows an expansion of the cow-milk profile in Figure 1, showing the ability of GC×GC to separate three components (A, Benzoic acid; B, Decanal; C, Siloxane) that would have coeluted in a 1D analysis.



broken (for example during the necessary washing stage), offer limited sensitivity, and may also suffer from capillary-action carryover effects. Sampling onto a relatively large volume of monolithic poly(dimethylsiloxane) (PDMS) addresses all these issues, and has been shown to give good results for the sampling of milk in two configurations—fitted around a glass-coated magnetic stir-bar (11), and likewise on a stainless steel probe (12) (used in this study). The analytical performance of both sampling formats is comparable, but the probes are far easier to manipulate, and are also amenable to automation. In both cases the volatiles are desorbed within a thermal desorption tube, followed by trap-based preconcentration. Recent work (13) has shown that probe-based sorptive extraction can extract a similar number of compounds from milk as headspace-SPME, but with an emphasis on lower-volatility compounds with lower polarity (which have a greater affinity for the PDMS sorptive phase).

Irrespective of the sampling approach, analysis of volatiles usually proceeds by GC–mass spectrometry (MS), but as in the case of many foods and beverages, the presence of structurally similar compounds in milk aroma profiles often gives rise to coelution with regular one-dimensional GC configurations. This is usually only tackled by long (but expensive) columns, or by multiple analyses that decrease productivity. To address this, food analysts are increasingly using GC×GC, which allows efficient separation of homologues and isomers, and can enhance analyte capacity by up to an order of magnitude (14).

This study demonstrates an approach to the analysis of volatile compounds in four types of liquid milk, by combining probe-based immersive sorptive extraction with GC×GC. A reverse-fill/flush flow modulator avoids the cost and logistical issues associated with thermal modulators using liquid cryogen (15), and parallel detection by time-of-flight (TOF)-MS and flame ionization detection (FID) enables both easy identification of unknowns and straightforward quantitation. To the best of our knowledge, this is the first reported example of milk analysis by high-capacity sorptive extraction combined with GC×GC.

Experimental

Samples: Whole cow's milk, goat's milk, soya milk, and almond milk were purchased from a local supermarket. A 10-mL measure of each sample was placed in a 20-mL headspace vial with 2 g of NaCl, to improve extraction efficiency by decreasing the solubility of analytes.

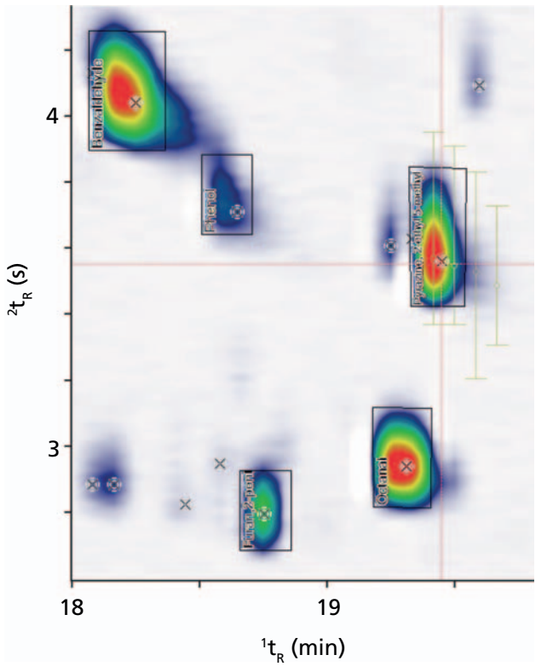


Figure 3: GC×GC–TOF-MS colour plot of cow milk showing the definition of boundaries around target peaks: from left to right, benzaldehyde, phenol, 2-pentylfuran, octanal, and 2-ethyl-5-methylpyrazine. These boundaries are subsequently used to assist quantitation using the FID dataset.

Immersive Sorptive Extraction: PDMS sampler: Inert HiSorb probe (Markes International); time: 60 min; temperature: 35°C.
Thermal Desorption: Instrument: TD100-xr (Markes International); focusing trap: "General-purpose". HiSorb probes were inserted into empty inert-coated stainless steel TD tubes.
GC×GC: Flow modulator: Insight (SepSolve Analytical). PM: 5.0 s.
TOF-MS: Instrument: BenchTOF-Select with Tandem Ionization (SepSolve Analytical): Simultaneous acquisition of 70 eV and 12 eV data; mass range: *m/z* 35–500.
Software: ChromSpace GC×GC software (SepSolve Analytical) for full instrument control and data processing.

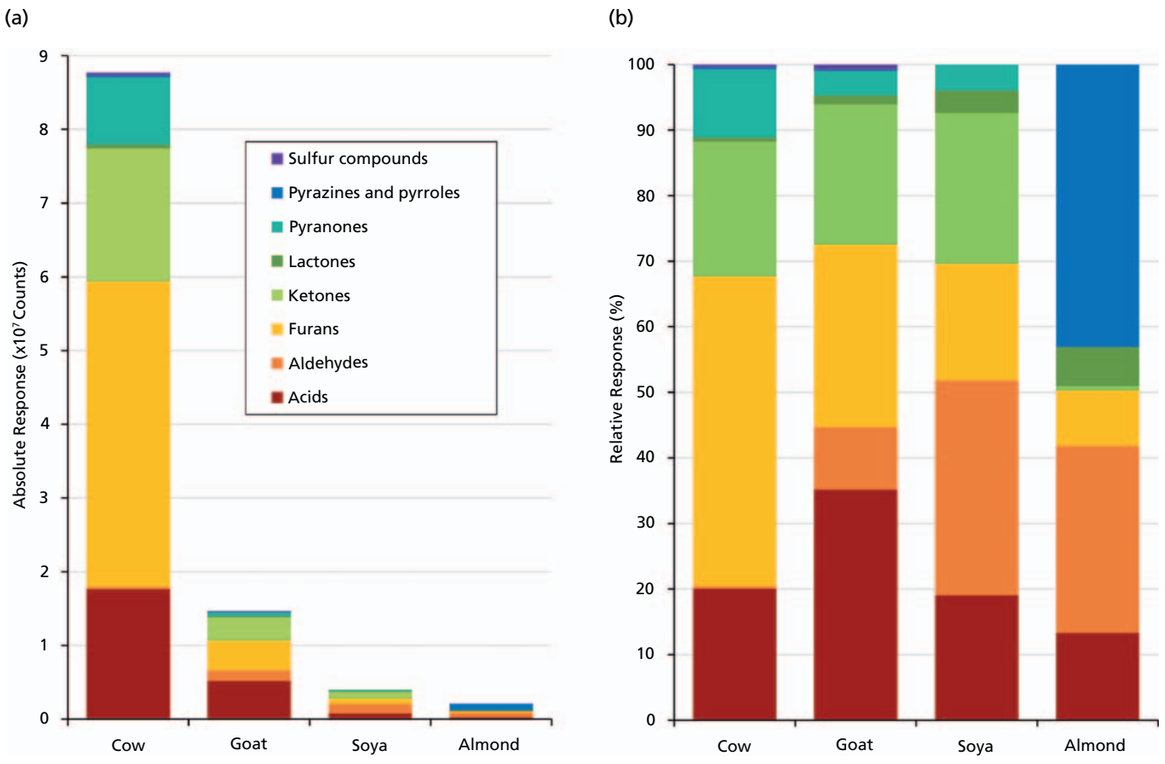
Results and Discussion

Sampling and Analytical Considerations:

The high-capacity sorptive extraction probes used in this study consist of a short section of PDMS located near the end of a stainless-steel probe, which is immersed in the sample (Figure 1). Agitation with gentle heating is then sufficient to ensure that analytes are effectively absorbed into the sorbent volume in a reasonable time frame. A key consideration is the relatively large sorbent volume (65 µL compared to 0.5 µL for SPME), which, combined with secondary focusing by thermal desorption (TD), results in higher sample loadings and therefore greater sensitivity across a wide analyte range.

The GC×GC–TOF-MS colour plots obtained from the four milk samples are shown in Figure 2, showing the excellent separation

Figure 4: Comparison of the compositions of the milk products, based on GC×GC–FID and grouped by chemical class: (a) as absolute responses; (b) as relative responses. Where a compound could be placed in more than one category, priority is given to cyclic structures.



achieved. A common issue with any sampling technique employing polymeric silicone stationary phases is the presence of siloxane contaminants in the chromatogram, but the second separation in GC×GC ensures that the aroma and flavour compounds of interest are well-separated from these (Figure 2, inset).

Analysis by TOF-MS is ideal for research or investigations into unknown contaminants, but routine quantitative analysis is best performed using FID, and in this study a dual TOF-MS/FID setup was used. Quantitation using this approach involves first identifying compounds using TOF-MS, with tightly defined boundaries being drawn around the

identified peaks (Figure 3). This collection of boundaries, known as a stencil, can then be transferred to the FID dataset, enabling rapid quantitation of all the target peaks.

Sample Comparison: Figure 4 shows the relative abundances for the various chemical classes resulting from the application of this approach to two dairy milks (cow milk, goat milk) and two non-dairy milk substitutes (soya milk, almond milk). A total of 57 compounds were identified across the four samples, with 45, 39, 20, and 19 being found in cow, goat, soya, and almond, respectively—only 10 compounds were present in all samples. As well as this difference in compound diversity, it is immediately apparent from Figure 4 that the cow milk showed a much greater overall response compared to the other samples, which largely derives from greater responses for the acids, furans, ketones, and pyranones. In contrast, the goat milk had a markedly weaker response, and the two non-dairy milk substitutes were weaker still.

Medium-chain fatty acids are important components of dairy products, and decanoic acid was the most abundant of this group in both the cow and goat milks, followed by octanoic acid (linear and branched-chain C_8–C_{10} acids have been noted to impart a distinctive "goat-like" flavour [16]). However, both samples contained a substantial quantity of acetic acid, and in the cow milk this dwarfed the other acids present by a ratio of 3.5 to 1, which may indicate a degree of rancidity.

Medium-chain esters have been reported to be predominant odorants in raw milk but of reduced importance in pasteurized milk (17), and in agreement with this no compounds from this group were detected in these two pasteurized samples. However, some cyclic esters were observed, of which the most predominant were δ-decalactone, δ-dodecalactone, and δ-octalactone ("lactonic/sweet/coconut/fruity" [2]). Methyl pyruvate made a significant contribution to the response in the cow's milk, and may be the result of fermentation (18).

Heterocyclic compounds were a major component of the cow milk sample, with 17 furan derivatives and five pyranones present, including as major components the maltol, isomaltol, and 2-furanmethanol ("burnt/caramellic" [19]); compounds of this group have been found in sterilized concentrated milk (1). Ketones are also important flavour contributors, and in the goat milk heptan-2-one ("blue cheese, spicy" [1,20]) and nonan-2-one ("mustard-like, spicy" [1]) were identified. The latter two compounds are also reportedly responsible for "UHT flavour" (2). Amongst low-boiling sulfur compounds, dimethyl sulfone was found in both the cow

and goat milks, in accordance with its identification as a significant odorant in these two products (21).

Compared to the dairy products, the soya milk had a distribution of abundances between the compound classes that is not too dissimilar, albeit with much higher responses for some medium-chain aldehydes. Amongst these, nonanal ("sweet, floral, green, grass-like" [1]) predominated, making an interesting comparison with an earlier study of soya milk headspace (22), which as expected found the more volatile congeners (pentanal and hexanal) to dominate. In contrast, the almond milk showed a quite different profile, with a distinguishing feature being the presence of a range of pyrazines, some of which are known to impart a "toasted almond" flavour (23). One of these is 2,5-dimethylpyrazine, noted to be partly responsible (along with 2-methoxy-3-isopropylpyrazine) for a "musty, potato-like" aroma defect in milk (24).

Conclusions

In this study, it has been shown that combining high-capacity sorptive extraction and GC×GC can enable the characterization of the complex flavour profiles of milk and related products, allowing useful cross-sample comparisons to be made. Compared to SPME, the sampling protocol is more robust for immersive extraction, while retaining the ability to sample a wide range of chemical classes. In addition, the use of flow-modulated GC×GC provides high chromatographic resolving power without the need for liquid cryogen, while software tools streamline data analysis from simultaneously acquired TOF-MS and FID datasets.

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Rebecca Preston graduated from Teesside University in 2014 with a first-class honours degree in forensic science. Following this, she worked at LGC as a human testing scientist, which involved extracting and analyzing anabolic steroids and new psychoactive substances from human matrices. In early 2017 she joined SepSolve, where she supports internal sales and coordinates the development of applications within the laboratory.

Laura McGregor received an M.Chem. in chemistry from the University of St Andrews, UK, followed by an M.Sc. in forensic science at the University of Strathclyde, UK. Her Ph.D. in environmental forensics, also at the University of Strathclyde, focused on the chemical fingerprinting of environmental contamination using advanced techniques such as GC×GC–TOF-MS. In her current role, she specializes in the application of GC×GC and TOF-MS to challenging applications.

David Barden studied natural sciences at the University of Cambridge, UK, and remained there for his Ph.D. in synthetic organic chemistry, which he received in 2003. A placement at Wiley-VCH, Germany, was then followed by seven years as a journals editor at Royal Society of Chemistry Publishing, UK, before beginning his current role as copywriter in 2011.

E-mail: **hello@sepsolve.com**
Website: **www.sepsolve.com**

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Best Practices for Analyzing Pesticides and Their Metabolites in Environmental Samples

When a company wishes to commercialize a new pesticide, they must conduct environmental studies and develop analytical methods capable of detecting the pesticide, and its metabolites, in soil and water samples. The methods must be robust and rugged, for easy use in routine analysis. James Stry, a principal investigator at FMC Agricultural Solutions, recently talked to *LCGC* about best practices he and his team have established for developing such methods, including approaches to meeting a variety of requirements of regulatory bodies, simplifying sample preparation, dealing with matrix effects, choosing an ionization method, and streamlining method development.

—Interview by Laura Bush, Editorial Director

Q. When you are developing environmental methods for the registration or re-registration with the U.S. Environmental Protection Agency (EPA) of a new pesticide, what exactly do you have to demonstrate?

A: We must demonstrate that the methods we develop extract the environmental residue of concern from soil or sediment and that the residue is accurately quantified. To demonstrate the performance of the extraction method, we treat soil samples with radiolabeled compounds and age them in conditions representative of the environment. Aging the treated soil or sediment allows for the environmental metabolites to form and for the soil to become representative of a sample collected from the environment.

We then extract the aged samples and analyze the radioactivity in the extract and the amount of radioactivity remaining in the soil. We test different extraction solvents, extraction techniques (for example, bead mill, sonication, or microwave), and the number of extractions conducted until close to all of the significant residue is extracted from the sample.

Once we have an extraction method, we fortify untreated control soil or sediment samples with known amounts of the analytes of interest. We then extract and analyze these samples and compare the concentration determined using the method to the known amount added to the control samples. The amount determined must be between 70% and 120% of the amount added for the method performance to be acceptable.

Q. In addition to meeting the requirements of the U.S. EPA, do you also have to meet requirements for other regulatory bodies, such as those of other countries, if your products will be sold abroad, or of individual states within the United States? If so, how do you balance meeting all the requirements?

A: In an attempt to minimize additional method development work, we develop our methods to meet the most conservative requirements and test our methods on soil collected from all parts of the world. Currently, the European Union (EU) has some of the most conservative requirements for environmental methods. These requirements are described in the SANCO 825 Revision 8.1 guidance document (1). Once validated, the environmental methods can be sent to support compound registrations anywhere. An example is the residue method for chlorantraniliprole in crops (2). This method was validated on 21 different crops at a limit of quantitation of 0.010 mg/kg. This method has been accepted by regulatory agencies around the world for data collection and maximum residue limit (MRL) enforcement.

Q. What are typically the biggest challenges in developing methods for detecting a pesticide and its metabolites in soil or sediment? What are the biggest challenges for detecting them in water?

A: Minimizing matrix effects is a major challenge when developing environmental methods. Since our methods are intended to analyze samples from grower fields, a suitable control sample will not always be available. Therefore, preparing standards in untreated control extracts may not always be an option. Moreover, the U.S. EPA prohibits the use of matrix-matched standards in all monitoring and enforcement methods (3).

Developing selective cleanup steps is one

way to deal with matrix effects. However, the number and diversity of metabolites in the methods and the low limit of quantitation (1.0 µg/kg or below) makes method development a challenging task. The diversity of metabolites makes it difficult to develop one cleanup procedure capable of removing co-extracts while keeping all of the analytes of interest in a single extract. Keeping all of the compounds in a single extract limits the selectivity of the cleanup procedure.

Developing chromatographic separations using ultrahigh-pressure liquid chromatography (UHPLC) columns or solid-core particles can help minimize matrix effects as well. The sharp peak shape these columns produce lowers the instrument detection level, allowing additional dilution of the extracts. Dilution is perhaps the best way to minimize matrix effects. Instrument manufacturers continue to produce faster, more sensitive, and more rugged instruments that have allowed us to simplify and streamline our methods. The speed of the newest generation of instruments allow for positive–negative switching without significant loss of sensitivity. This capability has removed the need to separate positive- and negative-ionizing compounds chromatographically or to analyze them in multiple chromatographic runs.

Water samples, although less complicated than soil or sediment, can be just as challenging. Water methods usually have a limit of quantitation of 0.10 µg/L or lower. For methods that include many metabolites, the analytes may need to be extracted from the water samples using solid-phase extraction (SPE) or a liquid–liquid partition step. Once the extract is concentrated, a solvent-exchange step may be needed before analysis. At the low levels analyzed in these methods, minor water contaminants can complicate quantitative analysis.

Q. Over the years, have you developed best practices or streamlined approaches to developing these methods?

A: When developing the extract purification procedures, we tend to use a systematic approach. Before extracting any samples, we test evaporation and reconstitution steps, liquid–liquid partitions, and SPE procedures. When developing SPE procedures, we filter the analytes through columns in a variety of solvents to determine when they are retained and when they are eluted. Once we retain the analytes on a cartridge, we wash the cartridges with progressively stronger solvents until we have a complete profile of the properties for all analytes. Having all of this information allows us to understand how the analytes behave and to piece together an efficient and effective cleanup procedure.

We prefer to include all of the known metabolites in the methods. If the compound is not detected in the terrestrial field soil dissipation studies or if it does not show any adverse ecotoxicology effects, it can be removed from the method. We refer to this approach as comprehensive method development. We validate our methods in this manner because the addition of a new metabolite to an existing method often results in completely reworking the method, which can be time- and resource-intensive.

Q. In environmental analysis, a challenge is often the complex matrix, which requires effective sample cleanup and preparation. Do you have "go-to" sample cleanup or preparation approaches for certain classes of products?

A: When developing our methods, we usually start with the most difficult soil or water samples. The idea is that if the method works for the most complex samples it will perform well for the other samples. For soil samples, we start with a high clay, high organic matter soil. We have found this combination results in an extract that is very difficult to purify and analyze. For water samples, we usually start with a pond-water sample.

The methods developed are based on the analytes included in the analysis. When developing methods for polar metabolites, some of the reversed-phase polymer SPE cartridges can be very effective at concentrating the sample extracts. We usually try to develop cleanup procedures that complement the separation and detection methods. If we are using reversed-phase LC we will try to develop an ion-exchange cleanup. If we are using an alkyl SPE cartridge (such as C18 or C8) for the cleanup step, we will develop a separation using a phenyl or biphenyl column. The overall goal is to minimize matrix effects by taking advantage of multiple physical-chemical properties of the analytes.

Q. What percentage of your methods use gas chromatography (GC) and what percentage use LC? Has there been any change in recent years in that balance?

A: Almost all of the methods we develop are LC methods. Many of our active ingredients are thermally labile, limiting the use of GC analysis. The inclusion of multiple metabolites to these methods also limits the amount of GC analysis we conduct. Often, the metabolites we are analyzing are small polar alcohols or weak acids that are not amenable to GC analysis without derivatization. Although derivatization procedures can be developed, they can be time-consuming and add complexity to the methods. It is also worth mentioning that relative to LC analyses,

we have generally observed more severe matrix effects when conducting GC analyses.

Q. What type of mass spectrometry (MS) detection do you typically use?

A: We typically use LC–MS/MS detection on triple-quadrupole instruments. Given that we are analyzing a limited number of known compounds using a reference standard, we can set up several multiple-reaction monitoring (MRM) transitions at the retention time of each analyte. In addition to being very sensitive, this approach allows for confirmation and quantitation during a single analysis. An added benefit is the availability of triple-quadrupole instruments in contract and monitoring laboratories. Using similar equipment allows for our methods to be transferred and revalidated at the laboratories performing the analysis with minimal modifications.

Q. When do you use atmospheric pressure chemical ionization (APCI) MS, rather than electrospray ionization (ESI)?

A: When developing a new method, we usually start with ESI because it is often more sensitive than APCI. If we determine matrix effects are affecting method performance, we attempt to develop a cleanup procedure that is efficient and effective at minimizing the matrix effects. If, because of the number or diversity of the analytes, the cleanup procedure is not effective, is not rugged, or is very complicated, we switch to the APCI ion source. Although less sensitive, APCI usually does not exhibit the same degree of matrix effects as ESI. Given the reduced sensitivity of APCI, we adjust the injection volume, aliquot factor, or the sample's final volume to reach the required detection level (2). Not all of the compounds we monitor can be ionized using APCI because of the increased temperature of the ion source and the gas-phase ionization mechanism. For compounds that are not amenable to APCI we return to ESI and look for new extract cleanup steps or better chromatographic separations until a robust method is developed.

Q. How do you balance the need for sample preparation methods that are effective but not overly complicated or time-consuming?

A: We need to strike a balance between the amount of sample cleanup, the time required to conduct the analysis, and the overall method performance. Cleanup can be time-consuming and tedious work. However, without adequate cleanup, a method will perform well for only a limited number of sample types because of matrix effects, or could result in false positives as a result of coeluted peaks. Reducing sample cleanup in

favour of longer chromatographic separations limits the number of samples that can be analyzed per instrument each day.

Because of the large sample size (2–5 g) required to produce a representative soil or sediment sample, we have not implemented the 96-well format or other automated sample preparation approaches. We have seen significant improvements in efficiency using bead mill extractors, however. Bead mill extractors show excellent extraction efficiency of incurred residues and require less solvent per sample, and all of the equipment used is disposable, reducing the probability of sample contamination. The biggest advantage of bead mill extractors is the amount of time required to perform an extraction. An extraction requiring 20–30 min on a wrist action shaker or in a sonicating bath can be completed in 2–3 min using a bead mill.

Our goal is to develop a method with an efficient and rugged cleanup procedure and a set of chromatographic conditions that allow for adequate sample throughput without compromising the accuracy of the analysis.

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James Stry is a principal investigator in the Regulatory Analytical Group at FMC Agricultural Solutions. Before joining FMC in 2017, he was with DuPont Crop Protection for 20 years. He is responsible for monitoring the global regulatory environment as it pertains to the residue and environmental analysis of crop protection compounds. In addition, he also maintains an active laboratory programme developing new residue and environmental methods to meet regulatory requirements. Stry graduated from the State University of New York at Buffalo (USA) with a Ph.D. in physical chemistry.

E-mail: **James.J.Stry@dupont.com**

Enhancing PLGA Characterization with Multi-Angle Light Scattering and Differential Viscometry

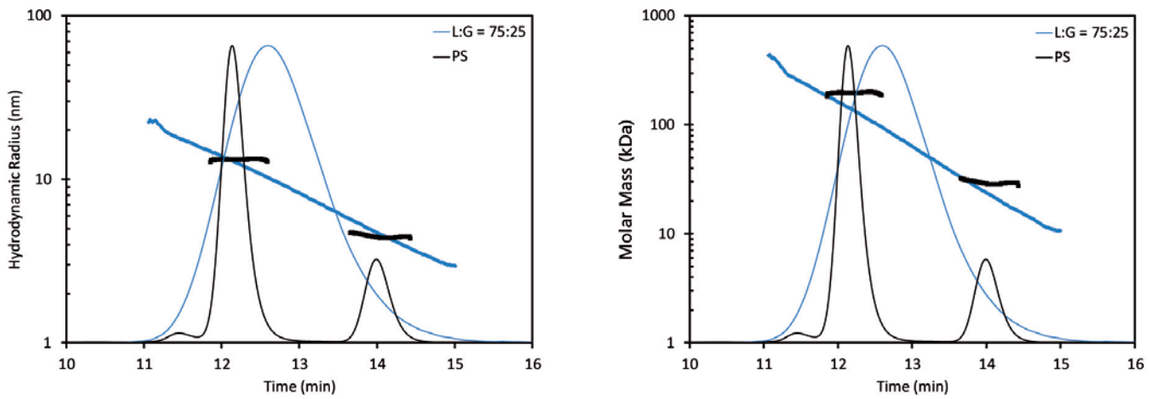
Sophia Kenrick, Wyatt Technology Corp., Santa Barbara, California, USA

The functional properties of polymers, such as poly(lactic-co-glycolic acid) (PLGA), relevant to drug delivery and biomedical devices, are governed by the molecular properties of molar mass, composition, conformation, and branching. This article demonstrates how such polymers are fully characterized, quickly and absolutely, using gel permeation chromatography (GPC) with multi-angle light scattering (MALS) and online viscometry.

Poly(lactic-co-glycolic acid) (PLGA) is a widely used polymer for drug delivery and biomedical devices, including sutures and prosthetic devices, because of its biocompatibility and biodegradability. As a drug delivery vehicle, PLGA extends the release of its payload to reduce dosing frequency, and as of 2016, there were 15 FDA-approved PLGA- or PLA-based drug products available in the US (1,2).

The drug-release profile is controlled by the overall molar mass and ratio of lactide to glycolide (L:G ratio). For example, drug delivery vehicles commonly make use of 50:50 L:G ratios to control the drug release profile (3–5). PLGA with higher L:G ratios tend to degrade more slowly because of the hydrophobic character of the lactide side chain (2). Therefore, characterization of L:G ratio and structure

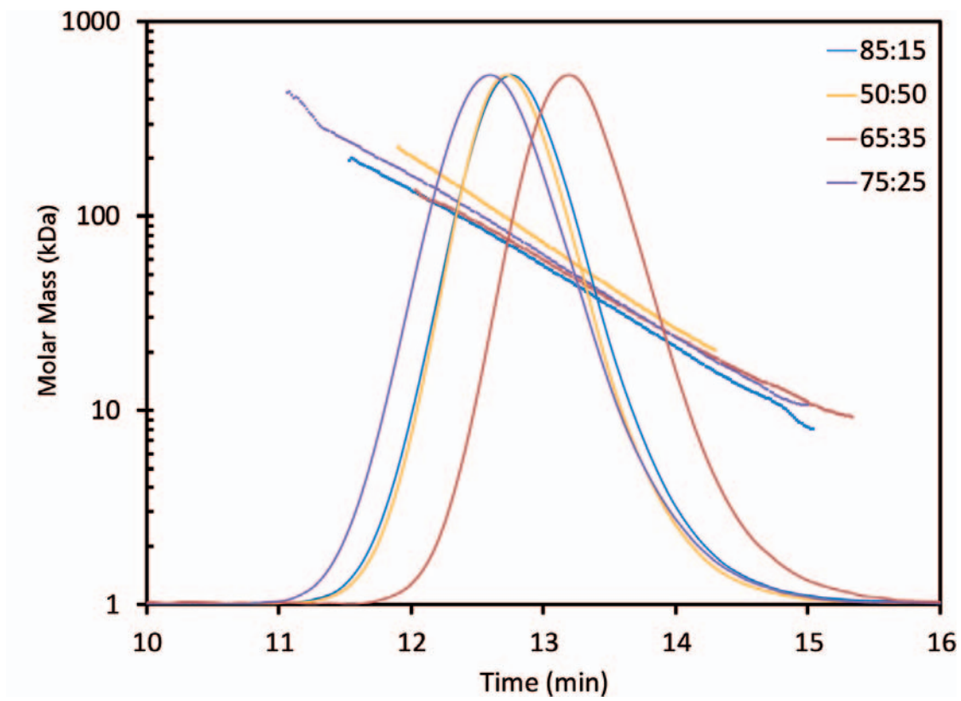
Figure 1: Measured hydrodynamic radius (left) and molar mass (right) overlaid on the light scattering chromatogram for a typical PLGA sample (blue) compared to a set of polystyrene standards (black). The hydrodynamic radius as a function of elution time indicates ideal GPC separation. The molar mass measured by MALS does not rely on the elution time of a molar mass standard and reveals differences in conformation or density of PLGA relative to the polystyrene standard.



is crucial for developing safe and effective therapeutics. This article describes the characterization of PLGA samples with distinct L:G ratios using gel permeation chromatography (GPC) coupled with multi-angle light scattering (MALS) and intrinsic viscosity (IV) measurements. Application of GPC-MALS-IV includes identification and characterization of PLGA samples with unknown L:G ratios, quality control of different lots of material, and comparison among manufacturers.

In a GPC-MALS-IV method, MALS directly measures the molar mass of the eluting polymer, regardless of elution time and without relying on molar mass standards; differential viscometry enables characterization of the polymer's intrinsic viscosity, hydrodynamic size, and conformation. The combination of MALS and IV provides the widest measurement range and highest sensitivity, quantifying molar masses from a few hundred g/mol up to 10^9 g/mol and radii below 1 nm up to ~1 µm. The relationship between intrinsic viscosity [η] and molar

Figure 2: Light scattering chromatograms for PLGA molecules with varying L:G ratios. The measured molar mass by MALS is overlaid for each chromatogram.



mass is often displayed using a Mark-Houwink–Sakurada (MHS) plot, whose slope and intercept are used to determine branching parameters. Linear polymers in thermodynamically good solvents typically exhibit MHS slopes around 0.5, and extended conformations can increase the slope up to a value of 1 for rod-like structures.

Branching increases the polymer density because monomer units are added to the branches and not just the ends of the monomer. The result is a polymer with higher molar mass at the same overall R_g or [η] as its linear analogue. Polymers with long chain branching (LCB) exhibit a lower slope in an MHS plot than their linear analogues. Short chain branching (SCB) also creates a dense structure with lower intrinsic viscosity than a linear polymer of the same molecular weight. However, the overall conformation (MHS slope) remains similar to the linear analogue because of the short branch lengths; thus, SCB phenomena are visualized as parallel MHS plots. For PLGA, conformational analysis using MHS plots clearly differentiates among the L:G ratios tested and may help elucidate their functionality.

Table 1: Molar mass moments, intrinsic viscosity, and slope of the Mark-Houwink-Sakurada plot as measured by GPC-MALS-IV. Values are average and standard deviation of three measurements.

L:G ratio	M_w (kDa)	M_n (kDa)	Đ=M_w/M_n	$[\eta]_z$ (mL/g)	MHS slope, a
50:50	69.0 ± 0.2	45.2 ± 0.2	1.53 ± 0.01	50.1 ± 0.4	0.57 ± 0.02
65:35	36.2 ± 0.3	25.0 ± 0.7	1.45 ± 0.03	41.1 ± 0.3	0.69 ± 0.03
75:25	64.0 ± 0.4	37.3 ± 1.4	1.72 ± 0.05	69.7 ± 0.4	0.68 ± 0.01
85:15	52.8 ± 0.3	33.9 ± 1.4	1.56 ± 0.05	64.9 ± 0.3	0.71 ± 0.01

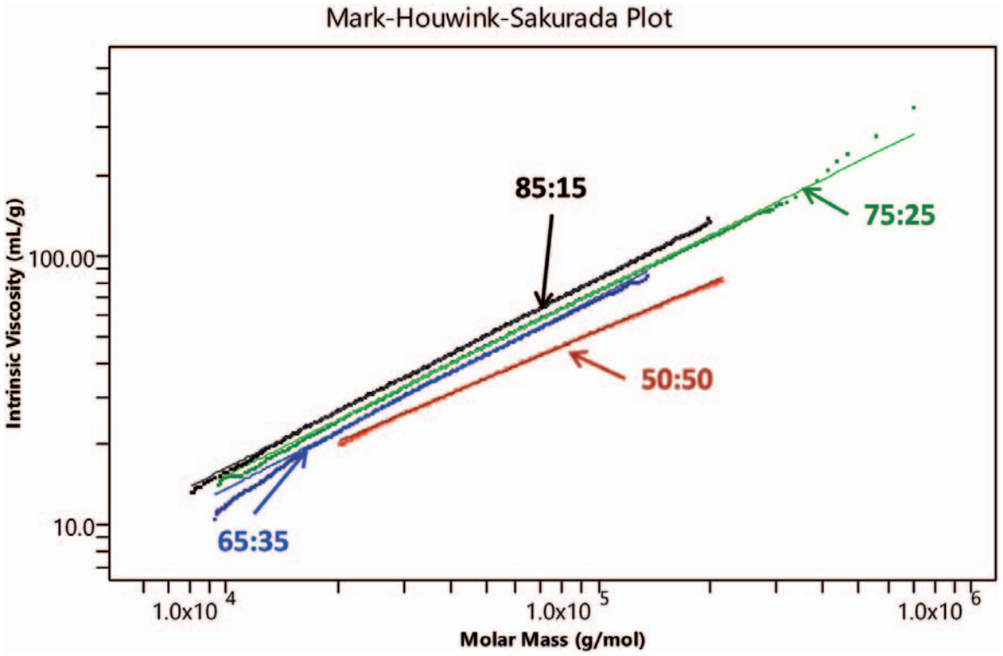
Methods

PLGA standards with varying L:G ratios (Sigma) were analyzed via GPC-MALS-IV. The separation was performed using an Agilent 1100 autosampler and two 7.5 mm × 300 mm, 5-µm PLgel mixed-C columns (Agilent) with THF as the mobile phase at a flow rate of 1 mL/min. Samples were prepared at 5 mg/mL in THF, and 100 µL of each sample was injected onto the column. The effluent from the columns flowed through a DAWN multi-angle light scattering detector, ViscoStar differential viscometer, and Optilab differential refractometer (Wyatt Technology Corporation). The light scattering, concentration, and viscometry data were collected and analyzed using ASTRA software (Wyatt). A constant refractive index increment (*dn/dc*) of 0.049 mL/g was used for the analysis of all PLGA samples.

One major advantage of incorporating MALS and IV with GPC is that the measurement is independent of elution time. This is critical for PLGA analysis because the polymer may not share the same conformation as a "typical" molecular weight standard.

Figure 1 shows a typical chromatogram for PLGA overlaid with data for polystyrene

Figure 3: Shifts in slope and intercept of the Mark-Houwink-Sakurada plot indicate differences in the conformation of the PLGA molecules as function of L:G ratio.



standards. At each eluting slice, the molar mass, concentration, and intrinsic viscosity are measured directly, and the hydrodynamic radius is calculated from the intrinsic viscosity. The measured hydrodynamic radius vs. elution time indicates the separation is ideal: the PLGA is eluting according to its hydrodynamic volume and is not interacting with the column (Figure 1, left). However, estimating the molar mass of PLGA based on its hydrodynamic volume (or elution time) would significantly overestimate the molar mass of the PLGA (Figure 1, right). Clearly, the PLGA is less dense than the polystyrene standard since molecules of the same hydrodynamic size have a lower molar mass relative to polystyrene.

Results

The GPC-MALS-IV data provided key insights into the molar mass, size, and

conformation of the different PLGA molecules. Figure 2 shows typical chromatograms for PLGA molecules with four different L:G ratios, and the molar mass moments are summarized in Table 1. Although the 75:25 LG ratio elutes first, its molar mass is actually less than that of the 50:50 PLGA, which elutes second. Moreover, despite nearly identical elution profiles, the molar mass measured by MALS for the 50:50 LG ratio is 30% greater than that measured of the 85:15 LG ratio. This mismatch in measured molar mass and elution volume points to differences in density and conformation among the different types of PLGA.

Intrinsic viscosity measurements provide additional detail regarding polymer size and conformation. Although all of the PLGA molecules were linear, they exhibited different conformations based on their L:G ratios. The MHS plot in Figure 3 highlights key differences for each type of PLGA.

First, with increasing glycolic acid content, the PLGA becomes more dense. Thus, for a given molar mass, a 50:50 L:G ratio produces the most compact conformation and smallest hydrodynamic size of all the L:G ratios tested. Second, the slope and intercept of the MHS plot conveys additional information about the molecular conformation. The slope of 0.57 for the 50:50 PLGA suggests a random coil,
while the slope of ~0.7 for the other three samples suggests a more extended conformation.

Despite the nearly identical slopes for the 65:35, 75:25, and 85:15 polymers, the conformation data do not overlay. Instead, they form parallel lines in the MHS plot. The parallel conformation plots are often indicative of short-chain branching, but in this case the differences among the molecules are more subtle. Since these PLGA controls are unbranched, the increase in density at higher L:G ratios is caused by the extra methyl side chain in the lactide monomer, which is not present in the glycolide monomer. This difference, although small, produced parallel MHS plots with good reproducibility across multiple sample injections, highlighting the robustness of the GPC-MALS-IV measurement.

Conclusions

Combining MALS and differential viscometry with GPC enables direct measurement of the molar mass and intrinsic viscosity of PLGA samples and highlights significant differences in conformation among the different L:G ratios. Even though all the tested samples

were linear, each L:G ratio produced a distinct set of Mark–Houwink parameters and distinct elution profile.

Importantly, these differences would not be evident by analytical GPC alone. GPC-MALS-IV characterization can be further extended to classify unknown PLGA samples based on known standard conformation and also to evaluate branching and other macroscopic structural differences.

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Sophia Kenrick is senior applications scientist at Wyatt Technology where she supports multiple applications for Wyatt instrumentation, especially in the field of molecular recognition and biomolecular interactions. Sophia received a bachelor's degree in chemical engineering from Arizona State University (Arizona, USA), a doctorate in chemical engineering from the University of California, Santa Barbara (USA), and has been with Wyatt since 2010. She is also the Dean of Light Scattering University, Santa Barbara campus.

E-mail: skenrick@wyatt.com
Website: www.wyatt.com/Polymers

Automated Multicolumn Purification of a Histidine-Tagged Protein

Katie McLaughlin and Candice Cox, Bio-Rad Laboratories, Hercules, California, USA

Approximately 40% of recombinant proteins that are purified use a histidine tag for easy capture. This article covers how to automate the purification of histidine-tagged proteins and how purification conditions can be optimized to an automated four-step purification scheme that uses affinity-, ion exchange-, and size-exclusion columns. Using a multistep purification scheme removes the manual steps that cause loss of precious proteins and take more time, like dialysis, collection, and reinjecting samples. The final purification scheme reduces a 3–4-day process to 11.5 h from start to finish, all while improving reproducibility, yield, and comparable purity.

Scientists purifying proteins frequently run multiple methods over multiple column chemistries to generate a sufficient amount of sample for their experiments. These individual column purifications, each with their own running conditions and method, are followed by multiple sample fractionations and fraction pooling. A significant amount of time is spent on developing these methods and running the subsequent steps necessary for the purification. A well-designed multidimensional method combines optimized methods for the individual column chemistries, resulting in an automated and highly reproducible multistep procedure.

Engineering a polyhistidine-tagged protein is a commonly performed molecular biology technique where users benefit from a quick and more efficient purification process. This article presents the development of an automated multidimensional method used to purify an

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Figure 1: Six scouting runs of the pre-elution wash from 0–25%B in 5%B increments. With increasing %B the leading shoulder of the 280 nm peak decreases without significant decreases in the GFPsf 495 nm trace up to 10%B. At 15%B significant GFPsf elution can be seen during the wash step and at 20%B all of the GFPsf has been eluted. A_{280} (–); A_{495} (–); %B (–).

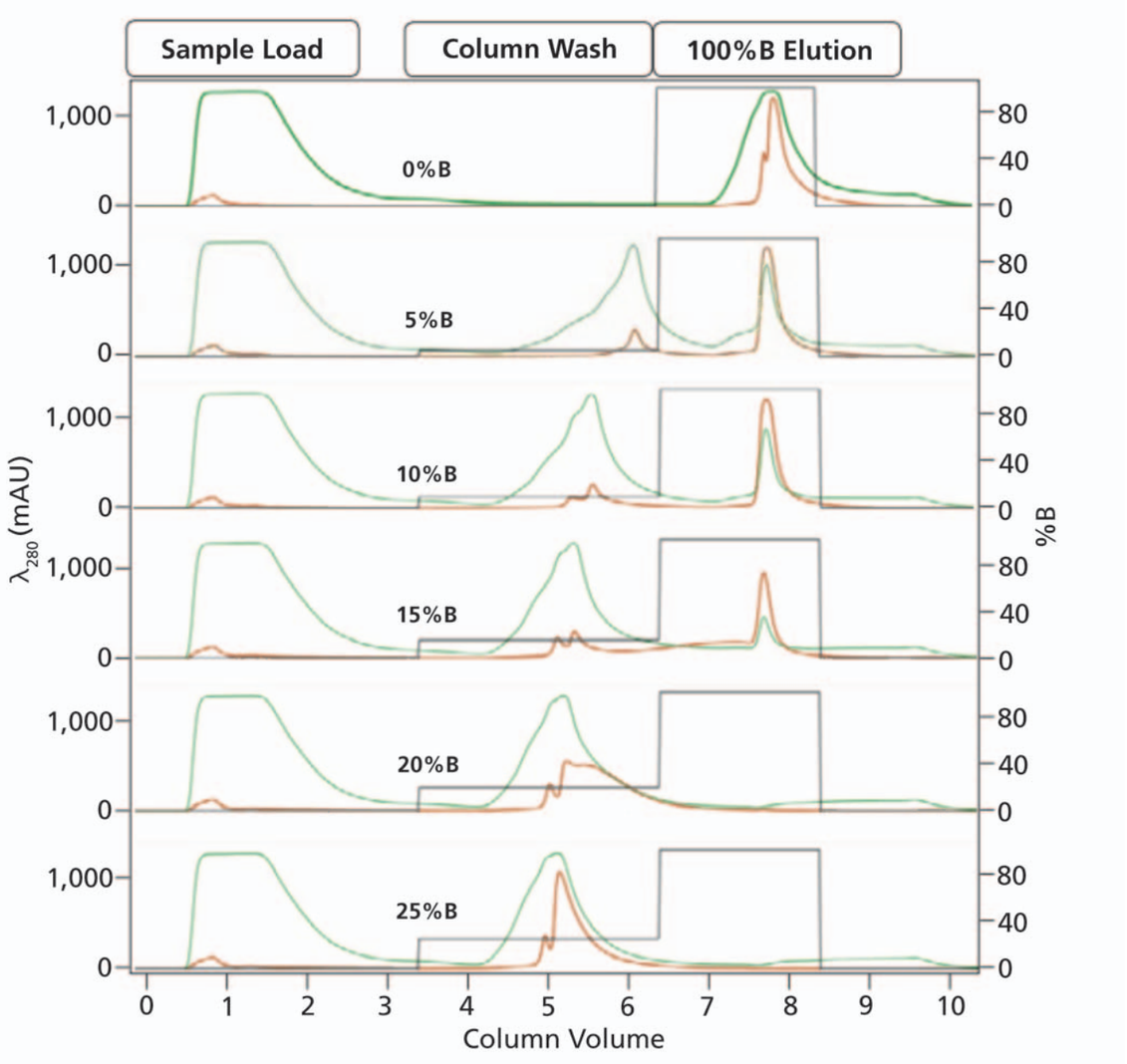


Figure 2: A buffer blending module was used to generate the pH scouting runs using Tris buffer for the ENrich Q Column scouting. (a) pH scouting runs using Tris pH 7.5, 8.0, and 8.5. Tighter GFPsf binding was demonstrated by the increasing amounts of salt required to elute the protein as the pH increased from the theoretical protein pI of 6.2. (b) Using pH 8.0 from the pH scouting, the linear gradient %B endpoint was scouted again starting at 20%B and increasing to 70%B in increments of 10%B. A GFPsf peak shift to the linear gradient begins at 50%B and reaches completion by 70%B. A_{280} (–); A_{495} (–); conductivity (–).

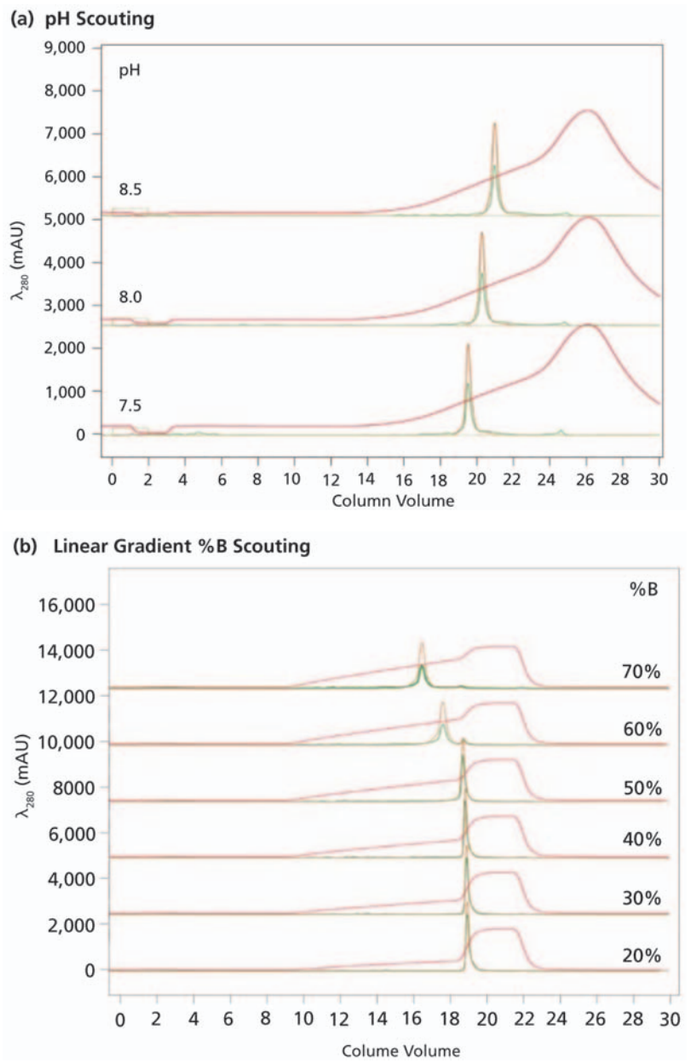
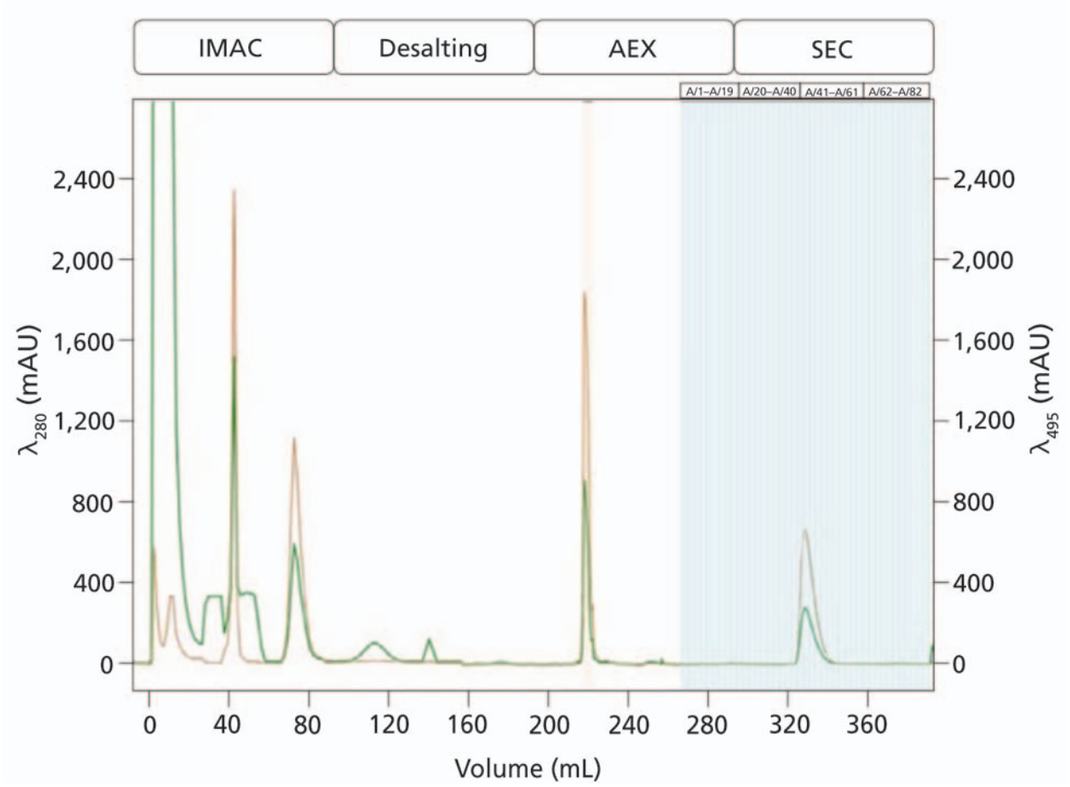


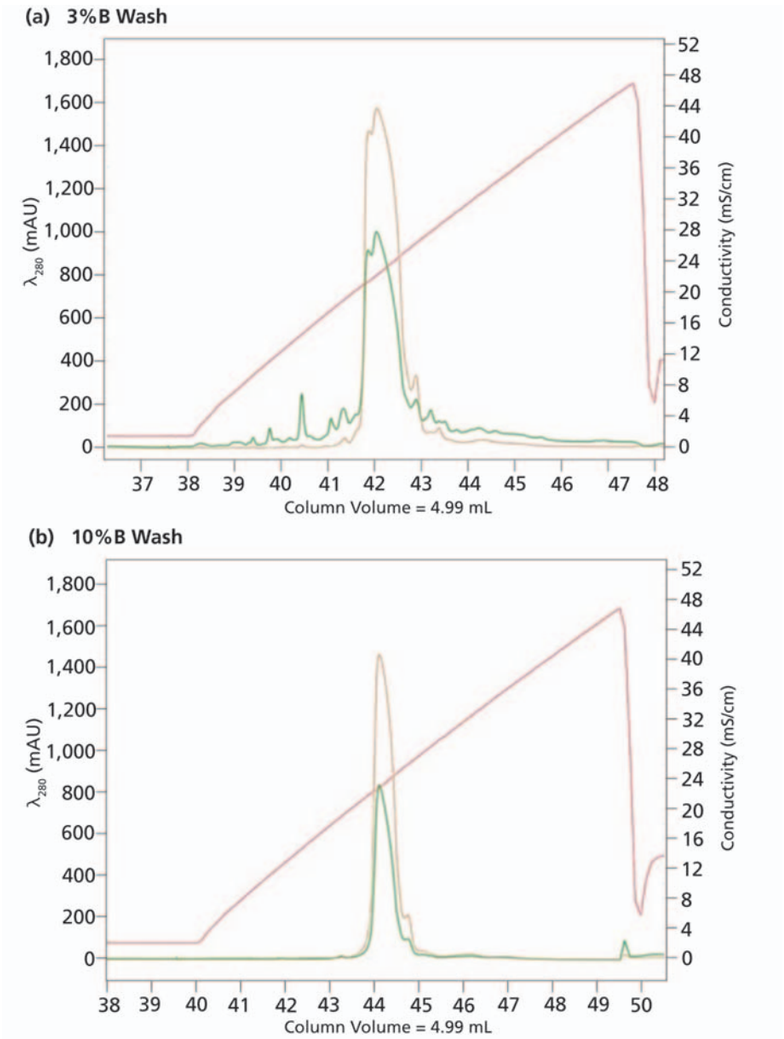
Figure 3: Clarified GFPsf lysate (2 mL) was loaded onto a 5 mL Nuvia IMAC Cartridge using the sample pump. The column was washed with 10%B and the target protein was step-eluted and stored in a 10 mL static loop. This was loaded from the loop onto the 50 mL Bio-Gel P-6 Desalting Cartridge, eluted isocratically, and collected into a 20 mL static loop. The 20 mL protein sample was loaded onto a 1 mL ENrich Q Column and eluted with a linear gradient. Using threshold collection, the eluate was shuttled into a 5 mL static loop, loaded onto a 120 mL SEC column in a single injection, and eluted isocratically in 5 mL fractions. The blue lines represent final fraction collection of the purified protein product. A_{280} (–); A_{495} (–),



N-terminal small ubiquitin-like modifier-tagged C-terminal polyhistidine-tagged superfolder green fluorescent protein (SUMO-6xHis GFPsf), referred to as GFPsf hereafter.

Using Ni2+ immobilized metal affinity chromatography (IMAC) as the capture step in a purification workflow produces sufficiently purified material for some applications. However,

Figure 4: Chromatograms of an automated IMAC purification emphasizing the Enrich Q Column linear gradient elution. (a) Pre-elution column wash phase during the IMAC purification at 3%B. The small shoulder peaks show coeluting impurities that may risk a more contaminated sample. (b) Optimized 10%B pre-elution column wash phase during the IMAC purification step. The lack of small impurity peaks on the leading shoulder allows for a low threshold value to be used for collection of the eluate to the loop resulting in maximum recovery and purity. A_{280} (–); A_{495} (–); conductivity (–).



many workflows also incorporate intermediate and polishing steps to increase purity of the final product. GFPsf is a commonly purified recombinant engineered protein. The method development of each single-column step can easily be optimized for purity and resolution, and then incorporated into a multi-column method.

The outlined multidimensional method uses the following column chemistries: Ni2+ immobilized metal ion affinity (IMAC), desalting–size exclusion (SEC), anion exchange (AEX), and size-exclusion (SEC) to effectively automate the purification process and improve recovery and reproducibility.

Methods and Results

System Configuration: A three-tier NGC Quest 10 Plus Chromatography System (Bio-Rad Laboratories) was used, configured with a sample pump, multiwavelength UV detector, two buffer inlet valves, two column switching valves (CSVs), and a single outlet valve. The sample pump on the system was used to load sample during the single-step optimizations and for the IMAC purification during the multidimensional method. A multiwavelength UV detector was used to monitor GFP at both 495 nm and 280 nm during development and for recovery calculations. One of the CSVs was substituted with a buffer blending valve module for pH scouting during the AEX column optimization process but placed back into the system for the multidimensional method.

Sample Preparation: *Escherichia coli* lysate containing GFPsf was clarified by centrifugation at 10,000 rpm for 10 min at 4°C and kept on ice.

IMAC Capture Optimization: Using a step gradient for the IMAC (IMAC step gradient template in ChromLab Software [Bio-Rad Laboratories]), 2 mL of clarified lysate was loaded onto a 5 mL Bio-Scale Mini Nuvia IMAC Cartridge (Bio-Rad Laboratories) for each run and was equilibrated with 50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole pH 7.5. The elution buffer B was 50 mM sodium phosphate, 300 mM NaCl, and 125 mM imidazole pH 7.5. To better understand the binding and elution profile of GFPsf on the cartridge, the scouting feature within the software was used on the 3%B pre-elution column wash phase to generate a series of ten runs with increasing %B column wash steps (0–45% at 5% increments). Figure 1 shows a subset of those runs with an overlay of 0–25%B column wash where contaminating proteins are predominantly being eluted up to 10%B as seen by the decreasing front shoulder of the 280 nm elution peak. At 15%B, the GFPsf begins to elute from the column as seen by the decreasing intensity of the 495 nm peak. Based on these data, the 10%B pre-elution wash and an elution volume of 6 mL were determined to be optimal and used to generate the multidimensional method.

Desalting and AEX Intermediate Optimization: Using a 50-mL Bio-Scale Mini

Bio-Gel P-6 Desalting Cartridge (Bio-Rad Laboratories) the IMAC buffer B with imidazole was removed, and the protein was placed into a low ionic strength buffer suitable for loading onto a 5 mm × 50 mm, 1-mL ENrich Q anion exchange column (Bio-Rad Laboratories). IMAC-purified protein (6 mL) was loaded onto the cartridge and eluted by an isocratic flow of AEX buffer A to a final sample volume of ~14 mL. Anion Exchange Conditions: A linear gradient (an anion exchange step gradient template in the software used) was used to load the desalted GFPsf (~14 mL) onto a 5 mm × 50 mm, 1-mL ENrich Q anion exchange column (Bio-Rad Laboratories). The optimal pH value of Tris buffer was determined using the scouting feature within the software to determine the ideal binding strength and elution profiles; pH scouting runs used Tris pH 7.5, 8.0, and 8.5 (Figure 2[a]). As expected, an increase in binding strength was observed as the pH moved further away from the pI of the protein (pI = 6.2). The final %B concentration in the linear gradient step of the elution phase was scouted against, creating a series of runs from 20–70%B (Figure 2[b]). The scouting data shows a shift in the elution peak during the 50%B endpoint that continued until the entire GFPsf peak was in the linear gradient during the 70%B linear gradient step in ~2 mL elution volume.

SEC Polish Optimization: A 16 mm × 600 mm, 120-mL SEC column (GE Healthcare) was used as the final column in the purification workflow. The purified protein was eluted with an isocratic flow using 25-mM $NaPO_4$ + 150-mM NaCl, pH 7.2 at 0.5 mL/min; no optimization was necessary.

Discussion

Scientists purify proteins to obtain samples necessary for their downstream experiments. It is commonly a multiday, labour-intensive process involving repetitions of single-column purifications, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) visualizations, and fraction pooling to get to the desired level of purity and yield. Much time is spent optimizing the binding and elution steps for each individual column. Once the ideal conditions are determined, the method can be routinely followed for subsequent purification runs. Automating this process frees up a significant amount of time for other research activities. Additionally, small batch-to-batch variations between purification runs or by different individuals performing or monitoring the purification are largely eliminated.

One noteworthy observation during the initial IMAC purification was the importance of the %B pre-elution column wash. Weakly or nonspecifically bound proteins washed off with just a small amount of imidazole in the wash. One benefit of using a GFP analogue is the ability to monitor only the protein of interest at 495 nm

using multiwavelength detection, an easier way to comparatively analyze runs without depending on SDS-PAGE visualizations. In addition, using a GFP analogue that absorbs at 495 nm prevents interference with other possible coeluting proteins and the imidazole in IMAC buffer B, which absorbs at 280 nm, enabling more accurate peak integration and recovery statistics.

Elution volume is important to consider for any multidimensional method. A good starting point is to use a volume ~2× the column volume (that is, a 2-mL loop for a 1-mL column) because the volume of the column needs to be accounted for even in reverse column elution circumstances. The exception here is desalting columns. Assuming the entire eluted IMAC sample needs to be injected for desalting, a 50-mL desalting cartridge would be required for the eluted IMAC protein stored in the 10-mL sample loop. Running a cartridge with a 10-mL sample loop shows an elution volume of ~15 mL. Since an AEX column follows desalting, a larger volume presents no issues because the protein binds to the column and is concentrated in the process.

Ion exchange offers several different variables to scout against but two of the most common are pH and %B endpoint of a linear gradient. Figure 2(a) shows a simple pH scouting. As expected, the protein binds more tightly with increasing pH and increased displacement from the theoretical pI of 6.2. Peak integration shows a 2% difference in peak area for the three runs in both the 495 nm and 280 nm traces (data not shown) indicating no real binding or elution differences under the various pH conditions. For pH continuity with the PBS used for the IMAC purification, buffer at pH 8.0 was selected.

With this information, the elution gradient endpoint was scouted against. Eluting with a linear gradient typically gives the greatest chance of separation from contaminants. Figure 2(b) shows the GFPsf elution peak starting to shift at the 50%B gradient endpoint, transitioning at 60%B with a small population of tighter binding GFP, and finally eluting by the 70%B linear gradient.

A dextran gel cross-linked with acrylamide column was used as the final polishing column in the purification workflow. Optimization was minimal because the sample volume dictates which SEC column will be used to ensure a single sample injection to maximize recovery. Optimal conditions that provided the best peak separations for individual steps were combined with elution volume information to create the multidimensional method.

Figure 4 shows the difference between following a standard IMAC–Affinity template 3%B column wash and the optimized 10%B column wash. This helps underline the importance of optimizing each individual step of the purification when constructing a multidimensional method, because choices on the first column affect the efficiency and recovery.

The final automated method took ~11.5 h to run from start to finish, a significant time saving compared to the traditional sequential approach, which would take 3–4 days per sample. Typically, SDS-PAGE is performed and visualized between each step, along with dialysis of the IMAC eluate overnight to remove buffer salts and imidazole. A large part of the multidimensional run is spent on equilibration and elution from a 120-mL SEC column during the traditional approach. Multiple injections on a smaller, more pressure-tolerant SEC column could produce purified protein in ~4.5 h, representing even greater time savings. Beyond time savings an automated workflow increases reproducibility between purification batches by removing potential human error or variability. Ultimately, automated purification shows the most promise for consistent protein production at the laboratory scale.

Conclusion

The creation of an automated multidimensional purification method is often seen as a barrier to achieving truly automated protein purification. Several pieces of data are required prior to generating the multidimensional method. First, optimized conditions for each individual column in the purification must be established. These optimized conditions can be determined by using a variety of methods from simple univariate scouting to more complex multivariate analysis utilizing design of experiment (DoE). Regardless of which method is used, the information about variables such as optimal pH, %B elution, sample loading, and flow rate must be incorporated into the multidimensional method. With this information, the multidimensional method can be constructed from scratch or by using a prewritten 2D template, replicating its method phases with minor edits. Though the method creation in this study uses the example of an automated IMAC purification, most protein purification workflows can be converted into a multidimensional method.

Katie McLaughlin is a global product manager in the Protein Purification Marketing Group at Bio-Rad Laboratories. Katie has 10 years of chromatography experience, focusing primarily on resins and applications.

Candice Cox is a global product manager in the Protein Purification Marketing Group at Bio-Rad Laboratories. She has been with Bio-Rad for over 13 years and has held different marketing and R&D positions within the Life Science Group.

E-mail: **katie_mclaughlin@bio-rad.com**
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michael.tessalone@ubm.com

Associate Publisher
Oliver Waters
oliver.waters@ubm.com

Sales Executive
Liz Mclean
liz.mclean@ubm.com

Sales Operations Executive
Kim Evans
kim.evans@ubm.com

Editor-in-Chief
Alasdair Matheson
alasdair.matheson@ubm.com

Managing Editor
Kate Mosford
kate.mosford@ubm.com

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lewis.botcherby@ubm.com

UBM Americas
Hinderton Point, Lloyd Drive,
Ellesmere Port, CH65 9HQ, UK
Tel: +44 (0)151 353 3621
Fax: +44 (0)151 353 3601

North America

Vice President/Group Publisher
Michael J. Tessalone
michael.tessalone@ubm.com

Publisher
Edward Fantuzzi
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Sales Manager
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stephanie.shaffer@ubm.com

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laura.bush@ubm.com

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john.chasse@ubm.com

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Administation and Sales Offices
Woodbridge Corporate Plaza,
485 Route 1 South,
Building F, First floor, Iselin,
NJ 08830, USA
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