APRIL 2013 # 04

the Analytical Scientist

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Online this Month

Heating Up

Last month's editorial, "How Well Do We Measure?", raised some deep issues – and the comments section is aflame with rhetoric:

"It would be naïve to think this may be a self-correcting problem because in the future better-prepared and scientifically trained lawyers will in court be able to put testifying analysts on the hot seat. Van Geel seems oblivious to what to me is a far more insidious elephant in the room […] If one has to go before an administrative hearing you will have almost none of the protections you would be guaranteed in a trial, including proof of chain of custody of any admitted physical evidence and hearsay testimony".

Frank Van Geel responds:
"Robert Blackledge points out an important issue in the doping system and analysis. Sporters are confronted with numerous obligations by their organizations: they have to be available at all times, explain their whereabouts, have no serious ways of challenging the results […] I do hope this issue stirs up the much needed discussion within our community about our responsibility as scientists".

Read the full, passionate debate online and, more importantly, add your view: theanalyticalscientist.com/issues/0313/104

Mile High Club

Content at 30,000 feet? If you're the proud owner of an Apple tablet (big or small), then you just might need The Analytical Scientist App. You can flick and swipe your way through the contents of The Analytical Scientist and enjoy a rich and engaging multimedia experience to boot. Learn more about the editorial in the video interview and download the whole issue to read offline. link.theanalyticalscientist.com/app

The Small Print

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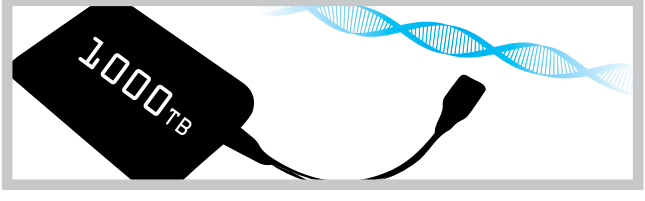
Digital DNA

On page 10, an interview with Nick Goldman reveals an exciting project that uses DNA to store digital information, such as MP3 and jpeg files. Online, you can find an additional article that delves further into both the story and the realms of science fiction. Read it online: theanalyticalscientist.com/issues/0413/207 or on the iPad app (see "Mile High Club").



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A newborn baby undergoes dried blood spot sampling for disease screening.
Image courtesy of March of Dimes (www.marchofdimes.com)

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the Analytical Scientist

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Is Industrial Research the New Gold Standard?

Considered for so long to be the poor relation to academia, industry researchers may well represent the best of science.

Scientific research is sound. The scientific method, based on careful experimentation and observation to test hypotheses, is robust. Its record – the scientific literature – is accurate and dependable, buttressed by the expert evaluation provided by peer review. We can confidently build on (the vast majority of) what's published to learn ever more about how the world around us works. Right?

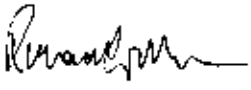
Wrong. We are realizing that there are huge cracks in the reliability of science and the scientific record. Just a year ago, a study co-written by a researcher at Amgen (1) reported that 47 of 53 "landmark" publications in cancer research, papers from distinguished researchers published in prominent journals, could not be replicated. The previous year, researchers at another drug company reported that "In almost two-thirds of the projects, there were inconsistencies between published data and in-house data" (2).

The scientific enterprise must face up to these damning statistics. It's not as though the problem is a one off. For example, a 1995 article entitled "Why Most Published Research Findings Are False" (3) attracted limited interest and generated no concrete counter-measures. In fact, The Reproducibility Initiative, described in last month's issue by Elizabeth Iorns, might be the first attempt to address the issue.

This lack of replicability has many causes but incompetence and outright fraud are not prominent among them, despite the occasional, sensational case. That's a mixed blessing. On the one hand, it's comforting that the vast majority of researchers are both competent and honest; on the other, it means that there are deep issues to be addressed, among which the cherry-picking of data, poor controls, inadequate number of repeats, non-publishable negative results, and bad experimental habits loom large. The reward system in academia exacerbates all of these problems.

This leads me to wonder if more reliable research comes out of industry, particularly analytical labs. Industry scientists have less emphasis on publishing, less pressure to generate a 'flashy' result and, given the demands of providing products or services, they are more focused on reproducibility. On page 44 of this issue, Lloyd Snyder nails the case for publication by industry researchers. To his arguments, might we add that industry scientists can teach academia a thing or two about the design and reproducibility of research? A comparison of the reproducibility would certainly be interesting.

Richard Gallagher
Editorial Director



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Lloyd Snyder

Lloyd Snyder has received international recognition for his wide-ranging contributions to chromatography, especially HPLC. "I first encountered gas chromatography in 1955, then switched to liquid chromatography in 1957, and HPLC, the premier technique for chemical analysis, in 1966," he recalls. Snyder, who recently retired, spent his entire career in industry. During that time he authored or co-authored of over 300 publications and nine books. *He discusses the role of publishing for industry research on page 44.*

Simon Kiddle and Hilary van der Hoff

Simon Kiddle is a Bristol-based European patent attorney and a partner at Mewburn Ellis. "I mainly deal with prosecution and opposition/appeal, but I'm also involved in due diligence work, strategic portfolio planning, and advising start-up companies." Simon has a degree in chemistry from Oxford University and handles patent work in chemical, biotechnology, pharmaceutical and nanotechnology fields.

"I studied biochemistry at the University of Oxford but followed up with research in immunology at the Institute of Molecular Medicine at the John Radcliffe Hospital in Oxford and in plant pathology at the Max Planck Institute for Terrestrial Microbiology in Marburg, Germany," says Hilary van der Hoff, who is now a European patent attorney and a Partner at Mewburn Ellis' Cambridge office. Hilary handles patent work in biochemistry and biotechnology, in particular plant science, immunology and antibodies. *See page 40.*

Richard Stadler

After a two-year post doc in the pharmaceutical faculty of the University of Munich, Richard Stadler joined the Chemical Toxicology Group at the Nestlé Research Centre (NRC) in Lausanne, Switzerland in 1992. A spell in Singapore as senior quality technologist saw Richard return to the NRC in 1998, where he became head of the Biomarkers and Contaminants Group. Transferring to the Product Technology Center in Orbe in 2004, Richard now holds the position of Group Expert for Chemical Food Safety. He is editor of the Food Additives and Contaminants Journal. *See page 36.*

Bill Kelly

Following a stint in military intelligence, Bill Kelly stumbled into biotechnology by recognizing a need for a deeper understanding of why scientists choose to purchase particular products for their labs. He co-founded BioInformatics LLC in 1994 to help lab suppliers better understand the needs of their scientific customers. Since 1997, the company has maintained a global expert network of scientists to facilitate communication between scientists and the suppliers who support their research. "The success of a scientist's research is partly dependent on having the right tools – we give them a voice in product design decisions that can ultimately lead to breakthrough discoveries." *See page 20.*

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Upfront

Reporting on research, personalities, policies and partnerships that are shaping analytical science.

We welcome information on interesting collaborations or research that has really caught your eye, in a good or bad way. Email:

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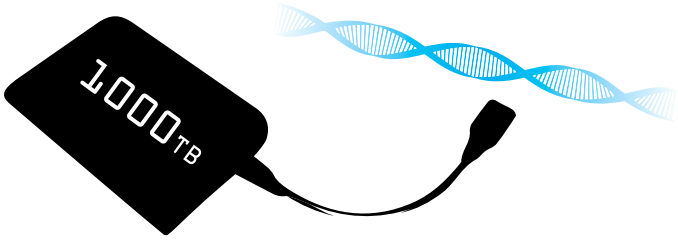

Digital Versatile DNA

Can deoxyribonucleic acid solve our spiralling digital data archive conundrum?

Hard disc drive space is now measured in terabytes not megabytes. And yet still we run out of space – or money. Perhaps the solution for long term archiving is best supplied by Mother Nature herself.

The Analytical Scientist caught up with Nick Goldman, from the European Bioinformatics Institute in Cambridge, UK, who has used DNA's resilient, efficient, and compact coding abilities to archive our digital life rather than our genetic one.

Binary to genetic – isn't that a bit of a leap?

"A code is just a code, if you know the system. Binary is not magic - it's just easier to have an on and off and nothing in between. All files have systems for encoding information into ones and zeros for storage, because that's what hard discs are good at. We decided to invent a new coding system that used the letters A, C, G and T instead of binary 1s and 0s."

Do you directly replace 1s and 0s with nucleotide bases?

"Actually, we read the files in bytes - or chunks of 8 bits, for example, in binary code, that could be: 0100100. We then re-wrote each of the possible 256 binary combinations (eight 1s and 0s) as unique five letter codes using A, C, G and T."

What did you encode into DNA?

"The files in question were: Martin Luther King's 'I Have a Dream' speech in mp3 format, a PDF of Watson and Crick's publication describing the structure of DNA, a text file of Shakespeare's sonnets, and a photo of EBI taken by me"

A recent paper in Nature gave DNA a half-life of 521 years in unfavorable conditions. How long could your storage DNA last?

"Bonds do break at a certain rate and the chemicals do degrade, of course, but it's really pretty slow. If you've got multiple copies and not all of them break in the same way, you can recover the data. DNA archives lasting tens of thousands of years is easily arguable and longer is not ridiculous."

You partnered with Agilent Technologies on the synthesis side – could you briefly describe how that works?

"It's called oligo library synthesis and it's like an inkjet printing process. But instead of firing ink onto paper, they fire chemicals containing DNA nucleotides at a glass slide where they are linked. They can very, very accurately address different spots on the slide and grow a chain according to our designed sequence. It's all automated such that the DNA is removed from the slide and supplied to us dried in a vial."

You sent your code to Agilent, they sent back the novel DNA, and then?

"The DNA was purified, amplified by PCR, and sequenced using an Illumina HiSeq – a world standard and well-understood piece of kit."

Courtesy of EBI

So, now you have raw data – the next part must be pretty complex…

"Biological experiments are messy and they don't produce beautiful clean data. Certainly, we know that errors can occur with DNA sequencing, and we assumed that the same was true of synthesis; we discussed it with Agilent and that is indeed the case. We attempted, therefore, to devise a coding system that was somewhat resistant to the kind of errors that were most likely to occur, for example, base repeats were avoided. Several layers of redundancy were built into the system for this purpose. To decode, the system takes the fragments of DNA, separates them out into indexing components, which contain information about the contents and location, and, using a majority voting system, rebuilds the file byte by byte. And when we compared the new decoded files with the originals in a formal bit by bit comparison, they were exactly the same."

Any surprising conclusions from the research?

"One of the things that we were really pleased about was people's realization of the fact that genomes are just digital information – like on your computer, and, in fact, they're interchangeable and we can go between the two and lose nothing!"

We are indeed living in a digital world and perhaps DNA is the newest, oldest code around. *RW*

Modular Protein Purification

Thoughtful yet simple product development proves that faster, higher, better aren't the only keywords for innovation.

Pittcon 2013 played host to innumerable innovations – enough to fill an entire issue – but of all the products on display, it was a seemingly simple chromatography system for protein purification that stood out from the crowd.

No doubt, Bio-Rad's marketing team created an excellent "campaign" (see images), but it was the thoughtfulness of the system – from nuts and bolts to software – that tailored the NCG modular chromatography system perfectly for biologists who have no real knowledge (or interest!) in chromatography. By simplifying the process with a number of innovative (and perhaps, in retrospect, obvious) additions to both hardware and software design, chromatography becomes a useful tool rather than a challenge to be wrestled with. For example, LED lights on the hardware ("Point-to-Plumb") indicate where tubes should be connected and confirm that you've just mistakenly configured your system to dump your precious protein into the sink…

But modular was the buzzword. Pumps that can be removed for maintenance (outside the cold room), in a system that can be expanded once you've made a breakthrough and need to ramp up purification. In fact, a Bio-Rad representative quipped that the modular system's "tier expansion" would be well-understood by ladies wishing to add space to their closet – though somewhat stereotypical, the nods from female onlookers proved the statement highly astute.

An early adopter at UC Davis, California, said of the system: "less time spent training and more time spent purifying" – perfect common sense. *RW*

Every Dog Has Its Day

A new "sniff" test for explosives uses direct, real-time vapor detection in a bid to put our canine colleagues out of work in the screening of people, baggage and cargo.

A team at the Pacific Northwest National Laboratory (PNNL) has developed a new method that could revolutionize how explosives are detected, using ionization chemistry at the front end of a standard commercial mass spectrometer. Rather than collecting explosive particles by surface swipes for later analysis, the system samples air directly for explosive vapors at ambient temperature and pressure. The new system easily detects vapors given off by a fingerprint-sized sample of RDX (Research Department Explosive), a low-volatility compound used in many explosives, at levels below 25 parts per quadrillion (1) - visit http://youtu.be/29ZYhxpfTQA to see the device in action.

Atmospheric pressure chemical ionization (APCI) is the key to the novel approach. Robert Ewing (pictured), a senior research scientist at PNNL, described how the chemistry was tailored to enhance ionization efficiency and selectivity for RDX. "Through the ionization process, a nitrate ion is formed – likely resulting from the production of ozone and NOx in the electrical discharge. The nitrate ion has a very high electron affinity and it is improbable that charge transfer will occur with other species. RDX is ionized by forming an adduct with the nitrate ion, whereas many other chemical species do not form such adducts, thus providing selective ionization for RDX". Notably, the ionization also works well for other explosive compounds that can form similar adducts, such as pentaerythritol tetranitrate (PETN), nitroglycerine and tetryl.

During development, the RDX vapors were ionized in a reaction region with a variable reaction time, which was controlled by either flow (atmospheric flow tube; AFT-MS) or an electric field (atmospheric drift tube; ADT-MS) in two different systems configurations. Each system gave similar, supporting results, but does one offer an advantage over the other? "The AFT-MS may provide a less complicated device wherein a small pump can be used for both sample collection and the movement of air to control reaction times," says Ewing.

With an eye on commercialization, Ewing has already started to think about moving from the experimental phase. "The major challenges in

Images courtesy of PNNL

making the equipment portable and usable in the field are associated with the mass spectrometer; the size and power requirements of the pumping system may be the biggest hurdle since smaller pumps limit the orifice size and thus reduce sensitivity," he notes. In the experimental system, a triple quadrupole MS was used to provide ion confirmation and additional selectivity. "The AB Sciex API-5000 is very sensitive to low ion currents resulting from the long reaction times used in the AFT or the ADT." Moving forward, Ewing is confident that other triple quadrupole MS systems will also work well as long as they have sufficient pumping capacity and a small instrument footprint. "We will be actively engaged in integrating the vapor sampling technology with one such instrument through the end of this calendar year," he says.

Comparisons are unsurprisingly made to the canine olfactory system, but how sensitive must the system be to surpass those capabilities? "The sensitivity of canines is unknown – although they have been "sniffing" explosives for a long time... This work represents the first direct vapor detection of explosives so it should be comparable to the canine response. Further research in this area will be required," concludes Ewing.

Cost is another consideration entirely, but given the general downwards direction of MS system prices and the rising cost of dog ownership (2), the tipping point may well be in sight! *RW*

For more information, check out the video produced by PNNL: http://youtu.be/29ZYhxpfTQA

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Family Affair

Günther Laukien, founder of Bruker Physik AG, posthumously receives 2013 Pittcon Heritage Award.

'Twas the night before Pittcon, when Frank Laukien, president, CEO and chairman of Bruker Corporation, accompanied by his daughter, stepped up on stage to receive the Pittcon Heritage Award on behalf of his father, demonstrating that the German giant is still very much a family affair.

Günther Laukien (1924 – 1997) was recognised for "delivering potent new research tools through his pioneering advancement and globalization of NMR and other important analytical technologies". Indeed, beyond NMR, Bruker's worldwide growth through the 1970s allowed them to expand into mass spectrometry – technology that dominated Pittcon press releases from Bruker and competitors alike.

Günther Laukien is the latest in a line of prestigious inductees into the Pittcon Hall of Fame, which includes: Genzo Shimadzu Sr. and Jr. (2012), for their early innovations at Shimadzu; George and John Hatsopoulos and Arvin Smith (2011) for their parts in the meteoric rise of Thermo Electron; Walter Jennings (2010) for his work in gas chromatography, later to the benefit of Agilent Technologies; and Alfred Bader (2009) for establishing the Aldrich Chemical Company. Amgen, Bio-Rad, Horiba, Perkin-Elmer… Going back through the last 11 years, the winners of the Pittcon Heritage Award put context behind many of the analytical powerhouses we know today. *RW*

Talking of Heritage...

Recording the impact of chemistry on society, the Chemical Heritage Foundation's participation in Pittcon adds valuable, historical depth.

The 2013 Pittcon Heritage Award ceremony was made all the more noteworthy because, for the first time in its 64-year history, Pittcon was hosted in Philadelphia, home of the Chemical Heritage Foundation (CHF), which co-sponsors the award. Thomas Tritton, president and CEO of CHF, made a most gracious welcome to his city and the CHF museum, which hosts many of the landmark instruments that pepper the histories of the Heritage Award winners. Somehow, the circle was neatly completed.

The CHF is currently seeking a 1967 Finnigan Model 1015 GC-MS (the below photo of which surely reflects the "Mad Men" generation). If you happen to have one gathering dust somewhere in an attic (we've already checked ours), please contact jenniferl@chemheritage.org. *RW*

Read more about the CHF and its museum at www.chemheritage.org.

Based on a photograph courtesy of Robert Finnigan

Hindsight

Meeting Expectations

This year's Symposium on MicroScale Bioseparations introduced a new format aimed at optimizing the scientific experience. How did it go?

Earlier this year, Jeff Chapman and James Landers, co-chairs of the 29th International Symposium on MicroScale Bioseparations (MSB2013), set out a six-point plan to maximize the value of their conference (see theanalyticalscientist.com/issues/0213/305). The event took place in March, and we've gone back to see whether it succeeded. Here, Jeff Chapman responds to questions.

What was the overall impact of the new format?

Our intent was to provide an engaging dialogue on frontier science in microscale bioseparations. From the delegate comments we received, it was very successful. I believe the new format has helped rejuvenate this meeting, it provided a unique forum to facilitate deep discussion.

What were the major plus points?

I'll mention three. First, expanded discussions. Researchers come to a meeting to discuss science, but all too often the Q&A periods are shortened. By building in extra time and fostering communication of expectations between the speakers and chairs, all authors were provided with good feedback, as well as stimulating novel ideas for future exploration. Delegates were quick to jump in, and I think most scientists felt they really had an opportunity to discuss their work.

Second, the confidentiality provisions that we implemented helped create an environment of openness. Most scientists were comfortable about bringing their unpublished work into the dialogue.

Third, speaker diversity and quality. Each session chair was given the responsibility of selecting abstracts to populate their session using a double-blind process from a peer-reviewed pool. We introduced an expanded two-page abstract with data and figures, which allowed the reviewers to assess novelty and quality. The net result was a series of new and interesting lectures, one quarter of which came from students – highlighting that it's not who you are but rather the quality of the abstract you submit that will get you a speaking spot in our conference.

Were there any lessons learned the hard way?

In future, we need to start the planning much earlier. With only a ten-month window, we did not have adequate time to introduce the new meeting format to enough people. *RW*

Join the discussion

Do you have comments on this strategy or suggestions on how to get the most from scientific events in your field? Please comment online: theanalyticalscientist.com/issues/0413/204

In My View

In this opinion section, experts from across the world share a single strongly-held view or key idea.

Submissions are welcome. Articles should be short, focused, personal and passionate, and may deal with any aspect of analytical science. They can be up to 600 words in length and written in the first person.

Contact the editors at edit@theanalyticalscientist.com

Yes, We Have a Quality Manager

Why lab SOPs are cumbersome and often ignored, and what can be done to improve them.

By Peter Kootstra, Co-owner of Lab-Q Academy, Amsterdam, The Netherlands.

The Editorial in last month's issue of this magazine dealt with the single most important topic for us as individual laboratory scientists and as a field devoted to analytics, namely, quality. As the Ford Motor Company slogan from the 1980's put it, "Quality is Job 1". Frank Van Geel's critique did not focus on the word, but quality is at the heart of his concerns.

When you ask analytical scientists what they think about quality, they always respond that it is very important. So far, so good.

But they invariably continue by stating that, in their company or institute, quality implementation is very bureaucratic and that they are fortunate that there is a dedicated quality manager.

If you ask how many pages their Standard Operating Procedures (SOPs) run to, a typical response is around 20. And instead of using it, they have a scrap of paper in their pocket with about five lines of instructions; either they follow this note or they do it by heart, having done it so many times before.

That is not a recipe for quality.

Why are SOPs so long that nobody ever bothers to read them? The simple answer is division of responsibility. It's the quality manager's job to create and perpetuate the system, and the analyst's job to get on with their task without paying any heed to ISO/IEC 17025 (the general requirements for the competence of testing and calibration laboratories), ISO 15189 (requirements for quality and competence of medical laboratories) or any other International Standard. And since only the quality manager has read the International Standard, it is his or her non-expert interpretation of the clauses that is followed. In all likelihood, the quality manual has been copied from another similar laboratory or bought over the Internet with the name of the new laboratory pasted into the original file.

Some companies take the approach of hiring a quality consultant to help put the manual together. This seems like a good move on the face of it, but with no affinity to the organization or its needs, quality implementation is destined to turn out in a similar, substandard, way.

Interpretation of the clauses of International Standards is difficult. Consequently, the standard written procedures will contain a lot of controls – the quality manager and the consultant will naturally think it better to be on the safe side. The result? Those long, bureaucratic procedures that the lab staff don't see the need for, and a quality manual that spends its life on the shelf while the analysts refer to their 'cheat sheets'.

In this process, the quality manager performs most of the internal audits to check whether the staff members are following the written rules. It's in everyone's interest to fudge this, so that the

"In all likelihood, the quality manual has been copied from another similar laboratory or bought over the Internet with the name of the new laboratory pasted into the original file."

entire organization convinces itself that (a) for quality there is a quality manager and (b) that he or she is implementing a quality management system.

In fact, none of the standards encourage you to set up such a bureaucratic system.

To build a genuine quality management system, the whole laboratory needs a thorough knowledge of the ISO standard. This does not start with the quality manager but top management; and it doesn't end with the quality manager, but with the most junior member of the lab staff. Analysts must understand that producing results in a transparent and traceable way will help them to improve their methods and will reduce their workload. The quality system must be an integral part of the work, not a bolt-on.

If you are in the analytical business, you owe it to yourself, your colleagues, your customers – and your quality manager – to implement the best system. And remember, it is never too late to improve.

Instrumental to Success

An illustration of how working with customers throughout development increases the effectiveness of new products.

By Steve Cohen, RDE Life Sciences Director, Waters Corporation, Milford, MA, USA.

How can a company, in any sector, tell if a product resonates with prospective customers while it is still in development? I believe that the best way – perhaps the only way – is to get customers involved from the word go. Here's an example from my company, Waters, of having customers actively participate in implementing and evaluating a sophisticated LC-MS system. In fact, this example even originates with a customer.

In 2004, we were approached by John Engen, then at the University of New Mexico. John is a pioneer in hydrogen-deuterium exchange mass spectrometry (HDX-MS), which is used to assess the solvent accessibility of protein amide protons. In HDX-MS, protein samples are placed in deuterated buffer to exchange amide protons with deuterons. After a specified time, the exchange is quenched by acidifying the sample at or near 0°C. The protein is then digested with the acid-stable protease pepsin, and the peptides separated and analyzed by LC-MS. The technique provides insights on protein stability, dynamics, the effects of additives, binding sites, and protein-ligand interactions.

Excessive back-exchange prior to MS was causing John problems. This process occurs readily during sample processing and analysis. Our Acquity UPLC system, which yields fast, efficient separations, limited back-exchange: the problem was solved.

The story could have ended there, but John, now at Northeastern University, felt that other avenues for improving HDX-MS were ripe for development. He encouraged Waters to engineer a cooled sample preparation module that was compatible with UPLC, to replace ice baths; and he suggested that this module should interface with a robotic sample-handling module. Chemist-engineers in Waters' Instrument Research Group, first Keith Fadgen and later including Michael Eggertson and Martha Stapels, produced a prototype in short order that provided the necessary thermal control in a user-friendly configuration.

There was clearly commercial potential in this. Our next step was to involve a number of customer sites to help identify and iron out problems prior to delivery to paying customers. The application chemists in an instrument company normally work with readily available standard samples. Working in customers' labs allows us to analyze more exotic samples, for example, protein biopharmaceuticals, that offer far more insight into the typical use of the instruments after purchase. In this case, certain proteins yielded carryover of hydrophobic peptides that both interfered in subsequent runs and gave incorrect exchange values in the initial run. With staff from Waters and our customers working together, modifications in the chromatography provided a between-sample column

wash that eliminated the problem. Further suggestions for improvements from customers extended to modifying the layout within the cooling module to improve access to key components, improving serviceability.

A significant roadblock that emerged was that, while sample analysis was streamlined, the system produced massive amounts of data. Initially, the ability to efficiently process and analyze this data and to visualize the results in a meaningful and timely fashion was missing. It became a critical element of the development process. Our solution was to adapt existing data-processing routines from proteomics workflows for use with HDX data and peptic peptide analysis. A novel processing method was developed that decreased processing time by several orders of magnitude, reducing what originally took months to hours and allowing complete data processing in approximately the same time that it takes to acquire the data. Based on suggestions from one of

"Initially, the ability to efficiently process and analyze this data and to visualize the results in a meaningful and timely fashion was missing. It became a critical element of the development process."

Excitement on the Smallest Scale

Microrheology is in its early stages, but it has the potential to expand the range and enhance the application of rheological characterization. Here's how.

By Steve Carrington, Product Marketing Manager – Rheology, Malvern Instruments, Malvern, Worcestershire, UK.

Those of us who treasure rheology recognise that it offers a unique perspective on material properties at all levels – from an academic understanding of how underlying molecular dynamics and microstructure drive complex bulk material deformation and flow properties, to more applied measurements that can optimise product performance or troubleshoot processing problems. Rheological characterization supports the formulation of highly sophisticated personal care products, for example, and foods that deliver market-leading consumer appeal at competitive cost.

Conventional rotational rheometers are highly sophisticated instruments that enable changes, sometimes over decades, to the critical control parameters that determine viscoelastic properties, such as applied stress, strain and frequency. However, mechanical rheometry has fundamental limits, primarily arising from the effects of mechanical inertia, which prevent complete characterization across all complex fluid types.

Imagine that you could apply rheometry on a micro-scale and remove the inertia limitations, enabling you to perform high frequency measurements that capture short timescale dynamics of low viscosity formulations. Imagine a drive force of such low applied stress and sensitivity that the onset of molecular aggregation or denaturation processes can be followed, within the linear regime of the most highly strain-sensitive systems. Imagine the ability to probe different material length scales, from bulk properties down to mapping localized spatial dynamics at a microstructural level. Then consider the sample volume for this thought-experiment – of course, that's on a micro(litre)-scale too – a real positive for high value, scarce materials such as biotherapeutic proteins or novel engineered polymers. Make this thought-experiment real, and you have microrheology.

The term microrheology describes a range of techniques that extract the rheological properties of soft materials by measuring and analyzing the motion of colloidal tracer particles dispersed in the sample. Passive microrheology exploits thermal diffusion of the tracers, whereas active microrheology drives probe particles using laser or magnetic tweezers. My focus is on passive dynamic light scattering (DLS) microrheology, where the average motion of an ensemble of tracer particles undergoing Brownian motion is tracked by light scattering. The way in which a particle diffuses is intimately linked to the rheology of the dispersion medium. For Newtonian fluids, the relationship between viscosity, particle size and particle diffusion is given by Stokes-Einstein, but for viscoelastic fluids it is far more complex. It has been the extension of the Generalized Stokes-Einstein Relation (GSER) to Non-Newtonian fluids, and in particular the

our collaborating customers – the biotechnology company BiogenIdec – we also enhanced data visualization and statistical analysis to produce a package for the comparability studies that are an essential part of the biopharmaceutical development process.

These instances of customer insight were dependent on the customers actually working with prototype instruments. If we had waited for feedback until after commercialization, the system would have been clearly deficient, critical aspects would have caused field failures, and customer frustration from slow data processing would have seriously compromised our success. Instead, the early test site program provided valuable feedback that greatly enhanced the system, which we successfully introduced in 2011.

Needless to say, the mutually beneficial interaction between instrument developers and customers is at the heart of everything that Waters is doing.

"Currently, microrheology sits firmly in the research laboratory, and while the promise of the technique is palpable, there are potential pitfalls for the unwary."

linking of particle diffusion with linear viscoelastic moduli, that has formed the basis of modern microrheology (1).

Currently, microrheology sits firmly in the research laboratory, and while the promise of the technique is palpable, there are potential pitfalls for the unwary. The choice of probe particle is critical and requires careful investigation and assessment – from suitable tracer chemistry to minimize sample interactions, to tracer size and concentration to ensure robust data. Sample preparation is vital for microrheology, and elements of this can be involved and non-trivial.

The rewards resulting from this careful sample preparation, however, are substantial: a quick and easy rheological test using tiny sample volumes. An ideal screening tool in fact. Just as importantly, microrheology allows the behaviour of samples to be assessed in ways that conventional methods do not. For example, we've used DLS microrheology to look at the onset of gelation in protein solutions caused by denaturation and aggregation (2), and to extend the characterization of dilute polymer solutions into the high frequency regime.

While I find microrheology an absorbing, and potentially valuable proposition, the technique is still in embryonic form and it is collaborations across the scientific community, including those working at the forefront of commercial product development, that will provide a thorough assessment of its value. Consider this an invite to get involved!

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The S-Word and US Research

What short- and long-term impact will sequestration have on the US scientific community? And is there a silver lining in the opportunity to re-assess research priorities?

By Bill Kelly, President and Co-Founder of BioInformatics, LLC, Arlington, VA, USA.

Sequestration, the automatic, across-the-board spending cuts to reduce the US federal budget by $85 billion, has gone into effect. At the time of writing, the FY 2013 budget-setting process is in flux, with a bitter negotiation process getting under way. As if the issue wasn't complicated enough, both the House and Senate are required to pass a budget resolution for FY 2014 by April 15.

Amidst this turmoil, scientists who rely on federal support for their research are, understandably, concerned. To give an example of the impact, the National Institutes of Health (NIH) budget will be cut by $1.6 billion over the next seven months and NIH has already notified contractors and grant recipients that their funds may be affected. The National Science Foundation (NSF) and other agencies supporting research will face budget reductions of comparable magnitude.

A few days before the sequestration took effect, my company, BioInformatics LLC, a market research and advisory firm in the life science industry, surveyed more than 300 US academic, government and pharmaceutical scientists to gauge the impact of the planned cutbacks in federal funding for research. Our goal was to collect information for companies that manufacture the "tools of science" about how the budget cutbacks would affect the sales of instruments and consumables sold to US research labs.

The survey indicated that lab suppliers can expect lower sales as a near-term reaction to the budget sequestration – especially from the government and academic sectors. However, only one-quarter of academic and government life scientists said that they will consider deferring or postponing capital equipment and/or instrument purchases this year. This is obviously good news for commercial suppliers. But the survey also revealed scientists' deep concern over the longer-term implications of the sequester.

"Sequestration 'makes about as much sense as performing brain surgery with a sledge hammer'"

The survey data suggest that most researchers are committed to keeping current research programs moving forward with funds already in hand, while reducing long-term spending through hiring freezes, lab closures, project cancellations and reductions in new labs. The cost reductions that survey respondents are most likely to take are precisely those that will have a long-lasting effect on the life science market. Scientific research isn't something that can be turned on and off – it is a long-term investment. Budget uncertainty begins to erode the supporting infrastructure, drives researchers out of the field and threatens the US leadership position in the biosciences.

Scientific societies, advocacy groups and scientists themselves have been extremely vocal in denouncing the sequestration and equally adamant that funding should not only be restored but increased. Many also agree that the heavy handed nature of the cuts hurts valuable research as hard as programs perhaps less worthy and deserving of reductions or elimination. One survey respondent commented that sequestration "makes about as much sense as performing brain surgery with a sledge hammer".

Sitting under this dark cloud, is there something to feel positive about? Well, Congress and the Administration will soon begin their debate over the FY 2014 budget and with this comes the hope of a more rational mechanism for attaining deficit reduction while preserving investments in critical research.

As US public sector budgets shrink, the scientific community must articulate its priorities and more effectively communicate the value of the research that supports these priorities. Scientists should not be expected to justify their research in terms of the societal and economic benefits they will deliver in return for public support, but it is not unreasonable to suggest that they play a role in developing methods to assess and report the impact of their research.

Lastly, many respondents to the survey were critical of the current system by which NIH grants are awarded, particularly in that it tends to fund those who are already funded and creates a generational divide between scientific "haves" and "have nots". The financial pressure of the sequestration and future spending reductions could provide the impetus for a system that rewards innovation and "out of the box" thinking and trains the next generation of scientists.

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GOOD NEWS

Our revolutionary Dried Blood Spot (DBS) autosampler is almost ready! The Flow-Through Desorption (FTD™) technology inside enables automation of the entire workflow for DBS analysis by LC-MS/MS systems without any manual intervention. Come and get an exclusive preview at our booth at ASMS (#147), HPLC (#A13), AACC (#4557) or scan the QR-code for more information.

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Spot On

Dried Blood Spot (DBS) sampling is being adopted in drug development, paediatric healthcare, and, more recently, patient compliance. We describe our positive experience with the platform, as well as the many advantages – and ongoing concerns – of this simple, stable, and versatile technology.

By Sangeeta Tanna and Graham Lawson

Collecting drops of blood from the heels of newborn babies and depositing them on specially prepared Guthrie cards, where they dry, is an established form of sampling. Analysis of the DBS extract has been used for decades as a method to screen newborns for the occurrence of specified diseases (see "Dried Blood Spot 101" on page 24).

Early DBS screening tests only needed to demonstrate the presence or absence of certain traits and were consequently qualitative in nature. However, in the past few years, improvements in the sensitivity of analytical instruments, especially mass spectrometers, have resulted in the drive to exploit the potential to quantify components extracted from dried blood spots, which in turn has unearthed significant challenges (see "Confronting Concerns" on page 26).

High-Resolution Inspiration

Our own early research on DBS methods for the analysis of captopril (a heart failure treatment drug for neonates) was certainly not trouble free. Initially, our work seemed to disprove everything we had read about drug stability in DBS samples. We were unable to detect captopril – even in the calibration samples! We then discovered that captopril rapidly reacted to form a dimer, probably in the extraction solution, and we were using the wrong mass range on the mass spectrometer. To prevent this reaction, we discovered how to pre-treat the sample card with a stabiliser solution to detect captopril. And though problems with our work continued, they did not relate to the DBS system per se. Our experience taught us two things: don't believe all you read in the literature and, if possible, select an analytical system that allows re-interrogation of the data at a later stage.

It was these latter thoughts that led us to investigate the potential of high mass accuracy/high resolution MS (HRMS) to match the compound specificity associated with multiple reaction monitoring (MRM) tandem mass spectrometry typically used to analyse blood spots. In MRM analysis, ions with a preselected mass that is characteristic of the analyte are fragmented in a collision cell yielding product ions with masses characteristic of the chosen drug. These ions are monitored by the second mass spectrometer. This combination of m/z values provides specificity but data collected per analysis are limited to the abundance of the preselected product ions – that is to say, the initially chosen target drug. In HRMS analysis, mass measurements are determined accurately to 1ppm of the chosen mass. It is this m/z accuracy that provides the specificity of the technique, providing you are looking for small molecules (<400 relative molecular mass). Importantly,

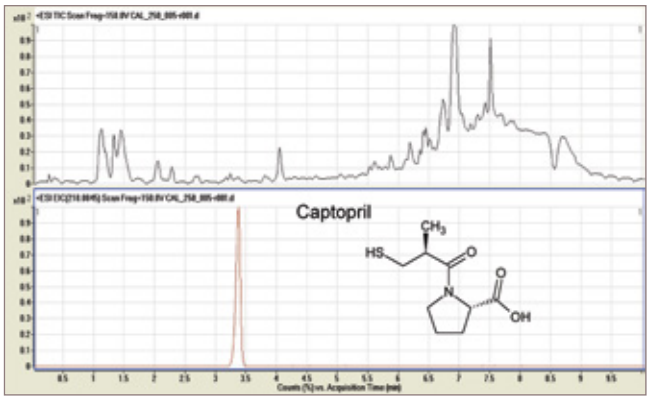


Fig 1: A comparison of the total ion trace (top) with the data specific for captopril (bottom trace) from a DBS extract.

the data collected in each HRMS analysis include the total ionisation from the sample (see Figure 1, top trace), which can subsequently be "mined" for the set mass (see Figure 1, bottom trace). The full data are retained and can be re-interrogated for other information at a later date. We coupled an Agilent 1290 LC to an Agilent G6530 A Accurate Mass qToF mass spectrometer, which produces searchable data. Not only did the qToF provide improved specificity and detection capability but it also provided the opportunity to monitor all ions in a single run. This data can be reprocessed subsequently, in the light of new ideas, without the need to repeat the experiment, which is a huge advantage in the research field.

The captopril project demonstrated the ability to quantify levels of the therapeutic drug in samples from neonatal patient's blood (1). This was possibly the first reported quantification of the drug in neonatal blood samples and the potential for this approach to improve patient care in the UK was reported to the National Institute for Health Research.

Cardiovascular non-compliance

As our captopril project was finishing towards the end of 2010, the 'polypill' or 'superpill' for the treatment of cardiovascular (CV) disease in adults was making the headlines. The older one of us started to pay more serious attention.

CV disease is the biggest single killer in the UK and there is evidence that around 60% of patients on CV prescriptions are taking their medication incorrectly (2), which sparked a thought: "Could this be the golden opportunity to see if DBS sampling is able to assess compliance to prescription and more importantly to see if patients can produce usable DBS samples at home?" Such a test appealed greatly to clinicians who said it would help them make more informed decisions and thereby improve CV therapy. To succeed, the process had to be able to detect the drug

Dried Blood Spot 101

Introduced by Robert Guthrie in the 1960s, dried blood spot (DBS) sampling involves taking small drops of blood from either a finger prick (or heel prick in neonates) and depositing them on specially manufactured absorbent card where they are allowed to dry. Once dry, DBS cards can be readily transported by post for analysis since the components of the blood remain unchanged for several days, even at room temperature. For analysis, a portion of the blood spot is removed from the card and placed in a solvent to extract the analyte(s) of interest. Therein lies the elegance and ease of the DBS sampling system: no specialist collection, no liquid blood, and no refrigerants. Guthrie card samples have seen widespread and routine use for neonatal screening of metabolic disorders, such as phenylketonuria, sickle cell disorders, and HIV infection.

To be a truly useful sampling system, DBS must be used in conjunction with analytical techniques capable of detecting the low levels of analytes present in just a few micrograms of dried blood. Indeed, the combination of DBS with state-of-the-art instrumentation such as liquid chromatography–tandem mass spectrometry (LC-MS/MS) has led to widespread use of the technique in clinical and drug discovery applications. Whilst this combination has become synonymous with DBS bio analysis, there are many other analytical techniques that can be used to obtain valuable information from a DBS sample, as shown in Table 1.

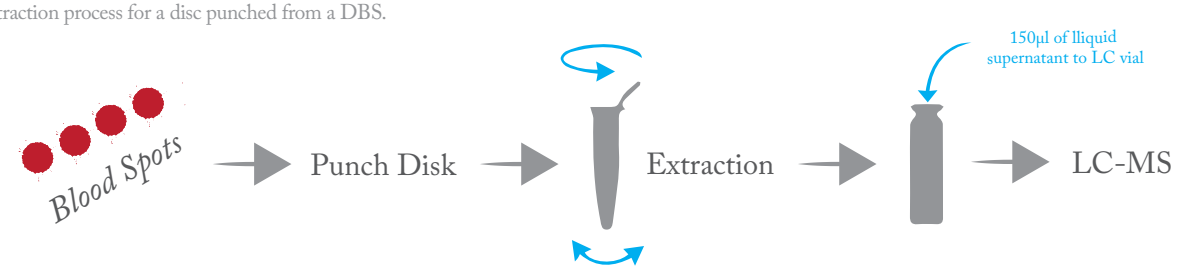
Certainly along with improvements to analytical sensitivity comes the drive to fully quantify components extracted from dried blood spots. Under these circumstances the extraction process assumes a major importance. Punching a fixed diameter disc from the spot provides a level of quantification but at a cost – the time and effort needed to deal with the punched disc (see Figure 2) and the possible loss in sensitivity as a result of not using the whole sample. Automation of the punching process and disc handling can help but the process is still complex. An alternative is to simply extract a fixed volume of the spot by passing a solvent through the spot whilst it is still on the card, which can be achieved by clamping two tubes opposite each other, on either side of the spot, and passing a fixed volume of solvent through that part of the spot. Provided there is no significant loss of solvent into the card, automatic extraction/quantification becomes possible. The advent of sophisticated instrumentation capable of quantifying the levels of analytes in DBS samples has led to the recent surge in interest in this sample collection technique.

Table 1: Selected examples of different assays used with DBS samples.

Assay Method	*Application*
Immunoassay	NBS
GC-MS	NBS
GC-MS/MS	TDM
LC-UV	NBS/TDM
LC-MS	NBS/TDM/DMPK
LC-MS/MS	NBS/TDM/DMPK
ICP-MS	Elemental analysis
PCR-DNA	NBS/Forensic

Notes: NBS =newborn screening, TDM = therapeutic drug monitoring and DMPK = drug metabolism/pharmacokinetics

Fig 2: A simplified schematic of the manual extraction process for a disc punched from a DBS.



or its metabolites in the blood sample at any time up to 24 hours after taking the dose. Quantification was not essential as the detection of the target species in the blood confirms the drug was taken. However, in principal, quantitative determinations and knowledge of the absorption, distribution, metabolism and elimination properties of the drug would allow an approximate time when the medicine was taken to be determined. The project divided neatly into two phases. The initial phase was the proof of principal where suitable detection routines for the top three CV drugs prescribed in the UK were developed and DBS samples collected under controlled conditions. A method for the determination of Bisoprolol, Ramipril and Simvastastin in DBS samples was developed and validated. Extracts from the blood spots were analysed using an LC-qToF mass spectrometer operating in HRMS mode. This analytical method successfully identified adherence to these drugs amongst a group of control volunteers (see Figure 3). Within this group no false positives from other CV drugs were detected. Currently, in phase two, volunteers are asked to take their own DBS samples and present them for analysis. Initial results for CV drug determinations confirmed the original concept and we are now setting up a multi-volunteer trial to confirm the ability to identify patients who are not taking the prescribed medication. Some initial results have indicated a greater level of adherence than expected but also suggested possible medication errors.

The change in research direction from paediatrics to adults proved to be inspired; volunteers with CV problems were readily available from staff within our own institution and news of this work soon reached the local radio station. Following a live radio interview during which the presenter offered to provide samples, we had volunteers chasing us to know when they could help. Our research also struck a cord internationally. The British Council in China sponsored trips to Chongqing and Wuxi to allow us to showcase our research. During a recent return visit to our laboratory the leader of a delegation from Chongqing requested that his own levels of CV drugs be tested while he waited! The trips to China gave us two other incredible experiences. Imagine being on an exhibition stand on a crowded Saturday, explaining where you work and what you do to visitors. Now shift this experience to central China to one of the largest cities in the world and imagine the opportunity – or perhaps challenge – at the 10th China Chongqing Hi-Tech Fair. It is amazing how much interest we encountered from a wide range of age groups and backgrounds. The second event was an invitation from the Nanjing 'Cafe Scientifique' to present our research to the public. This took place in a huge area in a major retail mall and was open to the general public. The audience and the responses were incredible despite the inevitable delays for translation.

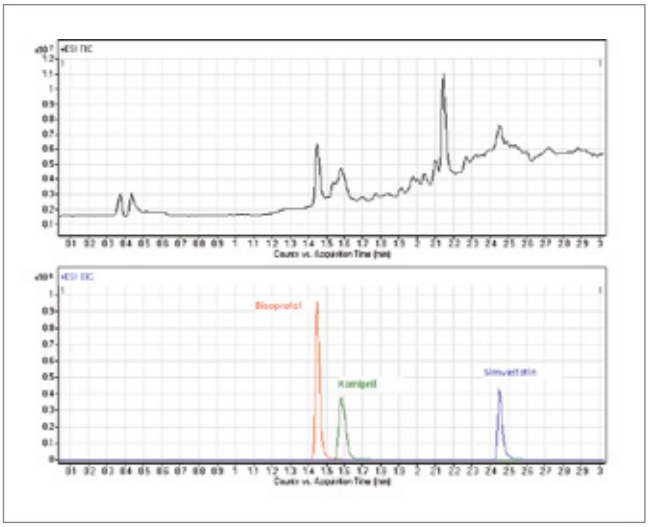


Fig 3: A comparison of the total ion trace (top) with the data specific for bisoprolol, ramipril and simvastatin (bottom trace) from a DBS extract.

Table 2: Opportunities for Dried Blood Spot Analysis.

Application	*Benefit*	*Challenges*
Newborn screening	More inherited diseases identified	Medical care not available for some
Child medication	Better use of medicines in patient care	Lack of suitable resources in some healthcare systems
Therapeutic drug monitoring	Personalized medicine	Ethical constraints
Environmental monitoring	Better control of exposure	Getting samples before and after exposure
Forensic analysis	Lifestyle indicator	Lack of reference samples/materials

As shown in Table 2, there are many applications of the research pioneered at DMU. The use of the LC-qToF MS identifies the presence of some 634 compounds in the extract from a single blood spot including caffeine, the residues of medication and potentially illegal drugs (see Figure 4), which indicates that this approach could be be used as a lifestyle indicator. Blood left at the scene of a crime could be collected using this methodology and although it is not yet known if blood can be as unique as a fingerprint, it is another direction currently under exploration in our research.

Principal Drivers for DBS Research

- **Regulatory drive and industry acceptance for better paediatric medication.** It is now recognised that regarding a child as a scaled down adult to calculate a medicine dose may not be appropriate, leading to possible treatment failure or overdose. Evidence-based studies on the safety and efficacy of medicines in children are therefore required. It is unethical to take a 5-10ml blood sample from a baby with a circulating blood volume of only 250ml, i.e. the volume of a mug of coffee. Therefore, DBS sampling is currently the only way forward.
- **Ease of sample collection.** In the healthcare sector the sample can be collected via a simple finger or heel prick. Specialists are not required and there is minimum patient discomfort.
- **Reduction in the number of animals used in drug discovery experiments by the pharmaceutical industry.** The dramatic reduction in the volume of blood needed for an individual measurement has meant that more samples can be taken from a single rodent. Furthermore, better data quality results from the reduced sample variability.
- **Reduction in sample transport costs.** Current medical trials are multi-centre and multi-ethnic, which implies samples being transported around the world. The ability to maintain sample integrity as a dried spot on a card at room temperature has major cost benefits.
- **DBS samples pose less of a biohazard risk to handlers than liquid blood samples.**
- **DBS is suitable for both preclinical (animal) and clinical (human) samples.**
- **Benefit of experience with Guthrie cards.** Health service providers and regulatory authorities are already familiar with a similar successful methodology in newborn screening and the expansion to other applications should be less problematic.
- **Potential for automation.** Healthcare service providers are always looking for ways to reduce costs; a patient sample on a fixed shape card provides the optimum sample for automation.

Confronting Concerns

With many potential benefits, it is easy to understand the drive to develop systems and methods acceptable to the regulatory authorities but several difficulties have arisen.

Haematocrit

The major assumption in the quantification of data from a dried blood spot is that the blood is uniformly absorbed throughout the body of the spot on the paper. Furthermore, it had been assumed that all blood samples had the same absorption characteristics for cards of the same material. If this were true, then the removal of a fixed diameter disk from a blood spot would remove a fixed volume of blood to enable drug levels to be reproducibly quantified. Unfortunately, the size of the spot produced is controlled by the haematocrit level (or erythrocyte volume fraction) in the blood, which can vary quite considerably – especially for newborns. As the haematocrit level increases, the same volume of blood takes up less space, meaning that the disk sample contains more blood and a subsequent bias in the level of drug detected.

There are several possible solutions:

- Measure the initial volume of the blood sample before it is placed on the card and then analyse the entire spot in the laboratory. This does, however, negate one of the basic attributes of the sampling system – the ease of sample collection.
- Match the haematocrit levels in the calibration samples to patient samples. Clearly for routine processing of many patient samples this approach would be far from ideal.
- Develop sampling matrices that mitigate or eliminate the haematocrit effect.

Work is underway in several groups within Europe and the USA to address the hematocrit problem, with several card vendors claiming victory – not everyone is convinced.

Internal Standards

It is good analytical practice, where a multistage process is used, to add a known amount of an internal standard (IS) to the original sample to indicate any quantitative changes resulting from subsequent

analysis. This fact raises two important questions for users of DBS analysis: At what point should the internal standard (IS) be introduced into the sample? And what exactly should be used as the IS? The IS signal, resulting from the known level added, acts as a reference for the other species monitored. Therefore, the IS should ideally be added to the blood spot sample before it dries on the paper, which is not a viable option; several alternatives have been considered. The most common option is to add the IS to the extraction solvent used to remove the analyte from the dried blood. In this case, the IS is not incorporated into the blood during the drying process and therefore the extraction conditions are not duplicated. Pre-treating the card with the IS prior to sample collection or spraying the card with the IS solution after the blood spot has dried are other alternatives. The pre-treated card appears to offer the closest to ideal as the blood absorbs some of the IS before clotting is complete, which should lead to more characteristic extraction conditions.

Ideally the IS should be as similar to the analyte as possible and the use of stable isotopic variants has become common. This approach ensures that the separation processes are identical, even up to the point where both analyte and IS enter the MS ion source at the same time. It is here where problems may occur. Excess levels of the IS may affect the ionisation process in the electrospray source to give a reduced signal for the analyte compared with other species that may be recorded in the same run. To reduce this impact, either a low level of the isotopically modified IS can be used or a similar compound with a different elution time can be chosen as the IS, but there is still much discussion and debate concerning the best way forward.

For the immediate future, the above challenges are such that regulatory authorities are still demanding data from whole blood or plasma studies to validate DBS results. And certainly, until regulatory acceptance endorses the validity of this form of sampling, there will be continued reluctance from some bioanalytical scientists to accept it as a suitable method for quantitative determinations.

Fig 4: The computer processed analysis indicating the presence of at least 634 individual compounds present in the DBS extract.

The determination of CV drugs is an example of the much wider application of DBS sampling. The methodology for monitoring various small molecule therapeutic drugs has now been expanded to include proteins and peptides, which may be part of a move towards personalized medicine – a topic that presents itself more and more frequently.

We have been fortunate that our DBS research, focusing on the 'appliance of science' to real healthcare applications, has earned us several accolades, including the Royal Society of Chemistry Analytical Methods Prize in 2010, but perhaps the full potential of DBS samples has still yet to be realised. Unused spots from newborn screening could represent a valuable resource, if they could be ethically released – an area of great contention in some parts of the world. As DBS sampling and analysis gains wider popularity, recognition and acceptance, investigation of the platform for other biological fluids e.g. tears, saliva and cerebral spinal fluid (CSF) could open up whole new areas of research.

Sangeeta Tanna is a pharmaceutical bioanalyst and Graham Lawson is an instrumental forensic analyst, both in the School of Pharmacy at De Montfort University, Leicester, UK.

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The Tools of Proteomics

The proteome, which is the set of proteins expressed by a genome, cell, tissue or organism at a given time, presents immense analytical challenges – but offers substantial rewards. Here, current approaches to proteomics, their strengths and their shortcomings, are explored.

By Amanda B. Hummon and Norman J. Dovichi

Just as proteins are the third component in the flow of genetic information after DNA and RNA, so proteomics represents the third challenge temporally in the comprehensive analysis of living systems, after genomics and transcriptomics. It is also the most complex of these challenges. Proteins are much more diverse and difficult to quantitate than nucleic acids, and unlike DNA, their expression varies in both time and space.

Knowledge of protein sequence and abundance has many important roles in biotechnology and medicine. One major application is biomarker discovery, for which the availability of patient samples is an essential resource. The hypothesis behind biomarker studies is that a disease, such as cancer, leaves tell-tale markers in serum that can be used for diagnosis and prognosis. Perhaps the best-known marker, prostate specific antigen (PSA), illustrates the complexity of the field. PSA was developed as a prognostic indicator for patients after treatment for prostate cancer. Its subsequent widespread use as a screen for the general population was not adequately validated and is now widely discouraged.

Proteomics is also used to identify cellular networks that are deregulated in disease. Such studies compare primary tissue from diseased and healthy individuals, and the goal is to identify potential therapeutic targets.

Another application is the quality control of recombinant therapeutics. These protein-based drugs are produced in cell culture. The host cell, which is often the Chinese hamster ovary (CHO) cell line, is engineered to produce the desired therapeutic. The cells or cell culture is then harvested, lysed, and taken through a set of affinity purification steps. However, residual proteins from the host cell line remain, albeit at trace levels. The host cell proteins represent potential allergens, and it is vital to both identify the contaminants and determine their abundance.

One class of recombinant therapeutics is antibodies, which are generated from polyclonal antibodies against the target molecule. Antibodies represent the exception to the rule that genomic sequence informs protein sequence within an

organism. The complex immunological system shuffles the sequence for antibodies, so that each immunological cell contains a unique genetic sequence for the antibodies that it produces. As a result, it is necessary to perform de novo protein sequence determination for target antibodies.

Finally, post-translational modifications decorate proteins with a wide range of functional groups. These functional groups play a diverse role in modulating a protein's function. Phosphorylation is the most commonly studied post-translational modification because of the availability of robust tools, the well-understood role that phosphorylation plays in modulating protein function, and the simple nature of the modification. It is not uncommon to see studies where the phosphorylation status of a large fraction of the proteome is studied. In contrast, another major post-translational modification, namely glycosylation, has received much less attention. This lack of attention does not reflect a lack of importance, but rather the complexity of the modification and the primitive status of analytical tools.

"The vast majority of proteomics is based on the bottom-up protocol, where the complex mixture of proteins from a cell, tissue, or organism is enzymatically digested into a very large mixture of peptides."

Historical Perspective

Although it is considered to be the younger sibling of genomics, protein sequence analysis is in fact older than DNA sequencing. It originates in the late 1940s/early 1950s with Sanger's sequence determination of insulin and Edman's development of the isothiocyanate degradation reaction. Those technologies are labor intensive, slow, and require large amounts of starting material. It was quite rare to determine the entire sequence of a protein by use of Edman's chemistry. Instead, that primitive technology was used to generate the sequence of a small peptide created from the protein, perhaps consisting of a dozen or so amino acids. The genetic code was then used to create probes for the corresponding gene. Libraries created from fragments of the genome would be screened for the target gene, which would then be sequenced.

As we described in the inaugural issue of The Analytical Scientist, genomics underwent explosive growth and maturation over the past twenty years. One legacy of this is the complete genome sequence of a very large number of organisms. That genetic information, in turn, has been used to populate databases with expected protein sequences for those organisms. It is no longer necessary to synthesize oligonucleotide probes corresponding to the sequence of a peptide and then screen a library of genetic fragments to identify the gene of interest. Instead, the peptide sequence is screened in silico against the genetic sequence. The availability of complete genomes has been an extraordinarily valuable resource in proteomic studies.

To maximize the potential of connecting genetic and protein sequences, efficient methods for generating sequences from short peptides was needed and in the 1970s mass spectrometry was identified as the ideal tool. The main challenge was to get large, rather non-volatile proteins or peptides into the gas phase for ionization and analysis. A number of approaches were investigated, including ultrasonic nebulization and secondary ionization mass spectrometry. These were superseded by the development of electrospray ionization by Fenn and MALDI (Matrix-assisted laser desorption/ionization) by Tanaka and Hillenkamp in 1988. Together, these approaches opened up the use of mass spectrometry for high-throughput proteomic studies.

Three additional advances were required for widespread proteomic studies. First, as we noted in our article in the January issue of The Analytical Scientist, genomic sequence databases approached reasonable size in the 1990s. Since then, whole-genome sequencing studies for many organisms have populated databases with the entire cast of protein sequences.

Second, beginning with the pioneering effort of Yates and colleagues around the year 2000, the development of efficient database search engines automated the identification of those protein sequences. At this point, we knew what we were looking for.

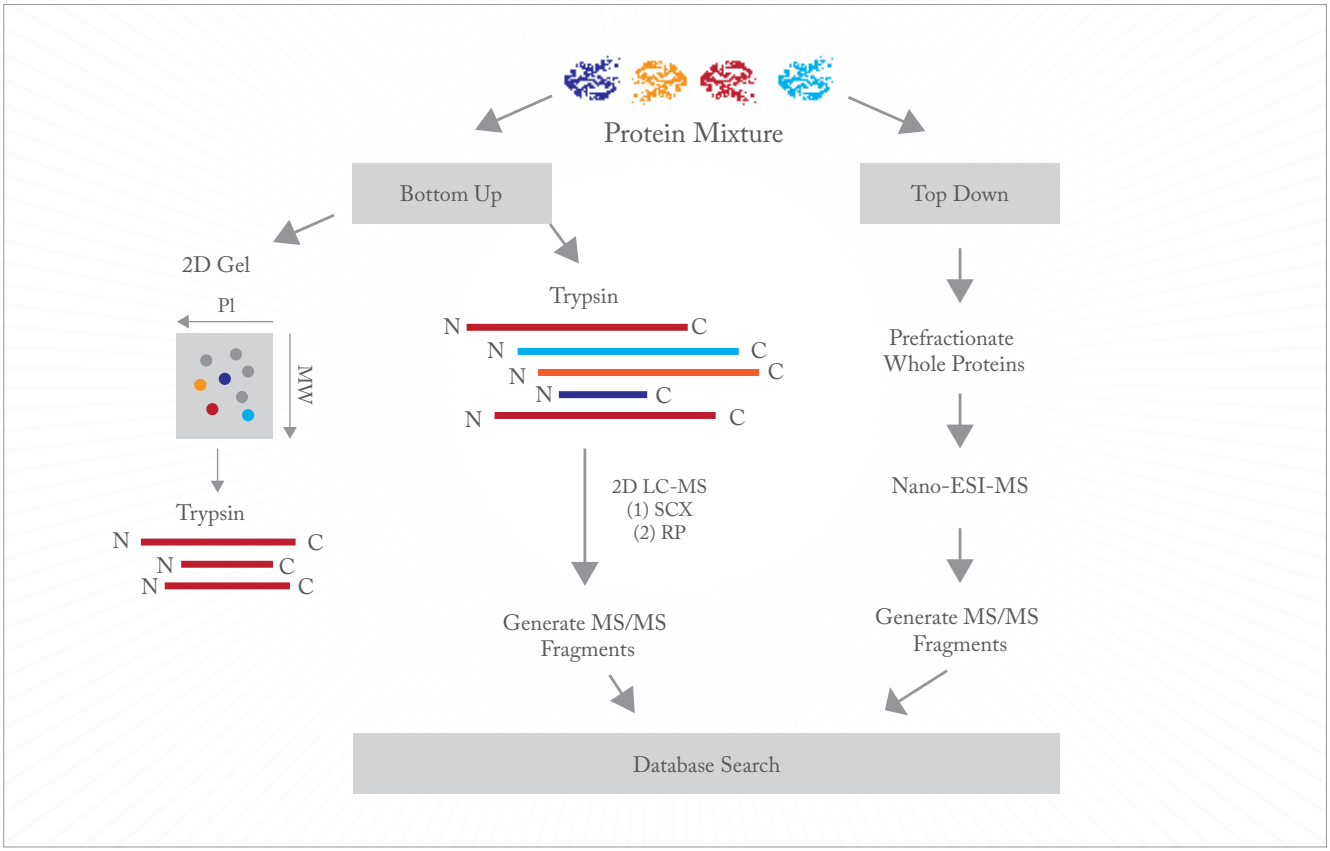

Fig 1: Schematic diagram of proteomics

And third, beginning with the iTRAQ (isobaric tags for relative and absolute quantitation) chemistry of Aebersold and colleagues in 2000, a suite of reagents and tools was developed to undertake quantitative proteomics, enabling us to understand changes in protein abundance that accompany disease and development.

Proteomic studies are commonly divided into top-down and bottom-up (see Figure 1). Top-down proteomics ionizes and introduces intact proteins into a high-resolution mass spectrometer. This approach has the advantage of capturing all posttranslational modifications within the protein but suffers from the disadvantage of needing to ionize, fragment, and interpret the resulting data for a very large molecule.

The vast majority of proteomics is based on the bottom-up protocol, where the complex mixture of proteins from a cell, tissue, or organism is enzymatically digested into a very large mixture of peptides. Trypsin is the proteolytic enzyme of choice. This enzyme is available at high purity and is relatively inexpensive. It cuts proteins at lysine and arginine residues. Since these amino acids are relatively common, each protein is cut into many peptides, which are typically five to 30 residues in length. While this mass range suffers from significant interference from the matrix used in MALDI, it is very well suited to analysis by electrospray ionization, which is most commonly used. Determination of most of the peptide sequence, along with knowledge of the parent ion's mass, narrows identification to a small number of possibilities in database searches.

Early proteomic studies were focused on identifying proteins within a given sample, essentially providing a parts-

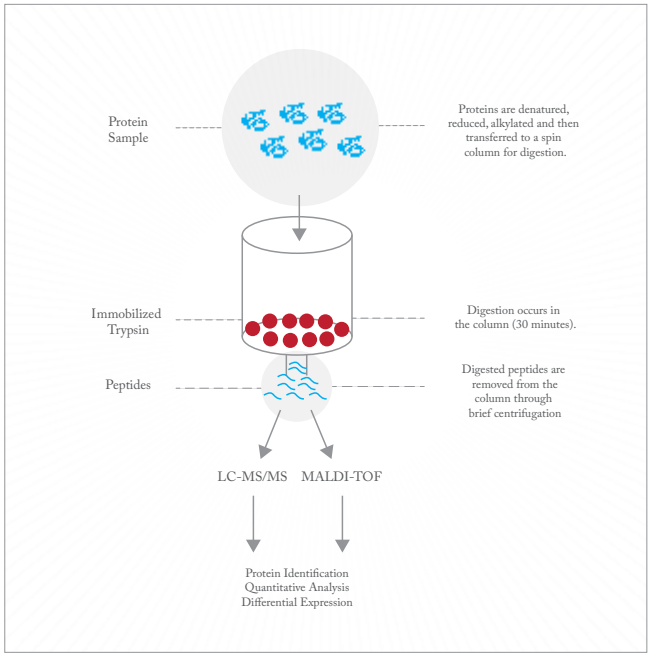

Fig 2: Immobilized trypsin procedure. Proteins are denatured, reduced and alkylated as appropriate before digestion. The protein digestion solution, containing the protein to be digested, is prepared and added to the immobilized trypsin resin. The protein digestion solution is incubated with the resin for 30 minutes. Digested peptides are removed by centrifugation and then prepared for downstream analysis.

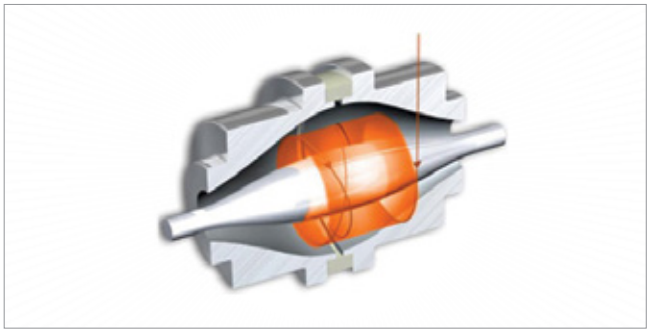

Fig 3: The Orbitrap consists of an outer, barrel-like, electrode and a coaxial inner, spindle-like, electrode that form an electrostatic field with quadro-logarithmic potential distribution. The image current from dynamically trapped ions is detected, digitized and converted using the Fourier transform into frequency and then mass spectra. To see a video of an Orbitrap in action, go to: planetorbitrap.com. Image courtesy of thermoscientific.com.

list of the proteins present. A modicum of quantitative information can be gleaned from such a parts list, most commonly by simply counting the number of occurrences of peptides from a target protein. More recently, differential isotopic labelling has been used to determine changes in protein abundance between two or more samples. For example, protein abundances from normal and diseased tissues can be compared to identify enzymatic and signalling pathways associated with the disease.

Modern bottom-up proteomic studies consist of four stages. First, the protein samples are digested and isotopically labelled. Second, the resulting complex peptide mixture is subjected to one or more rounds of separation. Third, the sample is ionized and analysed by the mass spectrometer. Fourth, the resulting data is analysed and interpreted. Below, we consider recent advances in each of these four areas.

Sample preparation

Trypsin digestion is performed for anything between 30 minutes and 24 hours. It's a trade-off: digestion periods at the shorter end reduce the analysis time and minimize auto-digestion, but at the expense of incomplete digestion, which results in missed cleavages, while longer digestion time leads to more complete digestion but at the expense of more auto-digestion and longer overall analysis time. Trypsin may be immobilized on a solid support, which dramatically increases the concentration of enzyme used in the reaction while minimizing auto-digestion of the enzyme (see Figure 2). Immobilized trypsin provides roughly an order of magnitude improvement in sample throughput, which is of value in, for example, control of recombinant therapeutic protein production. Speeding digestion is particularly important in the bio-pharmaceutical industry, where rapid proteomic analysis is vital for quality control of therapeutic proteins produced in cell cultures. The goal is to provide information rapidly enough for it to be used for process control.

Separations

The mixture of peptides present in the tryptic digest of a complex proteome is far too complex for direct analysis by mass spectrometry. Instead, the mixture must be separated to simplify the sample sprayed into the mass spectrometer. In the overwhelming majority of cases, this separation

is achieved by gradient-elution reversed-phase liquid chromatography. This separation mode employs solvent systems that are compatible with electrospray ionization, and that provide outstanding separation resolution.

The resolution of chromatographic separations improves dramatically as the size of the stationary phase particles decreases. However, the pressure required for the separation also increases as particle size decreases. The development of technology for routine ultrahigh pressure liquid chromatography has resulted in remarkable improvements in the separation of proteolytic peptides for mass spectrometric analysis.

Virtually all automated proteomic analyses employ liquid chromatography to separate peptides before analysis. However, there are hints that capillary electrophoresis may prove of value in proteomic analysis. We have developed a novel electrospray interface, which uses extensive sample pre-fractionation in combination with rapid capillary electrophoretic separations; combined with high-speed and high-resolution mass spectrometers, this provides an intriguing alternative. In the largest published comparison of capillary electrophoresis with UPLC separation, the two techniques achieved an essentially identical number of protein identifications in an identical analysis time. In this analysis, which was the study of a bacterial secretome, the two techniques had roughly fifty percent overlap in the proteins that they identified, suggesting that they probe complementary portions of the proteome. As an aside, this study also demonstrates that no single technique is capable of resolving all components within a complex proteome, and that many situations will benefit from combination of two or more techniques.

"The development of technology for routine ultrahigh pressure liquid chromatography has resulted in remarkable improvements in the separation of proteolytic peptides for mass spectrometric analysis."

Mass analyzers

The development of high-speed and high-resolution mass analyzers has revolutionized proteomic research. One class of instruments, the Orbitrap, is quite striking in its performance (Figure 3). These instruments routinely achieve 100,000 mass resolution (at m/z = 400), produce tandem spectra at 10 Hz, and achieve low attomole detection limits for parent ions.

This area of research is very competitive, and new generations of mass spectrometer appear every three to five years. We will undoubtedly see continued advances in mass analysis for many decades.

Quantitative proteomics

Early proteomic studies delved deep into the proteome, with the goal of detecting a large fraction of the proteome predicted from the translation of open reading frames in the genome. However, such parts lists are of limited value, since they do not directly provide information on the change in abundance of proteins between samples, such as those from healthy and diseased tissue.

A number of strategies have been developed for quantitative proteomics, with many more under development. Most methods employ isotopic labelling of primary amines found on lysine residues or the N-terminus of peptides, or sulphurs on reduced cysteine residues, using specific reagents. Two or more samples are treated with different isotopic labels, the samples are then pooled and analyzed using conventional chromatographic and mass spectrometric techniques. Software is used to identify the isotopic signature from the mass spectra. Reagents can be as simple as formaldehyde, which introduces an isotopic signature, to the iTRAQ chemistry that employs reagents that reveal the isotopic signature only after fragmentation during tandem mass spectrometry.

Alternatively, label-free methods can be employed to quantify protein abundance. These include spectral counting, where the number of peptides identified for a protein provides a surprisingly simple estimate for rough quantitation. Much higher precision is provided by multiple reaction monitoring (MRM), also known as selected reaction monitoring (SRM) (see Figure 4). This approach employs a triple quadrupole or

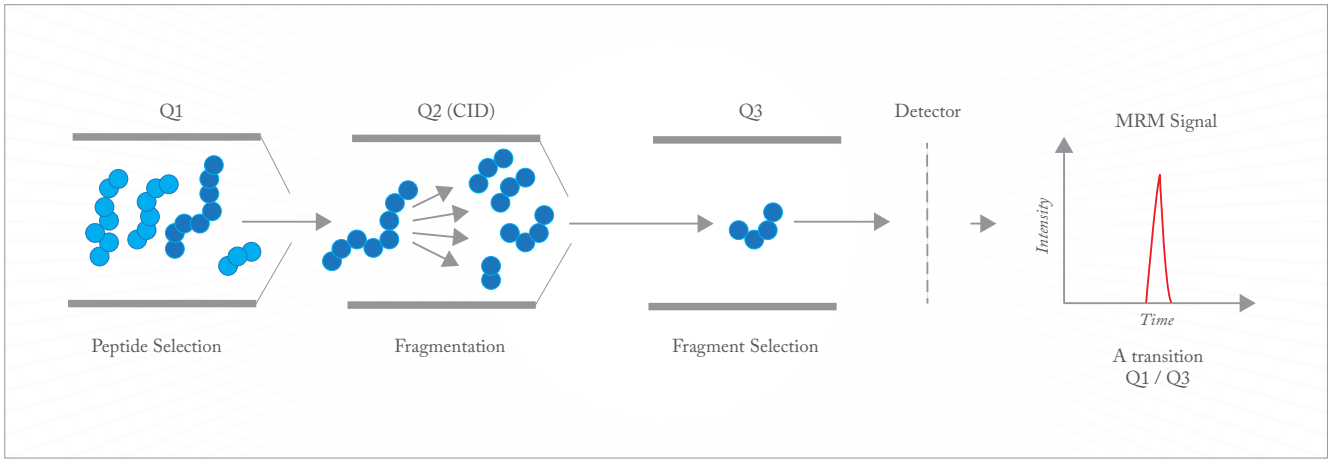

Fig 4: Multiple reaction monitoring (MRM). Based on N. R. Kitteringham et al. J. Chromatogr. B, 877, 1229-1239 (2009).

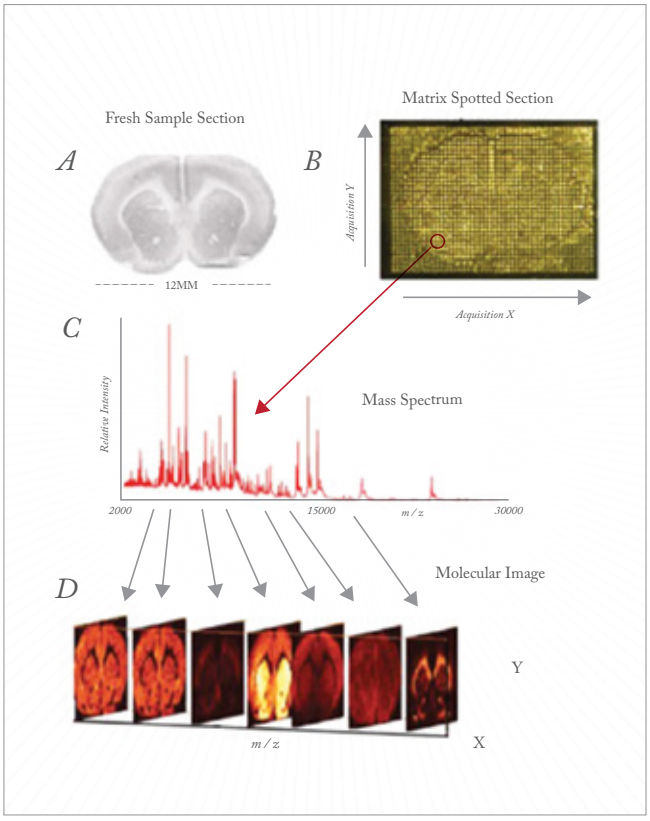

Fig 5: Imaging mass spectrometry. From Seeley & Caprioli, PNAS, 105, 18126 (2008)

a Q-trap instrument. The device is programmed to isolate a specific parent with the first quadrupole, to fragment that ion in a low-pressure cell, and then monitor the intensity of a specific fragment ion. MRM/SRM provides up to four orders of magnitude linear dynamic range and sub-attomole detection limits. It is only useful when the target peptide has been identified and when that information is available for construction of a calibration curve.

Post-translational modifications

As mentioned previously, most proteins have functional groups added after translation. These modifications play a vital role in determining the protein's function, cellular location, and lifespan. Three hundred different types of post-translational modification have been described, of which only a few have been studied in detail at the proteome level.

Phosphorylation analysis has received the most attention, in part because of the importance of phosphorylation in protein function but also because of the relative simplicity of the analytical challenge. Most phosphoproteomic analyses employ affinity reagents, such as metal ions held on a stationary phase (the technique is known as ion mediated affinity chromatography, or IMAC), to selectively enrich phosphorylated peptides from tryptic digests. The captured peptides are eluted and analyzed by liquid chromatography coupled with tandem mass spectrometry. Analysis of the fragmentation spectrum is used for high-throughput identification of phosphorylation sites.

Despite the many advances made in phosphorylation analysis, challenges remain. Highly acidic peptides also are captured by IMAC columns and, perhaps more importantly, phosphate groups can cleave or migrate during fragmentation: both of these confound the analysis.

Current techniques excel at identifying sites of phosphorylation but are less useful in ascertaining the extent of phosphorylation at a particular site. The main issue is that the phosphorylated and unphosphorylated versions of a peptide vary in their charge, and hence in their ionization efficiency. Calibration of the ions' responses is a challenge for high-throughput studies.

Glycosylation, an equally important post-translational modification, is much more challenging for current analytical tools. While glycosylation modifications can be quite simple, for example, consisting of a single sialic acid, glycans are produced in a bewildering variety of structures. Their analysis usually proceeds by enzymatic cleavage of the glycan from the peptide, followed by mass spectrometric analysis. This has two limitations: first, the relationship between the glycosylation site and the glycan's structure is lost; and second, mass spectrometry has challenges addressing isomeric structures.

"While it is now relatively straightforward to delve deeply into a proteome, doing so for quantitative analysis remains time consuming and expensive."

Conclusions

Proteomics is at a much earlier stage of development, and represents a much greater analytical challenge, than genomics. We estimate that proteomics will require two to three decades of development before quantitative analysis of whole proteomes, including post-translational modifications, becomes routine.

While it is now relatively straightforward to delve deeply into a proteome, doing so for quantitative analysis remains time consuming and expensive. The identification of post-translational modifications, and determination of the relative abundance of those modifications within a single sample, present complex challenges.

One area of promise is in situ proteomics. It is now possible to visualize the microscopic distribution of metabolites and small peptides using MALDI imaging (see Figure 5). However, performing proteomic analysis with high spatial fidelity across a tissue is a formidable task. Among the issues are the uneven deposition of MALDI matrix on the tissue and the use of a relatively large laser beam that averages the signal across several cells. Other challenges include the identification of detected ions and the modest sensitivity that the approach has.

Other factors that dog proteomics are superficially trivial, yet remain unsolved. For example, there are huge banks of archived human tissues available across a range of diseases but most of these samples are intractable for the current generation of analytical tools. Samples are generally fixed in formalin or frozen. Formalin cross-links primary amines, and these cross-links are difficult to reverse without sample loss and damage. And many of the frozen tissue banks use OCT (optimal cutting temperature) media. This contains large amounts of polyethylene glycol, which generates a complex mass spectral background signal that interferes with the analysis of tryptic digests.

Top-down proteomics – the analysis of intact proteins – simplifies a number of steps in protein analysis. However, it requires very high-resolution instruments and large-abundance protein samples. Advances in instrumentation, sample separation, and data analysis are required to make top-down proteomics a routine tool.

As we noted at the beginning of this manuscript, proteomics plays an important role in several areas of pharmaceutical research. First, differences in protein expression between healthy and diseased tissues provide targets for drug development. Second, the preparation and quality control of recombinant therapeutics requires proteomic techniques with a wide dynamic range to identify impurities in the presence of high abundance therapeutics. Finally, the characterization of protein abundance variance during development and disease progression will provide a deep understanding of basic biology in health and disease.

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Antimicrobial Abuse

In response to the over-dependence and misuse of antimicrobials in food producing animals, analytical testing and surveillance is being stepped up. Here, current best practice and likely directions for monitoring antimicrobials is described.

By Richard H. Stadler

The presence in food of antimicrobial residues at levels above legal limits is of major concern to manufacturers. It raises questions about the efficacy of controls along the food chain, introduces doubt as to the validity of the manufacturer's food safety standards, and can lead to significant economic loss in the case of product withdrawal or, even worse, recall. More importantly, it can negatively affect the health of consumers.

The sheer variety of classes and the number of antimicrobial compounds that require attention presents a tough analytical challenge. They include many substances that are regulated and others that are banned in animal farming, such as chloramphenicol, nitrofurans and nitroimidazoles. Despite the fact that antimicrobials are strictly regulated in most developed countries, incidences of residue contamination in food are not uncommon, whether in violation of maximum residue limits (MRLs), or simply "detectable" in the case of banned drugs where no limits are set and the analytical method's detection capability is decisive.

For further discussion of trends, see "Antimicrobial Trends and Risks" online: www.theanalyticalscientist.com/issues/0413/403-2

Farm and slaughterhouse screening

National control and enforcement laboratories frequently make use of inexpensive screening methods as a first check for drug residue compliance. Where indicated, these are followed up using confirmatory techniques, such as mass spectrometry (MS). Statutory screening programs require high throughput methods that are validated according to specific guidelines and predefined criteria, for example, as set out in the European Union's 2010 Community Reference Laboratories Residues Guideline on the validation of screening methods. However, despite the guideline, the EU does not harmonize screening methods and the effectiveness of monitoring depends heavily on the methods used at any particular site. Traditional qualitative/semi-quantitative screening tests based on microbial inhibition are widely used. Several non-commercial kits, such as (a) EU Four Plate Test, (b) Nouws Test, (c) Calf Antibiotic and Sulfa Test (CAST), and a number of commercial kits, including (a) Premi Test, (b) Eclipse 50, (c) Charm Kidney Inhibition Swab (KIS), (d) Delvo SP NT, have been developed – and some fully validated – for performance in a variety of matrices, such as milk, meat, organs and fluids.

These broad-spectrum microbiological tests allow for multiclass detection and are cost-effective, but they should also meet certain performance criteria; specifically, their detection capability should be at or below the MRL with low false positive rates prior to employment in routine operation. Pikkemaat and colleagues compared the performance of three microbial growth-inhibition screening methods in routine testing in slaughter animals and concluded that there was a lack of sensitivity and high false positive rates for the EU Four Plate Test (on kidney) and Premi Test (on muscle), respectively. The Nouws Test showed adequate performance for tetracyclines below the MRL, but in kidney failed to detect a gentamicin MRL violation (1). Modifications to the kits protocol (solvent extraction) may improve the performance of microbial inhibitor tests in some cases (2).

"Recent developments in rapid tests are towards multiplexing, with technologies that can cover multiple classes of antibiotics."

In dairy farming, upstream checks avoid bulk tanker rejection caused by a potential issue at a single farm. At the farm level, simplification of the test is crucial, as is speed and ease of operation. Essentially, a visual read-off with a "PASS or FAIL" decision (ideally close to or at MRL) within minutes is required. A diverse range of immuno and protein receptor tests designed for particular classes of antibiotics are now commercially available. For example, the BetaStar (a rapid lateral flow assay for the determination of beta-lactam residues in milk from Neogen Corp) has been optimized to achieve a result within two minutes (two incubation steps), which is ideal for conducting on-farm checks (3).

However, recent developments in rapid tests are towards multiplexing, with technologies that can cover multiple classes of antibiotics. In fact, the development of rapid, higher performance methods that can detect several contaminants simultaneously is being addressed in the European seventh framework project CONffIDENCE (www.conffidence.eu). This initiative encompasses many different food matrices – seafood, fish feed, dairy products, meat, honey – and a broad selection of chemical contaminants, including antimicrobial classes, such as tetracyclines, sulphonamides, quinolones, coccidiostats, and banned drugs, such as chloramphenicol. Technology platforms range from simple lateral flow devices, flow cytometry, optical

Table 1. Application of HRMS techniques for targeted and untargeted veterinary drug analysis in different food matrices.

HMRS	*Matrix*	*Screening*	*Ref.*
DART-Orbitrap MS	Milk, chicken feed	Antiparasitic drugs, coccidiostats, benzimidazoles	9
TFC-UPLC-Orbitrap MS	Honey	Wide spectrum veterinary drugs	10
Orbitrap MS	Meat	Anabolic steroids	7
Orbitrap MS	Animal tissues, honey	Wide spectrum veterinary drugs	11
Orbitrap MS	Cultured fish	Wide spectrum veterinary drugs	12
UPLC-Orbitrap MS	Bovine urine	Steroid hormones, beta-agonists, Wide spectrum veterinary drugs	13
UPLC-Orbitrap MS	Bovine hair	Steroid esters	14
Orbitrap MS	Milk and milk products	Wide spectrum veterinary drugs	15
LTQ-Orbitrap MS	Meat	Wide spectrum veterinary drugs	16
LC-ToF MS	Aquaculture	Sulfonamides	
LC-ToF MS	Meat, fish, egg	Wide spectrum veterinary drugs	17
UPLC-ToF-MS	Calves	Anabolic steroids	18

Note: DART = direct analysis in real time, TFC = turbulent flow chromatography.

and electrochemical biosensors, up to more sophisticated "omics" approaches, such as ambient MS and near infrared. An important offshoot from this project is the development of test kits that enable the detection of multiple antimicrobials in a single strip at or close to the MRL. An example is the BEE4SENSOR (Unisensor, Belgium), a dipstick format test that enables the detection of tylosin, chloramphenicol, the main sulfonamides and (fluoro)-quinolones in honey. Similarly, the 4Sensor is a competitive antibody-based assay in a multiplex dipstick format for the detection of beta-lactams, tetracyclines, dihydrostreptomycin, streptomycin and chloramphenicol in milk in a single operation.

Many different multi-screening antibody-based kits are available in plate format but these require a reader and are therefore inconvenient for farm use. A further promising technology uses flow cytometric immunoassays with microspheres or beads functionalized by size, color, florescence or magnetism. Flow cytometry is far better suited to multiplexing than dipstick-based tests and, depending on the test format, up to 100 different assay combinations can be handled at high throughput (4, 5).

"Many different multi-screening antibody-based kits are available in plate format but these require a reader and are therefore inconvenient for farm use."

The range of rapid test kits and screening approaches available today has increased speed and lowered costs but they do not provide full coverage of all antimicrobial residues of interest. Surveillance plans and programs must consider country-specific legislation and approval status, as well as typical antimicrobial use patterns in each geographic region, whilst not forgetting that banned compounds may still be in circulation.

The shift towards non-targeted approaches

Positive results in a qualitative or semi-quantitative rapid test kit or screening test need confirmatory analysis by a validated method. Over the past two decades, a plethora of chromatography–tandem mass spectrometry (LC-MS/MS) methods have been developed to detect and quantify residues of antimicrobials down to low parts per billion in all relevant food and feed matrices.

Tandem MS instruments have become more affordable, their performance improves continuously and LC-MS is considered the technology of choice for targeted analysis of one or several classes of antimicrobials. To enhance speed and

shorten tedious clean-up steps, techniques such as automated turbulent flow on-line LC-MS/MS look very promising, with reports of the successful analysis of 36 compounds from seven classes of antimicrobials in a single run for milk (6).

To better anticipate risks, laboratories are now gradually shifting towards non-targeted screening tools in the analysis of veterinary drugs and growth-promoting agents. Several recent publications focus on the use of accurate-mass full-scan high-resolution (HR) MS, such as time of flight (ToF) and Fourier Transform Orbitrap MS. Table 1 illustrates some applications, both targeted and untargeted, covering a variety of compound classes and matrices.

HRMS instruments show promise for antimicrobial screening in all relevant matrices, characterized by high throughput and fast data acquisition, and also have the potential to be used in quantitative analysis. The main challenge for HRMS is that higher resolving power may be required to achieve adequate sensitivity for the detection of banned compounds. A recent report comparing the performance of LC-MS/MS versus Orbitrap MS in the analysis of over 30 steroids showed that Orbitrap MS, operating at a resolution of 50,000 FWHM (full width at half maximum), compares well with LC-MS/MS in terms of selectivity and specificity, but showed somewhat inferior sensitivity for certain compounds (7).

Next steps: globalization and "omics"

Food globalization and increasingly complex supply chains exacerbate food safety and traceability risks – as exemplified by the recent horse meat scandal. The €15 m Agrifood GPS project (agrifoodgps.sharepoint.com), which began in January 2012 and runs until December 2016, is addressing untargeted screening in dairy, meat and fish products using HRMS with nuclear magnetic resonance (NMR) and Raman spectroscopy. In a recent paper, Ruiz-Aracama and colleagues from RIKILT applied untargeted metabolomics (NMR, UPLC-ToF/MS) to differentiate organic and conventional chicken feed (8).

Such platforms, based on "omics" principles , allow for rapid profiling of foods and increased detection of "unknown" chemical contaminants. Coupled with well-developed spectral libraries, data processing software and bioinformatics tools, these new approaches will play a key role in our early warning system for potential food safety crises caused by unwitting incompetence or outright fraud.

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Business

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Patent Pitfalls

There is little advice available on the pitfalls that await the unwary scientist filing a first patent application or general advice to those put in charge of managing the intellectual property for his or her start up. Here, we identify some of the most common mistakes made when patent applications are prepared and filed – and how to avoid them.

By Simon Kiddle and Hilary van der Hoff

In technology-rich companies, such as those in diagnostic or analytical science fields, intellectual property is likely to mean patent applications. Simply put, patents protect technical inventions and this means the applications of science, rather than the underlying scientific principles.

Once an invention has been made, the first questions to answer are "can we patent it?", "who should write the patent application?" and "what information will they need?". If there is some clever new science involved in the invention, then the answer to the first question is invariably yes, as long as no one has let the cat out of the bag by disclosing the invention to the public. As patent law compares the invention defined by the claims of a patent application with what is already known (the "prior art"), a previous disclosure of your invention will usually prevent your patent from being granted, or at least limit the scope of the claims that can be obtained. Here patent law is very unforgiving as the prior art includes any type of disclosure made anywhere in the world, provided it was available to the public. This includes a description of your invention in Sanskrit deposited in the public section of a library in Mongolia. However, more commonly, the disclosure will be a poster presentation made by one of the scientists at a recent conference or a description put up by the CEO on the company website to generate interest in a new technology.

Another common error comes about when a patent application is being prepared in parallel with submitting a scientific paper for review by a journal. It is common now for papers, once accepted, to be electronically published on the journal's website. If this happens before the patent application is filed, the paper will count as prior art. Knowing in detail what disclosures have been made by the research group or the company is therefore necessary before one can work out whether an invention is patentable and what sort of claims might be obtained.

In the rush to file a patent application, it is important not to overlook the question of the invention's commercial relevance. While most good research has some angle or another that might be patentable, by no means do all of those possible patentable inventions have any commercial value. Many worthless patent applications are filed for technology that is never developed into a commercial product. The value of a patent is that it allows you to exclude other people from your invention, thereby improving your competitive position in the marketplace or generating income through selectively licensing the invention to others. Therefore, before filing a patent application you should ask yourself whether someone else would actually want access to the invention. If nobody else would want to make the invented product or run the invented process anyway, then a patent is unlikely to generate a financial return for the company.

Of course, at the time of filing a patent application, the invention is typically in the early stages of development and it can be difficult to predict its potential value. Crystal ball gazing to work out how an idea will turn into a commercial product can be a challenging problem for scientists in academia or start up companies. Patent attorneys are adept at bridging this gap, or at least in keeping the wording of a patent application sufficiently open to cover changes made in development.

Now, as patent attorneys, you may think that we have a vested interest in recommending that you use a patent attorney to help write your application. It is true that some scientists can make reasonable attempts at writing patent applications. However, in most cases, a

patent attorney can improve a patent application and help to avoid some of the things that go wrong in drafting applications. It stands to reason that if it takes three or four years to qualify as a patent attorney, and many more to become experienced, then you are unlikely to master writing patent applications in an afternoon.

It is a surprisingly common phenomenon for a patent application not to cover the intended product due to mistakes or oversights made at the time it was prepared. Other times, a poorly written patent application may cover one embodiment of an invention but fail to extend to other aspects, allowing competitors to evade the patent by making trivial modifications.

When a patent application is being prepared it is important not only to describe and define the invention, but also to include fall-back positions that can be brought into play when the application needs to be amended. It is usual to amend patent applications during their examination at the patent office, for example to ensure the claims are clear of prior art or to update the claim scope as the commercial direction of the invention evolves. The drafter of the patent application must anticipate the need for amendments and include suitable wording for them in the original application, otherwise it will not be possible to introduce such amendments when they are needed later on.

A great misconception is that the contents of a forthcoming scientific paper can simply be pasted into a hurried patent application, filed at the patent office to secure a filing date before publication of the paper, and then handed over to a patent attorney for tidying up afterwards. This approach is doomed.

Patent law allows an applicant to

Patent Tips Quick Six

1. Understand that any previous disclosure of your invention often prevents your patent from being granted, or at least limits the scope of the claims.
2. Be aware of electronic publishing by some journals – if it occurs before the patent application is filed, the paper will count as prior art.
3. Do not overlook the question of the invention's commercial relevance.
4. It is not possible to master the art of writing patent applications in an afternoon; skimping on time, effort and expense is truly a false economy.
5. Don't drip feed information or withhold important details – this will complicate and slow down the patent-writing process.
6. If you need to retain some information as a trade secret, provide details to a patent attorney for advice on whether the information is necessary in the patent application.

file a further patent application for the same invention within 12 months of filing a first application and to "claim priority" from the first, which means retaining the original filing date for the invention. The snag is that priority can only be claimed for subject matter that is the consistent between the first and second patent applications, and the patent offices of many countries assess this requirement rather strictly. All too often, the patent attorney will have to extensively rewrite the text for the new application, if it is to have any hope of leading to a worthwhile patent, but the rewriting means that the new application will differ so much from the first one that it can only claim the all-important priority date for very narrow embodiments of the invention, if at all. All of this means that drafting that first priority application is critical to the success of a patent application. Skimping on time, effort and expense at this point is truly a false economy, albeit one that might not come to light for some years.

So having settled on using a patent attorney to help to write your first patent application, the question then is how do you maximise your bang for the buck? The main point is one of efficiency. The process of writing a patent application is a collaborative one, and the scientist's role is fundamental in ensuring it runs smoothly. If time is taken to fully explain the invention to the patent attorney and to provide all the relevant information in the beginning (including whatever is known of the prior art, a description of the invention, and what the future commercial plan may be) the patent attorney will be able to determine a suitable approach at the outset and to prepare the patent application accordingly. Drip feeding information or withholding important details, on the other hand, will complicate and slow down the process. Occasionally, researchers are tempted to gloss over certain details of an invention, either because the invention is not fully worked out or because the researcher is afraid of giving away too much information in the patent application. This is risky, not only because it may waylay the attorney, who is trying to get to grips with the key elements of the invention, but also because a patent that misses out important details may later be invalidated for insufficient or misleading disclosure. When it is desirable to retain some information as a trade secret, it is best to provide the details to the patent attorney, who will be able to advise on whether the information is needed in the patent application or not.

The lure of the profitable patent motivates the development of many new and improved technologies and, in turn, the dissemination of a growing body of technical information in patent publications. For some companies and inventors, the combination of a patentable invention, sound commercial strategy and skilled patent advice leads to great wealth and success. Indeed, a company's strongest assets may be its patents. However, for every valuable patent there are many more that absorb rather than generate funds. The pitfalls discussed in this article are a mere handful of the many reasons why some patent applications flounder, whether through bad decisions or just bad luck, but we hope it goes some way towards guiding scientists and those managing research on a path towards profitable patent can become a reality.

Simon Kiddle and Hilary van der Hoff are both partners and patent attorneys at Mewburn Ellis LLP. www.mewburn.com

Webinar

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Event Overview:
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Profession

Leadership
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Publish, or Perish the Thought?

Research papers are the bread and butter of an academic's career, but a different mindset prevails in industry. Should it? Here are the views of a prominent industrial scientist.

By Lloyd Snyder

I have been involved with industrial research for almost 60 years, and I still recall the first time I set out to submit a paper for publication. The response from management was: "If this stuff is really important, why are we giving it away to our competitors? And if it isn't really important, why would you want to publish it?" That nicely summarizes the quandary I want to address here.

As an industrial researcher, there are various reasons or incentives to proceed with publishing a paper, reflecting the interests of both the author and the company. The considerations are comparable to whether to visit the bathroom or not: it starts with an urge, after which timing and location are critical. In the case of publishing, the timing of submitting a paper will often be determined by the importance of confidentiality, and the place (that is, the company where the research was carried out) may be a dominant consideration. Let's look at these three parts of the question.

The urge: why publish?

Publication might be driven by a desire to:

- Share information with the scientific community
- Enhance professional status
- Promote a particular idea, product or procedure.

The advancement of science and technology rests on shared results; research scientists generally feel a responsibility to be a part of that process. On that first occasion and subsequently, I hoped that my own research might influence the way other scientists carry out their work, or more specifically, improve the way chemists make use of the technique of high-performance liquid chromatography (HPLC). Regardless of the quality of my work, it would have had little impact unless shared with other scientists. In the absence of other considerations, it is difficult to argue against sharing our discoveries with others.

Industrial workers as well as academics can benefit from their publications, especially today when most people will likely work for more than one company. These benefits include both recognition by one's peers (and a mention in our CV) and enhancement of the research. "Polishing" a story for publication necessarily improves it, and it also encourages more critical thinking for future use. There is also some benefit to the company in the creation of an image of professionalism and a good place to carry out research.

Publication can also be useful for exposing a new idea to a wide audience, encouraging some practice in the laboratory, or advertising a new product. For example, my own company developed software in the 1980s (DryLabR) that could simulate HPLC separation; this in turn meant that fewer experiments were needed to optimize the separation of a particular sample. We published a number of papers about the theory of computer simulation, its potential uses and limitations, and examples of its application. Publication in this case both advanced an understanding of our approach and brought its potential utility to the attention of would-be customers. However, there is a fine line between science and advertising, and this must be recognized if the paper is to be accepted by a peer-reviewed journal, as opposed to a trade magazine.

When to publish?

The first consideration is: wait until the fruit is ripe. I mentioned that my first paper in industry was initially turned down by management. When permission was finally granted a few years later, a much better paper could be – and was – written (presented opposite). Publication in an industry setting must also consider the

Reprinted from ANALYTICAL CHEMISTRY, Vol. 30, Page 32, January 1958

Two-Stage Gas-Liquid Chromatography

M. C. SIMMONS and L. R. SNYDER[1]
Houston Research Laboratory, Shell Oil Co., P.O. Box 2527, Houston 1, Tex.

▶ **An instrument arrangement has been devised for connecting two or more gas-liquid chromatographic columns in such a manner that preliminary cuts prepared by the first column can be charged directly to one or more secondary columns. This arrangement makes it possible to obtain detailed separation (and analysis) of various components in samples having a wide molecular weight range without the need for separate sample prefractionation by conventional distillation or gas-liquid chromatography techniques. Furthermore, separations can be obtained with this column arrangement which are not normally possible with previously described arrangements of single columns and multiple columns connected in series.**

In the petroleum industry the early promise of gas-liquid chromatography in the field of gas and light hydrocarbon analysis has been fully realized, and it is expected to become increasingly useful in the analysis of gasoline-range and higher samples. However, as the application of gas-liquid chromatography is extended to gasoline samples, problems are encountered which are inherent in the analysis of mixtures of wide boiling range. One problem arises from the fact that with such samples either the lower boiling compounds pass through the gas-liquid chromatographic column too quickly to be adequately resolved for analysis, or the higher boiling components require a prohibitively long time for elution.

Another problem arises from an inability to prevent remixing of separated bands when two or more columns of different type are coupled for series flow. Various column packings can be classified roughly as separating by either boiling point (nonpolar packing) or molecular type (polar packing). Hence, an advantage can be gained by connecting two columns of different type in series, one column containing a nonpolar packing and the other containing a polar packing. Those compounds which remain unseparated by the nonpolar column (compounds having the same boiling point) will be separated by the polar column (compounds of different molecular type). With mixtures of wide boiling range, however, separation in the second column almost invariably leads to a certain amount of remixing of a low boiling polar compound (aromatic or cycloalkane) with a higher boiling nonpolar compound (*n*- or isoalkane).

Figure 1 illustrates this point, using a hypothetical separation of six compounds by a one-stage, two-column, gas-liquid chromatographic unit, where the first column contains a nonpolar stationary liquid and the second column contains a polar liquid. In chromatogram 1 the incompletely separated bands are shown as they leave the first column. Compound pairs *a* and *b*, *c* and *d*, and *e* and *f* are unresolved. Chromatograms 2, 3, and 4 show that the second column resolves each of the unseparated bands from the first column (the molecular type of the compounds in each band being different). However, the composite separation (chromatogram 5) gives only four separate bands, compounds *b* and *c* and *d* and *e* being remixed in the second column.

For these reasons a two-stage, gas-liquid chromatographic apparatus has been constructed which connects a nonpolar column with a polar column in such a manner that preliminary cuts prepared by the first column can be either rejected or charged directly to the second column. Thus, the interfering low and high boiling components can be vented as they emerge from the first column, while only the cuts in the desired narrow boiling range are admitted to the second column for analysis.

The design of the two-stage, gas-liquid chromatographic unit was prompted by an analytical problem typical of those for which conventional one-stage gas-liquid chromatography is not well suited. It was desired to analyze rapidly various Platformer streams for the key components involved in C_6 conversion—namely, methylcyclopentane, cyclohexane, and benzene. These streams contain compounds boiling in the C_5 to C_8 range. Although one-stage gas-liquid chromatography permits such an analysis to be carried out, the time required to remove the heavier ends (C_7 to C_8) from the column after the C_6 components have been eluted and analyzed would be prohibitively long.

APPARATUS

Basic Operation. The basic oper-

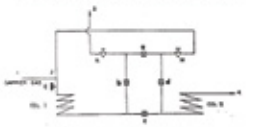
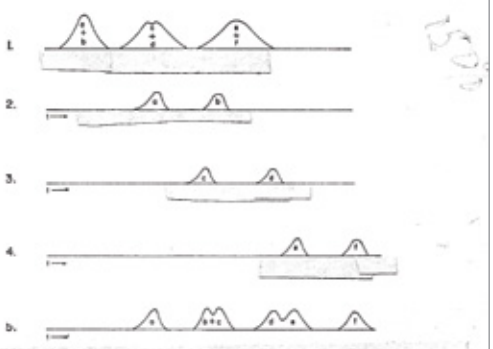


Figure 2. Schematic flow diagram of two-stage, gas-liquid chromatographic unit



[1] Present address, Union Oil Co., Bre
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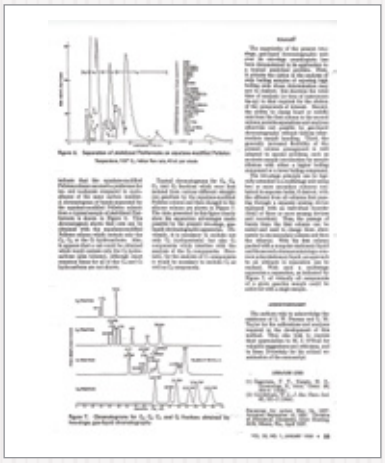


Extracts from Lloyd Snyder's first research paper.

competitive value of research results, and the need to exploit this information before competitors get hold of it. When patenting is appropriate, the work can be published within a year of a patent application. In other cases, it may be necessary to delay publication for a longer period. However, both the author and company should keep in mind that potentially publishable work cannot be kept hidden indefinitely.

Publication might also be delayed while the author ponders the next step in a research program. This is akin to picking all the fruit before the birds and squirrels arrive. Academics also want a leg up on their peers when opening up a new line of research, which argues against overly rapid publication by them.

Where was the work carried out?
Publication policies vary widely among different companies, usually for valid reasons. My company sold software to customers who needed to:

- Understand how it worked
- Have confidence in its accuracy
- Perceive its potential value.

A research publication could satisfy these different needs, especially when the claims in the paper had been validated in the process of publication (by reviewers working for the journal). Pharmaceutical companies similarly exploit the publication of clinical trials as a means of advertising new drugs. These same companies might be less interested in telling competitors about that drug during its development, just as we did not talk about our software until it was ready for commercialization.

There are general advantages in allowing some publication, as discussed below. But the kinds of companies that are most likely to encourage, and benefit from, publication are those whose lifeblood is the discovery and exploitation of new ideas. Such companies are also more likely to employ workers with a yen for publication. Similarly, industries that produce a tangible product (including software) are better candidates for research publication than companies in retail, finance, and so forth. In short, companies that employ highly trained scientists best fit the criteria for publishing.

The discoveries that are easiest for a company to publish are those that provide generic information that is widely applicable and not tied to a particular product. Happily, this is also most acceptable to journals. In my field, analytical chemistry, we were not involved with the development of hardware, and disclosures in areas like mine are usually easier to justify to management.

The situation today
I worked in industry for almost six decades. The first half was spent with four different companies, and the second half with my own company. Over the entire period I usually enjoyed both the time and resources to pursue ideas relevant to the raison d'etre of each company. Often the time spent was after hours, and frequently the idea was only distantly related to company business; but if I wanted to do something, usually I could. And by being persistent and patient, eventually a way was found to get my research (or most of it) published.

Do companies today offer a different response to publication than the one mentioned at the beginning of this article? More to the point, should they view research publications without the skepticism that I encountered all those years ago? I believe that the answer to this question is "yes", for the following reasons.

Increased worker productivity
Few would now argue against allowing workers as much freedom of action as possible, with an emphasis on the result and time of completion, rather than the means employed. The act of preparing a paper for publication can both enhance the interpretation of the research and lead to improved works skills. Encouraging publication should also have a positive impact on employee morale, at least for certain workers. It certainly did for me.

Encouraging new ideas
The prospect of publication should encourage workers to be especially open to new possibilities and/or new questions as they work. In some cases, this will suggest promising new lines of research as well as publishable results.

Professional bartering
A company environment that is open to publication is also open to the exchange of 'generic' information with companies that may be competitors. 'Generic' information refers to 'tricks of the trade' that can be helpful, without seriously compromising products that are currently under development. When companies are willing to exchange this kind of information, everyone benefits. But you can't take without giving. Publication carries this philosophy to a logical conclusion.

Better access to the labor pool
This is a traditional argument for allowing publication. It assumes that a certain kind of employee will be both essential to the company and motivated by the hope of publishing. When there is an excess of these people entering the job market, as at present, this argument becomes less forceful, but it would be a mistake to use the circumstances of today as a basis for tomorrow's policy.

To wrap up, work in industry followed by further research in my own company has enhanced my awareness of the role of publishing in a technically-oriented business. The benefit of writing papers was, at first, mainly that it was stimulating and personally rewarding. Later it also became a major factor in the success of our business. So, do I believe some companies should encourage publication? At a minimum the question deserves consideration, with attention to the above observations.

Lloyd Snyder is now retired and living in Orinda, California, USA.

GPC/SEC-MALLS analysis of Hyaluronic Acid

Hyaluronic acid is a polymer of disaccharides and can have up to 25,000 disaccharide repeat units. The molar mass range is from 5000 Da to 20 000 000 Da. Hyaluronic acid is mainly used in medical and cosmetic applications.

GPC/SEC is the method of choice for measuring the molar mass distribution of hyaluronic acid. True molar masses can be obtained when on-line multi angle laser light scattering (MALLS) is used in combination with a refractive index detector. The refractive index increment (dn/dc) for light scattering data evaluation can be determined on-line, offline using dedicated instrumentation or taken from literature.

Experimental Conditions

Eluent:	0.005M phosphate buffered saline, 0.069 M NaCl
Columns:	3 x PSS SUPREMA Lux 10 µm Ultrahigh (8 x 300 mm) + precolumn
Data acquisition:	PSS WinGPC UniChrom with Compliance Pack
Detectors:	SECcurity GPC1260 RI SECcurity MALLS SLD7000
Flow-rate:	0.5 ml/min
Concentration:	0.5 g/l
Injection volume:	100 µl

Results & Discussion

High molar mass samples require lower flow-rates and concentrations in GPC/SEC. The GPC/SEC conditions and columns have been optimized for high molar mass hyaluronic acid with respect to loading, flow-rate and column particle size and porosity. For sample preparation the water content of approx. 12 % has been taken into account.

Due to the lack of high molar mass calibration standards the use of light scattering detection is recommended. This also allows the measurement of true molar masses.

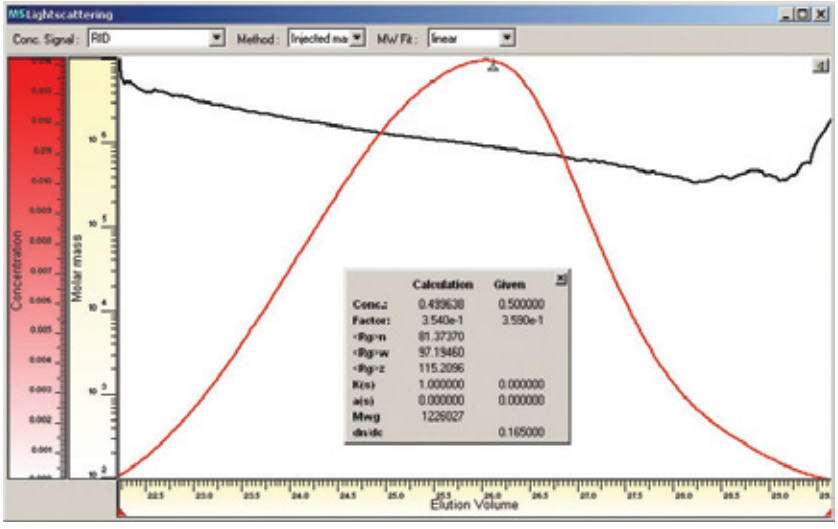
A refractive index increment (dn/dc) for hyaluronic acid of 0.165 ml/g has been used to evaluate the light scattering data.[1] Figure 1 shows the slice concentration measured by the RI detector as well as the on-line measured molar mass. Sample recovery (comparing Concentration Calculation vs. Given) was nearly 100% showing that all material eluted from the column.

Table 1: The molar mass averages of the hyaluronic acid sample obtained using a Pullulan calibration (RI only, apparent molar masses) and using light scattering. The light scattering Mw molar mass is 3 times lower than that obtained with a Pullulan calibration curve.

	*Pullulan Calibration**	*MALLS*
Mn [Da]	1 224 000	932 000
Mw [Da]	3 818 000	1 200 000
Mp [Da]	1 398 000	933 000

** Highest molar mass standard: Mp = 2 500 000 Da, P/N PSS-dpul2.5m*

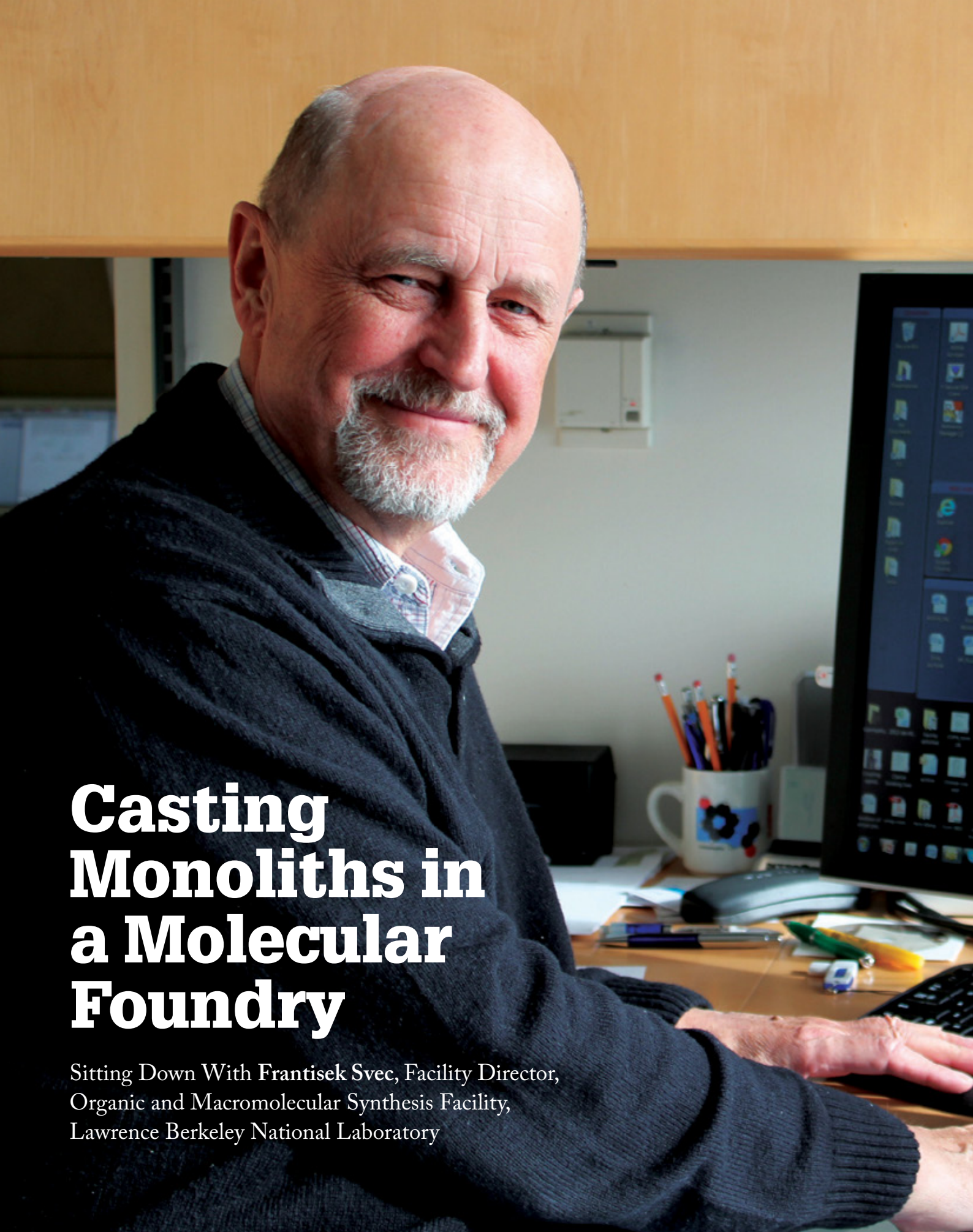
Figure 1: Slice concentration and molar mass obtained with a SLD7000 and an RI detector.



Literature:

1 *Lavrenko, Linow, Gornitz in Analytical Ultracentrifugation in Biochemistry and Polymer Science 517-531 Royal Society of Chemistry, Cambridge (1992)*

For more information, please see the full application note at: theanalyticalscientist.com/issues/0413/701



Casting Monoliths in a Molecular Foundry

Sitting Down With **Frantisek Svec**, Facility Director, Organic and Macromolecular Synthesis Facility, Lawrence Berkeley National Laboratory

When did you become interested in chemistry?

When I was in the fifth or sixth grade, my mother bought me a very basic book on chemistry for kids, called "The Secrets of Chemistry", in Czech, obviously. I loved reading it and started my own lab in our bathroom. It went well until I got a little permanganate into the laundry and stained everything, at which point I was exiled to the basement. But that was the beginning of my chemistry career.

Your training and early career were spent in Cold War Czechoslovakia. How did research opportunities and conditions compare with institutes in the West?

I was lucky to get a position at the Institute of Macromolecular Chemistry in Prague, which was relatively rich from licensing the technology for soft contact lenses. We performed research at a level that was competitive with our friends and colleagues abroad – in many ways better than the institute in Germany where I did a one-year postdoc. Our one big problem was that it could take several months to get particular chemicals, so we all kept large stores and indulged in horse-trading to get what we needed.

When and why did you move to the US?

In 1992. I made the move for two reasons: one, for a new challenge; and two, more prosaically, to enhance my collaboration with Jean Fréchet at Cornell University. Jean and I had been working together since the early 1980s but the cooperation was a little slow because of the distance. I'd visited Ithaca a couple of times since the Iron Curtain came down, and had even been awarded a grant by NIH to buy new equipment and hire a few people when I arrived.

While I was prepared for the scientific part of my new life, I wasn't raised here and found myself stumped by a number of things that even small kids know – new rules and new customs. In addition, I'd never had a credit card before, and it was very difficult to get one, because I had no history. Imagine life in the US without credit! It took me some time to adjust, but I am still here and still happy.

"I'd never had a credit card before, and it was very difficult to get one, because I had no history. Imagine life in the US without credit!"

How did your interest in separation science come about?

I started out as a polymer chemist – it featured in both my BSc and PhD. From the early 1970s onwards I was working with particles prepared by suspension polymerization in a variety of applications, such as catalysis, enzyme immobilization, and adsorption of metal ions. A dozen or so years later, we suddenly realized that the particles would be good stationary phases for liquid chromatography. It happened because I had a young chromatographer from the Soviet Union, Tatyana Tennikova, join my group for a year. She learned how to make the beads and in return taught me the tricks of chromatography. That was in the second half of the 1980s.

And not long afterwards you came up with the monolith…

It was my 'sternstunde' (great moment). Tatyana's boss, Professor Belenkii, had come up with a theory that, in ion-exchange and reverse phase chromatography of large molecules, the length of the column was not crucial. But he didn't have the means to demonstrate it experimentally, since making short packed columns is close to impossible because you always have some channelling. We had done some work with macroporous layers – membranes – that don't have channelling problems and could contain glycidyl methacrylate, which is a reactive monomer. So, we could prepare all the derivatives he was dreaming about. It worked like a charm. We then enlarged the number of chemistries available for these separations – ion exchange, reverse phase and so on. It was a completely new approach. In fact, when we submitted a manuscript to Journal of Chromatography in 1989, it was rejected because "no-one would be interested in such materials". Eventually, we did get it published, in the Journal of Liquid Chromatography.

When did monoliths take off?

When I spoke with another two pioneers in this area, they'd had similar experiences. One was Stellan Hjertén: when he tried to publish his paper, he had a similar problem with the reviewers, but because of his stature in analytical chemistry he got it published. The other was Nobuo Tanaka. He came up with the idea of making monolith from silica and submitted a paper to Analytical Chemistry in 1993-94, which was rejected. Despite also being well-known, he didn't get published until 1995. So, it was not easy to break through the barrier of the "old guys" who were defending

the typical packed columns, but eventually it happened. Now, everybody understands that monoliths are an option that chromatographers have available, and the number of papers is growing rapidly.

Can you describe the Molecular Foundry? How does it work and what is your role in it? How does it impact on your personal research?

The Molecular Foundry is a US Department of Energy (DoE)-funded initiative, one of five research institutions devoted to nanotechnologies. It is a purpose-built six-floor facility, with each floor dedicated to a different area of nanoscience. Mine, the sixth, is devoted to organic and macromolecular synthesis.

A feature of the institute is that 50 percent of our time is spent on our own research, and 50 percent on working with guest users. People from all over the world submit proposals on what they want to do here and we select the best projects which we carry out with the users. It is very rewarding to have a constant stream of new ideas and projects, and it's always fun to work with new people.

In my own research, I incorporate my background in polymer chemistry into the development of monolithic materials. We are experimenting with various applications, such as enzyme immobilization – I've never seen immobilized enzymes working that fast – and the rapid separation of large molecules, unique in chromatography. For some years we have also worked on combining monoliths with nanoparticles. Well, we are a nano facility! Now, we are also experimenting in new shapes or formats. Thin layer chromatography is showing a lot of promise. It's cheap, simple, doesn't need high pressure or expensive equipment, and you can do it at home. We think it can be used for separations, and are using it in reverse phase, in ion exchange, and in hydrophilic interaction mode. We are also making thin layers for separation in two dimensions, and starting to think about three dimensions.

"From the early 1970s onwards I was working with particles prepared by suspension polymerization in a variety of applications, such as catalysis, enzyme immobilization, and adsorption of metal ions."

The monoliths have been commercialized, by BIA Separations, a company in Slovenia. They sell monoliths in a variety of shapes and sizes, with radial flow columns up to 8 litres in size for use in biotech applications.

You also have a couple of major commitments outside of the Molecular Foundry.

I have been Editor-in-Chief of the Journal of Separation Science since 2004. I enjoy it and scan all submissions, several per day. We are currently publishing about 400 papers per year, that's 4000 pages in 24 issues. And we reject over 60 percent of the manuscripts received. The average time to first decision is 26 days, which authors appreciate. To achieve this I have 10 editors and am increasing this number to 12 as I speak, to cope with the workload. I handle some manuscripts, especially those on monoliths – that's my weakness.

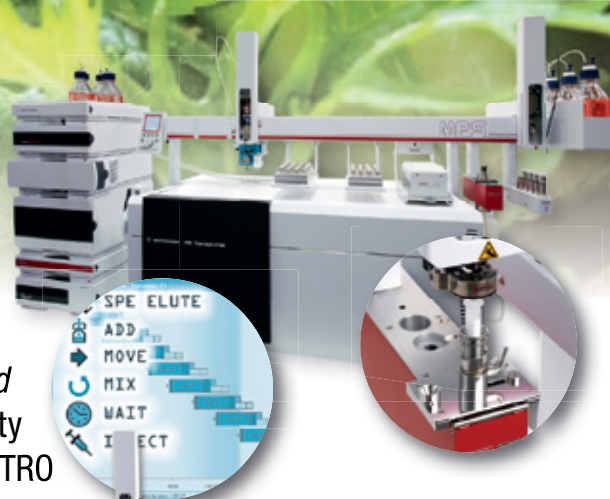
CASSS, an international separation science society, is my other big undertaking. I have served as president since 2003. In fact, I am only the fourth president in its 40 year-plus history. It started out as the California Separation Science Society, and the previous president Bill Hancock, got it to the level that it became well known in the separation world. We've built on that and become genuinely international. Now, CASSS runs 10-12 symposia per year, some larger (600-700 people), some smaller (100 people). One of the most popular is WCBP, a symposium on the interface of regulatory and analytical sciences for biotechnology health products, held in Washington, DC. Some of our meetings are so popular that we now organize sister events in Europe and Asia on the same topic, again bringing together the biotechnology industry and regulatory agencies. CASSS has around 6000 members and is free to join. We are in good shape, ideologically and financially.

That makes about 150% time commitment… do you have any spare time?

To fit it all in, I get to work at 6 am. But I do have hobbies too. I collect coins. I have all the coins of Czechoslovakia, the Czech Republic and Slovakia, since the country was created in 1918, which I am very proud of. It's quite an expensive hobby, but since I am essentially exchanging one type of money for another I figure that I shouldn't lose too much. I also enjoy skiing and the gym, and like to spend time with my wife, my daughters, and grandchildren.

Food for Thought

NEW APPLICATION:
Glyphosate / AMPA in Water, Tea, Grain...



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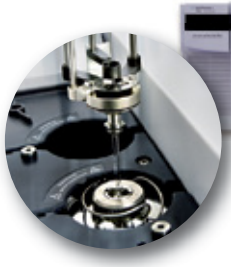

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