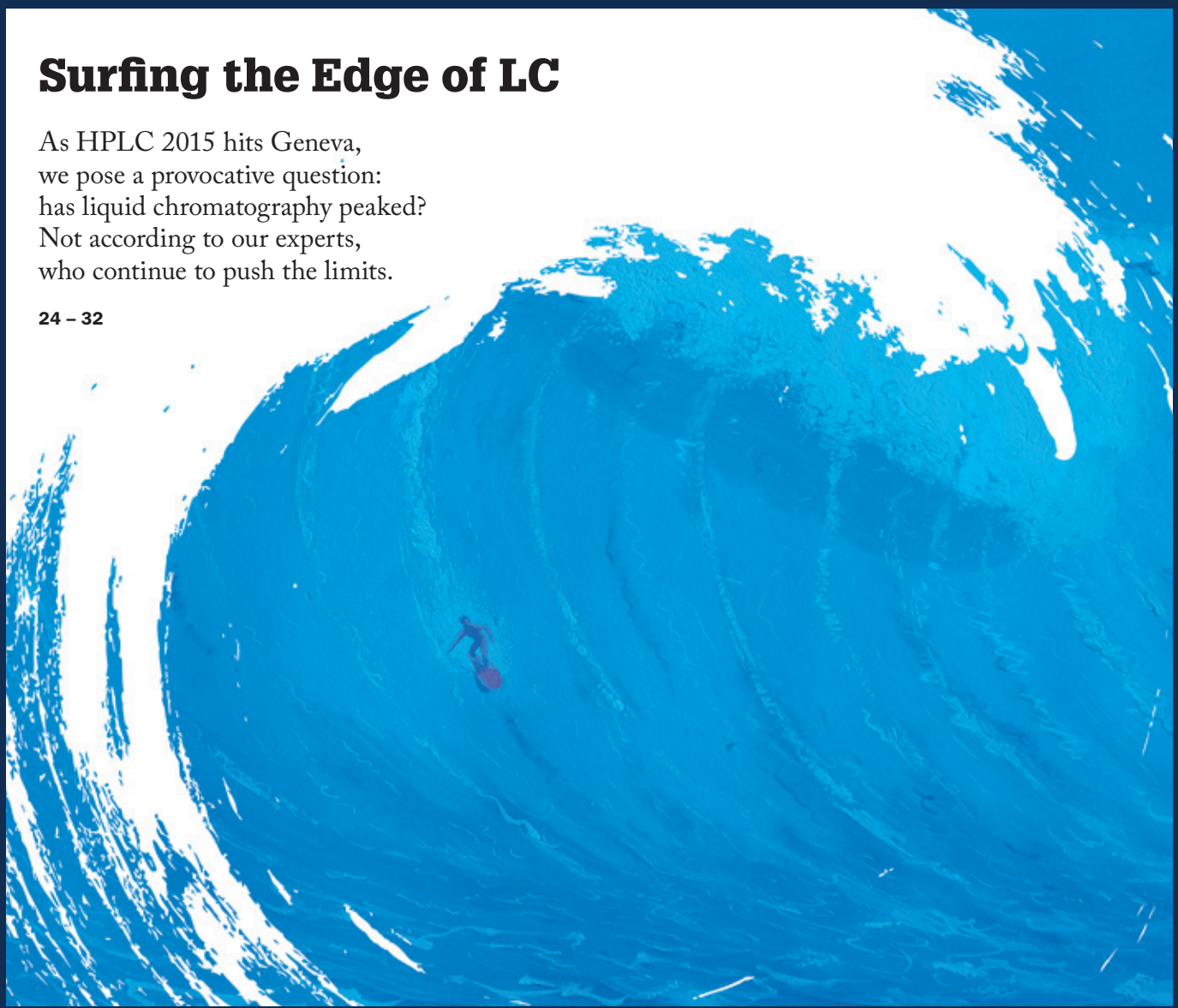
JUNE 2015 # 29

the Analytical Scientist

Surfing the Edge of LC

As HPLC 2015 hits Geneva, we pose a provocative question: has liquid chromatography peaked? Not according to our experts, who continue to push the limits.



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Peter H. Seeberger

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the Analytical Scientist


Andreas Seidel-Morgenstern

Meet the Winners

Peter H. Seeberger and Andreas Seidel-Morgenstern

Peter H. Seeberger and Andreas Seidel-Morgenstern of the Max-Planck Institutes in Potsdam and Magdeburg are the winners of the inaugural Humanity in Science Award for developing a method for the continuous flow production and purification of cheaper antimalarial medicines using plant waste, air and light.

They were awarded with a humble prize of $25,000 during an all-expenses paid trip to Pittcon 2015. On page 34, you can read more about their work and what inspired them to focus on such a high impact project.

Could it be you in 2016?

Analytical science has been at the heart of many scientific breakthroughs that have helped to improve people's lives worldwide. And yet analytical scientists rarely receive fanfare for their humble but life-changing work. The Humanity in Science Award was launched to recognize and reward analytical scientists who are changing lives for the better.

Do you know someone who's work has had a positive impact on people's health and wellbeing?

We are now accepting nominations for the 2016 Humanity in Science Award: www.humanityinscience.com

@Humanityaward Humanity in Science Award

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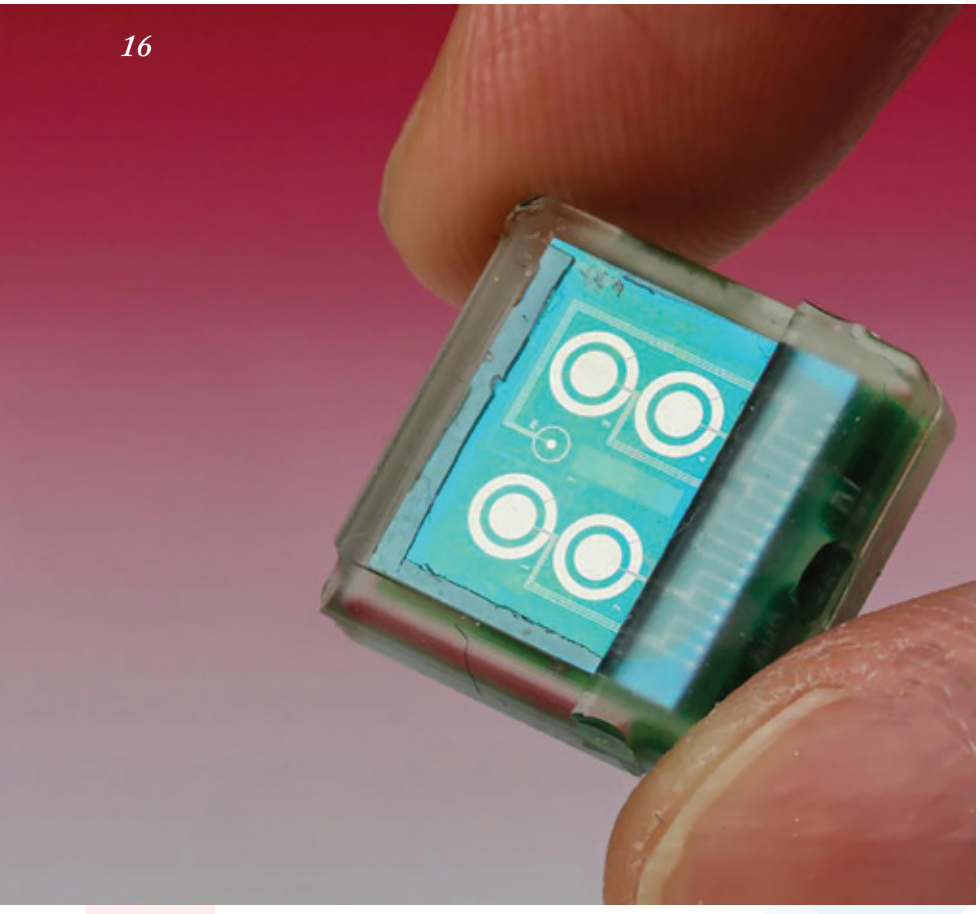

On The Cover

Surfing on the edge of liquid chromatography innovation.

Upfront

In My View

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the Analytical Scientist

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The Big Picture

In the quest for increased sensitivity or higher resolution, let's not lose sight of our objectives.

In a world of increasingly amazing technological marvels, it's easy to become obsessed with progress. ASMS 2015 in St Louis was a showcase for innovation – with impressive launches from companies big and small. There is no doubt that technology (hardware and software) drives overall advances in the analytical sciences – and science in general, for that matter.

And incremental improvements can also have a big impact – over time. As James Jorgenson notes on page 26 about the limits of liquid chromatography: "When I look at what's changed over the past year, I am always disappointed. However, when I look at what has changed over a decade, I am always amazed. How does the incremental accumulation of 10 years' of disappointments eventually become an exciting qualitative shift in performance?"

A good question. Answers are more than welcome...

But when I attend press conferences that simply cite increases in performance in terms of numbers (however big or small), Hans-Gerd Janssen's words often ring in my ears: "Performance should be fit-for-purpose and not necessarily a World Record attempt" (1). In some cases, clever solutions that help end users do their day-to-day job are more relevant. At ASMS this year, a number of "workflow" solutions moved us away from the numbers game, aiming to match such industry needs; for example, in biopharmaceutical analysis. Ironically, speaking to professionals in that industry, the big request is clear: "we need more sensitivity!" (Unfortunately, they also want it in an easy-to-use, robust package that rapidly pumps out extremely reproducible data...)

On page 50, Ruedi Aebersold offers a candid perspective from proteomics: "In many cases, the most important parameter is not how many proteins we see or quantify [...] but rather precision and reproducibility in the measurements we do make." He also notes that it *would* be great to see everything! Aye, there's the rub. Certainly, the ultimate in analysis would be the ability to measure everything at single-molecule sensitivity with 100 percent accuracy and reproducibility. But we're not there yet; until then, we need to consider the big picture in our own field. What is most important?

Surprisingly, a blog from best-selling author Seth Godin got me onto this topic (2): "High resolution is not the same as accurate [...] You don't need an electron microscope to figure out if a ball is round. (In fact, it will almost certainly tell you something less than useful.)" Godin is not a scientist, but he is often very thought provoking.

Rich Whitworth
Editor

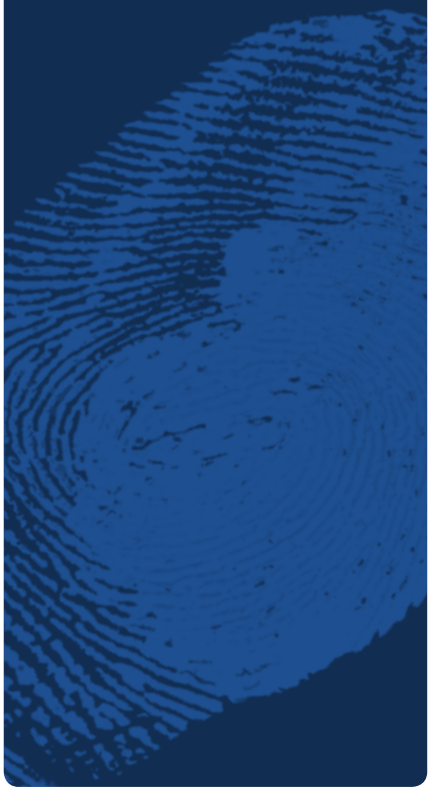
Reference

1. *Hans-Gerd Janssen, "My Ever-Expanding Analytical Toolbox", The Analytical Scientist (http://tas.txp.to/0615/toolbox)*
2. *Seth Godin, "High resolution is not the same as accurate", Seth's Blog (http://sethgodin.typepad.com)*

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:
rich.whitworth@texerepublishing.com



Fingerprinting mAbs

Using NMR spectroscopy to investigate the structure of monoclonal antibodies

Conventional wisdom says that nuclear magnetic resonance spectroscopy (NMR) measurements begin to fail for molecules that are larger than 30,000 Daltons without using more sophisticated isotope-labelling techniques, but that doesn't mean it's impossible. Using NMR, a team of researchers from the National Institute of Standards and Technology (NIST) have measured the 'fingerprint' of a monoclonal antibody (mAb) (1). A mAb's structural configuration could have profound consequences on safety and efficacy. Robert Brinson, a research chemist at NIST, tells us more about the work.

How did this project get started?

To aid in the development of methodologies for mAbs, NIST received a donation of an IgG1-kappa in its fully formulated state. It is being developed as a NIST standard reference material certified for concentration that is traceable to the Kg. NIST, along with many partnering institutions, is characterizing this material (which we call the NISTmAb).

What are the main challenges to fingerprinting mAbs?

The intact mAb is around 150,000 Daltons, and the Fc and Fab fragments are 50,000 Daltons. For comparison, aspirin – the classic small molecule drug – is 180 Daltons. The biologic Neupogen is 18,800 Daltons. A small molecule drug can be readily characterized, but this is not the case for a complex biotherapeutic protein. While the primary amino acid sequence may be known, one protein batch can be safe and another toxic. This is due to the higher order folding – the primary sequence folds back on itself into a secondary and tertiary structure, and quaternary structures.

The general purpose of our method was to develop and apply NMR spectroscopy as a higher order structure assessment tool to mAbs. Our goal was to show that this technique could deliver data that demonstrates highly similar or fingerprint-like similarity between protein lots, or between an innovator and a biosimilar.

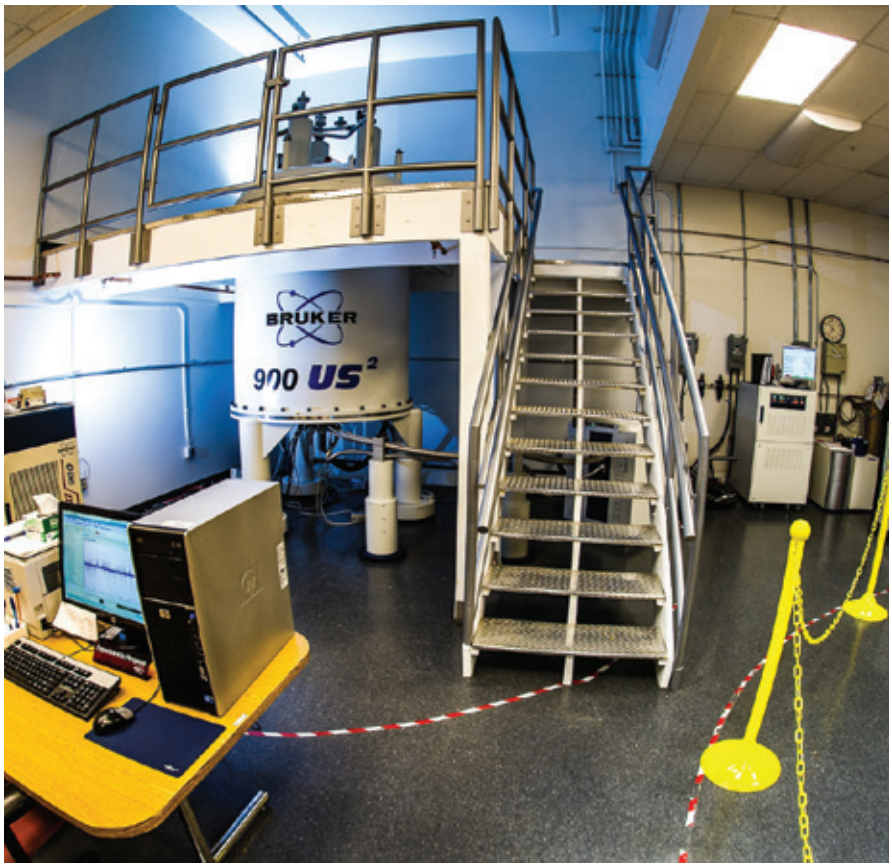
How did you approach the work?

Our goal as a lab is to push the practical limits of NMR spectroscopy. Within that framework, it was natural for us to attempt this type of characterization. We were pleasantly surprised, however, that we successfully collected data with such high quality on the intact NISTmAb.

We demonstrated that collecting the 2D ^{13}C,^{1}H NMR methyl fingerprint is feasible on the intact mAb. The methyl group has greater rotation than other functional groups, which leads to sharper peaks and therefore higher spectral quality. These groups are dispersed throughout the protein and directly report on how well the protein is folded.

Since NMR systems with lower magnetic field strength are more commonly found in analytical research labs, we divided the NISTmAb enzymatically into its two constituent Fc and Fab fragments so that the NMR mapping approach could be employed using more commonly available NMR systems. Importantly, we demonstrated that the two fragments generated from the full mAb showed no loss of structural information and that the sum of the fingerprint patterns of the fragments could be matched to the intact mAb.

It's also worth mentioning that our team used a combination of 'spin physics and sampling tricks' to successfully reduce the overall measurement time from multiple hours to around 30 minutes!



How does your approach differentiate itself from the "competition"?

Typical techniques include HPLC, SEC, DSC, FT-IR, CD spectroscopy – to name a few. A number of these exhibit high sensitivity but low to moderate resolution. They tend to look at overall structural elements and features but can miss detailed and important folding changes.

NMR allows assignment of signals at atomic resolution. In the case of this study, we are observing direct connection between individual hydrogens and carbons in the protein molecule. Any issues with higher order structure will be apparent from the NMR spectral read-out. That being said, I would like to caution that the NMR method should be considered as complementary to other analytical techniques.

What are the implications for drug development?

This measurement technique provides a robust means for a company to use for the characterization of the higher order structure of a protein drug product. This can be useful in pre-clinical and clinical setting by showing where the drug is acting and why. In a QC environment, NMR could potentially be used to evaluate multiple lots through statistical comparability methods and comparing biosimilars to innovator products.

Reference

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Bio Surfing

How to compare and contrast biosensor performance

Researchers working on a European Metrology Research Programme project called BioSurf have developed a reference biosensor surface that they believe will help to benchmark different biosensors by assessing accuracy.

"Diagnostic tests, as implemented in a point-of-care setting, have the advantage of providing rapid disease identification," says Alex Shard, a researcher at the National Physical Laboratory (UK) and one of the authors of the work (1). "The current issues revolve around sensitivity, reliability and quality control. Since many of the tests rely in some way upon having active probes attached to a surface it is important to be able to assess and verify how many probes are attached, how many are active and whether other species that interfere with the test have become attached to the surface."

Detection of biomolecules relies on flat surfaces or nanoparticles that are designed to specifically capture one type of molecule from a huge diversity of others. In many instances, researchers want to know if a biomolecule is present above a critical concentration; therefore, the sensitivity of the method used to detect it must also be known. The aim of the reference biosensor is to provide a surface that could be reproduced as part of a wide range of detection strategies to provide a benchmark for sensitivity.

The reference biosensor works by attaching two types of molecule to a gold surface: one is based on polyethylene glycol and resists non-specific attachment of biomolecules; the other has a biotin group at the end, to which avidin specifically binds. So far, the reference biosensor surface has been tested with serum proteins to confirm that there is little or no 'non-specific' binding.

"The key property of the surface we developed is its excellent repeatability in binding – and it appears to be robust to minor changes in surface composition. The amount of protein attached is now well understood and it can be used to compare the sensitivity of different methods," says Shard. "For example, we showed that quartz crystal oscillators are very sensitive to low amounts of protein attachment, but rapidly lose sensitivity as more protein binds. As the protein layer approaches full coverage, the sensitivity is reduced tenfold."

Development wasn't completely straightforward. Shard says that one of the key problems was measuring the concentration of the probe at the surface. The group developed a novel form of mass spectrometry to address this issue. "We were able to measure the concentration of probes with a detection limit ten times better than traditional methods. This was a major advance, since we were able to demonstrate that the biotin molecules were randomly spaced on the surface and that the manner in which avidin bound to the surface changed when the spacing between the biotin groups was about 5 nm, which is similar to the size of the avidin molecule," he says.

Now, Shard and the rest of the group are looking to investigate more forms of avidin and streptavidin to assess some of the more "intriguing" details of the response of the surface to different modes of binding. They are also adapting the surface to nanoparticle-based assays to investigate their sensitivity in colorimetric detection. *SS*

Reference

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To Eat or Not to Eat

You know the meat that's been sitting in your fridge for a while... is it still safe?

Looking to better spot meat spoilage, a group at the Massachusetts Institute of Technology have developed a chemiresistive sensor that measures the biogenic amines emitted by decaying meat to identify whether the food is still good to eat (1). The aim isn't just to avoid bad meat; trashing perfectly safe meat seems overly wasteful.

Lead researcher, Timothy Swager, John D. MacArthur Professor of Chemistry at MIT, explains, "Avoiding food waste is becoming a major social and political issue. Who can feel good about wasting food when there are starving or malnourished people in the world? I have been developing different types of chemical sensors for 30 years and the simplest sensors based on chemiresistors are now coming of age in terms of technology maturity and synergistic meshing with other technologies ("the Internet of Things"). Food quality and safety are really a high calling and I felt that this was a worthy target for our technology."

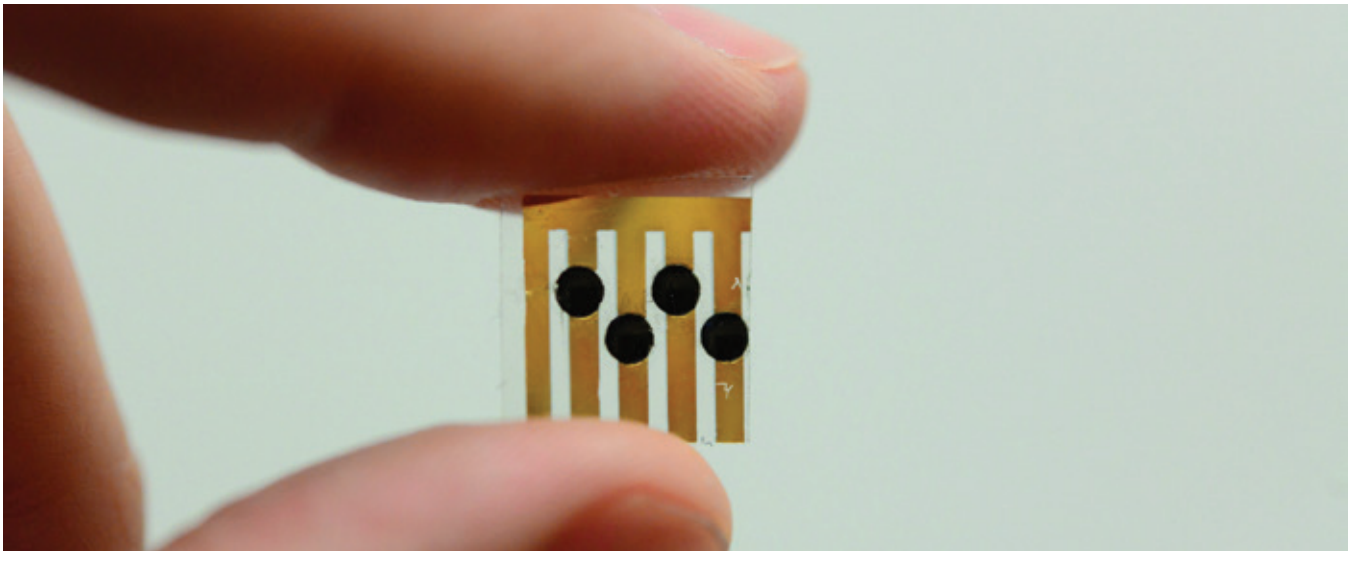
Back in 2012, Swager's group developed a sensor that could measure the ripeness of fruit by detecting ethylene, which promotes the process. For spoiled meat, the sensor – based on chemically modified carbon nanotubes – measures biogenic amines, which are produced by growing microbes and toxic to humans. "We have considerable sensitivity and should be able to detect early stages of spoilage. Sometimes meat is sold and is already compromised because of poor conditions in the packaging plants. We

should be able to detect ultra-trace levels that can head these things off early, as well as later stages wherein the meat is still safe but is beginning to be compromised," says Swager.

So far, the sensor has been tested successfully with chicken, beef, cod and salmon. Swagger expects to see differences between them, but at the moment the work is in the early stages. Chicken degraded faster than beef, and cod degraded faster than salmon, but when refrigerated all four stayed fresh over four days. Many people, however, may throw the meat out after only a few days. Other methods already exist for detecting off meat, but Swager says that his sensor has a big advantage: "Though our sensor isn't as accurate as some analytical techniques, it's inexpensive and can be integrated into packaging with appropriate encapsulation to avoid any contact or interaction with the food. Eventually, we expect sensors to be printed on the package," says Swager. *SS*

Reference

1. *S. F. Liu et al., "Single-Walled Carbon Nanotube/Metalloporphyrin Composites for the Chemiresistive Detection of Amines and Meat Spoilage." Angewandte Chemie International Edition (April 13, 2015).*



Advancing Paper Diagnostics

Can a stacking flow system offer more uniform results for immunoassay saliva tests?

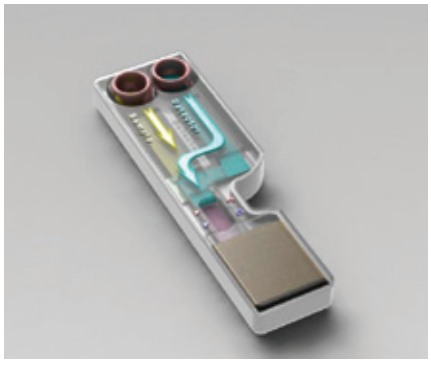
Humble paper-based systems play an important role in diagnosing infectious diseases in certain settings, but there is room for improvement in terms of quantification capability, particularly when it comes to testing saliva.

Jackie Ying, the executive director of Singapore's Institute of Bioengineering and Nanotechnology of A*STAR, says that conventional lateral tests found in paper-based kits are often complicated by the severe aggregation of detector particles when applied to test samples containing salivary fluid. In an attempt to solve the problem, Ying's group at A*STAR has developed a 'stacking flow system' that guides samples and reagents to the test strip through different paths, meaning that there is no need for the saliva sample to be pre-treated by filtration or centrifugation.

"The sample pad is placed at the bottom and in direct contact with the test strip, and the reagent pad is located above the sample pad. There is a flow regulator made of a liquid impermeable film inserted in between," explains Ying. "Salivary substances that interfere with the particle-based sensing system are removed via a fiber glass matrix before they make contact with the detection reagents, which greatly reduces the background. In addition, the stacking flow configuration enables uniform flow with a unique flow regulator, which leads to even test lines with good quantification capability."

According to Ying, when multiple streams are introduced into a test strip in a conventional 2D paper microfluidic network, typically only one stream can directly enter in the same direction as the test strip; other streams inevitably enter at various other angles because of spatial restriction. "Due to the laminar nature of the flow in a paper microfluidic network, liquid from different streams would flow in parallel along the test strip, resulting in non-uniform composition in the direction perpendicular to the flow. The stacking flow design avoids the problem by stacking flow paths normal to the test strip, allowing streams to enter in the same direction," she says.

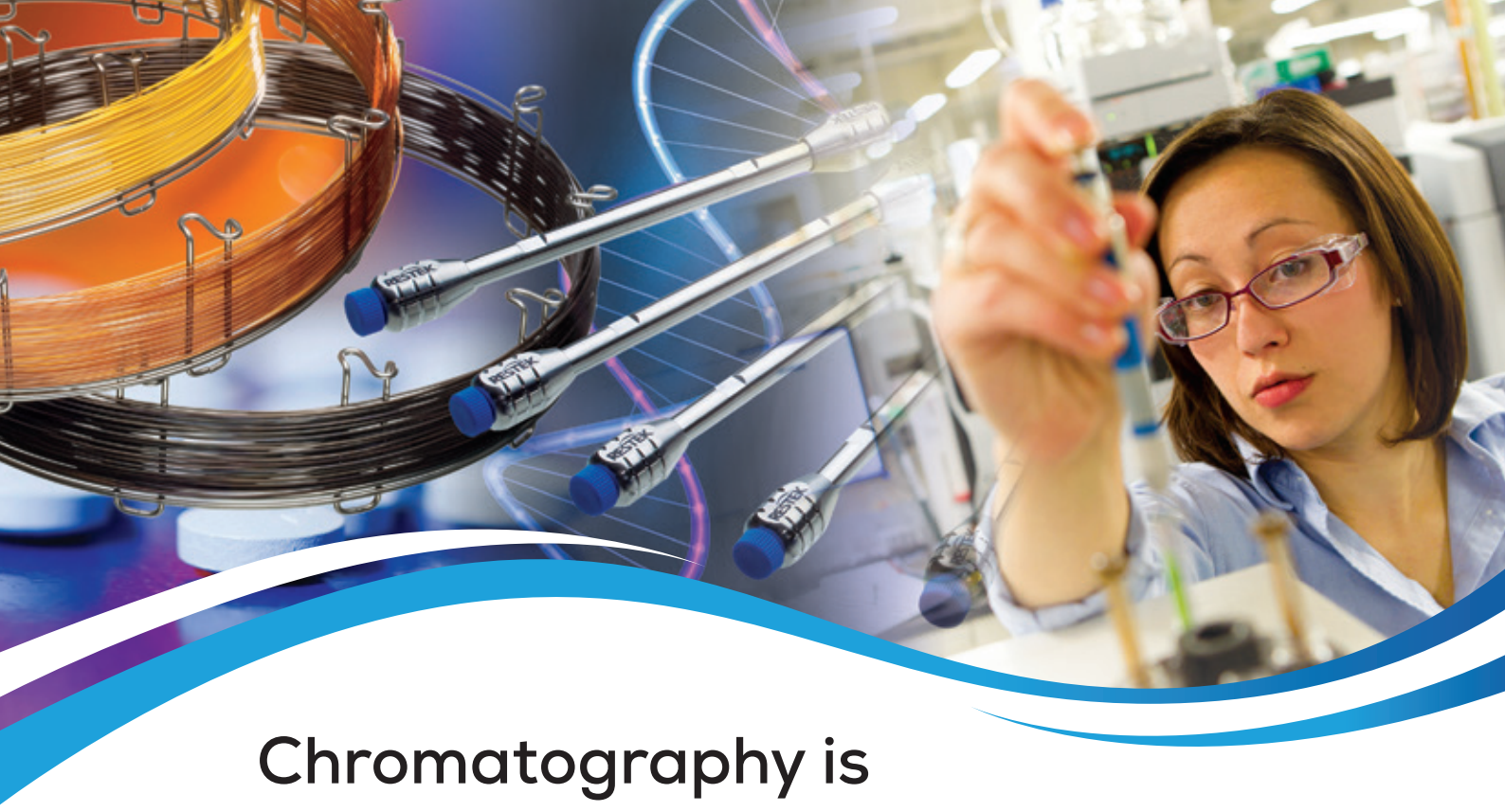
The system was applied to detect dengue-specific immunoglobulins spiked in the saliva samples. Dengue-specific IgG could be found at the onset of secondary infection in saliva samples; patients with secondary infections have a higher risk of developing dengue hemorrhagic fever or dengue shock syndrome, and discerning between primary or secondary infection could help better guide treatment.



Ying believes that the system could also be adapted to detect other infectious diseases, although the selection of the right paper materials will be crucial for different types of sample matrix, such as blood or urine. *SS*

Reference

1. *Y. Zhang, J. Baia, and J. Y. Ying, "A Stacking Flow Immunoassay for the Detection of Dengue-Specific Immunoglobulins in Salivary Fluid", Lab Chip, 15, 1465-1471 (2015).*



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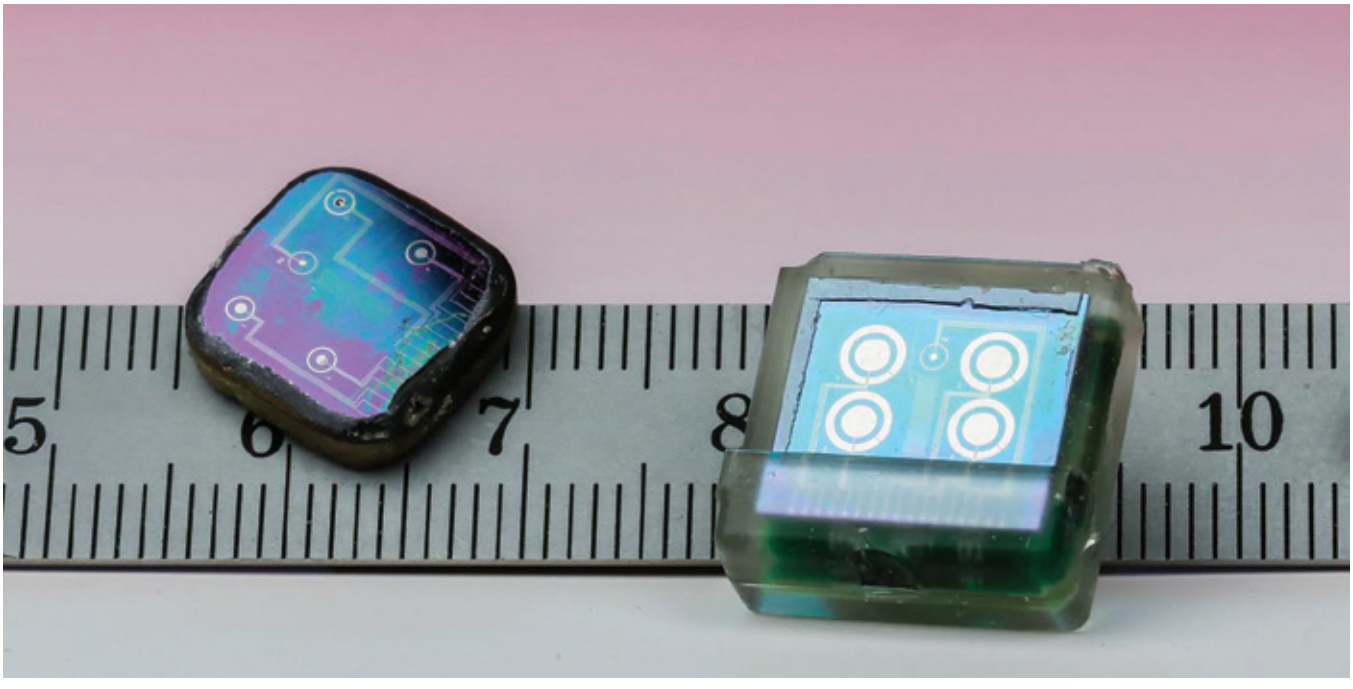
Simultaneous monitoring of multiple metabolites – with a chip beneath the surface of your skin

Researchers at École Polytechnique Fédérale de Lausanne (EPFL) have developed a 1cm^2 biosensor chip that, when placed under the skin, can simultaneously and continuously measure multiple metabolites, as well as parameters, such as pH and temperature, over a long period of time. In the short term, the team hopes the device will aid during translational medicine studies, but eventually they envision it being used to gather personal diagnostic data to monitor drug therapy.

According to Sandro Carrara, a scientist at EPFL, the sensor platform uses electrochemical biosensors based on active enzymes. "For the endogenous metabolites (typically correlated to the disease), we exploit the use of oxidases. For several disease biomarkers (for example, glucose for diabetes, cholesterol for cardiovascular diseases, or lactate for inflammations or sepsis), we can find the right oxidase that gives us the right specificity once immobilized on top of our electrochemical electrodes. In the case of exogenous metabolites (typically therapeutic compounds), we usually have two options: the drug itself may be electrochemically active or not," says Carrara. "In the first case, the compound is directly detected without an enzyme – selectivity is driven by a series of polymeric membranes that mask interferences from other electro-active molecules. For compounds that are not electrochemically active, we may use enzymes from the cytochromes P450 family. They are central proteins in our metabolism and catalyze almost all the pharma-compounds we injected."

Carrara claims that the chip's accuracy is comparable to that of glucometers available on the market for the daily monitoring of diabetic patients, although so far the device has only been tested in small animals. Lactate, cholesterol, bilirubin, glutamate, and dopamine, as well as several therapeutic compounds – metoxantrone, etodolac, etoposide, cyclophosphamide, ftorafur, ifosphamide, naproxen, filbuprofen, and paracetamol – have all been tested, but currently, a choice of four metabolites can be monitored simultaneously. The research team is hoping to expand function without enlarging the device, in addition to seeking an efficient and low-cost way to mass produce the chip. "Another improvement I'd like to see, which needs to be addressed through biotechnology, is more robust engineered mutants for the enzymes we can use to detect non-electrochemically-active therapeutic compounds," adds Carrara. The end goal is bold and simple: helping to prevent people from entering dangerous ranges for certain metabolites and increasing access to personalized medicine. *SS*



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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
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Bridging the Biomarker Gap

How to bring better diagnostic tests to the market to benefit patients.

By Rainer Bischoff, University of Groningen, Department of Pharmacy, Analytical Biochemistry, Groningen, The Netherlands.

Biomarker discovery and validation relies heavily on reproducible and robust analytical methodology. Separation science and notably high performance liquid chromatography (HPLC) are essential and efforts spent in developing more efficient and robust HPLC stationary phases, together with advanced mass spectrometers, have made modern biomarker research possible. Given that many clinical biomarkers are proteins, advances in protein bioanalysis and proteomics have also been critical in driving the biomarker field forward. Many new biomarker candidates are proposed for various diseases every week – but often from small-scale studies lacking statistical power.

Sorting through this mountain of information and prioritizing biomarkers for further validation is a challenge. And rather disappointingly, only a few biomarker candidates survive the validation phase in large clinical studies – even fewer enter commercial development and clinical application. This "biomarker gap" is recognized and major efforts are being deployed to professionalize biomarker discovery and validation. The recently founded Dutch Biomarker Development Center, a public-private partnership consortium, is a good example of the actions taken (http://biomarkerdevelopmentcenter.nl/).

I will talk about the challenges inherent to any biomarker discovery and development program with a focus on analytical science at HPLC 2015 in Geneva. I shall highlight pre-analytical factors that may bias biomarker studies, leading to discoveries that cannot be validated later on. I will exemplify this with studies on cervical cancer (1) and multiple sclerosis (2), and refer to other published studies where appropriate. While HPLC coupled to mass spectrometry holds great promise to gain a better understanding of the intricate changes that occur in protein and metabolite profiles in body fluids or tissue, it is vital that researchers are aware of the need for equally powerful data processing and statistical analysis approaches.

"Rather disappointingly, only a few biomarker candidates survive the validation phase..."

I'll also highlight some examples showing that data processing and statistical analysis alone may influence the final result considerably (3). I have no doubt that the trio comprising well-designed comparative clinical studies

addressing relevant disease-related questions, validated and robust analytical techniques, and reliable data processing and analysis forms the basis for successful biomarker research. Notwithstanding some setbacks, the field is alive and still holds great promise notably in the field of personalized medicine.

See you at HPLC 2015 in Geneva (www.hplc2015-geneva.org).

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2. *T. Rosenling et al., "The Impact of Delayed Storage on the Measured Proteome and Metabolome of Human Cerebrospinal Fluid", Clinical Chemistry 57, 1703–1711 (2011).*
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Capture Recapture

Isotope dilution mass spectrometry analysis highlights effective interdisciplinary collaboration – and its legacy should be at the forefront of modern research.

By Zuzana Gajdosechova, PhD student, TESLA group, University of Aberdeen, Scotland.

The elegance of isotope dilution mass spectrometry (IDMS) is its simplicity. The technique's accuracy is deeply rooted in advanced instrumentation, particularly within the finely tuned parameters of mass spectrometry (MS). Interestingly, this now broadly implemented technique was in use some 18 years before Joseph John Thomson was able to prove the existence of isotopes at the beginning of the 20th century.

In fact, the origin of isotope dilution analysis is based on an ecological study of sea fish by Carl Georg Johannes Petersen, who used the capture-recapture method for estimating plaice population (1). It was a simple idea: you capture a group of individuals, mark them and then release them back into their habitat. After sufficient time for marked and unmarked individuals to mix, the fish are captured once more. This time the catch contains marked and unmarked individuals and by calculating their ratio, you can estimate the population of the unmarked individuals relative to marked fish. And so yes, the approach provides good estimates for biological measurements, but it also earned George de Hevesy the 1943 Nobel Prize in Chemistry for his work in radiochemical isotope dilution.

Before the 1980s, IDMS was restricted to nuclear, geochemical and metrological applications. With the introduction of inductively coupled plasma (ICP) as an ion source for MS, the technique was liberalized with a pioneering application of post-column IDMS by Klaus Heumann's group in 1994 (2). That breakthrough led to rocketing use of IDMS in many diverse branches of chemistry.

Only 21 of all the pre-1940 elements in the periodic table are monoisotopic, which means that the window of opportunity for IDMS is wide open. In my view, we should all consider that opportunity by shifting slightly off our predefined paths to enrich our common knowledge. I believe that our own scientific struggle makes us innovative and broadens our capabilities for greater understanding; however, we must also take good care that our struggles do not kill our desire to know more.

"The evolution in IDMS has revolutionized research, but we should not forget that its origin is not in chemistry."

In the trace elements speciation group at Aberdeen, we quantify chemical species present within various environmental matrices. It can be challenging. One of the main problems is recovering the analyte. How many of you have also faced the loss of volatile analytes during sample preparation or its incomplete liberation from the matrix during derivatization? Honest answers only, please!

And what about incompatibility between calibration standards matrix with the matrix of the sample? Like many before me, I struggled with these issues during my PhD research. However, I was also fortunate enough to work with mercury, which has seven stable isotopes. IDMS was the light at the end of the tunnel.

Quantitative extraction of mercury species from animal tissue, however, proved to be rather challenging and when the list of tested possible variables was exhausted, I decided to

seek out species-specific IDMS. The basic principle of this technique, which highlights its superiority, is that once the isotope ratio between the endogenous and enriched species is altered, the quantification is virtually independent of the derivatization and extraction efficiency. In other words, whether you derivatize or extract 30 or 90 percent of the species of interest, the altered isotope ratio is going to be the same. Moreover, as the isotope ratio is the only measured variable when using IDMS, species quantification is relatively easy.

The evolution from radiochemical to triple-spike IDMS revolutionized research, but we should not forget that its origin is not in chemistry. Therefore, when you next face big challenges in research, try to reach out to other scientific disciplines. As the question we are trying to answer increases in its complexity, we often need to cross our own boundaries.

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Enlightening LEDs

The 2014 Nobel Prize in Physics, awarded "for the invention of efficient blue light-emitting diodes," has put LEDs under the spotlight, particularly as a stable, robust and efficient light source in analytical sciences.

By Mirek Macka, Professor of Analytical, Separation and Detection Sciences and Australian Research Council Future Fellow, University of Tasmania, Hobart, Tasmania, Australia.

Since the emergence of white LEDs for lighting applications in general, it was clear that such breakthrough technology would not only change many aspects of everyday life, it would also have a big impact on science. Harnessing their power for analytical science has been a continuous process for more than three decades (among the first breakthrough examples was a miniature LED-based oximeter introduced in the early 1970s).

Mainstream analytical use of LEDs emerged in the early 1990s and over the years they have shown many advantages over traditional incandescent and arc lamp lighting. Importantly for the analytical scientist, the robustness of solid-state emitter technology, small size, low cost (for well-established, mass-produced LEDs), and excellent stability (resulting in low noise in optical detection) make LEDs an ideal light source. In addition, LEDs can readily provide pulsed light (up to GHz frequencies), allowing larger light output in the "on" period, and enabling optical techniques requiring pulsed light source, such as wavelength multiplexing and time-resolved fluorescence.

Optical detection and imaging are the main areas for the analytical use of LEDs, and they are proving useful for analytical photochemistry, including photolithography in microfabrication and photopolymerization when using polymer monolithic stationary phases. LEDs also have many amazing applications in the broader area of life sciences, such as aiding tissue healing with near infrared (NIR) therapy, utilizing the pulsed capability of LEDs for fluorescence imaging of living cells, and reducing induced oxidative stress in cells... and the list goes on.

"LEDs produce quasi-monochromatic light, but what we really need is a broadband light source."

When assessing the potential of LEDs, we should really ask, "What *can't* LEDs do for the analytical scientist?" Clearly, LEDs have different properties to laser diodes. Apart from light coherence and directionality of laser diodes, their higher optical output compared to LEDs has often been to their advantage. Nevertheless, this is changing, as many new LEDs now achieve laser-like light output levels. And, what about the strengths and weaknesses of LEDs when compared with classical light sources?

LEDs produce quasi-monochromatic light, which can be advantageous, but in my view, what we really need is an LED that can provide a broadband

light source ranging from deep-UV (~200 nm) through the visible region to NIR. Such technology could replace bulky, fragile and expensive deuterium and similar lamps. A convincing proof of concept has been demonstrated for spectrophotometric detection; the technology exhibits lower noise and offers limits of detection that are several times higher than a standard D2 lamp (1). Will such a LED-functional equivalent of a D2 lamp become available in the future? Well, it will depend on two factors: namely, demand and the availability of deliverable technology. The first factor appears fulfilled. The second factor is trickier for me to judge as an analytical chemist, because it has everything to do with solid-state physics.

Clearly, it is possible to make LEDs with wavelengths at or below 200 nm, even when they are not yet commercially available for wavelengths below circa 230 nm. So, creating a broadband LED light source for deep UV might be a worthwhile challenge; however, this is where my judgment reaches its limits, so I'll stick to following future developments in IT, consumer electronics and other areas (including those used in greeting cards).

I believe we analytical scientists must follow LED developments in other areas and be ready to utilize them in our own research. For example, deep-UV LEDs with a wavelength below 250 nm have their uses for sterilization and water purification. Such use of light in 'non-analytical' applications is good for getting commercially available LEDs with enough optical power for analytical devices (especially those for fluorescence analysis) to market.

So, what is the future of LEDs and their use in analytical science? Bearing in mind their strengths and limitations, and their immense potential in many other areas, I think it is very bright indeed.

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Driving SERS into the Clinic

Raman spectroscopy has evolved into a powerful characterization technique for many applications. What more needs to be done to make it effective in clinical analysis?

By Mustafa Culha, Department of Genetics and Bioengineering, Yeditepe University, Istanbul, Turkey.

With the discovery of laser and subsequent advancements in laser and detector technology, the previously slow development of Raman spectroscopy moved into a higher gear. Raman spectroscopy has now proven its worth for analyzing biomacromolecules, including proteins and DNA, living cells, tissues, and microorganisms for detection and diagnosis.

Raman scattering, however, is a very weak process in which only one in a million photons is elastically scattered. An additional problem – autofluorescence – hinders the use of the technique in biological applications. Fortunately, in the early 1970s, a novel phenomenon was discovered where molecules in contact with (or in very close proximity to) noble metal surfaces, such as silver and gold, increased Raman scattering by up to 10^{11} times, which led to the development of surface-enhanced Raman scattering (SERS). In addition to enhanced scattering, SERS effectively quenches autofluorescence.

Although there is a lot of knowledge about SERS analysis of biological structures, in my opinion, there is a gap between research and clinical applications. Moreover, it is not going to be possible to translate the technique into real applications without understanding the needs and processes within a clinical setting.

For example, there are several issues that need careful consideration for sound interpretation of data gathered from a biological SERS experiment. First, the type of SERS substrate needs careful selection for the sample of interest. Should it be a nanostructured surface or colloidal nanoparticles, such as gold (AuNP) or silver nanoparticles (AgNP)? If the sample is a living cell, AuNPs or AgNPs can be a better choice. If the sample is microbiological, a surface or colloidal NP substrate is best.

After choosing the most appropriate substrate, it is important to test for reproducibility and applicability. The obtained spectral information should be evaluated by considering the selective interactions of the functional groups, such as SH and NH_2, with the noble metal surfaces, as these interactions define the environment.

For a decade, we have evaluated whether the technique can be used for clinical decision-making. We have analyzed living and dead cells, tissues, and microbiological samples using sample preparation methods developed and tested in our laboratories. We believe more has to be done to explore the potential of the technique because biological samples are not only very complex but also show variations from sample to sample.

Rapid identification of infectious microorganisms is critical for disease intervention in clinics. Although there are many studies demonstrating the proof of concept for utilizing SERS for fast microorganism identification, its capacity to identify them from clinical samples is not yet clear.

The complex nature of biological samples, such as blood and urine, is one of the major obstacles to decreasing the time needed for understanding the status of a sample. For example, in a urine sample, there could be several chemicals, including urea and creatinine, dissolved ions, white and red blood cells, and proteins together with infectious pathogens. These components may interfere with, or hinder, the SERS measurement without proper cleaning or separation, which of course increases analysis time. There are also several questions that need asking to determine the infection status of a urine sample. The first question seems obvious: is the sample infected or not? The numbers of bacteria in 1 mL of urine determine the answer, as only urine samples containing greater than 105 cfu/mL are considered to be infected. Then, we must ask which pathogen(s) is/are present? We then move onto asking if there is a marker that SERS can identify to show whether the urine is infected or not. Can the technique be used for the quantification of bacteria in the sample? Can the technique identify the pathogen?

We already know that SERS can identify bacteria, but further effort is needed to speed up the process from a complex sample. In my opinion, we are not that far from getting positive answers for some of these questions – and that will shorten the time needed to get SERS into a position where it can enhance clinical decision-making.

"There is a lot of knowledge about SERS analysis of biological structures, but there's a gap between research and clinical applications."

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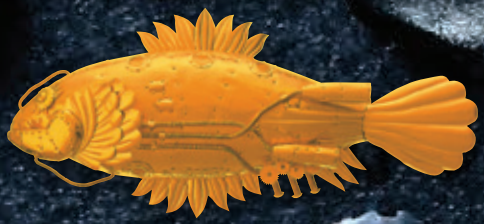

HPLC Columns

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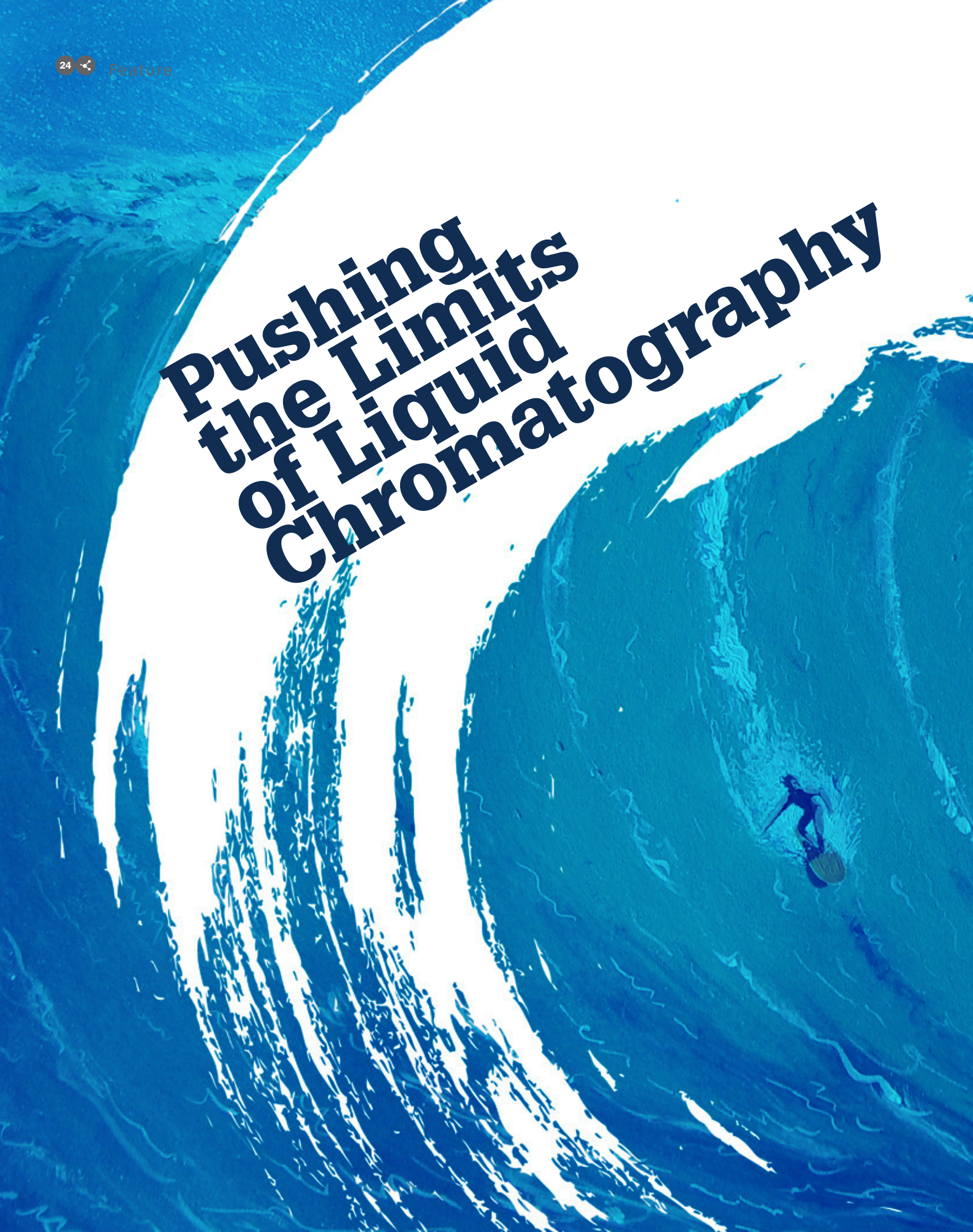


LC specialists in academia and industry – including Mary Wirth, Gert Desmet, James Jorgenson, Monika Dittman, and Fabrice Gritti – share a common and bold vision: to ensure that LC continues to be a platform for innovation rather than stuttering into stagnation. Here, our experts consider where we are, where we need to go, and how we get there.

Expanding LC Boundaries

We must inspire creative minds to keep LC moving forward

By Mary J. Wirth, W. Brooks Fortune Distinguished Professor, Department of Chemistry, Purdue University, West Lafayette, Indiana, USA.

Today, the pharmaceutical industry is a major user of liquid chromatography (LC), where stainless steel columns offer reproducibility and sensitive UV detection. The best commercially available LC columns for small-molecule separations now give about 50 percent more plates and faster separation times compared to 20 years ago. Even such a small improvement in resolution gained through the higher plate numbers is valuable for analysis of impurities and degradation products in pharmaceuticals – and the higher speed allows for faster methods development. Notably, separation speed has improved more than resolution.

The field has achieved these advances by decreasing the diffusion distance of the analytes, either with sub-2 µm fully porous or superficially porous particles. Both of these recent advances give comparable performance, and both advances were made on a sound theoretical foundation. For example, Jim Jorgenson and his group introduced sub-2 µm particles, and Jack Kirkland and co-workers introduced the superficially porous particles. Further reductions in diffusion distances will eventually give diminishing return, which means that diffusion distance will no longer be the limit. For large proteins, the best columns still give more peak dispersion than the best instruments, so some combination of packing heterogeneity, bonded phase, fittings and frits is apparently the main limit now.

Improving drug safety

As the primary users of LC are people in the pharmaceutical industry, both in drug development and in quality control, improvements in the field have essentially made drugs safer. Protein separations are a current and growing demand, both in the pharmaceutical industry, where protein drugs are the largest growth sector, and in proteomics, which is an integral part of biomedical research. Drug targets, cancer biomarkers, and diagnostics to monitor therapy usually involve proteins, and discovering these requires better columns due to the complexity of cell lysates and blood serum.

To that end, we need to think about the future and what steps we need to take. Advances require creativity, and one cannot really organize creativity. And, above all, we need to inspire creative minds to push the limits. Thankfully, there a number of people doing such pushing! For instance, Jack Kirkland continues to explore the limits of smaller diffusion distance, as well as the role of particle size, which affects packing homogeneity; Jim Jorgenson and Ulrich Tallarek (see next article) are addressing what underlies packing homogeneity; and Gert Desmet (see page 28) and his colleagues are trying to make the perfect LC column by micromachining.

There are many other efforts going on in the chromatography industry that are confidential, and we need to inspire more basic research. My own group, for example, is working on improving resolution in protein separations by improving packing homogeneity and avoiding the need for frits by using monodisperse colloidal silica.

Columns: still watching and waiting

Ultimately, the limit in any chromatographic separation is having the peak width determined only by diffusion of the analyte; that is to say, the instrument, the column, and other hardware contribute negligibly. This limit is only meaningful, however, if the separation time remains reasonable, since one could technically reach the diffusion limit by making the column length absurdly long. Therefore, the goal must be to reach the diffusion limit without making separation time or sensitivity worse than what we have today. Currently, the dispersion of the best columns is still quite a bit higher than the dispersion of the best instruments, so columns need attention, including the bonded phases. It is possible that connectors and frits also contribute to the dispersion.

Mass spectrometry, particularly top-down proteomics, demands higher resolution for protein separations by LC. Further, Fourier transform MS (FTMS) adds an additional constraint in column design because the mass resolution is dictated by the Heisenberg uncertainty principle. This means that the sharpest peaks in the time domain are no longer desirable since these would lower mass resolution. Instead, the sharpest peaks in the spatial domain are needed, with flow rate controlling the peak width in the time domain.

Daring to predict

Chromatography has changed very slowly in the past; for example, plate heights have dropped by about a factor of two over the last 20 years, and so, predicting the field five years from now is more daring than predicting over 10 years. Progress occurs slowly because the largest part of the LC market is regulated, meaning that change is not readily adopted. I think in 10 years we will at least be well on the road toward diffusion limited LC of small molecules, perhaps even with commercial products. For protein separation, it is certainly my own goal to enable diffusion limited LC-MS for top-down proteomics using capillaries. We have demonstrated that these can give diffusion limited separations that are fast, and the next goal is to do this with commercial instrumentation.

Have We Really Peaked?

Liquid chromatography is not just an analytical tool for today, it offers a great deal for the future.

By James Jorgenson, William Rand Kenan, Jr. Distinguished Professor, department of chemistry, University of North Carolina, Chapel Hill, USA.

Compared with 20 or 30 years ago, today's routine LC separations are much faster and have higher resolution. Columns are far better deactivated and much more base-tolerant and reliable. The biggest single benefit from these developments has been the increased productivity of the individual analyst.

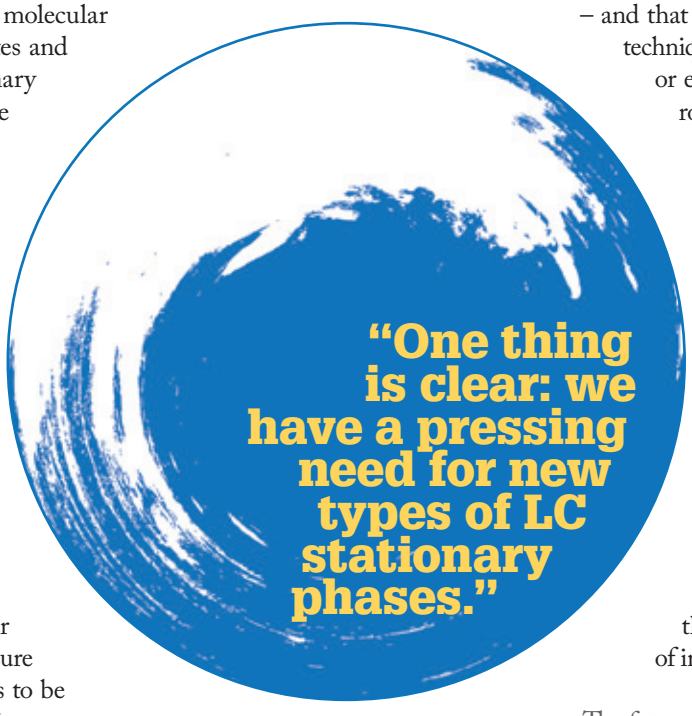
I won't mention names of individuals or vendors: it's not who you name, it's who you overlook and wish you had named. In reality, countless thousands of scientists, engineers and computer scientists have made important contributions to the development of LC. Identifiable important trends include the steady progress in developing and using smaller particles, the use of higher pressures, and development of instruments with decreased extra-column dispersion. Equally important are the improvements in silica support synthesis, more inert and hydrolytically stable bonded phases, and novel particle morphologies such as core-shell particles.

One of the sharpest tools in the box

The impact of LC is well recognized and it is regarded as an essential tool in the analytical toolbox. However, let's not forget that a lot of room for improvement and development remains. For

example, there is a real need – driven by proteomics as well as by protein-based pharmaceuticals – for better columns for separating intact proteins. Metabolomics is also driving the necessity for more capable stationary phases for retaining highly-polar metabolites, while using mass spectrometry (MS) compatible mobile phases. And, for the foreseeable future, nanomaterials and nanoparticles are poised to be important. Surely, LC offers much for the analysis and purification of nanoparticles; however, this may require improvements in modes such as hydrodynamic chromatography, size exclusion chromatography, or LC-related technologies, such as field-flow fractionation.

As we push the limits of LC, I would love to see more studies involving molecular dynamics simulations of analytes and their interactions with stationary and mobile phases. As people become more adept at molecular dynamics simulations, I think there is much we can learn about retention kinetics and equilibria. Smaller particles have been a steady trend in LC; and, I see no reason for that trend to stop. Also, the use of high temperatures in LC is interesting, but always must be taken on a case-by-case basis. The biggest concern is the thermal stability of the analytes. In addition, can we continue to aim for higher pressures? Why not? High pressure (unlike high temperature) tends to be benign in terms of analyte stability.



One thing is clear: we have a pressing need for new types of LC stationary phases. Classic reversed phase is wonderful, and will continue to be the workhorse method in the future, but it won't work for everything. There are a lot of questions that need answering, such as how do we best handle highly polar small molecules (for example, metabolites)? Are there possibilities for novel alternative mobile phases or mobile phase additives? Are there practical alternatives to water for higher-polarity mobile phases?

Risky business?

How can we as a community organize a dedicated effort to further advancing LC? Well, I can't think of anyone who would refuse more funding or collaboration for separations research. But in reality, collaborations tend to be best initiated from the bottom up by individual researchers. Furthermore, it is just as important for researchers in separations to interact with people in biochemistry, biology, materials science, engineering, synthetic chemistry, spectroscopy, and mass spectroscopy, as it is for them to interact with other people in separations. The most important thing is for grant awarding bodies to fund good people to do good research – and they shouldn't be afraid to fund risky research. Risky research usually fails, but it is also the source of unique new approaches...

As to limiting factors, in my opinion, we can never have enough resolution or peak capacity. We should never be satisfied with the status quo. There is no end to our imagination – and that opens up our horizons to other techniques; and how we work with them or embrace them. For example, the role of MS in analysis is constantly increasing. Any development within LC must maintain compatibility with MS. Top-down proteomics of intact proteins is increasingly viable as mass spectrometers become more powerful. We need highly effective columns for separating intact proteins coupled to these new mass spectrometers. There is a long way to go before the chromatography of intact proteins measures up to the great strides taken in the MS of intact proteins.

The future is full of promise, but...

When I look at what's changed over the past year, I am always disappointed. However, when I look at what has changed over a decade, I am always amazed. How does the incremental accumulation of 10 years' of disappointments eventually become an exciting qualitative shift in performance? I'm sure one factor is the occasional unanticipated paradigm-shifting development; the electrospray ionization source for MS, for example.

The introduction of monolithic columns as an intelligent stationary phase morphology is permitting greater freedom in design of column architecture. One of the questions that springs to mind about such developments is: can the dimensions of the physical features of successful and efficient monolithic columns be scaled down further to yield still greater efficiencies? The main

benefit so far of higher pressures and smaller particles has been greatly increased speed of analysis, with modest improvements in resolution and peak capacity. A second question then: can the benefits of high pressure be extended to longer columns with extraordinary resolving power?

It is difficult to predict what the next big new ideas will be, but a couple of things that cross my mind: will chromatography with slip-flow revolutionize separations, and will advanced 3D printing technologies permit direct fabrication of near-perfect chromatography columns? In reality, I don't know what the great developments of the next 10 years will be, but I am certain they will happen – and will be remarkable.

Marching Ever Onward

The evidence contradicts those who say liquid chromatography has fully matured.

By Gert Desmet, department head, chemical engineering, Free University of Brussels, Belgium.

In a recent survey looking back to 2002, my research group set out to compare optimal performances of LC. The starting date was well before the arrival of sub-2- µm columns. In those days, we found that the time needed to obtain 20,000 plates took about seven minutes, which with today's technology and techniques only takes 50 seconds (to measure a compound eluting with a retention factor of 10). And, in 2002 it took an impractically long 100 minutes to do an N=100,000 plates separation, whereas today we can do it in just over 20 minutes. It's impressive progress that contradicts those who say that chromatography is fully matured and so doesn't deserve further R&D.

LC has been – and continues to be – a platform for innovation. The first major breakthrough, for example, was the introduction of UHPLC inspired by the seminal work of James Jorgenson and promoted by the late Uwe Neue. The second big breakthrough was the reintroduction of core-shell particles around 2007. The latter breakthrough was somewhat serendipitous because the increase in efficiency compared to fully porous particles was much larger than could theoretically be expected, based on the reduction of the differential paths inside the particles alone.

Nothing stands still in LC. I think demands for faster and more efficient separations will continue hand in hand with the development of more efficient columns that reduce the time needed for method development – a very costly process in industry. We do need more efficient columns to support the current search for biomarkers; and, we need them for more general research, such as analyzing how the cells in our bodies are functioning and how we could cure them when something goes wrong. 2D-LC will certainly be needed to produce the required peak capacities for this type of research, but even then, the efficiency of the individual dimensions will first need to go up as well. And as biologists dig ever deeper into our bodies, aiming at single cell or even sub-cell level analysis, miniaturization of LC systems could become an important issue again.

Design for today, not the last century

Design is the limiting factor of today's instruments. They still have the same "hi-fi tower" design as those produced in the 1970s and 1980s. This form factor leads to such high levels of extra-column band broadening that we can say that our columns have become too good for our instruments, and we (or better, the instrument manufacturers) should do something about it to bring their designs into the 21st century!

Pressure is not an issue, as it seems theoretically and practically possible to run a column up to 3000 bar or so. As a matter of fact, Ken Broeckhoven and I are running a project on 2600 bar separation using normal bore columns and up until now things are going very well – we've had no explosions so far! So, in terms of mechanical strength there might be no fundamental impediment. What is trickier are the compressibility effects that make it more difficult to generate a precise flow rate, but perhaps there are ways to circumvent that as well.

No one can second-guess the future, but I do believe that we should be able to operate columns up to 2000 bar without any difficulty. I also think it should be possible to automatically couple up to three 10-cm columns packed with 1.5 µm core-shell particles within an integrated system that clamps the columns directly between the injector and detector to eliminate all connecting tubing.

Finally, because of the gradual decline in chromatography training for analytical scientists, we must improve the ability of instrument software to assist the analyst with decision-making. This – and more powerful instruments – will undoubtedly take our field even further forward.

Marking Progress

Monika Dittman (Agilent Technologies) and Fabrice Gritti (Waters) offer a manufacturer point of view on the practical limits of LC.

What is the current state of play?

Monika Dittman: In 1D-HPLC, using sub-2µm particles and (U)HPLC instruments operating at over 1000 bar, the time

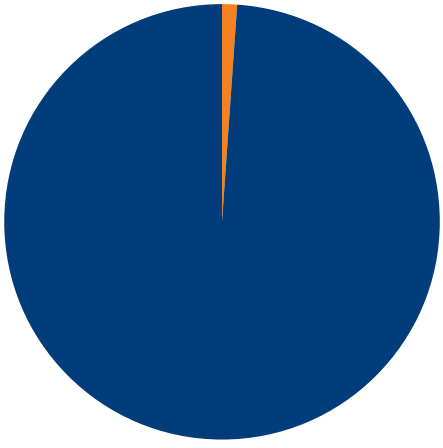
to reach a desired plate number has been reduced roughly by a factor of 5 (30,000 plates in four minutes with 1.8 µm particles at 1000 bar compared to 20 minutes with 5 µm particles at 300 bar) in routine analysis. The gain in performance can be used to obtain higher resolution in the same separation time (typically a factor of 3–4 on plate number or a factor of 1.5 in resolution/peak capacity), depending on the separation conditions.

These gains have enabled the fast separation of complex samples, and reduced the effort and time required for method development because a lack in selectivity is counteracted by increasing efficiency.

A further increase in operating pressure along with a further decrease in particle size could provide some additional improvement in speed and/or resolution, but 1D-HPLC eventually reaches a theoretical limit. Multidimensional LC can provide much larger peak capacities in a shorter time, which offers more power for very complex samples. And although it cannot be considered a routine technology right now, commercial solutions are available and being used by an increasing number of researchers.

Fabrice Gritti: Fast 1D-LC analyses of moderately complex samples and biomolecules is possible with short (5–10 cm) and narrow-bore (2.1 mm) columns packed with sub-2 µm particles. For the same level of efficiency, the analysis times in UHPLC have decreased by a factor of 10 relative to those observed in HPLC using 15-cm long columns packed with 5 µm particles. The volume consumption of eluent is also reduced by a factor of 10. The only downside is that system pressures have increased from 100-300 in HPLC to 400-1200 bars in UHPLC. The milestone for such an achievement was the emergence in 2004 of instrument pumps capable of delivering high flow rates at pressure of 1 kbar and the preparation of high strength sub-2 µm silica particles.

For all that, UHPLC rapidly touched its limits in terms of resolution because most standard UHPLC systems cannot provide more than 20 percent of the intrinsic efficiency of short (5 cm) and narrow-bore (2.1 mm) columns packed with sub-2 µm core-shell particles. System dispersion must be reduced further without precluding detection sensitivity (by diminishing the UV cell volume and path length) and speed (by shrinking the inner diameter of the connecting tubes). The transfer of United



Method Programming

Sample Processing

States Pharmacopeia methods from HPLC to UHPLC is also a critical issue because pressure and frictional heating affect retention (identification), peak width (resolution), and peak area (quantification) in UHPLC.

Since 2007, the lowest reduced plate heights of 2.1 mm to 4.6 mm i.d. analytical columns (capillary columns excluded) have been reached with core-shell packing materials (h=1.4) and 2.7 µm Halo silica particles. These developments mean there is no need for ultra-high pressures but it is still necessary to optimize standard HPLC instruments to reduce band spreading.

Resolving complex sample mixtures is routine practice using online and offline 2D (time x time) LC. Some commercial instruments provide customers with the possibility of 2D analyses, which plays an important role in polymer separation, in proteomics, and in genomics. Yet, 2D-LC faces some practical difficulties in terms of column orthogonality, solvent compatibility, sensitivity loss, and MS detection rate. For instance, the digest of host cell proteins having a dynamic range of concentration from one to more than a million cannot be solved satisfactorily by 2D-LC/MS.

Hyphenated LC/MSn techniques are now a routine analysis tool in any lab around the world. LC is still indispensable to fractionate complex mixtures, while MS enables identification of all the precursor and product ions. The limits of MS detection are quantification and the scan rate.

What contributed to these advancements in LC?

MD: Calvin Giddings laid the foundation for ultra-high pressure LC back in the 1960s when he predicted that gains in resolution/time could be achieved with higher operating pressures. Then, more than 20 years ago, James Jorgenson's group demonstrated extremely high column efficiencies when operating with 1 µm particles at very high pressures.

In 2004, Waters introduced the first commercial system that could operate at 1000 bar, together with 1.7-µm particle packed columns, which subsequently led to every major HPLC equipment vendor offering UHPLC systems with operating pressures up to 1500 bar. Although routine analyses are still performed under standard HPLC conditions, customers often buy UHPLC systems when replacing older equipment.

FG: I agree with Monika. Giddings' basic theory of chromatography has continuously pushed manufacturers to innovate and build new instruments (higher pressure, smaller system dispersion, 2D separation systems) and smaller particles to achieve faster separation and higher resolution performances by LC. Theory is obviously a major driving force for innovation.

The success of the sub-3 µm core-shell packing materials for analyzing small molecules is due to some unexpected properties of their beds. They are more radially uniform than those packed with conventional particles. This observation was not predicted by any theory of chromatography, which explains why most column manufacturers were initially reluctant in preparing such new materials. So, innovations can also result from pure luck! In contrast, Horvath predicted the use of core-shell particles for separating heavier molecules in the early 1960s based on the reduction of the average diffusion time across giant 50 µm particles.

In any case, technical achievements are delivering faster analyses, higher analysis throughput, and solving more complex sample mixtures than the old conventional HPLC techniques. But we must not rest on our laurels; separation science still faces numerous challenges:

- The complete resolution of protein mixtures in host cells for which the dynamic range of concentration extends from 1 to 10^7. This requires better isolation of the least abundant from the most concentrated proteins, more accurate quantification of the detected ions, and higher MS scan rates.
- The separation of glycan-binding protein for determining biological mechanisms.
- The separation of protein isoforms that differ by a few mutations of amino acids.
- A need to increase analysis throughput of chiral candidates during the drug discovery process demanded by pharmaceutical companies.

Where are we heading?

FG: Computerized molecular dynamics studies will further refine our established, basic theories of chromatography (Schure, Tallarek, and Mountain all talk about this). These studies enable a deeper understanding of the mass transfer and retention processes in LC from a molecular scale. In the end, they will consolidate or correct our old theories, and, in practice, they will enable the new design of the structures (kinetics or mass transfer) and of the chemistries (thermodynamics or selectivity) of separation systems for targeted performance.

UHPLC systems (injection, tubes, connections, detection) and column hardware (end-fittings + frits) will need redesigning to benefit fully from short (< 5cm) narrow-bore (1-2.1 mm i.d.) columns packed with 0.5 µm particles for analyzing biomolecules at low linear velocity.

Sub-1 µm particles are currently useless for the analysis of small molecules: that said, UHPLC systems would have to withstand pressures as high as 3 kbar to deliver optimum velocity. This could become a reality in years to come. Ideally, on-column injection/detection is required to eliminate system bandspreading. Ultra-low small injection volume (< 10 nL), short and small i.d. connecting tubes (< 50 µm), small volume detection cell (< 100

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nL) or on-tube detection, and improved column end-fittings need developing. Currently, we are just reaching the limits of miniaturizing "classical" UHPLC parts, operating in a range of flow rate from 0.1 to 1.0 mL/min with today's technology.

We must push research efforts for fully integrated LC-MS separation systems using either packed micro-channels or capillary tubes (100-500 µm i.d.) as separation media. The challenge with small volume separation systems is the detection sensitivity (too short path length). New detection techniques other than UV-Vis are therefore needed. Light emitting diodes are a solution (excellent monochromaticity and noiseless signal), yet they cannot cover a wide continuous range of wavelengths. In terms of speed, a larger number of compounds will be identified if the scan time of MS detectors is reduced. Injection, separation, and MS detection devices should be integrated to minimize losses of resolution and sensitivity.

What are the limits?

MD: With modern UHPLC instruments and small particles we have come very close to the limits that can theoretically be reached in 1D LC. However, very important improvements would include ease-of-use and maintenance, cost of ownership, overall system intelligence – those things that ultimately improve efficiency and reduce cost. With multidimensional LC, the complexity of instrumentation and applications had been limiting its extended use, but this is starting to change.

FG: The current limiting factors of UHPLC techniques in terms of resolution and speed are essentially instrumental: the column hardware affects the resolution power of short columns severely for the least retained compounds, small UV cells are reducing sensitivity, and very narrow connecting tubes generate too much backpressure for the current generation of pumps.

What other technologies/techniques will affect LC?

MD: We should expect to see MS-systems that keep pace with fast separations. In addition, we need intelligent software to not only generate data but also enable their meaningful use – one of the current bottlenecks is efficient and fast handling of data produced by ultra-fast, ultra-high resolution analysis.

Where do you expect LC to be in five years?

MD: MS will become more of a routine detection method (as in GC already) and multidimensional LC will become standard for medium complex samples. In addition, multidimensional LC-MS (or even multidimensional LC-ion mobility spectrometry-MS/MS) will become standard for complex samples.

As the majority of LC users will not be separation scientists but organic chemists or biologists the big challenge will be to create intelligent software solutions that support users in instrument operation, method development and data interpretation. This will be of particular importance for the success of multi-dimensional LC to be adopted as a routine technique.

FG: In five years from now, we should expect that UHPLC coupled to MS detection will finally play a major role in most academic and industrial laboratories. The high price of UHPLC technology, the difficulty to transfer well-established methods developed for HPLC to new methods for UHPLC, and the need for integrated MS detectors (everyone needs a LC-MS detector now, not a simple LC-UV instrument) holds back progress in the instrument market. SFC-based instrumentations (SFC-MS, LC-SFC-MS or SFC-LC-MS) will become available and friendly to use given the numerous advantages of supercritical CO^2 fluids (cheap and green solvent, fast mass transfer and high resolution, high analysis speed).

The boom in micro-fluidic devices will likely provide a new generation of high-resolution portable instrumentation for routine analysis. Nano-LC/MS at very high pressures should enable the use of relatively short capillary columns or micro-channels packed with sub-1 µm particles for the analysis of biological samples.

If we look further to 10 years, we may see the emergence of 3D-printing technologies capable of reproducing perfectly ordered column structures with a characteristic dimension smaller than a few µm. Fabricated from relatively cheap raw silicon material, these printed columns could well supplant the current silica-based particulate and monolith technologies.

Looking forward even further, the ultimate and unified analytical tool, which could solve any of the analysts' needs will feature in a single apparatus selected extraction units (by either liquid or supercritical fluids), selected injection devices (head space for GC analysis of volatiles, trap beds for SFC-based extracts, standard LC injectors), selected pump devices (for GC, SFC, or LC separations), switch-column valves (for 2D analyses), and MSn detection. All these technologies are currently available but as separate units for most. The fundamental, engineering, and technical challenges required to bring them altogether are obviously immense but not out of reach in the long term. At least, research efforts towards this direction should be funded and encouraged.

From an academic viewpoint, separation scientists are becoming a rare species even close to extinction after the passing of Giddings, Horvath, and Guiochon. Chromatography is no longer considered as an indispensable science instead is seen as merely a preparative tool. Yet, it should be remembered that most improvements made in GC and LC over the last 50 years were based on sound physical chemistry, material chemistry, and engineering. Future challenges in separation sciences and their new technologies to come will not escape this rule.

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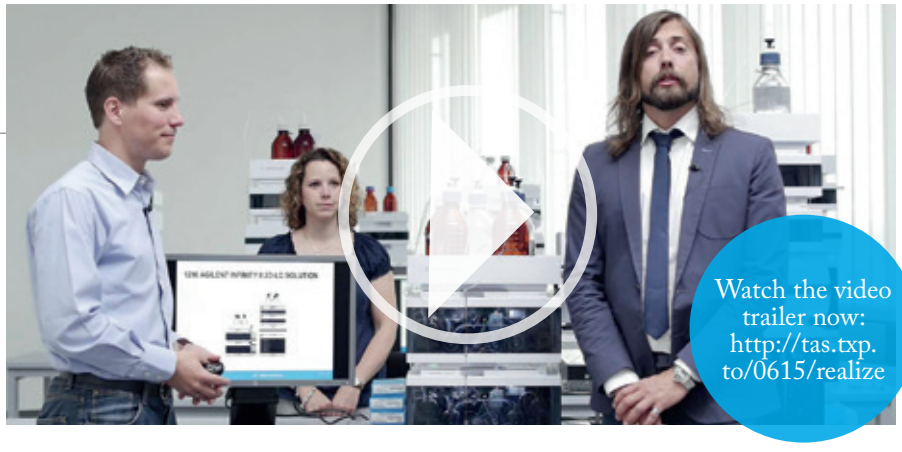

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Finding Humanity in Science

What drives someone to throw aside selfish pursuits to focus on projects with true philanthropic impact? Here, the winners of the 2015 Humanity in Science Award provide their answers.

The 2015 Humanity in Science Award was presented jointly to Peter Seeberger and Andreas Seidel-Morgenstern, directors at two collaborating Max Planck institutes in Germany. By coupling flow chemistry with advanced chromatography methods, Seeberger and Seidel-Morgenstern were able to manufacture artemisinin-based therapies – the most effective drugs to treat malaria – from plant waste material, air and light. The science is innovative and exciting, and the potential impact of their project – and the concepts born from it – could really shake things up in the pharmaceutical industry.

To explore the project in more detail, we highlight some key elements from Seeberger and Seidel-Morgernstern's submission to the Humanity in Science Award (www.humanityinscience.com):

"Today, the key active pharmaceutical ingredients of all artemisinin combination therapies are produced in one or two chemical steps from artemisinin (see Figure 1). The majority of artemisinin (~200 tons per year) is extracted from a plant (Artemisia annua) cultivated for the purpose and prices fluctuate with harvest yields [...]

"Seeberger initially developed the photochemical continuous synthesis of artemisinin from DHAA in 40 percent yield at 200g per day. Careful optimization of the reaction parameters of the continuous flow semi-synthesis resulted in a greatly simplified process and a significantly improved yield. Today, the process can be combined with continuous purification methods to obtain artemisinin of greater than 99.9 percent purity [...]

"To demonstrate the power of the fully continuous synthesis/purification regime, Seeberger and Seidel-Morgenstern developed a continuous three-stage, multi-column chromatographic/crystallographic purification method for α-artesunate (see Figure 2) [...]

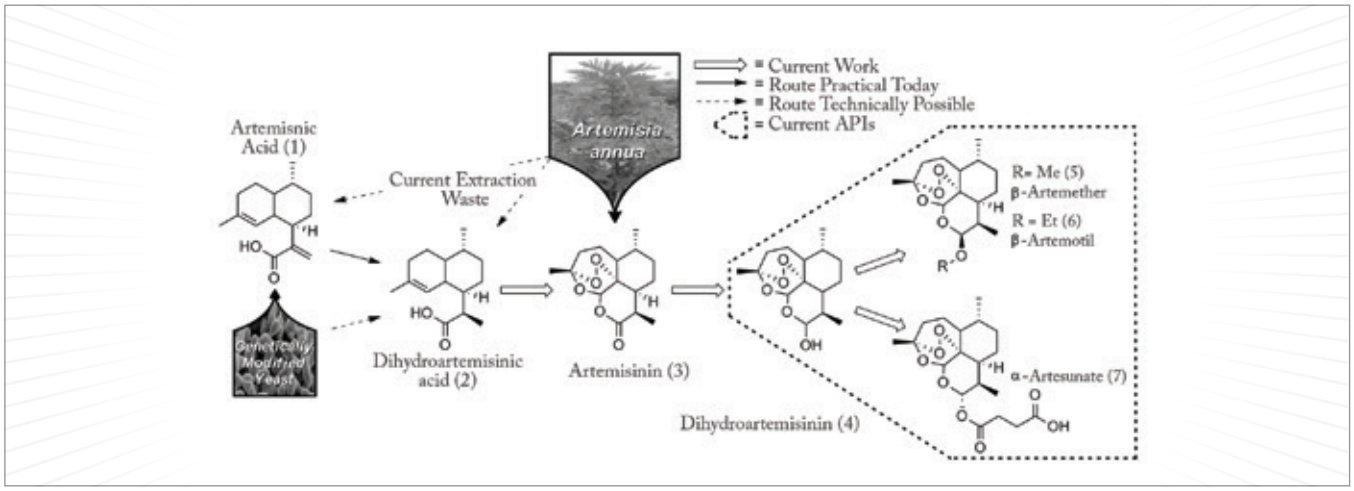

Figure 1. Production scheme of anti-malaria APIs from artemisinin obtained by extraction from Artemisia annua and genetically modified yeast combined with chemical modification. Dihydroartemisinin (4, combined with piperaquine in Eurartesim, Artekin and Duo-Cotecxin), α- artemether (5, combined with lumefantrine in Coartem), α-arteether (6, Artemotil), and α-artesunate (7, combined with amodiaquine in Coarsucam and ASAQ-Winthrop).

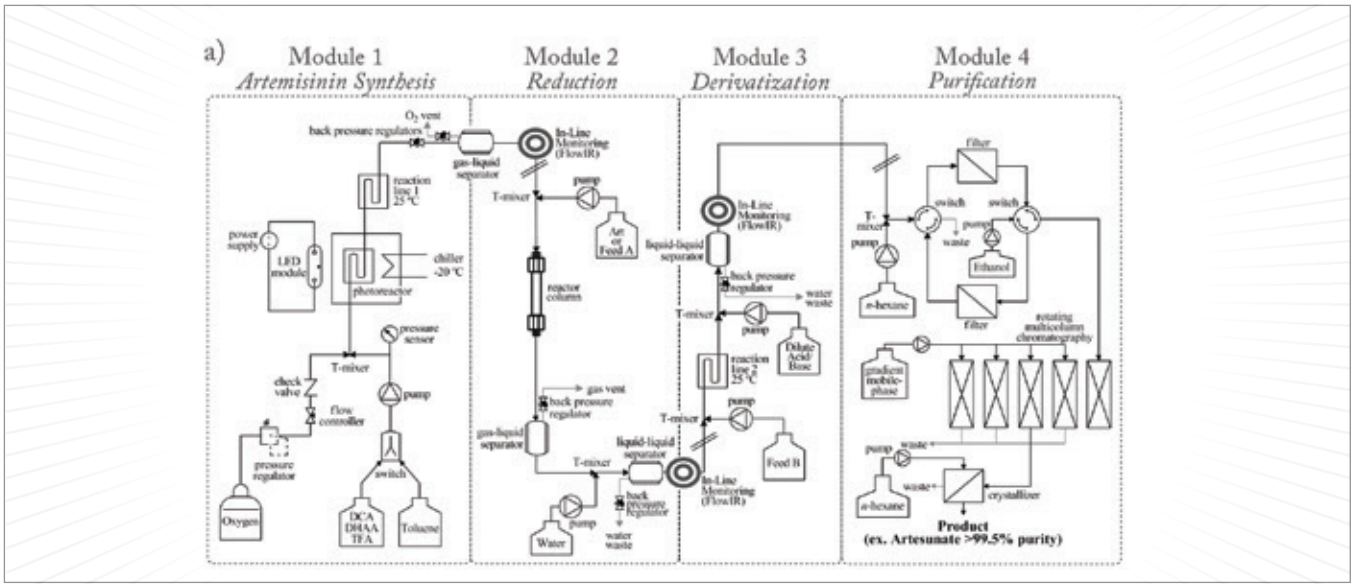

Figure 2. Four-module chemical assembly line system for the continuous synthesis and purification of artemisinin APIs. DCA: 9,10-dicyanoanthracene, DHAA: dihydroartemisinic acid, TFA: trifluoroacetic acid, Art: artemisinin.

"The process Seeberger and Seidel-Morgenstern developed is currently implemented in a pilot plant in Vietnam to enable the production of less expensive anti-malaria medications and to increase participation of developing nations in the value chain of drug production."

Now, let's focus on the personal stories of the duo that led the project to discover what seeds humanity in science.

You can read the full submission to the Humanity in Science Award online: tas.txp.to/0615/HiS.

Using Entrepreneurship and Chemistry for Good

Peter Seeberger, Professor and Director of the Max Planck Institute of Colloids and Interfaces, Potsdam, Germany.

How did you get into chemistry?

I grew up in Nuremberg in Bavaria and was the first member of my family to go to university. I guess I was a good high school student, because I qualified for the highest possible scholarship for Bavaria, something that is awarded to just a select few. I could have studied anything, but I chose chemistry.

I then had to do my mandatory national service in the German army, which further motivated my pursuit of chemistry – the armed forces were definitely not for me. I studied both chemistry and business to begin with, but I eventually focused on chemistry because it gave me the chance to stand out from the crowd. I studied chemistry for three years at University Erlangen-Nuremberg with a full scholarship. The program normally took five to six years to complete, but after three years I had finished and was nominated for a full graduate scholarship to go to the US for a year. I applied to both Berkeley and Colorado universities and ended up going to Colorado, which was great as I like skiing...

I finished my PhD in Colorado working with Marvin Caruthers – a member of the US National Academy who famously automated DNA synthesis and set up many companies, including Amgen. Working with him made me realize that doing very good chemistry could also help you to do very good biology. The idea of starting up companies was also interesting.

So you moved again?

Right. Merrifield won a Nobel Prize in Chemistry in 1984 for chemical peptide synthesis on a solid matrix and I thought I could do something similar for carbohydrates. To prepare myself, I applied to work with the best-known carbohydrate chemist of the time – Sam Danishefsky, professor at Memorial Sloan Kettering Cancer Center and Columbia University. He accepted me into his lab in New York, where I worked extremely hard – 18-hour days, seven days a week – for two years. I focused on developing methods for carbohydrate synthesis.

I'd already lined up a job in Germany as an assistant professor, but before I could accept it Danishefsky called me to his office at 1am on December 23 and asked me what I'd be doing after leaving his lab. He encouraged me to apply to Massachusetts Institute of Technology (MIT) – and I did. They invited me to give a talk and then I had a day-long interview. The following day I received an offer to be an assistant professor at MIT. I accepted.

Why was Danishefsky so keen to push you to MIT?

Danishefsky encouraged many of his people to apply to leading institutions. I'd published 12 papers with him and he seemed to think I would be a good match for MIT.

Moving to MIT was the best career decision I ever made, so I'm thankful to Danishefsky for pointing me in the right direction. Often, your choices in life are due to the influence of your mentors and role models. Without their influence, I would never have considered applying to top universities. When you come from Bavaria, places like Harvard and MIT are pretty far away – and not just in distance.

I did not know what to expect at MIT, but it worked out OK. I remember that in the first three days of starting my job, one of my colleagues asked me to go to the faculty lunch room and the provost said to me, "Young man, what do you think of your chances of getting tenure?" I replied that I had no idea, but I guess I was lucky because after about four years I was promoted to tenured professor at MIT at the age of 35.

Sometime later, ETH (the Swiss Federal Institute of Technology) in Zurich made me an offer I could not refuse. I'd been in the USA for 13 years and thought I would probably stay there my whole life. If I turned down ETH, I thought it would be difficult to return to Europe.

Initially, I didn't find life at ETH easy because I'd been Americanized both in the way I spoke English and in my etiquette. It was a learning process for both sides I think. I was there for six years, met my partner (who was a professor in Berlin) and had a daughter. The commute between Zurich and Berlin needed fixing.

The Max Planck society offered me a job to take over a directorship at the Institute for Medicine in Heidelberg. It was a good offer, but it would not improve my family situation (travelling between Heidelberg and Berlin is actually worse than travelling from Zurich to Berlin). However, the people at Max Planck were persistent and suggested that I join an institute in Potsdam where they would erect a new building for us. I have to say I was anxious about the move. When I left MIT it was one of the most difficult days of my life because I was not sure whether I made the right decision. I was in a similar situation and knew there would be a lot of things I would miss about Switzerland. That said, I have been lucky in life and felt it would turn out well.

What brought you such success?

First of all, you have to pick a good area to work in. Glycosciences is a fantastic area with seven Nobel Prize winners up until the early 1970s – and glycans are everywhere. The advances in molecular biology of the mid-1970s and the new found ability to manipulate DNA for proteins meant carbohydrates

"Often, your choices in life are due to the influence of your mentors and role models"

took a back seat and the technologies for enabling glycomics and glycobiology were lacking. I had expertise in DNA and peptide and carbohydrate chemistry that no one else had at the time. Many said that my idea for automated synthesis of carbohydrates wouldn't work, but it was a smart choice given my background.

I also work really hard – I'm very driven. I don't think I'm more intelligent than the next guy, but perhaps I am able to see interesting areas that enable long-term programs rather than just solving little puzzles.

And that approach fits in with your work at Max Planck where you are building platforms?

Yes – and that's how I got interested in flow chemistry, which is part of the work we received the Humanity in Science Award for. It's something I've been involved in since my days at MIT, where I remember hearing a talk by a physicist who had begun working on flow chemistry while he was in Germany. He talked about how you do chemistry in pipes instead of buckets, and that really appealed to me.

I started building systems and platforms. And we slowly began to get involved in medicine – after all, though my students are very well trained in carbohydrate chemistry, they need experience with drug molecules to improve their employment prospects! In fact, most of the people we train go into industry; more than 200 of them have left to get really good jobs. But I've also seen 47 professors come out of my lab.

It's fantastic to have the opportunity to convince talented young chemists to work with me. I give directions to make sure we get to a certain point, but the important work is done and implemented by the young scientists we train. If our students weren't as diligent, things could have gone in a very different direction.

What drove you to explore antimalarial drug manufacture?

Early on in your career, you want to make sure you publish the best possible papers. Then one day you ask yourself: "Do I want to publish another paper or do I want to make a real impact?" I knew that if I wanted to have impact on a global scale, I needed to work on something that could improve other people's lives, particularly those who have little chance themselves. So, that's why I started to think about things like malaria and HIV (we're also working on cheap antibiotics and anticancer medications). The artemisinin project was the first step in that direction. I'd usually work on vaccines – a cheap means to prevent disease – but with artemisinin, it became clear that the medication is there, but a huge part of the global population (the ones who most need it) can't afford to buy it. You can talk to people who travel to Africa and they will tell you that they had to lock up their antimalarial drugs to prevent the cleaning staff from stealing it. The inequality at the heart of the problem is just wrong.

I thought, "we can do something about this problem". But it wasn't enough to simply publish a new method – we had to take it further than that. It is very difficult to translate published research into something that can be implemented and taken to market; it's very frustrating to see good existing solutions that are simply not being implemented in developing countries. And it's sad that so many people are leaving Africa because of the bad conditions – the real solution is to help these people have better lives without them having to leave. I can only make a small contribution, but one of our goals is to convince governments to do something regarding technology, but even there I see some reluctance.

But much of the world is governed by economics – and that has to change, right?

When the artemisinin story first broke, it was all over the news. People said, "This is great, congratulations!" But without a lot of effort, it won't see the light of day because the big companies will try to bury it. Pharma companies are traded on the stock market so they have to make profits. One of the big questions of our times is how are we going to evolve drugs in the future? For example, I know that if any of us were investing in a pension fund, we'd expect the fund managers to make the best decision about putting our money into a drug company or into a car company. Is the drug company expected to give a big discount or free drugs to Africa? What about the car company? Does it have no obligation to give a discount? Right now, the system is not really geared towards improving health for a large part of the global population.

Unfortunately, I don't have a total solution to that problem – just a small piece of the puzzle. But wouldn't it be great if some really smart people stopped to think about changing the world of pharmaceuticals – not just how they are manufactured, but also how they evolve and how they are paid for. I do believe in a free market, but the population of poor people around the world is growing and being left behind – that can't be right. I don't think it's good to live in a world where very few people are very rich and a greater number of people have nothing. If I look back on my own life in Germany in the 1970s and 1980s, there were very few poor people and very few rich people. We see in many countries that the middle classes are being dissolved – the polarization of society. I don't think it would be fun to be part of these societies – even if you are rich.

Preparing to Change the World

Andreas Seidel-Morgenstern, Director of the Department of Physical and Chemical Foundation of Process Engineering, Max Planck Institute for Dynamics of Complex Technical Systems, Magdeburg, Germany.

Take us back to the early days...

I grew up and worked in what was East Germany – right up until I got my PhD, which I did in 1987 in the former Academy of Sciences' Institute of Physical Chemistry in East Berlin. It was an interesting time, but also very political. In fact, my scientific career was more or less over after I completed my PhD because I refused to become a member of the East German communist party. Later in life, I read my Stasi [The Ministry for State Security] file, which noted that I wasn't loyal and should not benefit from promotion. At the time, I did not really appreciate how strict the system was.

Then in 1989, the wall came down. It was a new world for me. I had my PhD and I wondered what I should do next. Because the "iron curtain" no longer trapped me, I contacted the Technical University in West Berlin where a colleague (who later became my boss) helped me become a more active member of the German chemical engineering community. Later, I thought I should move to an English-speaking country to see more of the world.

So you moved to the USA?

Right. My wife is a chemical engineer as well – we met in the old East Germany and we already had a family, but we were not married. One day she came home and said the company she was working for was collapsing, but there was an opportunity in Tennessee, USA. That evening I was thinking about a few papers I'd read from a guy in Tennessee – I could not recall his name so I searched my files and found him: Georges Guiochon, University of Tennessee, Knoxville.

The next day I wrote him a letter to ask if he had any positions open. About three weeks later the answer came back: "yes." I included a research project idea in the letter – a smart move – Georges liked it. He offered me a post-doc position and my

"Devising strong, widely applicable technologies is what interests us the most."

wife and I married to make it easier to move to the USA with the children as a family.

Why do you think Georges was so keen to work with you?

Georges' mathematical oriented views on chemistry and in particular chromatography meant he always had very good foreign experts. I guess he liked to work with anyone – foreigners included – that fit in with his strategy. I brought a new idea that was related to my PhD (how to calculate competitive adsorption isotherms) and I think he saw this field as a chance to enrich his own scope.

I could have stayed longer in the USA, but I applied for and received a grant from the German Science Foundation to support three-years at the Technical University of Berlin to do my habilitation. At the same time, Georges suggested that I should stay in the USA – this was a tough decision. However, we returned to Germany. We had young children and were still very poor; it was financially risky to remain in the USA – and my widowed mother was alone back home in Germany.

So, in 1992, we returned to Germany and I did a relatively rapid habilitation. I had many results so it was easy. Unfortunately, I did it too quickly because the grant rule agreement essentially said, "when you finish, we stop paying you". To make ends meet, I got a job with Schering in Berlin, but I wasn't there for very long; my boss encouraged me to apply for academic positions, noting an opportunity in Magdeburg, which I took.

And that was the connection to the Max Planck Society?

Yes. Three years later, there was an unexpected situation in Magdeburg. Germany was united and the Max Planck Society (supported by taxes) started to invest in the former East of Germany to ensure an even distribution of funding throughout the German states. Up until then, Magdeburg and the federal state Saxony-Anhalt had not received much support. The society decided to form a new institute on Dynamics of Complex Technical Systems, which now houses more than 200 people.

I became director of the institute in 2002, and we now have many projects in various engineering areas – chromatography is just one of them. By training, I am a reaction engineer and so we also do a lot of analyzing and quantifying reaction processes, which broadens our separation science based scope.

The Max Planck Society meets once or twice a year at annual meetings, and that's how I met Peter Seeberger. I quickly realized we were well matched; his group was strong in chemistry whereas we had expertise in designing continuous separation processes. We connected and now have quite a few stories to tell.

How do you work with Peter?

Peter's group focuses on certain target molecules and constantly comes up with new and fascinating chemistries. Very often, his

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reaction pathways are challenging (and very clever), but connecting them directly to our separation processes can be difficult. We started studying a simple model reaction. Then we worked intensively on artemisinin and artesunate. Currently, we are looking together for new target molecules to further contribute to this area.

My overarching goal is to develop universal methods that can be applied generically. Devising strong, widely applicable technologies is what interests us the most. Nevertheless, we are not naïve; we know that every separation problem brings its surprises. We need sufficient flexibility to fine-tune what we do.

The malaria story is wonderful. Our job was to establish a separation concept to isolate a valuable component from a complex mixture. And we have many other examples of similar problems in biotechnology. Typically, a single product will exist alongside many conflicting by-products. How can we structure the separation problem in a more generic way? If you think of chromatography, you could have a sample containing 100 components, but component 17 is your target. How do you get it? We consider this a pseudo ternary separation problem. One to 16 forms a big fraction before the target – the first fraction. The second fraction is your target – component 17 in a very narrow window, followed by another big third fraction (18–100). If you find a process that looks at the problem in the same way – for example, ternary simulated moving bed (SMB) chromatography – you can tune various pump flow rates representing the crucial process parameters to enable you to isolate any target from any mixture. Of course, in practice you have to connect several process steps together and you need to recycle streams if you want to be efficient and not lose valuable materials. That's characteristic of our way of looking at problems.

What would you like to leave behind for future generations?

We now have expertise in separating enantiomers and I would like to expand that knowledge into new chiral compounds. Agrochemistry, for example, is an interesting area. We apply large amounts of agrochemicals onto fields, but they are often chiral meaning that we could often be wasting 50 percent of these products simply because we don't understand their exact mechanisms and we do not have access to the active form. If we separate these mixtures into pure enantiomers, we will find that one will be more effective than the mixture. These are very cheap molecules compared with pharmaceuticals, so the agrochemical industry is not keen on using costly SMB technology, complicated columns, or high-pressure pumps. They need cheap separation technology and that leads us away from chromatography and into crystallization.

Now, we are working very intensively on a fantastic process called preferential crystallization that might be suitable for resolving the enantiomers of chiral agrichemicals. Imagine you have a solution containing two dissolved enantiomers in equal amounts. If you cool down the solution, the enantiomers would crystallize in the same way producing a 50:50 mixture solid phase. So, how can we separate such components by crystallization? The key is to cool very carefully – there is a window of maybe just two or three Kelvin below the solubility temperature, in which the solution will remain metastable. Although there's a driving force for crystallization, it's so small that we can keep the solution clear for many hours. We then seed this metastable solution with crystals of the target enantiomer and that will start the crystallization process. The seed crystals preferentially incorporate molecules of the same type due to stronger interactions. We are currently working on a fluidized bed process where we can demonstrate the feasibility and power of this technology. The process should offer a much cheaper solution for agrochemical targets.

That's a very specific process option but to summarize, I dream of creating and developing more efficient and cheaper separation alternatives that take greater advantage of molecular interactions between solid and liquid phases.

What do you find most scientifically rewarding?

From a scientific perspective, I like to to quantify processes. Organic synthesis chemists like Peter validate the success of their synthesis reactions using proven analytical methods. That's not enough for me. Quantification means finding ways to predict the outcomes of processes and to design them with respect to a specific objective; for example, to decide how big a reactor or a chromatographic column should be to do a certain job in an optimal manner. To reach such goals we need to know many details – the rates of the reactions taking place in the reactor, for example. To understand these rates, we need to understand molecular aspects that create them, which requires a broader perspective that bridges multiple scales.

Thermodynamics teach us that our fantastically organized planet is not a stable system – it can't last forever. The multiscale approach looks at this problem in a quantitative and systematic way, covering multiple time and length scales. It proceeds from molecular models, macroscopic kinetic a thermodynamic models, models for specific apparatuses, calculations on the plant level to simulations evaluating process impacts at the earth level and beyond. The challenge is to develop tools that transfer the knowledge to the next level without losing too much precision and resolution. This multiscale view, which attracts increasing attention within the various communities, needs more research on all levels. People who have expertise in connecting various scales will be important and highly sought after in the future.

In addition, I think that researchers should be happy to work intensively with young people who always ask new questions. Currently, I teach a lot and I like it very much. I fully support the classical view of Wilhelm von Humboldt that universities always need to balance and unite teaching and research. Indeed, besides doing good research, we should all endeavour to be good teachers.

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Reinventing GC-MS

At ASMS 2015, GC-Orbitrap™ technology was unleashed onto an expectant analytical community. Here's the backstory.

By Joshua Coon and Nicholas Kwiecien, Department of Chemistry, University of Wisconsin-Madison, USA.

Historically, The Coon Research Group has been focused on protein analysis with mass spectrometry. More recently, we've been interested in small molecule work in the field of metabolomics. It's pretty clear that quantifying small molecules can give a better correlation with biological phenotype than work further upstream. Moreover, until very recently, it was an area in serious need of new technology – and that's where our interest in coupling gas chromatography with Orbitrap technology started. As a group, we're very driven by new technology and its application to problems – especially when there's such a fundamental gap. Sure, you can already detect these small molecules pretty effectively with mass spectrometry, but more often than not, you can't understand their chemical formula. And it's very hard to go from signals in a spectrum to biological function, if you don't know what the molecule is... How can we identify these structures? Well, GC coupled with Orbitrap and its accurate mass capability seemed to be a great starting point to solve this problem.

Seize the gap

Clearly, there is a big difference between recognizing a gap and attempting to fill it. But fortuitously in the mid 2000s, we worked on a separate development project in collaboration with Thermo Fisher Scientific on electron transfer dissociation (ETD) for the Orbitrap, and we all recognized that it would be relatively straightforward to use that test system to try GC on an Orbitrap. The first 'Frankenstein's' system certainly wasn't practical, but it gave us data. In fact, it worked so well that another collaborative project was initiated to further investigate the potential. The short version of the story is that those initial efforts sparked Thermo Fisher Scientific's development cycle (led on the R&D side by Brody Guckenberger and Scott Quarmby) for the commercial instrument that was released at ASMS 2015: the Q Exactive GC™.

Of course, going from a proof-of-concept system to commercial instrument is in no way straightforward. And a big – often overlooked – part of the journey involves leveraging informatics. That's where Nicholas (Nick) Kwiecien stepped up to the plate. We were generating a lot of data – and if you knew what you were analyzing, you could get the right answers. But how do you go backwards? Nick expressed interest in trying to figure it out and came up with some outstanding ideas on how to leverage accurate mass to get back to structure.

For the past 50 years or so, people have been using GC-MS systems equipped with unit resolution mass analyzers – and that means there are a lot of great resources out there in terms of mass spectra repositories. The big question became: how can we leverage those resources? The answer led us to an innovative algorithm call high-resolution filtering (HRF), which is incorporated into the data processing software for the new instrument. HRF is uniquely enabled by the mass accuracy provided by Orbitrap technology and allows us to search existing reference databases with our acquired spectra in the same way as people have been doing for many years. But because we have such precise accurate mass, we can annotate every single peak in a spectrum using a simple combinatorial process. We take combinations of atoms from putatively identified molecules

and map those forward to peaks. The approach was extremely discriminatory against false positives, and should really increase the throughput of mapping unknowns back to structure.

Taking GC Orbitrap for a spin

We've taken on a large number of proteomics studies – thousands of different cell lines or hundreds of tissue samples – to try to understand how protein abundance varies from sample to another. Now, we can complement all of those experiments with deep and high-quality metabolome profiles generated by the Q Exactive GC.

Our first acquisition of a 1200 sample set showed that the correlation between the metabolome and proteome profiles is remarkably close. It turns out that it's much easier and faster to collect metabolome profiles GC-Orbitrap technology than it is to do proteomics. Given very large sample sets, we envision that our group – and many others – are likely to perform broad metabolome work to discover the most meaningful population subsets ahead of further work in the proteomics space.

With high quality data for both the proteome and the metabolome, you can investigate a small molecule with raised abundance and match it to the upregulated enzyme responsible. Such studies really allow you to understand function across the whole pathway at multiple molecular planes – from small molecule to protein.

Monitoring reactions

The folks at ASMS 2015 that we've spoken to seem very interested in acquiring the technology, you can almost hear them thinking how they can integrate GC-Orbitrap technology into their work. And certainly there have been lots of questions. Perhaps more interestingly, people who have not traditionally done metabolomic work (certainly, not in the way that we have done) appear to be seriously tempted by the possibilities. Indeed, there is a distinct air of surprise surrounding some of the corresponding proteome and metabolome results we've been able to show – especially at the scale we've worked on.

In our own lab there have been moments of surprise too. Frankly, we were quite shocked by how well the new instrument worked right out of the box. We'd been using the proof-of-concept system, which was not really capable of the sample throughput needed for our large-scale studies. So when we set up the new instrument and realized that the crew at Thermo Fisher Scientific had taken the GC-Orbitrap concept to a completely different level. The Q Exactive GC was a real surprise – in a very good way. Suddenly, we had the throughput to match the quality of the data.

People also seem really excited about the capability of the software tools mentioned earlier that are included with the instrument. I think our most fundamental contribution (besides providing a motivating force for instrument development) is offering the solution to deal with the data. I guess that sort of capability is on everyone's wish list – but previously we didn't have the right data to permit those kinds of algorithms. Now, we do.

Beyond metabolomics

Our group is very excited about the instrument's ability to map unknowns. But there are a lot of areas where scientists want to look for compounds that they already know – in pesticides and sports doping, for example. If you know what you're looking for, the system still offers many benefits. The accurate mass really boosts sensitivity, because you can pick out targets from chemical noise. It means you can achieve the level of sensitivity for target analysis that is approximately the same as the most sensitive GC instrument – the triple quad. But (and it's a big but) you can cover all the ions in the spectrum. Where sensitivity coupled with full scan capability is highly sought after, GC-Orbitrap technology will be of great interest.

From an informatics point of view, the fact that the data is so remarkably reproducible is also a pretty big deal. For our largest scale project to date, we had to cope with data files that were collected 45 days apart – but the runs looked the same. Such reproducibility really helps you gain access to meaningful results much faster – and it also facilitates the writing of custom code to analyze your data.

10th Anniversary

At ASMS 2015, Orbitrap celebrated its 10th birthday. Where will GC-Orbitrap technology be at its own party in 2025? Well, you can bet that the instrument will continue to improve over the next 10 years – that's just the trajectory of Orbitrap technology. At the same time, we're rapidly going to get a handle on unknown mapping and quantitation. Assigning identifications to unknowns is the current bottleneck in metabolomics (and a lot of other small molecule analyses) – and that's simply got to change. Accurate mass will allow people to go beyond current spectral libraries – and who knows how far software will have come by then? In terms of scale, today we're running 1000 samples and that's considered impressive. In 10 years, people won't be shocked by numbers 10 or 20 times bigger. And at that scale, you can almost force discovery.

As the technology rolls out, it's very likely that it will be used in areas that we cannot even envisage right now. Even talking to people at ASMS this year, exciting new ideas are already pouring forth; it's clear that once you introduce powerful new technology, the sky is the limit.

Video interview with Joshua Coon: tas.txp.to/0615/JoshuaCoon
To find out more: thermoscientific.com/HRAMGCMS

Solutions

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The (not so) EZ Journey to Improved GC Methods

In 2014, The Analytical Scientist Innovation Awards recognized our new and simplified EZGC® method development software. Here, we explore the complex roots that led to its creation.

By Chris Nelson, Jaap de Zeeuw, Jack Cochran, and Chris English

The Problem

Laboratories are constrained by requirements for extremely high sample throughput and rapid method development. How can a GC method-modeling program that was initially aimed at advanced users be repurposed to provide quick solutions to difficult separations?

Background

Restek's EZGC line of software tools for chromatography has a history dating back to the release of the original Pro ezGC DOS program, built in collaboration with Analytical Innovations in 1992. The first Pro ezGC software made it possible to predict retention times and optimize chromatographic methods without the need to analyze compound sets under many different conditions.

Built in the era of Intel 486 CPUs, the Pro ezGC tool was incredibly powerful for chromatographic modeling for its day. It contained algorithms that were capable of computing thermodynamic retention indices and complex chromatographic models quickly and accurately, while contending with the challenges of limited computer memory and space. Two products were initially available: the basic ezGC application, which modeled chromatographic separations based on pre-generated libraries of thermodynamic retention indices, and the Pro version, which allowed the user to create new libraries.

The Windows version of Pro ezGC software was released in 1994, and the product was built using some of the first object-oriented software for the Windows graphical user interface (GUI). And while the software was powerful for its time, it still had to contend with the limits of the systems it could run on. The thermodynamic index libraries were limited to 99 compounds, and the compound names themselves had character limits. In addition, the chromatographic models that were generated were limited to 100 compounds. But within those limits, the Pro ezGC program was able to perform impressively, evaluating thousands of models in a matter of minutes.

It was this power that was employed in labs around the world, including internally at Restek, where it is still used for the development of applications as well as optimizing new products. (The majority of the chromatograms found on our website were developed using Pro ezGC software.)

One notable application of the Pro ezGC program was the determination of the phase, film thickness, and conditions for columns that would eventually be used in the Philae lander's GC instrument. Robert Sternberg and his GC team at LISA, the Laboratoire Interuniversitaire des Systèmes Atmosphériques of the University of Paris XII, needed to develop methods to separate origin-of-life compounds in support of the European Space Agency's (ESA) Rosetta mission in its rendezvous with comet 67P/Churyumov-Gerasimenko (see tas.txp.to/0615/rosetta for more information).

LISA had very specific requirements in determining the columns to be included in the Philae lander. Power restrictions limited the analysis time as well as the average temperature of the analysis, and columns with a small inner diameter were needed to limit the consumption of carrier gas. Using Pro ezGC software they were able to select the stationary phases they would need by simultaneously adjusting film thickness, temperature, column length, column internal diameter, and flow. The results of the work required that

Restek manufacture custom columns based on LISA's Pro ezGC predictive modeling. The models closely matched the manufactured columns as published back in 1999 (1).

To be effective, Pro ezGC software requires the user to have a solid understanding of GC parameters. For example, the user must understand that lower starting temperatures are necessary when performing splitless injections, but a higher starting temperature is possible when operating in the split mode. Indeed, the user must answer several questions in advance because all parameters are chosen by the user, not the software; for example, is there a polarity mismatch between the compounds and the column? What carrier gas will work best with the instrument, and what is its maximum ramp rate? In other words, the speed of the Pro ezGC program is not helpful if injection port conditions, carrier gas choice, and GC limitations are not clearly understood.

As the years passed, the Windows platform changed significantly. Today, to run the Pro ezGC program in Windows Vista and 7, you need to use virtual machine. And it will simply not run in Windows 8. This new reality – coupled with the complex interface and the library size limits – was a key driver in our decision to update the software.

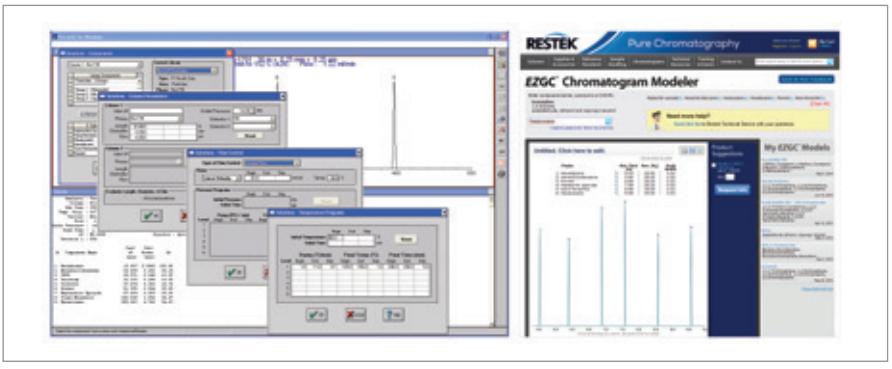


Figure 1: The single input field of the EZGC® chromatogram modeler replaced the complex, multi-form interface of Pro ezGC® software, creating a much simpler interface for the user.

The (New) Solution

In 2010, Neil Mosesman (head of Restek's Technical Service department at the time) convened a meeting with the Analytical Innovations team to determine a way forward for the Pro ezGC tool. Many ideas were proffered, from simply redoing the application for Windows 7 and above, to moving the whole application online. Funnily enough, the main concern voiced had nothing to do with the platform we chose, but instead with the target market for the new application; Pro ezGC software was not designed to help customers who work in high throughput labs that require instant column recommendations with column conditions.

The big question became: how do we re-purpose this admittedly complex tool to meet the needs of a new user base? Neil stepped in to help focus the problem: "What one thing do all of these users have in common?" The answer? "A list of compounds that must be separated." Suddenly, we had a solid basis for a new proposal; we want to take a list of compounds from the user and return a modeled result – with no user intervention. The model will be a "good" result, where good is defined as our best attempt to meet or exceed baseline resolution for all compounds requested on an appropriate phase. The resulting model would include a column recommendation (phase and dimensions), basic instrument conditions, and a recommended oven temperature program. The idea quickly caught on with the group and was reflected in our final project proposal.

Of course, implementing the idea was far easier said than done. Nearly two years of planning and development involved migrating code from the Windows platform to run on Linux web servers to take advantage of the increase in processing power and memory. In addition, we created and refined the tool's business logic and algorithms so that the software could produce a result quickly enough to match expectations set by the fast response times of modern websites.

And even with the pared down interface we were planning, there were significant usability issues that we needed to address. One hurdle was compound nomenclature, which required that compound names be routed through a synonym database to aid compound matches. Compounds could have up to a dozen different names and the program would have to accommodate any one of them. To further help customers hit the target, all the compounds in our database needed to have a phonetic key to help with alternate spellings or misspellings. And for customers who did not find their compounds matched in the Pro ezGC library, we performed a search in our chromatogram database as a backup (which was especially helpful with permanent gas requests where the best solutions are found on porous layer open tubular (PLOT) columns).

The end result – rebranded as the EZGC chromatogram modeler – was made available as a web application so customers would always have the latest version and never have to worry about the software becoming out-of-date. And the multiple dialog boxes and form fields of the previous interface were pared down to a single input field

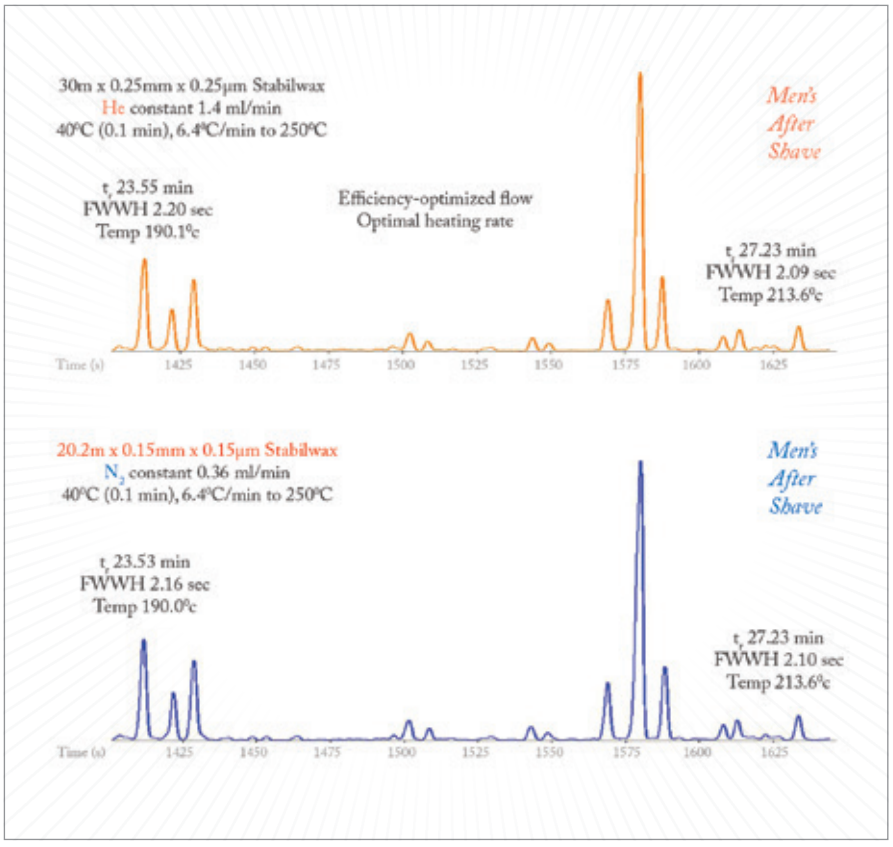


Figure 2: The conditions for using nitrogen carrier gas that were provided by the EZGC® method translator produced chromatography that is virtually identical to the original helium-based method. This is an example of how even novice users can generate new methods that allow them to move away from expensive and hard-to-source helium carrier gas.

(see Figure 1). Users need only enter a list of target analytes into the field and the software returns GC column and condition recommendations that are optimized for the user's specific analyte list, providing a significant leg up on method development.

The second app in the suite of tools – the EZGC method translator and flow calculator – was developed at the request of Jack Cochran, Director of New Business and Technology, who, along with Tom Kane, Director of Research and Innovation, oversaw the development of the tools and provided great advice on the interface and behavior of the application. The method translator was developed to bring back some of the functionality that was left out of the chromatogram modeler, while simplifying the interface and bringing a modern look and feel to a tool that chemists may use every day in their work. Customers could now take the conditions from a modeled chromatogram and tailor them to their needs while maintaining the elution temperature profile of the original model. We also paired the method translator with a flow calculator, and added the ability to move data between the two interfaces. The combined tool has many potential applications for method optimization and translation; it can be used to establish proper conditions when switching carrier gases, speeding up analyses, changing column dimensions, or moving from an FID to an MS detector.

Beyond the Solution

Restek's method translator was recently used by our own Jaap de Zeeuw to validate an idea that came to him in a dream (Jack says that Jaap does some of his best work while asleep!). The dream, which woke him up at 3am one night on a business trip to Singapore, was that using nitrogen on a 0.15 mm column should allow for a clean translation from a 0.25 mm column using helium, without needing to alter the temperature program or increase analysis time. He quickly tested and tuned his idea/dream in the method translator and then passed the idea by Jack, who followed up that weekend with some experiments on both a polar Stabilwax column (see Figure 2) and a nonpolar Rtx-CLPesticides column to verify the solution the software provided. Nearly identical analyses were obtained using nitrogen and the translated method, proving just how effective the software can be in aiding method development – or confirming the prophetic nature of dreams.

The journey from the powerful (but complex) Pro ezGC program to the newly reconfigured (and simplified) EZGC method development software has been an exciting one. Through the collaboration of analytical chemists and software developers, we were able to boil the input requirements of the original program down to a single essential question – what compounds do you need to separate? From there, further method optimization is possible, giving GC users a fast, easy-to-use tool for solving real-world problems.

Chris Nelson is a member of the web development team, Jaap de Zeeuw is International GC Specialist, Jack Cochran is Director of New Business and Technology, and Chris English is Innovations Laboratory Manager – all at Restek Corporation, Bellefonte, Pennsylvania, USA.

Reference

1. *C. Szopa et al., "Gas Chromatography for In Situ Analysis of a Cometary Nucleus: Characterization and Optimization of Diphenyl/Dimethylpolysiloxane Stationary Phases", J. Chromatogr. A 863, 157 (1999).*

In Search of Counterfeit Olive Oil using the Spark Spectral Sensor

By Miriam Mowatt

Background

Color is a great visual indicator of oil type, but it's not always possible to tell edible oils apart just by looking at them. With spectral analysis using a device like Spark, subtle differences among olive oil types can be observed (Figure 1). Spectral analysis brings more accuracy to color measurements than RGB sensor measurement techniques.

Monitoring Dilutions of High Quality Olive Oils

Dilution is a common way to counterfeit extra virgin olive oil. Figure 2 shows absorbance changes taking place with dilutions of the extra virgin olive oil. This is noticeable with the absorbance peak at 680 nm, which becomes very low at the lower concentrations of extra virgin olive oil. Spectral features from 380-500 nm also reveal the effects of dilution.

Discrimination between Real Extra Virgin Olive Oil and Adulterated Oils

Low-grade oils can be made to look like extra virgin olive oil through the addition of chlorophyll. For our testing, samples of sunflower and rapeseed oils were prepared with natural chlorophyll, extracted from spinach, to look like real olive oil. Visual differences among the adulterated oils were difficult to discern.

The addition of chlorophyll significantly changes the absorbance spectrum of these oils, which is notable in sunflower oil at 390 nm (Figure 3). When chlorophyll is added, the oil color is darker and "greener" than the original light yellow color.

Conclusions

A compact, low-cost spectral sensor like Spark can monitor olive oil to authenticate product quality and to detect counterfeit products. This was demonstrated for dilutions of extra virgin olive oil with cheaper oils, and for the addition of chlorophyll to cheaper oils to replicate the characteristic golden color of olive oil. Similar measurement methods could be scaled for laboratory analysis, integrated onto process lines or embedded into other instrumentation for quality control.

Tel: +1 727 733 2447
Email: Info@OceanOptics.com
www.oceanoptics.com

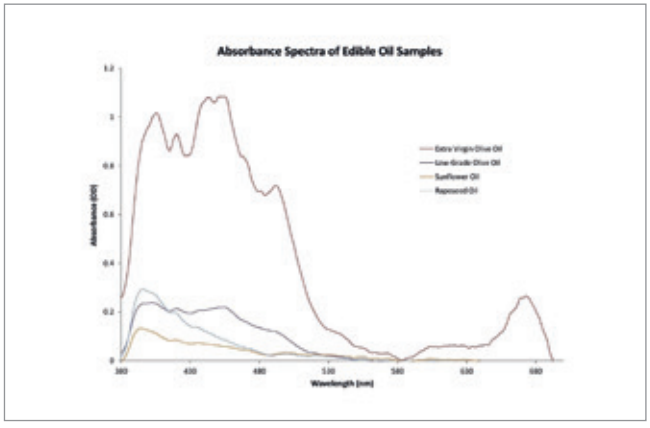


Figure 1 - Each type of oil has a unique spectral shape determined by the organic compounds present in the oil.

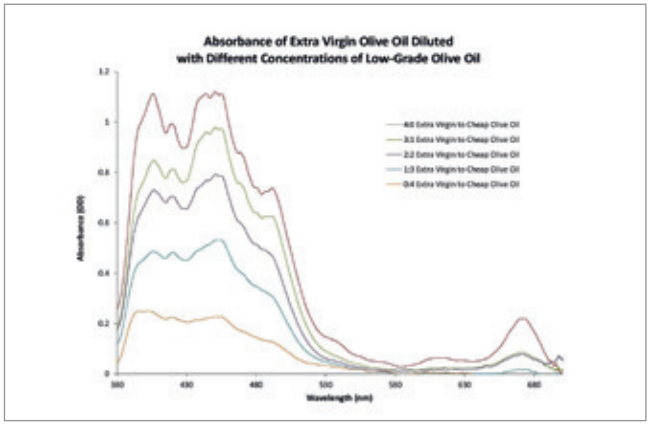


Figure 2 - Each ratio of extra virgin and regular olive oil was tested for absorbance using the Spark spectral sensor. The results show a significant change with each dilution.

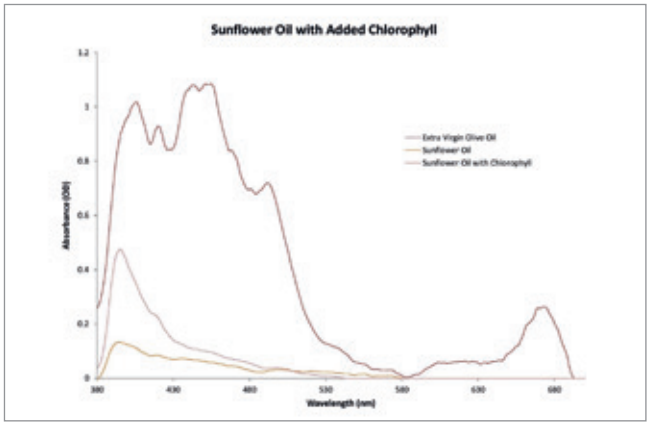
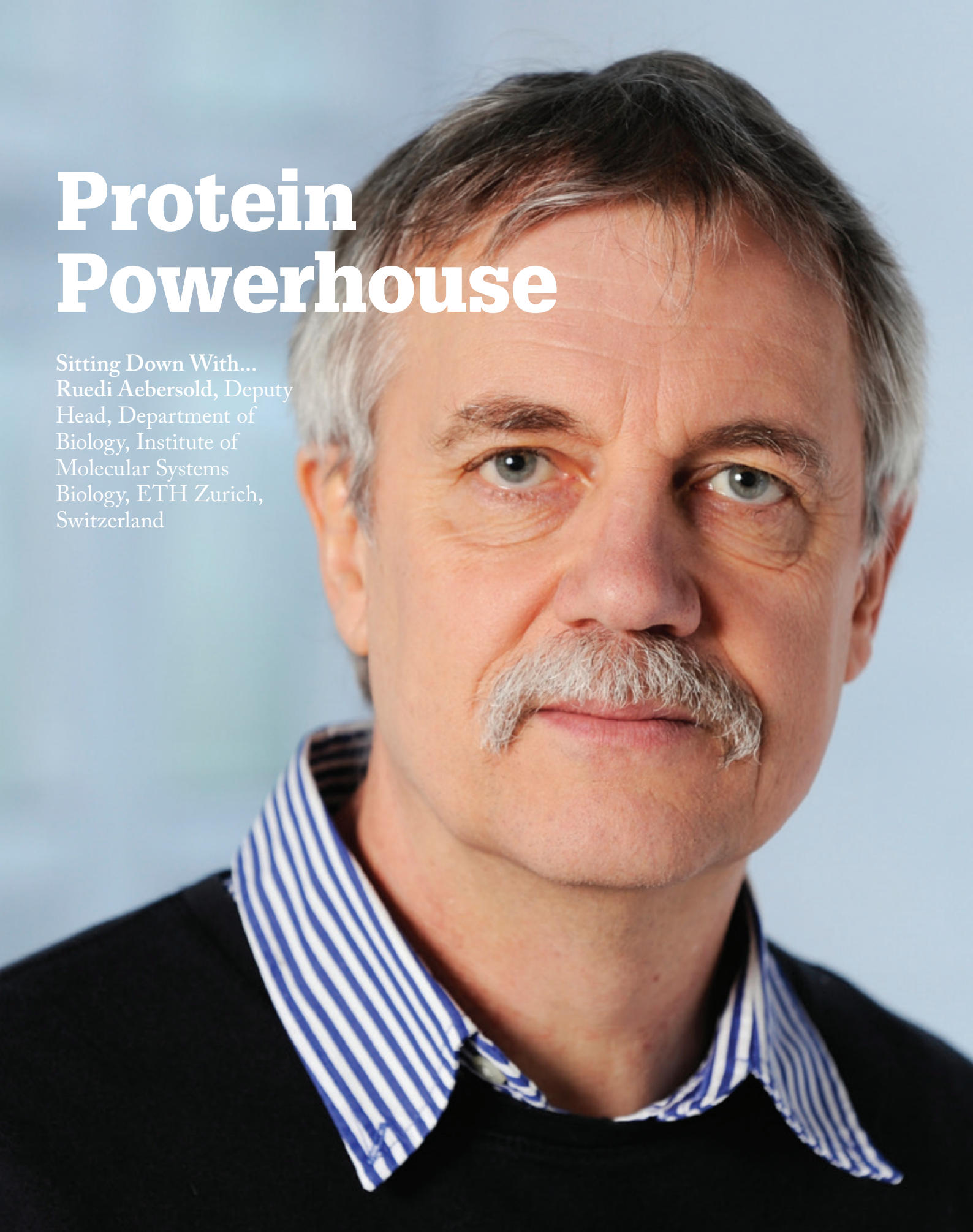


Figure 3 – Adulterants such as chlorophyll may be added to lesser-grade edible oils and passed off as premium-grade extra virgin olive oil. Spectral analysis helps to identify the adulterated samples.

Protein Powerhouse

Sitting Down With...
Ruedi Aebersold, Deputy Head, Department of Biology, Institute of Molecular Systems Biology, ETH Zurich, Switzerland



You are giving a keynote lecture at HPLC – how about a sneak preview?

I'll likely cover the new ability of proteomics to quantify proteins highly reproducibly across many samples, allowing a much better understanding of how biochemical systems function. One of the big, high priority questions is how genomic variability translates into different phenotypes, traits, and diseases. We can now measure genomic variability for whole populations with very high precision – we need to map that to function and abundances of proteins and enzymes in the cell.

How did you get into analytical science?

I read a book when I was younger that really opened my eyes to science. It explained everything from background microwave radiation to protein folding. The author had an amazing ability to put things in perspective. I ended up doing a PhD in the dark ages of immunology – it was an unpredictable field back then and the big questions didn't seem tractable. I started to shift focus to analyzing proteins using Edman degradation.

But you moved from one difficult area to another?

That's true! The light chain (200 amino acids) of an antibody took about half a year to sequence – a lot of manual (pretty boring) work. But it meant that we could relate biophysical measurements of monoclonal antibodies to affinity, sequence and specificity; the effort was worth it. But at around the same time, gene sequencing was really starting to take off. Suddenly, what took me half a year to sequence at the protein level took only days or weeks to do at the nucleic acid level. I remember suggesting to my supervisor that we switch to modern nucleic acid techniques. "No," he said, "we are a protein chemistry laboratory." I continued to work with proteins, which in retrospect was great because I got to optimize skills that were essential later.

So you moved into proteomics?

I realized that we were very limited in terms of techniques. I decided to join the most innovative laboratory of its the time in that areas – the lab of Lee Wood at Caltech – to help advance protein sequencing technology. I figured out how to take a band that was detected in the SDS gel and directly obtain amino acid sequence of the protein in the band, connecting protein sequencing methodology to the most commonly used tool for protein separation at the time.

Then mass spectrometry hit your field...

Right. In the late 1980s, two breakthrough methods came along: electrospray ionization and MALDI. All of a sudden, we could measure proteins and peptides by mass spectrometry very routinely. Questions quickly shifted to how you get proteins that usually occur in cells or tissue in a complex mixture into the mass spectrometer in a form that the protein can be measured. These were exactly the techniques I had worked on as a post doc; we knew exactly how to separate small amounts of proteins, enzymatically digest them, and separate the resulting peptides. Our head-start allowed us to make fairly fast inroads into the burgeoning field of proteomics.

What is your main contribution to proteomics?

The field was advancing pretty fast, with various pioneering contributions from great colleagues along the way – online LC-MS from Don Hunt's lab and SEQUEST from John Yates, for example. But the field needed to move beyond simply creating an inventory of proteins; we wanted to be able to quantitatively compare protein abundance in multiple samples. We knew we had a good solution. Initially, we introduced isotope coded affinity tag (ICAT) methods and then later came SILAC from Matthias Mann's group and a whole range of other labeling techniques.

How do you stay at the cutting edge?

You have to be driven by biological questions. We never approach a problem solely with an analytical goal, such as 10 times more sensitive detection of a peptide or the identification of a higher number of proteins from a specific sample. In many cases, the most important parameter is not how many proteins we see or quantify (though it would be great to cover everything!) but rather precision and reproducibility in the measurements we do make. We are focused on very high consistency across dozens or hundreds of samples – that's how SWATH came about. Initially, we could only precisely quantify 50-100 proteins with high consistency, via selected reaction monitoring; now, we can do several thousand and the data are becoming extremely informative. Of course, we want to dig deeper into the proteome – but while maintaining critical consistency.

What advice would you give to your 30-year-old self?

What I say to students and post docs: make connections earlier to scientific colleagues and peers. Go to conferences – make yourself known in the field; ultimately it is the network you operate in that is important, not just what you do. If you can build a network – inside and outside your field – you will achieve far more than being in isolation and trying to change the world alone. In short: network, open up and discuss things!

Some people seem worried that if they talk too early about a project or idea, someone will beat them to fame and glory. In my experience, it's extremely rare that someone has taken something, run with it, and scooped us. Usually, people provide feedback – there is enormous knowledge around. The chances of you being scooped by talking about your ideas is miniscule compared to the potential benefits you receive from feedback. I plea for people to share their ideas and openly discuss them. Then we can move forward together.

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- ✓ Breaking the limits of **metabolomics technologies**

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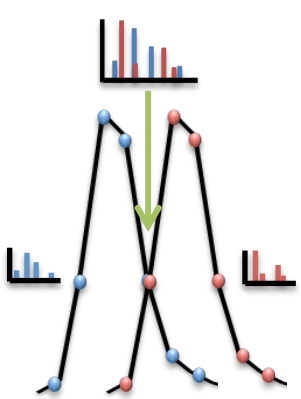

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