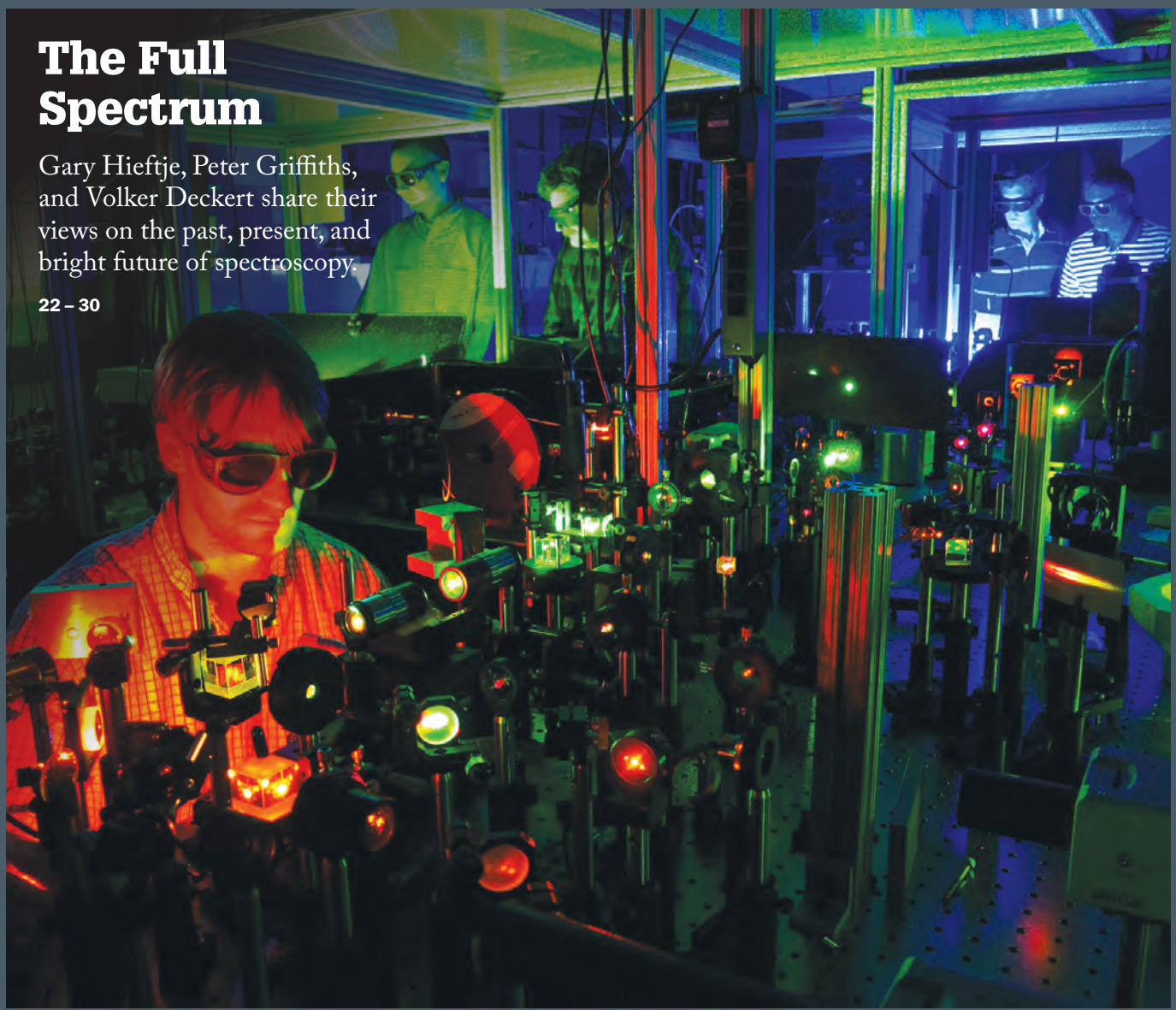
MAY 2014 # 16

the Analytical Scientist



The Full Spectrum

Gary Hieftje, Peter Griffiths, and Volker Deckert share their views on the past, present, and bright future of spectroscopy.

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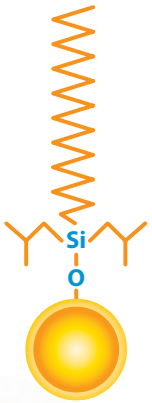

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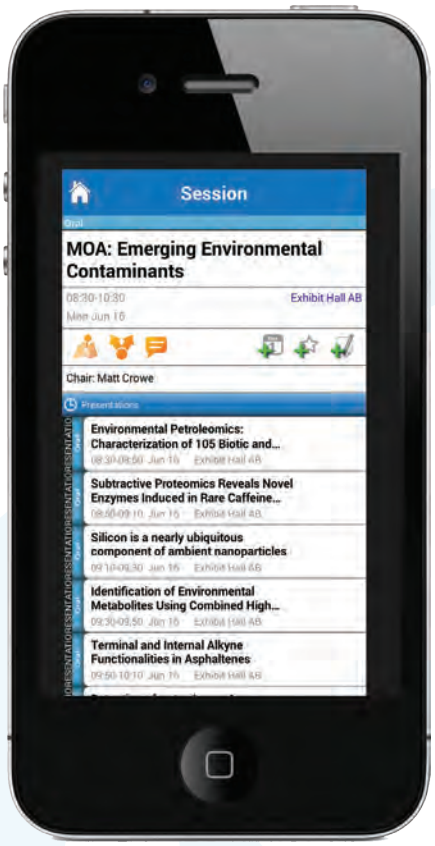
Online this Month

Pocket ASMS

App stores have gifted us a great many productivity aids, communication channels – and let's face it, pointless games – but the conference and symposium planners that are fast replacing hefty paper-based guides are starting to become shoulder-saving masterpieces.



The 2014 American Society for Mass Spectrometry (ASMS) conference is just around the corner (see page 14 for our Top Picks), so now may be a good time to get acquainted with the new app, available from Google Play and Apple App stores. This year's iteration follows the latest trend of adding personalized schedule information; by logging on, you can sync your device with the online planner. Very neat.

But two new features that really caught our eye were "Session Commenting" and "Attendee Messaging". Session commenting allows you to start discussions before, during or after poster presentations and lectures, truly embracing the social aspects of our Brave New World. Likewise, attendee messaging allows you to reach out to other logged on members to organize business meetings, poster discussions – or just lunch.

It's good to see that innovation at ASMS extends beyond the exhibitor booths and lectures into the apps that help us navigate our way around them.

Download for...

iOS: http://ativ.me/53y
Android: http://ativ.me/53z

Pandora's (Black) Box

Does analytical research mean anything? (tas.txp.to/0414/blackbox)

To quote Wolfgang Lindner: "Nowadays, fellow scientists tend to look on us analytical chemists as collaborators who generate data and, perhaps on some level, interpret it." I think that describes the sad reality of scientific research. A PhD (with or without post-doc experience) in the analytical field is meant to generate data using analytical techniques for the project he or she is involved in (the core responsibility) with luck, they might get a limited chance to go into analytical research or development.

Basically, the scientific community considers that analytical development (or new method development) is part of a large project and that there is no need for any analytical development (not even as a work package of the project!) due to their fear of other researchers sharing the credit. No wonder analytical research is no longer attractive and is on the wane... *– Arul Marie, France.*

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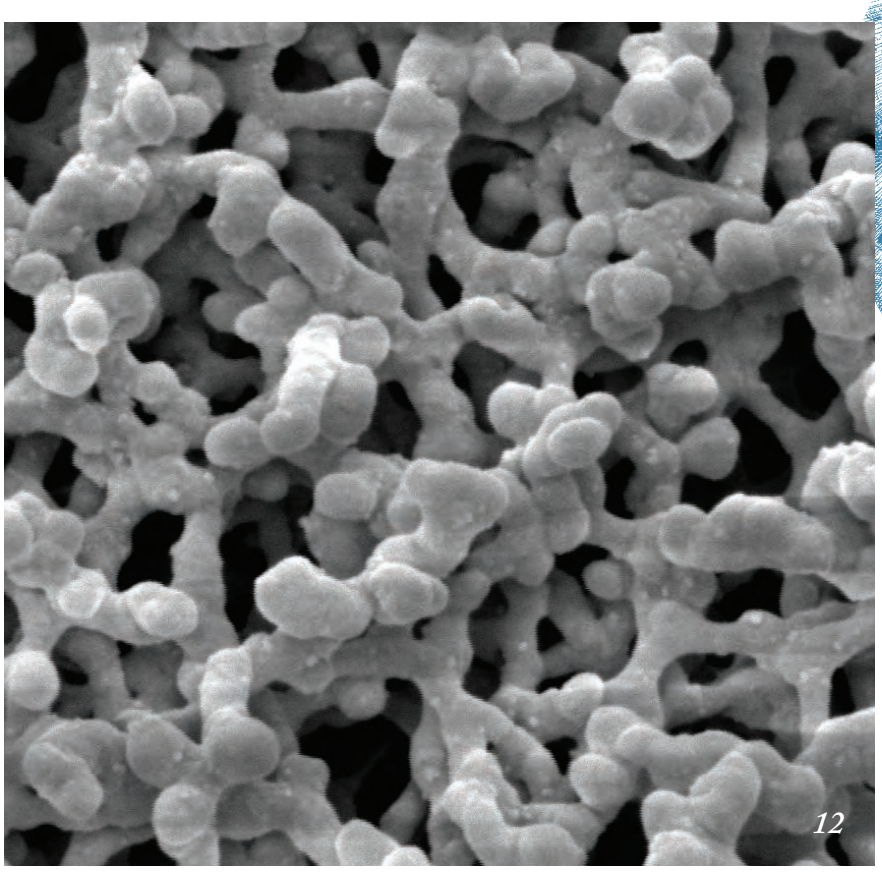
Contents


13


12


50

On The Cover

An integrated facility for the study of optical properties in a wide spectral range. Photo by R. Stewart McWilliams, Geophysical Laboratory, Carnegie Institution for Science.

Upfront

In My View

22

Features

Reports

Departments

Sitting Down With

the **Analytical Scientist**

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General enquiries:
www.texerepublishing.com
info@texerepublishing.com
+44 (0) 1565 752883
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Inspiration Particles, and Other Misconceptions

Where does inspiration come from? I mean the insight that leads you to a new invention or that suddenly makes you aware of the solution to a problem?

In the feature on spectroscopy (page 22), Volker Deckert, Peter Griffith and Gary Hieftje give us their personal (and sometimes unconventional) views on the past, present and future of the field, including the dead ends that they have encountered. One of the questions deals with the secret of success, and Deckert offers "inspiration particles", a term from Terry Pratchett's Discworld books, as his explanation. "People get hit by them, some more than others, and it leads to inventions and great developments", he told us with a smile. Of course, he does not think that inspiration particles exist, he knows it is mostly a matter of hard work, many failures, and some successes.

Another analytical scientist who knows this is Eli Hvastkovs, the assistant professor at the center of a storm in a US university. He told his students not to thank God in their personal graduation statements. Hvastkovs preferred that the students take personal responsibility for their success and failures, and I can only agree with his sentiments. It can be comforting to accept answers, such as inspiration particles, that make us stop questioning. But it is not science.

Elsewhere in the issue, other misconceptions are addressed. Hans-Gerd Janssen argues against the continuously growing number of methods for analysis. While university researchers are highly motivated to develop new approaches, Janssen says they must start to realize that it is not what industry specialists want. And Marcus Macht pushes back against the mainstream perception that we can figure out full proteomes through a straight-forward head-count of the protein present. Not so, he says; a true understanding needs to take full account of the complexities of these astonishing macromolecules. Macht's way forward is cooperation between the scientific community and instrument developers to solve the complexities of protein modification.

Each of these contributors sends us the message: "Do not be lazy scientists, we have some exploring ahead". What's in the full proteome? How do the components interact to make a cell work? How do particular cells then achieve particular tasks, such as a granulocyte going for an intruding bacterium? And so on…

Yes, we are getting closer to the big answers, but we'll have to keep on asking difficult questions and working hard to gain inspiration.

Just once in a while, however, when you are really tired of it all, inspiration can hit you out of the blue. I'm sure that you have experienced it. We don't fully understand Eureka moments yet, and it may take neuroscientists and psychologists many years to find out. But take my word for it: it happens to some more than others, and the answer is not inspiration particles.

Frank van Geel
Scientific Director

Contributors:

Igor Lednev and Justin Bueno

Building new instruments and developing new methods at the Moscow Institute of Physics and Technology gave Igor Lednev the experience that he needed to tackle some major practical problems in chemistry and biochemistry. After publishing over 160 peer reviewed articles on fundamental science, he and his students have developed a new method that could make a significant contribution to practical forensics.

Justin Bueno's pursuit of an advanced degree at the University at Albany, SUNY was determined mostly by coincidence. "I always loved watching documentaries on forensic science, but I was often left wanting a more in-depth description of the analytical techniques; luckily, Dr. Lednev had an opening in his research lab that meshed both analytical and forensic chemistries perfectly." The research performed by Bueno in Lednev's lab earned him a PhD in analytical chemistry in 2013. "I could have never dreamed that the project I started in 2009 would possess the great potential to be a tool that real world forensic investigators use."

Igor and Justin unleash their new forensic method on page 20.

Lawrence "Larry" Mason

When Larry Mason rejected a music scholarship to pursue his love of percussion and jazz, he opted instead to prepare for a legal education through undergraduate degrees in Economics and Political Science at the University of Illinois. His non-music undergraduate education took root and Larry continued on to obtained his law degree in 1989. "After nearly 25 years of helping businesses solve their litigation problems throughout the US, I take most pride in making the complex simple. I always leverage my non-scientific/technical background to achieve optimum results because if I can understand a complicated issue, I can more easily present it to juries and judges." When not in the courtroom, Larry hones his skills as an actor in community theatre productions throughout the Chicagoland area.

Larry gives his account of fear and loathing of science on page 44.

Craig Prater and Curtis Marcott

"When I was a child, I was given a large magnifying glass. When I wasn't trying to set fires with it, I spent a large amount of time looking at things that were too small for my unaided eyes to see," says Craig Prater. "This passion has continued through my professional life, developing instrumentation to reveal fascinating structures." Craig joined Anasys as CTO in 2007 to focus on nanoscale materials characterization.

Curt Marcott worked for over 28 years as an infrared spectroscopist at Procter & Gamble after which, he joined the spectroscopic consulting firm Light Light Solutions. in 2007. "Although I was very proud of the role I played in development of IR spectral imaging and its application to industrial problems, P&G managers were always asking me what it would take to look at smaller objects. Now in a 'retirement' job, I am really enjoying helping to make that dream a reality." Curt is also an adjunct professor at the University of Delaware and Miami University in Oxford, Ohio.

Craig and Curt take infrared spectroscopy to the nanoscale on page 17.

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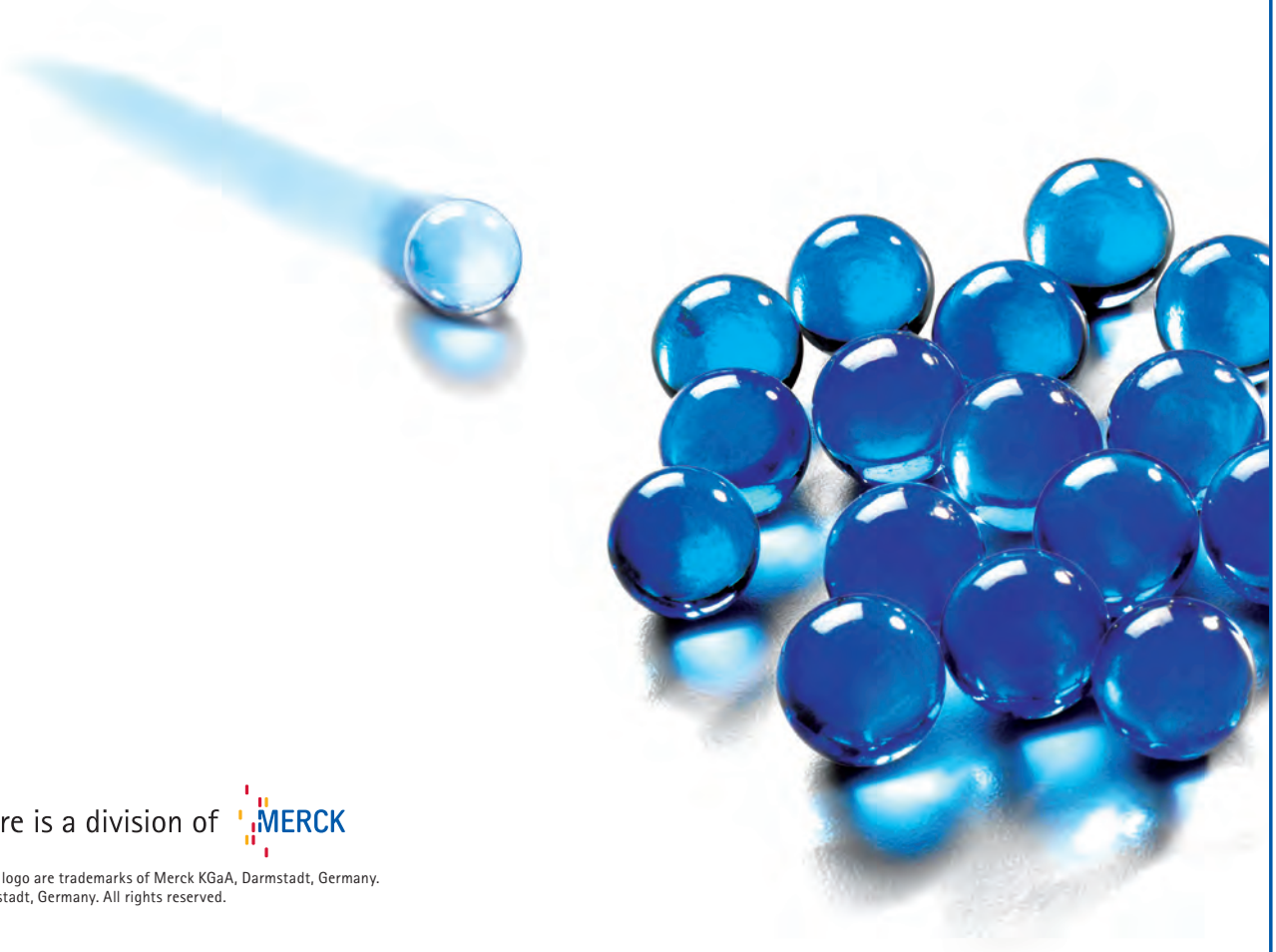

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Upfront

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

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

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Interred Tundra

Analysis of "dirty" ice from 3000 meters down reveals that ancient soil exists beneath the Greenland ice sheet

Researchers described themselves as "stunned" to find beryllium-10 (^{10}Be) isotope in ice core samples at concentrations orders of magnitude higher than they had anticipated. The finding indicates that a 2.7 million year-old soil still exists beneath the glacier, according to University of Vermont geologist Paul Bierman, lead investigator of a recent study (1).

Formed principally by cosmic rays, ^{10}Be accumulates on land through rainfall; the longer soil or rock is exposed at the Earth's surface, the more ^{10}Be it accumulates. Continental ice sheets tend to strip landscapes clean, so the team had expected the erosive forces at play to remove most, if not all, ^{10}Be and soil from the bed of ice below the summit. "We actually set up the experiment with multiple blanks and extra cleaning of labware so that we could detect very small concentrations," says Bierman.

The team measured organic carbon and total nitrogen in addition to ^{10}Be. The concentrations of C and N, measured using a Carla Erba elemental analyzer, point to organic material and the likelihood that a tundra-style landscape once existed before ice coverage. So, Greenland really was green once upon a time.

The ^{10}Be analysis was somewhat more complicated. The samples, along with a spike of ^{9}Be, were fluxed over a natural gas/oxygen flame in a mixture of potassium hydrogen fluoride and sodium sulfate, which renders the beryllium and potassium soluble. "We then isolated the beryllium, purified, dried and oxidized it to beryllium oxide, and analyzed isotopically using an accelerator mass spectrometer at Livermore National Laboratory," says Bierman. (For video footage of the analysis in action, see tas.txp.to/0514/tundra.)

The study is a logical extension of Bierman's long-standing and broader interest in understanding the rate at which landscapes change over time and space. "I want to know how glaciers, particularly ice sheets, work: where they erode and how rapidly they do so. Greenland is an easily accessible ice sheet about which we know some things but not others," he says. "The new results tell us that over the last 2.5 to 3.0 million years, the ice sheet has protected

the soil beneath, which means continuous or nearly continuous ice cover since then." Bierman concluded a university press release (2) with the comment that, if the human race continues on its current trajectory, "the ice sheet will not survive. And once you clear it off, it's really hard to put it back on."

References

1. *P. R. Bierman et al., "Preservation of a Preglacial Landscape Under the Center of the Greenland Ice Sheet", Science, published online 17 April 2014. DOI:10.1126/science.1249047*
2. *http://tas.txp.to/0514/Vermont*

Cutting that Red Tape

A report from the US National Science Board (NSB) has denounced excessive bureaucracy faced by researchers and urges a "focus on the science"

"Regulation and oversight of research are needed to ensure accountability, transparency and safety," said Arthur Bienenstock, chair of the NSB Task Force on Administrative Burdens. "But excessive and ineffective requirements take scientists away from the bench unnecessarily and divert taxpayer dollars from research to superfluous grant administration."

The NSB is the policymaking body of the National Science Foundation (NSF), which is the independent US federal agency that supports fundamental research and education across all fields of science and engineering.

In December 2012, the NSB task force issued a request for information to identify the federal agency and institutional requirements that contribute most to the administrative workload of principal investigators. The response from over 3,100 individuals indicated that the main bureaucratic offenders were financial management, the grant proposal process, progress (and other) reports; human subjects research and institutional review boards, and research involving animals and institutional animal care and use committees.

Armed with these data, the task force issued a report, "Reducing Investigators' Administrative Workload for Federally Funded Research" (1). Running to 98 pages, the report included three key recommendations:

- to limit proposal requirements to those that are essential for evaluating merit
- to ensure that reporting focuses on outcomes
- to automate payroll certification for effort reporting.

Addressing other bugbears, the NSB also proposed an evaluation of animal research, conflict of interest, and safety/security requirements, and indicated that universities should review their internal processes to speed up protocol approval.

The report acknowledges that many of the issues raised are not new, but notes that a clear inability to address them has continued to waste precious research dollars at a time when funding is already scarce. Its main objective is to allow researchers to "refocus their efforts on scientific discovery and translation." *RW*

What causes you the most grief in the lab? Let us know by commenting online.

Reference

1. http://nsf.gov/pubs/2014/nsb1418/nsb1418.pdf

Nano-labels for "Sour" Oil

Carbon black nanoreporters can warn oil producers of unacceptable levels of hydrogen sulfide

Just one percent of hydrogen sulfide in oil results in "sour crude," which is toxic and corrosive to both pipelines and transportation vessels. Prior knowledge of its concentration is therefore of great value in oil fields. To that end, researchers at Rice University, Texas, USA, have designed nanoreporter molecules based on carbon black (see Figure 1) that can detect levels of hydrogen sulfide "downhole" (1). We caught up with consortium leader James Tour to find out more.

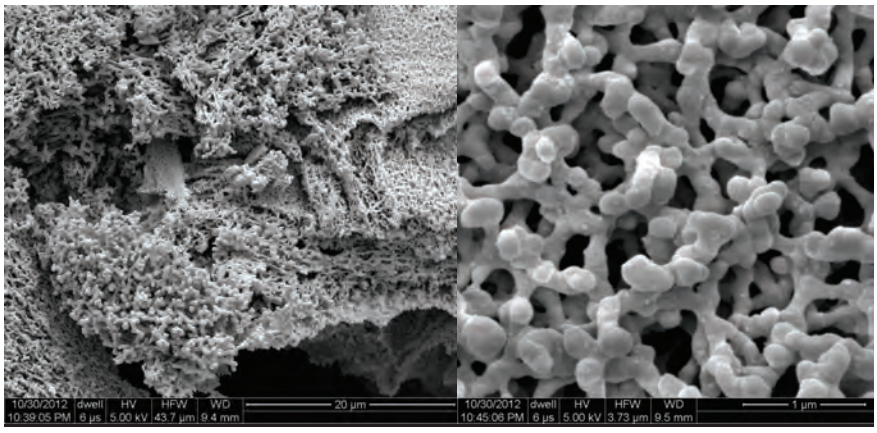

Figure 1. Electron microscope images shows carbon black nanoparticles modified with addition of polyvinyl alcohol for downhole detection of hydrogen sulfide. (Credit: Tour Group/Rice University)

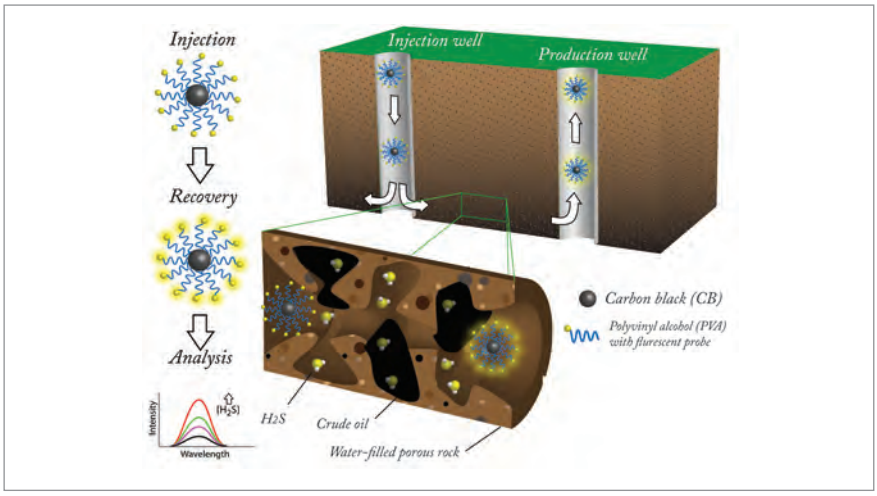

Figure 2. Nanoreporters are injected into the well where they react with any hydrogen sulfide, which causes the particles to fluoresce. The intensity of fluorescence tells producers how "sour" the oil reservoir is. (Credit: Chih-Chau Hwang/Rice University)

What inspired the design of the nanoreporters?

We used our knowledge of what had already been done in biology and what the needs of downhole environments are, and coupled the two together using nanotechnology. The design of these nanoparticles for passage though oilfield rock was developed swiftly because we had a year of experience in building carbon nanoparticles for passage though the human body – we already knew how to inhibit adhesion to surfaces. Nanotechnology – the building of entities in the 1-100 nm size region – is something we've have been doing for about 25 years. In short, it's really all about the convergence of expertise within our labs.

How do the nanoreporters work?

When the nanoreporter particles are exposed to hydrogen sulfide (H_2S), the H_2S reacts with the addend on the nanoparticles and causes them to fluoresce – the higher the H_2S concentration, the greater the fluorescence. When the nanoparticles emerge from the ground with the flow, fluorescence intensity can be measured using a handheld spectrophotometer (see Figure 2).

What prompted the research?

The concept fits well with local interests in Houston in oil and gas, as well as my own area of nanotechnology. It was actually part of a proposed study to the Advanced Energy Consortium; we have two other papers in the area wherein we detect and quantitatively assess downhole oil using a complementary sensor.

What are the main challenges in applying the technology in the field?

Getting the industry to try something new. And having a chemical company make the materials – it's not hard but it is a new process...

What next?

We will continue to develop methods to (i) better assess downhole oil quality and quantity, (ii) improve geolocation of the oil downhole – that is to say understanding where is it precisely, and (iii) facilitate removal of stranded oil.

References

1. *Chih-Chau Hwang et al., "Carbon-Based Nanoreporters Designed for Subsurface Hydrogen Sulfide Detection", ACS Applied Materials & Interfaces Article ASAP. DOI: 10.1021/am5009584*

Courtesy of www.sciencecartoonsplus.com

Cambridge, 1953. Shortly before discovering the structure of DNA, Watson and Crick, depressed by their lack of progress, visit the local pub.

For God's Sake

Was an assistant analytical chemistry professor mistaken to instruct graduates not to "thank God"?

According to The Daily Reflector, East Carolina University's Eli Hvastkovs recently wrote an email to chemistry majors that outlined personal statement guidelines for departmental graduation. One of the bullet points apparently stated, "You can't thank God. I'm sorry about this – and I don't want to have to outline the reasons why."

Evidently, other university officials did want to know the reason why and quickly responded by telling students to disregard the email. Provost Marilyn Sheerer stated in the counter email, "These statements can be your personal expressions and as such the University will only limit these expressions as permitted by applicable First Amendment law." She continued by expressing regret for the miscommunication, which had not been approved by the appropriate university officials, and asserted that personal statements should bring about a forum for student expression.

Sheerer also confirmed that the only statements prohibited were those that either violated state statutes on hate crimes or were disruptive to the campus (including inciting a riot!).

And, of course, given that this is a university after all, personal statements that exceeded the word limit or missed the deadline would also be struck of the list... *RW*

Learning Without Doing Equals Shortcoming

Removing practical assessments from science education is illogical, say UK chemical engineers

In the UK, Ofqual (the Office of Qualifications and Examinations Regulation) is charged with maintaining standards and confidence in qualifications. Ofqual has decided to remove the practical examination and grading component from biology, chemistry and physics A-levels (taken by 16-18 year olds) from the 2015 new academic year, much to the annoyance of the Institute for Chemical Engineers (IChemE). IChemE's chief executive David Brown said, "… it seems illogical to remove practical assessments from science, when it remains at the heart of grading in other subjects."

Brown continued, "Chemical and process engineering employers need graduates and apprentices with strong practical skills as well as sound knowledge and understanding."

As George Whiteside notes in last month's feature, enjoying the day-by-day activities of a profession (including "washing dirty dishes") is often more important than aspiring to reward or great success. By removing practical assessments, students are likely to focus more heavily on written work rather than the exciting (and challenging) aspect of experimentation, which could deter them from continuing into graduate level education. "The changes will not help students who we know are inspired and motivated by doing science […] And they will not help universities, colleges and companies who already struggle to recruit people with the practical experience they need," said Campaign for Science and Engineering's (CaSE) director, Sarah Main.

The UK Chemical Industries Association "Policy Issues" report notes that 90 percent of chemical businesses surveyed in the UK had some difficult in recruiting science graduates. In addition, 69 percent of companies felt that they were training their staff in skills that should have been learned at school, college or university (1). *RW*

What's the status of school education where you are? Let us know by commenting online: tas.txp.to/0414/education.

Reference

1. *www.cia.org.uk/Policyissues/Skills.aspx*

ASMS on Tour

On June 15, the 62nd ASMS Conference on Mass Spectrometry and Allied Topics sails into Baltimore

ASMS does not fill its program with "invited" sessions, but instead uses program chairs to select the very best abstracts from over 3,500 submissions to "showcase interesting and novel science" – but that's still a lot of presentations.

The sessions are arranged to minimize overlap with similar or complementary topics, but it can be tough to decide (see Pocket ASMS on page 3 for more help).

To show the breadth of topics at ASMS – and to start you off with your planning – we present two "Must-see" presentations for each day:

Monday

> Environmental Petroleomics: Characterization of 105 Biotic and Abiotic Petroleum Transformation Products 4-Years after the Deepwater Horizon Disaster (Ryan P. Rodgers, Exhibit Hall AB, 8:30am)

> Mobile Autonomous Underwater Mass Spec and Sampler System – Opening up the Entire Underwater Chemical Space (David Fries, Room 307-308, 3:50pm)

Tuesday

> Progress in the Development of Structures for Extended and Lossless Ion Separations and Manipulations (Richard D. Smith, Room 309-310, 8:30am)

> Coupling Atomic Force Microscopy with Biological Mass Spectrometry for High Spatial Resolution Imaging (Suman Ghorai, Ballroom IV, 3:30pm)

Wednesday

> Surprising New Ionization Methods for Mass Spectrometry, Mechanistic Insights and Potential Practical Utility (Sarah Trimpin, Room 307-308, 9:50am)

> Living Without Our Daily Bread – Towards Solutions for Sufferers of Gluten Intolerance (Michelle Colgrave, Ballroom I, 2:30pm)

Thursday

> Lifeline-S.O.S: "Crowd Curation" of Unidentified GC-(EI)MS spectra through Social Online Spectrometry (Manor Askenazi, Ballroom III, 10:10am)

> Transient Sample Introduction with Laser Ablation Coupled to an ICP Distance-of-Flight MS (Elise A. Dennis, Room 307-308, 4:10pm).

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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 edit@texerepublishing.com

My Ever-expanding Analytical Toolbox

New methods that focus on increased sensitivity or resolution are being developed on an almost daily basis. But are they the right methods and do we even need them? I say "no and no," and here's why.

By Hans-Gerd Janssen, Science Leader Analytical Chemistry, Unilever Research Vlaardingen, and Professor of Biomacromolecular Separations, van 't Hoff Institute for Molecular Sciences, University of Amsterdam, The Netherlands

To fully understand the point I would like to make, one needs to understand how an analytical laboratory works in industry and how the university equivalent functions – and to recognize the disparity between them. In academia, analytical research largely was – and still is – very technique oriented. Indeed, the academic analytical scientist often works on a single technique with objective of making a new discovery or some developmental breakthrough. The actual use of the technique – the application – is not very relevant; the techniques and theories developed are applied to a single sample just to fill the last (and least important) figure in the manuscript.

But in industry, how different the situation is. Except for instrument manufacturers, industry is not performing analytical research. Rather, industrial analytical chemists are trying to figure out how to use existing methods to solve problems. Emphasis in the previous sentence should be heavily applied to the word 'existing'. Unlike academics, who are terribly disappointed if they find an article describing an idea they just had and which they thought to be original, industrial researchers are very happy to find a solution to a problem in literature.

In fact, the best industrial analytical scientists are excellent thieves; immediately able to recognize relevant methods that can be "borrowed", slightly adapted, and "sold" as new developments to their superiors. In addition, a good industrial analytical scientist is also like a skilled plumber in that both need a huge toolbox filled with relevant tools and techniques. When confronted with a novel analytical (or plumbing) question, the real professionals can dive into the toolbox, select the tool that is most appropriate to solve the problem, and successfully apply it. However, this is just the part of the industrial analytical chemist's job that is becoming increasingly difficult. The toolbox is ever expanding. New methods are developed, with new column chemistries, separation modes, detection devices, derivatization strategies, data processing routing... need I go on? There are simply too many tools and unsurprisingly the toolbox is getting far too heavy to be easily carried around. We do not all share the skills of the magical Mary Poppins. Certainly, one needs an excellent memory to remember which tools are in the box – and an even better memory to remember how to operate them all.

The big question is, do we really need all the tools in the toolbox?

Can we not make do with just a few methods, each with wide compound or problem coverage? When thinking about the answer to these questions, please also bear in mind that for most users of analytical tools, the methods do not have to deliver the best performance. Performance should be fit-for-purpose and not necessarily a World Record attempt. An unfortunate complication here is that the parameters that industry finds extremely relevant, for example, reliability and ease of use, are rarely considered by academic technique developers. Capillary electrophoresis may well be an excellent and superior method given its basic figures of merit, but it is a technique best avoided in the real world. Classical HPLC methods might be slower, less efficient – take your pick of negative features – but at least they work.

"The big question is, do we really need all the tools in the toolbox?"

In my view, we have too many methods. And new methods that are only marginally better than existing methods are being added as I write. In reality, we do not need more methods that are slightly faster or slightly more sensitive. What we need are fewer methods with a broader coverage of compounds, matrices and, ultimately, questions. There is no one tool that can solve all problems. But, in industry, we definitely need to slim down the toolbox. Of course, these chosen few methods may not be the best method for each question. But that's not a problem as long as they provide clear, fit-for-purpose answers – both reliably and rapidly. Generic methods and techniques are the future.

Applying Infrared Spectroscopy at the Nanoscale

How to meet today's demands for nanoscale chemical analysis in materials and life sciences.

By Craig B. Prater, Anasys Instruments, Inc., Santa Barbara, CA, USA, and Curtis Marcott, Light Light Solutions, LLC, Athens, GA, USA

Infrared spectroscopy and microscopy rapidly analyze materials to create fingerprints that identify chemical species and create spatially resolved maps of chemical composition. Both techniques are used routinely in academic and industrial research settings but infrared microspectroscopy suffers from the limitation that, in practice, the spatial resolution is limited by optical diffraction to around 10-30 μm.

Extending IR spectroscopy beyond optical diffraction limits is a critical unmet need. Governments and industries have invested billions of dollars in developing novel materials and devices at nanoscale dimensions, far below the spatial resolution limit of conventional IR spectroscopy. And in the life sciences, conventional IR microspectroscopy has generally been limited to the scale of cells or larger, lacking the ability to perform chemical analysis at the sub-cellular level.

The recent combination of atomic force microscopy and IR spectroscopy (AFM-IR) offers a solution. Researchers have obtained IR spectra with spatial resolution somewhere between 50 and 500 times better than conventional Fourier transform IR microspectroscopy. The AFM-IR technique works by using the tip of an atomic force microscope (10-100 nm in diameter) as a nanoscale detector of local infrared light absorption.

In more detail, the AFM tip acts as a near-field detector of the photothermal expansion that occurs when IR laser pulses are absorbed by chemically-specific molecular vibrations in the sample. IR absorption spectra are created by measuring this photothermal expansion as a function of the wavelength of incident laser light. Chemical composition images can be obtained by mapping this photothermal absorption across a region of a sample with the laser tuned to an absorption band corresponding to a chemical species of interest. Because the photothermal expansion is detected locally with the AFM tip, the spatial resolution is not limited to the optical diffraction limit of conventional IR spectroscopy.

The implications of being able to apply the powerful chemical identification

technique of infrared spectroscopy at the nano scale in the materials and life sciences are far-reaching. Recent publications using AFM-IR have encompassed materials fields such as polymer blends, composites and multilayers; electrospun fibers; pharmaceuticals; plasmonics; fuel cell membranes, and semiconductors. In engineering polymeric materials, for example, materials developers use multiple polymeric components and a wide range of additives (for example, carbon fibers, nanocrystalline clay, carbon nanotubes, graphene) to achieve the desired functional properties. AFM-IR is becoming a critical tool for the chemical characterization of material component distribution and the interphase region between polymer domains and various additives.

Meanwhile, in the life science fields, AFM-IR has been used to map triglyceride vesicles in bacteria for biofuel production, to reveal protein/mineral concentration in bone, and to map lipids in skin.

Recently, the sensitivity of the AFM-IR technique has been enhanced, making it possible to perform IR nanospectroscopy on samples down to a thickness of a single molecular monolayer and with a spatial resolution of around 25 nm. This has been demonstrated on both self-assembled monolayers and biological membrane samples.

Clearly, spectroscopy's ability to chemically characterize samples in the materials and life sciences ultimately depends on the correct interpretation of the acquired spectra. Fortunately, AFM-IR measures the same fundamental property as conventional IR spectroscopy, namely the amount of absorbed infrared light. As such, the same techniques used to analyze conventional FT-IR spectra are directly applicable to AFM-IR spectra. In fact, AFM-IR spectra have been used to reverse-engineer laminated polymer multilayer films and polymer blends by taking advantage of IR materials databases and conventional IR interpretation expertise.

However, in several cases AFM-IR spectra reveal features simply not visible in bulk spectra. Conventional IR spectroscopy averages over regions that are many microns to many millimeters across and the resulting absorption peaks may be broadened due to the many molecular orientations and local environments within a bulk sample. On the other hand, AFM-IR measures regions that are small enough to reveal chemical heterogeneity that is averaged out over larger length scales; this reveals local molecular orientation or shifts in band height/shape due to molecular interactions at sub-micron length scales.

Together, the sensitivity and new information afforded by improved spatial resolution are enabling AFM-IR to open a whole new window on the chemical morphology.

Diving into Proper Proteomics

Let's not boast of "complete proteomes" or "protein coverage" until we correct our ignorance of post-translational modifications. Proteome analysis is more than just identifying and counting proteins.

By Marcus Macht, Director of European Applications at Bruker Daltonics, Bremen, Germany

When the term "proteomics" was invented back in 1994 at the Siena conference, the idea was to describe all proteins produced by a given organism at a given state. At that time, analytical technologies, such as 2D gel electrophoresis, already allowed us to visualize in excess of 1000 individual spots, each representing one or sometimes more proteins. With the increasing use of mass spectrometry, it has become obvious that these spots do not all belong to different proteins but rather that many of them share a common protein sequence and behave differently due to post-translational modifications. Thus, the number of actual different protein sequences was significantly lower than first thought. Recent successes in genome sequencing revealed that the number of genes in humans, for example, is also much lower than originally expected – around 22,000 individual genes are currently accounted for; for yeast, it's more like 6,500.

With our ability to identify and quantify several thousands of proteins in a single LC-MS/MS experiment, people tend to state that, "we can identify a complete proteome" and there are publications that indicate "the percentage of proteome covered." However, I think we need to be extremely careful with such statements for the following three reasons:

1. Identification is a tricky task. In the majority of the experiments, bottom-up technologies are used, which actually identify peptides – not proteins. Protein identity is inferred by the statistically most likely assemblage of peptides into a protein sequence. Mutations or species-dependent sequence variations can only be covered if the particular peptide is detected during the experiment, which is often not the case.

2. In quantitative analysis it is essential to know what is actually being quantified. Quantification of a protein's abundance in a multiple reaction monitoring (MRM)-based experiment using two peptides reveals absolutely no information about abundances of different protein species that differ in their modifications, so long as these modifications are not part of the quantified peptides.

3. Post-translational modifications (PTMs) in large-scale studies are usually neglected. While phosphorylation is still quite often a focus in proteomic analyses, large-scale glycosylation is rarely investigated. Further modifications, such as methylation or acetylation, have not really been covered in large-scale studies at all and remain completely unknown (save for a few individual proteins). There are numerous good reasons for this, such as a lack of enrichment possibilities and technical complications in the analysis and interpretation of the data, but what it means is that the number, location and regulation of huge numbers of modifications remain opaque.

One could ask, "Is that so important?" The answer clearly has to be, "Absolutely!" We are currently using systems biology to try to understand the regulating networks that exist between proteins and metabolites. Let's take as an example a patient taking warfarin. This drug, which acts against blood clotting, is still commonly used in the treatment of thrombosis or lung embolism, although you may know it as a rat poison (it's action here: critical internal bleeding). Patients using warfarin have reduced blood coagulation and sometimes suffer severe side effects. Given its dramatic effect, we would surely expect to see the action of warfarin in the proteome, right? Actually, the answer is yes – but only when we look very carefully indeed. While it is relatively easy to monitor the effect of the drug, its mode of action is to suppress the formation of g-carboxyglutamate modifications on several blood coagulation factors. Without the modifications, these proteins are not functional. Analysis of these PTM's is complicated, requiring at least a semiquantitative determination of modifications in peptides that are small and of low pI. Given that they originate from proteins that are highly glycosylated and show a tremendous amount of sequence variants, this is no small task. And yet, without this information, a comparison of treated and untreated patient proteomes would be utterly meaningless.

If we want to make claims about proteome analysis and coverage of "complete" proteomes, quantitative as well as comprehensive PTM information must be taken into consideration. In my view, there is still some distance to go before we can characterize a complete proteome, an endeavor that must be supported by both analytical instrument vendors and the scientific community. The temptation to pick low-hanging fruits like high throughput protein ID or protein based quantitation is clearly there, but complementary techniques to bottom-up technologies, such as 2D-gel based methods, reveal post-translational differences relatively easily. Right now, it is only by applying these additional complementary techniques that we can truly reveal the "complete proteome".

Firearm Forensics

Gun crime is not going away, but current forensic tools are limited at best. We believe that attenuated total reflectance (ATR) imaging could fill a big gap in the crime scene investigator's armory.

By Justin Bueno and Igor Lednev, Department of Chemistry, University at Albany, State University of New York, USA.

According to the Center for Disease Control, firearm related shootings were responsible for over 68 percent of homicides and were one of the three leading causes of injury-related deaths in the United States in 2011. Shootings were also the second leading cause of law enforcement officer deaths in 2010. Given these facts, we believe that a rapid, high-throughput analytical tool for the detection and analysis of trace firearm evidence is long overdue.

Forensic investigators usually rely on the collection of some sort of ballistics evidence, whether it is the actual firearm, cartridge case or projectile, to link a suspect to a firearms crime. Tool mark and impression identifications match physical markings left on cartridge cases or projectiles to markings on the firearms. While this technique is the most popular tool used by ballistic investigators, it is subjective by nature; a tool mark examiner is required to interpret certain observations. These comparisons are susceptible to high rates of error (over 10 percent) and variability because different examiners have different levels of training and experience. Given these shortcomings, we feel that tool mark examinations are not appropriate to determine whether ballistics evidence matches materials recovered from the suspect. Rather, the ideal tool would target crime scene shooting incident reconstruction through gunshot residue (GSR) analysis.

Although GSR trace evidence is associated with each and every firearm discharge, there is no current analytical technique for the elucidation of forensically relevant information which a crime scene sample has to offer. Current techniques are only suitable for GSR particles originating from the primer of the ammunition and target just a few heavy metals. Unfortunately, GSR samples originating from "heavy metal free" or "green" ammunition are void of these metals and thus current methodology is not appropriate for the analysis of these sample. Therefore, we believe there is a need in the forensic science community for a more specific method of GSR detection.

Our approach uses attenuated total reflectance (ATR)-imaging, which targets a much wider range of chemicals including GSR particles originating from both the primer and propellant of the ammunition (2), for the rapid detection of GSR. ATR-imaging requires absolutely no sample preparation (it simply requires pressing some common double-sided office tape against a surface populated with GSR) and, most importantly, it is non-destructive, which allows for multiple analyses or re-analyses to be performed on the sample – the ability to preserve forensic evidence is always of paramount importance.

ATR imaging combines the specificity of vibrational spectroscopy (measuring the molecular fingerprint of the analyte) with the convenience of automated microspectroscopic mapping. ATR imaging uses a germanium crystal in contact with the sample surface, which offers increased spatial resolution compared with other spectroscopic mapping techniques, meaning that we can lower the limit of detection – or decrease the size of GSR particles that can be resolved on the tape substrate. Chemical maps can be generated from the spectroscopic images that clearly visualize detected GSR particles that are hidden when a visual inspection is used.

When fully developed, the combined approach of tape-lifting and ATR imaging will allow forensic investigators to rapidly collect and detect GSR particles from the body or clothing of a suspected shooter. If GSR is detected, it has the potential to link the suspect to the crime scene, whilst its absence could equally exonerate an innocent person.

Where are we now? Well, we need to study the specificity of the approach with regards to potential false-positive contaminants. In addition, we must examine the effect of chemical composition of the propellant, primer, projectile and cartridge case, as well as the type of firearm and firing mechanism, on the spectroscopic signature of GSR. Beyond GSR analysis, we believe that the technique could have many applications in counter-terrorism and homeland security, including explosives detection. Unfortunately, there is no escaping the fact that violent crime will continue indefinitely and we analytical chemists must play our part in the battle against it.

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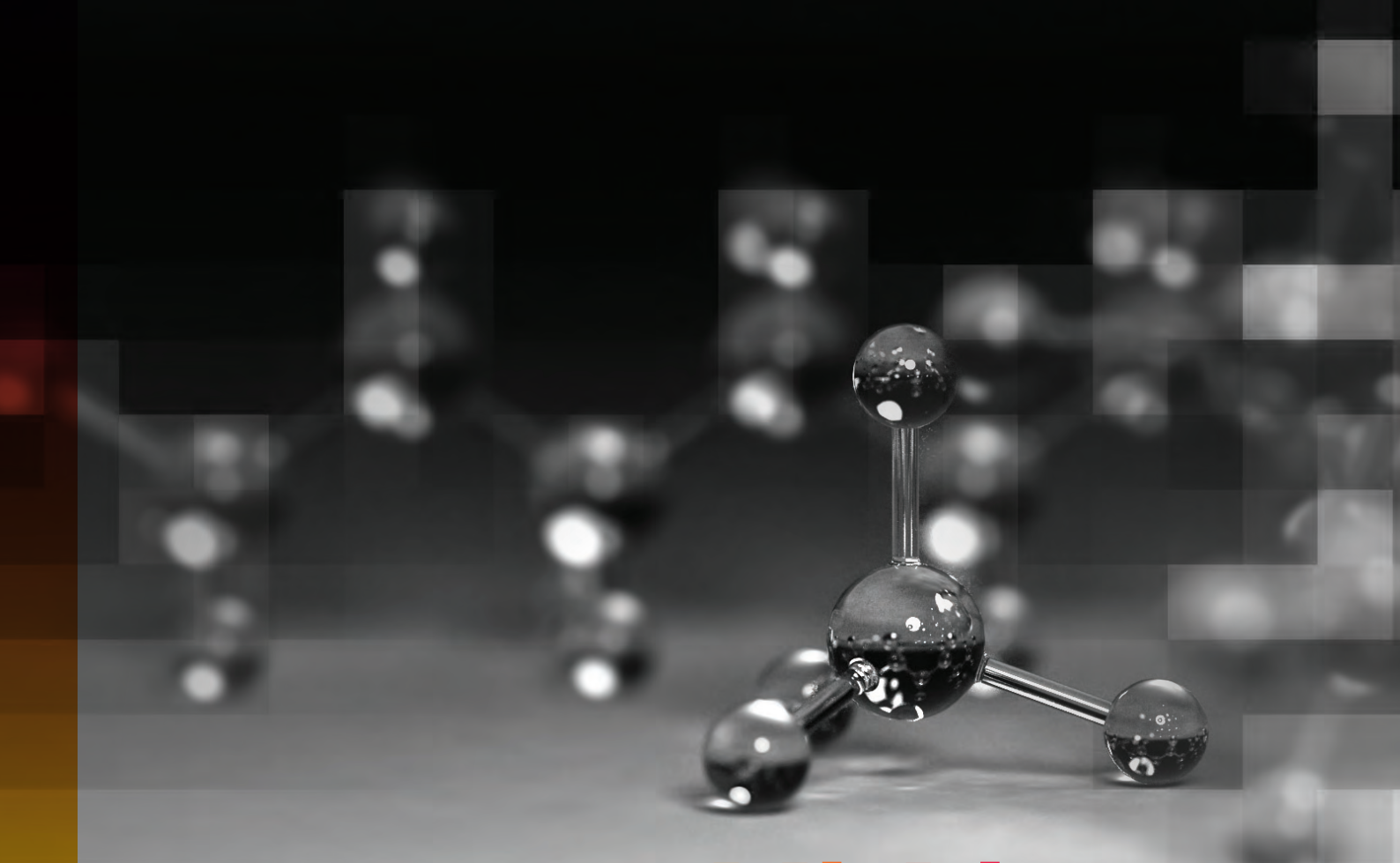

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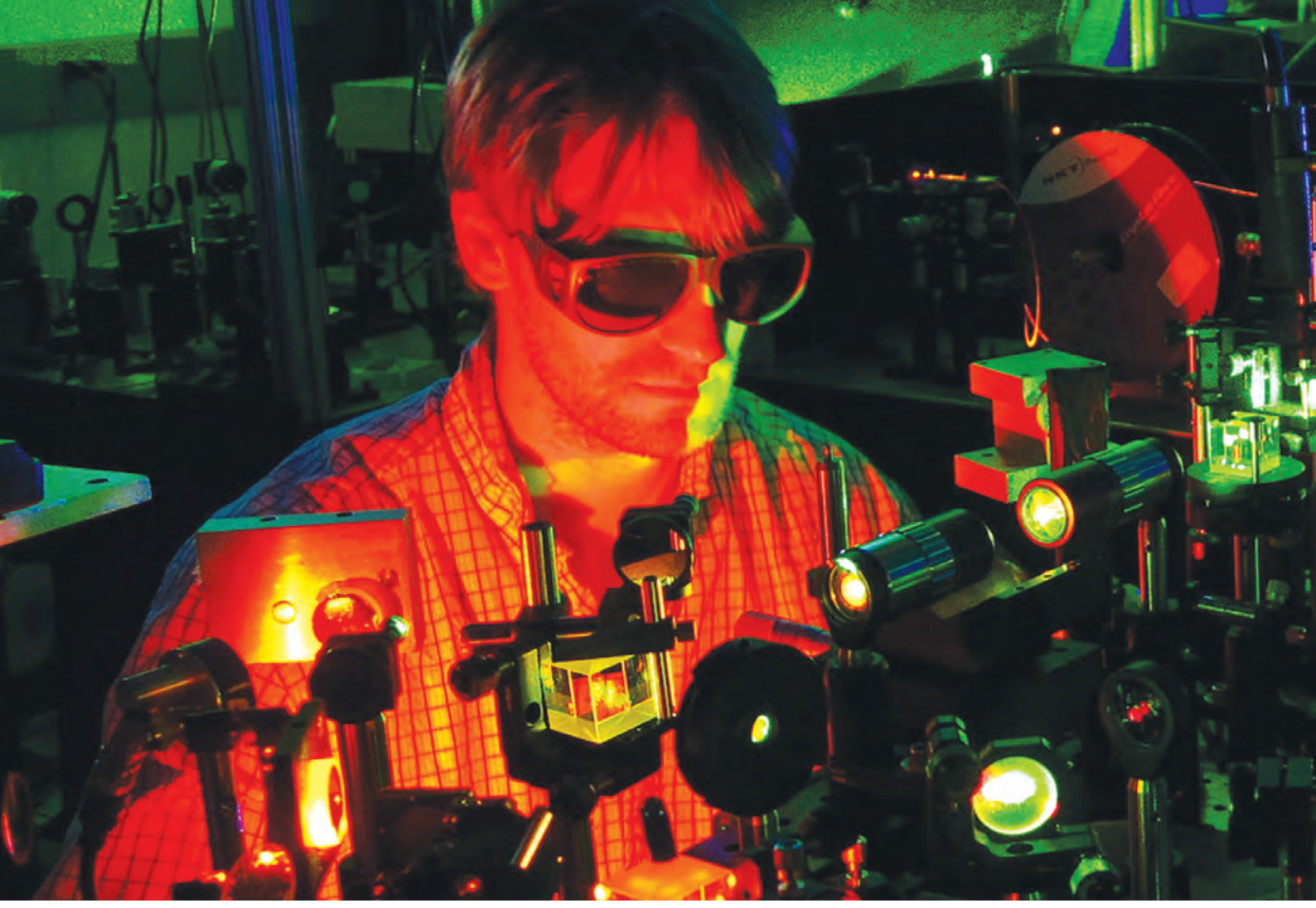
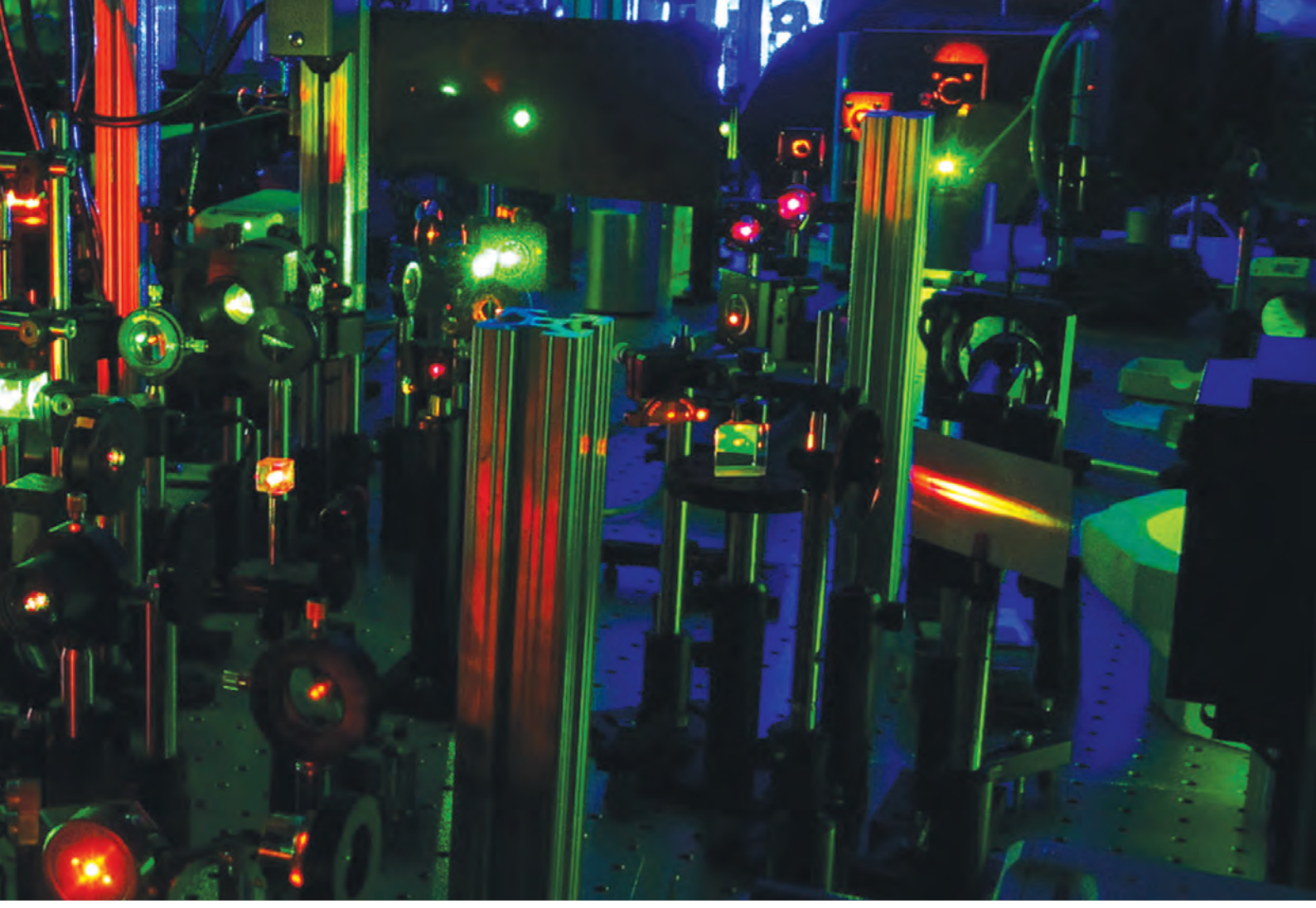


R. Stewart McWilliams, Geophysical Laboratory, Carnegie Institution for Science.

Three Gurus of Spectroscopy

What has driven spectroscopic techniques into their current prominent position in a plethora of application areas? Here, Gary Hieftje, Peter Griffiths, and Volker Deckert combine over 120 years of experience to assess past successes – and failures – and to offer a glimpse of our light-filled future.



Where have the last 50 years taken spectroscopy?

Gary Hieftje: Generally, over the last 50 years we've been moving towards extremes: faster, smaller, higher resolution, higher power, lower noise. Indeed, improvements in instrumentation (including sources, detectors, and spectral-sorting devices) have been significant – laser technology is a major player there. Furthermore, increased coupling with other methods (separations, other spectrometric methods, electrochemistry, and so on), have pushed potential application boundaries.

More specifically, near-field methods have seen significant development. And, in terms of data collection, multiplexing (via multichannel systems) has given us the ability to capture entire spectra at once; multidimensionality has allowed us to take advantage of all the characteristics of electromagnetic radiation (frequency, amplitude, phase, polarization), and the dimensionality of information-rich methods, such as NMR, have been copied across various techniques.

Volker Deckert: I think the main general trend at present is miniaturization, as noted by Gary. Instruments are getting smaller and smaller; hand-held spectrometers are a common sight at exhibitions. Another trend over the last 20-30 years has been the combination of spectroscopy and microscopy. This advance allowed for smaller sample volumes and different kinds of imaging techniques. Personally, I think this trend towards imaging really did the trick – seeing is believing! And distinguishing a compound from its direct neighbor directly without further invasive sampling is a major feature of today's optical spectroscopies.

Peter Griffiths: Fifty years ago, the field of spectroscopy was dominated by atomic spectroscopy. I believe that it is fair to say that molecular spectroscopy has now assumed the more dominant role, largely because of the remarkable development of FT-IR, Raman and near-infrared spectrometers – and the many problems in analytical chemistry that require measurement of molecular (rather than atomic) spectra. Therefore, I will concentrate on molecular spectroscopies here. And in light of my training and expertise, I will largely narrow my responses.

The Gurus

Gary Hieftje is distinguished professor and Robert & Marjorie Mann chair at the Department of Chemistry, School of Public and Environmental Affairs, and School of Informatics, Indiana University Bloomington, USA. Gary's research interests include the investigation of basic mechanisms in atomic emission, absorption, fluorescence and mass spectrometric analysis, the development of instrumentation and techniques for atomic methods of analysis, on-line computer control, the use of time-resolved luminescence processes, and the use of stochastic processes to extract basic and kinetic chemical information.

Peter Griffiths is emeritus professor of chemistry at the University of Idaho. His research interests involved many applications of vibrational spectroscopy in analytical chemistry. His group pioneered the use of diffuse reflection spectroscopy in the mid-infrared, the coupling of various types of chromatograph (GC, HPLC, SFC, TLC) to FT-IR spectrometers, surface-enhanced infrared absorption and Raman spectroscopy, and open-path atmospheric monitoring by FT-IR spectrometry. Peter was formerly editor-in-chief and is now second editor of Applied Spectroscopy.

Volker Deckert holds a joint position at the Institute of Physical Chemistry at the University of Jena, Germany, and the Leibniz Institute of Photonic Technology, also in Jena. He obtained his Diploma and PhD from the University of Würzburg, Germany, working on difference-Raman spectroscopy. As a postdoc at the University of Tokyo and the Kanagawa Academy of Science in Kawasaki, he worked on non-linear and time-resolved laser spectroscopy of photo-induced isomerisation reactions. While at ETH Zurich, he started working on the development of high spatial resolution techniques for Raman spectroscopy, a topic which has followed him to Jena, where, in particular, he explores the possibilities of the technology to investigate structural changes of bio-related compounds with nanometer resolution.

What are the major impacts of spectroscopy – in analytical science or society?

PG: Developments in spectroscopy (both hardware and software) have allowed the composition of reactants, intermediates and products in industrial processes to be determined and controlled in situ, with high accuracy and in very short times. The result has been improved product reliability and decreasing costs. An obvious example of this can be seen in process analytical technology (PAT) in the pharmaceutical industry. We are also getting close to the point where several different forms of spectroscopy will be used for medical diagnosis.

VD: As a spectroscopist, I would like to say that spectroscopy plays a major role in both analytical science and society. After all, direct information about the molecular structure is possible. The truth is more complex though. Try to investigate a mixture and you learn to appreciate separation technology; and if you can afford to lose material, mass spectrometry can deal much better with mixtures as well. What is most interesting, in my opinion, is the fact that the different technologies in optical spectroscopy are constantly challenging each other in terms of sensitivity, spatial resolution and application fields. Whenever Raman spectroscopy came up with improved light sources or novel detector concepts, IR spectroscopy answered with Fourier transform technology or with focal plane arrays. Similar stories can be told for other optical spectroscopies and, as a result, we have now instruments that are tremendously more sensitive than 10-20 years ago – and, in many cases, specialists aren't even needed to operate them.

GH: Let me take you back even farther than the first question. Seventy-five years ago, the only spectrometric method that was available in most routine-analysis laboratories was the Dubosc colorimeter (based on a technique invented by Jules Duboscq in 1870). Today, spectroscopic methods are pervasive and employ virtually all methods of spectral measurement (absorption, emission, fluorescence, scattering, acoustic, polarimetric, and others) and for a host of measurements (elemental, molecular, kinetic, chromatographic detection, remote sensing...). One of the most common methods of spectrometric measurement is the NIR method for measuring oxygen saturation via a probe clipped to a patient's finger. Many other laboratory-based or bedside measurements also rely on spectrometry.

"As a spectroscopist, I would like to say that spectroscopy plays a major role in both analytical science and society. After all, direct information about the molecular structure is possible."

Many other sorts of common optical devices (polarized sunglasses, xenon and LED headlights, intruder-detection systems for home protection, collision-avoidance systems in automobiles, laser scanners at grocery checkouts, Lidar devices for measuring auto speed or passing through "red lights", terahertz airport scanners) are all reliant on spectroscopy to some degree.

Becoming rather more abstract, several impressive natural phenomena – red sunsets, blue skies, the aurora, and lightning – are essentially dependent on spectroscopy!

Where are the strongholds in spectroscopy?

PG: Any list will omit important names, nevertheless, I'll present some of the groups that are doing cutting-edge research in vibrational spectroscopy today. Raman spectroscopy: our fellow guru here, Volker Deckert at the University of Jena (TERS); Duncan Graham, University of Strathclyde (SERS, SERRS). Infrared Spectroscopy: Rohit Bhargava, University of Illinois (biospectroscopy, microspectroscopy); Alexandre Dazzi, University of Paris (nanospectroscopy); Naomi Halas, Rice University (surface-enhanced vibrational spectroscopy).

VD: There are definitely too many to mention here and you will probably get as many different answers as the number of people you ask... In terms of fluorescence spectroscopy, certainly the development of sub diffraction limit imaging in Göttingen or Janelia Farm are most impressive. Here in Germany, Stefan Hell (developer of STED) and Eric Betzig (developer of PALM) are the major protagonists. In these cases the technology could almost immediately be applied in biology and medicine. In other fields of spectroscopy, I would not speak of strongholds as such, the fields have matured over a long time, so there are many places doing high quality research.

GH: I can answer this best with a couple of (non-comprehensive) lists from the US. In the past: Illinois (Malmstadt), Michigan (Willard), Wisconsin (Walters), Iowa State (Fassel), Purdue (Pardue, Amy), North Carolina (Reilley, Eisenhour), Cornell (Morrison).

Today: Purdue (Lytle, Wirth, Simpson), North Carolina (Ramsey), Indiana (Hieftje), Illinois (Sweedler), Texas (Holcombe), Notre Dame (Dovichi), Arizona (Denton).

In terms of topics: near-field methods, imaging, atomic spectrometry, sensors, single-molecule sensing, nonlinear and two-dimensional methods, high-resolution techniques, excited-state kinetics, fast instrumentation, and correlation are all likely to continue to be hot.

How did those you have mentioned become so successful?

VD: In parallel with the increasing complexity of the molecules or systems that were investigated, the demand for higher time resolution or spatial resolution was a major driving force in instrumental development. In order to

"It is not surprising that many of the top analytical chemists have training in physical chemistry, physics or an allied discipline."

perform the desired, the researchers simply had to be several steps ahead of current commercial offerings. And with such advanced instrumentation, you can do things that others cannot do. Sounds easy, doesn't it?! But perhaps it's even easier than that – just like Terry Pratchett's Discworld novels, where some people are hit by "Inspiration Particles"...

PG: I believe that in every case that I have listed above, the principal investigator has made significant advances in the theory of the technique in addition to the superb experimental achievements for which he or she is better known. It is not surprising that many of the top analytical chemists have training in physical chemistry, physics or an allied discipline. (Truth in advertizing: my doctorate is in physical chemistry!)

GH: Usually, I think success comes as a result of one or two highly innovative, driven faculty, whose new ideas and personalities draw colleagues and students to them.

Where are the great openings in spectroscopy?

PG: Perhaps the most important area in which vibrational spectroscopy is starting to show promise is medical diagnosis. Wong and his colleagues at the National Research Council of Canada showed that the mid-infrared spectra of exfoliated cervical cells showed evidence of extensive structural changes during carcinogenesis (1). Regrettably, the results were shown to be strongly dependent on the patient and it has taken many years of further work by a plethora of other research groups, of which I will single out the current groups of Max Diem at Northeastern University and Rohit Bhargava at the University of Illinois, to show that mid-IR and Raman spectra may allow various stages of cancer to be identified.

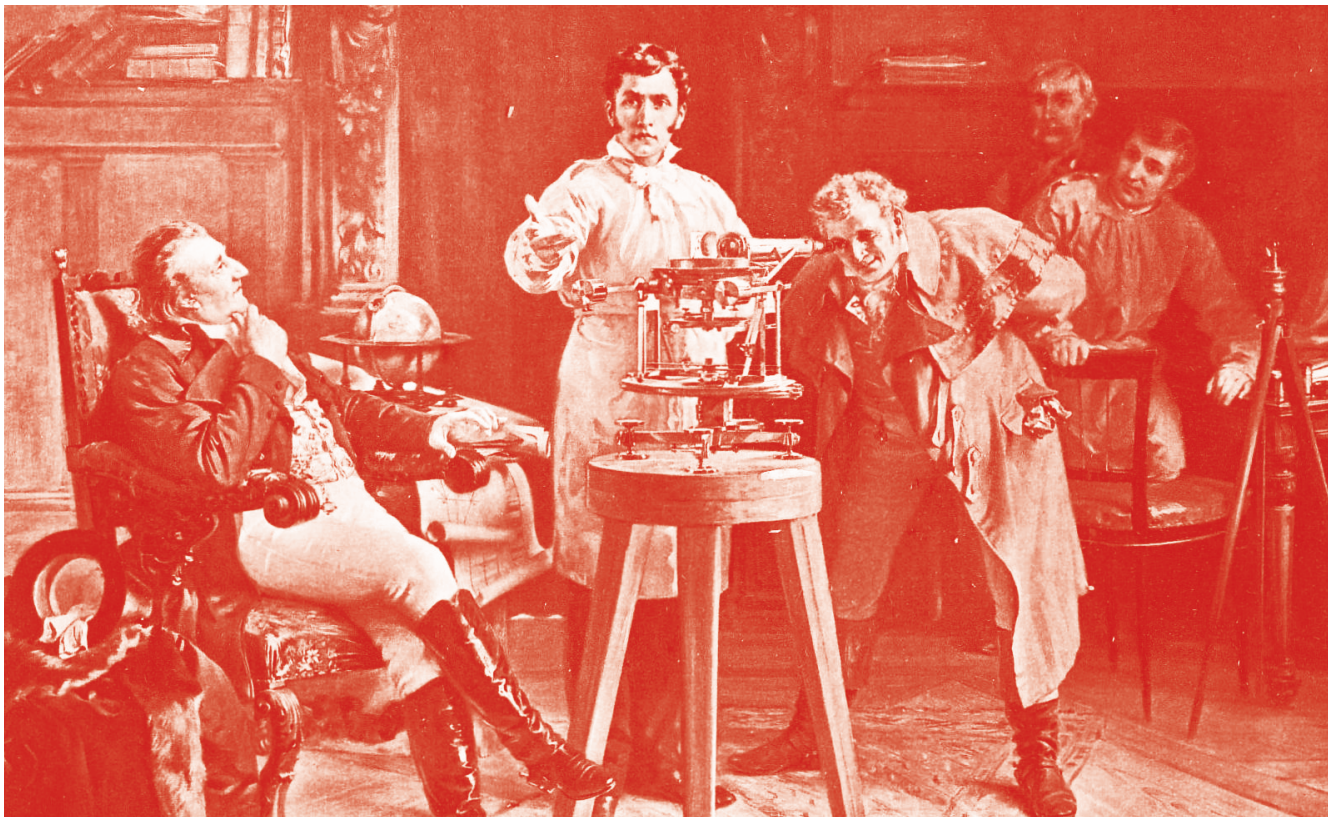
GH: The list of great openings is pretty long:

- Lasers
- Near-field methods
- Holographic gratings
- Charge-transfer array detectors
- Interferometers, FT methods
- Atomic absorption
- ICP emission and mass spectrometry
- Modern Raman spectrometers
- MCT detectors for IR
- Digital micromirror arrays
- Electro-optic and acousto-optic modulators.

VD: I would consider the wealth of applications that are now in reach all offer great openings. In particular, portable instruments that allow us to point spectrometers at virtually everything is probably the greatest opening.

How about dead ends?

GH: Dead ends? Interesting question… Widely tunable lasers seem to be one; though there have been many lasers, covering many wavelengths, none are easily tunable over a broad range. Diode-laser atomic absorption and fluorescence; great strides were made early, prompting some to predict that diode lasers would replace continuum sources, hollow-cathode lamps, and electrodeless discharge lamps for AAS and AFS.



Joseph von Fraunhofer demonstrating the spectroscope. From a painting by Richard Wimmer.

Mass-spectrometer (Finnigan MAT251) for measuring stable carbon (12C/13C) and oxygen (16O/18O) isotopes in carbonaceous shells.
Isotope laboratory of Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany. The engineer is checking the magnet for deflection and separation of the isotopes. The MAT251 was controlled by an Apple II computer (right).

However, lack of tenability and poor performance in the UV still make them largely impractical. Also, atomic fluorescence spectrometry, although fundamentally superior, has been killed by entrenched atomic absorption. And genome sequencing by individual base-pair optical scanning was simply too slow.

VD: Ask any scientist "how many dead ends?" in his or her career and the answer is likely to be, "more than great openings." The problem with both great openings and dead ends is that you don't really see them coming (ask Marvin Minsky about the confocal microscope...) Many ideas and concepts are worth trying, but in the end can prove to be flawed. The so-called aperture near-field optics concept to overcome the diffraction limit is such an example from my field. In principle, it can be applied to any optical spectroscopy, but sensitivity – and, more importantly, reproducibility issues – made it very difficult to handle. Interestingly, this dead end resulted in the development of a different concept: scattering or apertureless near-field optics, which can be dubbed as a great opening (so far!)

PG: One example of what appears to be a dead end is the non-invasive measurement of blood glucose by NIR spectroscopy. Well over $200 million has been spent on research to show the feasibility of such measurements without real success... Work is still continuing on this topic and some promising results are starting to be reported so, perhaps ultimately, this work may lead to a useful application.

I can also give an example of an incorrect result that has actually led to the development of an important technique – it can be found in the first report of surface-enhanced infrared absorption (SEIRA) (2). In this work, a thin layer of silver was deposited on the surface of a ZnSe internal reflection element and a few molecular layers of p-nitrobenzoic acid (PNBA) were deposited on the silver surface. After evaporation of the solvent, three strong bands were observed between 2800 and 3000 cm^{-1}, which the authors ascribed to PNBA. Regrettably, the bands that were observed in the spectrum were due to aliphatic C-H stretching modes and PNBA has no aliphatic C-H groups. Nonetheless, this paper became "reference number one" in many subsequent papers on SEIRA…

What are the opportunities and challenges?

GH: There are plenty of challenges (or opportunities, depending on your point of view):

- Low-noise, wide-bandwidth single-photon detectors
- Tunable lasers for the entire UV-Vis wavelength range
- Broad-scene imaging at nanometer spatial resolution
- Bright non-laser sources for IR
- Low noise, ultra-wide bandwidth analog amplifiers
- The need to develop a method for measuring sample erosion on the nanometer scale.

PG: It may have become apparent from my response to the previous question, but I believe that both the biggest opportunity and challenge is in the field of medical diagnosis, especially cancer detection. The concept of being able to probe a tissue in situ rather than waiting several days for the result of a biopsy could revolutionize histopathology. From an instrumental standpoint, the developments in nanospectroscopy, i.e., the measurement of both mid-infrared and Raman spectra at a spatial resolution well below the diffraction limit, are very exciting. Deckert, our co-writer here, has reported the measurement of tip-enhanced Raman spectra at a resolution of better than 20 nm, so that the spectra of a few base pairs of a strand of DNA can be acquired, and several TERS instruments are now commercially available. In the last decade, AFM-IR measurements with close to this spatial resolution have been reported by Dazzi's group in France (commercialized by Anasys Instruments in the USA) and by Keilmann and Hillenbrand in Germany (commercialized by Neaspec in Germany.) The challenge in TERS and AFM-IR measurements is to improve the spatial resolution to the atomic level; this will probably require smaller, more rugged AFM cantilevers as opposed to more sensitive spectrometers.

VD: Many optical spectroscopies are or can be made non invasive, which allows non-destructive detection or detection from a safe distance. These properties are ideally suited to medical diagnosis and many groups are currently developing systems for that purpose. Personally, I am most impressed by attempts in fast classification of bacteria by Raman spectroscopy. My colleague Jürgen Popp and his co-workers are developing strategies to identify bacteria on the strain level in less than two hours (something that takes several days of standard bacterial cultures). This advance really could change medical treatment of sepsis, when literally every minute counts. The biggest challenge here is likely to be the lack of synergy between scientific instrument development and the actual clinical environment. Physicians are used to robust and easy to use instruments. Spectral interpretation is definitely not on that list. Hence, apart from the actual instruments, automated interpretation and classification of the data is required.

"In the area of atomic spectrometry, people are still searching for a 'better ICP'. It is becoming well recognized that the ICP is inefficient and error-prone."

How can spectroscopists continue to make a substantial contribution to science and/or society?

VD: The answer here is pretty much in the line with my previous statement and Peter's earlier thoughts. I think medical diagnosis will have a big impact on the further development of spectroscopy and, hopefully, vice versa. But such developments need time and patience (good for patient safety, bad for rapid progress). Clearly, immediate breakthroughs in the field of clinical diagnosis are not to be expected. In view of changes in society, there is an increased need to remotely monitor patient status or perform quick screening in cases of emergency. Here, optical spectroscopy will surely have its fair share. After all, current smart phones already contain all the main parts required for spectroscopy: a light source and a detector. Include a laser pointer and you can do some basic fluorescence. The Star Trek Tricorder is almost here!

PG: Spectroscopists have never been very good at promoting their developments. Some important developments remain a well-kept secret while others are announced too early. Better ways of disseminating important developments in spectroscopy are clearly needed but I am not really the right person to say exactly how this should be accomplished. Perhaps that's where The Analytical Scientist could help?

GH: We need to encourage new small instrument firms that employ breakthrough technology. In other words, the ongoing and increasing consolidation of instrument companies into large entities essentially discourages innovation. Let's face it: there is little incentive for a large firm to adopt new technology that will compete with its own existing offerings.

And, by the way, the recent change in US patent law goes against this trend. Before, the demand was "first to invent". Now, the US has followed most of the rest of the world in giving rights to the "first to file". Unfortunately, this change legislates against innovation and publication by universities. The coin of the realm in universities is publications rather than patents; it compromises a student's chances for a job and a faculty member's chance for tenure if a publication is delayed until a patent is secured… But that's another story

What dramatic improvements in spectroscopic instrumentation could you cite that have led to some of the current levels of performance?

PG: To answer this, I would like to go back several decades. The development of FT-IR spectrometers in the late 1960s and early 1970s allowed many of the limitations of grating spectrometers to be overcome. Today, nobody thinks about using a monochromator for mid-IR spectroscopy any longer, except as a polychromator in combination with a focal-plane array detector. Two developments in the 1980s can be cited as being largely responsible for the current popularity and high performance of Raman spectrometers: first, charge-coupled device array detectors replaced photomultiplier tubes and second, notch filters allowed the second monochromator to be eliminated. Today, hand-held FT-IR and Raman

spectrometers are available with performance that would have been unthinkable on the bench-top only a few decades ago. I should also draw attention to the corresponding advancements in chemometrics and the development of sophisticated software that have enabled many applications that would otherwise be impossible on a routine basis.

VD: How far back do you want to go? Lasers are common today, but certainly without them most modern optical spectroscopies would be either impossible or extremely slow. Detector technology development has been somewhat more continuous; however, the introduction of CCD detectors to visible spectroscopy was a huge improvement. Quantum efficiencies of >90% and the ability to electronically record the whole spectrum dramatically increased the speed of detection. When used as pure imaging devices, the same advantages allowed for the fast acquisition of fluorescence images, for example.

Interestingly, the systematic combination of spectrometers and microscopes happened quite late in the case of infrared and Raman spectroscopy. In particular, the collection efficiency benefitted from the use of microscope objectives and, more importantly allowed mapping of larger areas and, consequently, the distinction of local variations.

GH: I would cite both array-detector technology and near-field methods as examples. Both now abound in commercial instrumentation and have dramatically improved performance and range of application. Similarly, nonlinear spectroscopic methods are being applied widely by biologists and biophysicists and are finding their way into the marketplace.

In the area of atomic spectrometry, key examples would be the improvement in plasma sources (for example, ICP, glow discharge, and more recently the atmospheric-pressure glow discharge), the development of electrothermal vaporization (L'Vov and others), the introduction of hollow-cathode lamps for atomic absorption (Walsh), and the invention of background correction methods for atomic absorption.

Do you see other landmarks on the horizon?

VD: If one could transfer the concept of energy dispersive detectors to the visible range with enough spectral resolution, it could be the most dramatic change in spectroscopy of all. You could get rid of the spectrometer itself as such a detector could directly detect the energy of incoming photons. I like that idea: spectroscopy without a spectrometer, as Rick Russo might say!

GH: Volker's idea is a great one, and in fact was pursued by Tomas Hirschfeld during the time he was at Block Engineering. The problem, of course, is that in the spectral ranges used in "optical" spectroscopy, the photon energies are comparatively low, on the order of single eV. As a result, to obtain even modest resolving power requires resolution on the order of thermal energies. To my knowledge, Tomas didn't employ cooled detectors, so the idea should be revisited.

Another invention on the horizon is the use of "fly's eye" lenses for imaging and for miniaturization of spectrometric instrumentation. And, as mentioned earlier, the translation of the multi-parametric methods now common in NMR into the optical regime.

In the area of atomic spectrometry, people are still searching for a "better ICP". It is becoming well recognized that the ICP is inefficient and error-prone, the latter caused by interelement (matrix) interferences. The causes for those interferences are finally being worked out, and methods for their detection, recognition, and control will follow. Similarly, the ICP requires lots of power, lots of argon, and generally substantial amounts of sample material. There are studies underway on small sources that operate in the open air or with air as the support gas, at modest power levels, that require only small amounts of sample, but which rival ICP performance...

PG: The development of quantum cascade lasers (QCLs) could open up new types of measurements in the mid-infrared region. At present, QCLs are expensive and the wavelength range is limited. But given a "killer app," where hundreds or even thousands of instruments could be applied, the cost of these lasers could be reduced dramatically.

I have worked in the field of vibrational spectroscopy for 50 years and it has always amazed me that just about every year at least one major development has been reported. This trend will undoubtedly continue. I just wish that I was smart (and young!) enough to see what tomorrow's developments will be.

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Two-dimensional Bioanalysis

Bringing biopharmaceuticals and biosimilars to market with 2D-LC

By Koen Sandra, Gerd Vanhoenacker and Pat Sandra

In recent years, the top ten pharmaceuticals sales list has been extensively populated with protein therapeutics, such as monoclonal antibodies and recombinant proteins, that are used to treat various life-threatening diseases, including cancer and autoimmune diseases. A number of these blockbuster biopharmaceuticals are coming off patent in the near future, a fact that has resulted in an explosion of activity in the biosimilar or "biogeneric" market.

Irrespective of whether a company is developing innovative biopharmaceuticals or copycat biosimilars, a detailed characterization must be performed. Indeed, product characteristics need to be very closely monitored prior to clinical or commercial release. With complexity far exceeding that of small molecule drugs, the characterization of protein biopharmaceuticals represents significant analytical challenges, typically involving a wide range of analytical techniques and methodologies (1).

Peptide mapping is a commonly used characterization methodology as it provides great detail of the molecule under investigation. Let's take a monoclonal antibody of 150 kDa as an example; a trypsin digestion will generate over 100 peptides all with varying physicochemical properties over a wide dynamic concentration range. The complexity associated with these digests demands the very best in terms of separation power. Compared with one-dimensional separations (1D-LC), two-dimensional LC – and especially comprehensive 2D-LC (LC × LC) – drastically increases peak capacity as long as the two dimensions are orthogonal and the separation obtained in the first dimension is maintained upon transfer to the second dimension.

Orthogonal combinations for 2D-LC based peptide mapping include strong cation exchange and reversed phase LC (SCX × RPLC); hydrophilic interaction chromatography and reversed-phase LC (HILIC × RPLC); and reversed-phase LC and reversed-phase LC (RPLC × RPLC) with different pH levels in the two dimensions (2-4). The highest orthogonality is obtained with SCX × RPLC and HILIC × RPLC, because the separation mechanisms are completely different. However, RPLC × RPLC is particularly interesting; excellent solvent compatibility in both dimensions makes it the most rugged, but it still offers very high peak capacity (driven by high plate numbers in the individual dimensions) and great orthogonality, which is due to the zwitterionic nature of peptides, such that major selectivity differences are achieved when performing separations on RPLC at pH extremes.

Figure 1 shows the 2D-LC tryptic peptide maps of two production batches of the monoclonal antibody trastuzumab using RPLC × RPLC. Trastuzumab has been marketed as Herceptin since 1998, and is still being used in the treatment of HER 2 positive breast cancer. The peptide map provides a wealth of information that allows identity and purity to be assessed. Trastuzumab's identity is spread over 62 tryptic peptides (20 light chain and 42 heavy chain) of which the majority is baseline resolved. Several of these tryptic peptides contain amino acids that are prone to modifications, such as deamidation, isomerization, oxidation, and so on. Such product related impurities can have an influence on both the safety and efficacy of the product and need to be closely monitored.

The benefits of 2D-LC technology with respect to such modifications are highlighted in Figure 2, which shows a zoomed view of the 2D-LC peptide map of a non-stressed and pH stressed originator. A deamidation event is substantially increased upon stressing trastuzumab at pH 9 for three days. This modification, which occurs on an asparagine located within the third tryptic peptide of the light chain, is already observed at around 10 percent in the non-stressed sample. Peak identity was obtained using MS and modification sites were revealed by MS/MS.

The precision associated with the RPLC × RPLC methodology combined with UV-detection (retention time RSD < 0.2% and area RSD < 5% for n=5) makes this very a powerful approach to demonstrate comparability between production batches, as demonstrated in Figure 1, and between originator biopharmaceuticals and biosimilars.

So, the big question is: where in the biopharmaceutical development pipeline do we see the true benefit of 2D-LC? It's fair to say that in early stage development, one dimensional-LC-MS is certainly an option; RPLC columns that provide peak capacities well in excess of 500 are widely available nowadays – when used in combination with high-resolution mass spectrometry, a

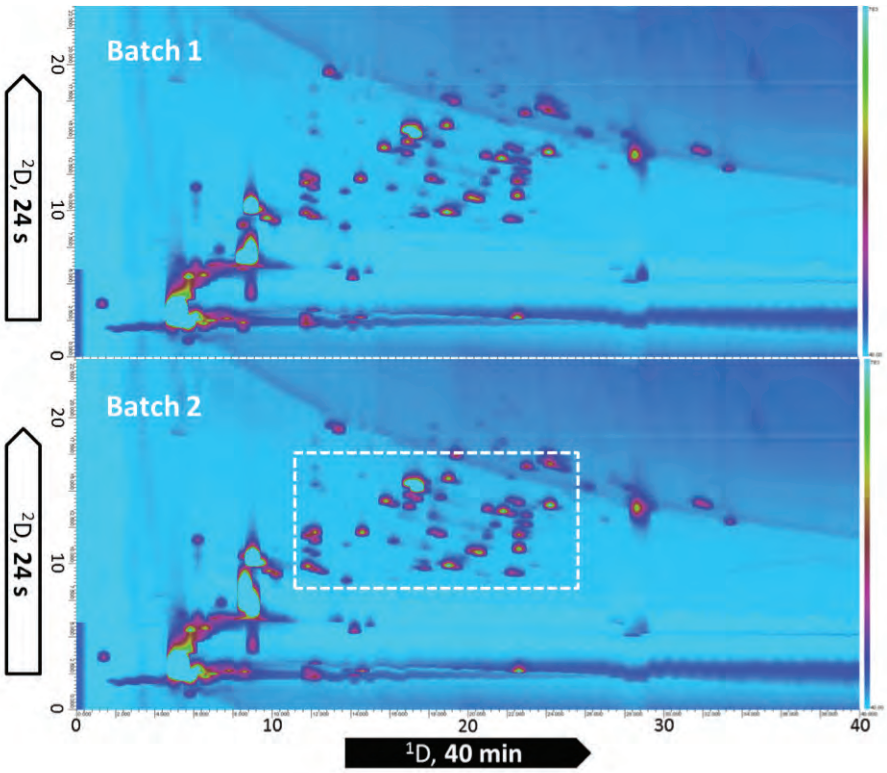

Figure 1. 2D-LC peptide map of two Herceptin production batches. First and second dimension separation consist of reversed-phase LC operated at high and low pH, respectively. Fractions were transferred from one dimension to the other using a dual-loop interface. UV detection was performed at 214 nm.

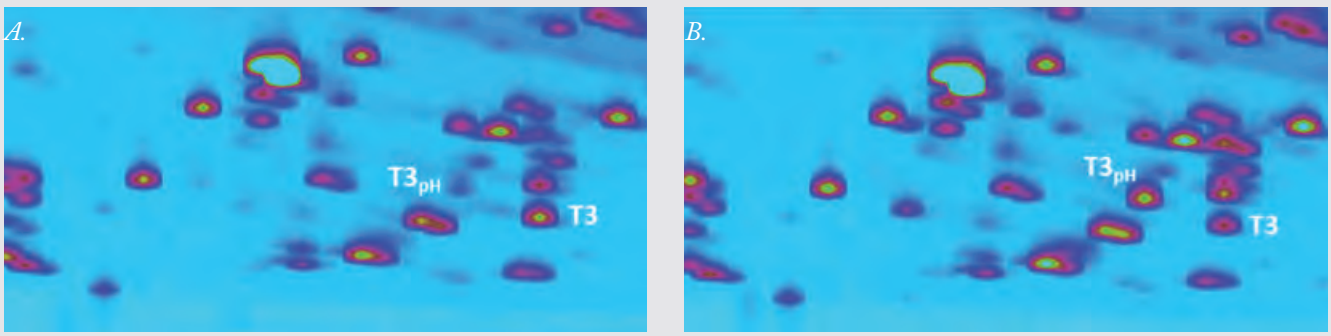

Figure 2. Zoomed view in the 2D-LC peptide maps of a non-stressed (A) and pH stressed (B) Herceptin production batch demonstrating asparagine deamidation in the light chain of the monoclonal antibody. T3 refers to the third tryptic peptide counting from the N-terminal side of the light chain. $T3_{pH}$ refers to the deamidated variant.

powerful characterization engine emerges. However, as we move along the development pipeline and enter the clinical or commercial release phases, MS preferably has to be replaced by UV detection and the enhanced resolution offered by 2D-LC comes into its own. Certainly, the appearance of commercial instrumentation represents a major step towards more widespread use of 2D-LC in biopharmaceutical analysis.

Koen Sandra is R&D Director, Gerd Vanhoenacker is LC Product Specialist/ Manager, and Pat Sandra is Founder and President, all at the Research Institute for Chromatography (RIC), Kortrijk, Belgium.

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Back to School for Pharmaceutical Analysis

Deeper consideration of individual analytical unit processes offers a more systematic approach to method development, ensuring that we don't forget the basics in an increasingly sophisticated world.

By W. Franklin Smyth

Back in 1970, when I took my PhD in analytical chemistry at The Queen's University of Belfast, the emphasis was primarily on "chemistry". In particular, I was working on electrochemical methods of analysis for pharmaceutical compounds. I've spent my entire career either teaching analytical chemistry or conducting pharmaceutical analyses, travelling widely in Africa, Australia and Europe. I finally settled at the University of Ulster, where I am currently an active Emeritus Professor in the School of Pharmacy. Over the years, my interests have changed somewhat – I became more heavily involved in bioanalysis and moved towards mass spectrometry-based techniques – but despite these changes of direction, chemistry has always been at the center.

I consider myself an "old school" analytical chemist, and I've noticed that all around me the "chemistry" part of my field is less and less obvious – analytical chemistry has become analytical science (as emphasized by the title of this very magazine!). That's not entirely a bad thing – it means we've embraced a much wider scope in terms of science and applications. The problem is that you can't simply forget about the exciting and interesting chemistry occurring (nowadays very much in the background) to enable a particular analytical method.

Teaching of pharmaceutical analysis is generally carried out via the subdivisions of techniques. The field divides naturally into spectroscopy techniques, for example, infrared (IR) or ultraviolet (UV) spectroscopy, and nuclear magnetic resonance (NMR); chromatography techniques, for example, thin layer chromatography (TLC), gas chromatography (GC) techniques, and high performance liquid chromatography (HLPC); and electrochemistry techniques, for example, potentiometry and polarography. Specific applications are typically addressed within the confines of each particular technique.

However, over the years I have come to organize the teaching of "the analytical method" slightly differently. Instead of focusing on individual techniques, I advocate considering the individual unit processes (representative sampling, preliminary sample preparation, separations, and so on) that make up the overall method. The journey begins with the definition of the problem: how do I find analyte A in matrix X? That could be as simple as finding an active pharmaceutical ingredient in a tablet with one other excipient; it could be as complex as trying to separate several drug degradation products and metabolites at very low concentrations in a hair or plasma sample. But in either case, I've always stressed the importance of the different unit processes and how each one plays its part in getting the best analytical result to solve the problem. It's a rather more systematic way of approaching method development that really highlights the fact that you not only have to understand the instrumental technique you're using, but also the origin of the sample and how best to handle that sample type for that chosen instrumental technique. I suppose it's my way of coercing students into a deeper consideration of the chemistry – and other processes – involved.

In this article, I offer examples from my past experiences to emphasize the importance of each unit process. Along the way, I also hope to highlight some interesting chemistries that have been invoked to produce the best results from a particular instrumental technique.

The unit process approach

I've stated that a complementary approach to a techniques-based teaching program is to more deeply consider the unit processes of pharmaceutical analysis. So, what are the unit processes? They are:

- the choice of technique
- the method used to obtain a representative sample
- the preliminary treatment of the sample
- the separation of desired constituent(s) from each other and from interferences
- the measurement (and its statistical assessment)
- calculation of the result.

Pharmaceutical analysis is concerned with the identification (qualitative analysis) and/or determination (quantitative analysis) of, mostly, organic substances in either simple matrices (drugs dissolved in solvent) or complex matrices (drugs and metabolites in blood, urine or hair) – see sidebar 1980s Pop Quiz for two examples. Clearly, a pharmaceutical analyst must be capable of designing, performing and interpreting measurements within a diverse world of problems. Extracting meaning from the results is the final essential piece in the puzzle.

The choice of technique

There are three points to consider with respect to technique choice. One, it is dependent on the methodology and

"I suspect a few of you have encountered a destroyed column after an unsupervised novice injected blood (or some other incompatible matrix) directly into your instrument..."

instrumentation available in the pharmaceutical analytical laboratory tasked with solving the problem. Two, the chosen method must give results within the necessary practical parameters, such as time and cost per sample analyzed. Three, the method must be suitable for its intended purpose – all the steps needed to generate qualitative and quantitative analytical results must be validated.

With regard to the last point, a three-step approach commonly adopted by the pharmaceutical industry is outlined below, using HPLC as an example.

- System validation: the wavelength accuracy of the detector, the flow and compositional accuracy of the pump, and the precision and sampling accuracy of the autosampler must be assessed.
- Method validation: the performance of the analytical method, including sample preparation, must be verified. Accuracy and recovery, precision, selectivity/specificity, linearity/calibration curves, limits of detection, limits of quantitation, ruggedness, robustness and stability must all be evaluated. The number of parameters that have to be assessed depends on whether the information is to be used for contract analyses, regulatory submission or laboratory accreditation.
- System suitability testing: a routine sample analysis must be carried out daily using the validated instrument and method. Usually, two or three of the validation criteria suffice to check the validity of the system, for example, precision of amounts, resolution between two peaks, and the tailing factor in HPLC.

Representative sampling

Liquid sampling is relatively simple if the solution is homogenous but for solid samples a more detailed consideration of what is or is not representative is needed. Let's use the European pharmacopoeia (PhEur) UV assay for chloramphenicol capsules as a typical example:

"Dissolve a quantity of the mixed contents of 20 capsules containing 0.2 g of chloramphenicol in 800 ml of water, warming if necessary to effect solution, and add sufficient water to produce 1000 ml. Dilute 10 ml to 100 ml with water and measure the absorbance of the resulting solution at the maximum at 278 nm. Calculate the content of $C_{11}H_{12}Cl_2N_2O_5$ taking 297 as the value of A (1 percent, 1 cm) at the maximum at 278 nm."

So, in this example, 20 capsules are considered representative of a given batch. "Is my sample representative?" – if you've not asked yourself that question, then exactly what are you measuring?

Preliminary sample treatment

Of course, the sample type defines the required preliminary treatment. Certainly, the efficient dissolution of solids is a prerequisite to further unit processes (as noted above for chloramphenicol dissolution, "warming if necessary to effect solution"). Yes, this is very basic science, but it can sometimes be forgotten in the rush to start injecting and analyzing. Biological samples can be much more complex. For plasma, anticoagulant must be added to the blood sample to prevent clotting. On the other hand, in serum analysis, the blood sample is allowed to clot, then centrifuged. The protein is then precipitated using perchloric acid and, finally, reversed phase HPLC of the aqueous supernatant can be conducted for drug and metabolite analysis. Once again, relatively simple steps – but I suspect a few of you have encountered a destroyed column after an unsupervised novice injected blood (or some other incompatible matrix) directly into your instrument… Once again, if we haven't even considered this unit process, we could inhibit our success in later steps. The assessment should encompass whether something new been developed that could speed up or improve preparation. It's easy to continue blindly following outmoded techniques, if the question never enters our mind.

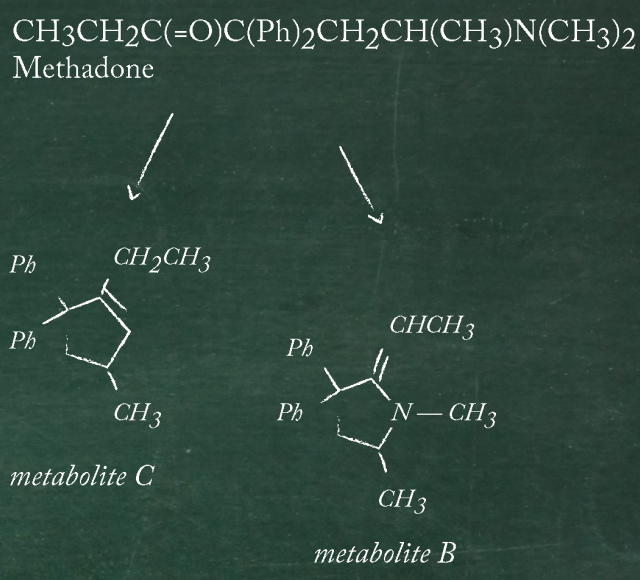

Figure 1. Methadone and its two metabolites (B and C).

Separation

Separation techniques abound, each with advantages and disadvantages. There's a lot to consider here, so I've provided a couple of examples that I've come across over the course of my career that I think are nicely illustrative. The question here again is, "Does a newer and better alternative exist?"

The GC analysis of methadone and metabolites (see Figure 1) illustrates the use of solvent extraction prior to instrumental analysis (2).

The following steps typically are typically performed in advance of GC-flame ionization dector (FID) analysis:

- Plasma pH is adjusted to 9.8, after addition of internal standard.
- Solvent extraction of neutral species into n-butyl chloride.
- Back extraction of protonated species into 0.2M HCl
- Separation of aqueous phase, made alkaline with 60% NaOH.

The final solvent extraction step is carried out using chloroform into which the neutral species are extracted and, after evaporation to dryness, the residue is dissolved in a small volume of chloroform prior to a portion being injected on a GC column with 3% SE-30 stationary phase and a temperature of 200 °C. In this example from 1972,

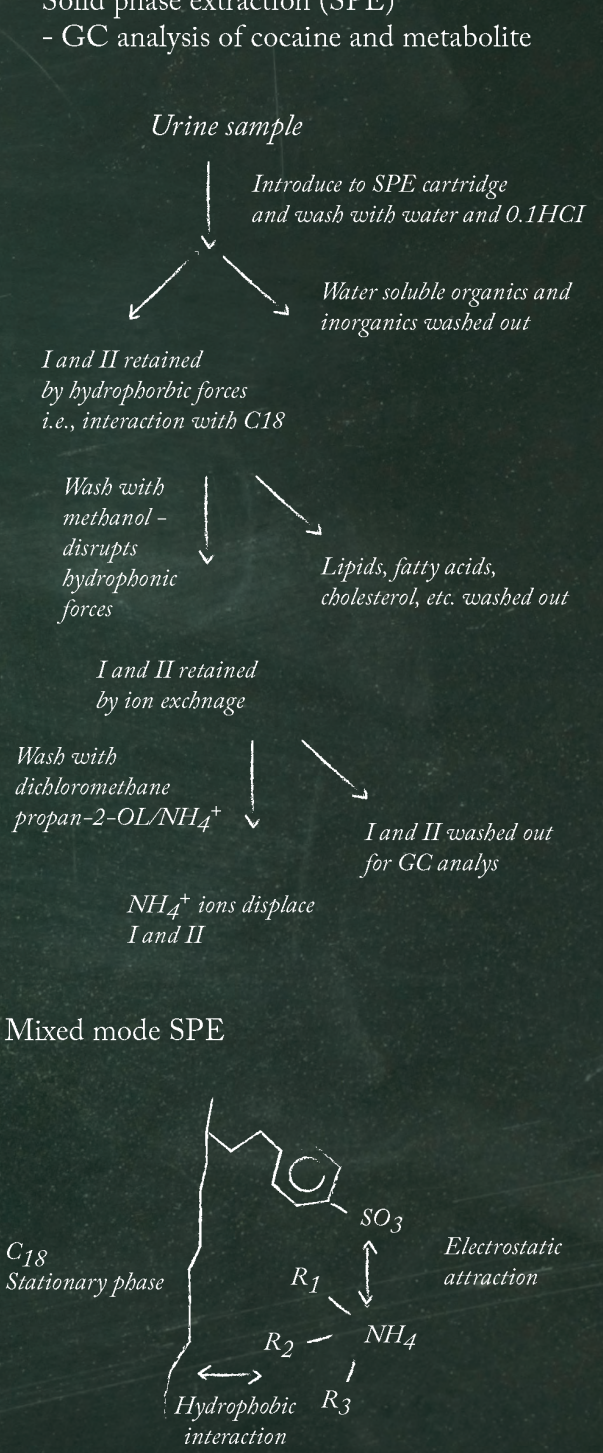

Figure 2. Solid phase extraction (SPE) and GC analysis of cocaine (I) and its metabolite, benzoylecgonine (II).

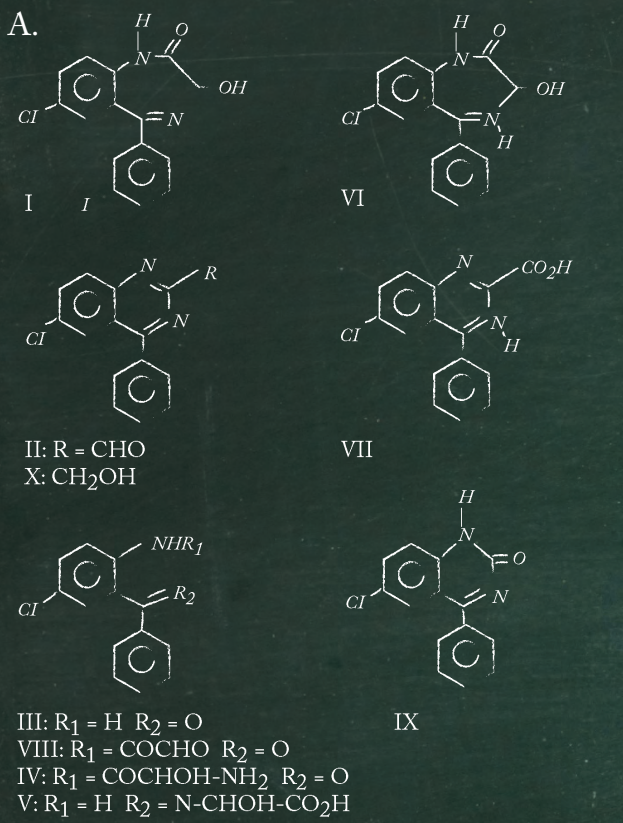

Figure 3. (a) Oxazepam and its degradation products. (b) Proposed ESI-MSn fragmentation pathway of oxazepam and 6-chloro-4-phenyl-2-quinazolinecarboxaldehyde (the aldehyde degradation product).

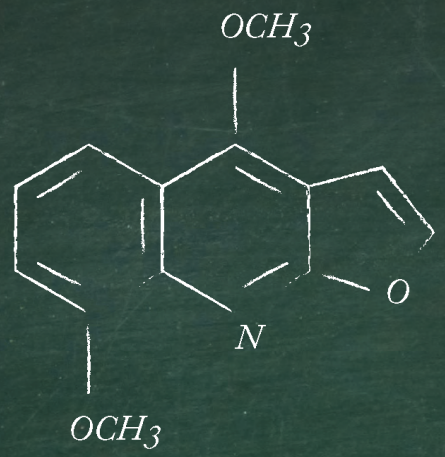

Figure 4. γ-fagarine.

recoveries are greater than 95 percent and are obtained with retention times of 2.3 min for metabolite C, 3.2 min for metabolite B, methadone at 4.7min and 8.8 min for the internal standard.

Can this be improved upon?

Solid-phase extraction (SPE) has taken over from solvent extraction in many cases because it negates the disadvantages of individual operator variations, hazardous solvents and relatively unselective extraction. There are many variations (for example, the wash processes and solvents used) that are dependent on the analytes to be extracted. SPE has the additional benefit of automation potential and can be used online with HPLC.

A good example of SPE use can be shown in analysis of cocaine and its principal metabolite, benzoylecgonine ahead of GC (3), which is illustrated in Figure 2. Note the interesting dual-retention chemistry used in mixed-mode SPE.

Chromatographic techniques are used extensively to separate desired constituents from each other and interferences. In pharmaceutical analysis, it is not uncommon to be able to detect a drug and its process impurities or degradation products in a single scan. For example, in 1995 Husain et al. reported that reversed phase HPLC analysis of ciprofloxacin and process impurities DCFA, Q acid, CPA, CFA could be carried out in a single scan lasting only 7 min (4).

Even earlier, Reif et al. published a stability indicating assay for oxazepam (I) and seven of its degradation products (II, III, VI, VII, VIII, IX, X, see Figure 3) down to 0.1% levels, in a single 20 min scan using reversed phase HPLC analysis (5).

I can offer a personal example of a complex combination approach that may be required in certain instances. My colleagues and I reviewed the of phytochemicals in selected plants using a combination of separation processes followed by electrospray ionization ion trap mass spectrometry (ESI-MSn) and LC-ESI-MS (6). Dereplication (or counterscreening) aims to isolate unknown bioactive candidates from known bioactive chemicals. Crude extracts of selected plants were subjected to column chromatography, the resulting fractions being tested for their bioactivity towards organisms, such as methicillin-resistant-Staphylococcus aureus (MRSA). The bioactive column chromatography fractions were further separated by preparative TLC to yield a series of bands, which were subjected to LC-ESI-MS and ESI-MS^n. The resulting retention times, molecular masses, and fragmentation patterns were used alongside Chemnet and in-house quinoline databases in the dereplication process to elucidate the structure of some of the compounds by comparing them with known structures of natural origin. Some molecular masses and the corresponding fragmentations did not correlate with any known compounds thus revealing potentially novel natural products that could be investigated on a larger scale and could ultimately find application as new drugs against MRSA and other multi-drug resistant microorganisms.

To illustrate the dereplication process, let's look at the identification of γ-fagarine (see Figure 4) in the plant Ruta graveolens. An $[M+H]^+$ ion for TLC band 5 was observed at m/z 230. This ion fragmented at MS^2 to generate a product ion at m/z 215, indicating the loss of a methyl radical. The product ion then lost 28 u at MS^3 to generate an ion at m/z 187. On referring to the quinoline database, γ-fagarine gave identical fragmentation as observed for this ion. Furthermore, LC-ESI-MS analysis revealed that the retention times of the compounds also match at ca 10.2 min.

1980s Pop Quiz

I've selected a couple of problems that I faced in my career. I present the solutions we used back then. Test yourself by thinking about how you would deal with the following scenarios today.

Problem A

The veterinary pharmaceutical levamisole (immunostimulant, antihelminthic) can contain 6-phenyl-2,3-dihydroimidazo[2,1-b]thiazole as a toxic impurity (see Figure 1 for structures). How could the quality control unit of a manufacturer ensure that levels remain below a certain threshold value using a simple, inexpensive and rapid test?

Our method: Perform TLC (chosen for speed and cost effectiveness) on 10 µL of the suspect raw material made up to 5 percent w/v on silica gel with toluene and glacial acetic acid as the mobile phase, visualizing with potassium iodoplatinate solution (brown, blue and purple spots with many basic (as opposed to acidic) drugs. Conduct TLC on 10 µL of pharmacopoeia standard made up to 0.025 percent w/v using the same conditions.

Our solution: If the TLC spot from the suspect solution is more intense than the spot for the standard solution, there is a 0.5 percent or higher impurity level.

If you are familiar with the current European pharmacopoeia, you may know that 6-phenyl-2,3-dihydroimidazo[2,1-b]thiazole and four additional impurities from manufacture and degradation must now be analyzed by HPLC with UV detection at 215 nm to ensure compliance.

Problem B

How can clinicians quickly and easily quantify the amount of anticancer drug dacarbazine, normally administered at 250 mg per day to a patient undergoing chemotherapy, to assess the best dosing regime?

Our method: Sample blood, allow to clot and centrifuge. The clear serum is subjected to protein precipitation with, for example, $HClO_4$. Protein-free serum is then analyzed by reversed phase HPLC-UV.

Our solution: Under the above HPLC conditions, dacarbazine elutes at 14 min (separately from biological interferences), permitting its quantitation and hence providing the clinician with data on which to base administration of the drug (1).

What methods did you suggest?

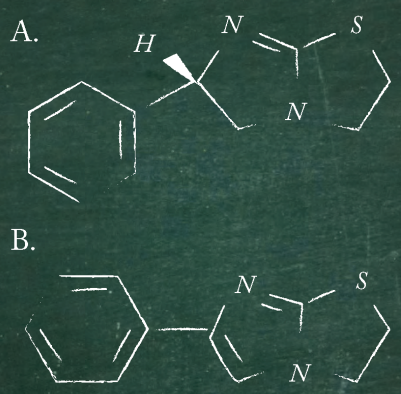

Figure 1. A. levamisole B. 6-phenyl-2,3-dihydroimidazo[2,1-b]thiazole

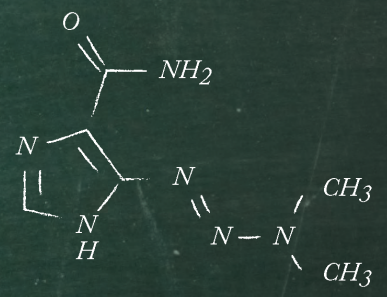

Figure 2. Dacarbazine

Measurements and their statistical assessment

Chemical measurements, such as titration, are still used to a certain extent in PhEur assays – particularly in industrial quality control, but instrumental measurements (for example, LC with diode array detection) are now very widely used in pharmaceutical analysis – we've already covered several examples of those above.

In terms of statistical analysis, in addition to standard deviation, three other commonly used tests in pharmaceutical analysis are:

1) *Q* test, for removal of insignificant data. To apply a test for bad data, the data are arranged in order of increasing values and *Q* is calculated by dividing "gap" by "range", where gap is the absolute difference between the outlier in question and the closest value to it. In terms of *Q* tables, if *Q* (experimental) is larger than *Q* (critical), the questionable value is rejected.

2) *t* test, for comparison of means of data sets. If there is an accepted value for the result based on extensive previous analysis, *t* (experimental) can be calculated and compared with *t* (critical) in the *t* table. If *t* (experimental) is smaller than t (critical) for the appropriate number of degrees of freedom, then the difference between the means is not significant.

3) *F* test, for comparison of the precision of data sets. The *F* test is used to test for differences among sample variance. Like the Student's t test, an *F* value is calculated and compared to one in the *F* table.

Correct statistical analysis is worth a feature in its own right. I covered it in detail in my book (7), but there is a plethora of sources available to provide a refresher course.

Webinars

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"In our modern world of faster, more accurate, higher resolution – and automated – instrumentation, it can be easy to forget the basics."

Needless to say, given its importance, it should always be subject to a significant degree of consideration.

Generating results

In terms of identification or qualitative analysis, let's go back to the oxazepam example. We used semi-preparative HPLC to separate and isolate oxazepam and its degradation products and characterized them using ESI-MSn (8). Structures of proposed degradation products and their fragmentation patterns were supported by ESI-QTOF-MS/MS investigations to obtain unequivocal confirmation. The proposed ESI-MSn fragmentation pathway of oxazepam and the degradation product 6-chloro-4-phenyl-2-quinazolinecarboxaldehyde are given as an example in Figure 3b. ESI-QTOF-MS/MS confirmed the m/z 269 signal as $C_{15}H_{10}N_2OCl$ (1.5ppm) and the m/z 241 signal as $C_{14}H_{10}N_2Cl$ (1.0ppm).

For quantitative analysis, calibration curves, standard addition methods, internal standard methods, and internal normalization method are all used readily. A particularly good example of the internal standard method can be found in a 1990 paper on the analysis of fluoxetine and its metabolite in serum by HPLC (9). Some things never seem to go out of fashion!

I hope with this article that I have either triggered some memories or opened some eyes on the absolute need to consider the individual unit processes of methods. Interestingly, I was searching on Google the other day and noticed that in organic synthesis, chemical engineering and many other fields, overall methods are approached in the form of unit processes. Just as I've described above, it's all about splitting up an overall method into discrete operations or steps. In our modern world of faster, more accurate, higher resolution – and automated – instrumentation, it can be easy to forget the basics. Sure, if you find that your trusty LC-MS isn't sensitive enough to detect a new analyte of interest in a particular matrix, you could race to your nearest instrument vendor for the latest and greatest innovation. Or you could take a step back and reconsider each unit process in turn: the use of a different preliminary sample preparation step or the addition of some interesting solid phase extraction chemistry could be just the trick to enable detection. In short, let's not lose sight of the basics in our rush to embrace sophisticated technology.

W. Franklin Smyth is Emeritus Professor of Bioanalytical Chemistry and Professor of Pharmacy at the School of Pharmacy and Pharmaceutical Sciences, Faculty of Life and Health Sciences, at the University of Ulster, Coleraine, Northern Ireland.

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Webinars

Virtual Events

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Increase GC Speed without Sacrificing Resolution: The Principles of Fast GC

Event Overview:
Providing a background to the basic theory behind Fast GC, this webinar will highlight the practical aspects of actually making it work. Popular applications, such as BTEX, PAHs, FAMEs, volatiles, and semivolatiles, will be shared as real world examples.

Date: June 25th – 11am EDT / 3pm GMT

Register Free at:
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Speaker
Michael D. Buchanan
Product Manager, Gas Separations

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Advances in fracking contamination analysis

Measuring Fracking Contamination: Rapid and Reliable Determination of Methane and Other Dissolved Gases in Water

Event Overview:
This webinar will look at new high throughput test methods that use robust and automated headspace and GC instrumentation for the quantitative determination of dissolved gases in ground, waste, and drinking waters.

Date: June 26th – 8am PDT / 11am EDT / 3pm GMT

Register Free at:
http://tas.txp.to/0514/thermo/webreg

Speakers
Massimo Santoro, *GC and Single Quadrupole MS Marketing Manager*
Andrea Caruso, *GC Application Chemist*

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Business

Economic drivers
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How broad are the legal duties of analytical laboratories and what do recent developments in US law mean for labs around the world?

By Lawrence Mason

Among the many legal developments over the past six months or so, two US court decisions stand out for their likely implications for the scientific community.

The first decision, Landon v. Kroll Laboratory Specialists, Inc., was decided by the New York Court of Appeals and is significant for its holding that a lab has a duty to report test results accurately. More specifically, the court determined that misreporting can support a damages claim by an alleged drug user against the lab.

In Landon v. Kroll, the complexity stemmed from the fact that the lab had no contractual relationship with the alleged drug user. Plaintiff Landon had been convicted of a crime and sentenced to five years of conditional probation, with one of the conditions being that he submit to random drug testing. The county's probation department had a contract with Kroll to conduct the test using oral fluid samples. Kroll performed the analysis and reported a positive test result. Later the same day, Landon secured an independent blood test, which produced a negative result. However, the probation department brought proceedings against Landon on the basis of Kroll's result.

And so, Landon filed a suit alleging that Kroll had negligently issued its test report and acted "as part of a policy of deliberate indifference to his rights." The scientific basis for Landon's claim was that the screen test cutoff level Kroll used – 1 ng/ml – was substantially lower than that level recommended by Orasure (the maker of the analytical device) and the United States Department of Health and Human Services Substance Abuse and Mental Health Services Administration (SAMHSA) of 4.0 ng/ml. The complaint alleged that Kroll had failed to disclose these differences in its report. The complaint further claimed that, despite New York State Department of Health Laboratory Standards requiring samples to be subject to confirmatory testing through the use of gas chromatography-mass spectrometry, Landon's sample was not subject to any type of confirmation test before Kroll reported Landon's positive test result.

Systemic negligence

Landon alleged the additional criminal proceedings against him were the result of "systemic negligence in Kroll's substance abuse testing practices." He asserted that he was required to serve an extended probationary term, which resulted in a loss of his freedom as well as emotional and psychological harm and monetary loss (for example, attorneys' fees).

The primary question before the court was whether Landon's claim could go forward. The court noted that Kroll performed exactly as the county had required in its contract. Since the lab's contract was with the county, the issue became whether Kroll owed a duty of care directly to Landon such as would support his claim under tort law. A tort is a civil wrong that is recognized by law as grounds for a lawsuit.

In reaching its decision, the court noted that although the existence of a contractual relationship by itself is generally not a source of tort liability to third parties, there are certain circumstances where a duty of care is assumed to individuals outside the contract. The court stressed that the duty to avoid harm to others is distinct from the contractual duty of performance. The court found the alleged harm to Landon was not remote or attenuated because it was his own biological specimen that was the sole subject of the lab's testing and he was directly harmed by the positive test result.

Defining "duty"

However, Judge Pigott, writing a two-judge dissent against the court's majority opinion, stated that the definition of "duty" used was too broad because the relief Landon sought would be better directed at the probation department than the lab. Judge Pigott noted that the lab complied with its contractual

obligations and it was the probation department's use of the test result, not the way the lab conducted it, that caused the plaintiff's alleged harm.

While Judge Pigott's dissent is not precedential authority, it may be persuasive to other courts evaluating a lab's duties. It remains to be seen whether other courts will follow the New York Court of Appeals majority opinion or the dissent's contrary reasoning. In any case, it is important for analytical laboratories to be mindful that they could be at risk to third parties in negligent testing cases.

"Don't ask me. I only work here…"

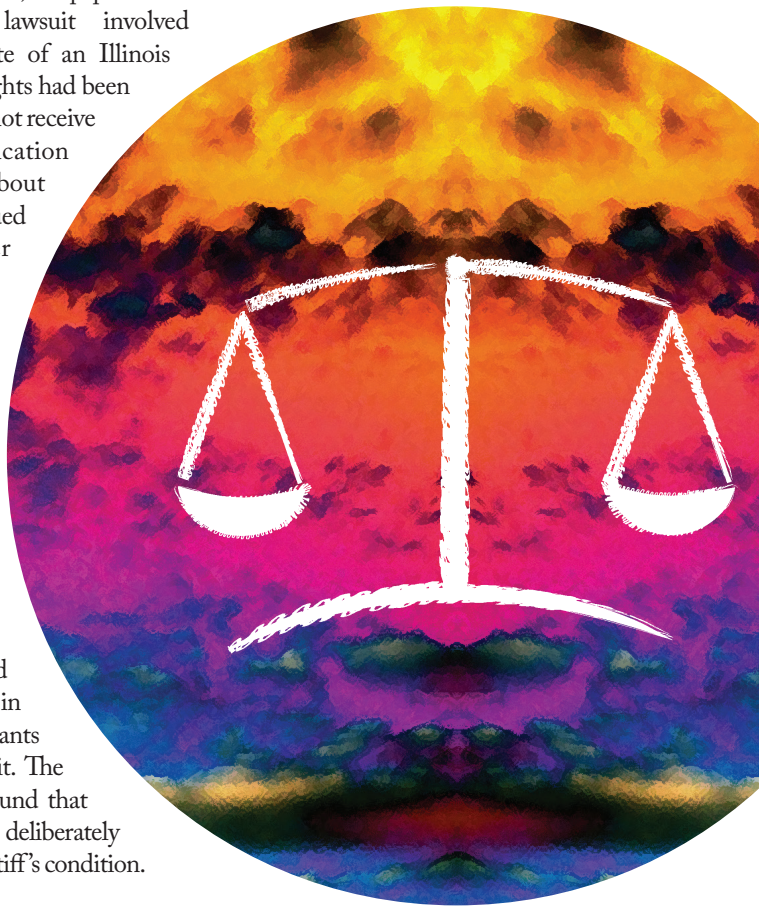
The second decision comes from the United States Circuit Court of Appeals for the Seventh Circuit and is perhaps of more broad interest, serving as a portend of change.

Disturbed that the magistrate judge, the district court judge, the plaintiff's lawyer, and the defense lawyer all accepted as medical fact something that could not have been true, the Jackson v. Pollion court devoted the bulk of its opinion to chastising not only the judges and lawyers in the case before them, but the legal profession generally.

"This lapse is worth noting because it is indicative of a widespread, and increasingly troublesome, discomfort among lawyers and judges confronted by a scientific or other technological issue," Circuit Judge Richard Posner wrote in the opinion, to which Circuit Judge Frank Easterbrook agreed.

The opinion was particularly critical of the magistrate judge and district judge in the case, suggesting that they should have made "some investment in learning about the condition." Posner, writing for the majority, noted that the lower court judges could have used their authority under Federal Rule of Evidence 706 to appoint a neutral expert witness, required the plaintiff to present expert evidence, or just consulted a reputable medical treatise. In noting that procedural wrangling and protracted discovery had caused this "plainly meritless suit" to drag on for more than four years, the court indicated that "[a] stronger judicial hand on the tiller could have saved a good deal of time, effort, and paper."

The underlying lawsuit involved a claim by an inmate of an Illinois prison that his civil rights had been violated when he did not receive hypertension medication for a period of about three weeks. He sued a nurse practitioner and a correctional counselor, alleging that they had been deliberately indifferent to his serious medical condition and, as a result, had subjected him to cruel and unusual punishment. The lower district court judge granted summary judgment in favor of the defendants and dismissed the suit. The district court judge found that neither defendant was deliberately indifferent to the plaintiff's condition.



Undisputed reliance on science

Within the first paragraph of its opinion, the Seventh Circuit affirmed the district judge's ruling, saying that it was "so clearly correct as to not require elaboration by us." While the opinion could have stopped there, Posner instead chose to continue writing multiple pages of dictum, which is an authoritative statement without any binding effect. The court's dictum criticized the judges and lawyers involved for their scientific lapse concerning the plaintiff's claim (that his three weeks without hypertension medication caused him to suffer loss of vision, nose bleeds, headaches and lightheadedness, and exposed him to the possibility of a stroke or even death).

Noting that the plaintiff was an otherwise healthy 22-year-old man and that the single reading of his blood pressure during the three-week period showed it to be only slightly elevated above normal range, the opinion indicated that the lack of medication could not have produced the symptoms – "The proposition . . . has no support in the record or the medical literature," the opinion noted. Despite this, the magistrate judge found that,

could have established that his period without medication was "objectively serious." Likewise, the district court judge concluded that the plaintiff "suffered from an objectively serious medical condition." The Seventh Circuit opinion stressed that the lower court judges could have decided the case without ruling on any medical questions, relying entirely on the lack of evidence of deliberate indifference by either defendant. "But if they were going to venture an opinion on the 'objective seriousness' of the plaintiff's 'medical condition,' they had to get the condition right – which was not hypertension but the medical consequences, in fact negligible, of a three-week deprivation of medicine for mild, early-stage hypertension."

Significantly, Posner commented that the "discomfort of the legal profession, including the judiciary, with science and technology is not a new phenomenon. Innumerable are the lawyers who explain that they picked law over a technical field because they have a 'math block.'" He also noted cause for concern at the extraordinary rate that scientific or technological advances featured in litigation. "The legal profession," Posner concluded, "must get over its fear and loathing of science."

In his concurring opinion, Circuit Judge William Bauer confessed that he is one of those lawyers who chose law over medicine because of a lack of interest in the clinical aspects of medicine, but stated that he would have concluded the opinion after the first paragraph, without discussing lawyers' fear of science. "I think that the opinion made the necessary legal point when it said that the record shows that summary judgment was clearly the right decision." Bauer wrote. "That's where I would stop."

While the Seventh Circuit's decision does not have direct precedential effect, it could signal the beginning of a new era in which judges, juries and lawyers set aside their fear of scientific and technical issues, with intent to challenge all aspects of science. How would that affect you?

Lawrence "Larry" D. Mason is a senior shareholder of the national civil litigation law firm of Segal McCambridge Singer & Mahoney.

Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution

Two-dimensional Liquid Chromatography: HILIC x RPLC-MS.

Gerd Vanhoenacker, Koen Sandra, Isabel Vandenheede, Frank David, Pat Sandra, Udo Huber.

Introduction

Biopharmaceuticals such as therapeutic monoclonal antibodies (mAbs) are becoming increasingly important in the treatment of various diseases. Peptide mapping is a commonly used technique for their comprehensive characterization and purity determination. The complexity associated with mAb digests demands the highest separation power. Compared to one-dimensional separations, comprehensive two-dimensional LC (LC×LC) will drastically increase peak capacity.

Results and Discussion

This article describes the application of HILIC×RPLC to the analysis of tryptic digests of trastuzumab using the Agilent 1290 Infinity 2D-LC solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS System. A combination of HILIC in the first dimension and RPLC in the second dimension should provide good orthogonality for compounds such as peptides in an LC×LC setup. This orthogonality and complementarity has been proven in various reports, but mostly using a stop-flow or an offline LC×LC approach. The LC×LC peptide map of trastuzumab is shown in Figure 1. The contour plot was generated with the MS total ion current data. The spots were identified by matching the experimentally acquired data on the theoretical trastuzumab sequence at high mass accuracy (< 5 ppm) using Agilent MassHunter Bioconfirm software. The resulting separation shows good orthogonality between both dimensions.

The applicability of the developed method was evaluated with stressed and non-stressed samples. Trastuzumab was subjected to forced degradation conditions prior to digestion. Comparing stressed with non-stressed data should reveal degradation products. Figure 2 shows the extracted ion plots for peptide T41 and its oxidation product. From the comparison of non-stressed and oxidized samples, it is clear

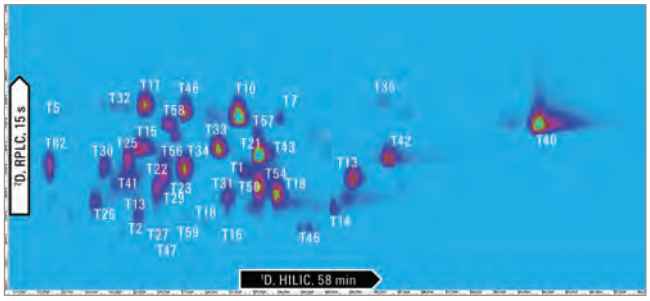


Figure 1. LCxLC contour plot for the analysis of a tryptic digest of trastuzumab.

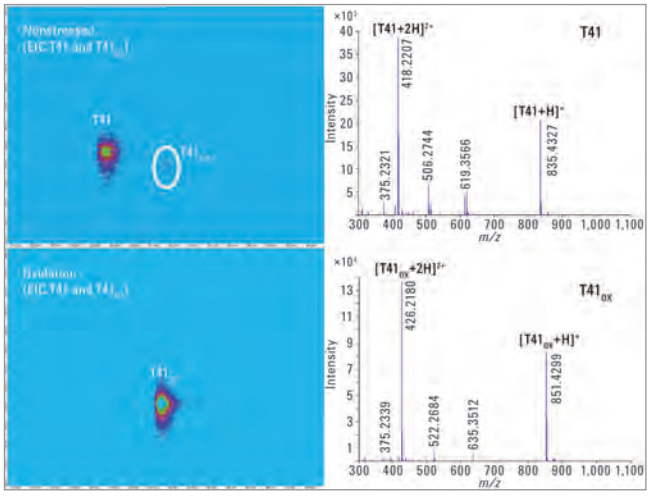


Figure 2. LCxLC extracted ion contour plot for the analysis of a tryptic digest of nonstressed and oxidatively stressed trastuzumab.

that the non-stressed trastuzumab already contains small amounts of the oxidation product. The inserts in Figure 2 show the mass spectra.

Conclusion

The Agilent 1290 Infinity 2D-LC solution coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS system is ideal for the comprehensive analysis of monoclonal antibody digests. The possibilities of LC×LC coupled to a high-end mass spectrometer for detailed peptide mapping analyses are illustrated with some representative results on stressed and non-stressed trastuzumab digests.

Agilent Technologies Inc.
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Agilent Technologies

Rapid and Robust Subunit Domain Mapping of Monoclonal Antibody Based Biotherapeutics

Highly specific enzymes for middle down approach - rapid mass spectrometric analysis of antibody based biotherapeutics using FabRICATOR®

Fredrik Olsson, Sylvia Koivunen

Digestion with the FabRICATOR® (IdeS) enzyme, which specifically cleaves IgG under the hinge region, followed by reduction results in 3 distinct mAb subunits (LC, Fd and Fc/2) in the range of 22-27 kDa. Optionally, selective Fc-deglycosylation can be performed using IgGZERO™ (EndoS) or GlycINATOR™ (EndoS2) to further reduce heterogeneity. Either of the two enzymes can be co-incubated with FabRICATOR ® during the digestion step. They facilitate characterization of posttranslational modifications like glycan heterogeneity, oxidation, lysine clipping and a rapid confirmation of amino acid sequence. The method can be applied for tasks like clone selection, process development, formulation and stability studies of monoclonal antibody based bio therapeutics such as MAb's, Fc-fusion proteins, biosimilars and ADC's.

Here we present a two-step process, which can be easily automated, for the preparation and analysis of antibody subunits by ESI-QTOF. By using this method for applications like sequence verification by accurate mass, sample preparation time can be reduced to 60 minutes. In the first step the mAb were cleaved using 1 unit of FabRICATOR ® /µg IgG (0.5-10 µg IgG / µl). After 30 min incubation at 37°C the reduction agents TCEP and Gd-HCl were added to a final concentration of 50 mM and 4 M, respectively and incubated for 30 min in room temperature. Samples were investigated by LC-MS using standard chromatography including desalting, combined with an UHR-ESI-QTOF (maXis 4G, Bruker) additionally automated acquisition, analysis and result generation, BioPharma Compass (Bruker) was used.

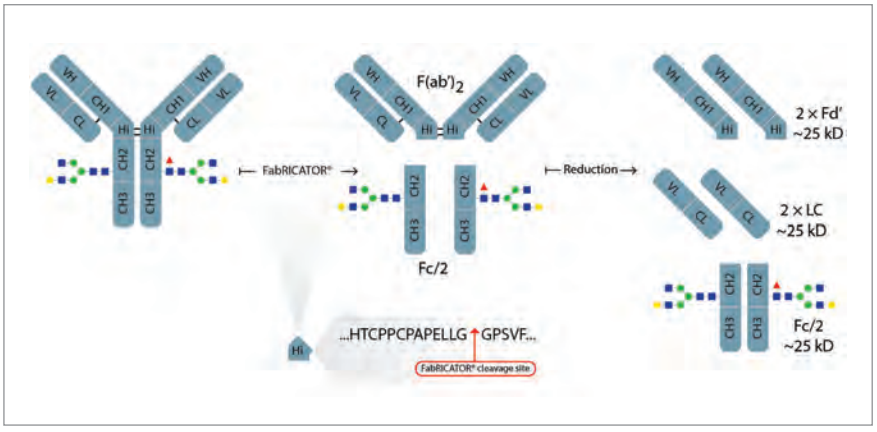

Figure 1: Two-step, 60 minutes process for the preparation and analysis of antibody subunits by ESI-QTOF.

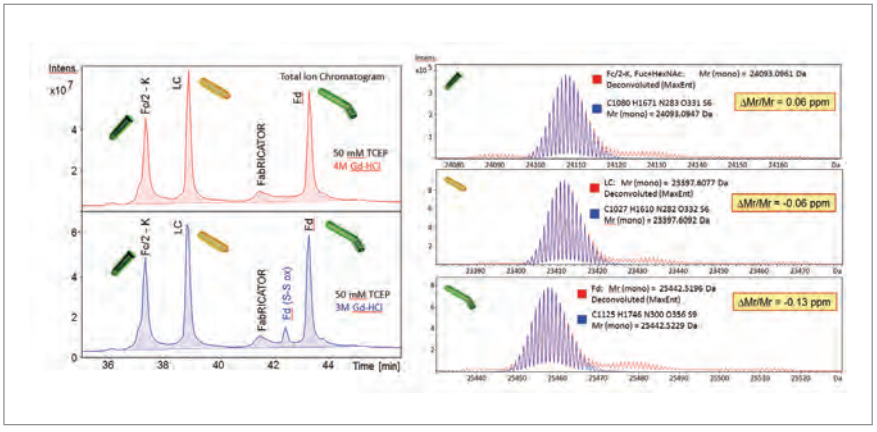

Figure 2: Left: Comparison of two TICs from samples with 4M and 3M Gd-HCl; 0.5 µg on column. Right: Measured deconvoluted MS spectra of the tree antibody subunits and comparison with expected mass and isotopic pattern.

Summary

Using the presented protocol, complete workflows of sample preparation, data acquisition, data analysis, and report generation can be automated saving time and money. Additionally, compared to traditional peptide mapping the short incubation at neutral pH and limited number of steps reduces the risk of sample preparation induced modifications commonly observed with tryptic digests.

Please visit us at ASMS booth 126

Saccharide and Polysaccharide Analysis

Introduction

Polysaccharides are very important in nature, occurring in food (starches in rice, wheat, etc.) and plants (cellulose). Some polysaccharides are also produced commercially, for example, dextrans, which are manufactured through the fermentation of sugar solutions. These are higher molar mass polysaccharides. Dextrans are used in clinical and technical applications, where molecular weight is critical in determining the properties of the final product. Accurate determination of the molecular weight distribution is vital.

Experimental Conditions:

Eluent:	*$NaNO_3$ 0.1M*
Columns:	*PSS SUPREMA 5 µm 3 x 100Å (8 x 300 mm) + precolumn*
Data acquisition:	*PSS WinGPC UniChrom*
Detectors:	*SECcurity GPC1200 RI*
Flow-rate:	*0.25 ml/min*
Concentration:	*4 g/l*
Injection volume:	*5 µl*
Sample Fig 1:	*Dextran T1, Glucose*
Sample Fig 2:	*Disaccharides*

In addition, low molar mass saccharides are also very common in food, such as fruits, honey and sweets. Examples for low molar mass sugars are mono- (glucose, fructose), di- (lactose, isomaltose, trehalose) and trisaccharides (maltotriose, isomaltotriose). The separation and identification of low molar mass polysaccharides is a challenge as the compounds have the same chemical formula and only small differences in structure, e.g disaccharides maltose, isomaltose, gentiobiose cellobiose and trehalose $C_{12}H_{22}O_{11}$.

Results & Discussion

A high resolution and therefore a good separation on the column, is necessary for a precise analysis. This is particularly important when new analytical LC coupling methods like GPC/SEC-ESI-MS are used, as the MS detector requires the columns to have a much higher resolution power within an overall smaller column volume.

The new SUPREMA column, with a reduced particle size of 5µm, offers a significant improvement in performance compared to 10µm materials and provides outstanding additional resolution, especially in the low molecular weight area, which is a major consideration when analyzing oligomeric polysaccharides.

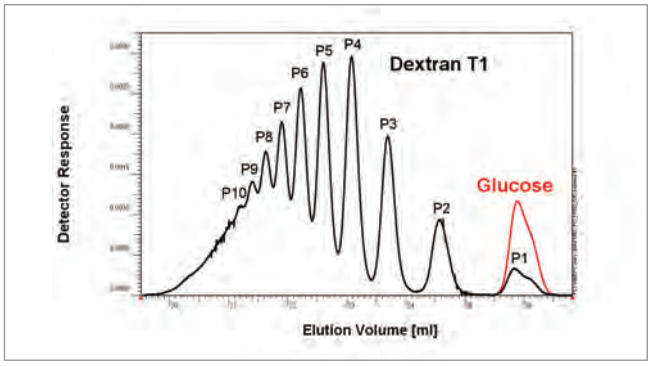

Figure 1: Overlay of elugrams of a glucose (red curve) with a low molar mass Dextran T1 (black curve)

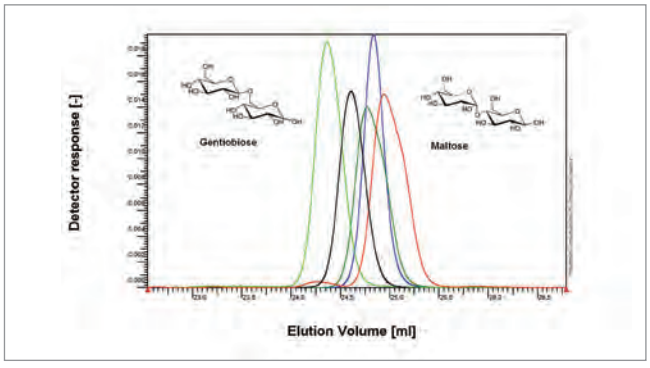

Figure 2: Overlay of elugrams of isomaltose (black), maltose (red), gentiobiose (green), cellobiose (dark green) and trehalose (blue).

The analysis of dextran T1 shows the separation power when a combination of three SUPREMA 5µm 100Å columns is used. The oligomers in the low molecular weight are able to be resolved up to P10. A glucose separation is overlaid, as a reference.

The analysis of different disaccharides shows the ability to separate compounds with the same chemical formula and with only small differences in structure and hence size in solution.

PSS SUPREMA 5 µm columns can be used for numerous neutral and anionic aqueous applications in the molecular weight range between 100 Da to around 5 million Da. The columns are available in analytical (ID: 8mm) and micro (ID: 4.6mm) dimensions with different porosities. Linear or mixed columns are also available.

A Little Respect

Sitting Down With Ellen Miseo, instrumentation and applications consultant for Analytical Answers and adjunct assistant professor at Bentley University, Massachusetts, USA.

You arranged a session at Pittcon on Women in Spectroscopy. How did that come about?

It came out of a number of things that have happened over the past couple of years. One was that a young woman who's a member of the Society for Applied Spectroscopy suggested that women needed a place to discuss career paths and so on, and proposed starting a group called SASSY. Another was that at Pittcon 2013, I met up with a group of female friends for lunch. What some of the younger women were saying about life in graduate school horrified me, especially in this day and age. The third was a very engaging article about Phyllis Brown, the first HPLC chromatographer working on complex molecules. She was interviewed by a young female associate professor, and I found the interaction between them fascinating. Lastly, the timing seemed right from a personal perspective: I have a daughter who's just about to graduate in chemistry and wants to do a PhD, and my son's fiancée just finished a Masters in chemical engineering.

It stuck me that all of these young women would really benefit from hearing how other women became successful. I wanted to bring together stories to show that academia isn't the only option, and I wanted them to learn about the challenges that women face.

How did it go?

I was incredibly happy with the session. Attendance was good and many participants agreed that we should do it again next year (a proposal has been submitted to Pittcon). There was also a positive response from some of men that I know, which is when I was sure that it was the right thing to do. Outside of Pittcon, our LinkedIn group, "Women in Spectroscopy", follows a similar principle, offering a closed forum for women to ask questions and share their views.

You've faced gender discrimination yourself. Could you speak about that?

When I was discriminated against, I was blindsided by it. I spent a long time saying to myself, "This can't be discrimination!" because it had never happened before; every previous position was accompanied by a very positive, professional attitude. But following acquisition of the company I was working for, it was obvious that the new owners were treating women as second-class citizens. They weren't respected for their knowledge and it was obvious that strong women were not welcome to participate in the business. I could see that it was a problematic and pervasive attitude. It started at the top and no one had ever challenged it.

Does these issues exist in both academic and business settings?

Yes – but in different ways. There have been a lot of studies published on how tenure comes right at the primary childbearing age. Biologically speaking, a woman has to take time off. A man can have a baby, take two weeks paternity leave, and be back in the lab 12 hours a day. A woman can't do that. In industry, there are other issues: some people question committment and if there are two people vying for the same promotion, underhanded tactics may come into play.

There's a phrase: if you're not part of the solution, you're part of the problem. What can the non-acting majority do to help?

Simple recognition that these things happen would be a good start. And if someone is in a position to call out a colleague, then I urge him or her to do so. People need to be willing to say, "I'm not going to let this happen."

I don't think it's so much training as awareness that is needed. That's what the unconscious (or sometimes covert) nature of the problem demands. At professional conferences, I've seen the tone of a male-only group change when a woman joins the conversation, and it's happened to me, but it doesn't seem to be intentional.

Positive discrimination (affirmative action) irks me; meritocracy must be upheld. A big problem is that our society starts discouraging females at a very young age from getting into the sciences. That needs to change and requires a grassroots effort.

If you could change one thing, what would it be?

There needs to be more acknowledgement of the fact that women bring different and valuable perspectives to bear. I'd like to see women receive the same level of attention and respect as their male colleagues.

I don't really want to be the woman who's saying that women need to be given more opportunities in analytical chemistry, though I appreciate I've indicated that partly here, because you asked! All I'm really trying to do is point out that issues that I thought were long gone are still present. I was the victim of discrimination myself, and I strongly feel that women need to educate themselves that there are still gross inequalities out there. I want them to speak up and stand up for themselves.

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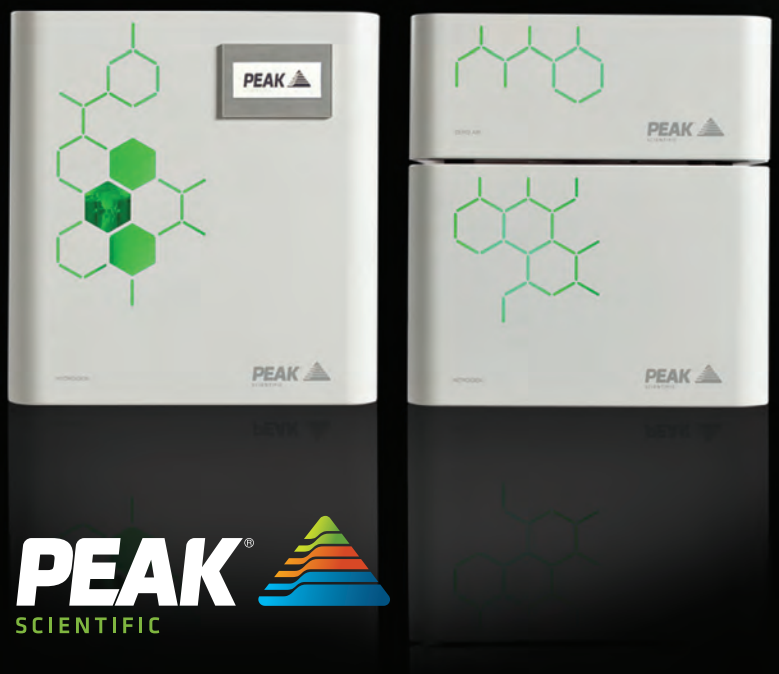

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