JUNE 2013 # 06

the Analytical Scientist

Toying With Athletes

We assess the collateral damage in WADA's war on doping

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Online this Month

World War WADA

Do you have a strong opinion on doping in sport?
What do you think about the World Anti-Doping Agency's strategy?
We want to start an open discussion on the issues raised in these pages (Editorial, page 7; "Toying with Athletes", page 22).
Add your perspective by commenting at: theanalyticalscientist.com/issues/0613/401 or by contacting the editors at edit@texerepublishing.com.

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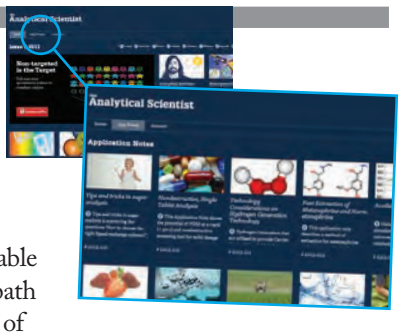

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"We may sit in our library and yet be in all quarters of the earth"
- John Lubbock, 1st Baron Avebury.

Video

This month, we sat down with Georges Guiochon (see page 50), who had many a tale to tell from his long and fascinating career. Watch the video online: theanalyticalscientist.com/issues/0613/601

Survey

What are your chromatography needs and preferences?
Swap 10-15 minutes of your time for the chance to win an Apple iPad in a prize draw. Then you'll be able to enjoy the wonders of The Analytical Scientist iPad edition...

Astronaut App

We can't confirm that UK astronaut Major Tim Peake will be taking The Analytical Scientist app aboard the International Space Station in 2015, but given that scientists are already cooking up experiments - from material science to bacterial virulence – that will require full engagement of his analytical brain, he might want to check it out beforehand…
theanalyticalscientist.com/app

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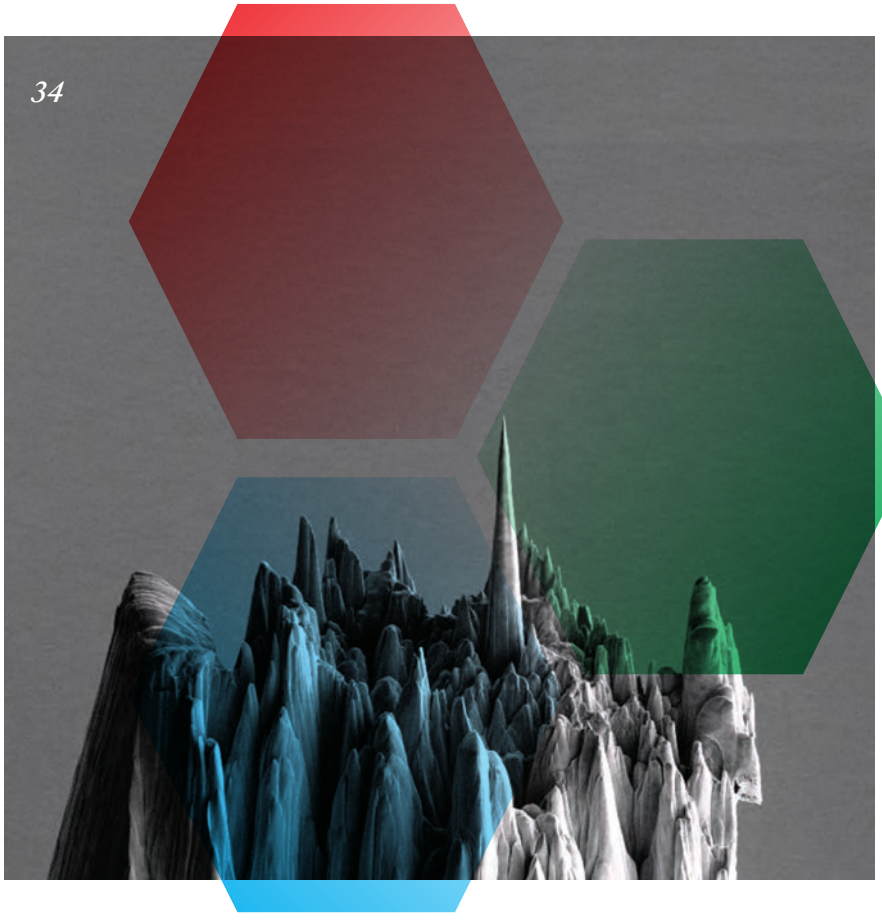

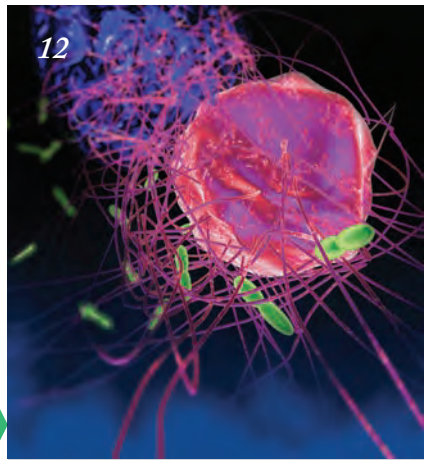

On The Cover

Concept: Racing cyclist figurines illustrate the claim that the World Anti-Doping Authority is toying with athletes.
Image courtesy of Alain Quenderf

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Play Fair

The governing body's all-out war against athletes guilty of doping fails to uphold the ideals of sport – or of basic justice.

The World Anti-Doping Agency (WADA) recently released a program to improve quality at the 32 analytical labs contracted to conduct its analyses (1). While this may be an honest endeavor to increase effectiveness, the report also serves to turn the spotlight away from how WADA operates itself. That can't be allowed to happen.

As illustrated by several experts in this magazine (see page 22), WADA's policy is best described as a war against doping: the ruthless tracking and punishment of all dishonest athletes. If WADA was able to identify and penalize undoubted cheats with surgical precision, this war would be legitimate. But it cannot. There is major collateral damage: honest athletes are being incorrectly identified and handed severe penalties. This simply is not justifiable.

Children who drank cough medicine, footballers who ate tainted meat, and athletes who used 'party' drugs are false-positive cases – false-positive in the sense that these were not attempts to improve sportive performance and have absolutely nothing to do with dishonesty in sport, per se. In some countries, half the 'positives' are recreational drug detection: while potentially of interest to drug enforcement agencies, these should not fall within in the remit of WADA. The list of banned substances is enormous, but scientific evidence to support any performance-enhancing benefit is often lacking. For others, there is no threshold value, so that even a picogram identified could have dire consequences for the athelete concerned.

In contrast to court cases (2), there is no requirement for transparency from WADA on analytical procedures or proof that the quantity of banned substance found is performance-enhancing. And it is effectively impossible for athletes to challenge a decision.

WADA has reacted poorly to feedback from the outside world. Questions over statistics, concerning threshold levels or the effectiveness of blacklisted compounds, have been met with letters from lawyers. Instead, WADA should embrace interaction with external experts.

WADA's (rather good) magazine is called Play True (3). We urge them to Play Fair, and we invite them to respond to criticisms.

And what about us? According to Douwe de Boer, a former head of IOC and WADA labs, it is the social responsibility of analytical scientists to ensure that athletes are given a fair chance. I agree.

Frank van Geel
Scientific Director

References

1. *www.wada-ama.org/Documents/World_Anti-Doping_Program/Reports-Assessments/2013-05-12-Lack-of-effectiveness-of-testing-WG-Report-Final.pdf*
2. *theanalyticalscientist.com/issues/0313/how-well-do-we-measure/*
3. *playtrue.wada-ama.org/*

Fiorenzo Omenetto

Along with colleague David Kaplan, Fiorenzo (Fio) Omenetto, Professor of Biomedical Engineering at Tufts University, has pioneered the use of silk as a material platform for photonics, optoelectronics and high-technology applications. Omenetto, who continues to investigate novel applications, was named one of the Top 50 people in tech by Fortune magazine, in a class that also featured Steve Jobs, Jeff Bezos, and Shigeru Miyamoto.

Find out how Omenetto's silk research got started on page 16.

Stephanie Rizk and Bryan Tweedy

"I was always a bit on the nerdy side," says Stephanie Rizk. "My first boyfriend asked me out by writing a program on my graphing calculator." Stephanie is manager of Sci-Mind, a learning product from the American Chemical Society (ACS), having previously worked in theater management, at non-profit consulting firms, and executive education programs. "Working with scientists is a definite thrill because there is always something more to learn," she says. Co-author Bryan Tweedy decided on an alternative career in science after his BS degree in biochemistry. He has worked in publications, industry relations, and technical training within ACS. "I've had the opportunity to meet and work with some of the most influential people in the chemical enterprise," he says.

Read their Profession article on professional training on page 42.

Johannes Lenstra

Hans Lenstra has been at the Faculty of Veterinary Medicine, Utrecht University since 1990. His research focuses on investigating the genome, evolution, and genetic diversity of domestic ruminants. After contributing to the identification of species-specific repetitive DNA elements and the development of species-identification tests, he is now enthusiastically engaged in genomic analysis of livestock breeds to create molecular reconstruction of the history of domestication.

Get Johannes' view on how to prevent "cheat meat" on page 17.

Amit Kumar Mandal and Amrita Mitra

"I was more inclined to be a teacher than a scientist," says Amit Kumar Mandal of the St. John's Research Institute in Bangalore, India, "but a fascination with the correlation between molecular structure and function dragged me into research." His was drawn to mass spectrometry because the kinetics of a chemical reaction can be visualized through it. "When I realized that structural biology can be solved using isotope exchange-based and ion mobility-based mass spectrometry," he says, "my excitement touched the roof." For Amrita Mitra, student of, and coauthor with, Mandal, "Structural biology is a fascinating field. My work involves mass spec-based proteomics to explore the urine proteome to identify candidate biomarkers for prostate cancer."

Mandal and Mitra discuss the next step for structural proteomics on page 18.

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Upfront

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Wound Healing in Diabetes

Applying an inclusive analysis that requires minimal sample manipulation to generate robust biomolecular signatures from diabetic wound fluid

Chronic ulcerative wounds are a distressing and common complication of diabetes. To help determine the processes that prevent normal wound repair in diabetic patients, the McLean Research Group based at Vanderbilt University, Tennessee, used ion mobility-mass spectrometry (IM-MS) to analyze wound fluid (a complex brew of byproducts of the wound repair process) from sponges implanted into the wounds of diabetic and control rats. Here, Kelly M. Hines, lead author of the group's recent paper (1) answers questions about the research.

What drove your interest in biomedical signatures?

The ion mobility-mass spectrometry (IM-MS) approach keeps the wound fluid intact. This allows a broad scale and untargeted investigation of the interactions between multiple types of molecules involved in the wound healing process. The approach spans the transition from untargeted to targeted analysis: differentially expressed molecules are revealed in the untargeted data set, while the guidance of statistical analysis and further experiments are performed in a targeted fashion to elucidate and validate their identities.

How did the results compare with your expectations?

It was interesting to observe biomolecular species in wound fluid that are similarly differentially expressed in the serum and blood of diabetics. Lysophoshatidyl choline (20:4), cholic acid and the protein S100A8 were associated with diabetes in previous studies, while S100A8 has been implicated in the wound healing process and diabetes.

Why was UPLC-IM-MS chosen as the analytical tool?

For the initial untargeted analysis, electrospray ionization (ESI) was used, and ultra-performance liquid chromatography (UPLC) was introduced for the subsequent targeted analysis. The separation of multiple classes of biomolecular species in the IM dimension is greatly beneficial for the analysis of complex samples because it allows us to integrate various omics analyses in the same experiment with very little sample preparation. For example, it allows lipidomics, metabolomics, and proteomics to be performed simultaneously. Omics analyses by MS only typically requires extensive sample preparation to deplete the undesired species from the biological samples, reducing the native complexity of the sample. Using IM-MS, sample preparation procedures were reduced to dilution and desalting (for ESI).

UPLC analysis was performed in a targeted manner to elucidate the source of the molecular species m/z 355 and 373, which were suspected to be in-source fragments of a common precursor. These species were shown to elute in the same chromatographic peak, and switching the instrument polarity from positive to negative ionization mode revealed the common precursor to be cholic acid.

What statistical analysis did you use?

The MarkerLynx XS software package from Waters. A combined scan method aligned the mass spectra, which were then normalized by the constant sum method.

Partial least squares-discriminant analysis (PLS-DA) was used to visualize group differences and orthogonal PLS-DA was used to generate S-plots, from which the features contributing the most to the group differences were revealed.

Were there major challenges with the stats?

The most challenging aspect was data processing, but not necessarily due to the size of the ESI-IM-MS data set. Ideally, statistical analysis would be performed on data aligned by ion mobility drift times and m/z. However, most conventional software packages rely on peak alignment by chromatographic (liquid or gas) retention times and m/z, and are not yet suited to perform peak alignment by drift times. This is disappointing, as the ion mobility data could not be fully incorporated into the statistical analysis of the wound fluids. As IM-MS becomes more popular, the biostatistics software will likely be developed, which is also an active research pursuit of our group. We anticipate being able to extract even more information from these datasets in the future.

How can the findings be applied?

The untargeted approach enables this platform to be used as a discovery tool; tracking treatment studies are one possible avenue to explore. In this particular study, the molecular species differentially expressed between the diabetic and nondiabetic groups, as well as between the diabetic groups at different time points could be useful in this regard.

And biomedical signatures generally?

The IM-MS based approach to identifying biomolecular signatures of disease could be applied to any number of discovery-driven and targeted endeavors due to the unbiased nature of the analysis. In addition to wound fluid, our group has looked at human breast cancer tissue extracts, cerebrospinal fluids, cell lysates, serum, and microbial extracts. *RW*

References

1. *K. M. Hines et al., "Biomolecular Signatures of Diabetic Wound Healing by Structural Mass Spectrometry," Anal. Chem., 85, 3651 (2013) (dx.doi.org/10.1021/ac303594m)*

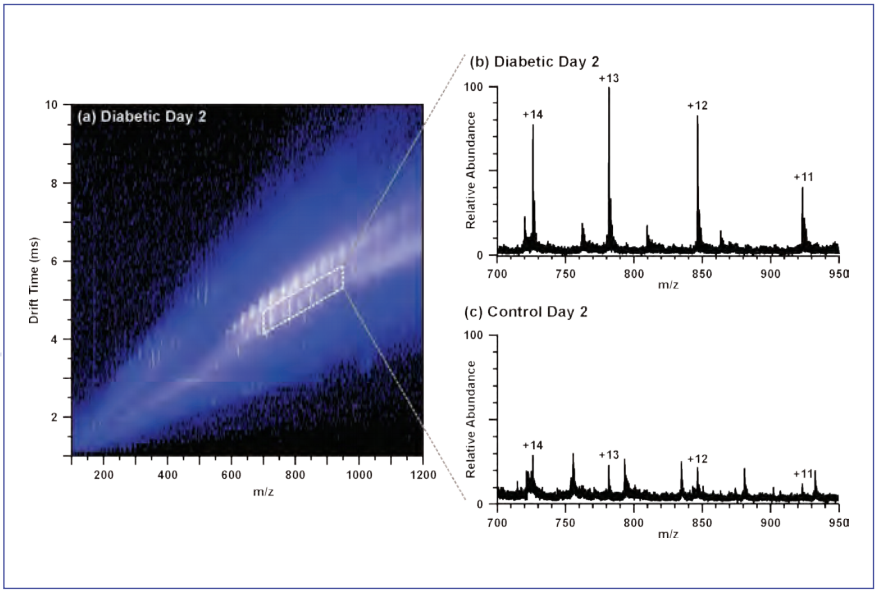
The Experiment

Sample Collection and Preparation

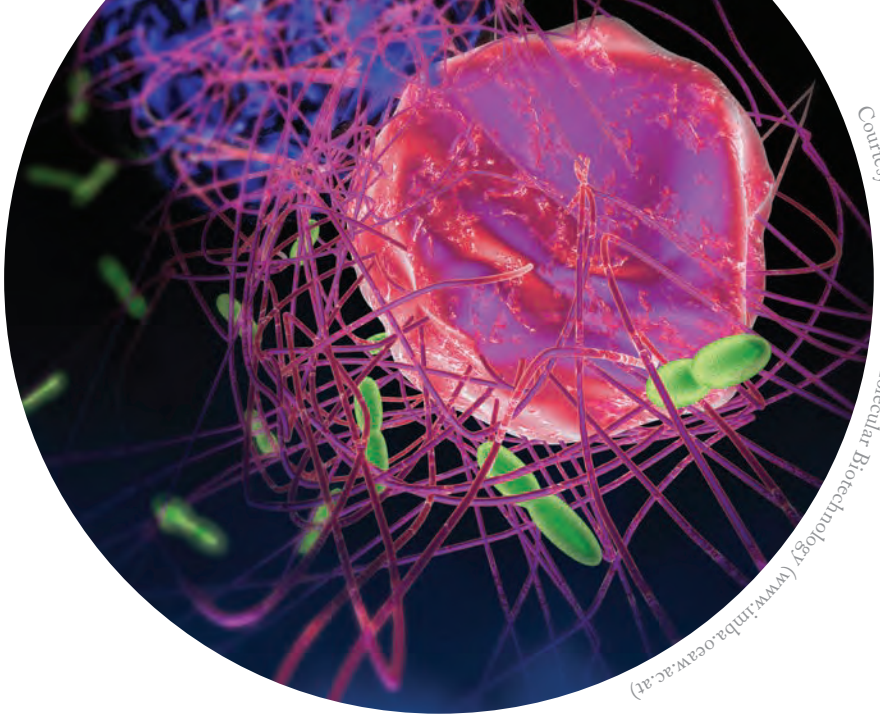
Implanted sponges were removed and centrifuged to collect wound fluids. For IM-MS analysis, wound fluids were diluted, desalted, dried and reconstituted in 1:1 methanol: water for electrospray ionization.

System

UHPLC: NanoAcquity UPLC (Waters), Acquity HSS C18 column (1.8 µm, 1.0 x 100 mm). The UPLC analysis was performed online with a Synapt G2 IM-MS (Waters), which utilizes electrodynamic traveling waves and nitrogen gas for ion mobility separations. Mass analysis was performed with orthogonal time-of-flight (oTOF) MS operating in single reflectron mode. The four-dimensional data set comprised of retention time, drift time, m/z and intensity.



(a) Three-dimensional ion mobility-mass spectrum of diabetic rat wound fluid collected two days after sponge implantation. The dashed white box in (a) contains a 10 kDa protein, which was differentially expressed between diabetic (b) and control (c) wound fluids. Collision induced dissociation of signals corresponding to this species yielded a partial protein sequence from which the protein S100-A8 was identified. S100-A8 has been implicated in the onset of inflammation in both diabetes and wound healing. Figure courtesy of Kelly M. Hines and John A. McLean, Vanderbilt University.



Courtesy of The Institute of Molecular Biotechnology (www.imba.oeaw.ac.at)

Immune System Chat Room

How hundreds of extracellular signaling proteins secreted during immune responses were discovered – and what it tells us about the whisperered conversations between cells

Felix Meissner is lead author of a recent paper (1) that describes how we can now listen to the full conversation between immune cells and not just the bits we like to hear. Meissner, who works in Proteomics and Signal Transduction at the Max Planck Institute of Biochemistry in Martinsried, Germany, briefly explains the research and its significance.

Why? We were curious whether our mass spectrometry technology was sensitive enough to compete with antibody-based tools, such as ELISA, to determine cytokine levels expressed by macrophages when exposed to a bacterial compound. Unexpectedly, we detected way more secreted proteins than we ever thought, enabling a systematic analysis of signaling adaptor functionality. [Signal adaptor proteins are accessory to main proteins in a signal transduction pathway].

What? We chose TLR4 signaling which is a relatively low complexity example of protein secretion. It is well studied and several secreted proteins were already known, so it offered a great system to benchmark our technology.

How? We used UHPLC (Easy nLC) coupled to a Q Exactive Orbitrap MS and a nanoelectrospray ion source (Thermo Scientific) – it's just the best combination! We analyzed the raw data files with our in-house software – MaxQuant (www.maxquant.org). For statistical hypothesis testing and data presentation, we used a combination of R programming, our in-house software, Perseus, and Graphpad's Prism.

What next? Now comes the really interesting part… We expanded our research and analyzed protein secretion in response to other pattern-recognition receptor ligands and also in real infection settings. We were very surprised by the fact that known secreted proteins, such as interleukins, cytokines and growth factors, comprise only a minority of the secreted protein content. Given that in biology things happen for a good reason, this observation implies that there are many more proteins with extracellular functions. We are now testing these unexpected extracellular proteins for the capacity to modulate immune function. *RW*

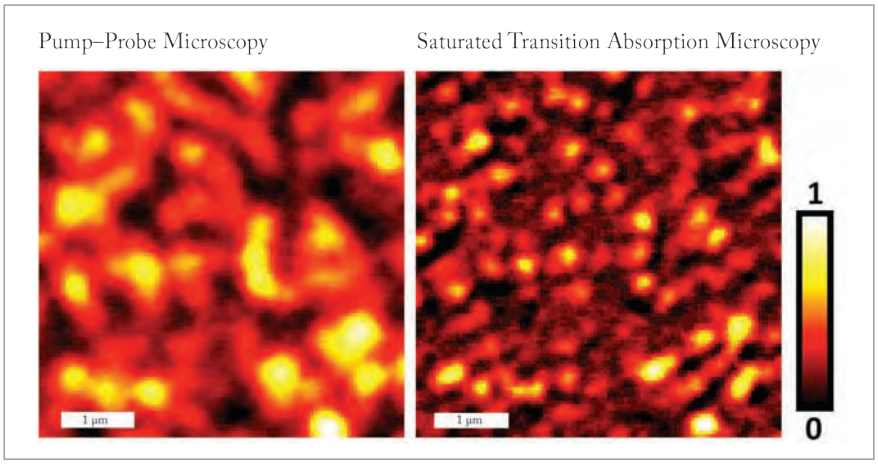
Further information: bit.ly/13URdBB
Full research paper: F. Meissner et al., "Direct Proteomic Quantification of the Secretome of Activated Immune Cells," Science, DOI: 10.1126/science.1232578 (2013).

Imaging Below 100 nm — without Fluorescence

Saturated transient absorption microscopy (STAM) opens a new door into the nanoscopic world of biomedical and nanomaterials research

The diffraction limit for non-fluorescent species – the fundamental limit of optical imaging resolution – has been breached. Researchers at Purdue University, Lafayette, Indiana, imaged graphite nanoplatelets about 100 nm wide (see image) (1). Conventional optical microscopes cannot resolve objects smaller than around 300 nm.

The group has developed a new iteration of super-resolution optical microscopy called saturated transient absorption microscopy (STAM). According to Ji-Xin Cheng, an associate professor of biomedical engineering and chemistry at Purdue, the project was initiated a few years ago. "We developed pump-probe



microscopy to study nanomaterials, and published our first paper in PRL (2)," he explains. "At that time, we were able to distinguish semiconducting versus metallic nanotubes; however, our resolution was not sufficient to separate adjacent nanotubes. This constraint triggered us to push the limit of resolution in label-free imaging".

In very simple terms, the addition of a third doughnut-shaped laser to the already complex pump–probe microscopy setup creates a reduced focal centre, which enabled the breakthrough.

The diffraction limit has been broken before, ushering in the advent of super-resolution optical microscopy, but native materials had not been imaged. "Up until now, far-field super-resolution studies have been based on fluorescence in a labeled specimen," Cheng says. "Ours is based on inherent absorption and is label-free." In fact, the doughnut shaped laser in the new system is based on that used in stimulated emission depletion (STED) microscopy, developed by Stefan Hell, now director of Max Planck Institute for Biophysical Chemistry in Goettingen, Germany. STED demonstrated the potential of super-resolution microscopy using fluorescent labels back in 1999. But because STAM is label free, the signal is obtained directly from the material itself, providing a more detailed understanding of its nanostructure. It means that STAM can be applied in new areas, including, Cheng says, "the electronic properties in nanodomains of 2D nanomaterials and the properties of single nanostructures in situ, for example, in a solar cell."

Graphene is an attractive candidate for study using STAM due to its photo-stability, which is a fundamental requirement of the technique. Proteins and lipids are likely targets in the biomedical space.

Cheng and colleagues hope to drive resolution down to 10 nm. "We are not there yet," he said in a previous interview, "But a few schemes can be applied to further increase the resolution of our system." *RW*

For a video presentation on STED: http://bit.ly/113eJYU

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Know Your Onions

Researchers tackle elucidation of sulfur-containing metabolites in plant extracts with Fourier transform mass spectrometry

Call it Onion-omics: Kazuki Saito and Ryo Nakabayashi, along with colleagues from the RIKEN Plant Science Center, Yokohama, Japan, EW probing bioactive compounds of the esteemed bulb and their potential medical application (1). "Chemical assignment is the most crucial issue facing mass spectrometry-based metabolomics," says Saito, of the RIKEN Center for Sustainable Resource Science. "Most detected peaks are assigned as 'unknowns'."

Elemental composition can provide useful information for the discovery of medically-relevant specialized or secondary metabolites, such as polyketides, flavonoids, alkaloids, and sulfur-containing metabolites (S-metabolites) that are not directly involved in the normal growth, development, or reproduction of an organism. "These secondary metabolites are important natural substances with unique biological activities that can open up innovative paths in drug development," Saito explains.

To start identifying some of those 'unknown' peaks, the group exploits the 'spectral fingerprint' of heteroatom-containing compounds using high-resolution mass spectrometry (HRMS). Saito admits that, while strategies that extract secondary metabolite groups by capturing specific features from metabolome data do exist, there is plenty of room for improvement. "In most cases, these secondary metabolites consist of C, H, N, O, and/or S. A first step toward better assignment of peaks is to precisely distinguish monoisotopic ions of N-, O-, and/or S-metabolites and then determine specific chemical

information, such as the elemental composition and structure of the ions," he says.

The group grew onion bulbs in standard and in carbon-13 atmospheric conditions. Comparison of the two HRMS data sets could, in theory, reveal the complete atomic make-up. And by comparing the data with that of known compounds, 67 sulfur-containing ions were identified. To obtain the data, liquid chromatography coupled to Fourier transform ion cyclotron resonance-mass spectrometry (LC-FTICR-MS) with C13 labelling was used. "LC–FTICR-MS has ultra-high performance on mass accuracy (< 1 ppm) and resolution power (> 250,000 FWHM) and can separate naturally occurring, stable isotope-labeled ions. Other HRMS platforms cannot fulfill those conditions," he says, noting that the FTICR-MS system (commonly known as FTMS) used in the study, a Bruker soraliX, readily combines with LC systems from several vendors.

FTMS accelerates research by eliminating the need for multiple stable isotope-labeled plants. "The performance from [FTMS] provides a minimal number of candidates for elemental composition, meaning that we only needed single stable isotope-labeled plants". To further speed up the cataloging of compounds, the group plans to add NMR into the mix, creating an automated structural assignment system.

Saito is optimistic about the impact of the approach: "Heteroatom-targeted chemical assignment, coupled with modern approaches in natural products chemistry – for example, LC-SPE-NMR-MS – will undoubtedly enable great advances in the isolation and structure elucidation of targeted metabolites in plants and other organisms." *RW*

Reference

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New Generation

In May, emerging analytical professionals gathered at a UK conference of the same name to present and network – and to compete for The Analytical Scientist's first ever prize.

With topics including equine medicine, airport security, and illegal drug precursor trends, a clear aim of Emerging Analytical Professionals was to explore diverse analytical fields. The event, held in Leeds, UK, featured 'Bright Sparks' presentations and poster pitches that offered early career delegates the chance to shine. The stand-out Bright Sparks presentation, "Keep off the Grass", focused on the extraction and detection of synthetic cannabinoids – look out for an upcoming Solutions article in the near future.

Congratulations go to Ásta Pétursdóttir, from the University of Aberdeen, who won The Analytical Scientist Poster Pitch Prize. She says of her efforts: "To make a poster pitch memorable, it needs to stand out. My way is to weave in a little humor; I had to convince people that arsenic and seaweed are exciting enough to leave the delicious cakes and coffee for." Ásta is currently trying to understand how arsenosugars and arsenolipids are produced in seaweed. "There is a relatively high concentration of total arsenic in seaweed, and that's what awakened my interest. Seaweed takes up toxic arsenate (structurally similar to phosphate) via membrane transporters," she explains. "I use a novel combination of HPLC coupled to simultaneous online detection by ICP-MS and ESI-MS, which gives both quantitative and structural information." *RW*

If you're an 'emerging analytical professional' and interested in becoming a committee member, email: asnenquiries@hotmail.com For updates on Emerging Analytical Professionals 2014, visit www.mmsconferencing.com

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

Hallway Talk

Casual conversations with colleagues from a different field can spark ideas, and even new areas. Here's how interdisciplinary research on the form and function of silk got its start.

By Fiorenzo Omenetto, Professor, Department of Biomedical Engineering, Tufts University, Medford, MA, USA

Sometimes hallway talk is perceived as a waste of time – deadlines, deliverables, grant writing, and the inevitable battles with font sizes and embedded movies have become very serious matters from which we cannot be distracted. But I have a particular affection for hallway interaction because it led to a decisive shift in my research. It is an understated, powerful catalyst for interaction – just like lunches, coffee breaks, or having a beer. After talking in the hallway, some go back in their office and close the door, while others leave their's open.

As an applied physicist working on femtosecond lasers and nonlinear optics for the better part of a decade, joining a Biomedical Engineering Department was a step into unfamiliar territory. A conversation with a colleague that was largely about Boston/New York Sports rivalries (no comment) ended up with me being handed a small plastic-looking disc made of silk. This was to be used as a tissue engineering scaffold to generate a cornea and it needed small holes for cells to grow into so that, eventually, the cornea would grow in a dish. We intended to add the holes with femtosecond machining but when doing so, the laser spot was nowhere to be found on the surface of the disc. We quickly realized that the silk surface was so smooth that there was no scattering whatsoever. This opened up an unlikely marriage of optics and silk that neither of us would have independently thought of and that quickly expanded into a materials transformation effort that continues to grow today.

Silk proteins are a unique family of biopolymers because of their structural and biological properties. From a materials science perspective, silks spun by spiders and silkworms are the strongest and toughest natural fibers known. Native silkworm silk can be simply boiled to extract the constituent protein. The resulting water-based silk solution can be used as the building block for a multitude of material formats, ranging from fibers, blocks, gels, mats, or plastic-like stable films with control of thicknesses down to below ten nanometers and patterned to form diffractive optics and 3D photonic crystals. These films are formed by simple casting of purified silk solution which crystallizes upon exposure to air, without the need for exogenous cross-linking reactions or post processing.

Most importantly, silk is processed in an all-water-based, room temperature, neutral pH environment. Further, silk materials are edible and biocompatible, along with the mechanical toughness and stability described above. They have precisely tunable mechanical properties, and can have programmable lifetime. Lastly when forming materials, any dopant that is added to the solution is stabilized and its function maintained in the material forms described above.

This is a particularly rich context for

materials, heralding a "silk renaissance" that brings together form (through the multiple material formats) and function (through material sustainability, biocompatibility, easy doping and biological activation). This coexistence of form and function transforms the context for materials, enabling unusual devices or adding value to existing ones. Among these are edible metamaterials, implantable optics, and resorbable electronics. Analytical devices can be designed out of a biocompatible and implantable material, with the chemistries needed for detection stabilized safely in the silk matrix. This principle was used, for example, to stabilize blood within a diffraction grating that "sensed itself": when the device is formed (by casting a blood-silk solution on a diffraction grating master, allowing the solution to dry and then lifting off the free-standing doped film), the hemoglobin remains active in the silk film and continues to respond to the external environment. This is one of the many possibilities that can be imagined when form and function come together.

"It is an understated, powerful catalyst for interaction – just like lunches, coffee breaks, or having a beer."

Lots of exciting interdisciplinary possibilities exist as new material properties enable familiar techniques and devices to operate in contexts where they usually do not.

At the root of this is the human connection between people that have the pleasure of reimagining things through their disciplinary experience. It would not have happened but for that dialogue where experiences were shared and came together seamlessly – in a hallway.

How to Beat Cheat Meat

Unscrupulous traders are substituting meat from undesired species. We have the tools to stop them, but do we have the political will?

By Johannes Lenstra, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Food forgery is an age-old problem that has recently resurfaced with a vengeance (see page 44). Today, meats are being labeled as coming from animals that we like to eat, but they might be something else entirely. Instead of beef, cheap meat from the noble horse is used; tasteless turkey substitutes for chicken; pork is served to people whose religion forbids them to eat it; even the involuntary consumption dogs, cats and rats has been reported. And it is not just meat: cheap fish is being colored and served as salmon. Italian mozzarella cheese is labeled 'di bufala' (from expensive water buffalo milk) when it has been made using normal milk, and on and on...

In most cases, these practices do not harm our health: meat is meat. You may also wonder why horses should be exempt from consumption. However, consumers have the right to choose and to be protected against the fraudulent food.

Despite the recent headline-grabbing cases, species substitutions are readily identifiable. In fact, protein-based or immunochemical species identification tests were developed 30-40 years ago and rapid, sensitive and versatile DNA-based assays have been available for 20 years. Several projects for improving species detection and traceability tests have been sponsored by the European Union and hundreds of reports have appeared in the scientific literature.

Our research group was one of the first to develop DNA-based identification tests. It proved not too difficult. DNA is abundant in most meat samples, even after curing, boiling, baking or autoclaving. And all of the methods tried, including hybridization to species-specific satellite DNA; restriction-enzyme cleavage of amplified mitochondrial DNA, and quantitative PCR with species-specific primers, have worked as predicted. There are now ready-to-use kits available, based on quantitative real-time Taqman PCR with primers and probes that match the mitochondrial DNA of the species to be detected. This approach can be automated, allowing thousands of samples to be processed at minimal cost. These assays are quantitative, although the species detection is not since the amount of DNA per gram of tissue is highly variable and one species may be detected with a higher

sensitivity than another. However, as qualitative tests, they robustly identify species substitutions or admixtures. Other tests, based on multiplex DNA, generate DNA fragments with lengths dependent on the species and can be used to detect several species simultaneously.

So, with sound detection methods available, why are we still being cheated on our meat? The answer is that the tests have never been introduced as part of routine food inspection and are used only on suspect samples. And the reason for this? Funding. Priority is given to food-related problems that more directly threaten our health. The past decade has witnessed a number of scares, including several livestock epidemics, new and dangerous human

"Identification tests were developed 30–40 years ago and rapid, sensitive and versatile DNA-based assays have been available for 20 years."

Structural Proteomics Takes Aim at Disease

A combination of HDX-MS and IMS will help to explore the basis of functional failures in proteins linked to medical conditions.

By Amrita Mitra and Amit Kumar Mandal, Clinical Proteomics Unit, Division of Molecular Medicine, St. John's Research Institute, Bangalore, India.

Unlike the genome, which is relatively static, the proteome is dynamic, constantly changing in response to intracellular and extracellular environmental signals. Modern mass spectrometric techniques, electrospray ionization and matrix assisted laser desorption ionization, are capable enough to take the "molecular elephants" of the proteome into gas phase, thereby studying them individually and collectively in hetero-molecular assemblies (1, 2).

This has huge implications for medicine. In patients, a variety of factors have a bearing on protein expression and post-translation modification, including the disease itself, pharmacological interventions, genetic factors and environmental variables. High throughput analysis of the proteome in a limited clinical sample is already feasible and ongoing improvements to increase resolution, sensitivity, mass accuracy and analysis speed of mass spectrometers along with the advent of higher order dimensions in separation will permit proteomic analysis of virtually any challenging clinical sample in a single step in the near future.

So what is the next quantum step? Proteomics need not be restricted to a study of primary structure. It can be extended to assess the thermodynamic stability of the three-dimensional structure of proteins, finally to arrive at an understanding of molecular mechanisms through structure-function correlation.

The discovery of protein structures has – since alterations in the chemical structure of hemoglobin in sickle cell anemia was achieved in the 1950s (3) – made it possible to assign disease to the functional disorder of a few atoms. However, structural analysis has been confronted with three issues. First, using any spectroscopic tool has required pure protein in large quantities, which is difficult to achieve with clinical samples. Second, due to complex milieu of molecules in the cell, in vitro stability parameters might not be exactly the same as those that occur in vivo. And third,

"Proteomics can be extended to assess the thermodynamic stability of the three-dimensional structure of proteins."

most spectroscopic methods that monitor molecular properties are not molecule-specific; rather, they are functional group-specific making it impossible to have structure-function analysis at the residue levels of a protein in vivo.

I believe that the solution to

pathogens, such as Enterotoxigenic E. coli (ETEC) and influenza variants, and forbidden contaminants, such as dioxin and antibiotics. Even the detection of genetically-modified organisms (GMOs) in food production, which in America is no issue at all, has taken priority over species fraud in some European countries. Thus, attention has been distracted from checking species origins of foods. This has opened the door to fraudulent traders, who never hesitate to step in.

Authorities should be aware that the safety of food can only be guaranteed if we are sure of the species it comes from. It's time to marshal modern DNA technology and clamp down on food cheats once and for all.

"In patients, a variety of factors have a bearing on protein expression and post-translation modification"

these problems lies with hydrogen deuterium exchange-based mass spectrometry (HDX-MS). By exploiting the permeability of heavy water (D_2O) through cell membranes, HDX-MS has been used to measure the thermodynamic stability of intact protein structure in vivo (4). HDX-MS has also been applied in vitro to establish structure-function correlations at the residue level of proteins in non-covalently bonded complexes by studying the kinetics of isotope-exchange patterns (5).

Ion mobility spectrometry (IMS) adds a further dimension to structural proteomics research by separating molecules on the basis of shape. The combination of HDX-MS and IMS can be used to explore the underlying mechanisms of protein folding and protein-ligand interactions in normal and disease situations. In our view, an investigation of protein function through structure-function correlation in vivo using isotope exchange-based mass spectrometry would be an important scientific breakthrough, one that connects us back to the origins of molecular medicine with the discovery of the molecular basis of sickle cell anemia in 1949 (6).

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Rising Above the Helium Shortage

Worldwide helium demand far exceeds current production levels. For many gas chromatography applications, the switch to hydrogen is painless. What's stopping you?

By Ed Connor, GC–MS application specialist, Peak Scientific, Inchinnan, Scotland.

Since the 1950s, helium's inert nature has made it the go-to carrier gas for the majority of gas chromatography (GC) applications. Despite GC's relatively low consumption of helium – estimated at less than two percent of the worldwide total (1) – laboratories now struggle to find a guaranteed supply and face stark price rises caused by worldwide refinery equipment failure and shutdowns (2), with scheduled maintenance of natural gas refineries causing further disruptions. The impact is compounded by increasing demand for helium from newly industrialized nations, such as China.

Helium, nitrogen, hydrogen, argon, and air can all be used as GC carrier gases – selection is mainly determined by the type of detector used. In some cases, helium can be substituted by hydrogen or nitrogen, both of which are readily available and relatively cheap. So why have they not been widely used as alternatives to helium until now? The short answer is that, in the past, there was no market pressure forcing chromatographers to adapt.

That has all changed. Now, GC manufacturers have started addressing the helium shortage in earnest. Bruker has released its Scion GC-MS systems specifically designed for use with hydrogen; Thermo has tested all of its GC products for hydrogen compatibility and has been highly pro-active in addressing the conversion of its GC systems to hydrogen through webinars and seminars, even producing a toolkit to assist customers in method conversion; and Agilent, with the largest GC market share, has hosted webinars, produced a carrier gas flow calculator app, and developed gas saving solutions to reduce helium consumption for applications that can't use an alternative carrier gas source.

Anyone considering a move away from helium will have encountered the van Deemter curve, which shows the relative carrier efficiencies of nitrogen, hydrogen and helium. What this curve tells us is that nitrogen can perform better than helium and hydrogen, but only at very low velocities. Helium and hydrogen perform similarly at medium carrier gas velocities, with hydrogen actually outperforming once things really speed up.

The performance of hydrogen at higher carrier velocities provides clear advantages to those labs looking to increase sample throughput without compromising on sample quality (3). But one major issue facing labs using US Environmental Protection Agency (EPA) methods is that hydrogen is simply not an option in certain cases. Until these methods are approved for use with hydrogen, the hands of those analysts are tied.

Some chromatographers have raised concerns over the potential reactivity of hydrogen with analytes and chlorinated solvents, however, in their recent webinar on helium to hydrogen conversion, Thermo Scientific found no problem with MS spectra or identification of compounds (4).

There are also safety concerns from some quarters about the explosive nature of hydrogen, though many labs have been using hydrogen as the flame gas for GC with flame ionization detection (GC-FID) for years. Once hydrogen reaches 4% volume in air, it reaches its lower explosion limit (LEL; the upper explosion limit is 75%) and can undergo auto-ignition. However, being less viscous than helium, it more readily escapes. Unless a large quantity is suddenly released into the environment, the danger of reaching the LEL is relatively low. One alternative to hydrogen cylinders is a hydrogen generator, which produces the gas from de-ionised water. A hydrogen generator will typically contain less than a litre of gas at any one time, whilst being capable of supplying the requirements of a small lab.

For the foreseeable future, helium will be required for certain GC applications. However, as a finite resource, it will become more difficult to source with a resulting rise in price. Given hydrogen's cost effectiveness and availability, coupled with the fact that it is the best 'like-for-like' alternative to helium, I believe it should become the carrier gas of choice in many GC applications.

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Image courtesy of Alain Quenderf

Toying with Athletes

WADA is on a mission to rid sports of substances that impart an unfair advantage. Many observers, however, feel that their approach is deeply flawed and potentially harmful, both to sport and to individual athletes. Here, a group of four analytical scientists set out their opposition to WADA's strategy and provide recommendations for a better way forward.

By Rich Whitworth and Frank Van Geel

The stated mission of the World Anti-Doping Agency (WADA) is "to lead a collaborative worldwide campaign for doping-free sport". This is (arguably) a laudable goal but a vociferous group of analytical scientists is far from satisfied with efforts to achieve it, questioning the effectiveness of anti-doping programs, the injustices created in their implementation, and the non-collaborative approach that WADA has taken.

Here, four of the critics respond to a series of questions in what we hope will be the start of an open discussion on the need for anti-doping control, what its goals should be, and how these goals can best be accomplished. WADA has been offered the right to reply to criticisms and to contribute more widely to the debate, and we invite you to have your say at theanalyticalscientist.com/0613/401.

What are your major concerns over anti-doping approaches as they stand today?

It would be easy to imagine that the anti-doping system works perfectly well: cheating athletes are caught and punished, and sports are fairer as a result, right? This expert group does not think so.

It is statistically challenged

Klass Faber: "Current practice is not state-of-the-art; sub-optimal methods are being used for data analysis. Instrumentation is not the problem. What is being done with (or to) the measurement results, that's the problem. The criteria for a positive result, that is, the decision limits, are not statistically underpinned. There is not a single test for which the risk of false-positives and false-negatives is known. That worries me a lot."

The Panel

Douwe de Boer
- Analytical biochemist
- Independent anti-doping consultant and expert witness in legal sports cases
- Active in the field of anti-doping analysis since 1986
- PhD in Pharmacy (analysis of anabolic androgens in urine samples), from an International Olympic Committee (IOC)-accredited lab
- Technical and scientific director of the IOC- and WADA-accredited Portuguese anti-doping lab in Lisbon (1998-2004)

Klaas Faber
- Chemometrician specializing in the use of optimal methods for data analysis and validation of final results
- PhD in chemometrics and post-doc with Bruce Kowalski, one of the founders of the field (1994-1996)
- Forensic and food research institutes advisor
- Private chemometrics consultant since 2002
- Independent anti-doping expert since 2006

Peter Kootstra
- Analytical chemist specializing in laboratory accreditation (for example, ISO/IEC 17025)
- Broad knowledge of analytical methods used in anti-doping laboratories
- Independent consultant on laboratory quality
- Fifteen years experience in residue analyses (veterinary drugs); 25 years worldwide experience in laboratory accreditation
- Expert witness in doping case

Bob Blackledge
- Analytical chemist specializing in forensic field
- Retired in 2006 after over 35 years in forensic science. Still active as a consultant
- 28 years experience in forensic laboratories of the US Army and Navy, working on analysis of suspected drug items
- Experienced in laboratory and individual certification
- Expert witness in over 200 court cases

It's a closed system

Peter Kootstra: "The whole system is closed, which is maintained under the auspices of independent accreditation bodies. The results I have seen and the discussions I have held with scientists from doping laboratories do not make me happy. There is a lot of confirmation bias (the tendency to favor information that confirms your beliefs), under the guise that 'every athlete is guilty, we just can't prove it'."

There is no right to a fair trial

Bob Blackledge: "I am neither pro nor con as far as the need for monitoring of possible sports doping by competitive athletes goes. But individuals pulled over by the police for traffic violations, ticketed for illegal parking, or charged with driving under the influence of alcohol or drugs (all actual crimes) have far more rights to a fair trial before a jury of their peers than an athlete charged with doping."

It is non-uniform

Doewe de Boer: "Worldwide, results must fulfil certain minimum criteria. However, some laboratories perform much better than those minimum standards and, therefore, create non-uniformity. The effect is a kind of regional bias in anti-doping control."

What are your views on the effects/perceived effects of doping substances?

Unlike pharmaceuticals, the physiological effects of which are heavily researched and supported by rigorous trials, substances used in doping are often poorly understood. Even within this group of experts there is a wide range of opinions on the impact of doping substances.

There is a lack of evidence

De Boer: "Evidence-based studies in respect to sport performance-enhancing effects and health effects are often missing. They may be under- or overestimated, but because of a lack of adequate studies, this can't be confirmed or denied."

There is a lot of propaganda

Faber: "Of course most compounds do have a performance-enhancing effect - but so does beet juice. The health risks often appear to be exaggerated. Think, for example, of the

notorious epogen (EPO) or of growth hormone: Bernat López has strongly questioned the dangers of both (2, 3). Just recently, WADA issued a warning for GW1516, announced as the 'new EPO', yet there appears to be no evidence of its detrimental effects (4). Is emphasizing dangers that are not corroborated by scientific evidence an act of good faith, erring on the side of caution, or is it propaganda?"

The placebo effect is a factor

Kootstra: "There are only a few compounds that will have an effect, depending on the sport. Anabolic steroids will have some effect in sports where power is needed. Beta-blockers help reduce tremors when you need a steady hand. Most of the effects are psychological – merely a placebo."

The ratchet effect is a concern

De Boer: "Unfortunately, anti-doping control does not solve the problem of doping, but rather shifts it to the abuse of sometimes more dangerous substances. Pharmacological substances still under investigation, not yet approved for clinical use, or even disapproved for clinical use, are becoming of interest."

Do you understand what ends up on the prohibited list, and why?

Given that the latest doping drugs are designed to be undetectable, the historical (and very long) list of prohibited substance must be constantly updated and evaluated. But this group of experts believe that significant question marks hang over the choices made, and those that make them.

Legacy and politics play a big role

De Boer: "The list of prohibited substances and methods originated in the 1960s, without clear objective reasoning. Only afterwards was the reasoning objectified, with criteria written to extend the list. In the early days, the list was evaluated and extended by the International Olympic Committee (IOC) mainly based on scientific grounds, but WADA's current list is also influenced by political arguments. Politicians have more influence on WADA than they ever had within the IOC, because they finance 50 percent of WADA's budget. For that reason, a relatively low threshold for a cannabinoid was maintained on the prohibited list longer than could be justified scientifically. Only very recently, was its low threshold upgraded from 15 ng to 150 ng/ml of urine."

There are contradictions

Faber: "It is not always clear why a substance is banned (understatement!). Conversely, it is not clear at all why pain medication is allowed. How can you have clean sport if pain medication is allowed? It is certainly performance enhancing. What's wrong with telling athletes to stay at home, regain their natural strength, and then compete? Also, pain medication leads to many addicts and sport casualties, notably in American football (5)."

Are the standards for anti-doping laboratories appropriate?

All anti-doping laboratories must be certified by both ISO and WADA. But the existence of standards does not always guarantee reproducibility, if standard operating procedures (SOPs) are not followed (6).

It is impossible to say

Kootstra: "Difficult to address since most required data are not available. Doping laboratories are inspected by employers of other doping laboratories…"

There are clear violations

Blackledge: "I am only quite familiar with one case, Floyd Landis and the 2006 Tour de France. Clearly, there were procedural violations of WADA rules, ISO requirements, and the lab's SOP. A supervisor at a WADA-certified laboratory in Paris had checked an instrumental data page and then used a rubber stamp "ASSURANCE QUALITÉ - LNDD". And yet lower on the page a technician had heavily marked over in black ink an original number (unknown) and changed it to '5'. What explanation is there for the fact that the lab found exogenous testosterone at a rate three times greater than other WADA-certified labs?"

There are insufficient guarantees of quality

De Boer: "The rules are defined in the International Standards for Laboratories (ISL) and are based on all relevant ISO requirements and regulations. National accreditation

bodies verify if local anti-doping laboratories are following the ISL, but cannot guarantee uniform quality. The analytical scientists themselves inside anti-doping laboratories play a very important role in assuring the required scientific level."

Do athletes who test positive get a fair trial?

Analytical science is not infallible, indeed margins of error must be tolerated in all scientific data. For athletes, false positive tests for doping are a huge concern (while the 'victims' of false negative results are unlikely to complain). Our panel holds that athletes who challenge a test result are treated unrighteously.

Lack of access to documentation is unethical

Faber: "They do not, for a fact. Anti-doping regulation is such that the laboratory result is assumed to be reliable. One can only appeal on procedural grounds, that is, was the test properly carried out? Moreover, the defence does not receive sufficient documentation to challenge a test result; that has also been arranged in anti-doping rules. How convenient!"

'Strict liability' severely limits defense

De Boer: "As long as the principle of strict liability is applied to athletes (meaning that they are responsible regardless of whether they were aware or not), 'fair' has a very special meaning. This is especially so if one measurement and, in theory, even one molecule, is enough to sanction an athlete. Anti-doping authorities reveal as little detail about their analytical strategies as is legally required because of potential abuse of those details by dishonest athletes. Unfortunately, the inability to access all relevant details also severely limits the ability of honest athletes to defend themselves. The anti-doping authorities hide themselves behind regulations, which they themselves set up and write."

Duplicates are not handled appropriately

Kootstra: "The B-sample analysis is a duplicate of the A-sample – same laboratory, same method, same instrumentation, different analyst. It almost seems like there could be no other result. Validation reports, QA control, instrument parameters – none of these can be inspected by the athlete or their representative. No discussion is possible."

You would fare better to be on a criminal charge

Blackledge: "The table below compares the criminal justice system (of the US) with that of doping hearings:"

Characeteristic	*Criminal Trials*	*Sports Doping Hearings*
Burden of proof?	On the prosecution	Defence must prove innocence
Right to an attorney?	Yes	No, if cannot afford
Right to a jury trial?	Yes	No (three-member panel from WADA list)
Proof of chain of custody?	Yes	No (panel decides on admission)
Hearsay testimony?	No	Yes (decision by panel)
Reanalysis by another lab?	Option for defence	No (only the sameWADA-certified labs)
Rebuttal testimony?	Yes - after passing voir dire	Not permitted by WADA lab analysts
Lab's proficiency tests?	May question past performance	WADA will not divulge

What should be done to improve anti-doping control?

A number of improvements are suggested by the commentators, including altering the thresholds at which substances are considered to be cheating. A consensus exists that more openness in the system, in the form of real engagement with anti-doping stakeholders, may be the key to a brighter future.

First, clarify the WADA mission

Blackledge: "Before changes can occur at the laboratory and analyst level, there must be changes at WADA. This can begin with the mission statement: WADA and its accredited laboratories must only be concerned with monitoring those substances that can have a positive effect on athletic performance. For example, can it be clearly shown that marijuana use positively affects athletic performance? Also, there must be established cut-off levels for every banned substance. Below these levels, any indications of their presence should be considered neither capable of improving performance nor an indication of an athlete's intention to cheat. For example, traces of clenbuterol were found in a urine sample from the cyclist, Alberto Contador. Whether these traces were or were not the result of eating meat obtained from Spain should not be the question. The question should be whether clenbuterol, at that level, could have enhanced his athletic performance."

Open up a bit

Faber: "The inhabitants of doping control laboratories can do better by opening the window to the outside world. I would argue that the best science is not being delivered. More importantly, about 50 percent of the convictions are questionable because thresholds are not being applied. The use of party drugs outside competition is expressly allowed in anti-doping rules; it is the negligible trace found in-competition that leads to a conviction. Drink a few alcoholic beverages on Friday and played a match on Saturday? Not a problem, because there's a threshold for alcohol. Smoked cannabis on Wednesday, and played a match the following weekend? Positive test and a sanction!"

Get athletes, analytical scientists involved

De Boer: "In theory, athletes are stakeholders of WADA and therefore have influence on the development of regulations. In practice, they are not well organized or well represented. Analytical scientists within anti-doping laboratories also limit themselves to regulations and claim that it is not their task or responsibility to sanction. However, if analytical progress is pushing identification limits lower and lower, the science will play an increasingly important role. Analytical scientists are also stakeholders in WADA and can exert their influence and provide proper advice – while doing so, they should keep the dilemma of fair chance in their minds. This is a social responsibility, rather than their analytical responsibility (which, I would argue, they are already fulfilling adequately)."

Analytical scientists need to say "no"

Faber: "The laboratory personnel should not assist in nonsense convictions. These labs have become production lines: 'you ask, we deliver'. The exonerating part of the proof currently being omitted, on a global scale, is simply mind-boggling."

What do you think?

Do the issues raised make you sceptical about anti-doping effort, or do you feel that WADA is on the right track? If you work in another field, how do the criteria compare to doping tests? If you work in a WADA-certified lab, what's the inside view?

Comment online at theanalyticalscientist.com/0613/401.

Rich Whitworth is Editor, and Frank van Geel is Scientific Director, of The Analytical Scientist.

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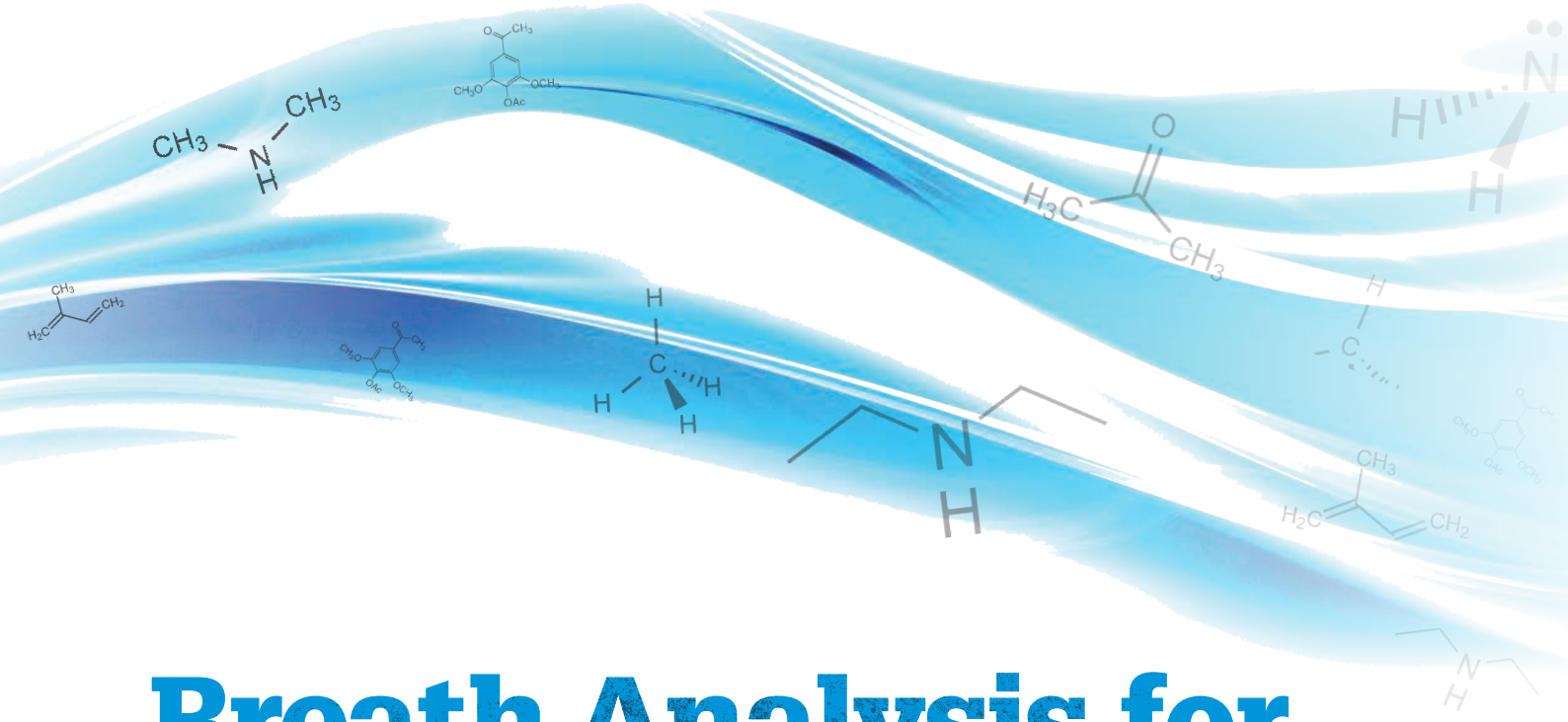

Breath Analysis for Disease Diagnosis

Jumping on the breath biomarker bandwagon is tempting, but a number of failures to observe the fundamental rules of good science have cast doubt over the technique. Here, the authors describe best practice, from collection to clinical evaluation.

By Lisa Spacek and Terence Risby

The current resurgence of interest in breath analysis follows directly from Nobel Prize winning discoveries of "nitric oxide as a signaling molecule in the cardiovascular system" and identification of significant concentrations of nitric oxide in human breath. Because the analysis of exhaled breath is relatively easy to perform, it's ever more important to encourage high quality investigation. The use of breath analysis in clinical diagnosis requires careful attention to be paid to:

- Breath sampling
- Breath analysis
- Biomarker discovery
- Clinical evaluation of potential breath biomarkers.

Failure to consider each of these important factors will limit their subsequent utility and increase the likelihood of "garbage in and garbage out".

Researchers who proposed that breath nitric oxide could

be used to evaluate airway reactivity in patients with asthma have paid careful attention to each of these factors and, as a result, the US Food and Drug Administration (FDA) has approved breath nitric oxide as a diagnostic tool to monitor asthma therapy.

Breath Sampling

Collection of a representative alveolar breath sample under controlled conditions is an a priori requirement for successful breath analysis research. Ideally, breath should be sampled when the concentration of carbon dioxide reaches a plateau and it is important that the patient does not hyperventilate. Normally, the depth and frequency of breathing are under autonomic control. However, when asked to provide a breath sample, the patient often transitions from autonomic breathing to conscious breathing and consequently hyperventilates. The easiest way to minimize hyperventilation is to ask the patient to breathe at a defined frequency (for example, 10 times per minute) and a defined tidal volume using visual and audio prompting. This protocol for breathing is known as paced breathing.

Sampling multiple breaths improves the accuracy of the resulting analyses and breath samples can be collected in inert gas-sampling bags, made from Tedlar, Teflon, Mylar, etc. The requirements for these gas-sampling bags are that they must not adsorb breath molecules and have volumes that exceed the amount of breath to be collected by at least a factor of two. This volume restriction minimizes backpressure from the collected gas that will variably restrict exhalation. Similarly, the inlet to the sampling bag should have sufficient dimensions to minimize pressure drop across the inlet. Evacuated polished stainless steel cylinders or thermal desorption tubes packed with adsorbents or solid phase microextraction (SPME) tubes, are alternative media used for the collection of breath samples. Sampling with these devices is performed indirectly using constant flow or constant timed gaseous diffusion. The advantages of these latter devices are that the study subject experiences no resistance to breathing. Moreover, the breath samples are easily transported in these devices from the sampling site to the laboratory.

"Normal cellular biochemistry can only be induced or suppressed by abnormal physiology and although disease states may appear to be producing unique molecules, these results may only be a reflection of the detection limit of the analytical method."

Breath Analysis

After collection, samples of breath can be analyzed with any analytical chemistry technique with specialized inlets. Many published studies have used standard analytical chemistry methods, such as capillary gas chromatography (GC) with sensitive, selective detection systems based upon optical spectroscopy or mass spectroscopy, since capillary GC can separate complex breath profiles and make biomarker discovery easier. Two-dimensional capillary GC improves the separation of the complex breath matrix further.

Many of the more modern analytical methods based upon absorption spectroscopy, selective ionization mass spectroscopy with ion-molecule reactions, electrochemistry, and chromatography, now have sufficient speed, sensitivity, and selectivity to allow real-time breath analyses to be performed – and analysis can be based on a single breath. Therefore, sampling protocols have been developed that have the patient breathe at a constant flow rate of exhalation by imagining they are trying to whistle a musical note. Flow can be monitored indirectly by monitoring mouth pressure. Visual prompting of mouth pressure allows the subject to breath repeatedly at the same flow rate.

Biomarker Discovery

The molecular profile of any breath sample will be defined by the concentrations and identities of the volatiles in inhaled air and in blood. The volatiles in air can be derived from both ambient and indoor sources. The sources of the volatiles in blood are:

- Molecules and/or their metabolites that have been inhaled
- Molecules and/or their metabolites that have entered the bloodstream via the skin
- Molecules and/or their metabolites that have entered the bloodstream from ingested foods or beverages
- Molecules produced by foreign cells, such as viruses, bacteria, fungi, and yeasts
- Molecules produced by tissues in the body including the mouth, nose, sinuses, airway, and gastrointestinal tract.

The majority of alveolar breath (99.995 percent) consists of nitrogen (78 percent), oxygen (13 percent), carbon dioxide

(5 percent), water vapor (4 percent); the inert gases and the remainder (<50ppm) is a mixture of as many as 1000 different compounds. The rates of excretion of molecules in breath are directly related to rates of ventilation and cardiac output. The physical and chemical properties of molecules also affect their rates of excretion. If a molecule is lipid-soluble, it may be stored in tissues not well perfused by blood, such as adipose tissue, and thereby be released more slowly than a similar molecule with hydrophilic properties that is not stored.

In general, the concentrations of endogenously-produced molecules are lower in breath than the concentrations of molecules from exogenous sources. Unique molecules in breath can only originate from the ingestion, inhalation, or dermal absorption of exogenous substances or be metabolically produced by foreign cells (bacteria, viruses, or eukaryotic organisms). Normal cellular biochemistry can only be induced or suppressed by abnormal physiology and although disease states may appear to be producing unique molecules, these results may only be a reflection of the detection limit of the analytical method.

Clinical evaluation

The use of breath for disease diagnosis first requires that the concentration profiles of breath molecules for normal healthy human subjects be established and these studies must include such variables as age, gender, ethnicity and body mass index. A methodological approach entails univariate analyses where each one of these characteristics is compared to breath biomarker values. This process will define breath measurement confounders, or participant characteristics that change measured breath values. It may be that an adjustment accounting for one or more of these characteristics is necessary before comparisons can be made. For instance, standardization of any biomarker molecule that is measured in exhaled breath can be corrected by average concentration of mixed expired CO_2. This serves to adjust for anatomic, physiologic, and instrumental dead space based on each individual participant. If comparisons of the results of breath analysis are to be used to study therapeutic intervention or to compare different study subjects, it is important to standardize concentrations of breath molecules to oxygen consumption or carbon dioxide production.

To recognize potential confounders, well-described cohorts of participants identified as "healthy" or "diseased" will need to provide breath samples that are evaluated for validity and reproducibility. Large study groups are required so that unknown confounders can be detected and explored. Participants should be identified who exhibit either significantly high or low breath biomarker concentrations that increased variability in a presumed homogenous population. Further research is needed to clarify our understanding of attributes that contribute to breath biomarker variability. Potential confounders, such as dietary intake immediately and within eight hours of breath collection, and mouth rinses done immediately prior to breath collection, must be evaluated. Collection and analysis of such data will refine the application of breath biomarker measurement in diverse populations.

The use of a particular breath molecule or group of molecules to ascertain disease can be implemented once a threshold, which separates healthy individuals from those with disease, is identified. The breath molecule will need to be compared to the current reference or 'gold standard'. Notably, a true gold standard test may not be available. The classification of disease versus healthy in dichotomous outcomes or multiple levels of disease severity will require sample sizes large enough to ensure adequate power and precision. Complete and accurate reporting of the accuracy of diagnostic tests is critical and guided by the Standards for Reporting of Diagnostic Accuracy (STARD) initiative first promulgated in 2003.

Despite its promise and the fact that there have been a large number of publications on breath analysis, only a handful of tests are used clinically, with a few others used for investigational purposes. Approaches to clinical breath analysis fall into two basic categories: tests based upon the quantification of metabolites in breath after administration of a labeled drug or substrate; and tests that quantify molecules in breath without any prior administration of a drug or substrate.

Labeled breath tests

Carbon dioxide, one of the major products of cellular respiration, is the most popular metabolite investigated. Briefly, substrates labeled with carbon-13 (^{13}C) are metabolized to ^{13}C-labeled carbon dioxide that can be separated spectroscopically from endogenously produced carbon dioxide by non-dispersive infrared absorption spectroscopy or isotope ratio mass spectrometry. The analytical limitation of this approach is the natural abundance (1.1 percent) of ^{13}C-labeled carbon dioxide. Breath tests based upon this approach require that the rate of metabolism of the ^{13}C-labeled substrate and the rate of excretion of carbon dioxide be well characterized. Typically, a set dose of a labeled substrate is given to the patient to evaluate a disease-related metabolic change. The concentration of ^{13}C-labeled carbon dioxide is quantified after a defined time. The labeled substrate is selected for a specific biochemical pathway that is related to a specific disease.

It is also possible to identify genetic polymorphisms of patients on the basis of the absence of a specific enzyme by using the required labeled substrate for this enzyme. The

limitation of tests based upon this approach is the ability to identify a relevant biochemical pathway that will provide disease-specific diagnostic information and the design and synthesis of a suitable labeled substrate.

Irrespective of the biochemical pathway under investigation, the breath test must be performed under carefully controlled conditions after a fixed fasting time. Time between the administration of the labeled substrate and the administration of the breath test must be defined. Currently, the urea breath test for *Helicobacter pylori*, an etiology of chronic gastritis and gastric ulcers, is the only FDA-approved labeled breath test.

Endogenous breath tests

The most widely used clinical breath test is based upon monitoring the concentration of unlabeled carbon dioxide, a major product of respiration. This test, know as capnography, is the instantaneous measurement of the concentration of carbon dioxide as a function of time. The capnograph provides real-time information for the excretion of carbon dioxide. The shape of the curve provides information on alveolar ventilation and respiratory patterns. Lung diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, affect these breathing parameters. Capnography is used to confirm the correct placement of endotracheal tubes (tracheal intubation) in all operating rooms, intensive care units and any situation that requires assisted ventilation. There are a number of companies that manufacture capnographs and most are based upon of the absorption of non-dispersive infrared radiation. In certain clinical situations, use of these portable and inexpensive devices has become standard of care and neglecting to use capnography can be construed as medical malpractice.

Assays based upon the determination of breath hydrogen or breath methane are the next most popular clinical tests. These tests are conceptually similar to the labeled breath test. Briefly, the concentration of hydrogen or methane is determined in the breath of fasting patients at defined times after the administration of a low dose of a specific sugar (fructose, lactose, glucose, dextrose, sucrose, d-xylose, sorbitol or lactulose). Elevated levels of hydrogen or methane after administration are indicative of irritable bowel syndrome and various sugar intolerances. Hydrogen and methane were initially determined using a specifically designed gas chromatograph but, more recently, hydrogen is monitored with membrane electrodes, which are hand-held and significantly less expensive.

The final breath test is based upon the determination of nitric oxide. The physiological origins of this reactive species are inflammatory cells in the oropharyngeal cavity and cells within the respiratory tract. Currently, breath nitric oxide is used clinically to monitor therapy for asthma. Initially, nitric oxide was determined by monitoring the chemiluminescent reaction with ozone. More recently, commercial breath nitric oxide monitors are based upon the absorption of mid-infrared radiation or use membrane electrodes. The latter monitors can be hand-held and less expensive.

In conclusion, clinical breath analysis remains in its infancy, despite the fact that its potential has been recognized since antiquity. Recent advances in instrumentation are inspiring a renaissance in breath analysis; in particular, the wider availability of real-time, portable monitors is enabling breakthrough research that can more easily allow the analysis of large numbers of human subjects.

"Despite its promise and the fact that there have been a large number of publications on breath analysis, only a handful of tests are used clinically with a few others used for investigational purposes."

Efforts to translate technical knowledge into clinical applications require expertise in several areas. Only dynamic multidisciplinary teams, consisting of device makers, breath analysis experts, and clinical researchers and clinicians (including statistical support), that bring together the specialized skill sets and problem-solving capability necessary can anticipate breakthrough success. Teams that lack any of these essential roles will not be successful.

Future research efforts must better define the pathophysiologic mechanisms to which breath analysis can be applied, such as the contribution of gut flora to health and disease. Additionally, clinical studies are needed to define disease states that clinical breath analysis can diagnose. The unique contribution that breath analysis brings to patient care and clinical diagnosis merits ongoing work to establish original applications of this innovative technology.

Dr. Lisa A. Spacek, is adjunct assistant professor in the Division of Infectious Diseases at the Johns Hopkins School of Medicine.

Dr. Terence H. Risby is professor emeritus of Environmental Health Sciences at the Johns Hopkins Bloomberg School of Public Health.

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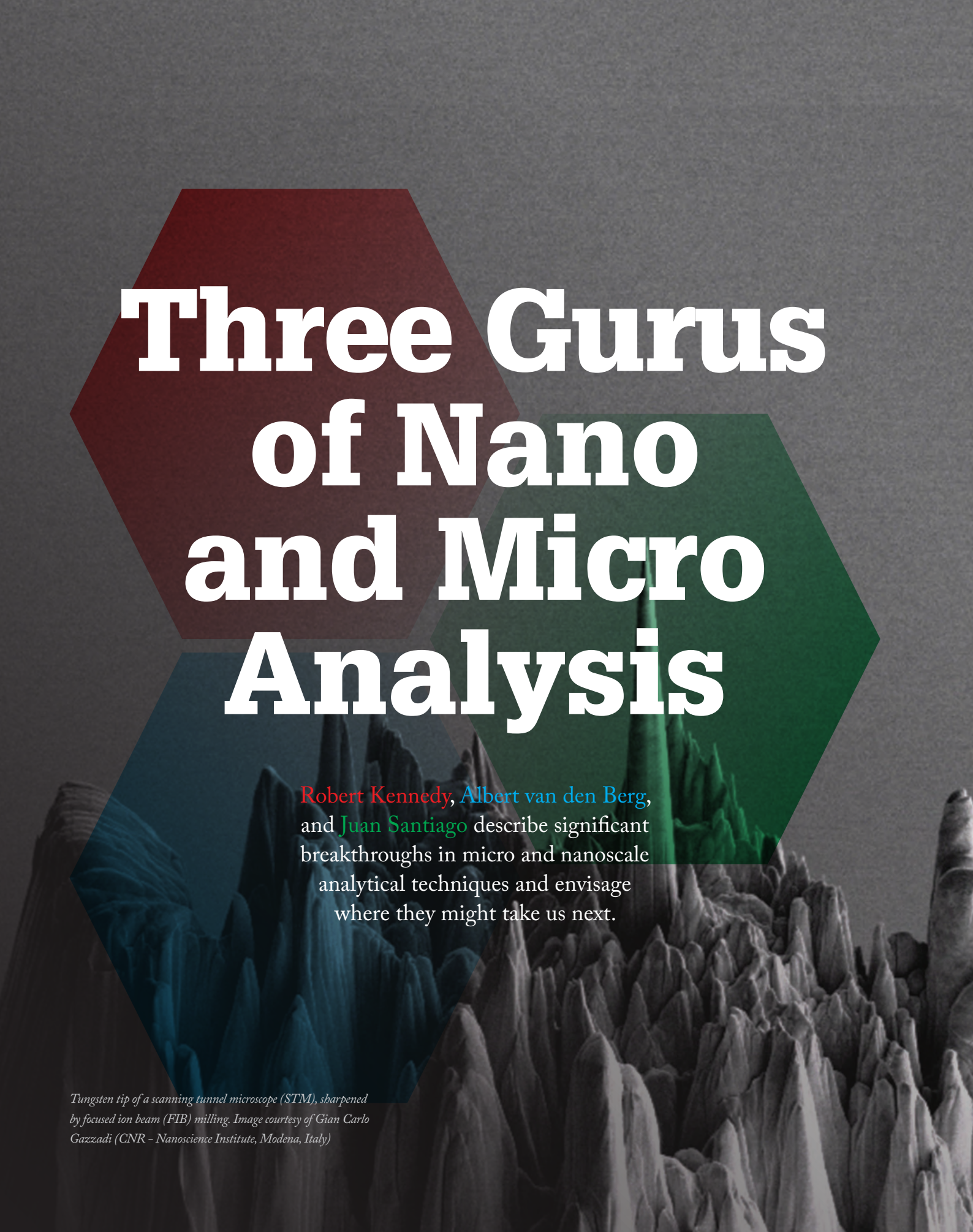
PEAK SCIENTIFIC

Three Gurus of Nano and Micro Analysis

Robert Kennedy, Albert van den Berg, and Juan Santiago describe significant breakthroughs in micro and nanoscale analytical techniques and envisage where they might take us next.



Tungsten tip of a scanning tunnel microscope (STM), sharpened by focused ion beam (FIB) milling. Image courtesy of Gian Carlo Gazzadi (CNR – Nanoscience Institute, Modena, Italy)

Robert Kennedy is the Willard Professor of Chemistry and Professor of Pharmacology at the University of Michigan. His research interests are analytical chemistry and its application to neuroscience, endocrinology, and biotechnology. His group developed instrumentation that couples sampling probes to capillary electrophoresis, capillary chromatography, mass spectrometry, and microfluidic assays for monitoring neurotransmitters in vivo. These methods have been used for studying changes in neurotransmitter concentrations associated with behavior and disease. Kennedy is Associate Editor of Analytical Chemistry and Director of the Microfluidics in Biomedical Sciences Training Program at Michigan.

Juan G. Santiago is Professor and Chair of the Thermosciences Group of Mechanical Engineering at Stanford University. His research includes the development of microsystems for on-chip chemical analysis, drug delivery, and sample preparation methods. Applications of this work include genetic analysis, drug discovery, and environmental monitoring. He is a Fellow of the American Physical Society, Associate Editor of the journal Lab on a Chip, and director of the Stanford Microfluidics Laboratory. Santiago has graduated 20 PhD students and advised nine postdoctoral researchers; 12 of these are now professors at major universities.

Albert van den Berg is professor and chair of the BIOS Lab-on-a-chip group at the University of Twente, Enschede, The Netherlands. His current research focuses on microanalysis systems and nanosensors, nanofluidics and single cells and tissues on chips. Applications in personalized health care, drug development and development of sustainable (nano)technologies are of particular interest to him. Van Den Berg has co-authored 250 papers, holds 10 patents, has participated in six spin-off companies, and is also Associate Editor of the journal Lab on a Chip. In 2011, he became a board member of the Royal Dutch Academy of Sciences (KNAW).

Which micro and nanoscale techniques are you particularly excited about and why?

I have been very intrigued by the use of nanoliter and smaller droplets as tiny reaction and assay vessels within microfluidic systems. This is important because although microfluidic systems allow small sample consumption, it has not been trivial to introduce small quantities or handle discrete samples easily. Droplet methods solve this problem.

My interest in the last four years has focused around an electrokinetic technique known as isotachophoresis (ITP). ITP is decades old but I believe that it holds great potential for new functionalities and new assays. My work has concentrated on applying ITP to the grand challenge of automated and efficient sample preparation, and to the challenging problem of accelerating reactions involving macromolecules like RNA or proteins.

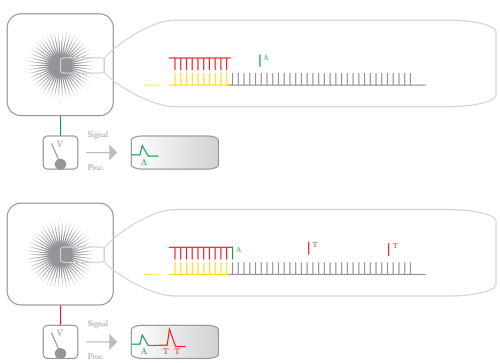
I am enthusiastic about the biomolecular preconcentration work using ion concentration polarization (ICP) originating from Jay Han's group (1). Although not fully understood, the method gives a tremendous preconcentration effect leading to much better detection limits. It is interesting because it is relatively simple to do, makes use of double layer and electrokinetic effects, has complex theory, and a variety of applications.

What has been the most significant advance in micro and nanoscale analytical techniques in recent memory?

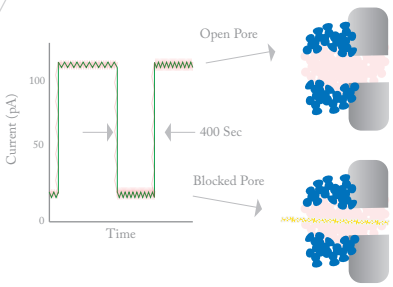
This is a tough one. I have to say that the initial work on microchip CE by Harrison and Manz (2) sparked a tremendous flood of research that has brought us to a high level of technical sophistication for microfluidic devices. Early work on measuring electrical effects of molecules through nanopores has also generated a lot of interest in using nanoscale devices for analysis. Finally, using nanoparticles as simple labels in assays, for example in work by Chad Mirkin (3), and early work on Quantum dots by Alvistatos and Nie (4) are both very important for nanoscale techniques.

I believe the greatest advances have been seen in microscale, not nanoscale, devices and techniques. Significant breakthroughs in the last decade include the advent, commercialization, and growth of droplet-based PCR systems (digital PCR) and on-chip electrophoresis devices for the size analysis of RNA and DNA, such as Agilent's Bioanalyzer system. Furthermore, microfluidics continues to plays an important supporting role in a wider range of applications, including deep sequencing and proteomics.

I will mention two. First, the new sequencing approaches are exciting. On the one hand there is the work now commercialized by Ion Torrent, where sequencing by synthesis is accomplished using a "good old" integrated pH sensor (ion-sensitive field-effect transistor (ISFET)) array. On the other hand, a lot of progress has been made with translocation and sequencing of DNA though nanopores. In both cases, there is a strong link with micro- and nanofabrication and nanoscale analytics. Second, there is a lot of activity using new materials, such as carbon nanotubes, graphene and nanoparticles, that improve surface area, create tiny nanopores or enhance optical detection techniques.



Ion Torrent



Nanopore DNA Sequencing

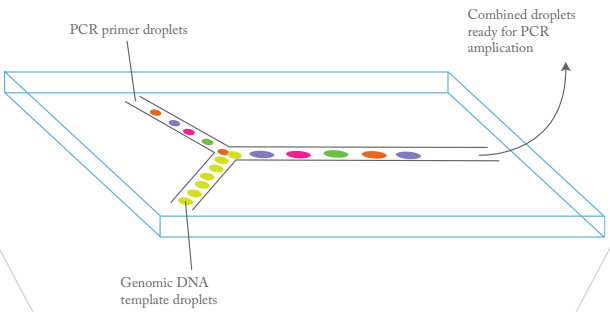
Which area is likely to see the highest uptake of micro and nanoscale technology and why?

There is very little infrastructure around single droplet or microfluidic systems. Everything you want to miniaturize or do on a small scale must be developed or mastered from another laboratory. This makes the whole research process slow-going at times. For example, we wanted to be able to remove aliquots from nanoliter samples, so we had to build an entire project around how best to do that. Probably the biggest challenge for the future is finding the "killer application". Nanoscale analysis can make some things better, but is it worth giving up the tried and tested?!

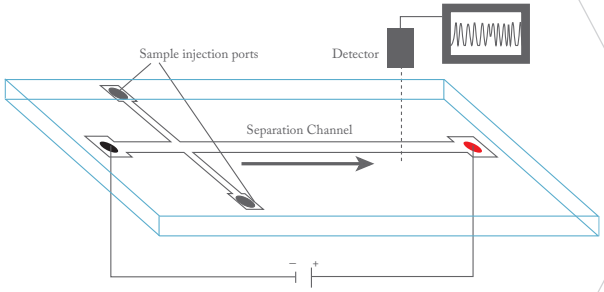
I believe that screening applications very well could be a major area. Also, clinical assays, for example, isolating cells for diagnosis and then analysis, will probably be another big area.

I believe the highest rate of uptake in the next five years will continue to be from researchers in the life sciences – as has been the case in the last decade. Clinical applications offer great potential and market volume, but clinical diagnostics typically require immense investments, significant development times, and risk. These are starting to arrive and poised to grow, but it will take money and time.

I would say that genomics – everything dealing with DNA sequencing – is profiting most from nanoscale technology. I am convinced that a $1000, or even a $100, sequencer will become available within the next 10 years. Besides that, I also strongly believe that distributed, point-of-care diagnostics will be realized by the use of nanoscale technologies. Finally, of course, (medical) imaging is one of the largest driving forces behind many nanoparticle research efforts.



Droplet-based PCR

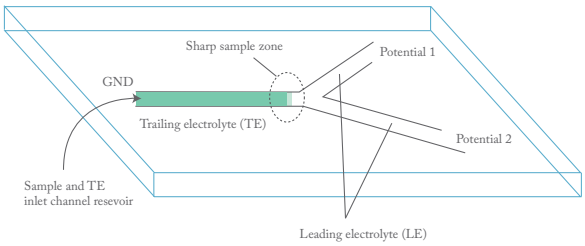


Microchip CE

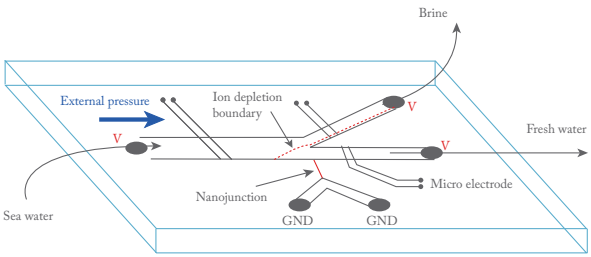
In your own research, could you please detail the enabling aspects of micro and nanoscale techniques?

Micron-scale microfluidic devices offer parallelization, integration, and control of multiple processes in a single device. In my work, we are combining electrokinetic sample preparation (from complex samples including blood, urine, and cell cultures) with assays based on enhanced reaction kinetics between target molecules and a variety of synthetic, fluorescently-labelled probes.

We use micro- and nanofabrication techniques with our MESA+ Nanolab. This allows, for instance, extreme control of microstructures to minimize peak broadening in classical separation systems. It also allows fabrication of ultraprecise arrays of nanopyramids and nanogaps leading to homogeneous, highly sensitive surfaces for surface-enhanced Raman scattering (SERS). Another aspect is economy of (small) scale: cheap microscale complementary metal–oxide–semiconductor (CMOS) transistors can be used for DNA sequencing. But nanostructuring also allows exact control of nanogaps between electrodes, enabling redox cycling sensing with extremely high sensitivity. Because of size similarity, nanochannels and nanopores are very interesting structures to study biomolecular interactions and sequence DNA.



Isotachophoresis (ITP)



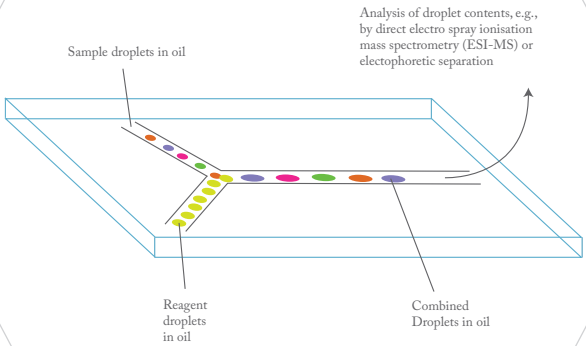
Ion concentration polarization (ICP)

How might some of the techniques you have used in your own research be applied in other areas?

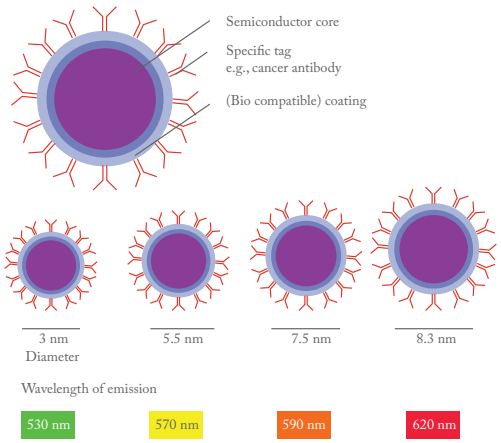
We and other groups have developed ways of doing high-throughput mass spectrometry on single droplets or large arrays of droplets. We have explored using this for screening, but it may also play a great role in enabling protein evolution and single cell analysis.

In essence, we have developed methods for rapid sample preparation, mixing, reaction, and separation. These are basic process steps for a wide variety of assays across genomics and proteomics. I envisage, for example, that ITP sample preparation could be a front-end for a wide range of techniques including digital PCR and sequencing.

We have recently started working with the microdroplet platform. This is a powerful tool because it creates picoliter confinements/environments that are useful for single cell analysis, single catalytic particle study, or single enzyme study. Furthermore, it is high-throughput; typically, 1000 droplets per second can be handled, as illustrated in pioneering work by Ismagilov (5), Weitz (6), Stone (7), Whitesides (8), Quake (9), Huck (10), etc. We believe microfabricated devices with integrated sensing will lead to a variety of disposable diagnostic devices for point-of-care applications.



Droplet assays



Quantum Dots

What are the major challenges in your micro- and nanoscale research?

One challenge is the increased importance of the surface chemistry of a device, and its effects on reproducibility and robustness of the assay. In many assays, we have tried to combat this issue by suppressing electroosmotic flow and non-specific binding to surfaces, while using electric fields and pressure-driven flows to control the motion of analytes in the bulk liquid.

Stable manufacturing is an important issue, as is making the connection between nanoscale and real life macrodimensions. And, as we become more prone to using top-down nanofabrication, maintaining the expensive infrastructure is a continuous and major concern. Apart from that, going to nanoscale also poses the question of how to bring analytes from, for example, millilitre samples to a nanometre-sized detector; sample preparation becomes the major challenge.

Where might nanoscale analytical techniques take us in the next 5 years? 10 years? 50 years?

5-10: I could see high throughput screening done at very low cost, based on utilization of nanoscale analysis, perhaps even bench-top screening done by individual investigators. In addition, routine single-cell isolation and analysis including proteomic and metabolomic analysis.
50: single cell dissection and surgery to remove select components for analysis.

5: improved chromatography; massively electrically readable DNA/protein arrays.
10: routine nanopore DNA sequencing.
50: continuous personal health monitoring for early diagnosis/preventive medicine.

Robert Kennedy:
kennedygroup.lsa.umich.edu

Juan G. Santiago:
microfluidics.stanford.edu/

Albert van den Berg:
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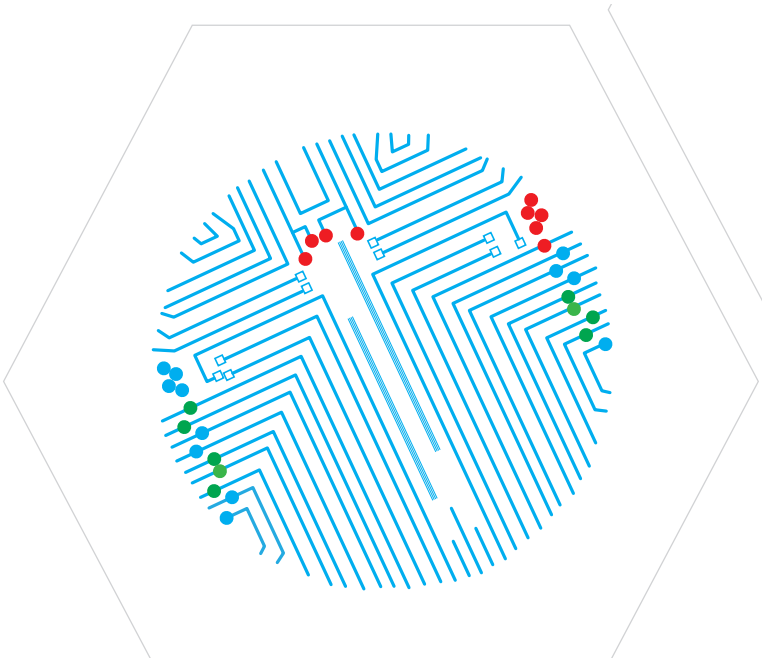

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Train for Success

You can still find formal training that provides quality, impact, and relevance in a world dominated by free content. Here's how.

By Stephanie Rizk and Bryan Tweedy

There's a famous maxim known to staff of the American Chemical Society: if you leave out books at the National Meeting, they will all walk away. Chemists are addicted to learning and knowledge – it's a lifelong obsession.

When was the last time you learned something new for your job? If you're working in science, your answer might be a moment ago or an hour ago. Learning is happening everywhere and all the time. In addition, scientists demonstrate a clear belief in the value of structured training, as evidenced by their attendance of technical sessions at conferences, short courses, and continuing education, in droves. These experiences allow scientists to step out of the day-to-day and focus on significantly advancing skills in a particular area, engaging in a deeper learning experience.

In recent years, however, significant conditions have emerged that threaten this basic principle of the structure of science.

The recession of 2008 changed the landscape of technical training for professionals in the US and Europe. According to Josh Bersin, Principal and Founder of Bersin by Deloitte, the economic recession has impacted training organizations on both sides of the Atlantic, with 11 percent cutbacks experienced by organizations in the US

in 2010. "While the cuts were steeper in the US [...] UK organizations are certainly feeling the pinch as well," he said (1).

Typically, training and travel budgets go to the chopping block early whenever there's a need for expense reduction. The actual price of the training is not the only issue: time away from the lab can decrease productivity in the short term and travel costs have greatly increased in recent years. Although these additional outlays have a negative impact on the return on investment of any training, good companies know that they must keep their staff fully trained to stay ahead of their competitors.

Many industries turned to online delivery of training because it removes the cost of travel and time away from the office. E-learning now represents $56.2 billion, out of an overall $200 billion spent on corporate training, according to Global Industry Analysts (2). But most online learning programs don't strongly support peer-to-peer networking, which is a highly valued component of in-person training and conference attendance. Another hurdle for online chemistry training was how to provide essential hands-on, interactive lab activities.

Meanwhile, there has been an explosion of free or nearly free content. Equipment manufacturers all have YouTube channels, many graduate school labs make instructional videos and there are loads of free webinars and courses on offer. Some organizations, including the American Chemical Society, record a portion of their conference presentations and provide them online after the conference. If you have a need for new knowledge, one approach is to spend all day on Google looking up articles, webinars, videos, and tutorials. But it is precisely this indiscriminate glut of available information that makes it so difficult to discover, evaluate, and utilize these sources as an effective replacement or complement to formal or informal training. At the ACS Department of Professional Education (ProEd) we recognized a clear need for flexible, high-impact training that was cost-efficient and that improved on the offerings available at the time.

A new approach to training

In 2010, one of us (SR) was reviewing evaluation data from a recent set of short courses. One comment that really got me thinking was this: "It would be great if we could send in our expectations beforehand and the course materials were designed with that in mind."

All of us have had that feeling – part of the class was very helpful, but you could have done without other segments. When a group of learners has varying backgrounds and levels of expertise, it is tricky for the instructor to provide material that all of them will find useful. Wouldn't it be great if the same set of learners were moving toward the same goal at the end of the class, but each was receiving only material that they was desired and needed? Truly an innovative idea, and one that our group has developed.

"That observation was one of the starting points for us," says John Miller, Director of the Department of Professional Education (ProEd) at the American Chemical Society. "And there were favorable conditions that we were able to take advantage of. Research showed that our customers were looking for learning that was on-demand and self-paced. But they wanted to be sure they were getting the best content from the best experts. Also, the technology had advanced enough that we could use a very affordable platform to support what we wanted to do."

ProEd has been running courses for chemists since the early 1960s, training many thousands through the years. Live online courses, which were basically online versions of the short courses, and on-demand courses, were introduced over the last decade, offering scientists the chance to get training at their own convenience. With a few years' experience of these programs under our belt, it was clear that there was still an unmet need. Scientists in our online courses were reviewing recorded portions of lectures after work, on their own time. But they weren't watching the lectures in order, or even in their entirety: they would watch a few minutes here, a few minutes there; they would skip entire portions. Clearly, the lectures as designed weren't meeting the needs of all types of scientists that were interested in that topic. There needed to be more flexibility and customization.

That's where new technologies have come in. While database systems that help organizations to manage training have been around for decades, until recently they were designed solely for universities and big companies that undertake large internal training programs. Organizations such as ours that offer training to a disparate group of customers were caught in an endless of cycle of IT development and customization to create systems easy enough to learn within and simple enough for a small staff to operate. Unfortunately, these systems couldn't change as fast as their customers did.

That changed ten years ago. The advancement of open-source learning management systems created a new breed of platforms with much higher flexibility and much lower administration costs. These platforms integrate with social media and use web services to share data with any number of external systems. For us, this was a pivotal development: it lowered the cost and technology barrier and allowed us to develop an entirely new online learning product, called Sci-Mind.

Sci-Mind, which was launched in 2012, integrates the best of traditional education, online learning and technology to create a dynamic online environment where individual lesson plans are tailored to each learner's professional needs and reinforced through their cohort experience.

Here's how it works:

- Before joining a Sci-Mind cohort, each learner completes a knowledge mapping exercise and works one-on-one with an ACS curriculum developer to receive a customized Learning Solution addressing their specific knowledge gaps in a given subject.
- Once the cohort begins, learners work at their own pace to complete their Learning Solution – all the while having direct access to a diverse pool of experts and peer-to-peer learning activities.
- Cohorts are kept small (no more than 15 students) and home in on a subject area in ways that make learning effective and engaging.
- Throughout the Sci-Mind experience, learners usually spend three to four hours per week focusing on assignments and online events, and have a six-week window to decide how and when they do their best learning.
- The cohort stays together for six weeks, and connects through group discussions, peer review of projects, and casual conversation. An alumni community allows them to maintain that connection to their peers and experts long after the session concludes.

The features of Sci-Mind include:

- Customization: the focus is on topics relevant to the learner
- Flexibility and Accessibility: wherever there is internet access, learners can access Sci-Mind 24-7 to participate or to complete Learning Solution activities
- Cost Effectiveness: time away from the office is minimized and no travel is required
- Innovativeness: the approach makes learning more dynamic and realistic, focusing on customized, individual learning with community reinforcement plus access to interdisciplinary experts
- Helpful progress reports (Learning Report and Manager's Report) to support job reviews and knowledge expansion, which enhances ROI and on-the-job time efficiencies
- Alumni/networking: part of growing one's career involves expanding one's network; Learners become part of ACS ProED alumni network that facilitates career-long professional networking and idea-sharing

"We think of Sci-Mind as an evolution of the kind of training we've done successfully for years," said Miller. "It takes the best features of those products and packages it in a way that works for today's industrial scientist."

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Stephanie Rizk and Brian Tweedy are at the Department of Professional Education, American Chemical Society, Washington.

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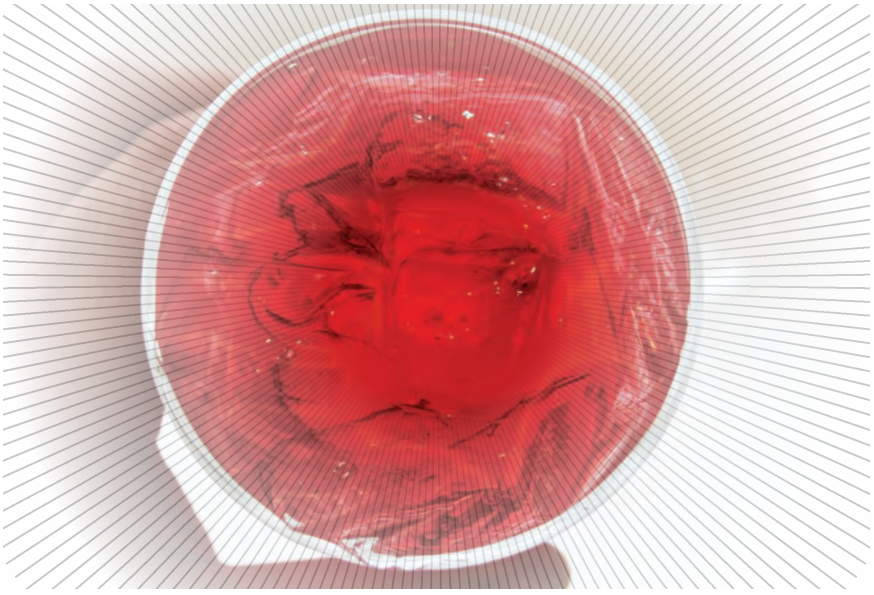
Juicing Up Food Fraud Detection

Direct sampling coupled with time-of-flight mass spectrometry points to a fraud-free food future for the consumer.

By Robert J. Packer and George Perkins



The Problem

How can we rapidly detect economically-motivated adulteration of food and deter fraudsters from committing future crimes?

Background

Food fraud has received increased attention in recent years, in part due to the highly publicized scandal in China in 2007 when melamine was added to pet food and infant formula (1), coupled with this year's discovery of rat meat disguised as lamb and horse meat in beef (see "How to Beat Cheat Meat", page 17). In the melamine case, opportunists exploited the long-standing system of payment for dairy products. Rather than payment by weight, the dairy industry has paid farmers and suppliers based on protein content for more than a century. Protein content is invariably measured by 19th century methods – Kjeldahl digestion or Dumas combustion – both of which measure nitrogen content and relate it to the amount of protein using a correction factor. The addition of high-nitrogen content compounds to dairy products generates a higher protein content reading and, hence, a higher price. Unfortunately, the added melamine crystallized in the kidneys of both pets and babies, affecting an estimated 300,000 infants, at least six of whom died.

Food fraud is not a new problem; adulteration of wines and spices was widespread in ancient Rome. Ahead of the melamine crisis, the impact of food fraud was mainly economic in nature and less newsworthy. The health impacts in China changed that perception and increased the focus of regulators and food processors. Now, industry leaders concern themselves over adulteration regardless of whether the adverse effects are to health or economics.

A major form of food fraud is counterfeiting, which takes a number of forms:

- Species substitution, for example, passing off mixtures of low-cost juices as high-cost juices.
- Incorrect organic claims, for example, labelling conventional milk as organic.
- Faking food provenance, for example, selling Indonesian vanilla as Madagascan.

The Solution

Traditionally, liquid chromatography

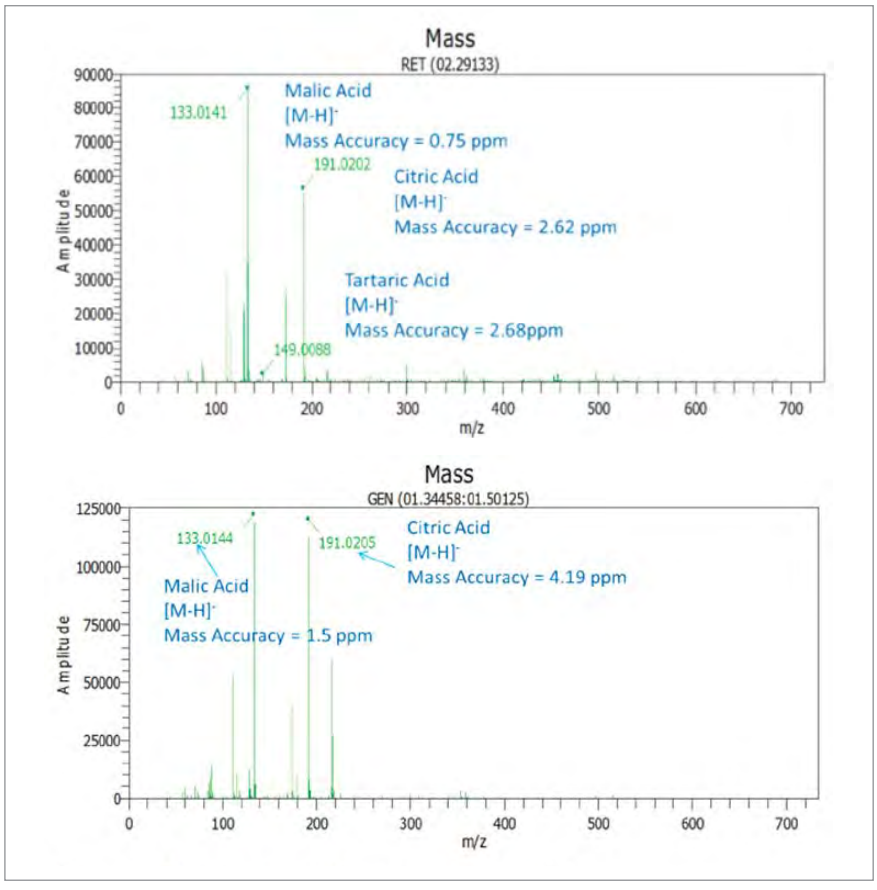


Fig 1. Spectrums of pure pomegranate juice (bottom) and pomegranate juice with 1% grape juice (top). Note the appearance of a tartaric acid peak (149.0088 m/z).

(LC) or gas chromatography (GC) have been combined with mass spectrometry (MS) to detect marker compounds as indicators for authentic or counterfeited products (2). An issue with these techniques is that they require a certain amount of sample preparation before analysis, such as homogenisation, extraction and clean up. This is then followed by lengthy separation time on a column before a result can be elucidated. The combination of direct sample analysis (DSA) and time of flight mass spectrometry (TOF MS) offers a rapid and simplified alternative. The technology allows for liquids or solids to be directly ionized into the mass spec without sample preparation or chromatographic separation. Powdered samples can be analyzed in a capillary tube with liquid samples pipetted onto a steel mesh and then analyzed.

Food-related samples from any source, natural or artificial, organic or conventional, contain a large number of components. The TOF MS allows for very accurate measurement of the mass of a given component in the mixture generated by the DSA. This makes it possible to identify, with a high degree of certainty, specific marker compounds that detect food fraud, without the need for prior separation. Combined with simple traffic light software that allow users with less familiarity with the technology to run it, this makes analysis of food fraud both more effective and more user friendly.

Case Study 1: Is this pomegranate juice pure?

Pomegranate juice has become popular in recent years, in part because of the perceived health benefits of its antioxidant compounds. Consumers increasingly prefer pure pomegranate juice over blends of pomegranate and other juices, although blended juices (with apple or grape) are more prevalent in supermarkets due to their lower cost (pomegranate juice is 5-10 times more expensive than apple or grape juice). A common fraud is to mislabel a blended product as pure pomegranate juice. Moreover, pomegranates are usually grown in countries with a tendency to produce high volumes of fraudulent foods, for example, Iran, Iraq and Georgia. Employing rapid and accurate detection techniques helps ensure that food processors and retailers can be confident in the authenticity of the products they buy and offer customers.

A common method of pomegranate juice adulteration is the addition of grape juice. Using the DSA TOF MS, the presence of even one percent grape juice in pomegranate juice can be detected by the presence of a small tartaric acid (2,3-dihydroxybutanedioic acid) peak (see Figure 1). Tartaric acid is absent in 100% pure pomegranate juice so the presence of tartaric acid indicates adulteration with grape juice.

Case Study 2: Is this milk organic?

The premium price of organic milk makes it a target for fraudsters. As such, regulatory bodies set strict rules on what constitutes organic milk. The US Department of Agriculture demands that organic milk must be:

1) Antibiotic free
2) Bovine Growth Hormone (BGH) free
3) Pesticide free (3)
4) Produced by cows that have had a diet of at least 30 percent pasture grass during a mandatory grazing season of no less than 120 days (4)

Rules 1-3 are often verified by either GC-MS or LC-MS/MS, but the fourth poses a more difficult challenge.

Recent publications reported the levels of minor acids in the milk. For example, one study used GC-MS to measure levels of phytanic acid in organic and conventional milk; as organically-raised cows eat more fresh green matter, they consume more phytol (a constituent of chlorophyll), which is broken down in the ruminant's stomachs to phytanic acid (5). Another study proposed using hippuric acid, which was also found in higher levels in grazing animals, in this case goats (6). The authors noted that ruminants discriminate on the vegetative status of the plants, preferring to eat shoots. Since vegetative activity increases the DNA level, they proposed, the fodder material in shoots increases the DNA content in an organic diet, producing a higher level of puric bases in rumen. Some of these bases are absorbed and metabolized to produce hippuric acid.

At PerkinElmer, we evaluated three organic and three conventional goat's milk samples, purchased from a local supermarket, using DSA TOF MS. All milk samples were subjected to the same preparation: protein precipitation followed by spiking with a known amount of deuterated hippuric acid standard (d5-hippuric acid) for quantitation. The overall results are shown in Table 1.

Table 1. Levels of hippuric acid in organic and conventional milk samples.

Type of Milk	*Hippuric Acid in Milk*
Organic Milk Brand 1	33.48 mg/L
Organic Milk Brand 2	36.73 mg/L
Generic Organic Milk 3	17.28 mg/L
Conventional Milk Brand 1	18.45 mg/L
Conventional Milk Brand 2	19.96 mg/L
Conventional Milk Brand 3	21.51 mg/L

For organic milk Brands One and Two samples, the levels of hippuric acid are high, averaging 35.11 mg/L; this is 1.75 times higher than that of conventional milk. The Generic Organic Milk sample shows levels of hippuric acid that are comparable to conventional milk, raising questions as to the organic standards applied in this case.

Although the results suggest a strong link between organic husbandry and hippuric acid in goat's milk, a wider study is required to provide conclusive proof. However, the results do suggest that pesticides, growth hormones, antibiotics and organic diet could all be assessed using a single instrument and technique to provide a definitive check for organic compliance.

Beyond the Solution

This work has shown that detection of marker compounds in combination with fast, minimal sample preparation techniques solves a number of food adulteration and authenticity problems. DSA TOF MS, with its ability to directly analyze solids, liquids, and powders, makes it an ideal technology to analyze multiple food types for known food fraud issues. Complementing this technology will be techniques to screen for new, undocumented adulterations – for the next melamine. These techniques use non-targeted screening, which, despite not having the sensitivity of TOF MS-based techniques, can alert a food processor if their incoming ingredients are outside the norm and should be stopped from entering the processing line.

It is conceivable that with non-targeted techniques, such as IR or UV supported by chemometrics at the goods-in bay and DSA TOF MS in the lab, novel adulterants can be stopped and then quickly identified without any danger of them reaching the final product and, ultimately, the consumer.

Robert J. Packer and George Perkins work in Global Food Solutions Execution at PerkinElmer, Shelton, Connecticut, USA.

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Fast Extraction of Metanephrine and Normetanephrine from Urine Using EVOLUTE® EXPRESS CX Prior to LC-MS-MS Analysis

Frank Kero and Victor Vandell, Biotage LLC, 10430 Harris Oaks Blvd., Charlotte, NC28269, USA

A new solid phase extraction technology has been developed to speed up sample preparation by eliminating steps in the extraction workflow. This application note describes the extraction of metanephrine and normetanephrine from synthetic urine using this 'load–wash–elute' approach with EVOLUTE EXPRESS CX 96–well SPE plates. Recoveries exceeding 80% and excellent linearity over the range 10–70 ng/mL are achieved.

Extraction conditions

Plate Configuration: EVOLUTE EXPRESS CX 30 mg plate p/n 601-0030-PX01
Sample Pre-treatment: Dilute synthetic urine sample with water (1:1, v/v).
Sample Loading: Load pre-treated sample (1 mL) at a flow rate of 1 mL / minute using positive pressure (PRESSURE + 96 Positive Pressure Manifold, p/n PPM-96). Note that plate conditioning and equilibration steps are not required.
Interference Wash 1: Water (1 mL)
Interference Wash 2: Methanol (1 mL)
Analyte Elution: Methanol/concentrated ammonium hydroxide (95:5, v/v, 1 mL)
Post Extraction: Evaporate extracts to dryness (SPE Dry Dual Sample Concentrator, p/n SD-9600-DHS) and reconstitute in 80/20 (v/v) mobile phase A/mobile phase B (500 µL).

HPLC Conditions

Instrument: Agilent 1200 Liquid Handling System (Agilent Technologies, Berkshire, UK)
Column: Organic Acids 150mmx 4.6mm (5 µm) (Restek)
Mobile phase A: 1% (v/v) formic acid in water
Mobile phase B: Acetonitrile

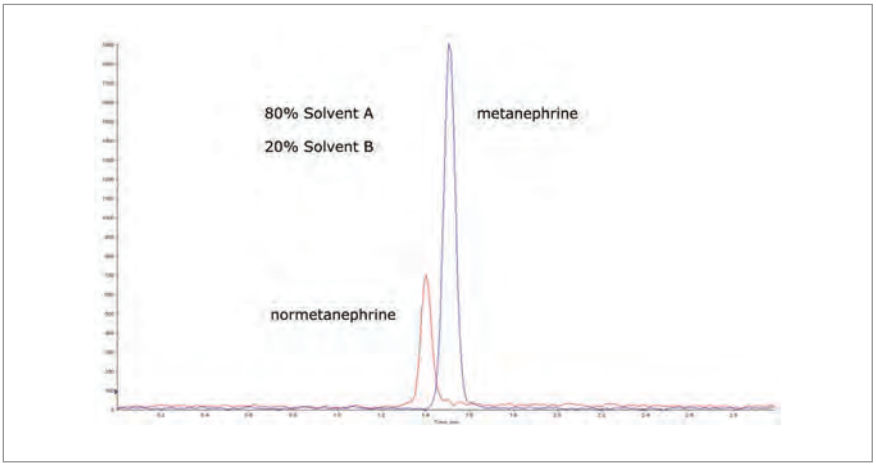


Fig 1: Extracted ion chromatogram for metanephrine and normetanephrine (20 ng/mL fortified urine sample)

Gradient				
Step	Time (mins)	Flow rate (mL/min)	%A	%B
1	0.0	1.0	80	20
2	3.0	1.0	80	20

Mass Spectrometry Conditions

Instrument: Applied Biosystems/ MDS Sciex 4000 Q-Trap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Turbo Ionspray interface.
Monitored transitions: metanephrine, 198>165; normetanephrine, 185>166

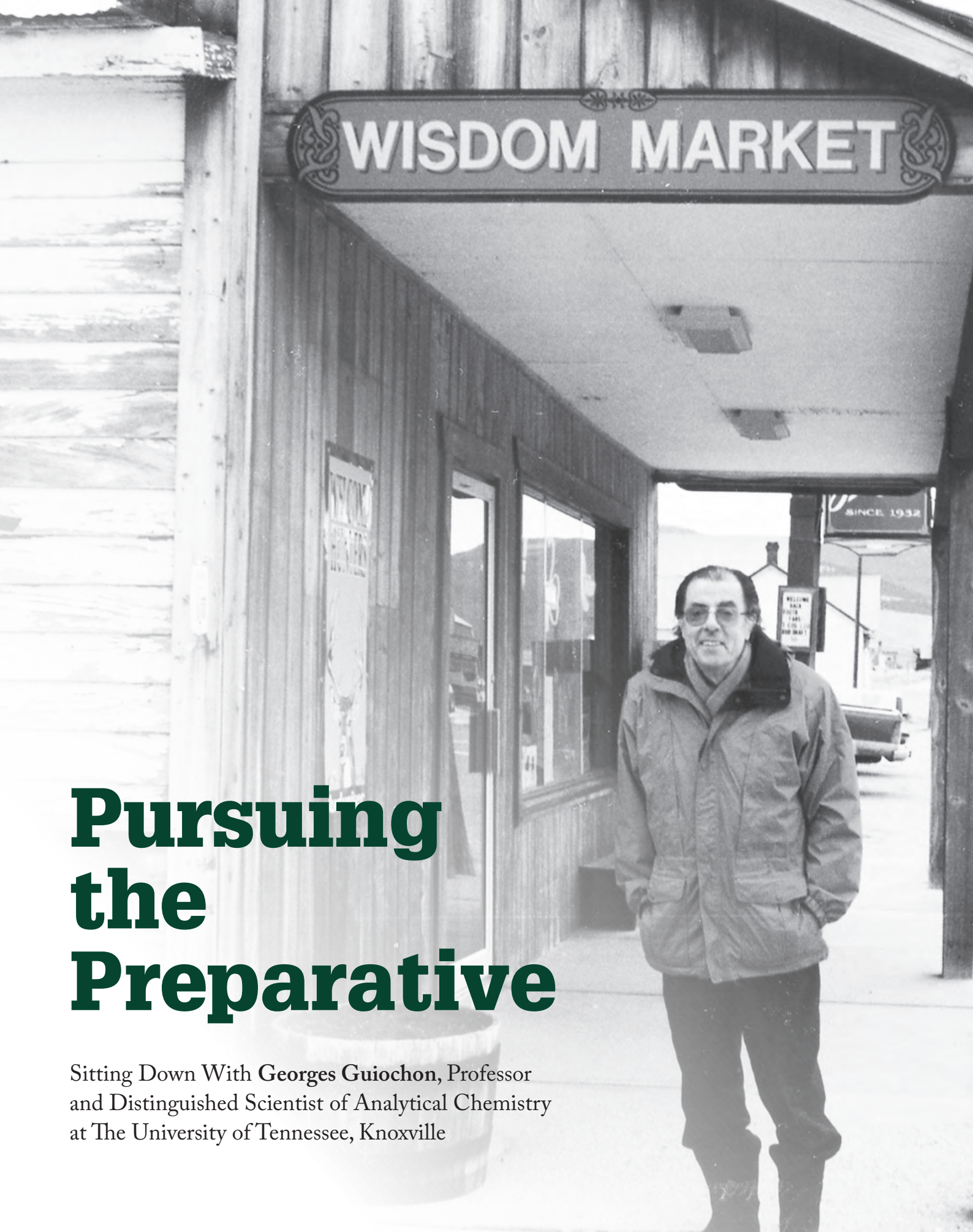
Results

Figure 1 shows the extracted ion chromatogram for metanephrine and normetanephrine from a 20 ng/mL fortified synthetic urine sample. The method demonstrated high (>80 %), reproducible recoveries of both analytes, with excellent linearity (r^2 >0.99) over the range 10-70 ng/mL.

Conclusions

The load-wash-elute procedure utilising EVOLUTE EXPRESS CX plates described in this application note proved fast, simple and effective for the extraction of the catecholamine metabolites, metanephrine and normetanephrine, from synthetic urine samples. Elimination of the traditional conditioning and equilibration steps speeds up the sample preparation process, without compromising on analytical quality.

Visit the literature database on biotage.com for more information.



Pursuing the Preparative

Sitting Down With **Georges Guiochon**, Professor and Distinguished Scientist of Analytical Chemistry at The University of Tennessee, Knoxville

How did you become an analytical chemist?

It's a strange story. In 1955, after I had graduated with an MS degree in engineering, a professor at the Ecole Polytechnique in Paris offered me a teaching assistant position. I was assigned to study the instability of ammonium nitrate, which had caused several disastrous explosions. About a year into the project, we were visited by a man who was received with respect by the professor. He told us that he thought the hoists on the boats unloading bags of ammonium nitrate were somehow catalyzing the transmutation of nitrogen into CO, and wanted us to study it. When he left I told my boss that it was a crazy idea. "You are right," he said, "but he is the son-in-law of the Prime Minister. You want a gas chromatograph to analyze the decomposition gasses of ammonium nitrate? Well, this is a way to get it!" He contacted the prime minister's office – this was in 1956, when there was very little money for scientific research – and within two weeks we had a beautiful PerkinElmer chromatograph. A year later we submitted a report showing that no CO was formed in the decomposition of ammonium nitrate. This drew an irrascible phone call from the government office informing us that the prime minister had changed and they had no interest in it. End of story. But it got me started in gas chromatography.

Where did you go from there?

My boss was close friends with a chemist-director of a big company that extracted perfume fragrances from flowers in the south of France. So we started to do gas chromatography with him and did a lot of interesting work.

In 1984 I decided to come to the US. There were too many smart people in LC and GC, so I decided to go into something that no-one else was doing seriously – preparative chromatography. Chemical engineers had no idea about the subtleties of the stationary phase, for instance, and analysts had no ideas about chemical engineering. I didn't have much idea either but I knew enough to marry them together.

Computers were starting to play a bigger role and I was able to solve numerically the mass balance equation for mass transfer in chromatography. I published a lot of papers and made my reputation with that.

Now I'm doing supercritical fluid chromatography.

What is the satisfaction in science for you?

Understanding phenomena, solving problems and training people.

Looking back on your long and prestigious career, what are your favorite moments?

Prestigious? I look at personal interactions, and helping people. A large part of the work on nonlinear chromatography was done with a guy from Iran who is now president of a US company. He first came to America in the late 1970s but went home after a year – it was at the time of the crisis at the US Embassy in Tehran. About a year later, a close friend of his was kidnapped and killed by the Iranian police and he returned. At the time I was the most recent professor appointed at Georgetown University so he was assigned to me and I quickly found that he was very smart with good mathematical training but completely unable to write in English. But his equations made sense and there was just about enough writing in between. I have brought people to work with me from Central Europe, Russia, Cuba, Iran, China, and that is what has given me the greatest satisfaction.

What trends do you see in chromatography today?

It seems that analytical chemistry in general and chromatography in particular are far less considered than they used to be in the academic world. A number of universities got rid of departments or divisions and there are now very few professors of analytical chemistry in the leading universities. That is bad because it is the science of most regulations – take EPA, take FDA, they have thousands of analytical chemists and spend huge amounts of money to control a third or more of the US economy.

What allowed the breakthrough in preparative chromatography?

You need to develop physical chemistry, thermodynamics and mass transfer chemistry to the point where you can predict the result of the separation and optimize the experimental conditions. And you can't do this without moving from linear to nonlinear, where the equilibrium between the stationary phase and the mobile phase depends on concentration. Generally people work in a concentration range where deviation from linear behavior is negligible, but beyond a certain point the velocity of the band depends on the concentration and instead of being a nice symmetrical band you obtain an asymmetrical band with a very sharp front and a long tailing peak. You cannot avoid this but you have to understand and be able to predict it. How far you can push it to still get pure product is the question.

What are the practical payoffs?

In the pharmaceutical industry, where a very pure product is needed, you can build columns which are up to five feet in diameter, containing a few million dollars' worth of packing material to produce significant amounts of pharmaceutical intermediates. The industry bought into this in the early nineties, and it has a become standard for compounds made by synthesis, particularly enantiomers, and for purifying peptides and proteins.

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