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Forensic examination of glass evidence
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Our first article ("The transparent witness: forensic examination of glass evidence at the Bundeskriminalamt" by Peter Weis) is a fascinating look into the use of laser ablation inductively coupled plasma mass spectrometry in forensic examination of glass fragments. These are often associated with crime scenes and easily "attach" to any people near them. Thus, they can be used to link criminals to their crime and to provide information on where a glass fragment might have originated.

Next, Ewa Sikorska and Igor Khmelinskii describe the "Glowing colours of foods: application of fluorescence and chemometrics in food studies". Whilst the major components of food are usually non-fluorescent, many minor food components that affect its nutritive, compositional and technological quality are fluorescent. Given its sensitivity, ease of use and non-destructive nature, this makes it useful in many applications around monitoring food processing and in fundamental food research.

Martin Byrdin and Dominique Bourgeois tell us about "The CAL(AI)^{2}DOSCOPE: a microspectrophotometer for accurate recording of correlated absorbance and fluorescence emission spectra". Using synchrotron radiation, the microspectrometer allows for the correlated investigation of absorption and fluorescence emission properties of nanovolumes of proteins under actinic illumination. The instrument should also have applications in wide range of applications in biology, chemistry, physics and technology in general.

More use of synchrotron radiation comes from Chris Kelley, Mark Frogley, Ann Fitzpatrick, Katia Wehbe, Paul Donaldson and Gianfelice Cinque in "Synchrotron infrared near-field spectroscopy in photothermal mode". They describe the capability of the near-field method to probe polymer microspheres within a protein matrix, and present the first infrared synchrotron radiation photothermal near-field Fourier transform infrared spectrum from within an individual biological cell. This establishes the feasibility of hyperspectral mapping at submicrometre resolution in a practical timescale.

Tony Davies is impressed with the NMR facilities at the University of Warwick, UK, and he and Steven Brown ask "What is the collective noun for solid-state nuclear magnetic resonance spectrometers?".

Peter Jenks makes a welcome return to the Quality Matters column telling us that "Change is in the air". Next year, a new version of ISO/IEC 17025 will be published, which is going to mean changes for all those involved in quality systems.

Finally, Kim Esbensen and Claas Wagner move "Into the laboratory… TOS still reigns supreme" and continue to stress that grab sampling is still an absolute no-no regardless of the size of the sampling device or the sample.

Glass fragments provide very useful forensic information. Their analysis by laser ablation inductively coupled plasma mass spectrometry and other techniques is described in the article starting on page 6.

Publisher
Ian Michael
E-mail: ian@impublications.com

Article Editor
John Chalmers
VSConsulting, Stokesley, UK.
E-mail: johnmchalmers@aol.com

Advertising Sales UK and Ireland
Ian Michael
IM Publications, 6 Charlton Mill, Charlton, Chichester, West Sussex PO18 0HY, United Kingdom. Tel: +44-1243-811334, Fax: +44-1243-811711,
E-mail: ian@impublications.com.

Americas
Joe Tomaszewski
John Wiley & Sons Inc.
Tel: +1-908-514-0776
E: jtomaszews@wiley.com

Europe and the Rest of World
Stephen Parkes and Kate Butler
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, UK,
Tel: +44-1243-770367 / -772018,
Fax: +44-1243-770432,
E-mail: stephen.parkes@wiley.com kbutler@wiley.com

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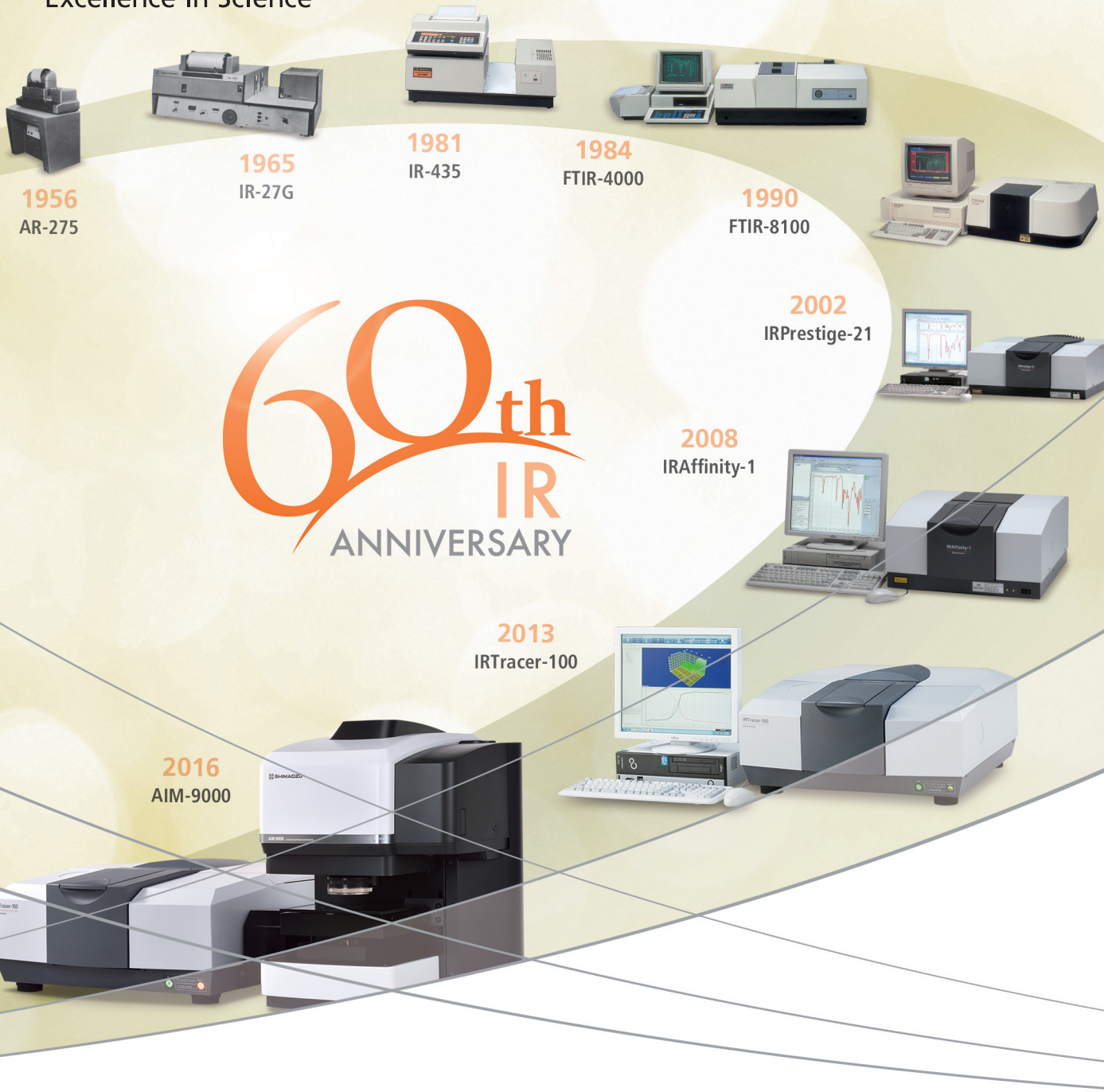

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ARTICLE

The transparent witness: forensic examination of glass evidence at the Bundeskriminalamt

Peter Weis
Bundeskriminalamt – KT 42, 65173 Wiesbaden, Germany. E-mail: peter.weis@bka.bund.de

Introduction: what makes glass important for forensic examinations?

Some people say that we live in the glass age, and there is a point to it, although it is equally true to say that we live in the plastic age, the silicon age or in the age of nano particles. However, our modern civilised world is loaded with glass objects—windows, bottles, doors, tableware, illuminants, decoration objects, touch screens and so on. Glass tends to get broken as a result of criminal activity—a perpetrator may break the glass of a window or a door in order to gain access to the place that will later be called a scene of crime, or a glass object, e.g. a broken bottle, may have been used as a weapon.

A combination of properties subsequently allows forensic examiners to use glass traces as evidence to establish a connection, e.g. between a scene of crime and a suspect. First, glass produces a considerable number of large to minute fragments when shattered. Second, small glass fragments tend to adhere to garments like the clothing of anyone standing close to the glass object while it breaks. Third, glass is a homogeneous material, which means that even microscopic fragments resemble, to a first approximation, the same chemical composition and intensive physical properties as the original glass object.

Forensic examination of glass traces

These three aspects would not be of much use if it was not for some sophisticated analytical methods capable of interrogating these miniscule pieces of evidence. The method that is most commonly used in forensic glass examinations is the determination of refractive index (RI). At first glance, this seems a bit incongruous, because more than 95% of the glass samples in case work are either container glass or float glass, both of which are soda-lime-glass, and have an RI between 1.510 and 1.530.

Therefore, a sophisticated method to determine the RI of glass samples with diameters of the order of some 0.1 mm or even smaller with very good precision and accuracy was developed, which is described in ASTM E1967 – 11a (*Standard Test Method for the Automated Determination of Refractive Index of Glass Samples Using the Oil Immersion Method and a Phase Contrast Microscope*).

A glass fragment is placed onto a microscope glass slide and immersed in silicone oil. The temperature of the oil and the glass fragment are automatically altered using a heating stage, which itself is mounted in a phase contrast microscope, illuminated with monochromatic light at 589 nm. The RI of the immersion oil changes with temperature to a much larger extent than the RI of the glass. At the "match temperature" the RIs of glass and oil are equal, and at this point, the glass fragment is nearly invisible in the phase contrast microscope. This temperature can be detected with high precision and translated into RI values using a calibration curve.

Measurement precisions in the order of $1–3 \times 10^{-5}$ can be achieved, while the RI variations within a window pane are normally in the order of $1–2 \times 10^{-4}$. Therefore, this method can be used to discriminate between different soda-lime-glass sources. Unfortunately, a probability of several percent remains that two randomly chosen soda-lime glass samples from different sources cannot be discriminated based on their refractive indices.

This is one reason why forensic glass examiners developed additional methods to further increase the evidential value of glass comparison analyses. One possibility is to measure the refractive indices not only at 589 nm, but also at one or two other wavelengths (e.g. 486 nm and/or 656 nm).

Another possibility is to reanneal the glass samples by heating them to ~550–600°C and allow them to cool down slowly using an oven with a controlled temperature program, thereby removing thermal stress from the glass samples, and measuring the RI again after this process. The difference between the refractive indices before and after the re-annealing process (Delta RI) provides information about the thermal history of

the glass, i.e. large Delta RI values indicate that the glass was thermally toughened (security glass), while smaller Delta RI values indicate that the glass originates, e.g., from a glass bottle or a non-toughened window.

Further methods focusing on the physical properties of the bulk material are density measurements and colour determinations. If recovered particles exhibit original surfaces, additional methods for examining the surface structure and curvature, and functional layers may be applied. In addition to these methods, the chemical composition of the glass samples can be utilised to compare glass fragments. The most frequently used techniques in this context are scanning electron microscopy/energy dispersive X-ray spectrometry (SEM/EDX), micro X-ray fluorescence (μXRF) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS).

Advantages of LA-ICP-MS

While μXRF and especially SEM/EDX are limited to the analysis of major, minor and only few trace elements, LA-ICP-MS provides enough sensitivity and linear range to enable the determination of trace and ultra-trace elements (limits of quantification are in the ng g^{-1} range for many elements) while determining main and minor element concentrations during the same measurement. This is important when comparing float glass samples, because the manufacturers control the main and minor elements very strictly, while the trace and ultra-trace elements are not added to the glass on purpose, but as contaminations in the raw materials.

Therefore, the discriminative power of chemical analysis using LA-ICP-MS is much better than using SEM/EDX or μXRF. Furthermore, irregularly shaped and contaminated surfaces do not compromise the measurements.

LA-ICP-MS glass analysis at the Bundeskriminalamt—a short history

At the Bundeskriminalamt (BKA), the potential of LA-ICP-MS for forensic glass comparisons was recognised at an early stage.[1] A quadrupole ICP-MS instrument was installed in 1993, and the first ultraviolet-LA Instrument (266 nm) in 1998. This combination of instruments was later replaced by more robust, reliable and sensitive successors (193 nm and 213 nm laser ablation microprobes, quadrupole ICP-MS spectrometers).

In addition to internal research activities, the Bundeskriminalamt was and is engaged in international networks promoting the application of LA-ICP-MS in forensic glass casework.

Within the EU-funded NITECRIME network (2001–2004; natural isotopes and trace elements in criminalistics and environmental forensics), a standard method for the quantitative determination of elements in float glass samples by LA-ICP-MS was developed together with research institutes and law enforcement agencies in Europe, Australia and North America. Within several collaborative exercises, it was proven that accurate, precise and robust elemental data could be obtained from different laboratories around the world using widely differing LA-ICP-MS instrumentation. Two dedicated, matrix-matched glass standards (FGS 1 and FGS 2) were developed in cooperation between the Bundeskriminalamt and Schott AG in order to improve the accuracy and precision of forensic glass analyses.[2]

Between 2008 and 2011, the project "elemental analysis of forensic evidence: workshop and working group" (EAWG), funded by the United States National Institute of Justice (NIJ) focused on the cross-validation and evaluation of the performance of μXRF, ICP-MS and LA-ICP-MS for the elemental analysis of glass[3] and on the evaluation of the performance of different exclusion criteria.[4] The work of this group also led to the publication of ASTM E2926–13 [*Standard Test Method for Forensic Comparison of Glass Using Micro X-ray Fluorescence (μ-XRF) Spectrometry*] and ASTM E2927–13 (*Standard Test Method for Determination of Trace Elements in Soda-Lime Glass Samples Using Laser Ablation Inductively Coupled Plasma Mass Spectrometry for Forensic Comparisons*).

A successor group started to work on the follow-up project "Strengthening the evaluation and interpretation of glass evidence using statistical analysis of collection sets and databases of refractive indices and elemental data (μXRF, ICP-MS and LA-ICP-MS)" in 2016.

False positive and false negative results, exclusion criteria and evidential value

After the application to a few cases between 2001 and 2004, the LA-ICP-MS method was included in routine glass casework at the BKA in 2005. In this context it was important to implement a robust exclusion criterion. To this end, two sample sets were analysed— a set of 62 float glass samples from different sources and 34 fragments (44 data sets) from the same glass pane. Possible exclusion criteria were evaluated to minimise false positive results for samples from different sources and to minimise false negative results for data from the same source.[5] A modified four sigma criterion was chosen for casework, which resulted in two false positive results in 1891 pairwise comparisons (error rate 0.11%) and 10 false negative results in 946 pairwise comparisons (error rate 1.06%).

Since 2005, the results from LA-ICP-MS analyses of control samples and non-matching recovered samples were collected, totalling 398 samples. We recognised 26 random matches in 79,003 pairwise comparisons, corresponding to an error rate of 0.033%. The difference to the test data set can be easily explained by the fact that this data collection from casework consists of various kinds of glass (float glass, container glass, non-float flat glass, specialty and household glass etc.) while the test data set is much more homogeneous and consists only of float glass samples.

Casework example 1: comparison analysis

The majority of glass cases are comparisons between samples from a known source related to the crime and samples that are related to a suspect.

A security van was stopped by a car. Two masked and armed men broke the windows of the transporter, put the driver in chains, stole the money from the back of the van and drove away with the car. The driver of the van remembered the brand, model and colour of the vehicle and suspected that it was a rental car.

The police then asked car rentals close to the incident whether they owned such a car, and found a suspect vehicle. When inspecting the car, a police officer found glass fragments in the profile of a tyre, and sent these to the laboratory together with fragments from the van's broken window. The examinations revealed that both glass sources could not be distinguished by RI measurements or by elemental analysis using LA-ICP-MS. Therefore, a possible connection between the scene of crime and the car could be established, and the person that rented this car on the respective day was confronted with these results.

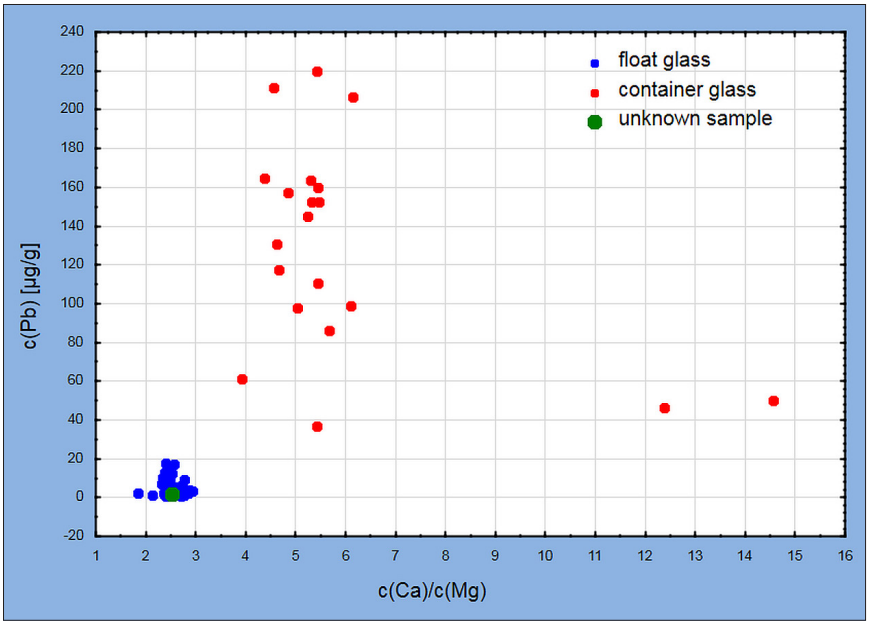

Figure 2. Pb, Ca and Mg concentrations of float glass, container glass and the unknown sample.

Casework example 2: classification of a glass sample

Questions concerning the classification of glass samples are less common, but are often interesting cases which involve some seldom used examination methods.

During the autopsy of a victim that had been murdered by a gun shot, a glass fragment was found within the dead body. The fragment was sent to our lab and we were asked from which object the fragment could have originated (Figure 1).

Refractive index measurements before and after re-annealing revealed a large Delta RI, indicating that the exhibit was thermally toughened glass, probably a security glass. Quantitative elemental analysis utilising LA-ICP-MS showed that the fragment has a composition that is in good agreement with other float glass samples. Figure 2 depicts the results of the analysis of the sample in question, of 20 container glass samples and of 62 float glass samples (lead concentration vs the quotient calcium concentration/magnesium concentration[6]).

The glass fragment was coated with dark enamel on one side. Examinations with SEM/EDX revealed that the coating contains, among others, the elements lead, chromium, cobalt and copper. Similar compositions are known to be used for car windows, nearly exclusively for non-retractable windows (front and rear windows, triangular windows at the sides of the car etc.).

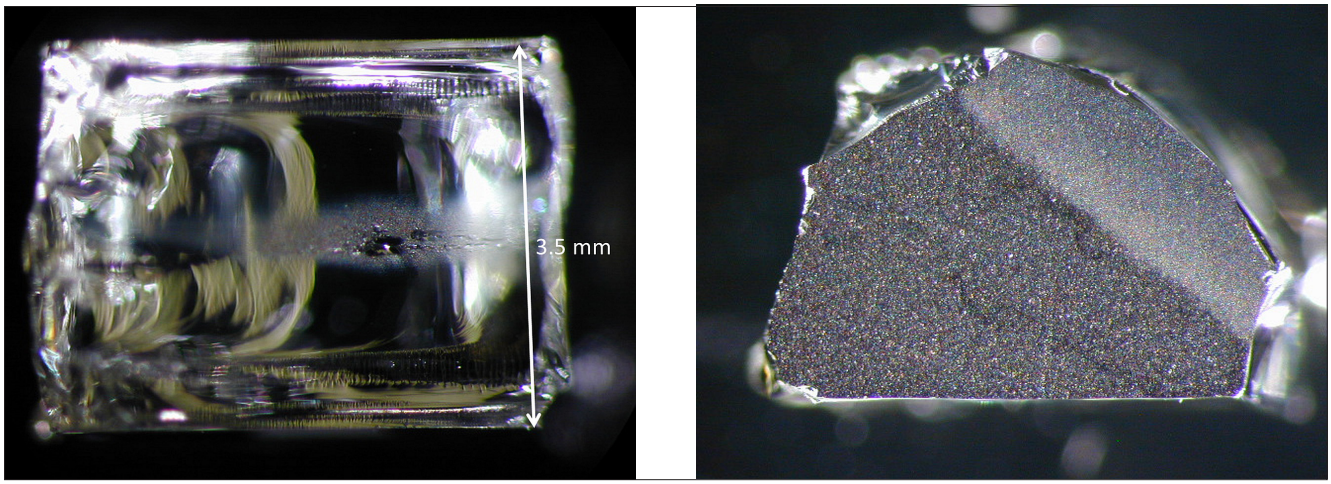

Figure 1. Glass fragment, side view (left) and coating (right).

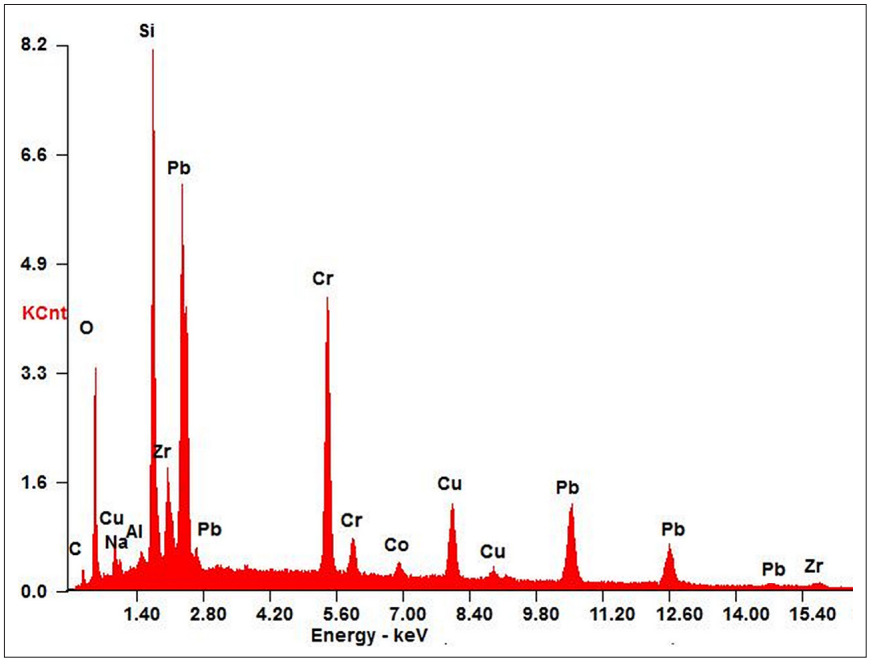

Figure 3. SEM/EDX spectrum of the enamel coating.

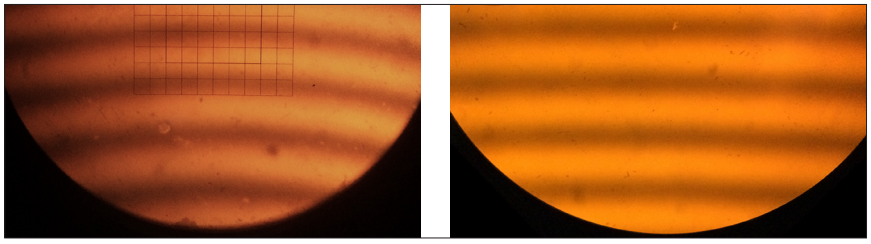

Figure 4. Interference lines of a slightly curved car side window (left) and a flat glass (right).

With the glass fragment including the original surfaces, it was possible to reconstruct the curvature of the original glass object by interference microscopy. A beam of monochromic light is divided by a semi-transparent mirror. One beam is reflected at the original surface of the glass fragment and the other at a very flat reference surface. The two beams are then recombined, resulting in an interference pattern that reveals the form of the original surface—flat glass results in straight, parallel interference lines, a light curvature results in slightly curved interference lines, and strongly bent glass surfaces (e.g., bottle fragments) result in a strong curvature of the interference lines.

The interference pattern received from the glass fragment in question showed light curvature, as would, e.g., be expected for a window of a car.

The results of the examinations indicate that the glass fragment from the body of the victim probably originated from the rear window or from a stationary (non-retractable) side window of a car. Since the fragment was found inside the body of the victim, the deadly shot was probably fired through such a car window.

Current and future developments

The application of LA-ICP-MS analysis is at present limited by the minimal required size dimensions of at least 0.4 mm in diameter and 0.1 mm in thickness, although many recovered fragments in casework are smaller and can therefore only be examined by less discriminating methods. Improved instrumentation like faster (e.g., time of flight, TOF) or more sensitive (e.g., sector field) ICP-MS instruments, laser ablation instruments with pulse durations in the femtosecond instead of the nanosecond range, and adopted analytical methods will enable the examination of smaller samples.

The interpretation of the results especially from elemental analysis data may be improved, i.e. by increasing the database on which these interpretations are founded and by employing more sophisticated statistical models.

References

1. W. Stöcklein, E. Kubassek, R. Fischer and A. Chadzelek, "The forensic analysis of float-glass characterization of glasses from international sources", *ENFSI EPG Newsletter* **3,** 8 (2009). doi: http://dx.doi.org/10.13140/RG.2.1.3294.3763
2. C. Latkoczy, S. Becker, M. Dücking, D. Günther, J. Hoogewerff, J. Almirall, J. Buscaglia, A. Dobney, R. Koons, S. Montero, G. van der Peijl, W. Stoecklein, T. Trejos, J. Watling and V. Zdanowicz, "Development and evaluation of a standard method for the quantitative determination of elements in float glass samples by LA-ICP-MS", *J. Forensic Sci.* **50(6),** 1327 (2005). doi: http://dx.doi.org/10.1520/JFS2005091
3. T. Trejos, R. Koons, S. Becker, T. Berman, J. Buscaglia, M. Duecking, T. Eckert-Lumsdon, T. Ernst, C. Hanlon, A. Heydon, K. Mooney, R. Nelson, K. Olsson, C. Palenik, E.C. Pollock, D. Rudell, S. Ryland, A. Tarifa, M. Valadez, P. Weis and J. Almirall, "Cross-validation and evaluation of the performance of methods for the elemental analysis of forensic glass by μ-XRF, ICP-MS, and LA-ICP-MS", *Anal. Bioanal. Chem.* **405,** 5393 (2013). doi: http://dx.doi.org/10.1007/s00216-013-6978-y
4. T. Trejos, R. Koons, P. Weis, S. Becker, T. Berman, C. Dalpe, M. Duecking, J. Buscaglia, T. Eckert-Lumsdon, T. Ernst, C. Hanlon, A. Heydon, K. Mooney, R. Nelson, K. Olsson, E. Schenk, C. Palenik, E.C. Pollock, D. Rudell, S. Ryland, A. Tarifa, M. Valadez, A. van Es, V. Zdanowiczq and J. Almirall, "Forensic analysis of glass by μ-XRF, SN-ICP-MS, LA-ICP-MS and LA-ICP-OES: evaluation of the performance of different criteria for comparing elemental composition", *J. Anal. At. Spectrom.* **28,** 1270 (2013). doi: http://dx.doi.org/10.1039/c3ja50128k
5. P. Weis, M. Dücking, P. Watzke, S. Menges and S. Becker, "Establishing a match criterion in forensic comparison analysis of float glass using laser ablation inductively coupled plasma mass spectrometry", *J. Anal. At. Spectrom.* **26,** 1273 (2011). doi: http://dx.doi.org/10.1039/c0ja00168f
6. P. Weis, M. Dücking, P. Watzke and S. Becker, "Discrimination of float and container glasses by elemental analysis using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) ", *ENFSI EPG Newsletter* **1,** 18 (2007). doi: http://dx.doi.org/10.13140/RG.2.1.2376.8722

ARTICLE

Glowing colours of foods: application of fluorescence and chemometrics in food studies

Ewa Sikorska[a]* and Igor Khmelinskii[b]
[a]Faculty of Commodity Science, Poznań University of Economics and Business, al. Niepodległości 10, 61-875 Poznań Poland. E-mail: ewa.sikorska@ue.poznan.pl
[b]Universidade do Algarve, FCT, DQF and CIQA, Campus de Gambelas, Faro 8005-139, Portugal

Introduction

Fluorescence spectroscopy is a well-established research and analytical technique used extensively in many disciplines. Fluorescence is a type of photoluminescence; a process in which a molecule, raised to an electronically excited state by absorption of ultraviolet (UV) or visible radiation, decays back to its ground state by emission of a photon with energy different from that absorbed. It involves transition between the excited and ground electronic states of the same multiplicity, usually $S_1 \rightarrow S_0$, occurring at relatively high rates, typically $10^8 s^{-1}$.

The main advantages of fluorescence over absorption spectroscopic techniques are its higher sensitivity and selectivity. Light emitted by fluorescence is detected against a low background, making fluorescence spectroscopy a much more sensitive technique than the absorption techniques, and one which is capable of even determining concentrations down to parts per billion levels.

A unique feature of fluorescence, distinguishing it from other spectroscopic techniques, is its inherently multidimensional character. Fluorescent properties of every compound are characterised by two basic types of spectra: excitation and emission, while not all of the absorbing molecules are fluorescent. These features contribute to higher selectivity of fluorescence as opposed to absorption spectra.

In recent decades, a remarkable growth in the use of fluorescence in food studies has been observed. Characteristic autofluorescence landscapes of products coupled with chemometric methods are used as fingerprints in non-targeted food analysis. In this article the applications of fluorescence spectroscopy to qualitative and quantitative analysis of foods are briefly reviewed.

Early studies of food fluorescence

The term "fluorescence" was introduced by Sir George Gabriel Stokes, physicist and professor of mathematics at Cambridge University, who in his paper entitled "On the Refrangibility of Light" described in 1852 the phenomena he called dispersive reflection, concluding that the wavelengths of the dispersed light are always longer than the wavelength of the original light. One of Stokes' famous experiments involved the study of emission from quinine. A quinine solution when illuminated with the visible part of sunlight dispersed by a prism remained transparent. However, when the quinine solution was placed beyond the blue part of the sunlight spectrum (corresponding to the UV region), the solution glowed with blue light.[1]

Quinine is an alkaloid, found naturally in the bark of the cinchona tree, widely known at that time as a treatment for malaria. Interestingly, a few years after Stokes' discovery, Mr Erasmus Bond used quinine as an ingredient of Quinine Tonic Water, which he produced and patented in 1858. In this way quinine turned out to be one of the first food components in which fluorescence was studied scientifically. Nowadays, fluorometric determination of quinine in tonic water is a widely used laboratory experiment, teaching undergraduate students the basics of fluorimetry, see Figure 1.

Since the Hg lamp with the Wood's filter became commercially available in 1925, visual observation of food fluorescence induced by UV light has been utilised in assessing food quality. The use of Wood's lamp was accepted as the US official method for detection of olive oil

Figure 1. Tonic water illuminated with visible light (left) and a UV lamp (right). The glowing blue colour is due to the fluorescence of quinine.

adulteration. This method used characteristic chlorophyll fluorescence present in extra virgin olive oil, detecting adulteration with refined oils even then at the level of 5%.[2]

The monograph *Fluorescence Analysis in Ultra-violet Light* published in 1959 reviewed the application of fluorescence for the analysis of a variety of food products, based on more than 400 references published since 1911.[3]

The improvements in both spectroscopic instruments and computers contributed to the development of applications of fluorescence spectroscopy to food analysis over the following decades. Intensive progress in this area started from the 1980s, when the first applications of multivariate data analysis methods for the analyses were reported.[4]

Recording fluorescence fingerprints of foods

Fluorescence may be measured directly on the samples, using right-angle geometries for diluted solutions with low optical densities, or using front-face illumination for opaque samples.[5]

Food samples are complex systems; therefore, conventional single emission or excitation spectra are often insufficient to fully characterise their fluorescent patterns. In such cases, multidimensional measurement techniques are used, see Figure 2.

In a system containing several fluorophores, the emission spectra depend on the particular excitation wavelength used for the measurements. Thus, to obtain comprehensive fluorescence characteristics, a set of emission spectra over the range of excitation wavelengths is recorded. The resulting excitation–emission matrix, known also as the total fluorescence spectrum or the fluorescence landscape, provides comprehensive information of the fluorophores in the system, being a unique fingerprint of the sample studied.

Alternatively, food may be investigated by synchronous scanning fluorescence measurements, involving simultaneous scanning of both excitation and emission wavelengths, with a constant difference ($\Delta\lambda$) between them. This technique is very useful for the analysis of multi-

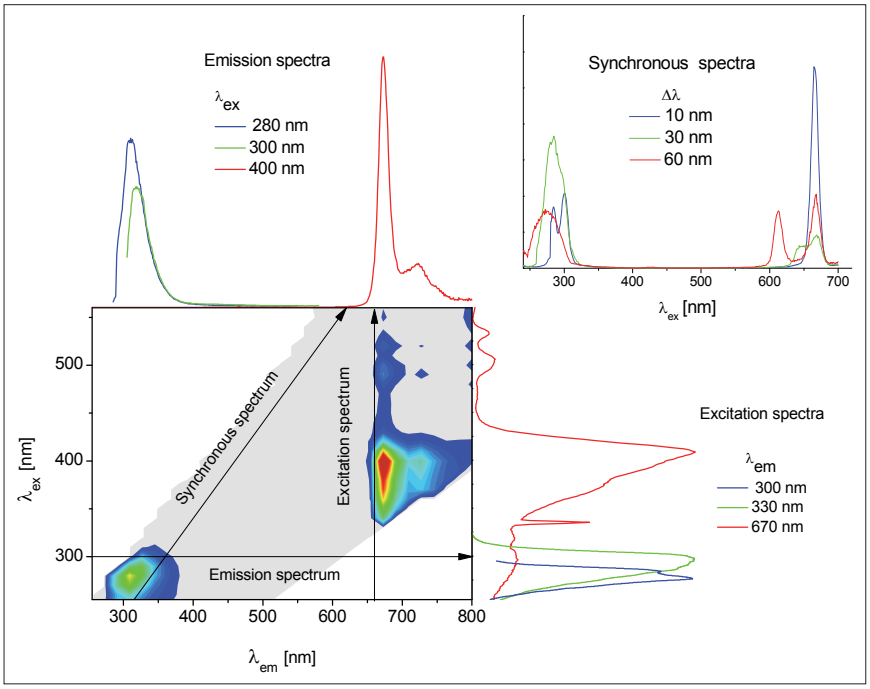

Figure 2. The different fluorescence spectra used as food fingerprints. Spectra of extra virgin olive oils are shown as an example from Reference 2 (Copyright E. Sikorska).

fluorophoric systems, because both excitation and emission characteristics are included into a single synchronous fluorescence spectrum. The main advantages of synchronous fluorescence spectra as compared to total fluorescence spectra are their improved selectivity and sensitivity, significant reduction in the spectral complexity and suppressed light-scattering interferences.[6]

The main characteristics of the synchronous fluorescence spectrum are determined by the $\Delta\lambda$ value. A set of synchronous spectra recorded at different wavelength intervals may be concatenated into a total synchronous fluorescence spectrum. In such, spectra fluorescence intensity is usually plotted as a function of the excitation wavelength and the wavelength interval. The relations between various kinds of fluorescence spectra of a virgin olive oil are presented in Figure 2.

Multivariate data analyses of fluorescence fingerprints

Multivariate data analysis methods are used to extract quantitative and qualitative information from the spectra. Analysis of a set of conventional emission, excitation or single-offset synchronous spectra arranged into a matrix usually starts with data exploration. This analysis does not require any prior knowledge of the explored data, being aimed at finding any patterns and trends in the sample set, and detecting outlying samples. The most commonly used method for data exploration is principal component analysis (PCA).

A calibration consists of building a relationship between the desired property of a sample and its fluorescence spectrum. The advantage of such an approach is the replacement of wet chemical measurements, which are usually slow and expensive, by non-destructive and fast spectral measurements.

The analytical problem for food quality assessment often involves assignment of a particular product to a specific category or discrimination between products from different categories. To perform such an analysis, supervised pattern recognition classification or discrimination methods are used, including the linear discriminate analysis, *k*-nearest neighbours, discriminate partial least squares regression, soft

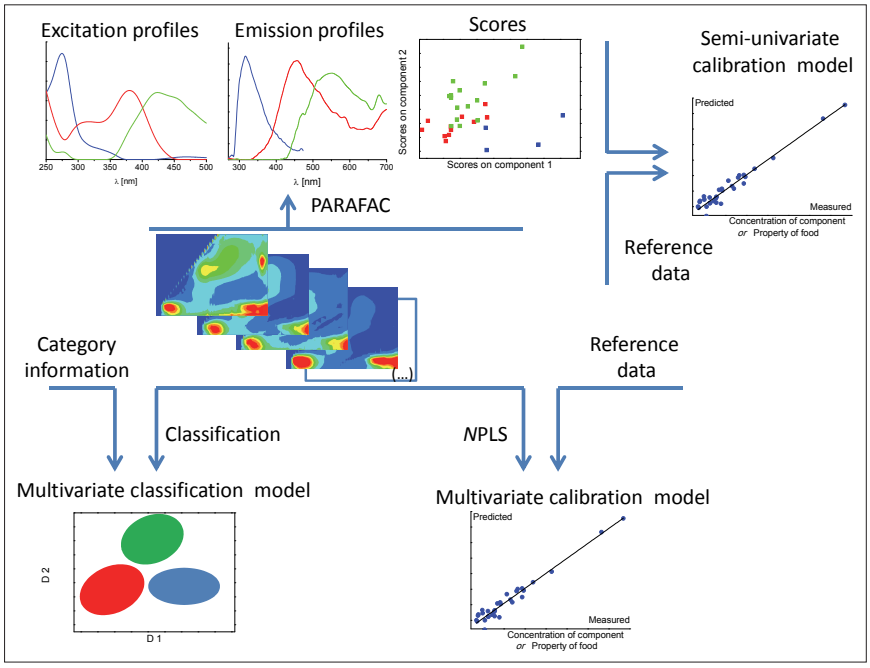

Figure 3. Schematic of the analysis of the excitation–emission fluorescence matrices by multi-way methods.

independent modelling of class analogy and artificial neural networks.

The multivariate regression methods most frequently used in fluorescence analysis are partial least-squares regression (PLS) and principal component regression (PCR).

Multi-way methods are used for the analysis of sets of excitation–emission fluorescence matrices, see schematic in Figure 3.

One of these, parallel factor analysis (PARAFAC) is used to decompose the fluorescence data into a number of components corresponding to the distinct fluorophores present in the samples.[4] This analysis provides estimates of the relative concentrations of each of the fluorophores and their respective excitation and emission profiles, facilitating identification of the fluorescent constituents. This approach, called mathematical chromatography, enables qualitative and quantitative analysis of the individual mixture components. *N*-way partial least-squares method (*N*-PLS) is used for the regression analysis of excitation–emission matrices.

Alternatively, excitation–emission matrices may be unfolded by arranging the data into two-dimensional matrices, and analysed using common multivariate methods.

Applications of fluorescence in food analysis

Fluorescence is usually observed in organic aromatic compounds with conjugated double bonds and rigid molecular skeletons. Note that the major food components are usually non-fluorescent. In contrast, many minor food components that affect its nutritive, compositional and technological quality are fluorescent. Food-relevant fluorescent compounds include aromatic amino acids, vitamins and cofactors, nucleic acids, porphyrins, polyphenols and alkaloids.

Note too that some products of food oxidation or deterioration also exhibit fluorescence. Fluorophores in foods also include process-derived compounds. Additionally, some food contaminants such as myco- and aflatoxins equally exhibit fluorescence. Therefore, fluorescence may be used to analyse all of these aspects of food quality.

The range of quality aspects of the food products studied by fluorescence-chemometrics is quite wide, including the application of both classification and regression methods. Fluorescence coupled with multivariate regression data analysis has been used for quantitative determination of chemical, physical and sensory food properties. Fluorescent techniques coupled with multivariate classification methods have been exploited to classify or discriminate foods according to different criteria. An important application is the assessment of food authenticity and adulteration, and discriminating between foods of different geographical origin or denominations of origin. In fact, fluorescence has become an analytical tool for process control in the food industry.

For example, *edible oils* contain fluorescent tocopherols, phenolic compounds and pigments of the chlorophyll group, along with oxidation and process-derived products that may contribute to their fluorescence.[2] Important applications for fluorescence in edible oils studies include: authentication of virgin olive oils, discrimination between their different quality grades and geographical origins, and detection of adulteration with low-grade olive oils or other vegetable oils. Oxidation of olive oil, its quality changes during storage, and the interaction between plastic food packaging and olive oil have also been studied using fluorescence. Minor component of oils, such as tocopherols and chlorophylls, were quantified using fluorescence.

Dairy products contain several intrinsic fluorophores, including aromatic amino acids and nucleic acids, aromatic amino acids in proteins, vitamins A and B_2, nicotinamide adenine dinucleotide, chlorophyll and fluorescent oxidation and process-derived products.[3] Fluorescence of tryptophan was investigated in dairy products, as an indicator of the protein structure. Fluorescence was used for discrimination of the cheese types, ripening stages and geographic origins. Fluorescence correlated with the rheological characteristics of various cheeses. Intrinsic milk fluorescence was used to evaluate the heat treatment of milk. Riboflavin fluorescence was used as a marker of photo-oxidation due to light-induced changes in cheeses and yogurts.

Fluorescence coupled with multivariate regression enables quantification of riboflavin in yogurts.[4]

Meat and fish autofluorescence originates mainly from collagen, adipose tissues, proteins and oxidation products.[5] Fluorescence of collagen in meat has been used for quantification of connective tissue and collagen, and correlated with tensile properties, tenderness and water-holding capacity. Fluorescence of oxidation products correlated with lipid oxidation and rancidity of meats. Fluorescence of dietary porphyrins was suggested as an indicator of faecal contamination of meats.

Fish fluorescence was proposed as a method for detecting bones in fish fillets and assessment of fish freshness. Fluorescence of fish oil was suggested as a screening method for dioxin contamination.

Cereals and cereal products fluoresce due to aromatic amino acids, ferulic acid and riboflavin.[4] Fluorescence was used for monitoring wheat flour refinement and milling efficiency and for classification of wheat cultivars.

Fluorescence of *alcoholic drinks* is attributed to several fluorophores. *Brandies* and *whiskies* fluoresce due to caramel, coumarins, tannins and other fluorescent compounds originating from wooden casks.[7] Fluorescence combined with absorption spectroscopy revealed grouping of single-malt whiskies according to their geographic area of production. *Beer* autofluorescence originates from aromatic amino acids, polyphenols and B group vitamins; contribution of the bitter acids was also suggested, but not fully confirmed.[4] Fluorescence was proposed for monitoring bitterness and determining the aromatic amino acids and riboflavin content in beer. *Wine* fluorescence shows contribution from polyphenolic compounds. It allows classification of wines according to variety, typicality and manufacturer. Recently, quantification of several phenolic compounds using wine fluorescence was also reported.

In fruit and vegetables, chlorophyll fluorescence was used as an important indicator for their quality during harvesting and post-harvesting periods.

Sucrose itself is not fluorescent, thus the fluorescence of the impurities was utilised in the analyses of sugar and sugar solutions. In particular, the fluorescent signals of the aromatic amino acids tyrosine and tryptophan and colorant polymers formed in Maillard reactions during the sugar processing were identified.[4] The classification according to production site and correlations to important sugar quality parameters were demonstrated by directly measuring fluorescence of crystalline samples.

Conclusions

One of the most important features of the fluorescence, common also to the other spectroscopic fingerprinting techniques from the practical perspective, is its ease of use and simplicity. Different chemical components or properties of the sample may be determined from a single spectral measurement. The non-destructive fluorescent measurements are performed directly on the untreated samples, thus avoiding time- and labour-consuming chemical treatment steps and fulfilling the requirements for green chemistry.

Fluorescence, characterised by its high sensitivity and selectivity and multidimensional character, may be superior or at least complementary to the absorption spectroscopy fingerprinting techniques in many practical applications, and also in the fundamental research of food systems.

References

1. B. Valeur and M.N. Berberan-Santos, "A brief history of fluorescence and phosphorescence before the emergence of quantum theory", *J. Chem. Educ.* **88,** 731 (2011). doi: http://dx.doi.org/10.1021/ed100182h
2. E. Sikorska, I. Khmelinskii and M. Sikorski, "Analysis of olive oils by fluorescence spectroscopy: methods and applications", in *Olive Oil-Constituents, Quality, Health Properties and Bioconversions*, Ed by D. Boskou, InTech, p. 63 (2012). doi: http://dx.doi.org/10.5772/30676
3. J.A. Radley and J. Grant, *Fluorescence Analysis in Ultra-violet Light*. Chapman & Hall, London (1959).
4. J. Christensen, L. Nørgaard, R. Bro and S.B. Engelsen, "Multivariate autofluorescence of intact food systems", *Chem. Rev.* **106,** 1979 (2006). doi: http://dx.doi.org/10.1021/cr050019q
5. R. Karoui and C. Blecker, "Fluorescence spectroscopy measurement for quality assessment of food systems—a review", *Food Bioproc. Technol.* **4,** 364 (2010). doi: http://dx.doi.org/10.1007/s11947-010-0370-0
6. Á. Andrade-Eiroa, G. de-Armas, J.-M. Estela and V. Cerdà, "Critical approach to synchronous spectrofluorimetry. I", *Trends Anal. Chem.* **29,** 885 (2010). doi: http://dx.doi.org/10.1016/j.trac.2010.04.010
7. V. Uríčková and J. Sádecká, "Determination of geographical origin of alcoholic beverages using ultraviolet, visible and infrared spectroscopy: a review", *Spectrochim. Acta A* **148,** 131 (2015). doi: http://dx.doi.org/10.1016/j.saa.2015.03.111

ARTICLE

The CAL(AI)^{2}DOSCOPE: a microspectrophotometer for accurate recording of correlated absorbance and fluorescence emission spectra

Martin Byrdin and Dominique Bourgeois
Institut de Biologie Structurale, Univ. Grenoble Alpes, CEA, CNRS, F-38044 Grenoble, France

The CAL(AI)^{2}DOSCOPE (Cryogenic Absorption/Luminescence Alignment Independent Alternative Intermittent Detection Optical μSCOPE) is a microspectrometer that was constructed with the aim to facilitate the correlated investigation of absorption and fluorescence emission properties of nanovolumic protein samples under modulatable actinic illumination.

Introduction

Microspectrometers are (sometimes bulky) spectrometers specifically designed for the study of microscopic samples. A variety of instruments have been developed that combine spot sizes down to sub-microns with spectral ranges from ultraviolet (UV) to infrared (IR) for various optical spectroscopy modalities such as absorbance, transmittance, fluorescence or vibrational spectroscopies. If, on top of being tiny, the samples are delicate objects such as protein microcrystals, other challenges than just tight and achromatic focusing arise: sample optical anisotropy, large refractive index and demanding environmental requirements make most commercially available devices unsuited. Therefore, at many places where microspectroscopy is required (for example at synchrotron sources to investigate protein crystals), home-made microspectrometers have been developed in recent years that strive to meet the above-mentioned challenges.[1]

At the European Synchrotron Radiation Facility in Grenoble, France (ESRF), a dedicated system based on three mutually aligned mirror objectives has been installed ("Cryobench")[2,3] that combines the absorption (A), fluorescence (F) and Raman modalities with the possibility of rotational adjustment of the sample (via a goniometric support), and the option to cool the sample down to 100 K due to a stream of gaseous nitrogen. Our experience with the powerful possibilities of this system, and the realisation of its limitations prompted us to design a next generation microspectrometer dubbed "CAL(AI)^{2}DOSCOPE" which is presented in this article. The biological samples we study (fluorescent proteins used as genetically encoded markers for advanced fluorescence microscopy) exhibit an intricate behaviour highly sensitive to their micro-environment and notably to their illumination history. As a consequence, their absorption and fluorescence (giving access to their photophysical properties) cannot be studied independently, but rather need to be followed (quasi-) simultaneously, requiring coordination in space and time. Existing solutions such as crossed beams (e.g. ESRF Cryobench)[4] or optics rearrangement (e.g. SwissLightSource)[5] are not fully compatible with these requirements. Therefore, we developed the alternative solution of a common optical path for both A and F, coupled to rapid switching of light sources and detectors by upstream mechanical shutters.

Design features of the CAL(AI)^{2}DOSCOPE

i) The CAL(AI)^{2}DOSCOPE was designed and fabricated by the opto-mechanics company Optic Peter (www.optiquepeter.com, France). The key feature of the instrument is an optical layout in which light beams for absorption, fluorescence and actinic illumination are arranged colinearly and pass through a common objective. This layout is inspired by the design of classical epi-fluorescence microscopes (see Figure 1 for a schematic view). It provides a solution to the complex alignment problems encountered with non-colinear beams, for which insertion of each new sample of finite thickness and specific refractive index compromises beam overlap (displacement, inclination) and hence requires reajustment. The use of a common objective for diverse excitation/observation tasks simplifies the issue of mutual excitation/observation alignment to that of proper tuning of the upstream semi-transparent beam coupling optics. Alignment of F and A thus becomes essentially sample-independent and can

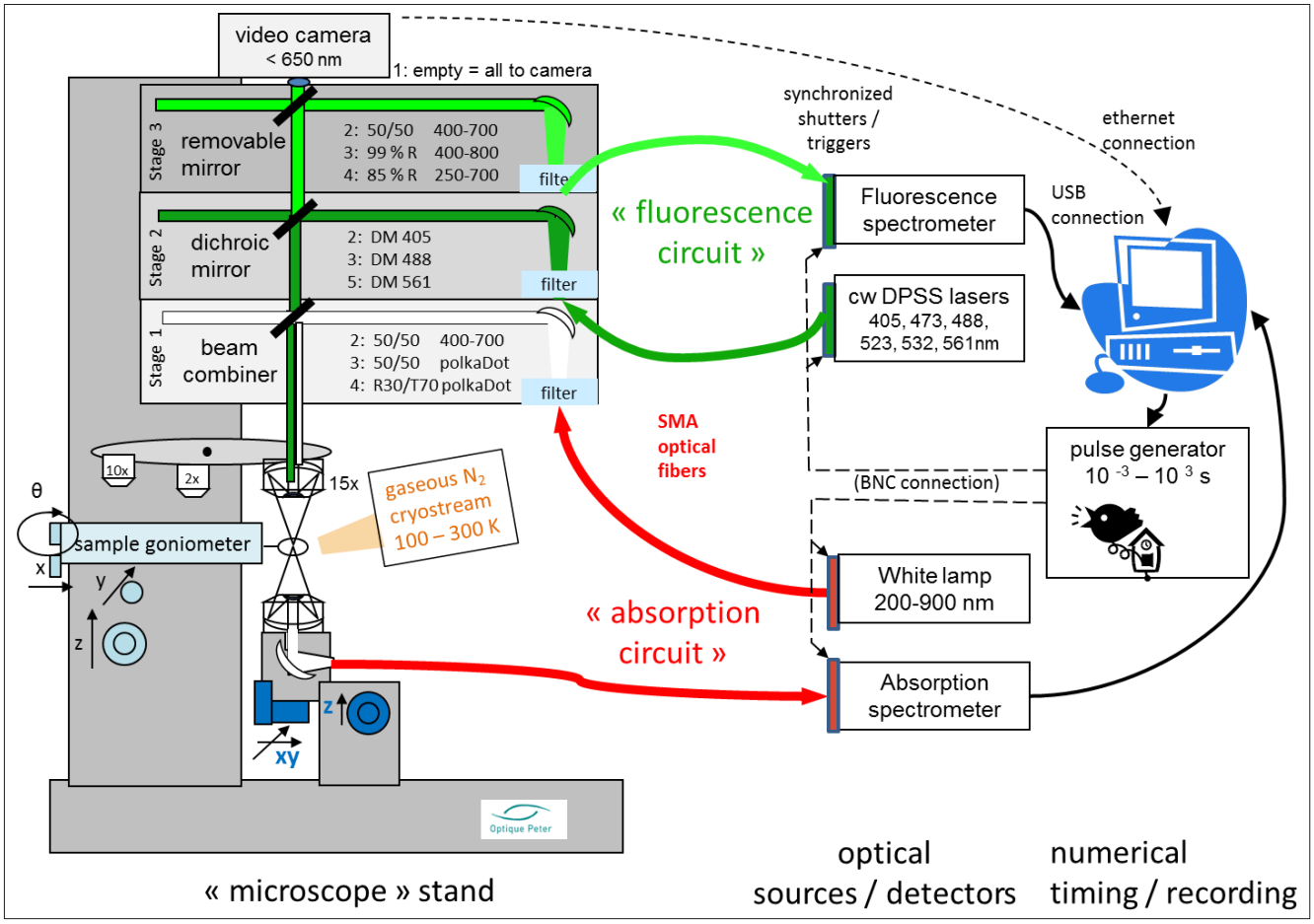

Figure 1. Opto-mechanical setup of the CAL(AI)^{2}DOSCOPE. The four light-blue knobs (x, y, z, θ) serve to position the sample with respect to the fixed top objective, the three dark-blue knobs serve to position the lower objective with respect to the sample (and hence the top objective).

be accomplished once and for all before any measurement series. Measurement of identical sample portions for A and F is thus optimised by design and light-induced colour changes or residual minor mechanical instabilities at the sample level no longer impact overlap.

ii) Although looking like a microscope from the outside (Figure 2), our device has features that are optimised for a spectrometer, notably to minimise chromatic aberrations. Thus, objectives are mirror objectives (Cassegrain design), the fibre collimators are off-axis parabolic mirrors and the beam splitters are optical sieves (polka dot design), ensuring a flat spectral response. In total, the absorption light path contains no less than seven mirrors: one per fibre coupling (×2), two per objective (×2), one per beamsplitter (×1), and consequently the overall optical throughput does not exceed a few percent.

iii) The highly rigid microscope stand ensures that the pre-aligned positions of the objectives and samples are kept stable over time. The stand does not incorporate light sources or detectors, except a camera for the observation of the alignment process and the documentation of its result. Instead, it houses four entrance/exit gates that can accommodate at will fibre-coupled devices such as two CCD spectrometers or any desired light source. Beside tungsten and halogen lamps, several diode-pumped solid-state (DPSS) lasers at selected wavelengths between 405 nm and 561 nm are aligned to a common path and can provide stable measuring/actinic light. Between each of the microscope's fibre ports and its common main optical axis, there is space for the insertion of 1-inch circular plates such as filters or polarisers.

iv) It is clear that for different excitation/detection wavelengths, different dichroic beam splitters are required and for different partition of light between the absorption and fluorescence paths, different reflectivities of the beam splitters are desired. In order to achieve this flexibility and to maintain the advantages of the pre-aligned layout, we introduced, again inspired by microscope design, four-position stages at the level of each of the beam splitters, thus allowing for one-click repositioning between pre-aligned elements via a slide bar (see Figure 2).

v) To avoid mutual perturbation between the A and F modalities, we use alternate detection. This is to say that at any time there is either laser illumination and fluorescence detection ON (and absorbance lamp and spectrometer OFF) or vice versa. To this end, mechanical shutters allow for temporal synchronisation between detectors and

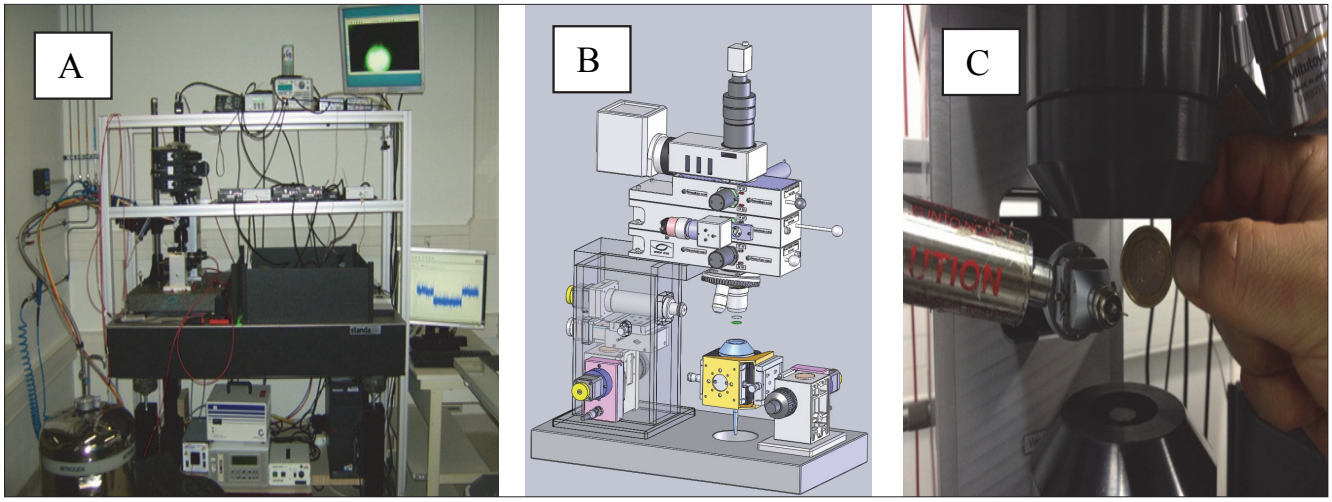

Figure 2. View of the instrument. A: Overall view: the microscope stand is on the left, the laser assembly on the right, the timing electronics on the top and the cryosystem is under the optical table. Of the two computer screens, the top one shows the camera image of the sample (for alignment) and the one on the right shows a kinetic trace from the fluorescence spectrometer. B: Sketch of the CAL(AI)^{2}DOSCOPE. C: Zoom into sample space: the space between the symmetric Cassegrain objectives is large enough to house the goniometric sample holder and the cryonozzle coming from the left. (The piece is 2€ with diameter 25 mm.)

sources. The shutters are synchronised via a Labview interface on the measuring computer that also receives the data from the spectrometers. Temporal resolution is limited in theory by the opening time of the shutters (about 1 ms), in practice more often by the integration time needed to obtain reasonable signal-to-noise ratios on the spectrometer chips (more like 10 ms). As a consequence, photo-physical processes that evolve at rates faster than ~100 Hz are currently beyond detectability. The quasi-synchronous detection opens the possibility to study the time course of photo-reactions in several types of immobilised samples, e.g. crystalline, liquid or embedded in polymer matrices, opening the way to a broad range of applications.

vi) In the actual layout, a significant part of the measuring light is lost, due to the presence of the beam splitters in the common optical path. An obvious improvement both in time resolution (switching speed) and signal amplitude (light intensity) could be obtained by using for A/F beam separation a rotating mirror or an acousto-optical device instead of a beam splitter, so that the totality of the available light could reach the active detector (both solutions do in principle exist, but would require further adaptation of the mechanical design). An important desirable development concerns automated sample positioning, possibly with the option of multi-well plate scanning, opening the door to high-throughput screening of multiple samples, e.g. variants of fluorescent proteins produced by directed evolution approaches.

Application

We look at the case of phototransformable fluorescent proteins: why do we need to study these proteins under different phases (crystalline versus solution), with A/F at the same spot at the same time, and, moreover, under actinic illumination?

The proteins that are studied in our group are so-called "phototransformable fluorescent proteins" (PTFPs): their A/F properties are sensitive to light and change with illumination, a phenomenon known in absorbance as *photochromism*. For many practical applications in bioimaging or biotechnology, light-induced changes in fluorescence (excitation/emission wavelength, quantum yield) are crucial. For example, fluorescence that can be optically induced, stopped or even toggled between ON and OFF states, provides a handle to distinguish individual chromophores (and important intracellular biological molecules labelled by them) and opens the door for advances in particle tracking, interaction studies or super-resolution fluorescence microscopy… Many of these exciting techniques are made possible by the switchability of protein fluorescence by actinic light, but the performances of these methods depend crucially on a precise understanding of the photo-physical processes exhibited by the fluorescent markers. This presents a considerable challenge and is the reason why our CAL(AI)^{2}DOSCOPE has to be able to follow as many parameters as possible simultaneously on one and the same protein sample spot: the one that is hit by the actinic beam causing the photophysics to happen. Intrinsic timescales of photophysical processes within PTFPs range from (sub)nanoseconds to hours. However, timescales relevant to advanced microscopy are typically those accessible by the CAL(AI)^{2}DOSCOPE. Photoswitching or photobleaching are examples of photophysical processes, the rates of which can be conveniently investigated in this way.[6,7]

Figure 3 shows an example study of a variant of the phototransformable fluorescent protein mEos2. Absorption at 490 nm and fluorescence at 512 nm are detected simultaneously under constant 488 nm actinic illumination superimposed to alternate light of 405 nm causing fluorescence OFF and ON switching. With advancing switching cycles, the amplitude and the ON/OFF contrast

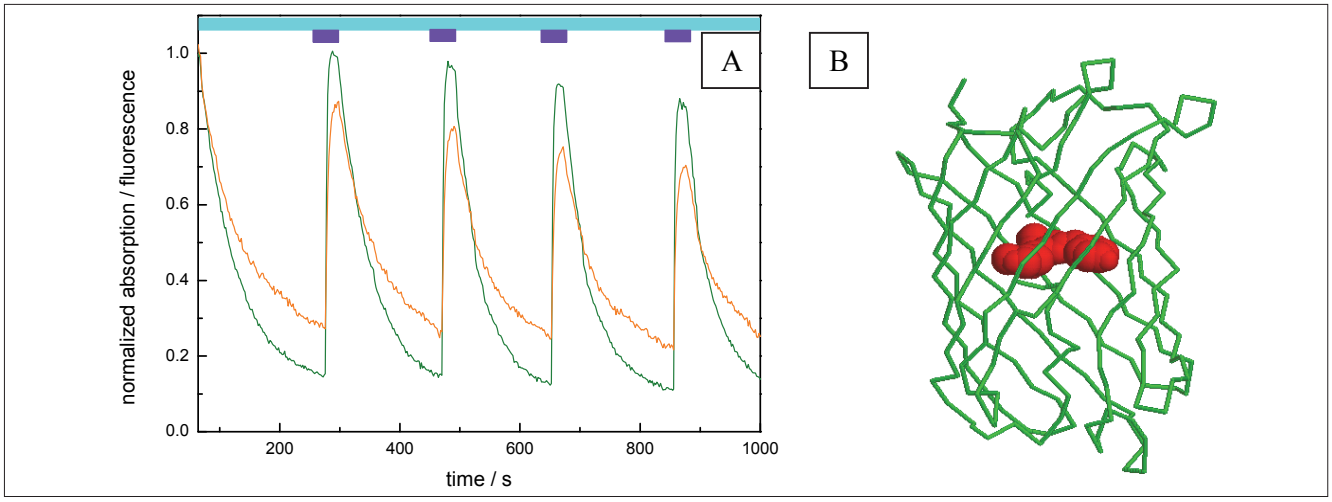

Figure 3. "Photofatigue" of a variant of the photoswitchable fluorescent protein mEos2 under OFF/ON cycles of actinic 405 nm light (violet bars) and permanent 488 nm fluorescence excitation (cyan bar). Panel A shows the temporal evolution of absorbance (around 490 nm, orange) and fluorescence (around 512 nm, olive) of a microdrop of an mEos2 mutant immobilised in a matrix of polyvinyl alcohol (PVA). The amplitudes are normalised for presentation on a common scale. Panel B shows the crystallographic structure of mEos2, the parent fluorescent protein with the green "barrel" structure housing the chromophore in red.

diminish, a phenomenon known as "fatigue". It can be seen that during OFF-switching, the fluorescence decays in a biphasic manner that differs from that of absorption, suggesting that the OFF-state, though not fluorescent, does still absorb at 490 nm. Another intriguing observation is that, towards the end of the ON-switching periods, fluorescence starts to diminish while absorption continues to rise. This subtle photophysical behaviour (hinting at a photobleaching mechanism induced by 405 nm light that now needs to be investigated in detail) would be difficult to detect with a standard apparatus and highlights the potential of the CAL(AI)^{2}DOSCOPE.

Conclusion

The CAL(AI)^{2}DOSCOPE incorporates in a single instrument multiple features that can only be found separately elsewhere:

- microscopic samples mountable on a motorised goniometric sample holder;
- camera-supported alignment with up to 15× optical magnification (numerically unlimited);
- quasi-simultaneous absorption and fluorescence spectroscopy on identical sample sections of down to 10 µm diameter;
- access to light-induced reactions (photo-physics, photo-chemistry) via programmable illumination of the same spot with various actinic sources; and
- controlled environment via continuous flow of gas of choice (air, argon, N_2) at temperature of choice for the latter (100–300 K).

Overall, the modular design of the CAL(AI)^{2}DOSCOPE allows for a maximum of versatility, adaptability and flexibility.

Although originally developed to investigate fluorescent protein photophysics, the instrument, thanks to its unique combination of features, should be useful in a much wider range of biological-chemical-physical-technological applications.

Acknowledgements

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References

1. A.R. Pearson, A. Mozzarelli and G.L. Rossi, "Microspectrophotometry for structural enzymology", *Curr. Opin. Struct. Biol.* **14,** 656–662 (2004). doi: http://dx.doi.org/10.1016/j.sbi.2004.10.007
2. D. Bourgeois, X. Vernede, V. Adam, E. Fioravanti and T. Ursby, "A microspectrophotometer for UV-visible absorption and fluorescence studies of protein crystals", *J. Appl. Crystallogr.* **35,** 319–326 (2002). doi: http://dx.doi.org/10.1107/s0021889802003837
3. D. von Stetten, T. Giraud, P. Carpentier, F. Sever, M. Terrien, F. Dobias, D.H. Juers, D. Flot, C. Mueller-Dieckmann, G.A. Leonard, D. de Sanctis and A. Royant, "In crystallo optical spectroscopy (ICOS) as a complementary tool on the macromolecular crystallography beamlines of the ESRF", *Acta Crystallogr. D* **71,** 15–26 (2015). doi: http://dx.doi.org/10.1107/S139900471401517X
4. T. Barros, A. Royant, J. Standfuss, A. Dreuw and W. Kühlbrandt, "Crystal structure of plant light-harvesting complex shows the active, energy-transmitting state", *EMBO J.* **28,** 298–306 (2009). doi: http://dx.doi.org/10.1038/emboj.2008.276
5. R.L. Owen, A.R. Pearson, A. Meents, P. Boehler, V. Thominet and C. Schulze-Briese, "A new on-axis multimode spectrometer for the macromolecular crystallography beamlines of the Swiss light source", *J. Synchrotron Radiat.* **16,** 173–182 (2009). doi: http://dx.doi.org/10.1107/s0909049508040120
6. C. Duan, V. Adam, M. Byrdin, J. Ridard, S. Kieffer-Jaquinod, C. Morlot, D. Arcizet, I. Demachy and D. Bourgeois, "Structural evidence for a two-regime photobleaching mechanism in a reversibly switchable fluorescent protein", *J. Am. Chem. Soc.* **135,** 15,841–15,850 (2013). doi: http://dx.doi.org/10.1021/ja406860e
7. R. Berardozzi, V. Adam, A. Martins and D. Bourgeois, "Arginine 66 controls dark-state formation in green-to-red photoconvertible fluorescent proteins", *J. Am. Chem. Soc.* **138,** 558–565 (2016). doi: http://dx.doi.org/10.1021/jacs.5b09923

ARTICLE

Synchrotron infrared near-field spectroscopy in photothermal mode

Chris S. Kelley,[a] Mark D. Frogley,[a] Ann Fitzpatrick,[a] Katia Wehbe,[a] Paul Donaldson[b] and Gianfelice Cinque[a]*

[a]Diamond Light Source Limited, Harwell Science and Innovation Campus Didcot, Oxon OX11 0DE, UK.
E-mail: gianfelice.cinque@diamond.ac.uk
[b]Central Laser Facility, Research Complex at Harwell, Didcot, Oxon OX11 0FA, UK

Near-field infrared (IR) spectroscopy is revolutionising molecular imaging by allowing vibrational spectroscopic analysis at the sub-micrometre scale. We recently developed the world's first near-field IR photothermal microscope using synchrotron radiation (SR), which uniquely spans a spectral range from the near-IR to the far-IR/THz. Here we demonstrate the capability of the near-field method to probe polymer microspheres within a protein matrix, and we present the first IRSR photothermal near-field Fourier transform infrared (FT-IR) spectrum from within an individual biological cell, which establishes the feasibility of hyperspectral mapping at sub-micrometre resolution in a practical timescale. Photothermal near-field spectroscopy provides both depth sensitivity and IR molecular specificity, which is ideal for organic matter/biological samples. In addition, SR gives seamless and ultra-broadband spectral coverage superior to the bandwidth of commercially available lasers.

Introduction

Until recently, the spatial resolution in infrared (IR) microspectroscopy was fundamentally limited to the scale of the wavelength due to optical diffraction and the limited numerical aperture of the microscope objectives. Even via synchrotron radiation (SR) IR beamlines, where a diffraction-limited microbeam is achievable,[1] or in a micro-attenuated total reflection (micro-ATR) geometry, where the high refractive index of, for example, a germanium crystal in contact with the sample enhances the magnification by up to four times, the spatial resolution in the information-rich fingerprint region of the mid-IR spectrum (500–1800 cm^{-1}) is still limited to several micrometres. To overcome the spatial diffraction limit, near-field techniques are needed and pioneering measurements based on tip-scattering[2] and photothermal detection[3] have demonstrated that, by using an atomic force microscope (AFM) as a local probe, IR spectra can be obtained at a scale of just tens of nanometres. These two revolutionary nano-IR approaches have opened up a huge variety of research avenues across many scientific disciplines.

The photothermal near-field method,[3] in which IR absorption is detected by the mechanical deflection of the AFM tip induced by the local thermal expansion of the sample, has several advantages: i) the IR spectrum produced is a true linear IR absorption; ii) it requires no numerical modelling of the signal to produce the spectrum; iii) it is not surface-limited in sensitivity since the whole volume of the sample is probed. Within their limited spectral range, latest generation IR laser sources can provide high quality near-field spectra in less than one minute and, with AFM tip resonance-enhanced techniques (RE-AFMIR), spectral signatures from nanoscale areas of samples as thin as a single molecular monolayer have been detected.[4]

We recently demonstrated that the brilliant, ultra-broadband, IRSR emitted by Diamond Light Source in the UK can be used to obtain near-field photothermal spectra by the RE-AFMIR method, at the sub-micrometre scale[5] within several minutes. In combination with the SR tip scattering approach first realised in the THz,[6] then across the mid-IR region[7,8] and now under development at several SR facilities worldwide, these scientific tools find unique applications where the spectral bandwidth available from laser sources is a limitation.

In this article we present new experimental RE-AFMIR results from two sample types demonstrating the unique capabilities of the SR-based method. First, we look at a sample of polymer microspheres embedded within a protein layer, from which we are able to obtain broadband spectra and IR images of buried beads, and then we discuss the first data obtained by near-field photothermal IRSR from within an isolated human cell.

Experimental

The SR resonance-enhanced AFM-IR system is schematically outlined in Figure 1. The broadband IR light generated from Diamond Light Source and collected by MIRIAM beamline B22[1] is collimated into a FT-IR interferometer (Bruker Vertex 80V). This is a Michelson set up where the IRSR beam is divided into two by the beamsplitter and reflected off two separate mirrors before being recom-

bined. The moving mirror (coloured blue in Figure 1) causes an intensity modulation of the broadband beam, where each optical wavelength is modulated at a unique temporal frequency. The FT-IR modulated SR beam is focused through a fast mechanical chopper, adding a further fast temporal on/off modulation to the beam intensity. The beam is then focused via a high numerical aperture Cassegrain IR objective (Agilent 15×, NA 0.65) through the IR transparent substrate onto the sample.

At IR wavelengths where there is an absorption band of the material, the absorbed light power causes a very small temperature change (of order 10 mKelvin) and consequent thermal expansion (sub-picometre) of the sample, which produces a deflection of the in-contact gold-coated silicon cantilever of the AFM (Nanonics MV1000). The AFM laser deflection signal from the position sensitive detector (PSD) is passed into a lock-in amplifier where only signals around the frequency of the chopper are amplified and therefore the weak, chopper modulated, deflection signal can be recovered within the environmental thermal background. This amplified signal still carries the FT-IR low frequency temporal modulation (interferogram), which is preserved in the thermal expansion of the sample, so the standard FT-IR software directly produces an IR spectrum comparable with conventional optical IR spectra.

Crucially, when the chopper modulation frequency is tuned to a mechanical contact resonance frequency of the AFM cantilever[4] the mechanical deflection is dramatically enhanced (of the order 100× for a typical Si cantilever); this is key to obtain the IRSR-induced spectra on a practical timescale of a few minutes. The inverse of this chopper modulated frequency defines the measurement timescale, the thermal diffusion radius and therefore the spatial resolution.[5] In these experiments we used a harmonic of the fundamental cantilever resonance, between 60 kHz and 100 kHz, corresponding to about 1 µm spatial resolution[5] for the materials studied here.

The dedicated set up is customised from a double infinity-corrected optical microscope (Nanonics) allowing both

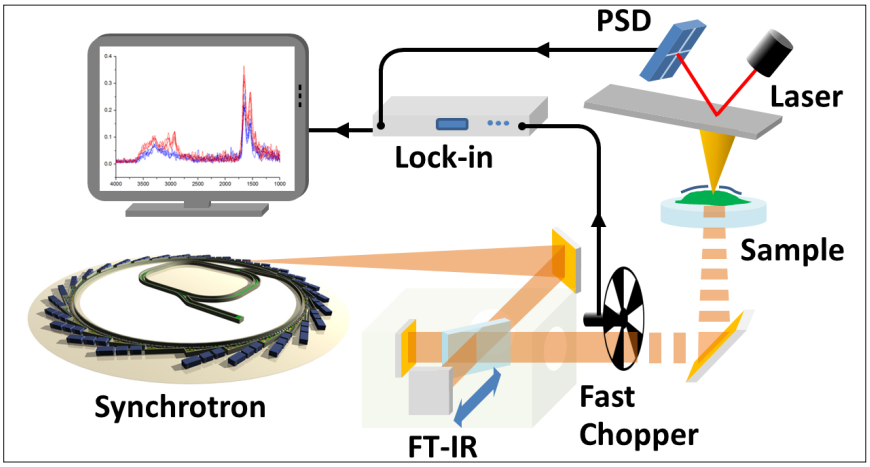

Figure 1. Schematic of the synchrotron RE-AFM-IR experiment.

top and bottom IR illumination and a visible camera view of the sample through identical Cassegrain objectives. The AFM is mounted on a large-range piezo-controlled x–y stage (PI), for precise positioning of the sample and AFM tip with respect to the IRSR microbeam focus. The sample is then scanned under the tip by the internal short-range piezo scanner of the AFM.

Polymer microbead samples were produced by mixing protein Bovine Serum Albumin (Sigma Aldrich) into a de-ionised water suspension of 1.04 µm polystyrene (PS) beads (Bangs Laboratories, Inc.), then drop casting onto a CaF_2 substrate and dried in air. The preparation was tailored to produce samples in which the BSA covered the microbeads in most areas. The cell samples were formalin fixed *H. pylori* infected human cells (Bio-Quick Corp.) on a ZnS substrate.

Results/discussion

Figure 2a shows an AFM topography image of the polystyrene (PS) beads/BSA sample recorded with an AFM tip of <100 nm (including gold coating). The height at some bead positions is less than 1 µm above the surrounding BSA indicating that the beads are at least partially buried, however, from this topographical data we cannot determine if there is BSA on top of the bead or not. SR RE-AFMIR spectra recorded in positions on and off the beads (positions A and B in Figure 2a) are shown in Figure 2b, with the chopper frequency optimally tuned to match the contact resonance in the two spatial positions. The spectrum obtained on pure BSA shows the expected IR bands for a protein, which are strong Amide I and II (1500–1700 cm^{-1}), Amide A (3300 cm^{-1}), and relatively weak C–H and O–H stretch bands (2750–3600 cm^{-1}). At the position of the PS bead, the strong C–H stretch bands of PS are observed along with a weaker shoulder due to the C–H bend (1425–1475 cm^{-1}) also characteristic of PS. The equivalent far-field IR absorbance spectra for individual BSA and PS samples[9] are shown in Figure 2c, confirming the expected spectral bands from each material.

Strikingly, in Figure 2b, the BSA is clearly contributing to the spectrum at the bead location (A) and the Amide I and II bands are even stronger than at the "background" position (B). This suggests that there is BSA covering the bead. It is worth noting that in this case a scattering near-field IR measurement would only be capable of detecting the BSA, and this depth sensitivity demonstrates one of the powerful advantages of the RE-AFMIR approach. Figure 2d shows the spectrally integrated RE-AFMIR signal recorded whilst scanning the topographic image in 20 minutes for the 10 × 10 µm scan. In this case the chopper frequency is tuned to the nominal contact resonance at the summit of the topography. This leads to some distortion of the RE-AFMIR image as the actual contact resonance varies with the sample to tip interaction changes.

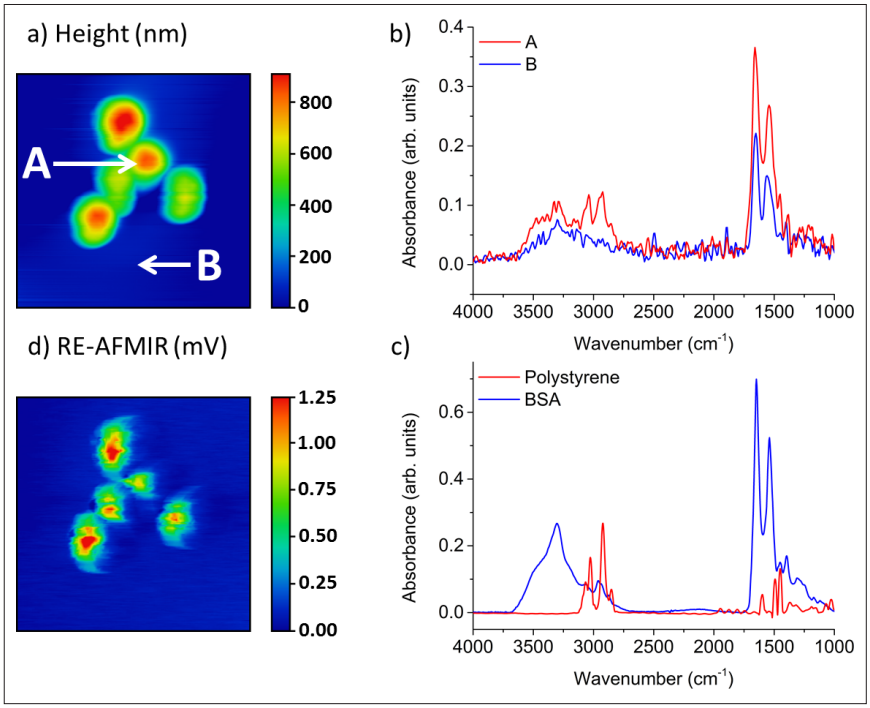

Figure 2. a) AFM topography image of PS beads embedded in BSA. b) SR RE-AFM-IR spectra recorded at A and B. c) Far-field (bulk) spectra of pure BSA and pure PS. d) SR RE-AFM-IR integrated signal map of the same area as a).

The ability of RE-AFMIR to resolve the chemical information from subsurface beads was confirmed by imaging a larger sample area, this time using a quantum cascade laser source (QCL, Daylight Solutions) tuned to the aromatic C=C stretch band of PS at 1601 cm^{-1} (Figure 3). The QCL was also mechanically chopped (50% duty cycle as with the SR) at a fixed frequency of the nominal contact resonance of the cantilever. The topography (Figure 3a) shows some broad undulating features, whilst the near-field IR signal (Figure 3b) in the same areas is essentially flat with well-defined individual beads now clearly resolved. In particular, the raised region A in the topography image is completely absent in the IR map and several buried beads-barely observable in topography (B, C) appear as strong, well spatially resolved features in the 1601 cm^{-1} absorbance map.

Large area RE-AFMIR imaging is also possible in reasonable times (tens of minutes) using the SR beam (Figure 2d) at present via the full spectrally integrated signal.

For the first time, we have been able to measure the SR near-field photothermal spectrum from a single human cell across the whole mid-IR region. This is expected to be a key area of application for the method, since the cellular organelles and structures are below the cell membrane. Figure 4 shows the AFM topography (a) and integrated SR RE-AFMIR signal map (b) of a single, fixed, bacterial infected human cell on a ZnS substrate. The typical cell IR spectrum, obtained in eight minutes, is clearly detectable above the background level of the substrate (shown for comparison) and with the expected features of biological matter[10] including Amide I and II dominated (1500–1700 cm^{-1}) by proteins, Amide A and B (3100 cm^{-1} and 3300 cm^{-1}) and C–H stretch (2800–3150 cm^{-1}) normally assigned to lipids, as well as the broad O–H band (*ca* 3000–3700 cm^{-1}). In addition, several bands in the fingerprint region corresponding to phosphates (DNA/RNA) and other groups are clearly evident; specifically, the symmetric and antisymmetric PO_2-stretches (1080 cm^{-1} and 1243 cm^{-1}, respectively)[11] and the CH_3 bend from proteins (1399 cm^{-1}). Some of the apparent noise in the 1250–1600 cm^{-1} region is likely due to residual atmospheric water vapour, which has not been corrected for. It is worth noting that no normalisation to the IRSR beam intensity spectrum has been performed, although this could be done for absolute quantification of the results. The FT-IR spectrum in Figure 4 demonstrates the feasibility of broadband SR RE-AFMIR mapping of a complete cell. In the current setup, at the spectral quality of Figure 4, a complete hyperspectral cell image over ca 15×15 µm square area will take approximately one day at 1 µm spatial resolution. As the method is still in its infancy there is room for significant improvement in data quality as well as acquisition time as the technique is developed.

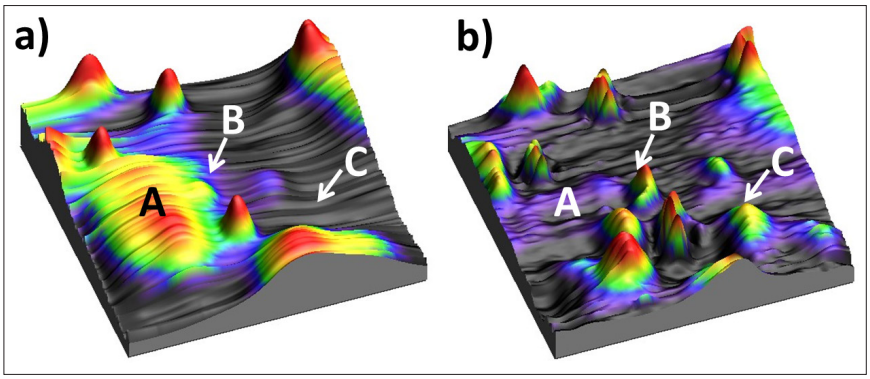

Figure 3. a) AFM topography of the PS bead/BSA sample. b) Single frequency RE-AFMIR image at the 1601 cm^{-1} band of PS obtained with a QCL IR source.

Conclusions

We have measured the world's first broadband synchrotron-based near-field photothermal infrared spectrum from a 1-µm region of an isolated biological cell in eight minutes, showing it is feasible to measure a complete hyperspectral map of a cell at this reso-

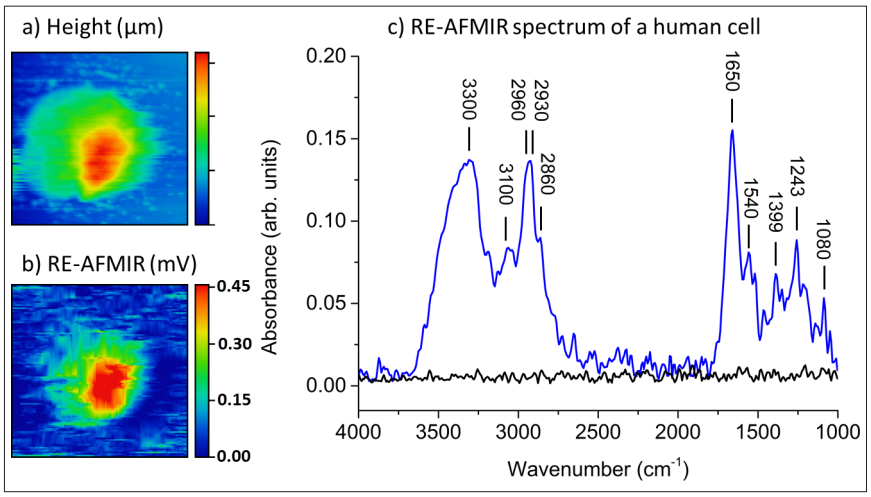

Figure 4. a) AFM topography of a single bacterial infected human cell. b) Integrated signal SR RE-AFMIR map acquired with the topography. c) SR RE-AFMIR spectrum from the cell in the region of peak integrated signal (blue), compared with background spectrum from the ZnS substrate (black).

lution within a day. Using polymer microbeads embedded in BSA, we have demonstrated the ability to spectrally resolve sub-surface features. This depth sensitivity and the direct, model-free FT-IR spectroscopy are clear advantages of the photothermal approach over the scattering based near-field method for many applications, whilst the use of synchrotron radiation gives access to a seamless ultra-broadband spectral range (Vis–THz) currently unmatched with laser sources. In the future we expect improvements in the technology to allow faster data acquisition rates and, using higher-resonance frequency cantilevers, spatial resolution approaching 100 nm.

References

1. G. Cinque, M. Frogley, K. Wehbe, J. Filik and J. Pijanka, "Multimode InfraRed Imaging and Microspectroscopy (MIRIAM) beamline at Diamond," *Synchrotron Radiat. News* **24,** 24 (2011). doi: https://doi.org/10.1080/08940886.2011.618093
2. B. Knoll and F. Keilmann, "Near-field probing of vibrational absorption for chemical microscopy", *Nature* **399,** 134 (1999). doi: https://doi.org/10.1038/20154
3. A. Dazzi, R. Prazeres, F. Glotin and J.M. Ortega, "Local infrared microspectroscopy with an atomic force microscope tip used as a thermal sensor", *Opt. Lett.* **30,** 2388 (2005). doi: https://doi.org/10.1364/OL.30.002388
4. F. Lu and M.A. Belkin, "Infrared absorption nano-spectroscopy using sample photo-expansion induced by tunable quantum cascade lasers", *Opt. Express* **19,** 19942 (2011). doi: https://doi.org/10.1364/OE.19.019942
5. P.M. Donaldson, C.S. Kelley, M.D. Frogley, J. Filik, K. Wehbe and G. Cinque, "Broadband near-field infrared spectromicroscopy using photothermal probes and synchrotron radiation", *Opt. Express* **24,** 1852 (2016). doi: https://doi.org/10.1364/OE.24.001852
6. U. Schade, K. Holldack, P. Kuske, G. Wüstefeld and H.W. Hübers, "THz near-field imaging employing synchrotron radiation", *Appl. Phys. Lett.* **84,** 1422 (2004). doi: https://doi.org/10.1063/1.1650034
7. P. Hermann, A. Hoehl, P. Patoka, F. Huth, E. Rühl and G. Ulm, "Near-field imaging and nano-Fourier-transform infrared spectroscopy using broadband synchrotron radiation", *Opt. Express* **21,** 2913 (2013). doi: https://doi.org/10.1364/OE.21.002913
8. H.A. Bechtel, E.A. Muller, R.L. Olmon, M.C. Martin and M.B. Raschke, "Ultrabroadband infrared nanospectroscopic imaging", *Proc. Nat. Acad. Sci.* **111,** 7191 (2014). doi: https://doi.org/10.1073/pnas.1400502111
9. Data reproduced from Thermo-Fisher Scientific Spectral Libraries.
10. G. Bellisola and C. Sorio, "Infrared spectroscopy and microscopy in cancer research and diagnosis", *Am. J. Cancer Res.* **2,** 1 (2012).
11. P.T.T. Wong, E.D. Papavassiliou and B. Rigas, "Phosphodiester stretching bands in the infrared spectra of human tissues and cultured cells", *Appl. Spectrosc.* **45,** 1563 (1991). doi: https://doi.org/10.1366/0003702914335580

TONY DAVIES COLUMN

What is the collective noun for solid-state nuclear magnetic resonance spectrometers?

Antony N. Davies[a,b] and Steven P. Brown[c]
[a]Strategic Research Group – Measurement and Analytical Science, Akzo Nobel Chemicals b.v., Deventer, the Netherlands
[b]SERC, Sustainable Environment Research Centre, Faculty of Computing, Engineering and Science, University of South Wales, UK
[c]Department of Physics, University of Warwick, Coventry, CV4 7AL, UK

A few weeks ago saw the University of Warwick in the UK Midlands host a very successful two-day conference for the AkzoNobel analytical science community with attendees from the UK and Europe. It is not my intention to advertise any particular location for running conferences or to present anything of the content of the meeting, but as part of the event we enjoyed interacting with some of the staff of the superb facilities the University has available to support non-Warwick-based researchers to use supporting Open Innovatione.

Background

Amongst some excellent science seen on our tour, the solid-state nuclear magnetic resonance spectroscopy (NMR) facilities at Warwick stood out; not only due to the large number of magnets, eleven (maybe the collective noun could be "Kettle" as used for many hawks?), but also the attitude and approach of the staff.[1]

Interestingly, the solid-state NMR facility is located within the Physics Department. This was described as essential due to the complexity of the mathematics involved (seems like a challenge, chemists!). These facilities are in addition to the strong NMR Spectroscopy presence in the Chemistry Department with four open access 300 MHz and 400 MHz instruments and four high-field instruments at 500 MHz, 600 MHz and 700 MHz.

Why is solid-state NMR so different?

For those who do not practise solid-state NMR, it is worth reviewing why this field is so different from its liquid-state cousin. The easy answer is "it isn't". The interaction between the nuclei of the atoms in the sample and the magnetic fields applied by the spectrometer and the superconducting magnet are the same. However, what makes solid-state NMR a field worthy of placing in a different faculty is time and anisotropy.

Within an NMR spectrometer, the electromagnetic field is strongly directional. It interacts with the rotating nuclear spins as long as there are isotopes which contain an odd number of protons and/or neutrons, which give an intrinsic magnetic moment and angular momentum to the nuclei. As the applied field is directional and the magnetic moment of the nuclei is also directional, you would expect to have different interactions depending on whether the fields align or not; similar to a polarisation experiment in optical spectroscopy. This is where time comes in. In a standard solution-state NMR experiment, the sample molecules are rapidly moving around compared to the time scale of the experiment. This averages out the anisotropic interactions so you normally get nice narrow signals to interpret. In solid samples the motion is no longer present, so the different orientations of the molecules in the solid sample to the applied field cause a large broadening of the observed signal.

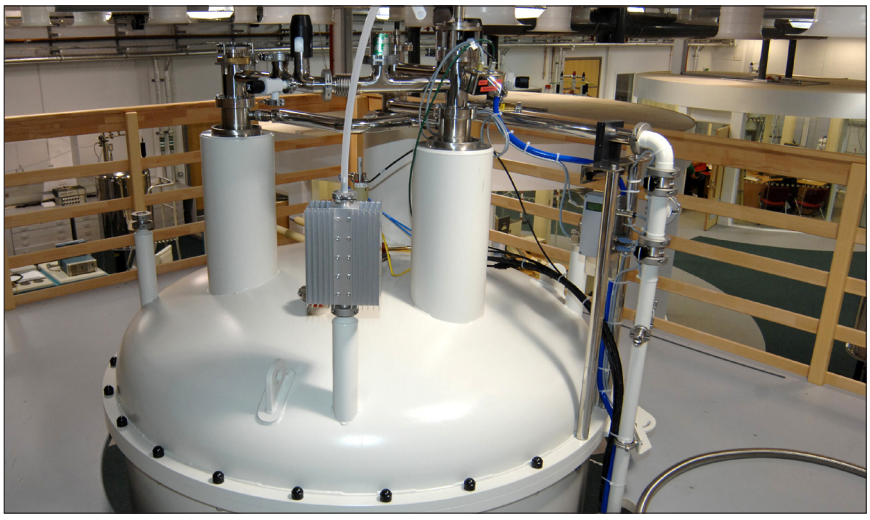

Figure 1. The UK's National Facility's 850 MHz wide-bore magnet hosted at Warwick.

This is not to say that this broadening necessarily has to be avoided, as it is carrying interesting structural orientation information about the sample being analysed. If you are looking for liquid-NMR-like narrow peaks for your sample interpretation, there is a simple experimental way to eliminate the broadening by imitating the averaging motion in solution-state NMR: rapidly rotate the sample. This so-called magic angle spinning succeeds in averaging out some of the broadening, but is only partially successful. However, in the age of the Internet, this column cannot be allowed to descend into a beginner's guide to solid-state NMR, so let us move on to why solid-state NMR is such an important facility to have maintained as a state-of-the-art Open Innovation resource.

Applications

As suggested above, the root causes of the broadening which are lost in conventional solution-state NMR can be useful in solid-state NMR, providing structural information on the solid being measured. The higher field strengths give access to a wider variety of more complex experiments probing macromolecular and domain information often unavailable by other techniques. These include interactions between nuclei across space rather than through bonds, crystal domains and defects, hydrogen bonding, and supramolecular structures. Although biological applications are often headline-grabbing, many industrial materials science questions are capable of being addressed through solid-state NMR.

Clearly, the complexity of the systems, and especially the dynamic biological systems, require advanced computing and modelling capabilities as well as the necessary hardware. All this investment would be wasted if a vastly more important foundation were absent. The most precious resource of all is the scientific staff capable of operating the technical side whilst displaying the people skills to successfully communicate and interpret the needs of their varying customer base. I found it interesting that one of the duties of the facility manager—alongside all the technical responsibilities you would expect in such a role—was "Promoting a positive and welcoming feel to the Facility". This has often been overlooked when staffing and operating national-scale facilities in the past. The successful long-term use of such facilities often relies on the building of strong open relationships between the staff and their customers whether in industry or "rival" academic institutions.

With EU-originating funding playing a key role in the establishment and ongoing world-class delivery of this facility, it is sobering to read the eloquent submission to the House of Commons Science and Technology Committee in August on the potential effects of the Brexit vote.[2]

Computing

The results of the various experiments are usually processed using the vendor software. However, for a facility aligned with the Open Innovation agenda I was interested in how the data is distributed and made available to their customer base. Non-critical data is served to the user community through a dedicated data archive server off-spectrometer. Those wishing to process the data themselves can do so by downloading their data to their local IT environments and work using the standard vendor software at their own locations. Many research institutions do not have their own NMR data processing capabilities and, where this is the case, the facility will assist in providing access to the necessary software.

For industrial partners or those with heightened secrecy requirements on IP or other grounds, their own data dissemination routes are set up to meet their specific needs as necessary.

Conclusions

Six years have passed since the UK 850 MHz Solid-State NMR Facility was original opened and they are currently halfway through their current 3+2-year funding period (Figure 3).

The facility has quite a complex management and oversight structure, which is very transparent in its operations. Their 2015 report, for example, publishes their Customer Satisfaction Survey which, as someone tied up in evaluating such exercises over recent years, reads very well.[3] I think the future of this facility post-Brexit with the timing of their subsequent funding rounds and broad international scientist population may well serve as a potential bell-weather for a post-Brexit UK science landscape. They need to be able to keep up with, for example, the investment available to their colleagues in a country with a much lower GDP such as the Netherlands highlighted in 2013.[4] It would be a tragedy if they end up scratching around for cash to keep the facility open rather than concentrating on delivering world-class science like our colleagues in the uncertain world of Canadian High Field NMR facilities.

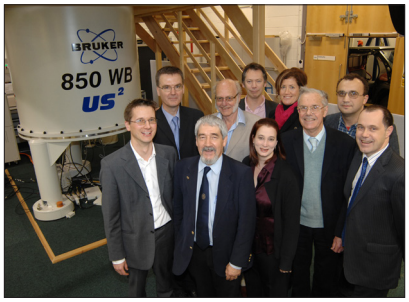

Figure 2. UK NMR nobility turnout for the original symposium celebrating the opening of the UK facility in 2010. Steven Brown is the scientist smiling happily on the very left of this group, to his left is the then chief executive of EPSRC, who are the principal funding agency (with partial support from BBSRC), with others compromising the then National Management Committee.

So, going back to the original conundrum at the top of the article, maybe we should be looking at proposing "Wisdom" as the collective noun (after a Wisdom of Wombats—for those who love the added alliteration!). Hopefully, wisdom will also shape UK government funding policy in a post-Brexit world and they will deliver on their promises that overall funding for such facilities will not suffer and we will not see the smile wiped off Steven's face.

References

1. http://www2.warwick.ac.uk/fac/sci/physics/research/condensedmatt/nmr/
2. http://www2.warwick.ac.uk/fac/sci/physics/research/condensedmatt/nmr/850/HoC_EU_UK_850_MHz_Solid_State_NMR_Facility_160829.pdf
3. http://www2.warwick.ac.uk/fac/sci/physics/research/condensedmatt/nmr/850/annual_reports/uk_850_mhz_solid_state_nmr_facility_report_2015.pdf
4. A.N. Davies, "GHz NMR!", *Spectrosc. Europe* **25(3),** 18–19 (2013). http://bit.ly/2dfh5GL

QUALITY MATTERS

Change is in the air

Peter Jenks
The Jenks Partnership, Newhaven House, Junction Road, Alderbury, Salisbury, Wiltshire SP5 3AZ, UK.
E-mail: peter@jenks.info

Just as in the political arena, the world of chemical metrology is poised on the brink of fundamental change: a new International Standard, ISO/IEC 17034, General Requirements for the Competence of Reference Material Producers was published on 1 November 2016: this means that over the next three years all existing certified reference material (CRM) producers, presently accredited to a combination of ISO REMCO Guide 34 and the International Standard ISO/IEC 17025, General requirements for the competence of testing and calibration laboratories, and together sometimes referred to as the "Gold Standard", will have to migrate their accreditations to the new standard.

In 2017, a new and fully revised version of ISO/IEC 17025 will be published. This means all labs presently accredited to ISO/IEC 17025 will, over the next couple of years, have to review their quality systems against the new version of the standard. We will look at the differences between the old and new versions in a future edition!

As regular readers of this column will know, one of the requirements of ISO/IEC 17025 is that wherever possible CRMs be used by accredited laboratories for the calibration of analytical systems, the validation of analytical methods and as a part of on-going quality assurance.

Since the early 1990s, as more and more testing laboratories sought and achieved accreditation to ISO/IEC 17025, demand for CRMs increased beyond the ability of the national metrology institutes to respond. Accordingly, commercial production of CRMs gathered pace, but at that time there was no formal route to accredit producers. It was felt that accreditation to a combination of ISO/IEC 17025 and ISO REMCO Guide 34 would at least ensure CRM production could be accredited whilst a new standard was developed.

It took far longer than expected, almost 10 years, to work out quite how to migrate an ISO REMCO Guide into an ISO/IEC Standard, but in 2014 ISO REMCO and ISO CASCO agreed a procedure that resulted in an *ad hoc* ISO CASCO Working Group AHG3 being established. The first meeting was held in Geneva in December 2014. Progress was relatively rapid and the new ISO/IEC standard was published at the end of 2016, preceeded on 26 October 2016 by the adoption of the new Standard by European Committee for Standardization (CEN, French: Comité Européen de Normalisation), thus paving the way for it to become a European Standard. Also, in late 2016 ILAC agreed to develop a global mutual recognition agreement (MRA) for reference material producers accredited to ISO/IEC 17034: this should come into effect during 2018. At the same meeting ILAC agreed with ISO that there would be a three-year transition with accreditation to ISO Guide 34 ending on 31 October 2019.

In the UK, UKAS has started a detailed gap analysis comparing the old Guide 34 with the new Standard to help prepare accredited laboratories, and also in early 2017 UKAS will start training for evaluators and peer evaluators from other accreditation bodies. This preparation phase means that accredited producers can be prepared to migrate to the new standard during 2018. Any producer that does not migrate in the second year will be informed that if they do not complete accreditation to the new standard by 31 October 2019 their accreditation will lapse.

During early 2017, UKAS will add accreditation to ISO/IEC 17034 to their ISO/IEC 17011 Scope, so that by the end of 2017 they expect to be accredited to offer accreditation to the new standard. It is worth note that the revised version of ISO 17011 includes the concept of accreditation to a flexible scope, so that all accreditation bodies can, if they wish, offer accreditation to ISO/IEC 17034 with a flexible scope. As there has been much debate within the accreditation community about the use of a flexible scope in association with accreditation to ISO Guide 34 it remains to be seen if there will be universal uptake of this aspect.

The new ISO/IEC 17034 standard contains one very significant change from the old combined accreditation, and facilitates one crucial development.

The change

It has been generally accepted that until now a CRM producer must hold the dual accreditation of ISO Guide 34 + ISO/IEC 17025: to hold an ISO 17025 accreditation required an analytical laboratory, so all CRM certification bodies had to have an accredited analytical laboratory. The new ISO/IEC 17034 standard allows the CRM certification body to use third party analytical laboratories; provided they are accredited to ISO/IEC 17025 and the scope of their accreditation is appropriate. So with the publication of the new standard, the

door is opened allowing organisations previously unable to achieve accreditation as a CRM producer to enter the market place.

Demand for CRMs is growing, rapidly. This growth, in significant double digits, is driven by the accreditation of more and more laboratories to ISO 17025 and this is, in turn, a consequence of a never ending drive to analytical quality. That new producers will be more easily able to achieve accreditation should extend the availability of CRMs, which is to the advantage of all striving to achieve good analytical data.

The development

Although accreditation to the combined "Gold Standard" is well accepted by national accreditation bodies in Europe and North America it has not been able to achieve an ILAC global MRA covering CRM production as some national accreditation bodies have not accepted that it is possible to accredit to a Guide. As mentioned above, the arrival of ISO/IEC 17034 and the accreditation of CRM producers to this standard paves the way for an ILAC MRA, facilitating the use of CRMs everywhere.

Into the future

These changes will bring an additional workload to national accreditation bodies and their clients and much will cascade down to the technical auditors, without whom accreditation would not be possible. Satisfying the technical aspect of a surveillance visit can be a daunting challenge and this is something that we would like to help you with. It gives me enormous pleasure to be able to announce that my long-time colleague, Alan Nichols, has agreed to join the "Quality Matters" team and write on the use of RMs and CRMs from the perspective of an accreditation body technical auditor.

I have known Alan since the early 1990s when he ran the Reference Materials Division of Radian Corp., a small specialist business in Austin, Texas, in the USA. Alan had graduated from the University of Texas in Austin and took his profound interest in organic synthesis to Radian. Back in the 1980s, demand for environmental reference materials was growing and Radian won a contract with the US Environmental Protection Agency (EPA) to produce reference materials and Alan was responsible for the synthesis of many compounds of environmental interest. Working in partnership with Cambridge Isotope Laboratories Inc., Alan pioneered the synthesis of many stable isotope labelled dioxins and furans. Radian went on to replicate this success developing reference materials for forensic analysis, which is when I first met him in Germany in 1994.

In the late 1990s, seeking a new challenge, Alan moved to the United States Pharmacopeia where after a spell in marketing he took over the production of USP Pharmaceutical Reference Substances.

In the late 1990s, Alan concluded that life in Washington, DC, was not everything and moved to Laramie, Wyoming, USA to join RT Corporation: at that time I was working very closely with RTC, running their European subsidiary, and with Alan and Bob Rucinski, RTC's owner, had come up with a concept to develop a range of secondary certified reference substances, traceable to the USP Primary RS and certified for purity as a CRM. This innovative programme attracted the interest of Sigma Aldrich Corp, now Merck KgAA, and in February 2011 RTC was acquired by Sigma Aldrich. So Alan and I joined Sigma Aldrich.

In 2012 Alan was asked to relocate to the Sigma Aldrich Supelco facility in Bellefonte, PA, USA, to upgrade the RM production facility to meet the requirements of ISO Guide 34 and ISO/IEC 17025 and achieve accreditation to the "Gold Standard". Achieving an accreditation is one thing, upgrading the thousand or so RMs to CRM status is another, but this was done by late 2015 and in early 2016 Alan retired. He now works as a metrology consultant and Lead Assessor for ISO/IEC 17025, ISO Guide 34 and ISO/IEC 17043.

Alan has an unmatched experience in both CRM production, accreditation and quality management: I look forward to his future contributions to this Column.

SAMPLING COLUMN

Into the laboratory… TOS still reigns supreme

Kim H. Esbensen[a] and Claas Wagner[b]
[a]KHE Consulting, www.kheconsult.com
[b]Sampling Consultant—Specialist in Feed, Food and Fuel QA/QC. E-mail: cw@wagnerconsultants.com

This column has so far treated primary sampling from lots in all shapes, forms and nearly all sizes—but the lots treated have all been *larger* than the typical sample on the laboratory bench. The main lesson from the previous 11 columns was simple and powerful: all types of lots in this size range and all types of materials can be sampled based on the exact same principles, codified in the Theory of Sampling (TOS). Lot size, material, form… in a crucial sense do not matter, all that matters is the degree of heterogeneity that has to be counteracted by the sampling process. With one exception, however, lots that move… *dynamic lots*. The arena of process sampling will be treated in full in just time, but here-and-now the focus shall be on completing the realm of *stationary lot* sampling, by closing the lot size range. With this and the next column we are finally moving into the laboratory focusing on smaller and smaller lots. It does not matter that occasionally some of these operations will take place in the field (think of a large primary sample conveniently being split down in the field with obvious transportation or other advantages). For systematic convenience we shall treat all stages and operations performing sample splitting etc. as taking place in the laboratory—without loss of generality.

Representative sampling—a scale invariant endeavour

Sampling of small lots of the size typical of the work taking place on the laboratory bench, sub-sampling, sample preparation, final aliquot extraction… all involve a greater-or-lesser part of sampling of the same kind as has taken place before the lot in question arrived in the laboratory (indeed sub-sampling is nothing but sampling). The unifying principles promulgated by TOS are all with one aim—to make sampling from heterogeneous materials as simple as possible with the imperative of being representative, at all scales. This is simply a continuation of applying the same TOS principles all the way from the primary lot to the final analytical aliquot. Thus, one of the most powerful unifying governing principles in TOS is that representative sampling is *scale invariant*. The physical dimensions of the sampling tools change so as to be commensurate with the lot size. However, the essential issue is that the sampling process at all times (and scales) is 100% focused on how to counteract heterogeneity (Figure 1).

Another of TOS' simplifying principles is that any reduced mass of the original lot (of course preferentially a representative sample thereof) can be viewed as a lot in its own right. This means that at any sampling stage one *may* temporarily view the current lot as a "primary lot" from which to extract a primary sample (if this is advantageous). This "local viewpoint" is obviously *not* a statement meant to disregard the full pathway, on the contrary. But this local focus leads directly to the understanding that one is faced with exactly the same challenges as when facing the original (larger) primary lot. The local lot is still heterogeneous with all the same ensuing issues… in fact it is only the size of the contemporary lot that is different, nothing else. The sampler is still facing

Figure 1. The laboratory—the realm of the spatula. At this ultimate stage of the long sampling pathway from the original lot, significant sampling errors can still crop up, mostly due to a faulty understanding that if the material *appears* homogenous this justifies grab sampling, spatula sampling. NOTHING could be further from the truth, however.

SAMPLING COLUMN

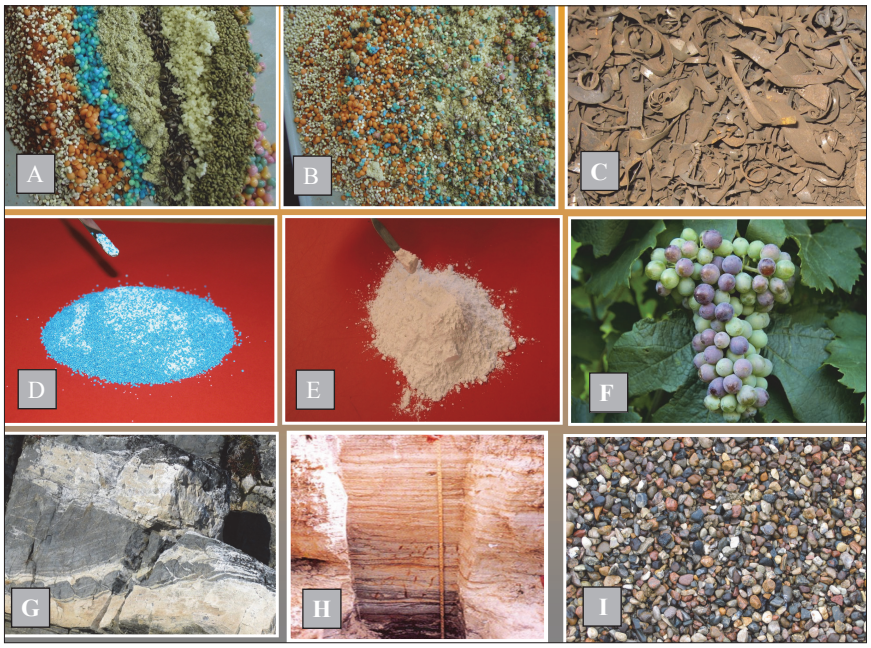

Figure 2. The phenomenon of heterogeneity is intrinsic to each lot material (always displaying both a compositional as well as a spatial component, CH and DH). For this understanding, which determines the necessary sampling process (composite sampling always—never grab sampling), it does not matter what came before. It is actually immaterial that of the "lots" depicted only panels F, G and H are primary lots—all other panels depict samples from a particular later stage in the generic "from-lot-to-aliquot" pathway.

Figure 4. Sampling tool size is set to match the lot size first, but the key function of any sampling tool is always to counteract the material heterogeneity present (CH, DH); see also Figure 3.

the same fundamental problem as when facing the original lot size: how to extract a representative sample from a heterogeneous sample? The lot in question just happens to be smaller than its original precursor.

But since the problem is identical, so are the optional solutions: representative sampling is *scale invariant*. It is only the sampling tools that will have to change their physical dimensions—everything else remains identical. There

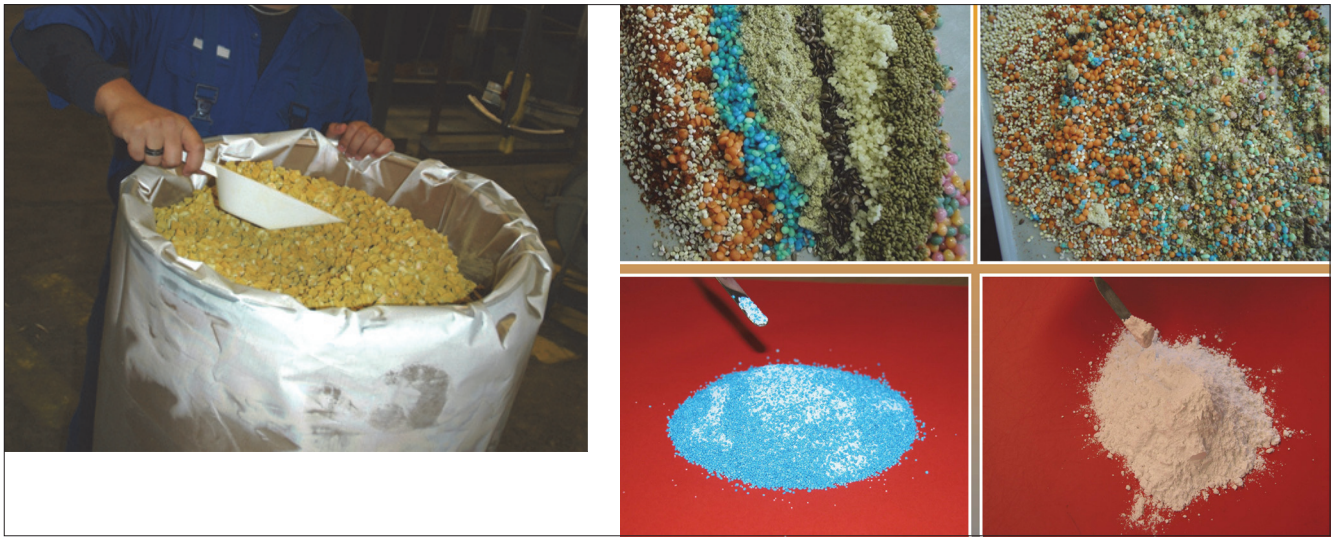

Figure 3. Mesoscale grab sampling (left) vs micro-scale in the lab. (right)—what is the difference? This is the wrong question, at the wrong time and at the wrong place (scale)—what matters is that heterogeneity follows suit all the way to the final aliquot extraction. It truly does not matter whether the human eye can discern any material heterogeneity, or not—60+ years of experience allows TOS to state categorically that all materials are significantly heterogeneous and shall therefore be treated accordingly. This insight actually makes all of sampling immensely easy: act as if all lots, all materials, at all scales are always heterogeneous.

SAMPLING COLUMN

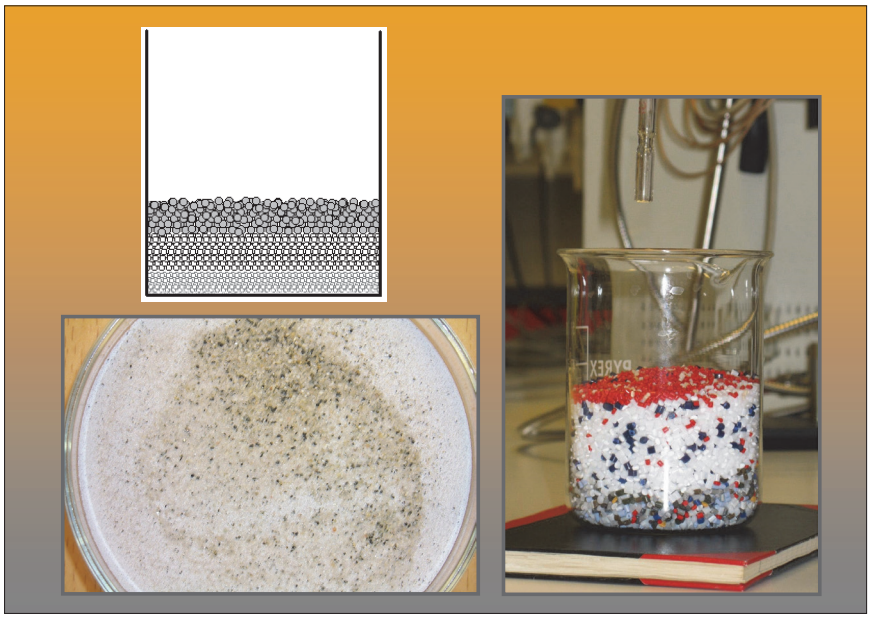

Figure 5. Even at laboratory scales, segregation may present serious heterogeneity problems. Composite sampling is imperative, with the critical proviso that all increments must cover (counteract) heterogeneity in the vertical direction (see previous *SE* Sampling column on "spear sampling"). The exact same principles apply in the laboratory as everywhere else.

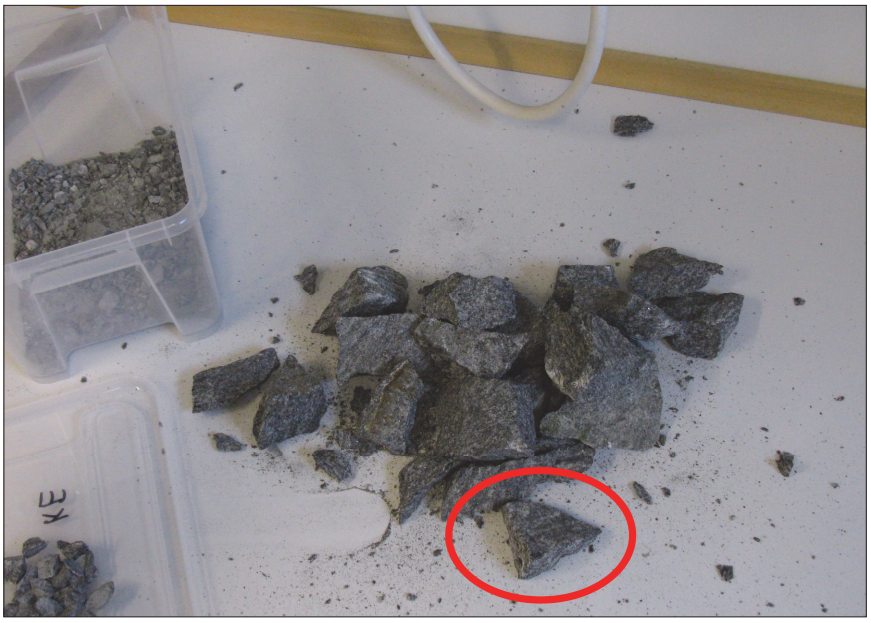

Figure 6. Perhaps the world's most misplaced sub-sampling call: in the process of crushing carefully collected 12 kg composite field samples (>16 increments, as illustrated), assisting students were told by the analytical laboratory head to "forget all this TOS stuff—the usual procedure here is to select a lump the size of what is needed for further treatment and only crush this mass instead of all the silly 12 kg" (the indicated lump is circled—20 g). Luckily the students involved were sufficiently competent w.r.t. TOS to have the courage to neglect such "advice". There is a (sacred) reason why the field composite samples weighed in at a minimum of 12 kg—specifically to counteract the very troublesome *field heterogeneity* encountered. The suggestion to skip the crucial full laboratory crushing stage would have produced a ~600 times smaller sub-sample (12,000 / 20), essentially grab sampling at this sub-sampling stage, which would have eliminated the primary composite sampling objective with a 100% certainty. The mind boggles at the incompetence of the analytical laboratory head!

may, or there may not, be a smaller heterogeneity in the lot now residing on the laboratory bench, depend on the preceding sampling process, i.e. whether some sort of *splitting* was invoked or to which degree *mixing* was part of a composite sampling procedure etc. This is actually not even an important issue—what is certain is that also all sub-samples of original heterogeneous lots are also themselves *intrinsically heterogeneous*. There can, therefore, be no slacking of the demands for representativity at *any* smaller scale than the size of the original lot (Figure 2).

These issues are emphasised with the aim to disallow any-and-all arguments that may be levelled in order to try to justify that different sampling demands reign at the significantly smaller laboratory scale or that different types of sampling equipment are needed, or acceptable, at this scale in the laboratory realm. Following the logic of TOS there can be no other demands to either procedures or equipment at the laboratory scale as at *all* higher scales. TOS's six governing principles and the four Sampling Unit Operations recognised by TOS are the only tools available with which to address sampling—at all scales (Figure 3).

This understanding has many, perhaps at first silly manifestations and consequences, e.g. a digger, or a front loader, with a one-ton front grabber is *identical* to a spatula! Identical in the way it may be used *wrongly* to perform grab sampling if only one increment is extracted and *wrongly* pronounced as a "sample". It is the faulty grab sampling procedure (one increment only) that is identical, albeit performed with radically different sized implements (one ton vs a few grams, perhaps). In this context a digger—is a front loader—is a spoon—is a spatula (Figure 4)!

As with all inherent sampling characteristics (governing principles, unit operations, sampling equipment) the task of the competent sampler is to look behind the superficial manifestations—to find out whether apparent sampling usages actually comply with the simple

SAMPLING COLUMN

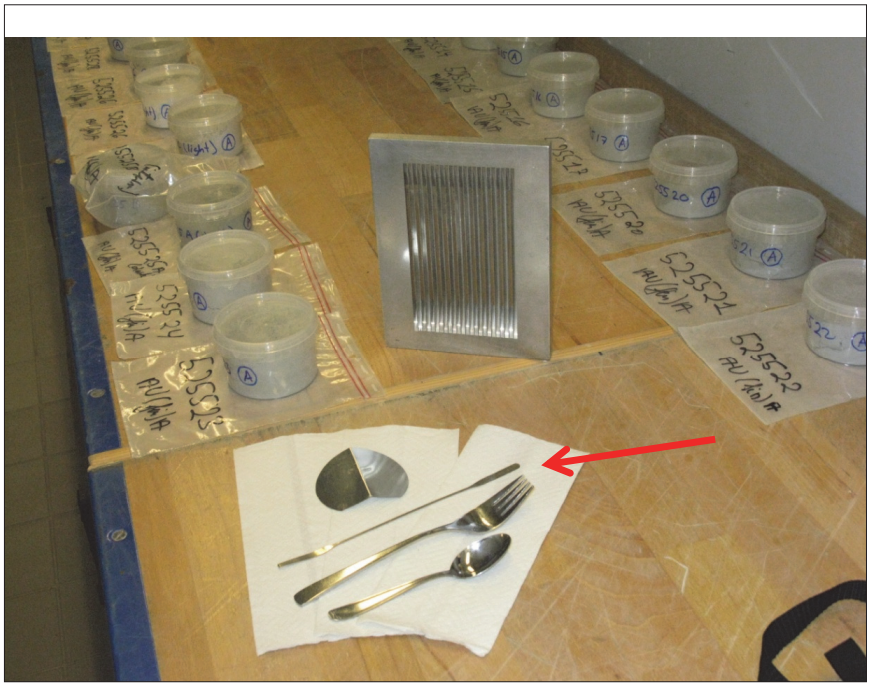

Figure 7. A show of futility. Shown here are genuine sampling and sub-sampling tools in a professional analytical laboratory. The issue was *which* tool is optimal for final analytical aliquot extraction: spoon vs spatula (the fork is supposed to be a mixing implement)? The mind again boggles at the incompetence revealed—grab sampling reigned supreme even at this ultimate, smallest scale.

(6 + 4) demands of TOS, or not. If not, it will never be possible to qualify a particular sampling process as representative, no matter how "ingenious", "smart", "labour-saving", "practical"… at first sight.

Size does not matter—only heterogeneity, and how to counteract it

Figures 5–7 give a few illustrations, all from the laboratory realm. Enjoy how there is absolutely no difference here with respect to examples presented in earlier columns relating to larger scales. Once the simplicity of the governing principle: "representative sampling is scale-invariant" has been comprehended in full, a massive empowerment ensues. Size never matters again—only heterogeneity.

It is fair to state, however, that this insight has not always been present in the gamut of scientific, technological and even occasionally in the sampling literature. A plethora of examples from the literature exist to justify the preceding harsh statement, but it suffices to present but a few spectacularly illustrative cases here. The matter presented above also occupies a central role in later columns under diverse headings, e.g. "Sampling Hall of Fame" and, perhaps more so, in "Sampling Hall of Shame", all in good time.

The example in Figure 6 points forcefully to the issue that there must always be a *unified* sampling responsibility all the way "from-lot-to-aliquot", of which more later.

Further reading

P. Bedard, K.H. Esbensen and S.-J. Barnes, "Empirical approach for estimating reference material heterogeneity and sample minimum test portion mass for 'nuggety' precious metals (Au, Pd, Ir, Pt, Ru)", *Anal. Chem.* **88,** 3504–3511 (2016). doi: https://doi.org/10.1021/acs.analchem.5b03574

K.H. Esbensen, "Materials properties: heterogeneity and appropriate sampling modes", *J. AOAC Int.* **98,** 269–274 (2015). http://dx.doi.org/10.5740/jaoacint.14-234

K.H. Esbensen and C. Wagner, "Theory of sampling (TOS) versus measurement uncertainty (MU) – a call for integration", *Trends Anal. Chem.* **57,** 93–106 (2014).

E. De Andrade, K.H. Esbensen, M.C. Fernandes and A.M. Lopes, "Amostragem representative para uma quantificao precisa de sementes geneticamente modificadas", Ed by P.S. Coelha and P. Reis. Agrorrural – Contributos Cientificos, pp. 604–615 (2011). ISBN 978-972-27-2022-9

L. Petersen, C. Dahl and K.H. Esbensen, "Representative mass reduction in sampling – a critical survey of techniques and hardware", in *Special Issue: 50 years of Pierre Gy's Theory of Sampling. Proceedings: First World Conference on Sampling and Blending (WCSB1)*, Ed by K.H. Esbensen and P. Minkkinen, *Chemometr. Intell. Lab. Sys.* **74,** 95–114 (2004).

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20–23 February, Hilton Head Island, South Carolina, USA. **PANIC 2017– 5th Annual Practical Applications of NMR in Industry Conference.** http://www.panicnmr.com.

27 February–1 March, Porto, Portugal. **5th International Conference on Photonics, Optics and Laser Technology (Photoptics 2017).** photoptics.secretariat@insticc.org, http://www.photoptics.org.

5–10 March, Chicago, Illinois, USA. **The Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (Pittcon 2017).** http://www.pittcon.org.

2–8 April, Vitoria, ES, Brazil. **14th Rio Symposium on Atomic Spectrometry.** Maria Tereza, riosymposium2017@ufes.br.

3–5 April, Dresden, Germany. **XXVI International EPR Seminar.** http://www.ifw-dresden.de/de/institute/institut-fuer-festkoerperforschung/events/xxvii-international-epr-seminar.

7–11 May, Konstanz, Germany. **15th European Workshop on Modern Developments and Applications in Microbeam Analysis (EMAS 2017) and 7th Meeting of the International Union of Microbeam Analysis Societies (IUMAS VII).** Mike Matthews, matthm@hotmail.com, http://www.microbeamanalysis.org/emas-2017.

8–12 May, Faro, Algarve, Portugal. **III International Conference on Applications of Optics and Photonics (AOP 2017).** Eduarda Jesus, Viagens Abreu, S.A., Av. Da República, 124, 8000-079 Faro, Portugal, eduarda.jesus@abreu.pt, http://www.optica.pt/aop2017/.

10–12 May, Potsdam, Germany. **4th European Conference on Process Analytics and Control Technology (EuroPACT 2017).** Nina Weingärtner, DECHEMA e.V., Theodor-Heuss-Alee 25, 60486 Frankfurt am Main, Germany, weingaertner@dechema.de, http://dechema.de/europact17.html.

19–20 May, Kyoto, Japan. **17th Symposium on Molecular Spectroscopy.** Yosuhiro Ohshima, ToykoTech, ohshima_@_chem.titech.ac.jp, http://regulus.mtrl1.info.hiroshima-cu.ac.jp/~molspec/e-index.html.

22–26 May, Strasbourg, France. **European Materials Research Society (E-MRS) 2017 Spring Meeting.** mrs@european-mrs.com, http://www.european-mrs.com/meetings/2017-spring-meeting.

4–8 June, Indianapolis, Indiana, USA. **65th ASMS Conference on Mass Spectrometry and Allied Topics.** http://www.asms.org/.

5–7 June, Jerusalem, Israel. **Mediterranean Conference on the Applications of the Mössbauer Effect (MECAME 2017).** http://www.medc.dicp.ac.cn/conference/mecame/.

11–16 June, Victoria, Canada. **9th International Conference on Advanced Vibrational Spectroscopy (ICAVS-9).** http://www.icavs.org/.

11–15 June, Copenhagen, Denmark. **18th International Conference on Near Infrared Spectroscopy (ICNIRS 2017).** icnirs2017@mci-group.com, http://www.icnirs2017.com.

11–16 June, Pisa, Italy. **Colloquium Spectroscopicum Internationale XL (CSI 2017).** http://www.csi-conference.org/.

19–23 June, Champaign-Urbana, Illinois, USA. **72nd International Symposium on Molecular Spectroscopy.** http://isms.illinois.edu.

19–22 June, Naantali, Finland. **15th Scandinavian Symposium on Chemometrics (SSC15).** http://ssc15.jimdo.com/.

19–22 June, San Diego, California, USA. **BIO International Convention (BIO 2017).** http://convention.bio.org/2017.

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