Available online at www.sciencedirect.com
SCIENCE DIRECT®
C. R. Physique 5 (2004) 907–920
COMPTES RENDUS PHYSIQUE
http://france.elsevier.com/direct/COMREN/

Fundamental metrology/Métrologie fondamentale

Metrology in Chemistry: considerations, approaches and developments on the applicability of methods of 'higher order'

Available online 5 November 2004

Presented by Guy Laval

Abstract

A systematic approach to the realization of global comparability through traceability to the SI of measurement results in chemical measurements has only been initiated since the establishment under the Inter-Governmental Treaty of the 'Metre Convention' of the Consultative Committee for Amount of Substance – CCQM – in 1993. In chemical metrology the measurand is not only defined by the general concept of the quantity to be determined but also by the specific analyte or component one intends to measure, the matrix environment and eventually the applied method. The whole measurement chain, from (sub-)sampling, sample preparation and treatment to final measurement, affects the final measurement result and the measurement uncertainty. Incomplete definition and understanding of the measurand is a major cause for wrong results and lack of comparability. Direct primary and primary ratio methods are to a certain extent in principle available, but in several cases not very practical or even not applicable. For the calibration of the whole measurement process one needs homogeneous, well characterized and defined pure calibrants or matrix CRMs. So, purity analysis is of fundamental importance. The field of metrology in chemistry is very wide, so only a few examples are described in this article. Considerations behind the use of different measurement procedures and methods of 'higher order', including primary methods, are given, with results achieved. ***To cite this article: R. Kaarls et al., C. R. Physique 5 (2004).***

Résumé

Métrologie en chimie : considérations, approches, et développements sur l'applicabilité des méthodes d'ordre supérieur. La mise en œuvre des comparaisons globales des résultats des mesures chimiques par leur traçabilité au SI n'a commencé que depuis la création, dans le cadre de la Convention du mètre, du Comité consultatif pour la quantité de matière (CCQM) en 1993. En métrologie chimique, le mesurande n'est pas seulement défini par le concept général de la quantité à déterminer, mais aussi par le composé spécifique que l'on veut mesurer, la matrice dont elle fait partie et, finalement, la méthode appliquée. La chaîne complète de mesure, depuis l'échantillonnage préliminaire, la préparation de l'échantillon et son traitement jusqu'à la mesure finale, affecte le résultat final de la mesure et son incertitude. Une définition incomplète et une compréhension imparfaite du mesurande est la cause majeure des résultats inexacts et de l'impossibilité de les comparer. Des méthodes primaires et des méthodes primaires donnant le rapport des quantités existent en principe, mais dans plusieurs cas sont difficiles, sinon impossibles, à mettre en œuvre. Pour étalonner une procédure complète de mesure, il faut avoir des matériaux ou des matrices de référence homogènes, bien définis et caractérisés. Le domaine de la métrologie en chimie est très vaste, si bien qu'on ne décrit dans cet article que quelques exemples. Des réflexions relatives à l'utilisation des différentes procédures et méthodes de mesure et des méthodes d'ordre supérieur, incluant les méthodes primaires sont données avec les résultats obtenus. ***Pour citer cet article : R. Kaarls et al., C. R. Physique 5 (2004).***

Keywords: Metrology; Traceability; Measurand; Primary methods; Procedures of 'higher order'; Purity analysis; Inorganic analysis; Organic analysis; Bio-analysis

Mots-clés : Métrologie ; Traçabilité ; Mesurande ; Méthodes primaires ; Méthodes d'ordre supérieur ; Matrices de référence caractérisés ; Analyse inorganique ; Analyse organique

1631-0705/2004 Published by Elsevier SAS on behalf of Académie des sciences.
doi:10.1016/j.crhy.2004.09.002

1. Introduction

Robert Kaarls[a,*,1], Martin J.T. Milton[b]

[a] *CCQM, Klaverwydenstraat 13,NL-2381 VX Zoeterwoude, The Netherlands*
[b] *National Physical Laboratory, Queens Road, Teddington, Middlesex TW11 0LW, UK*

The science of metrology is based on a number of practical principles that can be usefully applied to any type of measurement. Although these principles were largely developed for applications in the physical sciences and engineering, it is now recognised that some of the greatest challenges for metrology occur in the measurement of chemical and even biological quantities. Measurements of these quantities are very widespread and are not only of importance to trade but have such potential for influencing the quality of life within society and for underpinning the implementation of regulation that 'chemical and biological metrology' may yield some of the best examples of the benefits of metrology in the 21st century.

The agreement in 1875 by the nations signing up to the 'Convention du Mètre' initiated international activities aimed at providing access to comparable measurements of all types. Yet, when the Système International d'Unités (SI) was established in 1960 it was based on a set of six base units that were largely chosen to meet the needs of physical and engineering measurements. It was not until 1971 that the mole, the base unit of the quantity amount of substance, was established as the seventh base unit of the SI, its definition reading as follows: "*The mole* (*symbol* 'mol') *is the amount of substance of a system which contains as many elementary entities as there are atoms in 0.012 kilogram of carbon 12*." It is understood that reference is made to unbound atoms of carbon 12, at rest and in their ground state. When the mole is used, the elementary entities *must* be specified and may be atoms, molecules, ions, electrons, other particles, or specified groups of such particles.

This definition leads to a definition of the Avogadro constant, which is used universally in chemistry and is the essential scaling factor that links the atomic and macroscopic worlds.

Although the introduction of the mole into the International System of Units (SI) provided the basis for dimensional analysis within chemistry, a large majority of chemical measurements are of intensive quantities, such as amount of substance fraction (the fraction of the number of entities of one type to the total number of entities in a sample) or mass fraction (the fraction of the mass of entities of one type to the total mass of the sample). These intensive quantities are usually expressed in terms of the ratio of quantities expressed in the same units. Since the units can be cancelled, the quantities are dimensionless and the focus of practical scientists is taken away from the need to establish traceability to a base unit of the SI. The fact that many chemical measurements achieve their traceability to the mole by combining the results of measurements of the mass of samples with their relative molecular mass (RMM) has provided further reason why the mole is not always recognised as being the basis for chemical measurements.

The Metre Convention recognised the need to develop an international infrastructure for the whole range of chemical measurements in 1993 with the establishment of the CCQM (Comité Consultatif pour la Quantité de Matière). The first meeting of the CCQM stated that a realisation of the mole was not required and that since chemical measurements were often traceable directly to measurements of mass, volume or other quantities that it was preferred practice to make all of the measurements 'traceable to the SI'. It also identified the need for the development of primary methods of measurement [1] that were capable of making chemical measurements. They were characterised as being of 'the highest metrological quality' and able to operate 'without reference to standards of the same kind'. Although primary methods provide the basis for traceability, there are many important measurements for which practical and accurate primary methods are not available.

Within three years of its foundation, the CCQM identified the need for specific activities to improve the comparability of measurements within gas analysis, organic analysis and inorganic analysis. Subsequently, its activities have grown to encompass electrochemical analysis, surface analysis and most recently biological analysis. Additionally, it has developed joint activities with several other inter-governmental and international bodies, like in the areas of laboratory medicine and food analysis.

It has been recognized that, to a greater extent than in other fields of metrology, the whole process, starting with the preparation of the sample to be measured leading through to the final measurement, contributes to the final measurement result and its measurement uncertainty. In most cases the uncertainty components involved in the preparation of the sample are considerably larger than those caused by the measurement itself.

In general, the uncertainty components related to taking a sample outside the laboratory are not taken into account for the calculation of the measurement uncertainty in the final measurement result. However, the measurement uncertainty components involved in the sub-sampling, sample preparation (such as dilution, drying time and temperature, digestion, extraction, etc.) and the calibration of all measuring devices used in the analysis, are all taken into account for the final calculation of the measurement result.

* Corresponding author. President, Comité Consultatif pour la Quantité de Matière.
[1] E-mail addresses: rkaarls@euronet.nl (R. Kaarls); martin.milton@npl.co.uk (M.J.T. Milton).

In several cases one of the major causes of lack of comparability of measurement results in chemistry is caused by the fact that the measurand is not well defined or even not really understood. For example, the influence of the matrix environment in which the analyte to be measured is situated is not well understood, or instead of total extraction only partial extraction of the analyte has taken place. In many cases the measurement result is method dependent. If the measurement procedure is applied incompletely deviating results are obtained. Consequently in chemical measurements the measurand is not only defined by the general concept of the quantity to be determined but also by the specific analyte or component one intends to measure, the matrix environment and eventually the method applied. So, it is essential that the measurand has to be understood and defined completely and very clearly. In principle, it should be possible to write down the complete measurement equation including all influence parameters.

Calibration of the analytic chemical measurement device is normally done with pure elemental solution calibrants. To check the overall performance of the measurement process one makes use of a certified matrix reference material (matrix CRM). If needed, or desirable, a so-called recovery correction is made for the difference between the CRM value and the measurement result. In those cases the matrix CRM used is part of the calibration chain establishing traceability. So, in principle the measurement equation should include also this matrix CRM component. Scientific discussions on this issue are still continuing. It is, however, essential that the measurement equation is complete and completely understood.

This article has not covered the issue of 'commutability', which is of crucial importance, for example, in the field of clinical measurements. It is clear that an essential element in these measurements is that a matrix CRM used to verify the calibration chain behaves and delivers the same result as a real biological sample.

As purity analysis is essential for calibrating the chemical measurement process, we will discuss in this article some of the basic approaches for purity analysis.

Further we will present some examples of and the considerations behind the application of 'higher order' measurement procedures and methods in the fields of inorganic, organic and bio analysis. The scientific discussion on the definition and applicability of *direct primary methods* and *primary ratio methods* is not yet finalized and is thus still on going.

2. A practical approach to certifying the purity of single-component reference materials

Steven Westwood[a,2], Laurie Besley[b]

[a] *National Measurement Institute of Australia, PO Box 385, Pymble NSW 2073, Australia*
[b] *National Measurement Institute of Australia, Bradfield Road, West Lindfield, PO Box 218, Lindfield NSW 2070, Australia*

The role of a reference material in quantitative chemistry is an important one. Ideally the result of any quantitative chemical measurement should have traceability to the units in which the result is expressed. In practice this often means traceability to the certified reference material that is used to calibrate and validate the measurement method. The task of the reference material producer, therefore, is to certify the material in such a way that its property values are traceable to the measurement units, thus providing a traceability path for its end user, and to provide that user with a statement of the property value that has a known uncertainty. In the case of single-component reference materials, the challenge is then to measure the purity of the material using a technique whose result has the above characteristics.

The purity of a nominally pure substance can be established by either direct assay of the material (which we refer to here as the 'direct' approach) or by measuring the amount of substance fraction of every impurity, adding these together and subtracting the result from 1 (which we refer to as the 'indirect' approach). The 'direct' approach has an advantage in theory that it measures directly the property of interest, the concentration of the major component of the material. Because it is based on a single measurement, in theory also the calculation of the uncertainty of the measurement result is simpler. There are a number of candidate 'direct' methods. A traditional one has been freezing point depression, using either adiabatic calorimetry or differential scanning calorimetry. More recently other techniques, notably nuclear magnetic resonance spectroscopy, have also made claims. Each method has particular strengths and weaknesses. In practice, however, no single technique has yet been shown to be capable of covering all situations and the 'direct' approach runs the risk of serious error if the technique used is not sensitive to significant impurity components.

In contrast, the 'indirect' strategy of measuring the level of each of the impurities individually is more rigorous although it is much more time consuming and a robust estimation of the uncertainty of the result obtained is not straightforward. However, the challenge of quantifying each of the impurities is made a little easier by the outcomes of the processes that are used to purify the material to the stage where it justifies characterisation. Most purification processes are such that the major impurities

[2] E-mail addresses: steven.westwood@agal.gov.au (S. Westwood); laurie.besley@csiro.au (L. Besley).

remaining in the purified material are of a very similar chemical structure and composition to it, and are of a similar molecular weight. This means that often a technique such as GC-FID can be used to quantify all of the impurities in a single analysis of an organic substance. It also implies that measurements that are made in terms of mass ratios can be expressed in terms of amount of substance ratios and only a negligible increase in the uncertainty of the result will be introduced. There is one significant exception to this statement and that involves impurities that may be introduced by any use of solvents in the purification process, the most prevalent residual impurities, of course, being the solvents themselves.

Finally it should be noted that some candidate reference materials will contain impurities that are quite different to the main analyte because of the way they are produced. Such a situation is outside the scope of the current purity assessment protocol.

In what follows we describe a combination of both the 'direct' and 'indirect' approaches. This is the strategy used in NMI for the purity certification of single-component organic substances, a common type of pure-substance reference material, and which we believe offers the best solution to this challenge. In this approach the assigned purity value is based on 'indirect' measurements of individual impurity levels, but is cross-checked by 'direct' assay. The strategy is a revision and development of our initial approach described previously, [2].

2.1. The combination approach

Gas chromatography using a flame ionisation detector (GC-FID) and capillary columns is a well-established technique offering good resolution of the impurities, impressive limits of detection and excellent dynamic range, so that both the major component and the impurities can be determined in a single run. It forms the cornerstone of our approach. Weaknesses of the GC-FID method are that it can fail to detect both very volatile and non-volatile impurities, it does not measure moisture content and for low volatility compounds, care must be taken to demonstrate that artefact peaks are not introduced as part of the injection process. However, we believe that its combination with data from other techniques such as HPLC, NMR, DSC and TGA to correct for impurities not detected by GC-FID, and the use of elemental microanalysis as an overall check for serious omissions, constitutes a primary measurement method.

The algorithm used to calculate the purity and its associated uncertainty is simple:

$$X_{\mathrm{A}} = 1 - X_{\mathrm{GC}} - X_{\mathrm{Other}}, \tag{1}$$

where:

X_{A} = amount of substance fraction of the major component A,

X_{GC} = amount of substance fraction of impurities potentially able to be quantified by GC-FID,

X_{Other} = amount of substance fraction of other impurities not able to be quantified by GC-FID.

The term X_{GC} itself comprises three components:

$$X_{\mathrm{GC}} = X_{\mathrm{Det}} + X_{\mathrm{NR}} + X_{\mathrm{ND}}, \tag{2}$$

where:

X_{Det} = amount of substance fraction of impurities directly identified and quantified with GC-FID,

X_{NR} = amount of substance fraction of impurities that could be detected by GC-FID but were not resolved from the active compound by the chromatographic process,

X_{ND} = amount of substance fraction of impurities whose concentration is below the detection limit of the GC-FID apparatus.

We now proceed to examine each of the terms in Eqs. (1) and (2) and define a process to estimate the uncertainty associated with each of them.

2.2. Evaluation of X_{Det} and its associated uncertainty

X_{Det} is calculated by comparing the area under the GC peak of the impurities in question with the area under a peak corresponding to a known mass of the parent compound A, a measurement result that is thus traceable to the units of mass. The response of the FID to the impurities is assumed to be the same as that of the compound A. This is a reasonable assumption as the FID response is proportional to the number of carbon atoms in the analyte and is relatively insensitive to structural variations. We have already shown that the impurities are likely to be compounds of similar composition and molecular weight to that of the major component A.

What is more difficult is to estimate the uncertainty associated with this value of X_{Det}. We have based our estimates of this uncertainty on experimental measurements. We have carried out numerous GC-FID bias checks by gravimetrically mixing small amounts of representative impurities with a highly-purified main material and then measuring the indicated purity using GC-FID. The analysis of these data served to quantify both the bias and the precision associated with the GC-FID measurement of impurity levels. Regression analysis indicated that when analysing compounds of low volatility with our equipment the

GC-FID method slightly underestimated the gravimetric amount of impurity present. When we defined an empirical GC-FID correction factor F_{GC}, where

$$F_{GC} = \frac{\text{(impurity content (mass \%) from gravimetry)}}{\text{(impurity content (Area \%) from raw GC-FID data)}}$$

the data gave values for F_{GC} that had a mean of 1.07 with a standard deviation of 0.06. The detail of this work has been reported elsewhere [3]. In our current standard procedure, therefore, the relative peak area response determined for the total detectable impurities determined directly from the chromatogram is multiplied by 1.07 to calculate X_{Det} and that value is assigned a standard uncertainty of 0.06 times X_{Det}.

2.3. Evaluation of X_{NR} and its associated uncertainty

Typically the evaluation of X_{NR} is made by using either GC-FID with a different column type to that used to determine X_{Det}, or a completely different technique, such as high-performance liquid chromatography (HPLC). If no additional impurities are identified through this process, a single impurity is still assumed to be present at a concentration value between zero and the LOD of the additional method. The value given to X_{NR} is half the LOD of that method and its uncertainty $u(X_{NR})$ is that associated with a triangular distribution.

2.4. Evaluation of X_{ND} and its associated uncertainty

The inclusion of this factor is based on the assumption that a number of impurities might be present but remain undetected because their concentration is below the LOD of the techniques used, and that their combined effect would be significant. The limit of detection (LOD) of the GC-FID is typically 0.02%. The number of impurities assumed to be present is estimated on the basis of experience with a particular type of compound and the concentration of each impurity is assumed to be half the LOD, i.e. 0.01%. Thus if there are assumed to be two undetected impurities present, X_{ND} will be 2×0.0001, or 0.0002. The uncertainty of X_{ND}, $u(X_{ND})$, is assumed to be that corresponding to a triangular distribution, i.e. $u(X_{ND})$ will be $X_{ND}/2.45$.

2.5. Evaluation of X_{Other} and its associated uncertainty

The components contributing to X_{Other} are volatile impurities and, less commonly, non-volatile residues. The volatile residues are generally related to solvent used in the production or final stages of purification of the material. Thermogravimetric analysis (TGA) permits an initial quantification of these when their presence is detected. This result is cross-checked for consistency with data obtained by other techniques such as ^{1}H NMR analysis and elemental microanalysis. The uncertainty associated with the value is assumed to be that of the TGA method.

When no evidence of impurities of these types is seen it is difficult to establish a value and an associated uncertainty for X_{Other}. What is assumed in that case is that impurities exist at a concentration half that of the LOD for TGA, and that the uncertainty of that value corresponds to that of a triangular distribution.

2.6. Combination of all data

When all of the terms X_{Det}, X_{ND}, X_{NR} and X_{Other} have been quantified as described above, X_A is calculated from Eqs. (1) and (2). The purity result can be expressed in this way as an amount of substance fraction, or converted to a mass percentage. The standard uncertainty associated with this value is calculated in the usual way by the procedures of the ISO-GUM by taking the square root of the sum of the squares of each of the component uncertainty terms $u(X_{Det})$, $u(X_{ND})$, $u(X_{NR})$, and $u(X_{Other})$.

2.7. Cross-check by assay techniques

The total level of impurities is evaluated independently by using at least two assay techniques, chosen for their suitability for the material in question. HPLC, ^{1}H NMR, GC-MS and DSC are all employed singly or in combination. The result is a 'direct' value for the total impurity level. If this is consistent with the sum of the various impurity terms explored above, within the limits of the calculated uncertainty, the 'indirect' values are assumed to be the definitive purity measurements.

If the data from the 'direct' and 'indirect' methods do not agree, an attempt is made to identify the causes of the disagreement and correct the results appropriately. If the differences cannot be resolved, an unrecognised bias is assumed to be present in at least one of the results and a weighted mean of the data is taken for the certification value.

2.8. Conclusion

The combined method for quantifying the purity of a chemical substance gives the user both maximum confidence in the property value derived by it, and a highly-reliable estimate of the uncertainty associated with that value. It is a conservative approach, because it introduces minor allowances for impurities that have not actually been detected, but which experience leads us to believe are probably present in amounts below the detection limits. It is thus more likely to overestimate the level of impurity in the substance rather than to underestimate it. It also provides traceability of the result to the fundamental units of measurement. For these reasons we recommend it as the most appropriate method available for the characterisation of pure-substance reference materials.

3. Inorganic analysis

Mike Sargent[3]

Laboratory of the Government Chemist LGC, Queens Road, Teddington, Middlesex TW11 0LY, UK

Analytical chemistry originated with the inorganic analysis undertaken by alchemists investigating the possibility of producing gold and silver from base metals. As that aspiration grew ever more unlikely, the emphasis turned towards measuring the amount of each element in mixtures such as the ores or alloys of these precious metals. Just as is the case today, it was vital for purposes of trade that assays performed at different places or times should be in agreement. Without realising the implications, the chemists of the day applied the principles of metrology to meet this requirement.

Thus, classical inorganic analytical chemistry depended on traceable calibration of balances and volumetric glassware to achieve comparable data between laboratories. Analytical laboratories placed great emphasis on the origin of the calibrated weights and glassware used in their gravimetric or titrimetric analysis methods. They also recognised the need to safeguard the integrity of these calibrated artefacts which, even more than 100 years ago, formed part of a national 'weights and measures' infrastructure based on metrology. In many analytical laboratories, for example, a single set of balance weights would be externally calibrated and traceable to a national reference. These 'reference weights' would then be used within the laboratory for secondary calibration of other balance weights or volumetric glassware. This task was an important, routine aspect of laboratory operations and its importance was emphasised during the education and training of every chemist.

Classical analysts also recognised that metrology in chemistry is not concerned per se with the determination of mass and volume. They were acutely aware that their task was not merely to determine 'amount' but the amount of a specific chemical entity such as, for example, gold. Hence the chemist was required to correctly identify the chemical entity being measured and for this purpose a 'chemical standard' was necessary. This is also the fundamental requirement for metrology in chemistry. It was common practice in the analytical laboratory until quite recent times for the chemist to prepare such chemical standards in-house. A good knowledge of chemistry was essential to ensure that the chemical standards comprised the correct substance and were of sufficient purity for the purpose in hand. This activity was an important part of a chemist's training, which emphasised that any error in assessing the identity or purity of such a chemical standard would affect the reliability of measurements dependent on it. This, too, was an early application of the principles of metrology to chemical measurement.

Recognition of the value of applying the principles of metrology to underpin comparability of measurements in inorganic analysis saw a period of decline from the 1940s until almost the present day. The decline coincided with rapid growth in the application of instrumental methods for routine inorganic analysis; many analysts believed that with instrumental methods the concepts of metrology were no longer relevant to analytical chemistry. This situation was reflected in the growth of the chemical reagent industry, which increasingly supplied the chemical materials and calibration solutions needed by laboratories undertaking inorganic analysis. In order to ensure reliable reagents each supplier adhered to an agreed specification. These specifications were, however, largely local or sector-based and little attempt was made to adopt the concepts of metrology and traceability. Thus there was rarely an international basis for these materials and no single, international measurement infrastructure existed to underpin them.

This situation came about because calibration using chemical standards is complicated by the dependence of the chemical measurement process on the sample matrix. In classical inorganic analysis this problem was overcome by quantitative removal of the analyte from the matrix prior to measurement or by using appropriate chemistry to overcome matrix interferences. This approach was feasible in many cases because the number of analyses undertaken was quite small and, in general, analyte concentrations were relatively high. In routine instrument-based methods, many applications involve rapid trace analysis and, in

[3] E-mail address: mike.sargent@lgc.co.uk (M. Sargent).

addition, the instrumental determination is often not completely free of matrix interferences even after extensive pre-treatment of the sample. Hence calibration of the instrument using a pure chemical standard, even a traceable one, is on its own insufficient to achieve adherence to metrology principles. However, in the past 10–15 years, a number of national measurement institutes have attempted to overcome this problem through application of a technique known as isotope dilution mass spectrometry (IDMS) which was initially developed for elemental analysis during the 1950s [4]. As described below, IDMS has the potential to overcome the issue of sample matrix effects, allowing an unambiguous relationship between the analyte content of a complex sample and a traceable, chemical standard of the pure analyte.

A key advantage of mass spectrometry lies in its ability to use an isotopically enriched or labelled analogue of the analyte as an internal standard. This enables exact compensation to be made for errors at all stages of the analysis, from sample digestion/preparation through to the final measurement. In order to achieve this goal, the isotopic analogue is added to the sample at the very beginning of the analytical method and should come into equilibrium with the natural analyte without loss or isotopic fractionation. Initially, IDMS of inorganic analytes was most frequently performed using thermal ionisation mass spectrometry (TIMS). More recently, IDMS based on inductively coupled plasma mass spectrometry (ICP-MS) has become more prevalent, because ICP-MS requires much less sample preparation prior to analysis yet still provides results of the accuracy required for application to metrology.

The underlying principle of IDMS is quite straightforward [5]. An accurately known amount of the isotopic analogue is added to the sample as explained above. The ratio of the amounts of the two isotopes is measured on a portion of the sample using a mass spectrometer, so enabling the unknown concentration of the natural analyte present in the sample to be calculated. A number of approaches have been developed to achieve this but in a typical IDMS experiment the main stages are as follows:

(1) Characterisation of the isotopic analogue using a traceable natural standard by a ‘reverse IDMS’ analysis. If a certified isotopic analogue is used this information is provided in the certificate.
(2) Addition of an accurately known amount of the isotopic analogue to an accurately measured portion of the sample. This step is widely referred to as ‘spiking’ the sample.
(3) For inorganic analysis, dissolution of samples and destructive digestion of organic matter is usually necessary in order to attain isotopic equilibration. Sample preparation may also involve a suitable extraction or purification step.
(4) Introduction of an aliquot of the equilibrated spiked mixture into the mass spectrometer followed by accurate measurement of the isotopic ratio, i.e. the ratio of the signal responses for the ions resulting from the analyte and the isotopic analogue.

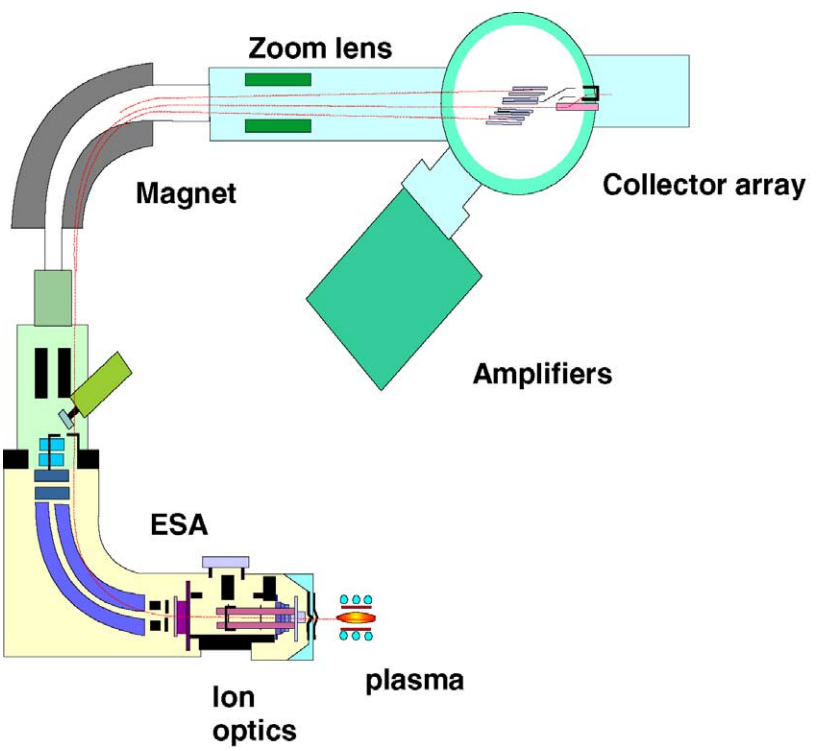

Fig. 1. Construction of a high resolution, multi-collector, inductively coupled plasma (ICP) mass spectrometer.

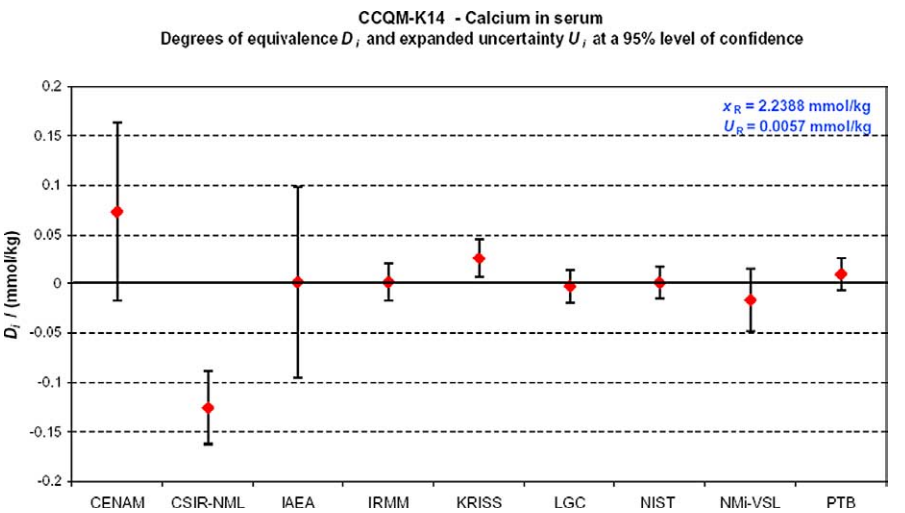

Fig. 2. CCQM key comparison for Calcium in serum. All participants used IDMS with scanning ICP-MS instruments except PTB (IDMS with TIMS) and IAEA (non-IDMS method).

Table 1
Typical ranges available for the best precision attainable for isotope ratio measurements

Type of mass spectrometer	Approx. range of optimum precision (% RSD)
Most TIMS	0.01–0.005
Recent TIMS	0.01–0.001
Quadrupole ICP-MS	0.2–0.1
Magnetic sector scanning ICP-MS	0.1–0.05
Multi-collector ICP-MS	0.01–0.002

(5) Calculation of the concentration of analyte from the isotopic ratio by comparison with the same ratio measured for the same ions in standard calibration mixtures. The calculation includes corrections for instrumental effects, such as mass bias and detector dead-time.
(6) Performance of a separate 'blank analysis'. This is required when using IDMS because any isotopic contribution to the mixture (from reagents, contamination, etc.) will affect the isotopic ratio and ultimately lead to a systematic bias.

Provided that the above stages are correctly undertaken, the ICPS result is directly traceable to the natural calibration standard of the pure analyte (or to the spike if an enriched material is used which is certified for the amount content of the labelled analyte). In addition, the uncertainty of the result will depend primarily on the uncertainty of the measured isotope ratio [6]. Thus the ability of the mass spectrometer to measure isotope ratios with the highest precision is critical if small measurement uncertainties are to be achieved by IDMS.

Until recently, the required isotope ratio precision was best achieved for inorganic analytes using TIMS instruments. However, advances in ICP-MS instruments have allowed their application to a wide range of measurements with sufficient precision for many metrological applications. Most such instruments use a single ion collector (detector) and must be scanned from one isotope to the next. Thus the two isotopes required for the IDMS experiment are measured sequentially. Instrumental changes during this scanning period limit the overall precision of the measured isotope ratio. Recently, multi-collector ICP-MS instruments have enabled simultaneous measurement of two (or more) isotopes. This provides similar levels of precision to TIMS, allowing the application of high accuracy IDMS measurements to inorganic analysis of a wide range of sample matrices. Table 1 illustrates typical ranges of the best precision attainable for isotopic ratio measurements with different types of mass spectrometer. Fig. 1 shows schematically the construction of a mass spectrometer of this type. In practice, many NMIs undertaking inorganic IDMS measurements find the scanning ICP-MS instruments suitable for their calibration services and the equivalence of their capabilities has been evaluated in a large number of international comparisons organised by the Inorganic Analysis Working Group of CCQM. The results of a typical key comparison are shown in Fig. 2.

4. Organic analyses

Willie E. May[4], Reenie Parris

National institute for Standards and Technology, Analytical Chemistry Division, Chemical Science and Technology Laboratory, ACSL Bldg. (227), National Institute of Standards and Technology, Gaithersburg MD 20899, USA

Measurements to assess the chemical composition of a material or to determine the amount of a particular substance in a material are critical in providing information needed to assure equity in trade, monitor and enhance industry's products and services, and to assess and improve public health, safety, and the environment (Fig. 3). A large number of these measurements are for organic chemical entities in a very diverse range of matrices (Fig. 4). In addition to this multiplicity of analytes and matrices, challenges associated with chemical measurements of organic measurands in 'real samples' include the need for accurately measuring 'practically nothing' in the midst of 'everything else' without the benefit of absolute or quantum-based methods and the multiplicity of methods being used.

For organic measurements, an essential first step is specifying the measurand which can range from a well-defined compound such as benzo[a]pyrene to a specific isomeric form of a more complex molecule such as Cardiac Troponin-I, or could be a measure of both free and bound forms of an entity as with 'total cholesterol', or a procedurally defined measurand such as 'total extractable hydrocarbons using a specific cited method', etc. Typically, for organic analytical measurements, the instrumental analysis technique is only part of the measurement process or 'method' required to determine an individual molecular entity. Usually, the method will include some, if not all, of the following steps: extraction of the analyte from the sample matrix; further isolation or fractionation based on class, size, etc.; transformation to afford better extractability, separation, or detection; chromatographic separation from similar chemical entities; selective detection of the analyte; and, transformation of signal into chemical information. These steps are typically part of any organic analytical measurement procedure whether performed in routine chemical testing laboratories or by National Metrology Institutes (NMIs) when providing calibration services or value-assigning CRMs. Based on intended use of the measurement results and fit-for-purpose measurement needs, chemical testing laboratories typically will employ methods that are fast and cost-effective and NMIs typically employ 'higher order' methods where emphasis is placed on methods of low bias, whose operation can be completely described and understood, and for which a complete uncertainty statement can be written in terms of SI units.

Fig. 3. A diversity of needs.

[4] E-mail addresses: willie.may@nist.gov (W.E. May); reenie.parris@nist.gov (R. Parris).

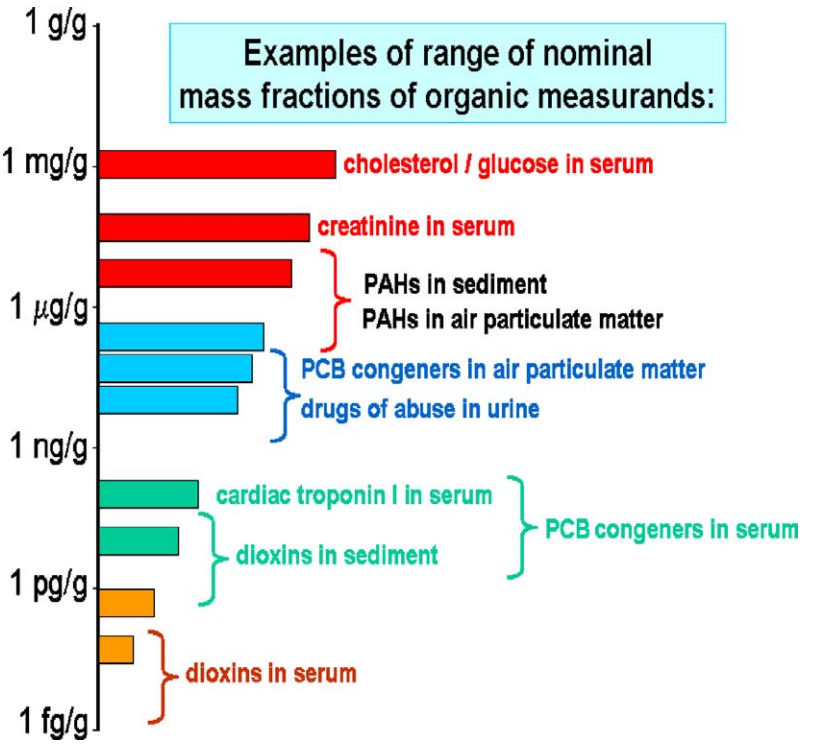

Fig. 4. Range of nominal mass fractions.

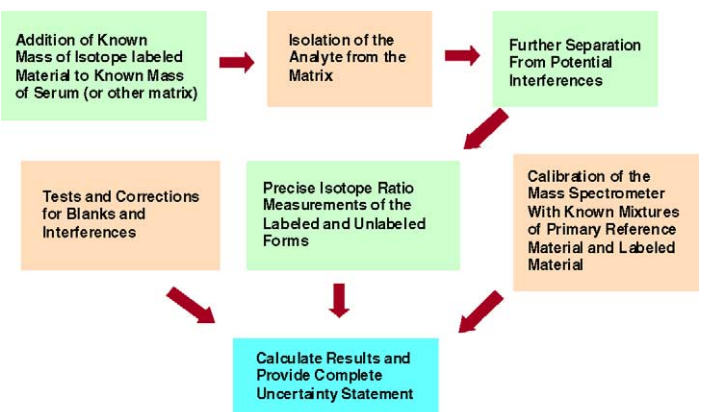

Fig. 5. General approach for organic analytical measurements based on isotope dilution/mass spectrometry.

Measurement methods should be designated as ‘higher order’ based on *demonstration* and not *designation*. Various instrumental techniques can provide very precise measurement results. However, the instrumental analysis is only part of the overall organic analytical measurement procedure as discussed earlier and is often not the major contributor to the overall uncertainty of the measurement. Although a method may be demonstrated as having ‘higher metrological qualities’ for one compound, at a particular concentration, and in a particular matrix, this does not necessarily mean that it provides ‘higher order’ results for all species, at all concentrations, and in all matrices.

Isotope-dilution/Mass Spectrometry, shown schematically in Fig. 5, is generally recognized as being a ‘primary-ratio’ method for analysis of organic entities in complex matrices.

In practice, the isotope diluent is only a nearly chemically identical internal standard that enables provision of very precise analyte-to-internal standard ratios for transformation into amount of substance units. In order for this transformation to be accurate, other factors that arise from other process steps must be critically evaluated, including:

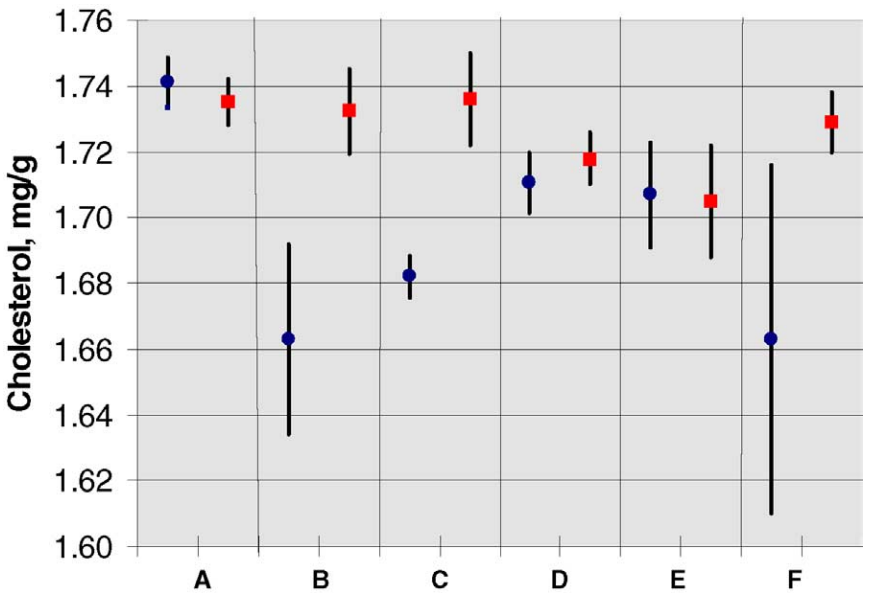

Fig. 6. CCQM: comparison of results for cholesterol in serum in 1999 Pilot Study ● (mean ± U: 1.700 ± 0.087 mg/g) and in 2000 Key Comparison ■ (CCQM-K6.b) (mean 1.726 ± 0.013 mg/g).

- Completeness of equilibration of isotope diluent with the matrix;
- Completeness of extraction of analyte as defined relative to the isotope diluent;
- Chemical interferences;
- Calibration errors;
- Instrument instability;
- Memory effects;
- Differences in fragmentation of the analyte and isotope diluent.

Fig. 6 shows results from the analysis of the same sample in a CCQM Pilot Study in 1999 and a Key Comparison in 2000 on the determination of total cholesterol in human serum unbeknown to the participants. The repeat sample was one of two analyzed in both comparisons.

Although each NMI used gas chromatography/isotope dilution mass spectrometry (with a C-13 isotope diluent as the internal standard) in both comparisons, the results of laboratories B, C, and F were considerably lower than those of Laboratory A in the pilot study. A meeting was held shortly after submission of the Pilot Study results for presentations of the methods used, followed by discussions and critical evaluation of the results and methods. After the discussions, it was agreed that results from these three laboratories were most likely biased low due to incomplete hydrolysis of cholesterol esters in the serum. These laboratories subsequently assessed the completeness of their hydrolysis, modified their sample preparation procedures accordingly, and participated in the 2000 Key Comparison, where the results of the overall study were much more comparable in general and their results were in excellent agreement with Laboratory 'A' that had more than fifteen-years experience in the 'higher order' determination of cholesterol in serum pools.

To facilitate responsible decision-making and confidence in measurement results, it is important that the results be comparable and not that the methods be the same. The results presented in Figs. 7 and 8 from CCQM-K27 demonstrate that comparable results can be obtained from critically evaluated methods based on GC-FID and those based on GC-ID/MS and titrimetry. This is not at all uncommon for samples that do not require a great deal of sample preparation prior to analysis.

In conclusion, mutual recognition and confidence in data are required to facilitate and underpin international trade and decisions regarding health, safety, commerce and/or scientific studies. The organic chemical measurement universe encompasses an enormous range of substances and materials for very diverse customer sectors with a range of measurement quality needs. Challenges associated with chemical measurements of organic measurands in 'real samples' include the need for defining the measurand, accurately measuring 'practically nothing' in the midst of 'everything else' without the benefit of absolute or quantum-based methods and the multiplicity of methods being used. Organic measurement accuracy needs vary widely across and within various sectors such as measuring the amount of substance to assign the value of a specific commodity, e.g., ethanol in wine; to assess the health status of an individual, e.g., cholesterol in serum; to determine the nutritional content in foods;

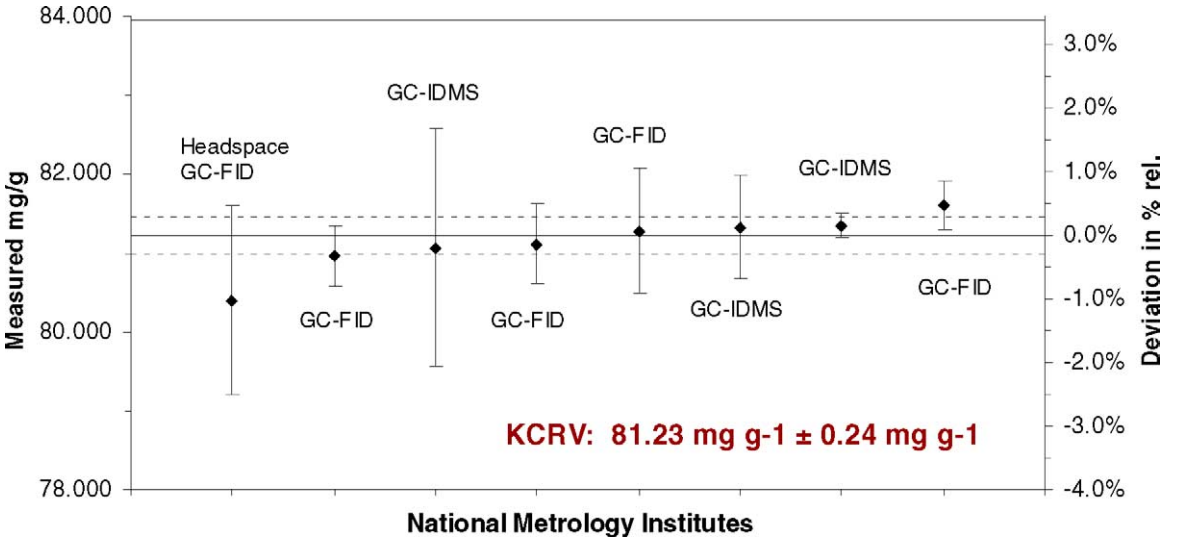

Fig. 7. CCQM-K27, Ethanol in Aqueous Matrix: Sample C, White Wine.

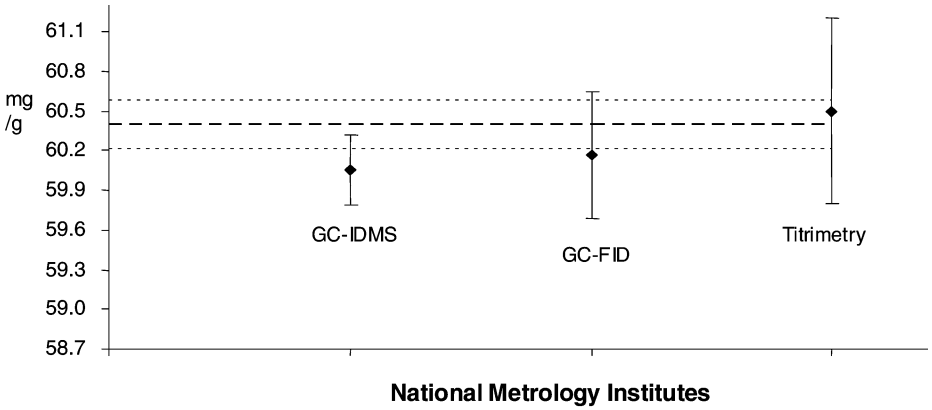

Fig. 8. CCQM-K27-subsequent, Ethanol in Aqueous Matrix, Level 4.

to assess an individual's use of illegal drugs; to characterize forensic samples for providing forensic drug testing; to determine contaminants in foods or the environment, etc.

It is our judgment that a 'higher metrological' approach is one in which there is a high degree of confidence in the accuracy of measurement results in that it has been *demonstrated* that all known or suspected sources of bias have been fully investigated or accounted for. The challenge for both analytical chemists and chemical metrologists will be to demonstrate that a given chemical measurement methodology be fit-for-purpose.

5. Bio analysis

Martin J.T. Milton[a,5], Gavin O'Connor[b]

[a] *National Physical Laboratory, Queens Road, Teddington, Middlesex TW11 0LW, UK*
[b] *Laboratory of the Government Chemist, LGC Limited, Queens Road, Teddington, Middlesex TW11 0LY, UK*

Many of the recent insights into the operation of biological systems such as the sequencing of the human genome, result from the development of innovative new measurement techniques. These techniques are already finding applications in the

[5] E-mail addresses: martin.milton@npl.co.uk (M.J.T. Milton); gavin.oconnor@lgc.co.uk (G. O'Connor).

pharmaceutical industry and healthcare, and there are emerging requirements for the development of a metrology infrastructure that can underpin their comparability and stability.

Biological systems present a unique set of challenges to measurement science. Unlike chemistry, where the behaviour of a molecule can largely be understood by reference to its atomic structure, the operation of biological systems cannot be readily determined simply from their molecular structure. Biological molecules also occur as heterogeneous mixtures of 'isoforms', each of which has a different activity. Consequently the concept of a 'biological' defined as "*an entity defined with respect to its biological activity*", is central to understanding the operation of measurements needed for effective regulation in this area [7].

For example, an understanding of disease development and consequently the discovery of new drug targets, is dependent on knowledge of the human proteome, which comprises over half a million proteins of which fewer than 30% have a known function. Proteins are involved in the majority of physiological processes, and their malfunction is often associated with the development of disease. A huge body of research is aimed towards the separation and identification of proteins, and the characterisation of their structure, function and activity. The behaviour of proteins is influenced not only by their chemical structure, but also by the extent to which they fold into structural motifs such as alpha helices and beta sheets ('secondary structure'); the packaging of these structural motifs into an overall three-dimensional structure ('tertiary structure'); and the interactions between protein sub-units ('quaternary structure'). Significant innovations in measurement science are required to make rapid and repeatable measurements of the primary, secondary, tertiary, and quaternary structure of proteins [8]. There are few techniques available that address the measurement issues of higher-order protein structure. Techniques such as NMR are applicable to smaller proteins, or protein sub-units, but many therapeutic proteins, such as monoclonal antibodies, are beyond current capability. X-ray crystallography requires molecules to be crystallised – a particular challenge for membrane-bound proteins, which are the targets of many pharmaceutical products. Finally, optical techniques, such as infrared, or Raman, vibrational spectroscopy, or circular dichroism absorbance spectroscopy, are popular for determining protein secondary structure. Currently, there is little comparability or traceability for these measurements – despite their use in the regulation of biopharmaceutical products.

Many of the techniques used to characterise the properties of protein populations, rely on tagging the 'probe' molecule or its 'target' with a molecule that is readily detectable spectroscopically. In most cases, the tag is either intrinsically fluorescent or radioactive. In these cases, the metrology challenges can be addressed through the methods used to standardise fluorescence and radioactivity. For example, a fluorescence measurement made with a spectrometer can be calibrated with a standard fluorescent tile with a certified value for its fluorescence spectral emission traceable to the established scales for diffuse reflectance. Although, this approach can give an accurate calibration of the instrument that is traceable to the SI, the practical differences between measuring surface, and solution-based fluorescence mean that there is limited applicability to biological applications.

Ultra-sensitive variants of fluorescence spectroscopy have been developed, such as Total Internal Reflectance Fluorescence (TIRF) Microscopy, which uses novel optical excitation methods to increase the detection sensitivity of fluorescence down to the level of small numbers of molecules. The fundamental drawback of fluorescence based approaches is the affect of the tag molecule on the biological behaviour of probe or target biomolecule. Therefore, there is a drive to develop new spectroscopic methods, which are intrinsically sensitive to molecular structure.

One such alternative approach, with the potentially greater flexibility is Surface Enhanced Raman Scattering (SERS), which has the potential to provide a rapid and ultra-sensitive technique for detecting single molecules [9,10]. This technique is based on the intrinsic Raman signature of the target molecule itself. The scope and exploitation of SERS has been limited because existing substrates and sample preparations have not been sufficiently reproducible. Consequently, research is focussed into the mechanisms responsible for these processes, in order to support their use in applications where comparability and reproducibility are required.

There are many examples where the techniques of chemical metrology have been applied successfully in biological applications. For example the use of mass spectrometry, combined with an isotope dilution method of analysis. The unique ability of this approach is that it enables the introduction of a structurally identical internal standard, prior to any sample manipulation, which enables traceable chemical measurements irrespective of the matrix.

This approach has been successfully applied to the quantitation of 'small' biological compounds such as cholesterol, glucose and creatinine in serum, (CCQM K6, K11, K12). However, its application to 'larger' biomolecules such as proteins and DNA is more difficult. The method is reliant on the availability of isotopically labelled analogues, of sufficient isotopic and chemical purity, where the enriched isotope and degree of labelling can be critical. Also the method, as applied to metrological measurements, relies on the availability of a well-characterised primary standard. Secondly, the method requires the separation of the individual molecular ion isotopes to enable the determination of the isotope ratios. For a biomolecule with a mass in the region of 50 kDa this is beyond the capability of most commonly available mass spectrometers. Arguably such an approach would offer the best chances of traceable quantitative measurement in the long term. However, the production of such standards would be prohibitively time consuming and costly, especially if the pace at which biotechnology is growing is taken into consideration.

These limitations can be overcome by carrying out protein quantitation using mass spectrometry is generally performed at the peptide level. Relative quantitation has been achieved by comparing peptides derived from proteins, which have been

isotopically altered. Both metabolic and chemical labelling of the proteins has been reported [11]. Metabolic labelling, whereby labelled amino acids are incorporated during natural metabolism, has the advantage of enabling the comparison of native peptides, either directly or by use of the labelled proteins as internal standards. This has many advantages in that the protein does not have to be altered by a 'Tag' molecule and can be pooled with the sample after minimal sample preparation. However, the metabolic labelling of anything larger than a small mammal is practically inconceivable. Chemical labelling generally occurs before or during proteolysis. Labelling before proteolysis normally entails the modification of specific amino acids. This has been combined with the addition of affinity Tags to improve sample clean-up prior to MS analysis [12]. Labelling during proteolysis, normally incorporating ^{18}O, has the advantage of being indiscriminate. However, differences in digestion between the samples will not be cancelled in this instance.

Absolute quantitation, by incorporating isotopically labelled synthetic peptides has been reported by a number of groups, [13,14]. The labelled peptides were designed to mimic those that were protein derived. Quantitation was realised via comparison to a standard curve. Such methods make many assumptions, about quantitative protein digestion etc., however they offer a feasible starting point for realising traceable protein quantitation in the future.

The reliance of genetic testing on PCR processes has resulted in MS having a minimal role to play in DNA quantitation, the majority of MS studies being applied to the characterisation of primer extension products for SNP profiling [15]. The direct IDMS analysis of DNA or intact oligonucleotides is prohibitive for the same reasons as those described for intact protein quantitation. However, preliminary studies have shown that digestion of an oligonucleotde to its constituent monomers (deoxynucleotide monophosphates) and the subsequent IDMS analysis of these can enable the amount of original oligonucleotide to be determined [16].

Many of the above processes are concerned with the quantitation of specific molecular sequences. However, in the case of complex systems the direct relationship between absolute amount, function and biological activity is not necessarily apparent. This therefore calls for a combined approach requiring structural and quantititative information to enable bio-metrological measurements.

References

[1] M.J.T. Milton, T.J. Quinn, Metrologia 38 (2001) 289–296.
[2] B. King, S. Westwood, GC-FID as a Primary Method for Establishing the purity of organic CRMs used for drugs in sport analysis, Fresen. J. Anal. Chem. 370 (2001) 194.
[3] S. Westwood, S. Davies, H. Wang, P. Harvey, B. King, Developments in the Certification of Pure Organic Substance CRMs, in: 9th Symposium on Biological and Environmental Reference Materials, Berlin, 2003.
[4] J.H. Reynolds, A mass spectrometric investigation of branching in 64Cu, 80Br, 82Br and 128I, Phys. Rev. 79 (1950) 789–795.
[5] M. Sargent, C. Harrington, R. Harte (Eds.), Guidelines for achieving high accuracy in isotope dilution mass spectrometry (IDMS), The Royal Society of Chemistry, Cambridge, UK, ISBN 0-85404-418-3, 2002.
[6] P. Evans, C. Wolff Briche, B. Fairman, High accuracy analysis of low level sulphur in diesel fuel by isotope dilution high resolution ICP-MS, using silicon for mass bias correction of natural isotope ratios, J. Anal. Atom. Spectrom. 16 (9) (2001) 964–969.
[7] C. Henry, Analytical Chemistry (1996) 68.
[8] H. Bayley, P.S. Cremer, Nature 413 (6852) (2001) 226–230.
[9] S. Nie, S.R. Emory, Science 275 (5303) (1997) 1102–1106.
[10] K. Kneipp, H. Kneipp, I. Itzkan, R.R. Dasari, M.S. Feld, Surface-enhanced Raman scattering and biophysics, J. Phys. Condens. Mat. 14 (18) (2002) R597.
[11] J. Lill, Proteomic tools for quantitation by mass spectrometry, Mass Spectrom. Rev. 22 (3) (2003) 182–194.
[12] T.J. Griffin, S.P. Gygi, B. Rist, R. Aebersold, A. Loboda, A. Jilkine, W. Ens, K.G. Standing, Quantitative proteomic analysis using MALDI quadrupole time-of-flight mass spectrometer, Anal. Chem. 73 (5) (2001) 978–986.
[13] Y. Oda, K. Huang, F.R. Cross, D. Cowburn, B.T. Chait, Accurate quantitation of protein expression and site-specific phosphorylation, Proc. Natl. Acad. Sci. USA 96 (1999) 6591–6596.
[14] D.R. Barnidge, E.A. Dratz, T. Martin, L.E. Bonilla, L.B. Moran, A. Lindall, Absolute quantification of the G protein-coupled receptor rhodopsin by LC/MS/MS using proteolysis product peptides and synthetic peptide standards, Anal. Chem. 75 (3) (2003) 445–451.
[15] J. Tost, I.G. Gut, Genotyping single nucleotide polymorphisms by mass spectrometry, Mass Spectrom. Rev. 21 (6) (2002) 388–418.
[16] G. O'Connor, C. Dawson, A. Woolford, K.S. Webb, T. Catterick, Quantitation of oligonucleotides by phosphodiesterase digestion followed by isotope dilution mass spectrometry: proof of concept, Anal. Chem. 74 (15) (2002) 3670–3676.