Recent developments in quantitative SERS moving towards absolute quantification

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SUMMARY

Surface enhanced Raman scattering (SERS) generates molecularly specific fingerprints of analytes and when the experimental conditions are carefully controlled this is highly quantitative. This review critiques the development of quantitative SERS from simple univariate assessment of single vibrational modes to multivariate analysis of the whole spectrum for improved quantification. SERS has also been developed for direct multiplex detection and quantification of multiple analytes and this is also discussed, as is the need for LC-SERS for analyte separation should multivariate chemometric approaches fail to effect quantification. Finally, to effect absolute quantification with SERS, the concepts of isotopologues is introduced along with the standard addition method (SAM) and suitable examples that have been developed and exploited these techniques are presented. We believe that SERS will be routinely used for quantitative analysis and it is only a matter of time before this technique translates from the laboratory to the clinical environment.

THE NEED FOR QUANTIFICATION

In all areas of science there is a need for absolute quantification of analytes of interest. This may be within the context of environmental science where the level of a toxicant polluting an ecological system must be below expected levels (usually set by a regulating authority in that country or region). Within food it is essential to know the levels of any pesticide or fungicide, as well as any chemical contaminant that may be present naturally or artificially by trying to make the food look more appealing [1]; for example, certain foods may contain the banned azo dye Sudan I which, along with Sudan III and IV, have been branded as category 3 carcinogens by the International Agency for Research on Cancer [2] and these dyes are often used to make chili or curry look brilliant red. Of course within medicine it is essential that key biomarkers of disease are assessed in a robust and reproducible manner – there can be no error in measurement here. By way of example, in clinical biochemistry when testing for Type II diabetes using the oral glucose tolerance test [3], the measurement of glucose in blood plasma must be reported in mmol/L (or mg/dL) and the normal glucose concentration range for a fasted individual is expected to be 3.3 to 5.5 mmol/L (60 to 100 mg/dL). In drugs testing accurately determining the level of a drug and its metabolites (so called xenometabolites) in blood or urine are needed either for illicit substance detection or for personalised medicine where it is important to know the pharmacodynamics of drug clearance.

Whatever analytical approach is used for measurement the output must be the same and must be reported in units that other scientists and non-cognoscenti understand. A cautionary reminder of what happens when this goes wrong is the Mars Climate Orbiter that on 23 September 1999 failed to insert into orbit around Mars, but rather crashed into the surface of this planet. This was because the design specification in the ground software was in US customary units (pound force-seconds) rather than SI units (newton-seconds); an expensive avoidable and somewhat embarrassing mistake [4].

Surface enhanced Raman spectroscopy (SERS) and the closely related Surface enhanced resonance Raman spectroscopy (SERRS) are analytical methods that are constantly being developed to measure a range of biologically-relevant molecules. These target molecules may be DNA, RNA or other nucleic acid sequences. While proteins and metabolites are also molecules that need accurate quantitative assessments and these too can be quantified using SERS-based analyses.

Whilst there are investigations that have reported SERS analysis directly on blood that could be used for stratification of people with disease from healthy individuals [5], as these are fingerprinting based and lack molecular resolution; that is to say, the SERS spectra from such a complex (bio)chemical mixture will be a combination of many molecules measured within these biofluids and so it is very difficult to assign bands to specific chemical entities without prior separation (e.g., by chromatography). We believe that the true power in SERS is for targeting molecules that are known to be useful for addressing a particular clinical acumen. Within this context these assays tend to be highly specific for

the determinand of interest and are used when a known biomarker has clinical utility.

Figure 1 describes a typical biomarker pipeline from concept to clinical delivery [6]. After the clinical decision is made to look for new biomarkers for patient stratification a biomarker discovery phase is first undertaken. This is typically performed with small numbers of individuals and so needs to be repeated in a second cohort of different people to validate the initial study. At this stage the biomarker (nucleic acid, protein, metabolite) is unknown and so this is analytically intensive as mass spectrometry (MS) usually linked to prior chromatographic separation (*viz*. GC-MS and/or LC-MS) is the technology used in these analyses; these typically take 30 min per analysis type. MS has great appeal here as, along with NMR spectroscopy (on isolated molecules), allows structural elucidation and hence identification of targeted molecules [7]. At the end of this process a list of candidate molecules is generated which needs to be validate in larger cohorts before ready for use within the clinic. At this stage in addition to larger sample numbers there is no need to use expensive analytical techniques like GC-MS, LC-MS or NMR as these lack sample throughput. What is needed is a technique that is easy to use, potentially portable for at patient or bedside use and one that has chemical specificity for the target molecules of interest and is of course highly quantitative. Such portable point-and-shoot techniques [8] are ideal for personalized medicine where there is a need for at home testing [9], and as indicated in Figure 1 this is where SERS is best used as it addresses all the needs in terms of speed, high sample numbers, and when developed appropriately is highly specific and quantitative for target analytes.

A BRIEF INTRODUCTION TO SERS AND QUANTITATIVE ANALYSIS

Surface-enhanced Raman scattering (SERS) is a vibrational spectroscopic method that was discovered in 1974 which uses a metal surface to boost the Raman signal overcoming its rather weak output [10, 11]; typically in Raman only 1 in 10^6 – 10^8 photons undergo an inelastic light scattering event so collection times are long. SERS utilises roughened metal substrates, which may be roughed electrodes, thin films or nanoparticles (NPs). With respect to quantification NPs colloids are mainly used as these allow averaging of the signal during collection and hence improved quantification [12].

The use of NPs gives rise to large enhancements of the Raman signal and these are typically in the order of 10^4 – 10^6, and when combined with an additional enhancement based on resonance between the analyte and excitation wavelength, even single molecule detection is possible [13]. There are also reports of single molecule detection in non-resonant molecules [14]. There is much debate on the underlying mechanisms that are responsible for SERS (see papers in Themed Collection: Surface enhanced Raman Scattering (2017) *Faraday Discussions* **205**, 1-626). The dominant process is an electromagnetic (EM) effect, and this occurs when the electrons on a roughened metal surface (usually of Ag or Au for SERS, although others are possible) are excited by an incident laser light creating a localised surface plasmon (LSP) and any analyte

that interacts with the LSP has an amplification of the EM field and hence enhancement of selective bands in SERS [15, 16]. Another contribution to SERS, although at a lower level, is the chemical effect. This is based on a charge-transfer mechanism when the analyte forms a chemical bond with the metal surface, and there is a transfer of electrons from the metal to the surface and *vice versa*. This gives rise to increased molecular polarisability and thus also enhances the Raman signal [17].

Although the exact mechanisms are yet to be fully elucidated, therefore one cannot yet readily go from a computational modeling approach to generate a theoretical SERS spectrum that predicts laboratory-based investigations, this has not stopped SERS being used in a vast area of applications. For an excellent recent review on where SERS is used within biomedical applications the reader is directed here [18].

SERS is based on Raman spectroscopy and Raman (as the underlying technique) provides a signal that scales linearly with the number of molecules under investigation. Thus quantitative analyses can be conducted based on the band area for a specific molecular vibration, as well as band position and band shape [19]. SERS has also been used for quantitative analysis (*vide infra*) and as is the case in inherent/normal Raman, the band area(s) for specific molecules of interest also increase when more molecules are present within the interrogation voxel. However, it should be noted that as enhancement is greatest when molecules are adsorbed directly onto the metal surface or less than 1 nm away from the surface, quantitation is dependent on analyte concentration and surface coverage, as well as orientation of the molecule on the metal surface which may affect the SERS spectrum due to relative distance of different vibrational modes within a molecule from the LSP. The slope of the calibration graph is different in the regions above and below monolayer coverage of the metal surface and this should be taken into account when optimizing a detection approach [20].

Therefore, in order for an analytical technique to provide a quantitative output, that can be used to determine the level of an analyte, with a real unit (i.e. mg/mL and not some arbitrary instrument response), it must first be calibrated. Calibration is commonplace for all detectors. Liquid chromatography-mass spectrometry (LC-MS), gas chromatography using a flame ionisation detector (GC-FID), or mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) spectroscopy, electrochemical detection (ECD), etc. are not quantitative unless the detector response is related to the concentration of an analyte under investigation. And the same is true for SERS.

The most common method of calibration used in SERS is to take a well-defined SERS/Raman peak and first calculate the peak height or peak area; peak area tends to give more accurate results but this depends on the baseline and therefore sometimes peak heights are preferred, where it is usually easier to subtract the background contribution from the baseline. This is then plotted against the concentration of a target analyte and a calibration curve is constructed. The number of points within the linear range of this calibration plot should be at least 5 different concentration levels of the target analyte. If a full

calibration is performed the calibration curve will be S-shaped: at low concentrations no analyte is detected and this is used to calculate the limit of detection (LOD) and quantification (LOQ) of the system (see Table 1 for definitions); above the linear range the signal will either plateau or start to decline. This is because, as described above, over a certain threshold the metal NPs used in SERS will be saturated and no more analyte will be within the LSP enhancement zone. In addition, if the aggregation conditions of the nanoparticles are not carefully controlled, large aggregates of NPs are formed that start to precipitate out of the colloid suspension also resulting in a decrease in response.

In order for peak areas to be reproducible then substrates with high fidelity and nanostructure uniformity must be generated and there are many factors when fabricating metal nanoparticles that need to be considered [21]. This is important and can be addressed with SERS substrates that are designed using rational means [22], or by exploiting statistical design of experiments (DOE) as demonstrated for the quantification of the illicit drug mephedrone [23], and the human β-blocker drug propranolol [24] as well as its quantification in human plasma, serum and urine using SERS [25].

Microfluidics can also be used to improve quantitative analysis with SERS as initially demonstrated by Keir *et al*. [12] who exploited these miniaturised devices for *in situ* nanoparticle production and aggregation. Popp and colleagues have also combined SERS with microfluidic devices as a general approach for quantitative analysis [26], which was recently demonstrated using the model analyte adenine [27].

Melamine is a substance that was added to milk to increase its nitrogen content and hence disguise the level of protein in watered down milk. This hazardous analyte caused the deaths of 6 infants in China and 300,000 victims in China alone were estimated to have been exposed to this poison, which was dispersed worldwide throughout the food supply chain [1]. It is perhaps unsurprising that given the importance of detecting melamine in milk, there have been several studies that have targeted SERS of this analyte [28-31]. Recently, Hu and coworkers developed molecularly imprinted polymers and reported an LOD and LOQ of 0.0165 and 0.055 mmol/L for the determination of melamine in milk [32, 33].

With respect to other food safety concerns, several potentially carcinogenic substances that may be present in food have also been targeted for quantitation with SERS and these include aflatoxins in maize [34], Sudan I spiked in chili powder [35] and Rhodamine B in chili oil [36]. Finally, although not hazardous to health the boar-taint compounds skatole and androstenone, which alter the flavor of meat, have also been recently detected and quantified in pork fat using SERS [37].

Finally, the detection of drugs of abuse is important and this has been shown using colloidal-based SERS for the quantification of the controlled substance tramadol [38] and the previously categorised legal high 5,6-methylenedioxy-2-aminoindane (MDAI) [39]. SERS has also been used to measure drug overdoses

from human saliva [40] as well as drugs of abuse [41, 42]. Clearly the detection and quantification of drugs is important and in contrast to the colloidal-based SERS used above this has also been possible using thin films produced via galvanic displacement [43], that have excellent reproducibility [44], as illustrated for the discrimination of a range of illicit materials [45].

MULTIVARIATE ANALYSIS FOR QUANTIFICATION FROM SERS

The univariate quantification detailed above works well when SERS peaks are present that are unique and are not crowded or overlapped with other spectral features. This may occur when an analyte is within a complex background (e.g. blood, urine or saliva) or when the goal is to perform quantitative multiplexed analysis of multiple analytes simultaneous. When this occurs more powerful algorithms are needed.

When univariate analysis fails it is prudent to use the whole spectrum, or a subset of the spectral features, as inputs to multivariate analysis (MVA) techniques. SERS data are by their nature multivariate and a typical spectrum from 200-3400 cm^{-1}, at just 4 cm^{-1} resolution will contain 800 discrete descriptors. These descriptors are the columns in the spectra or termed 'bins'. Clearly the interpretation of such high dimensional data is difficult. Thus the underlying theme in MVA is simplification of the multivariate data into something that is over low dimension and hence more readily interpretable.

For quantitative analyses two general types of approaches are use in MVA: these may be exploratory analyses or multivariate regression based; these are introduced below, before some examples of their use for SERS are provided:

Exploratory data analyses are used to look at the relationship(s) between groups of samples. In these algorithms no prior information is required and so these are *unsupervised* in nature [46]. This means that the only information provided is the input data matrix. For SERS spectra this will be a group of n samples by m descriptors (bins), these are referred to as X-data. The most common unsupervised learning method is principal component analysis (PCA), others include dendrograms and (Kohonen) self-organising (feature) maps (SO(F)Ms).

PCA is a well-known and very old chemometrics approach [47, 48] that is used to reduce the dimensionality of multivariate data whilst preserving most of the variance. The results of PCA are a series of scores and loadings. The first score (PC1) is extracted to explain the most natural variance in the data and subsequent ones are extracted in decreasing order of importance [49]. Scores are used to inspect the relationship between samples and for quantitative analyses one would expect to see linkage in the scores ordinations plots which are related to quantification-related trends in the data; linkage in PCA is seen when points representing samples with different concentrations of a target analyte line up (i.e., are linked), and this pattern/trend reflects the increasing concentration of the target analyte. Inspection of the loadings plots allows the important spectral features to be discovered and for quantitative analysis this

should resemble, to some degree, the pure SERS spectrum of the analyte that one is trying to quantify.

Not only do these exploratory multivariate approaches show quantitative trends in the data if they are present, but they can also be used to identify outliers. These outliers may fall outside the Hotelling T^2 95% confidence limit in PCA scores plots and this may be used to identify unusual samples that can arise from sample contamination or other analytical artifacts.

However unsupervised methods are descriptive in nature and require human interpretation. A much more powerful method of analysis is to use *supervised learning methods* that effect some multivariate regression (or mapping) of the SERS input data (*X*-data) onto the quantitative level of a target determinand (*Y*-data). The central feature of these methods is to ascertain whether SERS spectra can be correlated with a continuous variable (the quantitative level of a target analyte). Once calibrated, these methods allow quantitative prediction of unseen samples to this continuous variable. During calibration, a training set consisting of a series of training pairs is used, where each pair consists of a SERS spectrum (*X*-data) with the concentration of an analyte (*Y*-data) present with that spectrum. Usually a single analyte is investigated and the *Y*-data is a single *Y*-vector, however for multiplexing with, for example, 3 analytes to be measured simultaneous then for PLSR and ANNs the *Y*-data would be a matrix containing 3 columns; one for each of the analytes of interest.

Table 2 contains a summary of the most common multivariate chemometrics used for quantitative analysis, along with their key features. Partial least squares regression (PLSR) is a very common method which is largely used for linear regression [50]. Other methods such as support vector regression (SVR), which is a variant of support vector machines (SVMs) can in addition perform non-linear mapping. A more in depth description of these algorithms and related chemometric methods are found here [1, 51].

Although multivariate analyses have been used in other fields of analytical chemistry, their application within SERS for quantitative analysis is relatively recent. Most applications of chemometrics to SERS are dominated by PLSR and these include the quantification of thymine [52], the quantitative detection of the spore biomarker dipicolinic acid (DPA) extracted from bacterial spores [53], quantitative assessments of the neurotransmitters glutamate and γ-aminobutyric acid in serum [54] and the accurate measurement of the fungicides thiram [55] and carbendazim [56] using SERS.

The above methods exploit machine learning in that the algorithm is first taught with samples of known provenance (i.e. concentration(s) of target analyte(s)), before its performance for quantification is tested with 'unknown' samples; these samples are unknown to the algorithm but known to the operator testing the stability of the system. An attractive alternative is to use chemometric algorithms that perform spectral unmixing/deconvolution. Multivariate curve resolution (MCR) is a method that is used to resolve mixtures by determining the number of individual components within a sample and is often combined with

alternating least squares (ALS) for optimisation of the extracted component spectra [57]. This MCR-ALS approach generates two matrices: the first is a *C* matrix which contains concentration profiles and along with it is the *S* matrix which contains the corresponding pure spectra; which can be compared with the SERS spectra of pure analytes. For quantitative analysis from SERS spectra, MCR-ALS has been used to determine uric acid spiked into human urine at clinically relevant concentrations (0.11 mmol L^{-1}) [58], and within the biocatalysis field for following the enzymatic turnover of substrates into products [59].

MULTIPLEXED QUANTIFICATION FROM SERS OF MIXTURES

SERS assays for quantification of specific DNA sequences allows for the analysis of low levels of DNA labeled with SERRS reporter molecules [60], and this approach has better analytical sensitivity than fluorescence based assays for the measurement of DNA [61]. For two recent reviews focusing on the quantification of DNA sequences using SERS the reader is directed here [62, 63].

One of the earliest examples of multiplex detection using SERS was reported by Faulds and colleagues who used two different excitation lasers for SERRS (to show the additional effect of resonance enhancements) and combined this with five different dyes in order to achieve quantification of 5-plex DNA-detection system with detection limits of 10^{-12} mol dm^3 [64]. This system did not use any chemometrics but relied on having five different SERS peaks being resolved.

Quantification of multiple dyes using SERS starts to get more complex when a single excitation source is used and so the same authors turned to multivariate analyses. In a study using six different dyes these authors were able to detect whether a labeled oligonucleotide was present or absent using PLSR in a total of 2^6 (64 mixtures) [65], and this was also compared favourably with reanalysis of these data using Bayesian statistical methods [66]. More recently, Faulds and co-workers developed a novel quantitative assay for the simultaneous detection of three pathogens involved in meningitis using a combination of λ-exonuclease, SERRS and PLSR [67].

Detection of multiplex DNA sequences has also been reported using molecular beacon approaches using metal film over nanosphere (MFON) [68, 69] and silicon nanowire arrays (SiNWAr) with gold nanoparticles deposited onto the surface [70]. However both these approaches are negative assays that result in a decrease in SERS response upon target hybridisation and univariate analysis was used and quantification within the multiplex was not demonstrated.

The above assays use DNA hybridisation to detect and subsequently quantify a specific DNA sequence from a labeled reporter that is SERRS active. SERS from molecules within mixtures can also be developed for quantitative analysis of multiple analytes without recourse to chromatography. Alharbi *et al*. reported two studies investigating drugs and their metabolites. In the first study, ANNs outperformed PLSR in the quantification of the drug nicotine and its two major xenometabolites cotinine and *trans*-3'-hydroxycotinine [71]. This may have been

because optimal aggregation and hence SERS signal was effected using 3 different pH conditions, rather than salts. Optimal SERS signals were seen at 3 different pH which corresponded to the 3 different pKa (acid dissociation constants) of the 3 analytes in the mixtures, and so all three spectra were concatenated and used in the ANN analyses. In a further study SERS with ANNs using 3 outputs (*Y*-data) allowed for the quantification of caffeine and its major metabolites theobromine and paraxanthine [72], here a single pH environment was used for aggregation of the NPs and hence the chemometric analysis was simpler.

SERRS immunoassays have also previously been used for the specific detection of target antigen through selective antibody-antigen binding. A simple assay has been developed which utilises a SERRS labelled secondary detection antibody to detect directly the presence of the intracellular signalling protein p38 [73]. This demonstrated quantitative detection of a single target protein within an immunoassay; however, lower detection limits have been achieved by incorporating nanoparticles into the assay [74].

A SERS protein assay using gold nanoparticles functionalised with target antibodies and Raman reporter has been developed [75]. Nanoparticles were functionalised with a monolayer of bioselective detection antibodies by coupling through a strong Raman reporter molecule, DSNB (5,5 '-dithiobis(succinimidyl-2-nitrobenzoate)). The functionalised nanoparticles were captured onto a gold coated glass slide through an antigen immunoassay leading to quantitative detection of CA 19-9 and MMP-7 using spatial multiplexing in an array format [76]. Methods to identify a number of analytes positively *in situ* have also been developed [77]. A protein immunoassay that utilises SERS detection to detect four target antigens simultaneously has been reported for the detection of multiple proteins using self-assembled mixed monolayer gold nanoparticles through a sandwich type immunoassay [78]. SERS has also been shown to outperform conventional ELISA assays for the detection of MUC4, expressed in pancreatic adenocarcinoma cell lines and tissues [79], and for the detection of p53 and p21 [80], as well hydrophilically-stabilised gold nanostars for the quantification of the tumour repressor p63 in cancerous tissues [81]. Multiplexed detection of PSA, thrombin and mucin-1 has also been achieved using aptamer functionalized nanoparticles [82] and a magnetic separation assay based on the use of paramagnetic nanoparticles for the detection of West Nile and Rift Valley fever virus [83].

Above are examples of where the detection of multiple analytes is possible within a mixture directly. However, when the mixture is very complex (e.g., from a patients blood or urine), or when there are multiple chemical species competing for the metal surface used for SERS, then alternative strategies are needed.

Liquid chromatography (LC) is one method that has been recently coupled with Raman and signals boosted using SERS (a combined method called LC-SERS). Cowcher and colleagues showed that using isocratic elution, where the mobile phase remained constant, silver colloid could be fed directly into the eluent

stream from the LC. This subsequently underwent KNO_3 mediated aggregation and was channelled through a quartz capillary for Raman/SERS interrogation. Targeted purine bases (adenine, guanine, hypoxanthine and xanthine) could be quantified with limits of detection (LoD) of 100–500 pmol [84]. This work is notable as it established for the first time that LC-SERS could be performed on-line; at the time other workers had only achieved off-line analysis by deposition of the eluent into wells containing SERS substrates (see for e.g. [85, 86]). More recently, an improved on-line LC-SERS system using reversed phase LC, where enhanced analyte separation was possible by programming the mobile phase during elution, showed that it was possible to target and quantitate methotrexate and its major metabolite 7-hydroxy methotrexate in urine from human patients suffering from rheumatoid arthritis [87].

On-line methods based on Ag (or Au) colloids, while attractive, have the disadvantage that a continued supply of NPs is needed. By contrast, Schultz and colleagues have developed an attractive on-line system where the eluent from the LC is directed onto a SERS active surface using a sheath-flow interface that uses hydrodynamic focusing to confine analyte molecules eluting out of an LC column onto planar SERS substrates [88, 89]. These authors have shown that it is possible to detect peptides and 20 amino acids separated by capillary electrophoresis [90, 91] and to perform quantitative analysis of several metabolites [92].

THE USE OF INTERNAL STANDARDS AND ISOTOPOLOGUES

There are many factors that need to be considered when SERS is used for quantitative analysis. Three important factors are the number and degree of aggregation of the nanoparticles relative to the concentration of analytes within the interrogation voxel during analysis, how well the analyte binds to and orientates on the metal surface, and the power/fluence of the laser can vary (although we note that modern lasers do have improved stability). These are unfortunately inherently variable and hard to control with perfect precision, as small variations in either of these will alter the absolute signal output from SERS and hence the ability to perform accurate quantitative analysis.

One way to overcome these issues is to employ an internal standard (IS). One of the first examples of this was reported by Bell and colleagues who wanted to quantify dipicolinic acid (DPA; pyridine-2,6-dicarboxylic acid) [93]; DPA is important as it is a small molecule that is unique to *Bacillus* spores and so its detection and quantification can be an indication of potential anthrax attacks. However, in their study the SERS generated DPA signature from the symmetric ring stretch from pyridine evolved slowly over time due to the use of Na_2SO_4 as the aggregating agent. Therefore, to compensate for this temporal signal variation they used potassium thiocyanate as an internal standard and ratioed the pyridine to the CNS^- vibration and this significantly improved quantification [93]. Following on from this, Cowcher and coworkers also quantified DPA from a nitric acid extract from *Bacillus* spores and used glutaric acid as the internal standard (KCNS was not used as it would have acid hydrolysed in these

conditions producing CN!) [53]. Here the signal was also adjusted using a ratio calculation and they were able to quantify down to 7,000 spores (10^4 spores are required for inhalation anthrax [93]). Finally, when Bell's group combined this with meso-droplets supported on superhydrophobic wires this allowed for the exquisitely sensitive detection of DPA which was equivalent to just 10 spores [94].

The problem with the use of these internal standards is there is still unequal competition between the target analyte and the internal standard (IS) on the metal surface. This means that even for similar chemical structures the signal may vary as a function of overall concentration and will not work above saturation of the NP surface [95]; this means the ratios of analyte to IS are not linear (as illustrated in [53]). One recent study has gone some way in overcoming this issue of competitive adsorption by placing the IS molecules within the inside of core-shell nanoparticles. A method that improved the quantitative analysis of target molecules over a large concentration range [96].

Isotopologues are one potential solution to the above problem and these are molecules that differ from each other only in their isotopic composition. For example, by the substitution with a stable isotope such as ^{13}C for ^{12}C or ^{15}N for ^{14}N or as illustrated in Figure 2 the substitution of H with D. The substitution of hydrogen with deuterium is particularly attractive as the reduced mass of a functional group is very different as illustrated in the figure for the C-H to C-D substitution. This results in a shift in CH vibrations from *ca* 2800 cm^{-1} to *ca* 2100 cm^{-1}, and band ratios can then be made of the isotopologue to the natural isotope. This should have improved accuracy for quantification as the isotopic labelled molecules will compete in a very similar manner for the metal surface. Thereby the use of these internal standards will result in the reduction of any influences due to the number of nanoparticles within the analysis zone and fluctuations in laser fluence.

The use of isotopologues for SERS was first reported by van Duynes' group for systematic studies of single molecule detection of crystal violet [97]. For quantitative analysis, this approach is often termed isotope dilution surface-enhanced Raman scattering (IDSERS) [98], and has been used to improve quantification between different batches of nanoparticles [99] and to effect the absolute quantification of markers in serum [100] as well as human plasma [101], and for the analysis of nicotine content in electronic cigarettes [102].

When competitive co-adsorption/co-association with the metal surface exists between different chemical species within complex matrices, (e.g.) in clinical samples, then isotopologues may not fully compensate for this. In these cases additional sample pre-treatment may be needed which would include selective (solvent) extraction of the target analyte. Here we would suggest the isotopologue is added before the extraction is undertaken to adjust for any variability in extraction efficiencies from such complex mixtures.

STANDARD ADDITION METHOD FOR ABSOLUTE QUANTIFICATION

An alternative to using isotopologues or IDSERS is to use the standard addition method (SAM) for absolute quantification. The SAM is a well-established technique in analytical chemistry that can be used to effect absolute quantification of specific molecules within complex mixtures. Most SERS studies have quantified target analytes in simple matrices (e.g. water) and when more complex backgrounds are involved there can often be chemical species present which also compete for the metal NP surface needed for SERS and thus affect the ability to perform accurate quantifications.

As illustrated in Figure 3, SAM works by spiking known amounts of a standard into a sample of interest. This standard will be the molecule that one is trying to quantify. Plots of the peak area of a characteristic peak against the concentration of the standard spiked will yield a straight line. The equation of which is $y = mx + b$; where m and b are the slope of the line and y-intercept, respectively. When the sample contains no analyte/standard the intercept will be 0, by contrast when the sample already contains the analyte, then the intercept will be positive in the y-axis. From these plots (upper plot in Figure 3) the concentration of the analyte can be determined from the point at which the extrapolated line crosses the concentration axis (x) at zero signal (i.e., where $y = 0$ and thus $x = -b/m$ such that the concentration = b/m) [103, 104].

In a series of elegant papers showing the evolution of lab-on-a-chip (LoC) SERS devices combined with SAM, Popp and colleagues developed the LOC device [105] after which they illustrated that using SAM improved quantification for the target analyte congo red [106], before going on to show that LoC with SAM allowed for the precise determination of the levels of the antibiotic nitroxoline spiked into human urine [107]. This group has also combined MCR-ALS with SAM for the quantification of nicotine in the presence of cotinine and anabasine in spikes of human urine using SERS [108]. Finally, other workers also exploited the SAM with SERS to analyse real-world samples when PLSR and MCR-ALS failed, and showed that this approach was able to effect absolute quantification of uric acid from urine from pregnant individuals suspected of having preeclampsia [109].

CONCLUSIONS AND OUTLOOK

This review has critiqued the development and application of SERS for quantitative analysis. Over the last five years there has been an explosion of interest in SERS for quantitative analysis including the analysis of multiple analytes simultaneously directly on the sample without recourse to any prior fractionation or chromatography. Much of this success has been due to improved chemometric analysis and the adoption of multivariate analyses, which are still quite new to this field, as well as the implementation of classic analytical chemistry methods borrowed from other fields. Most notable here is the use of isotopologues or IDSERS as internal standards and the exploitation of the standard addition method.

Recently the applications of SERS for quantitative analysis has moved out of simple chemistry based on mixture analysis in clean, uncomplicated backgrounds (*viz*. water), to more complex scenarios where accurate quantification of a variety of molecules has been possible in different human biofluids including blood plasma and serum as well as urine and saliva. We believe that SERS has evolved and is no longer an emerging technology. We predict that in the next decade this technique will be translated from the laboratory to the clinical environment and SERS will be exploited for quantitative analysis of many different chemical species. In addition, due to its inherent portability SERS will be an attractive point-of-care technology for at home testing [9] and for personalised medicine where it may replace more cumbersome and expensive analytical methods.

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TABLES

Table 1. Terms and criteria used to assess a model's ability to quantify a target determinand accurately

Term	Type of analysis	Use/Definition
LOD	Statistics based	Limit of Detection: 3 × the standard deviation of the blank§
LOQ	Statistics based	Limit of Quantification: 10 × the standard deviation of the blank
R^2	Correlation coefficient	Assesses linearity in prediction from data used to *train* the model
Q^2	Correlation coefficient	Assesses linearity in prediction from data used to *test* the model
RMSEC	Error based	Root mean squared error (RMSE) in the calibration (C) data used to *train* the model
RMSECV	Error based	RMSE in the *cross-validation* (CV) data used during the calibration of the model to set certain internal parameters*
RMSEP	Error based	The prediction (P) RMSE of the *independent test set data* not used during calibration

§ The lowest amount of a substance detected for a specific peak. Defined as the detectable signal greater than the blank sample, with a 1% confidence limit [110].

* For PLSR this will be the number of latent variables (LVs) to use

Table 2 Common multivariate chemometric methods used for quantification of target analytes

Abb.	Algorithm	Key features
CCA	Canonical correlation analysis	• Assesses the relationship between two sets of variables *X* and *Y* • Latent variables (LVs) inform which features are correlated • Performs a *linear* mapping from *X* to *Y*
MLR	Multivariate linear regression	• Performs simple regression analysis on multiple input variables (*X*-data) • Performs a *linear* mapping from *X* to *Y*
PCR	Principal components regression	• Uses principal components analysis on *X*-data • Extracted PC are regressed against the target (*Y*) • Performs a *linear* mapping from *X* to *Y*
PLSR	Partial least squares regression	• Performs simultaneous regression on *X* and *Y* • Can be used to predict multiple variables (PLS2) • Performs a *linear* mapping from *X* to *Y*
SVR	Support vector regression	• Can use many potential kernel functions • Distribution of samples does not influence output • Performs both *linear* and *non-linear* mapping (dependent on kernel)
ANN	Artificial neural network	• Machine learning technique based on learning • Many different learning approaches • Converges to answer relatively slowly (compared to the other techniques in this table) • Very powerful *non-linear* mapping technique
MCR	Multivariate curve resolution	• Method used to resolve mixtures by determining the number of individual components within a sample • Generates two matrices: *C* = concentration profiles and *S* = corresponding pure spectra • Often combined with alternating least squares (ALS) for optimisation of the extracted component spectra

* *X* are input (SERS) data to the algorithm
Y is a vector containing the concentration of a target determinand

FIGURES

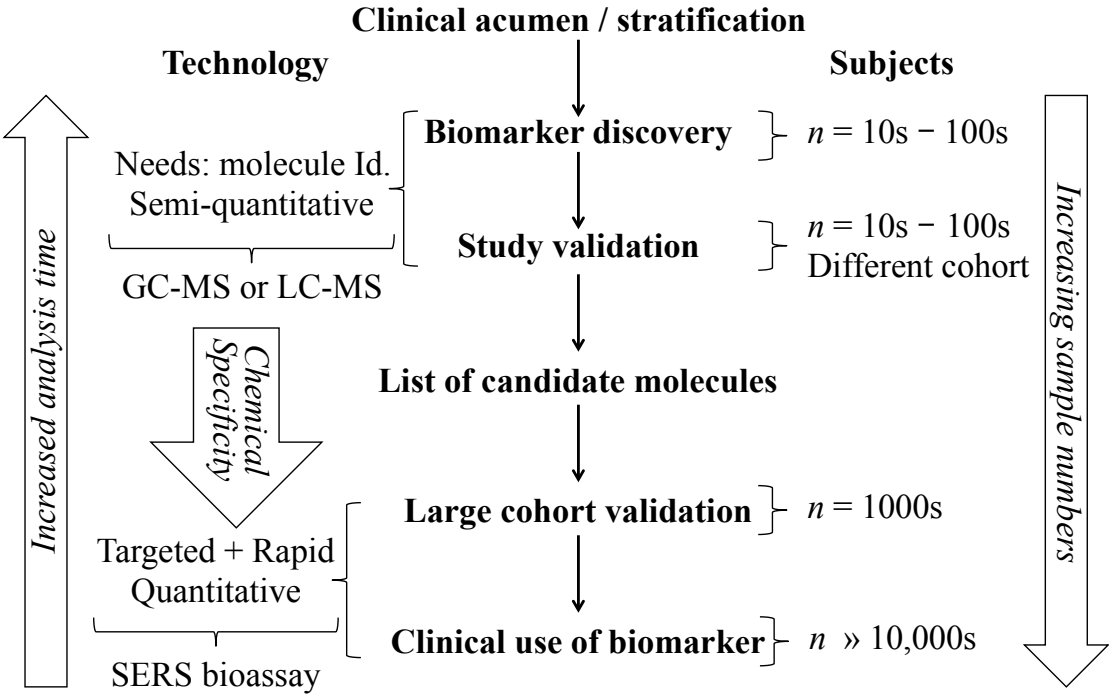
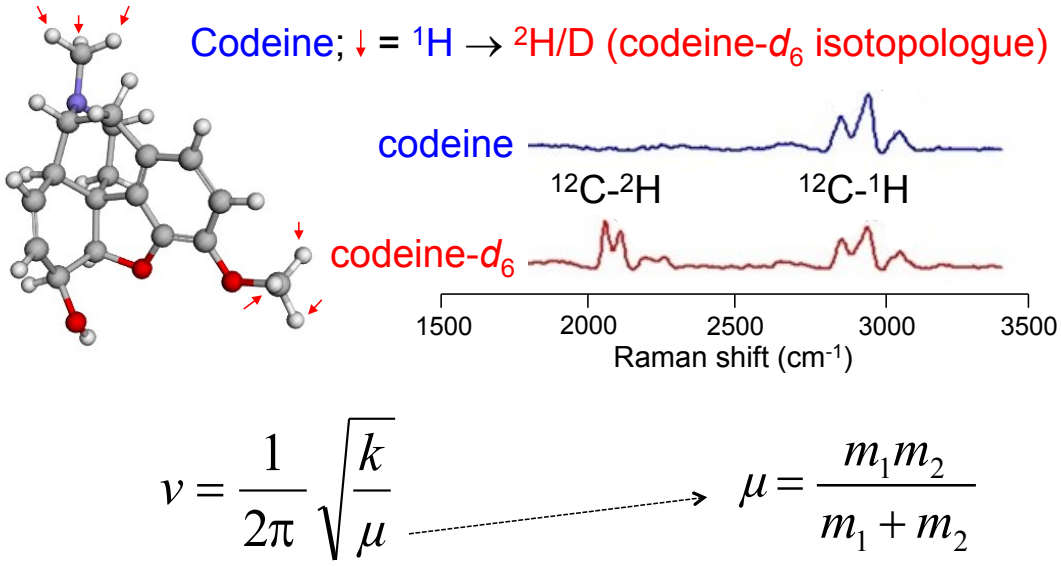


Figure 1 The biomarker discovery pipeline. This starts with the discovery of a new biomarker – DNA, RNA, protein, metabolite – that allows stratification of patients into (e.g.) patients with disease from healthy matched controls. Once candidate biomarkers are discovered then higher throughput and chemical specific analyses based on SERS bioassays are used for epidemiological assessment before delivery of the new biomarker into the clinic for use.



$$\nu = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}} \longrightarrow \mu = \frac{m_1 m_2}{m_1 + m_2}$$

Where: ν = vibrational frequency
k = force constant
μ = reduced mass

$\therefore$ ^{12}C-^{1}H, μ = 12/13 = 0.92
^{13}C-^{1}H, μ = 13/14 = 0.93
^{12}C-^{2}H, μ = 24/14 = 1.71

Figure 2 The use of isotopologues for absolute quantification using codeine-d_6 as an example.

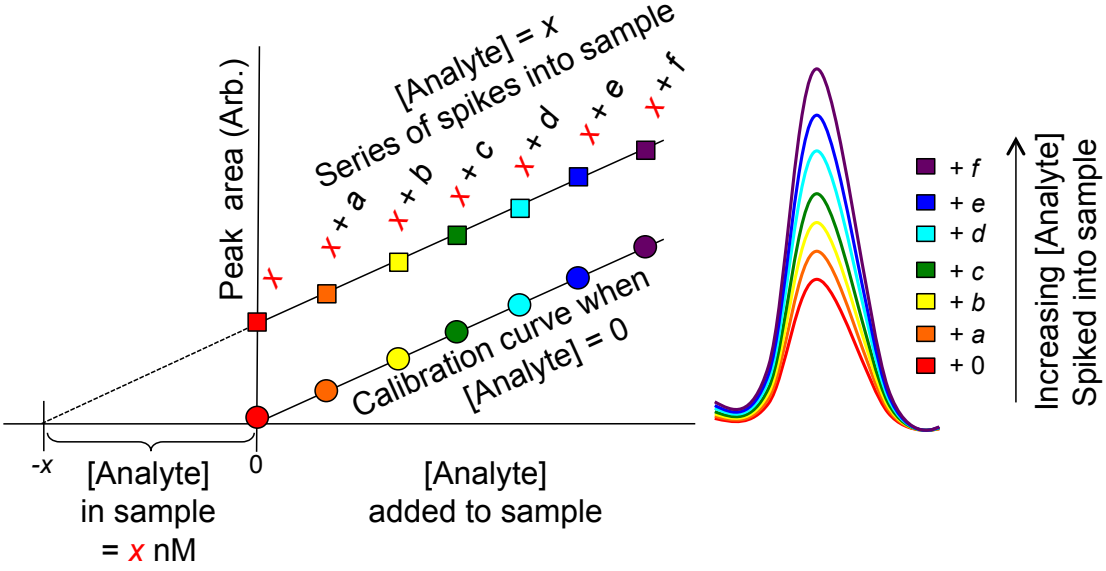


Figure 3 The standard addition method (SAM) for absolute quantification.