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Potential of Raman spectroscopy for the analysis of plasma/serum in the liquid state: Recent advances

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Abstract

There is compelling evidence in the literature to support the application of Raman spectroscopy for analysis of bodily fluids in their native liquid state. Naturally, the strategies described in the literature for Raman spectroscopic analysis of liquid samples have advantages and disadvantages. Herein, recent advances in the analysis of plasma/serum in the liquid state are reviewed. The potential advantages of Raman analysis in the liquid form over the commonly employed infrared absorption analysis in the dried droplet form are initially highlighted. Improvements in measurement protocols based on inverted microscopic geometries, clinically adaptable substrates, data preprocessing and analysis, and applications for routine monitoring of patient health as well as therapeutic administration are reviewed. These advances suggest that clinical translation of Raman spectroscopy for rapid biochemical analysis can be a reality. In the future, this method will prove to be highly beneficial to clinicians for rapid screening and monitoring of analytes and drugs in the biological fluids, and to the patients themselves, enabling early treatment, before the disease becomes symptomatic, allowing early recovery.

Keywords: Raman spectroscopy, Extended Multiplicative Signal Correction, Therapeutic drug monitoring, Disease diagnosis, Serum, Plasma, Partial Least Squares Regression

1. Introduction

Vibrational spectroscopic techniques, both Raman and Infrared (IR) absorption, have been extensively explored over the last two decades for obtaining the biochemical composition of bodily fluids in the field of biomedical analysis [1–10]. The sensitivity to detect subtle changes in the biochemical composition and ability to detect the presence of specific biomarkers or drugs makes vibrational spectroscopy an ideal tool for the early diagnosis of various pathologies [11–14] and therapeutic drug monitoring [15–17]. Both techniques are truly label free, rapid, cost-effective, easy to operate, non-destructive and provide the unique molecular fingerprint of the sample with minimal sample preparation steps. Such techniques are particularly attractive for routine analysis of biofluids, as they are easy to apply, require minimal sample preparation and are readily adaptable to analysis of various bodily fluids [1-5], potentially reducing clinical analysis time, and alleviating patient angst (figure 1).

Over the past decades, there have been numerous studies of analytes in biofluids using vibrational spectroscopy, and, in recent years, attenuated total reflection (Fourier Transform) IR (ATR-FTIR) has become popular for rapid screening of biofluids, particularly blood plasma and serum [13]. Notably, however, ATR-FTIR is predominantly conducted on dried droplets of bodily fluids, adding to the complexity of the measurement and the clinical workflow [18, 19]. In comparison, the prospect of using Raman spectroscopy for the label-free extraction of biochemical information from biological fluids is attractive from various perspectives; liquid sample analysis, no requirement for additional reagents, ease of use, speed, cost-effectiveness and low sample volume requirement. The application of Raman spectroscopy to biomolecules and even tissues was first demonstrated as early as the 1960s, and by the mid 1970s biomedical applications were explored [20–22]. Whole cell and tissue studies have been carried out on a range of pathologies [23–25] and *in vivo* studies [26, 27] have demonstrated the prospective for diagnostic applications. Raman microspectroscopy potentially lends itself naturally to the

analysis of liquid biofluids and such applications have attracted considerable attention in recent years. There have been, however, multiple strategies that are used to perform such real-time analysis and these options must be carefully considered to achieve optimum Raman spectra from liquid samples [28, 29]. Several studies have reported the proof-of-concept of liquid sample analysis using Raman spectroscopy [30–37]. However, no systematic validation and testing of protocols has yet been carried out to consider this technique for real-time clinical applications. This review will summarise the recent advances in the standardisation of measurement protocol for biological fluid analysis in the liquid state using Raman spectroscopy, in terms of the optimal wavelength and substrate, serum fractionation methods, data collection, pre-processing and post processing steps to obtain results with higher accuracy and sensitivity. Applications for both analysis of imbalances of high and low molecular weight serum constituent components of pathological and clinical significance, as well as therapeutic drug monitoring will be considered. Note, that the studies considered do not require signal enhancement techniques such as surface enhanced Raman spectroscopy, and therefore such techniques are not considered within the scope of the review. However, as they have been extensively explored in recent times, a brief comparison of the techniques to infrared absorption based techniques is provided.

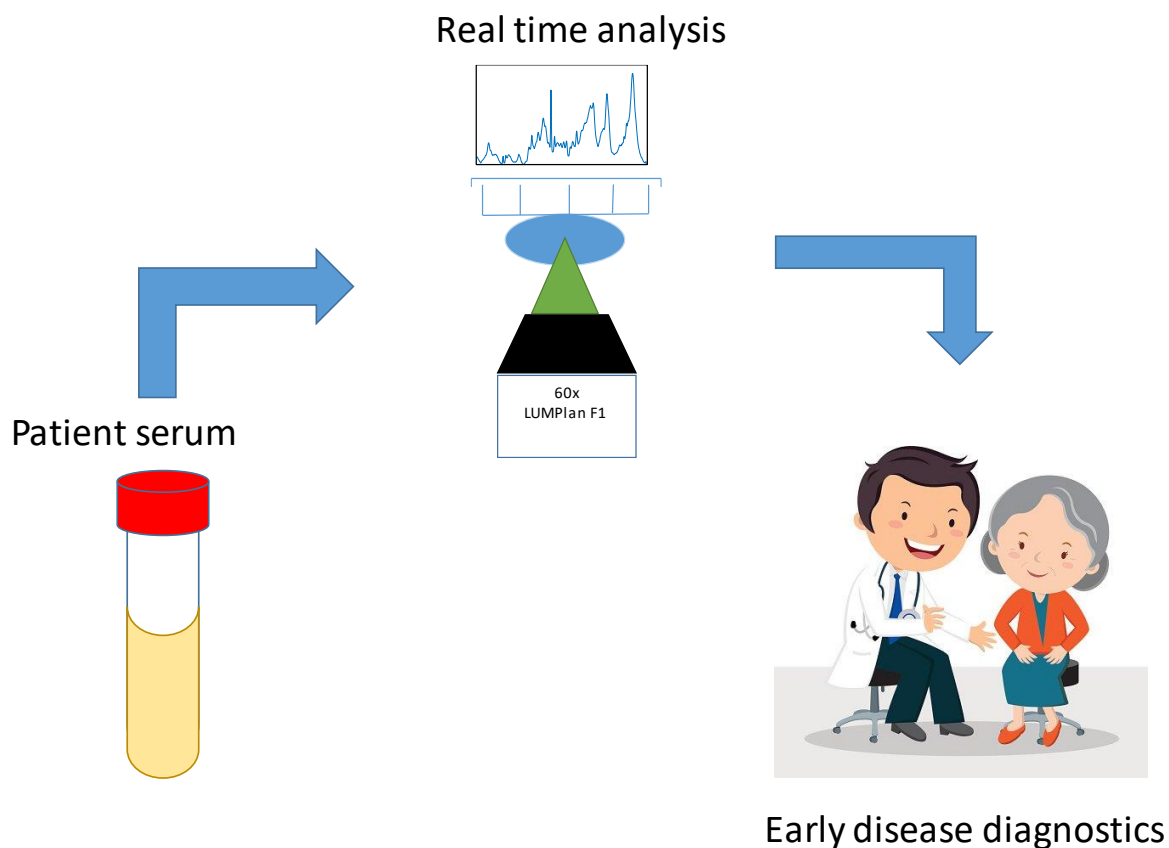


Figure 1. Early disease diagnosis, prognosis and treatment is possible with real-time analysis of patient serum using the inverted Raman spectral analysis.

2. Raman vs Infrared absorption spectroscopy

Although Raman and IR spectroscopy are considered complementary techniques, the fundamental physical phenomena governing them are very different, and thus result in distinct technical challenges to their clinical implementation [6, 38]. As IR spectroscopy is based on absorption due to electric dipole transitions associated with molecular vibrations, water cannot be used as a solvent, due to its intense absorption in the IR region due to the highly polar OH groups [39, 40]. IR analysis of bodily fluids has therefore been predominantly performed on air-dried samples, which leads to chemical and physical inhomogeneity due to the so – called “coffee ring” effect and thereby inconsistencies in the results obtained [18, 19, 39]. Raman spectroscopy is an inelastic scattering technique based on the Raman effect, i.e., the coupling

of the oscillating electronic polarisabilities of the molecular bond with the source electromagnetic field [41, 42]. The distinct advantage of this technique is that it is compatible with water-rich samples such as serum/plasma, as water molecules have a relatively low scattering cross section and do not mask the scattering from the solutes in the aqueous solutions [43–45]. Zhao *et al.*, developed a fast and reliable approach to identify and quantify liquid injectables in the liquid state for spurious/false-labelled/falsified/counterfeit medical products (SFFCs) using Raman spectrophotometer of 785nm excitation source [46]. Principle Component Analysis (PCA) combined with Classical Least Squares (CLS) chemometric methods were used to overcome the problems of the interference signals of glass containers and solutions, and weak signals from active pharmaceutical ingredients were finally extracted. Water was used as an internal standard for normalisation and CLS quantitation models were established. When Raman predicted values were compared with HPLC reference results, the relative error of eight doxofylline liquid injectable samples were within 5% and three low-concentration Levofloxacin in Levofloxacin Lactate and Sodium Chloride Injection samples were within 10%, demonstrating this approach to be a reliable and rapid screening method to detect SFFCs in liquid dosage forms. In order to extend this Raman spectroscopic set up for the study of biological fluids, further studies should be conducted on biological fluids to extract clinically relevant information.

Multianalyte, dried serum analysis has previously been reported using mid-infrared spectroscopy, for the simultaneous quantitation of eight serum analytes: total protein content, albumin, triglycerides, cholesterol, glucose, urea, creatinine and uric acid [47] and for simultaneous quantification of glucose and urea analytes along with malaria parasitemia quantification using ATR-FTIR [11]. As a direct comparison of the techniques of mid-IR and Raman spectroscopy, together with multivariate data analysis, for the quantitative analysis of serum, Rohleder *et al.* analysed the serum of 247 blood donors [36]. The IR analysis was

undertaken on dried droplets, whereas the Raman analysis was of the liquid serum and/or serum filtrates. Under their investigation for the quantification of glucose, urea, uric acid, LDL cholesterol, HDL cholesterol, total protein, cholesterol and triglycerides, Raman and mid-infrared spectroscopy delivered similar accuracies for the prediction of physiologically relevant analyte levels [36]. More recently, Parachalil *et al.*, undertook a similar comparison of Raman compared to ATR-FTIR, using identical sample preparation and analysis protocols, to quantitatively monitor diagnostically relevant changes of glucose [44], indicating that Raman spectroscopy in the liquid state can perform at least as well as ATR-FTIR, without the need for the drying step.

Notably, whereas many of the previous studies of liquid serum samples employed an upright microscopic geometry, that of Parachalil *et al.*, employed an inverted geometry, as previously demonstrated by Bonnier *et al.* [62]. Improved analysis of serum using Raman spectroscopy was reported when the sample was analysed in the inverted geometry using a water immersion objective with a 785nm laser and CaF₂ substrate. A drop of water is used to minimise the differences in the refractive indices between sample, objective and the substrate, and thus improve the optical coupling. However, the water drop does not contribute to the data collected, as it is outside the focus of the beam. As a much better cost-effective, clinically adaptable option, Parachalil *et al.* introduced a commercial, cover slip (of 0.16-0.19 mm thickness) bottomed vesicle (Lab-tek plate) as the substrate. The use of glass precludes the use of a 785nm source [48, 49], and thus a 532nm laser was chosen as the source, which provides a strong Raman signal of water with minimal background interference (Figure 2). This set-up also has the added advantage of providing high quality, consistent Raman spectra from a sample volumes as low as 1µL. Medipally *et al.* also reported the benefits of using inverted Raman geometry to analyse plasma samples of small volume (20µL) from prostate cancer patients

[50]. Note, enhanced methods such as Surface Enhanced Raman Spectroscopy (SERS) are not considered in this review.

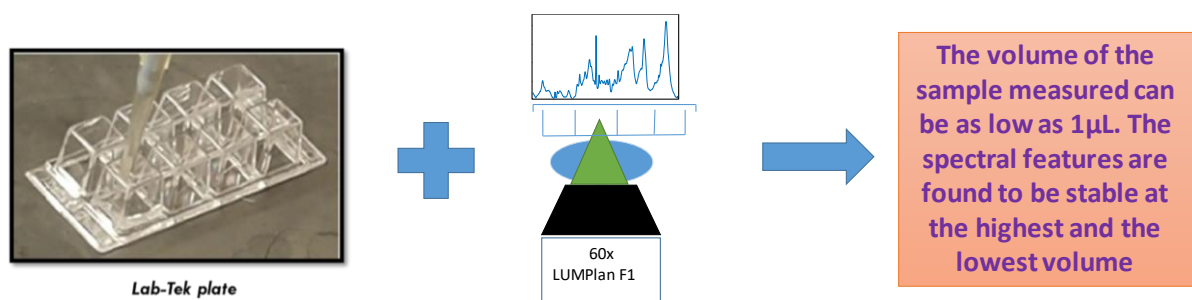


Figure 2. Thin glass bottomed Lab-Tek plate combined with inverted Raman analysis can collect spectral data from very low amount of samples (1µL) making it an ideal tool for clinical laboratory analysis. Spectra can be recorded in less than 1 minute.

3. Measurement of Plasma vs Serum

Human blood plasma or serum are the most commonly studied bodily fluids for disease diagnosis, biomarker discovery and therapeutic drug monitoring [51–57]. While plasma and serum are both cell-free fluids obtained from blood samples by centrifugation, they differ on the basis of whether clotting has been allowed or not. One question which inevitably arises in performing biochemical estimation of blood, therefore, is what is the right choice of medium to be used, plasma or serum? Plasma is commonly obtained as the supernatant layer after refrigerated centrifugation of blood collected with anticoagulants (such as potassium-Ethylenediaminetetraacetic acid, sodium-citrate and lithium-heparin) for 10 minutes at 2,000 x g to concentrate unwanted cells and platelets [58]. For serum preparation, whole blood is allowed to clot at room temperature for about 15–30 minutes, whereupon the clot is removed by refrigerated centrifugation at 1,000–2,000 x g for 10 minutes, often separated by a gel component to avoid contamination [58]. It is important to immediately transfer the supernatant (plasma or serum) into a clean polypropylene tube and maintain samples at 2–8°C while handling. If the samples are not analysed immediately, they should be stored at –20°C or

preferably lower [59]. The time delay between centrifugation and separation of the globular fraction and the improper storage conditions could negatively impact on the protein profile obtained [59]. Therefore, standardisation of sample collection, processing and storage protocols is crucial to ensure reproducibility and consistency in the results obtained. It is also recommended to avoid freeze-thaw cycles, because this may have detrimental effects on many serum components [60].

Blood serum and plasma are predominantly composed of water (~90%), minerals, organic substances and gas (oxygen, carbon dioxide) [60]. Proteins are the predominant molecular components of blood plasma, the remaining constituents being carbohydrates, lipids and amino acids. Serum albumin, globulins, fibrinogen and a handful of other abundant proteins account for 99% of total plasma/serum proteins, while the remaining 1% is composed of low abundance circulatory proteins [54]. Additionally, plasma or serum contain more than 114,000 known metabolites at varying concentration level (<1 nmol/L to mmol/L) [60].

Since most of the clinical analytical instruments are accurate for both serum and plasma, these two terms are used erroneously interchangeably in most clinical tests [7]. Notably, many studies that have been reported to be carried out in serum were in fact carried out in plasma [61–66].

Medipally *et al.* investigated the effect of different instrumental and sample preparation parameters to identify a combination that would reduce the overall acquisition time for recording spectra from blood plasma with minimal of sample preparation steps [50]. Out of the four different laser lines (785 nm, 660 nm, 532 nm and 473 nm) tested, only the 785 nm laser line gave a reliable biochemical signature of liquid plasma samples. Fluorescence was observed when the 660 nm laser line was used and a resonance Raman effect due to the presence of β carotene was observed when 532 and 473 nm laser lines were used. Plasma samples from 10

prostate cancer patients and 10 healthy volunteers were used in this study. A 96 well plate (cover glass bottomed) was used to hold samples of 20 μ L and the Raman spectra were recorded in the inverted geometry. Spectral preprocessing steps and principal component analysis – linear discriminant analysis (PCA-LDA) was performed in the R environment. The classification resulted in a sensitivity and specificity of 96.5% and 95% respectively. Although a cost effective approach to perform rapid analysis of liquid plasma is demonstrated in this study, no attempts have been made to reduce the effect of spectral interferences while using the 532 nm laserline.

In validating the protocols for analysis of bodily fluids using inverted Raman microscopic analysis using 532nm, Parachalil *et al.* utilised a simulated blood plasma mixture of albumin, fibrinogen, cytochrome C, and vitamin B12 [44]. The findings from this study with simulated plasma protein mixture show that the poorly soluble fibrinogen component obscured the systematic variations of the protein concentrations due to high degree of scattering. Mild sonication of the aqueous solution helped to improve the solubility of fibrinogen and significantly improved the Raman spectral intensity by minimising scattering effects. Since centrifugal filtration failed to separate fibrinogen from rest of the proteins, ion exchange chromatography had to be applied to separate the fibrinogen by altering its net surface charge. Although ion exchange chromatography is a quick method to separate proteins, this method has to be tailored for a specific protein depending on its charge and cannot be used as a 'one-for-all' separation kit for all the proteins. In terms of the applications of Raman Spectroscopy for analysis of blood content, it is therefore recommended that fibrinogen content should be extracted from plasma, and mildly sonicated for quantitation, whereas blood serum can be readily further analysed for High Molecular Weight Fraction (HMWF) and Low Molecular Weight Fraction (LMWF) quantification.

4. Serum Fractionation

As long ago as 1999, Berger et al. used near IR light at 830nm to perform Raman microscopic analysis of liquid whole human blood and serum samples to quantify the content of six analytes, namely glucose, cholesterol, triglyceride, urea, total protein and albumin [28]. The total acquisition time per sample was 5 min and the samples were continuously stirred in a quartz cuvette to minimise heating artefacts from high intensity laser. However, no attempts were made to fractionate the serum to deplete the HMWF analytes.

Centrifugal filtration devices have been utilised to improve the sensitivity of quantitative analysis by both Raman and IR spectroscopy, by separating the molecules according to their molecular weight [68]. The proteins that are highly abundant in serum dominate the spectral profile, and by the removal of these proteins (albumin and globulins,) the ability to monitor changes in the lower molecular weight fraction (LMWF) is enhanced. Pre-rinsing of the filter devices with 0.1M NaOH prior to plasma analysis is essential to avoid glycerine interference in the analysis [67]. The optimised washing and rinsing procedure includes spinning 0.5mL 0.1M NaOH at 14000×g for 30 minutes, followed by three rinses with distilled water by spinning 0.5mL distilled water for 30 minutes at 14000×g. Every 30 minute wash and rinse must be followed by spinning the device in the inverted position at 1000×g for 2 minutes, to remove the residual solution contained in the filter. After washing, 0.5mL sample is transferred to the filter and centrifuged at 14000×g for 30 minutes. The solution that flows out from the filter is the filtrate, which contains mostly water and molecules smaller than the pore size of the chosen filter. The remainder of the sample, known as the concentrate, is collected by placing the filter device upside down and spinning for 1000×g for 2 minutes. The resultant concentrate, ~50µL, contains molecules with molecular weight larger than chosen pore size, and is concentrated by a factor of ~10. This indicates the potential for the prediction of other biomolecules that exist within the LMWF with this method, and with further research, such

techniques could be translated into the clinical environment as a rapid tool for screening and monitoring.

In the study conducted using 247 blood donors by Rohleder *et al.* [37], a key was that the prediction accuracy of glucose and urea were improved by up to a factor of 2 by depletion of the HMWF using centrifugal filtration techniques. The processing protocol has been extensively explored for both ATR-FTIR and Raman analysis [39, 68] whereby the HMWF can be isolated in the unfiltered concentrate, while the LMWF filtrate can be further fractionated and/or concentrated. Notably, Bonnier *et al.* have highlighted the importance of appropriate rinsing of the filters to avoid contamination of the filtrate [67]. In the study of Rohleder *et al.* [37], Raman spectroscopy was used for quantitative analysis of serum and serum ultrafiltrate with an accuracy, within the range of clinical interest [37], by using 785nm laser as the excitation wavelength and a quartz cuvette to hold the sample. In this measurement setup, a minimum of 200 μ L sample was required. 10kDa centrifugal filters were used to deplete the HMWF from the serum and the spectra of glucose, urea and uric acid were recorded from the serum as well as the ultrafiltrate.

Parachalil *et al.* further demonstrated the suitability of Raman spectroscopy as a bioanalytical tool, when coupled with ultra-filtration and multivariate analysis, to detect imbalances in both HMWF (total protein content, γ globulins and albumin) and LMWF (urea and glucose) of the same samples of human patient serum, in the native liquid form [69]. Using a validated Partial Least Squares Regression (PLSR) method, the γ globulin and total protein analysis models, based on unfiltered patient serum, produced R^2 values of 0.88 and 0.82, and Root Mean Square Error of Crossvalidation (RMSECV) of 126 mg/dL and 115 mg/dL, respectively. Post fractionation of the patient serum samples by ultra-filtration using 100 kDa and 50 kDa filters, a similar analysis produced an R^2 value of 0.91 and RMSECV of 90 mg/dL for albumin, which is comparable to the values previously reported for a model of aqueous solutions of albumin

over a similar concentration range. In the case of urea, R^2 and RMSECV values of 0.90 and 70.40 mg/dL for the range of aqueous solutions of varying concentrations were achieved, and 0.92 and 1.73 mg/dL, for the low molecular weight (<10 kDa) filtrate of patient samples, when the full spectral range of 400-1800 cm^{-1} was employed. Reducing the spectral range of the analysis to 800 cm^{-1} to 1030 cm^{-1} considerably improved the prediction accuracy and sensitivity, resulting in an R^2 value of 0.97 and RMSECV of 1.14 mg/dL. In the case of glucose, a reduced spectral range from 1030 cm^{-1} to 1400 cm^{-1} was chosen to avoid interference from urea, resulting in an R^2 value of 0.84 and RMSECV value of 1.84 mg/dL in the filtrate from the same patient samples. Although both the proof-of-concept studies have been carried out on rather small populations (25 patient samples), they demonstrate that the method has potential for clinical implementation for early disease diagnostics from bodily fluids. In this work, ultrafiltration in conjunction with chemometric methods were used to overcome three problems: first, eliminating the interference from water and β -carotene; second, extracting the Raman signals from LMWF analytes; and third, solving the problems associated with multiple analyte variations in the serum. The sample preprocessing enables a fractionation and concentration of the different molecular weight fractions of the serum, enabling their analysis and quantification without the need for additional enhancement techniques. It has been demonstrated that, using Ag or Au colloids, intense and repeatable spectra are only obtained if the high molecular weight protein fraction is filtered out from the serum [70]. Enhanced signals of the low molecular weight fraction can then be obtained, although the process of addition of colloids would add to the workflow in terms of time and cost.

5. Data preprocessing

Data preprocessing, such as spikes removal, baseline correction, smoothing etc., aims to remove any perturbations to the spectra due to any distortions to the spectra which might arise during the measurement process (scattering, electronic noise). This pretreatment of data is critical to accessing the desired information without losing crucial information. The fundamental concepts and basic theory of most used chemometric tools in the pharmaceutical industry for pre-processing, processing and post-processing of the generated data have been detailed in the review article by Sacre *et. al* [71]. The majority of the commercial software, e.g. Labspec, includes real-time spikes correction, enabling visual inspection of the spectra, and/or manual spike removal when very few spikes are present. Some algorithms have been developed to perform automated spike removal in the case of a large dataset, for which manual correction is not possible [72–76]. The most commonly used smoothing algorithm for de-noising the spectra without losing much information is that of Savitzky-Golay [77]. Drifts in the baseline occurring due to scattering or fluorescence may be corrected using different approaches such as asymmetric least squares [78], mixture models [79], polynomial filter [80, 81] and the rubber band method [82]. In the study conducted by Medippally *et. al.*, rubber band baseline correction, Savitzky–Golay smoothing algorithm and vector-normalisation was performed on the raw Raman spectra of liquid plasma prior to post processing, in R based statistical software [50]. In studies aimed at early diagnosis of oral cancer using Raman spectroscopy by Sahu *et. al.*[83] and differentiation Meningioma by Mehta *et. al.*,[84], raw liquid serum spectra were baseline corrected using a fifth order polynomial function, smoothed using Savitzky-Golay algorithm and vector normalised in Matlab based statistical software. Fifth order polynomial background subtraction from the raw serum spectra and normalisation was also implemented in Matlab by Rohdeler *et. al.* [36] in their study to compare mid-IR and Raman spectroscopy in quantitative analysis of serum. Two different algorithms were used by the same group to

perform pre-processing of spectra of serum spectra of ultrafiltrate [37]. Spectra originating from ultrafiltrate were scaled to the area under the water Raman band at 1640 cm^2 after determining the area by a Gaussian fit with linearly decreasing background from 1550 to 1770 cm^2 . In the case of serum, spectra were scaled to the maximum intensity and a fifth order polynomial background was subtracted. In their study to detect alterations in glucose and lipid components in the serum using near-IR Raman spectroscopy, Borges *et. al.*[85] fitted a seventh-order polynomial function over the $400\text{-}1800\text{ cm}^{-1}$ region and subtracted from the raw serum spectrum to remove the unwanted background, providing an effective baseline correction, and spectra were normalised by the area under the curve prior to data analysis. Least squares fitting was used by Berger *et al.* to remove the background mathematically by subtraction of a sloped straight line from each serum spectrum without affecting the shape of the Raman peaks [86]. Jenkins *et. al.* subjected the raw Raman spectra of serum to wavenumber standardisation, background subtraction using a rolling circle filter algorithm and normalisation to the peak at 1004 cm^{-1} [29]. The phenyl alanine peak was chosen for normalisation as it is the sharpest and most intense peak within the serum spectra and it is observed that normalisation to this peak produced better diagnostic discriminatory results when compared to other normalisation methods such as vector normalisation.

Although the accepted dogma is that the contribution of water to Raman spectra is significantly less than to mid-IR spectra, water still has a significant contribution to the Raman signatures in the fingerprint region of bodily fluids, due to the OH stretching vibration at 1640cm^{-1} . Analysis of the ultracentrifugation concentrate reduced the relative contribution of the water signal [68] but further efforts to remove it by data preprocessing have been explored, to increase the relative contribution of the analyte. Kerr *et al.*, [87] have compared a number of commonly employed data pre-processing techniques, and, especially in the case where the background contains a known interferent, such as substrate or background, demonstrate the benefits of the

adapted EMSC model, which also contains a polynomial with linear and higher order components. Parachalil *et al.* adopted an adapted method of Extended Multiplicative Signal Correction (EMSC) [87] to subtract the known spectrum of water from their spectra, initially for the analysis of the HMWF in simulated plasma mixtures [44], and subsequently the measurement protocol was used to quantitatively monitor diagnostically relevant changes of glucose in liquid serum samples (spiked serum samples and patient samples), and the results were compared with similar analysis protocols using infrared spectroscopy of dried samples [43]. The analysis protocols to detect the imbalances in glucose using Raman spectroscopy were first demonstrated for aqueous solutions and spiked serum samples. As in the case of infrared absorption studies [40], centrifugal filtration was utilised to deplete abundant analytes and to reveal the spectral features of LMWF analytes, in order to improve spectral sensitivity and detection limits. After the depletion of the abundant proteins, the dominant water peak from the filtrate collected after centrifugal filtration using 10kDa can be removed by using the EMSC algorithm, and PLSR analysis applied to obtain a prediction model relating the glucose concentrations and the intensity of glucose features. Note, that the study introduced a water normalisation factor into the EMSC protocol, using the fitted co-efficient of the water scaling factor, which has the effect of scaling the analyte spectra, assuming a constant water contribution to all sample spectra. This step helps to considerably reduce the spectral variability of the Raman spectra of glucose recorded from 25 patient samples (Figure 3) [44].

The principle of EMSC for subtraction of a specific measurable background spectrum and the associated Matlab codes have previously been published by Kerr and Hennelly, 2016 [86], and their description is adapted in the following. The raw spectrum, S , consists of Raman spectrum of interest, R , a baseline signal, B , and the water signal, W .

$$S = R + B + W \text{ [86]} \tag{1}$$

The Raman spectrum of interest can be represented by a reference spectrum of the material of interest, r , and it can be assumed that R is the product of this reference spectrum and a certain scalar weight, C_r , which describes the concentration dependence [88,89]

$$R \sim C_r \times r \quad [86] \quad (2)$$

Similarly, a spectrum, w , is recorded from water directly in order to represent the spectral contribution of water in W , as the product of pure water spectrum and a certain scalar weight.

$$W = C_w \times w \quad [86] \quad (3)$$

The baseline, B , is now represented by an appropriate order of polynomial (N) as:

$$B_N = C_0 + C_1X + C_2X^2 + \dots + C_NX^N \quad [86] \quad (4)$$

where N is the order of polynomial and C_m for $m = 0 \rightarrow N$ represents various coefficients of polynomial. The EMSC algorithm is used to obtain estimates of the scalar values C_r , C_m and C_w . These estimates are obtained from an optimal fit of the various vectors in Equation 5.

$$S \sim [C_r \times r] + [C_w \times w] + [\sum_{m=0}^N C_m X^m] \quad [86] \quad (5)$$

The background corrected, concentration dependent analyte spectra, T , can be represented as:

$$T = \frac{S - [C_w \times w] - [\sum_{m=0}^N C_m X^m]}{C_w} \quad [86] \quad (6)$$

Note, that division by C_w has the effect of scaling the analyte spectra, assuming a constant water contribution to all sample spectra.

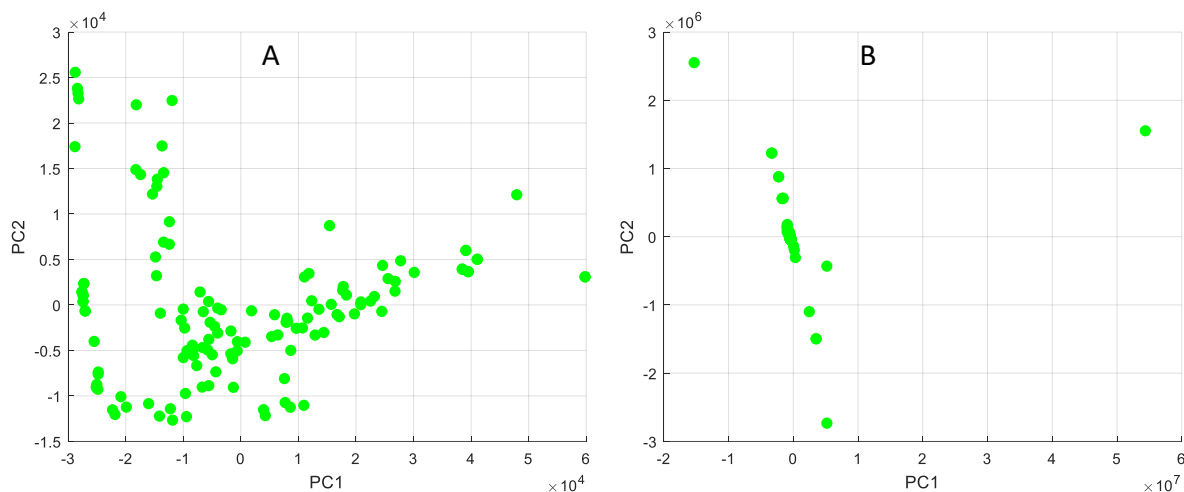


Figure 3. (A) PCA scatter plot of Raman data of the filtrate obtained after ultrafiltration of patient serum without scaling the analyte (glucose) spectra from the previously published study by Parachalil *et. al* [44] and (B) after scaling the analyte spectra to the water content. Figure 3B displays less scatter when compared to Figure 3A, indicating less variability among the spectra

However, although the EMSC algorithm removed the underlying water spectra effectively, interference from other LMWF analytes, namely, urea was noticed. Hence, a shorter spectral range from 1030cm^{-1} to 1400cm^{-1} was chosen for data analysis, as this region does not contain signature peaks of urea. Improved Root RMSECV was observed for Raman prediction models, whereas slightly higher R^2 values were reported for infrared absorption prediction models.

The adapted EMSC algorithm can also be employed to remove known spectral interferents. In several studies of human serum, β -carotene has been observed as a strong contributor to the spectrum, particularly when using 532nm or lower as source, as the scattering from the conjugated antioxidant species are resonantly enhanced [29, 50, 83]. The LMWF species are not easily removed by centrifugal separation [69], but their contributions can be effectively “digitally” removed using the adapted EMSC protocol.

6. Data Analysis

Data analysis protocols can be differentiated into classification protocols, largely employed for diagnostic purposes, and regression protocols, predominantly used for quantitation of identified analytes. The latter have been explored for both imbalances in intrinsic blood constituents, as well as for therapeutic drug monitoring.

Depciuch *et al.* collected Raman and FTIR spectra from dried and liquid serum from depressed patients and their analysis indicated that both methods provided equally valid results to discriminate the patients with similar accuracies [33]. The light source wavelength was 780 nm and the sample volume used was 1.5ml, which could be an impediment for clinical translation. Other similar studies conducted by the same group using the same set up on phospholipid-protein balance in human serum and qualitative, quantitative changes in phospholipids and proteins in animal depression models. The role of zinc deficiency induced phospholipid-protein imbalance in serum of the animal models suggest that both IR and Raman spectroscopic techniques could be used as effective tools to identify the changes in the blood serum [30–32]. To investigate similarities and differences between the serum samples of different types of depression in humans, Principal Components Analysis-Linear Discriminant Classification (PCA-LDC) was employed in their analysis of Raman and FTIR spectra from dried and liquid serum from depressed patients [33]. The results from both FTIR and Raman spectra unambiguously demonstrated that the levels of proteins and phospholipids are higher in healthy controls than in depressed subjects and that phospholipids affect the structure of proteins. Two measurement strategies were compared in order to determine the influence of water on the measured spectra; the first method entailed recording the water spectrum as back-ground, and subsequently automatically subtracting it from each serum spectrum, while the second method entailed recording a spectrum of the air prior to the spectrum of blood serum and subsequently the water spectrum was subtracted from the blood serum without air background. Both the methods provided identical serum spectra, suggesting that appropriate measurement of the

background and the subtraction of water signal had the greatest impact on the reliability of the results.

Jenkins *et al.* developed a high-throughput (HT) serum Raman spectroscopy platform and compared dry and liquid data acquisition of serum samples for liquid biopsy of 30 colorectal cancer patients and 30 matched control patients [29]. Using a stainless steel high throughput substrate that allows up to 40 samples to be loaded at once, and 785 nm laser as the excitation wavelength, the maximum sensitivity and specificity obtained for discrimination of colorectal cancer patients were 77% and 81% respectively. In this study, Raman spectra were subjected to routine preprocessing such as wavenumber standardisation, background subtraction using a rolling circle filter algorithm and normalisation to the peak at 1004 cm^{-1} . Partial least squares discriminant analysis (PLS-DA) was used to investigate causes of differences and variances within datasets. PLS-DA models were cross validated using k-fold cross validation with 5 folds. The sample volume required was 200 μL and the total time for the data collection was 12.5min. In a clinical setting, it would be advantageous to reduce the data collection time in order to avoid delay in analysis. When excitation wavelength of 532nm was used on the liquid platform, the specificity was found to be low, due to the prominence of carotenoids in the serum spectra. No measures were taken to remove the interference of carotenoids from the spectral data. The prominence of carotenoids due to resonance Raman was also reported by Sahu *et al.*, in an exploratory study for detection of oral cancers [83] and by Mehta *et al.*, in their study conducted to differentiate meningioma. Sahu *et. al.* used 532nm as excitation source and Raman spectra were collected by placing 30 μL serum samples on a glass slide and the laser was focused through a 50X Nikon objective. The spectra were vector normalised, baseline corrected, smoothed and subjected to PCA-LDA followed by cross-validation using leave-one-out cross-validation (LOOCV). Although this study reported spectral differences between DNA, changes in the plasma amino acid profiles and β -carotene levels across the analysed

groups, the strong bands of β -carotene could interfere with the detection of other analytes increasing the ambiguity in the results obtained. In the study conducted by Mehta *et al.*, 25 patient samples from healthy and meningioma groups were subjected to PC and PC-LDA followed by LOOCV cross validation, yielding classification efficiency of 92% and 80% for healthy and meningioma respectively. Passively thawed 30 μ L serum samples, on a calcium fluoride (CaF_2) slides were subjected to Raman analysis using 785nm laser excitation source. Borges *et al.*[85], collected blood serum from 44 volunteers to discriminate between altered and normal concentrations of glucose, total cholesterol, triglycerides, low density (HDL) and high density lipoproteins (LDL). Raman analysis was performed using 830nm excitation source for a sample volume of 100 μ L. The data collected was subjected to PCA yielding a classification efficiency of 77% for total cholesterol, 81% for triglycerides, 59% for HDL and 60% for LDL.

In terms of quantification of systematically variable analyte concentrations, multivariate PLSR analysis is often the method of choice. PLSR is most commonly employed to construct a model that can relate variations of the measured spectral responses to a systematic variation of concentrations of the target analyte [90–92]. The constructed model can then be employed to identify spectral factors which account for the maximum variation in predictors ‘X’ (spectral data) versus associated responses ‘Y’ (target values of protein concentration) [91]. The spectral data (X matrix) can thus be related to the target concentrations (Y matrix) according to the linear relationship $Y = XB + E$, in which B and E are matrices of regression coefficients and residuals, respectively. The PLSR model can be used to predict the outcome of varying concentration of analytes based on the spectral data. The model is validated using a rigorous cross validation procedure which evaluates its performance in accurately predicting analyte concentrations [44]. In the study of Berger *et al.*, PLSR prediction models were built, using leave one out cross validation (LOOCV), for each the six analytes after spectral background

removal, providing an root mean square error of 26mg/dL, 12mg/dL, 29mg/dL, 3.8mg/dL, 0.19mg/dL and 0.12mg/dL for glucose, cholesterol, triglyceride, urea, total protein and albumin, respectively [28]. A similar approach was employed by Parachalil *et al.* [43] to compare the predictive capacity of Raman microscopy for quantitation of glucose in human serum, compared to the similar ATR-FTIR analysis of Bonnier *et al.* [87] indicating that the RMSECV for Raman of 1.84mg/dL is comparable or better than that of 3.1mg/dL for ATR-FTIR.

Having removed the spectral interferences using EMSC, PLSR can independently identify spectral correlation associated with target analyte range. Cross-validation of the PLSR model is required to evaluate the accuracy, often cited in terms of the Root Mean Square Error of Cross Validation (RMSECV) [93]. The number of latent variables used for construction of the PLSR model is optimised by establishing the value that is equivalent to the minimum of the RMSECV. The R^2 value provides an indication of the correlation between the analyte concentration and spectral intensity, while the standard deviation (STD) provides an indication of the variation between each spectrum calculated from the same sample. Commonly, a multiple fold cross validation approach is employed to validate the robustness of the method. Typically, the set of observations is randomly divided into approximately equal size, e.g. 50% of the spectral data randomly selected as test set, while the remaining 50% is used as the training set [101]. The cross-validation process is then repeated multiple times (the folds), such that all observations are used for both training and testing, and each observation is used for testing exactly once. The results from the folds can then be averaged to produce a single estimation. The Root Mean Square Error of Cross Validation (RMSECV) is calculated from the multiple iterations to measure the performance of the model for the unknown cases within the calibration set.

In order to advance the studies of Berger *et al.*, [86] and Rohleder *et al.*, [36, 37] using the improved inverted microscopy modality, Parachalil *et al.* [69] also used the EMSC correction, ultrafiltration protocols and PLSR analysis to construct models for the quantitation of the whole serum (total protein, γ globulin), serum concentrate (albumin) and serum filtrate (urea and glucose) of patient samples, resulting in higher accuracy and sensitivity of analysis. The strategy demonstrated in this study enables the simultaneous estimation of total protein level and detection of imbalance in γ globulin concentration accurately from whole serum, without the use of any reagents and without destroying the sample being studied. The proposed method has many advantages over the routinely used biuret method, Turbidimetric Immunoassay (TIA) and Radial Immunodiffusion (RID), as the required sample volume can be as low as 10 μ L, it is rapid and non-destructive to the medium being studied, whereas the conventional methods are considered impractical due to the requirement of large sample volume and laborious sample processing steps [94–96]. Moreover, the linearity reported for a comparative study of RID and TIA is 0.59 [97], substantially lower than the R^2 calculated by Parachalil *et al.* [44]. Using filtration, the albumin was isolated from patient serum and the prediction model had a prediction accuracy significantly superior to that reported for the Bromocresol Green method (2.2 g/dL), used to determine albumin concentrations from cirrhotic patients [98]. It has previously been reported that selecting the spectral region from 1030 cm^{-1} to 1400 cm^{-1} improved the sensitivity and specificity for the prediction model of glucose from patient samples over the concentration range 52.5–434.2 mg/dL, and the technique was demonstrated to be at least as accurate as ATR-FTIR of similar patient samples [69], measured in the dried state and closer to the accuracy of colorimetric methods, 1.4 mg/dL urea [99] and 2 mg/dL glucose [100]. Similarly, higher prediction accuracy (RMSECV=1.14 mg/dL) was attained when PLSR analysis was performed on a reduced range for urea from patient samples over the concentration range 2.52–78.99 mg/dL, compared to the full range (RMSECV=1.73 mg/dL).

This standardised, optimised methodology was also applied to determine the Limit of Detection (LOD) and Limit of Quantification (LOQ) for therapeutic drug monitoring (TDM) in human serum, using the examples of Busulfan, a cell cycle non-specific alkylating antineoplastic agent, and, Methotrexate, a chemotherapeutic agent and immune system suppressant [101]. Ultrafiltration of the spiked human pooled serum with 10kDa centrifugal filter efficiently recovered the drug in the filtrate prior to performing Raman analysis. The drug concentration ranges were chosen to encompass the recommended therapeutic ranges and toxic levels in patients. Finally, prediction models were built by using PLSR and LOD and LOQ were calculated directly from the linear prediction models. The LOD calculated for Busulfan is 0.0002 ± 0.0001 mg/mL, 30-40 times lower than the level of toxicity, enabling the application of this method in target dose adjustment of Busulfan for patients undergoing, for example, bone marrow transplantation. The LOD and LOQ calculated for Methotrexate are 7.8 ± 5 μ M and 26 ± 5 μ M, respectively, potentially enabling high dose monitoring. Although SERS gives promising results in detecting drugs at low concentrations in biological matrices [16, 102], qualitative variations within the SERS substrate, and the interference of other biomolecules with the SERS spectra, makes quantification in real samples a challenging task [103]. The simpler approach, adopted here, of Raman spectroscopy coupled with concentration of the high and low molecular weight serum fractions using commercially available centrifugal filter devices and multivariate analysis technique ensures that the information in the fractions is effectively preserved, while enabling easy detection of the analyte concentration with higher accuracy than in the unprocessed sample. Better analysis of serum using Raman spectroscopy was observed when the sample was analysed in the inverted geometry using the water immersion objective with a 532nm laser as source with Lab-Tek plate as substrate [44]. A drop of water is used to minimise the differences in the refractive indexes between sample, objective and the substrate. However, the water drop does not contribute to the data collected, as it is

outside the focus of the beam. The promising results from the systematic studies conducted by Parachalil *et al.*[69] using this analytical set up combined with chemometric techniques such as EMSC to measure concentrations of HMWF, LMWF and drugs in human serum strongly indicates a highly significant correlation between predicted and reference concentrations. These results suggest that Raman can on its own can detect and quantify concentrations close to 1% without the aid of any enhancement method [37].

Considering the more general applications of the technique to a broader range of drugs or analytes, it is notable that both the LOD and LOQ for a given set up are determined by the STD of the measurement of the control (serum or filtered serum), and the Raman scattering efficiency of the analyte. To further illustrate this point, the LOD of vitamin B12, cholesterol, urea and glucose from liquid samples for this measurement protocol were also determined using the method previously reported by Parachalil *et al.* [101] (Table 1). Figure 4 displays a plot of LOD versus the maximum intensity per unit acquisition time, per unit concentration. An approximately linear, inverse relationship can be seen, which simply emphasises the maxim that the stronger the Raman signal of the analyte, the easier it is to detect. The correlation also implies, however, that a simple measurement of the analyte in aqueous solution could be employed to predict the LOD, and therefore the suitability of the technique for the therapeutic monitoring application.

Table 1: LOD of glucose [44], busulfan [101], methotrexate [101], cholesterol, urea [69] and vitamin B12 calculated from the PLSR prediction plot of these analytes, compared to the maximum Raman intensity of maximum peak per unit acquisition time, per unit concentration

Analyte	Intensity (arb. units)	LOD(mM)
Glucose	1000	0.0006±0.0005
Busulfan	19704	0.0008±0.0001
MTX	1600	0.00076±0.0005
Cholesterol	216718	0.0006±0.0001
Urea	540000	0.00033±0.00001
VB12	1266666	0.00014±0.00002

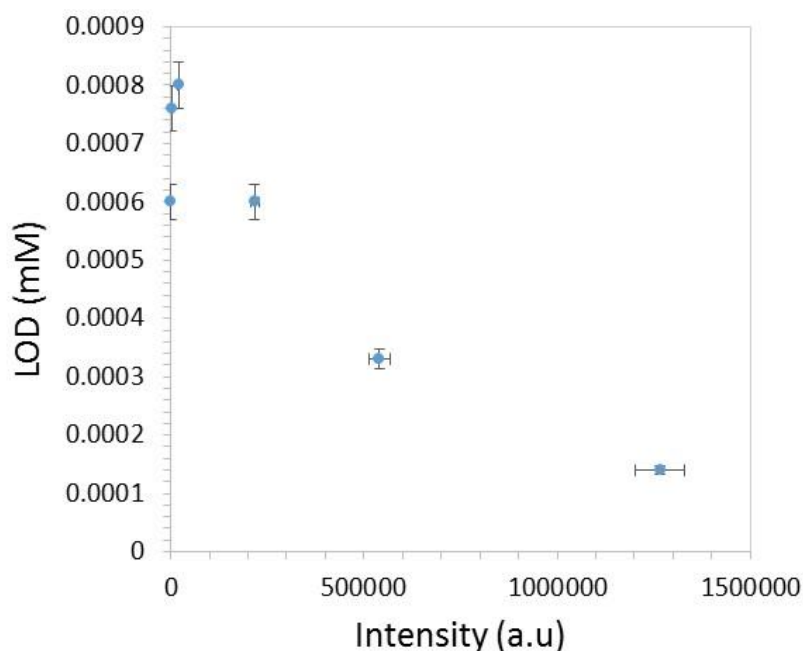


Figure 4. LOD of glucose (44), urea (69), busulfan (101), methotrexate (101), cholesterol, and vitamin B12 calculated from the PLSR prediction plot of these analytes, compared to the ‘maximum Raman intensity of maximum peak per unit acquisition time, per unit concentration’. The methodology used for calculating LOD was previously published (101)

7. Clinical Translation

Vibrational spectroscopic techniques have attracted a lot of attention as analytical methods of choice for analysing biological samples. The promise of the techniques is based on the abilities to objectively fingerprint the biochemical profile underlying early onset of disease in cells, tissues or bodily fluids [3, 6, 9]. The techniques have no requirement of specific reagent, and the minimal sample preparation means there should be fewer procedural errors and no major time delay in providing the results to the patients. In the case of FTIR, considerable progress towards using this technique as a routine diagnostic tool for disease diagnosis has been made. Recently, ClinSpec Diagnostic, based at the University of Strathclyde's Technology & Innovation Centre, won a £1.2 million investment to further develop a "revolutionary" blood test using FTIR which could potentially improve brain cancer survival rates through early detection of the disease [104]. Researchers from Monash University have similarly developed FTIR based techniques to analyse disease-causing pathogens in human blood [105]. Advances in instrumentation have made FTIR an ideal tool of choice for an increasing number of clinical application even though the sample drying step is potentially a huge drawback [19, 68, 106]. Notably, it is demonstrated that the Raman analysis protocol can yield accuracies which are comparable with those reported using infrared absorption based measurements of dried serum, without the need for additional drying steps. However, there remain challenges to fulfilling the requirements for clinical translation. Distortions in the spectra due to the water contributions, low detectability of the low molecular weight fraction analytes, lack of standardisation steps and financial factors could be the hindrance for clinical translation in the medical and clinical environment. Nevertheless, new strategies have been recently developed to address these potential limitations, namely the inverted Raman geometry, low cost (Lab-Tek plate) substrates, serum fractionation techniques, selective spectral region analysis and sophisticated data preprocessing (EMSC) and analysis (PLSR) techniques. These advances have maximised

the diagnostic accuracy and are cost-effective solutions that are likely to be adopted in a clinical setting.

Application of the adapted EMSC based algorithm helped to eliminate the contribution from water and scattering associated with the HMWF, and linear predictive models were built from the PLSR analysis. It is worth noting that PLSR seeks to correlate systematic variations in the spectroscopic profiles with the external target variable, in this case protein concentration. As such, the method is not inherently specific to any molecular variations, in the case where multiple species vary simultaneously over the same range. The accuracy of the proposed method is comparable to that of the most commonly used method for detecting albumin from biological fluids, the Enzyme Linked Immunosorbent Assay (ELISA) [107, 108], and the most commonly used gold-standard method i.e., the Clauss assay for fibrinogen [109]. The proposed approach can be expeditiously employed for early detection of pathological disorders associated with high or low plasma/serum analytes.

Bodily fluids are usually collected from a large number of patients in a hospital, potentially delaying the performance of the analysis and availability of results, which may in turn delay the therapy, and prolong patient anxiety. The accuracy of the conventional test kits that enable point-of-care testing can be poor, and they are can be avoided due to high cost [110,111]. The enormous advantage of employing this inverted Raman spectral analysis for real-time serum/plasma analysis in a clinical setting is that, in the face of emerging diseases, one could get an early diagnosis in a cost effective manner before the disease becomes symptomatic (Figure 1). Integrated with smaller spectroscopic instrumentation, implementation of such a screening/diagnostic tool in developing countries where conventional diagnostics methods are scarce and avoided due to high cost, would bring about a dramatic impact on population screening.

8. Conclusion

The potential advantages of employing Raman spectroscopy of bodily fluids for disease diagnosis apart from its high sensitivity and specificity are: low cost, no reagents are required, samples can be probed in the native state and rapid results can be obtained. Although there is a wealth of information to support the application of Raman analysis for bodily fluids, this approach is still considered young and upcoming in the eyes of medical community, thereby hindering its clinical translation. The examples summarised in this article attest to the cost effective, easy to use, reproducible method of Raman measurement protocol for detecting imbalances in serum/plasma proteins and low molecular weight analytes, as well as therapeutic drug monitoring. Significantly improved sensitivity of Raman spectroscopic measurements of blood samples in liquid form is achievable by means of serum fractionation and the implementation of chemometric approaches. In the case of clinical translation, there remain challenges to overcome before it can be a reality. Nevertheless, it has been demonstrated that options and alternatives are available to overcome these challenges in using Raman spectroscopy for liquid sample analysis, leading to a better accuracy and repeatability and thus a better sensitivity. Naturally, the next phase includes not only refining this method and improving the technical capabilities to match those of the current clinical needs, but also technical advances to translate them from research laboratory to clinical practice. In addition, to ensure further relevancy of this method, comparison studies between the gold standard of current diagnostic methods and the current method in a large multi-centred randomised clinical trials are required. Once these factors are taken into account, it is possible to envisage a routine platform providing clinical biochemical analysis at minimal cost.

Conflicts of interest

There are no conflicts to declare.

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