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Quantitative reagent-free detection of fibrinogen levels in human blood plasma using Raman spectroscopy

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Fibrinogen assays are commonly used as part of clinical screening tests to investigate haemorrhagic states, for detection of disseminated intravascular coagulation and as a predictor of a variety of cardiovascular events. The Clauss assay, which measures thrombin clotting time, is the most commonly
10 used method for measuring fibrinogen levels. Nevertheless, inconsistencies are present in inter-manufacturer reagent sources, calibration standards and methodologies. Automated coagulation analysers, which measure changes in optical density during the prothrombin time (PT-Fg), have found use in many hospitals. However, the PT-Fg method is found to give falsely elevated values due to varying choices of calibrants, reagents and analysers. As an alternative, Raman spectroscopy has previously been applied to
15 the analysis of blood and its various constituents to determine various analyte concentrations such as glucose, urea, triglycerides and cholesterol. In this study, Raman spectroscopy was investigated for its ability to accurately quantify fibrinogen concentration in blood plasma. Samples collected from 34 patients were analysed by Raman spectroscopy and the resultant spectra were fitted with a Partial Least Squares Regression model using target values obtained through a pre-calibrated Clauss fibrinogen assay.
20 Various spectral pre-processing methods were utilised to prepare data to be entered into a calibration model. A root mean square error of prediction of 0.72 ± 0.05 g/L was achieved with as few as 25 spectra. In this pilot study, Raman spectroscopy has been demonstrated to be a robust technique providing rapid and reagent-free quantification of fibrinogen levels in blood plasma and a potential alternative to the Clauss assay.

25 Introduction

The activation of fibrinogen or Factor I, a 340 kDa dimeric plasma glycoprotein synthesised in the hepatocytes of the liver, is involved as an acute phase protein in the pathogenesis of inflammation, tumour growth and various diseases. During
30 circulation, fibrinogen is degraded into various molecular weights, 69.7 % remaining in the original high molecular weight (HMW) form (340 kDa), 26.5 % as low molecular weight (LMW') fibrinogens (305 kDa) and the remaining 3.8% in the low molecular weight (LMW) configuration of 270 kDa, as a
35 result of the consecutive removal of a 35 kDa carboxyterminal polypeptide from one of the A α -chains.¹ Epidemiological studies have demonstrated elevated levels of fibrinogen in blood to be an independent risk factor for cardiovascular and coronary heart disease^{2, 3}, while low fibrinogen levels are seen as an important
40 diagnostic physiological variable in pathological conditions including various haemorrhagic states, disseminated intravascular coagulation, liver disease and during thrombolytic/defibrination therapy.⁴ Fibrinogen concentration has also been shown to be an independent prognostic parameter in patients diagnosed with
45 cervical cancer.⁵ Fibrinogen levels in plasma may be determined through various methods. These methods are based on different techniques of detection such as measures of clotting rate⁶ or clottable protein,⁷ turbidimetry (light scattering of the sample),⁸
50 immunological/antibody⁹ and heat/salt precipitation¹⁰. Assays based on clotting rates and changes in turbidity/light-scattering are most commonly used in clinical practice. The Clauss

fibrinogen assay, developed in 1957, measures the thrombin clotting time and remains the most frequently used technique for
55 fibrinogen measurement. The method entails the addition of excess thrombin to diluted plasma samples and the subsequent measurement of the clotting time. The recorded time is compared with a calibration curve constructed through the measurement of clotting times from a series of dilutions of reference plasmas of
60 known fibrinogen concentration, allowing the concentration in the test sample to be established. Prothrombin time-derived (PT-Fg) methods are currently used in approximately 50% of UK Haematology laboratories¹¹, their popularity being largely due to the introduction of coagulation automates. The assay measures
65 the turbidity and light-scattering properties after the complete clotting of the plasma occurs. The analyser requires calibration through the recording of the prothrombin time on a series of plasma dilutions of known fibrinogen concentration and the subsequent construction of a graph of the change in optical
70 properties against fibrinogen concentration. The optical response recorded from the test sample may then be converted to fibrinogen content. It has also been shown that the LMW and LMW' fibrinogens exhibit prolonged clotting times compared with HMW fibrinogen.¹²
75 Despite the popularity of the two assays, several issues have been raised regarding their reproducibility and standardisation. The Clauss assay is relatively time consuming with respect to calibration, requiring sufficient expertise to evaluate difficult end-points from high dilutions of standard plasmas, samples with low
80 fibrinogen content or those forming unstable clots.⁴ Analysers

relying on mechanical, tensile clot strength end-point detection may be affected by heparin therapy while photo-optical systems measuring light scattering changes may be vulnerable to slow fibrin polymerisation, turbid reference plasma samples along with the presence of any bile pigment or free haemoglobin.^{4, 13} Inconsistencies arise when fibrinogen levels are measured using commercial reagents sourced from different manufacturers. For example, thrombin strength, buffer composition (anticoagulant inhibitors and degradation products) along with calibration and dilution methods suggested by the various manufacturers vary significantly.¹⁴ Despite the availability of International Standard preparations for plasma fibrinogen, some commercial reference samples have been found to be incorrectly calibrated using internal laboratory plasma samples or their respective national standards.¹⁵ Substantial differences were also observed depending on the type of analyser used.

Raman spectroscopy measures vibrational modes in molecules and can be employed to characterise more complex mixtures of molecules. The spectrum of a complex biological mixture of proteins is composed of contributions from each of the major components in that mixture, and changes in the concentration of one or more components in that mixture will give rise to reproducible changes in the Raman spectrum. Both Raman and infrared spectroscopy, in combination with multivariate statistical techniques, have previously been investigated as a potential clinical diagnostic tool to differentiate between normal and malignant cells¹⁶⁻¹⁸ as well as tissues from the skin, breast, prostate, oesophagus and cervix.¹⁹⁻²³ Furthermore, it has been demonstrated that vibrational spectroscopy has the ability to quantify various analyte concentrations (total protein, glucose, urea, triglycerides and cholesterol etc.) in blood and its fluid derivatives.²⁴⁻³² In contrast to current assay approaches, which rely on physicochemical means of determining fibrinogen, vibrational spectroscopy provides detailed chemical information of the biological compounds, each possessing unique spectral signatures. Rohleder et al. compared mid-infrared spectroscopy to Raman spectroscopy with respect to the analysis of various blood serum constituents including total protein content²⁴ and the strengths and weaknesses of each technique were addressed (sample preparation, operational parameters etc.). Significantly it was found that Raman spectroscopy can quantify the total protein content with lower root mean square errors of prediction than corresponding mid-infrared spectroscopic results. While Raman spectroscopy has been used previously to establish total protein content in blood serum,^{24, 31, 33} the current pilot study attempts to distinguish and quantify the single component of fibrinogen in dried, undiluted, whole human blood plasma collected from patients. Being able to determine changes in protein levels in whole blood samples compared to serum has the potential advantage of minimising sample processing. Although fibrinogen is a minor component of the total protein content (~4%), the ability to correlate sample dependent variations in content, through a regression model relating changes in spectroscopic signatures to the gold standard Clauss assay, promises a potential alternative routine screening assay. Spectral data was therefore collected and modelled using a partial least squares regression algorithm, correlating values to those obtained in parallel using a pre-calibrated Clauss Fibrinogen assay.

EXPERIMENTAL PROCEDURES

Blood Plasma Preparation

Venous blood from 34 patients was collected in BD Vacutainer™ Plus plastic tri-sodium citrate tubes. After eight inversions, each tube was centrifuged at 1500g for 15 minutes to separate the plasma and cellular components. Samples were immediately stored at -20 °C until needed. Prior to measurement, the microtubes were thawed in a 37 °C water bath and 10 µL of plasma carefully spotted in 0.75 x 0.75 cm² squares onto pre-cleaned calcium fluoride windows (Crystran Ltd., UK).

Table 1. Performance of Clauss Fibrinogen Assay (HemosIL® Fibrinogen-C ACL Top Family)

	Sample Number	Range (g/L)	
Normal Range	119	2.38 – 4.98	
	Mean Fibrinogen (g/L)	CV % (run to run)	CV % (day to day)
Normal Control	3.03	4.5	5.5
Low Control	1.07	5.1	6.8
Linearity	0.35 – 10		

The plasma squares were then passively dried at room temperature for at least 4 hours in a desiccator filled with a silica gel desiccant. Fibrinogen content was determined using a pre-calibrated HemosIL® Fibrinogen-C ACL Top Family Clauss assay; performance values provided by the manufacturer are listed in Table 1. The fibrinogen content recorded from patient samples ranged from 0.60 to 8.18 g/L. Crystalline purified Fibrinogen from human plasma, used as a comparative control for the spectral measurements, and was purchased from Sigma-Aldrich®, UK.

Instrumentation

Raman Measurements

Raman spectra were recorded on a Horiba Jobin-Yvon (Villeneuve d'Ascq, France) HR800 LabRam Raman spectrometer (LabSpec V5.0 software) using a 785 nm diode laser source (~110 mW at the sample), focussed onto the sample through a 100x objective lens (NA=0.9, MPlan, Olympus). Raman scatter was collected in a back-scattered configuration through a 100 µm confocal aperture onto a spectrograph/Peltier-cooled Synapse CCD detector. A 300 line/mm grating was used to scan a spectral region between 400 – 1800 cm⁻¹, achieving a dispersion of ~1.5 cm⁻¹ per pixel. Calibration was performed with reference to the 520.7 cm⁻¹ peak of crystalline Si, and spectra were corrected for detector dark noise, system optics and system spectral response through the use of a Raman Intensity Correction Standard (SRM 2241, NIST). Spectra were recorded from a square array of 10 x 10 points (totalling 100) spaced 2 µm apart using 30 x 3 second acquisition periods per point. The array was collected from the homogenous middle of the dried plasma square.

FTIR Measurements

Parallel infrared absorption spectroscopic imaging was carried out using the same dried plasma squares on CaF₂ windows on a Perkin Elmer (Waltham, Massachusetts, USA) Spotlight 400N FTIR imaging system equipped with a linear array of 16 liquid nitrogen cooled MCT detectors. A 25 µm x 25 µm pixel

resolution was used to capture an average absorption image in transmission mode.

Data Analysis

A host of pre-processing techniques were trialled in various combinations on the raw dataset to produce an equally weighted input into the regression model for reducing prediction errors. Methods included combinations of a Savitzky-Golay smoothing filter, vector normalisation, linear/polynomial baseline offset correction and extended multiplicative scatter correction. All pre-processing methods were performed using Matlab™ v7.7 (Mathworks, Natick, Massachusetts) in conjunction with PLS Toolbox 5.22 (Eigenvector Research, Wenatchee, WA).

A common method for creation of calibration models for prediction of target analyte concentrations with vibrational spectra is Partial Least Squares Regression (PLSR).^{29, 31, 34, 35} The PLS model attempts to elucidate factors or latent variables (*LV*) which account for the systematic majority of variation in predictors '*X*' (spectral data) versus associated responses '*Y*' (target values of fibrinogen concentration). In other words, PLS uses the information contained in *Y* to determine the systematic multivariate signatures in *X*. This is achieved through the reduction of the variables corresponding to the wavenumber axis of the spectral data *X* into reduced number of spectral variables while simultaneously reducing variables containing associated target concentration information *Y* in each training sample. Latent variables that are best correlated with variations in target values allow the creation of a calibration model that can subsequently be tested using test data. The linear regression model is created by reducing the *X* and *Y* matrices into latent variables and scores via principal component analysis (PCA) such that the *X* matrix scores are regressed against the *Y* matrix scores in the model. The process can therefore be described mathematically as:

$$\mathbf{X} = \mathbf{TP}' + \mathbf{E} \quad (1)$$

$$\mathbf{Y} = \mathbf{UQ}' + \mathbf{F} \quad (2)$$

where; *X* and *Y* = Matrices of the predictors and responses respectively.

T = Matrix of *X* – scores

U = Matrix of *Y* – scores

P = Matrix of *X* – latent variables

Q = Matrix of *Y* – latent variables

E = *X* – residual errors

F = *Y* – residual errors

Cross validation is a technique enabling the assessment of the optimal complexity of a model (in this case the number of latent variables in the PLS model) thus allowing an estimation of the performance of a model when applied to unknown data. Cross validation involves a series of sub-validation experiments, which see the removal of a subset of objects (or spectra) from a dataset for testing (the test set), construction of a model using the

remaining objects in the dataset (the calibration set), and subsequent application of the resulting model to the test set. This ensures each sub-validation experiment robustly tests a model using objects which are independent of those used to construct the model. In this study, two cross validation methods were used for comparative purposes: Random sample cross validation, which chooses different random subset test sets determined through the random selection of *n/s* objects in the data set (where *n* is the total number of objects in the dataset and *s* is the predetermined number of data splits), such that no single object appears in more than one test set. This procedure is repeated for a user defined number of iterations. In this study, a split of *s* = 5 was chosen and the process was iterated 100 times. The second method is Leave-One-Out cross validation, involves the use of a single element from the data set being used as a test or validation 'set' while the remaining objects are used as the training set. This process is iteratively repeated so that every single observation in the data set is used once as the designated validation data set.

The SIMPLS algorithm³⁶ of the PLS Toolbox v5.22 (Eigenvector Research, Inc.) was implemented within Matlab. The training of the teaching data set was optimised by calculating the root-mean-square error of calibration (RMSEC) and the root-mean-square error of cross validation (RMSECV). The spectral data obtained from the 33 samples were split 60/40 into training and test sets respectively. The various RMSE were calculated according to equations 3, 4 and 5 below:

$$\text{RMSEC} = \sqrt{\frac{\sum_{i=1}^{N_{\text{train}}} (x_{\text{pred},i} - y_{\text{resp},i})^2}{N_{\text{train}} - LV - 1}} \quad (3)$$

$$\text{RMSECV} = \sqrt{\frac{\sum_{i=1}^{N_{\text{train}}} (x_{\text{pred},i} - y_{\text{resp},i})^2}{N_{\text{train}}}} \quad (4)$$

$$\text{RMSEP} = \sqrt{\frac{\sum_{i=1}^{N_{\text{test}}} (x_{\text{pred},i} - y_{\text{resp},i})^2}{N_{\text{test}}}} \quad (5)$$

Here, *x_{pred,i}* and *y_{resp,i}* correspond to fibrinogen content in sample *i* determined by Raman spectral data and the Clauss assay, respectively. *N_{train}* and *N_{test}* denotes the number of training (*N_{train}* = 20 x *n* spectra) and test samples (*N_{test}* = 14 x *n* spectra) respectively. The number of latent variables used for the PLS calibration is optimised by finding the value that corresponds to the minimum of the RMSECV. Using the training values, a model is constructed and the quality of fit is judged by how well

Table 2. Peak assignments³⁷ for the Raman spectra of blood plasma.

Peak Position (cm ⁻¹)	Assignments of Raman Vibrational Modes
540	Histidine ^a
621	C–C twisting mode of phenylalanine
642	C–C twisting mode of tyrosine
758	Symmetric ring breathing of tryptophan
829	Out of plane ring breathing tyrosine
853	Ring breathing mode of tyrosine
878	Arginine ^a
939	C–C stretching mode backbone (α -helix conformation)
958	CH ₃ deformation
1003	Symmetric ring breathing mode of phenylalanine
1032	C–H in-plane bending mode of phenylalanine
1082	C–N stretching mode
1128	C–N stretching mode
1158	C–C / C–N stretching mode
1175	C–H in-plane bending mode of tyrosine
1208	Tryptophan and phenylalanine n(C–C ₆ H ₅) mode
1271	Amide III (C–N stretching mode, mainly α -helix conformation)
1318	C–H deformation
1339	C–H deformation
1447	C–H deformation
1552	Tryptophan
1585	C=C stretching
1606	C=C stretching mode of phenylalanine and tryptophan
1616	C=C stretching mode of tyrosine and tryptophan
1659	Amide I (C=O stretching mode of proteins, α -helix conformation)

^a Tentative assignments.

it is able to predict the values from the test data set. The root-mean-squared error of calibration (RMSEC) and the root-mean-squared error of prediction (RMSEP) are used to evaluate the predictive capacity of the constructed model. The efficacies of the various pre-processing and validation methods were evaluated through the performance of the PLS model.

RESULTS

While water possesses a low Raman scattering cross section in the visible region, it is resonantly enhanced in the near infrared due to the onset of O–H vibrational overtones.³⁸ Furthermore, as it has only been mildly centrifuged, the protein rich blood plasma scatters significantly, introducing a large background to the spectrum which swamps the Raman signal of the solute.³⁹ Therefore dried samples were chosen over fresh/wet samples. However, the drying process resulted in inhomogeneous samples as constituents precipitated predominantly at the edge of the spotted square either through surface tension or molecular weight differences. Figure 1 shows an FTIR image illustrating this sample inhomogeneity in terms of spectrally averaged absorbance. These variable thicknesses produced Raman spectra of various intensities and backgrounds due to differences in sample density cross-sections.

To maintain a level of consistency, Raman spectra were always recorded from the middle of the dried spot as it was the largest area of consistent thickness and uniformity. Figure 2 shows the mean Raman spectrum of dried blood plasma (top) (smoothed, baseline corrected and normalised) together with a spectrum of crystalline purified Fibrinogen from human plasma (bottom) (Sigma-Aldrich®, UK) for spectral comparison. Total plasma proteins (albumin and globulin) are the most abundant species in blood plasma and hence spectra are characteristic of these protein base vibrations. The observed peaks primarily show the

tyrosine/phenylalanine twisting doublet at 621/642 cm⁻¹ while the tyrosine ring-breathing doublet can be seen at 829/860 cm⁻¹. The sharpest and most dominant peak at 1003 cm⁻¹ is attributed to the phenylalanine symmetric breathing mode while CH₂ bending appears at 1446 cm⁻¹ and the C=O stretching mode of Amide I at 1657 cm⁻¹. The Raman spectrum of crystalline fibrinogen contains similar features, but notably has strong contributions from tryptophan at 758 cm⁻¹ and 1552 cm⁻¹. This is surprising as fibrinogen does not have a high tryptophan content, but may be due to sample impurities or alignment of tryptophan moieties in the crystalline sample. A full list of peak assignments is provided in Table 2.

Spectral Pre-processing

Despite the improvements over the low signal-to-noise limitations associated with the analysis of wet plasma samples, the dried plasma films still exhibited inconsistencies evident in the variable backgrounds seen in the raw spectral data (Figure 3). Although fluorescence is a possible origin of such backgrounds, the collection of blood plasma involves gradient and centrifugation separation, which should have removed all cellular material, including red blood cells and therefore there should be no heme-proteins in the sample. Such backgrounds have also been demonstrated to be the result of Mie-like scattering of both the source laser and the Raman bands themselves which enters the spectrometer as uncollimated stray light, together with variable contributions due to spot to spot sample morphology.³⁷ Therefore, the application of spectral pre-processing or corrective methodologies was an integral step to ensure that robust and precise quantitative information may be extracted from the Raman dataset.

Smoothing of the spectra was carried out using a Savitzky-Golay filter⁴⁰ to remove any spurious peaks and as dried plasma films generally produced spectra with a relatively high signal-to-noise

ratio, a 3rd order, five data point window proved sufficient. Baseline offset correction was seen to be a crucial pre-processing step due to the large variation in background seen in the raw data. A polynomial baseline function was used and compared with an
 5 iterative, linear-nodal correction (written in-house). For polynomial correction, band-free regions between wavenumber ranges of $400 - 630 \text{ cm}^{-1}$ and $1502 - 1800 \text{ cm}^{-1}$ were chosen, while the linear-nodal correction was executed in both automated
 10 node selection mode as well as manually, using the same specified regions above. Vector normalisation is a standard method in which the total intensity of the spectral dataset is considered rather than individual peaks. This is achieved by calculating the sum of the

15 **Figure 1.** Average FTIR absorbance image showing inhomogeneous sample distribution. The scaled colour bar indicates that zero absorption occurs at 0 while maximum absorption occurs at 1.

20 **Figure 2.** Typical spectrum of human blood plasma (top) and spectrum of purified crystalline human fibrinogen (bottom).

squared intensity values of the spectrum and using the squared root of this sum as the normalisation constant. Each variable of a spectrum is then divided by this constant. Figure 4 shows vector
 25 normalisation applied to the averaged intersampled dataset. The data processing procedure has removed much of the variability of the raw spectra of Figure 3. All spectra were retained for the regression model to mimic as best as possible an automated sampling technique.

An alternative technique applied in order to remove

30 multiplicative effects from spectra was Extended Multiplicative Scattering Correction (EMSC).⁴¹ EMSC is a combination corrective algorithm dealing with both additive and multiplicative background effects of each sample spectrum originally developed for Fourier Transform Infrared spectroscopy.

35 PLS

Two different datasets were used as the observed predictors (X) for the PLS algorithm; one utilised the entire original spectral dataset (100 spectra per sample), while the other was composed of an average, intersampled matrix to explore model efficiency
 40 with a reduced, averaged dataset. The intersampling process was carried out through a four-point moving window average of the full dataset, reducing 100 spectra per sample to 25 averaged spectra. Intersampling, as we found, reduces the effect of outliers in the final result. Therefore, after random sorting of the datasets,
 45 the training matrices were 2040 and 510 spectra (60% split) for the full and averaged intersampled datasets respectively, with the remaining 40% split being designated as test matrices (1360 and

50 **Figure 3.** Raw spectra ($n = 3400$) obtained from all dried blood plasma samples.

Figure 4. Average intersampled dataset ($n = 850$) after smoothing, automated linear-nodal baseline correction and vector normalisation.

340 spectra respectively). Out of the 100 iterations, Figure 5
 55 shows an example of a single iteration of RMSEC and RMSECV against the number of latent variables from the PLS analysis showing, in this case the minimum RMSECV to occur at $LV_{\min} = 15$. Once the model algorithms were sufficiently trained, validation of the model performance was carried out using the

unseen, test matrices.

The fibrinogen concentrations from the 14 test sample sets were predicted by the constructed PLS model and evaluated against target values obtained by the Clauss fibrinogen assay. The random nature of the cross-validation procedures resulted in the generation of different values of $LV_{\min}$ and hence prediction errors. Therefore, the model was trained and executed 100 separate times (with associated randomisation and splitting of the data on each run) to produce minimum, maximum and average values of $LV_{\min}$, RMSEP and R^2 with associated variances

Figure 5. An example plot of the root mean square error of calibration (RMSEC) versus root mean square error of cross validation as a function of the number of latent variables. The optimum number of latent variables corresponds to the minimum of RMSECV.

DISCUSSION

The predictive performances of the models show a close correlation between the concentrations predicted by Raman spectroscopy and target concentrations established by the Clauss Fibrinogen assay. As can be seen from Figure 6, the wide scatter in predicted Fibrinogen concentrations using the full spectral dataset prevents an accurate classification of samples as either moderate but normal Fibrinogen concentrations (1.7 – 4.0 g/L as determined through clotting assays)⁴² or high clinically significant values (e.g. 7 – 9 g/L). Furthermore, it is notable that the best fit regression does not extrapolate to the origin. The average intersampled dataset ($n = 25$ per sample) visually appears to contain less data scatter with less influence from outliers resulting in a stronger correlation coefficients (Figure 7), thus improving prediction to ≤ 0.25 g/L over the full dataset (Table 3). Extrapolation towards zero fibrinogen content was also more accurate within the averaged dataset. As previously mentioned, the inter-sampling process was carried out through a four-point moving window average of the full dataset, which effectively reduced the influence of outliers. This may also imply that there are a threshold number of spectral replicates needed from a sample to accurately represent the biochemical content and to achieve quantification of a target constituent. However, on average, more latent variables or factors were required to explain variations in the spectral data and target values using the averaged, intersampled dataset (mean $LV_{\min} = 20$) than the full dataset (mean $LV_{\min} = 15$). In an attempt to improve the performance of the calibration

model using the full dataset, a filtering technique was developed for the removal of outliers. Firstly, PCA was employed to determine the principal component. The scores of each sample were then plotted onto a three-dimensional space and the mean centroid was determined from the resultant cluster. The sample(s) with the furthest calculated squared Euclidean distance from the centroid are deemed outliers and omitted from the dataset. The furthest three, five and ten outliers were removed in this fashion. However, predictive errors were actually slightly increased (data not shown). This indicated that the selected outliers, which were

Figure 6. Performance of the PLS calibration model and subsequent test set prediction using the full spectral dataset.

Figure 7. Performance of the PLS calibration model and subsequent test set prediction using the average intersampled spectral dataset.

deemed as such based on the loadings or intrasample variable relationships, still contributed in some way to the construction of the model.

The two cross-validation procedures chosen produced slightly different prediction accuracies. While Leave-One-Out cross validation is a commonly used technique, it has a tendency to produce overly optimistic results for larger datasets ($n > 20$) and be prone to the replicate sample trap (replicates of the same sample existing in both the training and the test set).^{43, 44} The random sample selection cross validation method provides more

realistic results and is more adaptable in that it may be used on many types of datasets with the advantage of avoiding the replicate sample trap through the use of a lower number of splits and an increased number of iterations. However, the random
5 nature of the latter method might lead to different researchers producing different PLS models of the same data.

Table 3. Results of calibration and prediction PLS models using the test set. Values are averaged over 100 runs each with associated standard deviations. (LOOCV = Leave-One-Out Cross Validation, RanCV = Random Cross Validation)

Average intersampled Dataset (n = 850, 25 spectra per sample)				
$nLV_{\min}$	RMSEC (g/L)	RMSEP (g/L)	R^2 Calibration	R^2 Prediction
20.	0.38 ± 0.04	<i>Polynomial Baseline, Vector Normalised LOOCV</i>		0.90 ± 0.01
		0.75 ± 0.05	$0.98 \pm 5E-3$	
20	0.41 ± 0.03	<i>Polynomial Baseline, Vector Normalised RanCV</i>		0.90 ± 0.01
		0.75 ± 0.05	$0.97 \pm 4E-3$	
20	0.40 ± 0.08	<i>Polynomial Baseline, EMSC LOOCV</i>		0.90 ± 0.02
		0.78 ± 0.06	0.97 ± 0.01	
18	0.44 ± 0.07	<i>Polynomial Baseline, EMSC RanCV</i>		0.89 ± 0.01
		0.78 ± 0.05	0.97 ± 0.01	
20	0.39 ± 0.06	<i>Linear-Nodal, Vector Normalised LOOCV</i>		0.91 ± 0.01
		0.72 ± 0.05	$0.97 \pm 6E-3$	
20	0.42 ± 0.02	<i>Linear-Nodal, Vector Normalised RanCV</i>		0.91 ± 0.01
		0.73 ± 0.06	$0.97 \pm 4E-3$	
21	0.38 ± 0.05	<i>Linear-Nodal, EMSC LOOCV</i>		0.91 ± 0.01
		0.73 ± 0.05	$0.98 \pm 6E-3$	
21	0.40 ± 0.02	<i>Linear-Nodal, EMSC RanCV</i>		0.91 ± 0.02
		0.73 ± 0.06	$0.97 \pm 2E-3$	
Full Dataset (n = 3400, 100 spectra per sample)				
$nLV_{\min}$	RMSEC	RMSEP	R^2 Calibration	R^2 Prediction
17	0.78 ± 0.03	<i>Polynomial Baseline, Vector Normalised LOOCV</i>		$0.83 \pm 8E-3$
		0.98 ± 0.02	$0.89 \pm 8E-3$	
16	0.81 ± 0.03	<i>Polynomial Baseline, Vector Normalised RanCV</i>		$0.82 \pm 9E-3$
		0.99 ± 0.02	$0.88 \pm 9E-3$	
17	0.80 ± 0.02	<i>Polynomial Baseline, EMSC LOOCV</i>		$0.83 \pm 9E-3$
		0.98 ± 0.02	$0.89 \pm 7E-3$	
16	0.82 ± 0.02	<i>Polynomial Baseline, EMSC RanCV</i>		$0.83 \pm 9E-3$
		0.98 ± 0.03	$0.88 \pm 6E-3$	
17	0.77 ± 0.02	<i>Linear-Nodal, Vector Normalised LOOCV</i>		$0.83 \pm 9E-3$
		0.97 ± 0.02	$0.90 \pm 6E-3$	
16	0.80 ± 0.03	<i>Linear-Nodal, Vector Normalised RanCV</i>		$0.83 \pm 8E-3$
		0.98 ± 0.02	0.89 ± 0.01	
18	0.78 ± 0.02	<i>Linear-Nodal, EMSC LOOCV</i>		$0.83 \pm 9E-3$
		0.98 ± 0.02	$0.89 \pm 6E-3$	
17	0.80 ± 0.01	<i>Linear-Nodal, EMSC RanCV</i>		$0.83 \pm 9E-3$
		0.98 ± 0.02	$0.89 \pm 4E-3$	

Concerning the pre-processing of the spectral data before initiating model training, it is recognised that baseline correction should be carried out before any normalisation or corrective procedures to avoid incorporating baseline fluctuations into the normalisation constant.⁴⁵ Comparative results are shown regarding the use of an iterative linear-nodal and polynomial baseline correction algorithm (Table 3 – Supplementary Data). While the linear-nodal correction brought all spectra down to a common baseline (zero), the polynomial correction method distributed spectra over a range of wavenumbers, hence the latter method producing $\approx 5\%$ larger errors in prediction. With respect to the linear-nodal method, the choice between automated node selection and user-specified nodes saw the former perform better in terms of the prediction errors produced. This may be explained in terms of the minute wavenumber shift of nodes in that they may not necessarily be located in the same region for all spectra during the correction. Hence the automated selection was a better choice to account for this.

For the correction of the inherent multiplicative effects of scattering seen in terms of prediction errors, vector normalisation out-performed EMSC in the averaged intersampled dataset. While EMSC visually converged spectra more efficiently, it imposed a detrimental effect in failing to preserve key spectral features contributing to the training of a better quantitative predictive model. To avoid inter-sample crossover effects, each sample set of spectra (n = 25 or 100) was either normalised or corrected with EMSC individually rather than applying the corrections over the entire dataset. As with baseline correction, the choice of method to correct for multiplicative effects appeared to be more significant when applied to the averaged intersampled dataset.

To enable a measure of the model performance versus the stated precision of the Clauss assay in a clinical context, the coefficients of variation of the observed prediction errors (CV_{RMSE}) were calculated (6) and evaluated against the standard deviation of the reference values (calculated from the mean and relative coefficient of variation %CV (7)) provided by the manufacturer (Table 1).

$$CV_{RMSE} = \frac{RMSE}{\bar{x}} \quad (6)$$

$$\%CV = \frac{\sigma}{\bar{x}} \times 100 \quad (7)$$

Where σ = standard deviation, $\bar{x}$ = mean

The precision of the reference assay is described by a standard deviation of 0.17 and 0.14 for day-to-day and test-to-test respectively, as specified by the manufacturer. This is compared to a CV_{RMSE} of 0.19 (best) and 0.21 (mean) as achieved by Raman spectroscopic measurements. These figures suggest that the Clauss assay slightly outperforms the Raman spectroscopic model with regards to precision or reproducibility in the measurement of fibrinogen concentration. Ignoring the influence of outliers, the model shows a calibration accuracy of approximately 1 g/L and 2 g/L for the test set. Nevertheless, the case is presented for the technique to be a potential analytical tool for a frequently required measurement. It is noted that the sample preparation was somewhat crude, and that the measurements were made on the thinnest regions of the sample. Improved sample preparation by for example spin coating could significantly improve signal to noise and spectral background. Ultimately, *in situ* measurements in wet samples would be desirable.

Attention has to be drawn to the patient samples, which featured almost 50% of the values below 3 g/L and only three values above 5 g/L, this reflects the normal healthy range of 2.2 – 4.3 g/L. However, this unbalance not only affected the correlation but also the errors associated with the constructed model. However, the choice was made to include all samples without dilution to maintain the reagent-free nature of the study, ensuring a robust comparison of the technique to the gold-standard assay. As this is a pilot study to test the sensitivity of Raman spectroscopy, a larger sample set of blood samples was not pursued. Of greater importance was to explore and establish the range of fibrinogen concentrations to construct a robust model. It is noted that calibration against a limited gold standard is a process that is in itself obviously limited. Ultimately, a cross validation against more precise proteomic techniques would be required.

Conclusions

This study investigated the potential of Raman spectroscopy to quantify the Factor I fibrinogen protein in undiluted, dried patient blood plasma samples. The use of dried blood plasma forgoes the issues related to background water spectral contributions, scattering, heating and biohazard safety. Also, the small volume (10 μ L) of plasma needed for analysis means the majority of the blood sample is retained for parallel tests. Partial Least Squares regression was employed to construct and train a calibration model using Raman spectra obtained from plasma samples as fitted to target fibrinogen values obtained from the Clauss assay. It was shown that the various spectral pre-processing methods chosen in this study affected model training performance and resultant prediction errors. The best predictive result (0.72 $\pm$ 0.05 g/L) came with the use of an averaged, intersampled dataset that had been smoothed using a Savitzky-Golay filter, baseline corrected using an automated linear-nodal algorithm and vector normalised. While the latter combination of methods worked best in this particular study, given a different analyte and/or instrumental setup the pre-processing techniques would no doubt be suitably modified. Although pre-processing remains an essential part of Raman spectroscopy, unwanted variability may

be introduced through over manipulation of spectral data, which is especially applicable in sensitive quantitative analysis. Thus the basic rule of thumb is to ensure to preserve spectral integrity through the use of only the minimum amount of pre-processing needed. While the accuracy of Raman spectroscopy failed to match that of the gold-standard assay in this study, a more balanced dataset or calibration using certified fibrinogen standards and controls would possibly improve performance towards clinical application. However, the number of spectral replicates and hence acquisition times needs to be reduced for the purposes of daily routine analysis. It is noted that calibration against a limited gold standard is a process which is in itself obviously limited. Ultimately, a cross validation against more precise proteomic techniques would be required. However, the study demonstrates that, as a quantitative tool, spontaneous, unenhanced Raman spectroscopy is a highly reproducible technique in the detection of single components in a complex biological matrix.

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