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# Monitoring Cell Culture Media Degradation using Surface Enhanced Raman Scattering (SERS) Spectroscopy.

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## Abstract:

The quality of the cell culture media used in biopharmaceutical manufacturing is a crucial factor affecting bioprocess performance and the quality of the final product. Due to their complex composition these media are inherently unstable, and significant compositional variations can occur particularly when in the prepared liquid state. For example photo-degradation of cell culture media can have adverse effects on cell viability and thus process performance. There is thus, from quality control, quality assurance and process management view points, an urgent demand for the development of rapid and inexpensive tools for the stability monitoring of these complex mixtures. Spectroscopic methods, based on fluorescence or Raman measurements, have now become viable alternatives to more time-consuming and expensive (on a unit analysis cost) chromatographic and/or mass spectrometry based methods for routine analysis of media. Here we demonstrate the application of Surface Enhanced Raman Scattering (SERS) spectroscopy for the simple, fast, analysis of cell culture media degradation. Once stringent reproducibility controls are implemented, chemometric data analysis methods can then be used to rapidly monitor the compositional changes in chemically defined media. SERS shows clearly that even when media are stored at low temperature (2-8 °C) and in the dark, significant chemical changes occur, particularly with regard to cysteine/cystine concentration.

**Keywords:** Raman spectroscopy, Surface Enhanced Raman Scattering (SERS), Biotechnology, cell culture media, photo-degradation, chemometrics.

## 1. Introduction

Cell culture media are an essential part of industrial mammalian cell culture, sustaining optimal cell growth and ensuring correct product formation. In many cases chemically defined (CD) media are now being used to avoid inconsistency issues associated with complex hydrolysates and other biologically derived media. CD media are usually complex aqueous chemical mixtures containing a range of amino acids, carbohydrates,

vitamins, inorganic salts and other supplements. One such example is eRDF, an enriched basal RDF medium containing higher amino acids and glucose concentrations which has ~60 components [1]. Media quality is absolutely critical to process performance, reliability, and reproducibility. However, it is known that media are not chemically stable [2] and undergo some slow rate chemical reactions when stored in the dark between 2-8 °C, the industry norm for storing liquid cell culture media. It is also well known that chemical changes induced by light exposure can adversely affect process performances and cell viability [2-8]. In addition, some unstable components like cysteine are of particular interest since it and its oxidation product, cystine, can have significant effects on protein aggregation [9]. Therefore it is critical importance to assess and monitor media stability particularly during process development where unknown/uncontrolled media variability can have very adverse consequences. Multi-dimensional fluorescence in combination with chemometric data analysis has been used to easily monitor light induced changes in media like eRDF [10, 11]. This was possible due to the fact that riboflavin and some of the significant photoactive compounds (Tryptophan / Tyrosine) in the media are fluorescent. Furthermore, several photoproducts were also fluorescent and so by use of chemometric tools like PARAFAC [12] and MCR [13] it was possible to track composition changes. However this technique was not able to detect changes affecting non-fluorescent original components or degradation products.

The use of Raman spectroscopy for media analysis can lead to a more comprehensive analysis of the media composition because nearly all molecular constituents of media have distinct Raman spectra and can easily be distinguished [14, 15]. Unfortunately, conventional Raman spectroscopy is not good for analytes present in low concentration ( $< \sim 0.5\%$  w/w) which is the case in most components in liquid cell culture media. One way in which sensitivity can be enhanced is to use Surface Enhanced Raman Scattering (SERS) [16-18]. SERS is the enhancement effect on Raman scattering observed when molecules are adsorbed onto nanosized noble metal structures, typically fabricated from gold or silver. SERS is potentially a very sensitive analytical method (down to single molecule) for complex biogenic materials analysis [16, 19, 20]. SERS measurements could provide valuable information about variances in media in terms of the non-fluorescing components which would then complement existing fluorescence EEM based measurements [2, 11, 19, 21, 22].

Here we show how SERS in combination with chemometric methods can be used to rapidly identify and monitor chemical changes in cell culture media under different storage conditions. However SERS is fundamentally difficult to implement reproducibly and thus careful experimental design and a high degree of care was required during the experimental work to ensure that the SERS data was both correct and reproducible. It was for this reason that a silver colloid SERS substrate was used, and additionally because it avoided carbonization problems that can be an issue with solid substrates. This method provided a rapid, simple, and inexpensive approach for media monitoring over extended timeframes, which should be of benefit for industrial biotechnology.

## 2. Materials and methods

2.1 *Materials:* eRDF was obtained from Kyokuto Pharmaceuticals Industrial (Japan). Sodium bicarbonate (>99.7 %), silver nitrate (99.999 % metal basis), sodium citrate (>99.0 %) were obtained from Sigma-Aldrich and used without further purification. eRDF stock solution ( $17.7 \text{ g L}^{-1}$ ) was prepared by dissolving 4.4248 g of eRDF powder and 0.2832 g of sodium bicarbonate in  $250 \text{ cm}^3$  of sterilized high purity water. The solution was immediately sterilized by membrane ( $0.22 \mu\text{m}$ ) filtration and then dispensed (1.25 mL aliquots) into sterile 2 mL translucent polypropylene eppendorf tubes before being placed in one of the four storage conditions: 1). RT-L: Room temperature ( $16.4 \pm 3.0 \text{ }^\circ\text{C}$ ) in the light; 2). RT-D: Room temperature in the dark; 3). C-L: Cold (Fridge,  $6.0 \pm 1.5 \text{ }^\circ\text{C}$ ) with light, and 4). C-D: Cold (Fridge) in the dark. All other details have been communicated previously [11]. SERS data were collected over 32 days (Day 0, 7, 11, 14, 18, 21, 25, 28, and 32). At every sampling point three samples were removed from each storage condition and immediately placed in the dark at  $4^\circ\text{C}$  to limit any further change in the samples during data acquisition.

2.2 *SERS, Raman Instrumentation, and data collection:* Silver colloid was carefully prepared using the Lee and Meisel method [10, 18]. Prior to preparation all glassware was thoroughly washed firstly with soap, rinsed with high purity water (HPW) from a Milli-Q® system, then isopropanol, HPW, and then soaked overnight in *aqua regia* before being rinsed again with HPW, until the pH of the rinsing water was neutral (pH paper). If multiple batches of colloid were being prepared over a week, the glassware was only rinsed with HPW between batches. Batches produced straight after cleaning of the glassware with *aqua regia* were systematically discarded as they had a wider particle size distribution and larger mean size. This effect was probably due to glass surface modification during cleaning, which changed the dynamics of nanoparticle formation. Subsequent batches made in the same glassware immediately afterwards, without cleaning, showed more consistent physical and SERS properties.

A silver nitrate solution was first prepared by dissolving 45 mg of  $\text{AgNO}_3$  in 250 mL of HPW and brought to the boil. When the temperature of the paraffin bath reached  $135 \text{ }^\circ\text{C}$ , 5mL of 1% sodium citrate (57 mg in 5mL HPW) was slowly ( $\sim 1$  drop every 4s) introduced to the boiling solution using a pressure equalizing dropping funnel. The reflux was maintained for 1 hour and the system was protected from the light at all times using aluminum foil. In practice, a color change, from clear and colorless to opaque olive/grey, rapidly after the start of the citrate addition indicates a successful preparation (absorbance maximum around 404 nm and full width half maximum of the absorbance  $< 100 \text{ nm}$ , see below).

UV-vis spectroscopy was used as the primary quality assurance tool as both the maximum of the Plasmon resonance and the FWHM of the absorption band are symptomatic of both nanoparticle size and distribution [23, 24]. Ideally, these measurements should be validated using particle size measurements at the same time, but this was not available in this instance. There are several published studies correlating both size and size-distribution with the UV-vis spectroscopic studies, both experimentally and from a theoretical standpoint.

Using this approach, reasonably reproducible colloid quality was achieved with an average absorbance maximum ( $\lambda_{\text{max}}$ ) and full width half maximum (FWHM) of  $404 \pm 2.5 \text{ nm}$  and  $86 \pm 15 \text{ nm}$  respectively (for the six batches used in this study). Batches 7 and 8 were

combined for this study, and this mixture was characterised by a FWHM = 86 nm and a  $\lambda_{\text{max}}$  (abs.) = 404 nm, which suggests an average particle size of ~20 nm [24-26]. While, the particle size is smaller than the 50 nm optimum for 785 nm excitation (in terms of SERS enhancement) as determined by Stamplecoskie *et al.* [26], it was appropriate for this application where signal reproducibility rather than enhancement factor was the critical issue. The only change observed between follow-on batches was a varying baseline in the Raman spectra of the pure colloid probably due to small changes in the particle size distribution (*see supplemental information, SI*). Finally, each colloid batch was contamination checked by Raman analysis immediately prior to use.

SERS spectra were collected with 785 nm excitation (1 sec. exposure, 8 cm<sup>-1</sup> resolution) using a RamanStation spectrometer (AVALON Instruments Ltd., Belfast) on 100  $\mu$ L sample volumes in a stainless steel wellplate [14]. The effect of sample to colloid ratio (SC ratio) and incubation time on the quality of the SERS spectra were investigated (*data not shown*, [10]). Ultimately, a 1:19 SC ratio was selected (*vide infra*) and every test sample consisted of 5  $\mu$ L media solution to which was added 95  $\mu$ L of silver colloid, which was then mechanically mixed using a micro-pipette. Each sample was measured immediately after mixing using the super macro point mode which involved the collection of seven Raman spectra (and backgrounds) from around the well center. Spectra were then co-added and each sample was measured in triplicate using fresh aliquots in different wells.

**2.3 Chemometrics and data analysis:** All calculations were performed using PLS\_Toolbox 4.0<sup>®</sup>, supplemented by in-house-written codes for Matlab<sup>®</sup> (ver. 7.4). Random spikes caused by cosmic rays were removed using an in-house written Matlab function. For the ageing experiment, any data containing cosmic spikes were immediately recollected. Classical principal component analysis (PCA) was carried out on SERS data that was baseline corrected (weighted least square algorithm) and normalized (unit area).

### 3. Results and discussion.

One of the most critical aspects in the development of a SERS based method for complex mixtures was to produce a robust sample handling procedure that delivers both signal reproducibility in terms of spectral profile and intensity, and a useful SERS enhancement with good signal-to-noise. We selected citrate reduced silver colloid [18] as this offers a good balance between ease of preparation and effective SERS response. The quality of the synthesized colloid was assessed by UV-vis spectroscopy in terms of Plasmon resonance band maximum and FWHM, which serves as an excellent diagnostic tool in lieu of measuring particle size distributions (*see supplemental information* and [24]). Once made these colloids were carefully stored in brown glass, acid washed bottles away from any direct source of light and used within 6 months of preparation. While these batches of citrate reduced colloid were suitable (as evidenced in the control sample measurements, *vide infra*) for this proof-of-concept study, we do recognize that the colloid batch reproducibility does not match the reproducibility achieved by silver colloids generated by the reduction of hydroxylamine phosphate [27]. Here to minimize the impact of colloid variation, unless stated otherwise, only one batch per experiment was used.



However, as important as the synthesis/storage controls, the key factors affecting the quality of the SERS spectra and data involved careful consideration/management of the sample-colloid mixture preparation step. To avoid reproducibility and background signal issues [24], we used the colloid as a suspension. Aggregation was not an issue as the eRDF formulation (17.7 g L<sup>-1</sup> soln.) contained high concentrations of aggregating agents like MgSO<sub>4</sub> (435 μM) and NaCl (105 mM). For complex media mixtures, the probability of different adsorption rates, affinities, and surface coverage for the different components was high and therefore one needed to carefully consider both the incubation time and sample-to-colloid (SC) ratio. In addition one had to account for colloid aggregation rate, which was modulated by some media components and, the time-dependent precipitation of large aggregates out of solution. The first factor increased SERS signal intensity, whereas the second reduced signal intensity.

**3.1 Spectral analysis:** Figure 1(top) shows the conventional Raman and SERS spectra of an eRDF solution (17.7 g L<sup>-1</sup>) and as expected the classical Raman spectra gave a very weak signal that was not very diagnostic [15]. SERS in contrast gave strong signals with multiple enhanced bands in the fingerprint region. Since eRDF was a complex mixture we get enhancement of multiple surface adsorbed species leading to this complex signal. The complexity of eRDF (with many SERS active compounds present), coupled with the different enhancement factors for each analyte made an accurate assignment of each band to a particular vibrational mode of a specific compound a challenging task (see *supplemental information* for more details). We suggest that, due to their structure and relatively high concentrations, most of the strong SERS signals originate from the amino acids [28-30] and vitamins present. The 1394 cm<sup>-1</sup> band was probably the amino acid –COO<sup>-</sup> symmetric stretch [31, 32], the ~914 cm<sup>-1</sup> band the C–COO<sup>-</sup> stretch, the weak band at 722 cm<sup>-1</sup> could be COO<sup>-</sup> deformation while the shoulder at 626 cm<sup>-1</sup> was probably due to COO<sup>-</sup> wag [32]. The C–S stretching of cysteine (present at 600 μM) was probably responsible for part of the 658 cm<sup>-1</sup> band [33], however, it was also possible that folic acid (present at 19.94 μM) also contributed to this band and to the peak at 962 cm<sup>-1</sup>.<sup>1</sup> The broad band around 1600 cm<sup>-1</sup> was the water O–H bending mode with contributions from other modes such as ring CC stretches of aromatic amino acids. The shape of this band in particular varies significantly (*vide infra*) according to the SC ratio employed. In the context of media change identification, it was less important to identify the specific source of each band, than to observe composition change. The identification of the specific species involved in media change would be better suited to high-resolution mass spectrometry methods.

**3.2 Time dependence of SERS measurements:** It has been shown [19, 34] that the incubation time<sup>2</sup> can influence the profile and quality of SERS spectra. To assess the effect of different incubation times on the SERS measurement of the eRDF solution, a series of SERS spectra were collected over a 20 min incubation period using an arbitrarily chosen 1:4 SC ratio (Figure 1). This showed that the main variation observed in the SERS spectra were increases in baseline intensity and enhancement with time (Figure 2a/b). The largest increase occurred

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<sup>1</sup> No specific vibrational mode was associated with these bands in the literature.

<sup>2</sup> Time between the moment when the sample is added to the colloid and time of the measurement.

during the first ~ 6 minutes, after which it slowed. The eRDF induced aggregation of the nanosized Ag colloid particles, caused at first a relatively large increase in average particle size, leading to very significant increase in the amount of Tyndall scattering from the sample, and thus an increased baseline. After this initial growth phase, the relative increase of aggregate size was much less and there were comparatively fewer particles present and thus the rate of increase in Tyndall scattering decreased.

When the changes in the intensity of the SERS signal due to analytes were investigated by plotting the integrated area of the baseline corrected spectra versus incubation time (Figure 2b), it was clear that the SERS enhancement followed the same trend as the baseline signal which indicated that the effects were related. This can be attributed to two factors: 1). as the colloidal particles aggregate, highly angled, contact junctions between particles were formed and these are known to generate dramatically larger SERS signals, and 2). the increased aggregate size resulted in a red-shift of the Plasmon resonance closer to the excitation wavelength which further increased SERS enhancement [35].

The other significant aspect of the SERS spectra was the fact that the spectral profile (Figure 2C) was largely unchanged over the 20 minute incubation time which indicated that media components interacted very quickly with the Ag surface generating a stable population of surface-bound analytes. This was supported by a PCA model of the raw SERS spectra (data not shown) where only one component was needed to explain 99.85 % of the spectral variance. This showed that SERS spectral changes were related only to absolute intensity and not due to any changes in the surface bound population of analytes. Similarly, a single component PCA model fitted using baseline corrected and normalised data explaining 99.84 % of the total variance. Since in both cases the explained variances were very similar and > 99.8 %, this meant that the difference between the spectra collected with different incubation times was only a change in scale (multiplicative factor/signal enhancement increase with time). However, it should be noted that the SERS spectra were only this well behaved when the sample/colloid mixture was re-suspended mechanically (with a micropipette) immediately prior to measurement. If this was not done, then the presence of multiple aggregating agents in the eRDF caused rapid precipitation of colloid aggregates out of the suspension. When the incubation experiment was implemented using different SC ratios (see SI) similar behaviour was observed for the baseline and spectral signal intensities. However, it was also noted that the spectral profile changed significantly as the SC ratio varied.

**3.3 Influence of SC ratio:** The profile of the SERS spectra depends dramatically (Figure 3a) on SC ratio and SERS signal intensity was strongest for small SC ratios with intensity gradually being decreased with additional colloid. What was more important for diagnostic applications was the large spectral profile change in the normalised spectra (Figure 3b). This indicated that there were very different surface analyte populations present and thus one needed to exercise caution in selecting the correct ratio. For a 1:199 SC ratio, the relative peak intensities were much more comparable across the spectral range which might suggest that there was a much more diverse population of analytes, all of which contributed relatively equally to the SERS spectrum. Since, eRDF comprised of many different molecules with varying affinities for the Ag surface, the provision of excess colloid enabled more analyte binding and enhancement. For example the lower affinity compounds like folic acid (bands

at 690, 1186, 1510, and  $\sim 1595\text{ cm}^{-1}$  see SI) now become visible [36]. In contrast, for the larger ratios (*e.g.* more sample), it was the higher affinity components (*e.g.* cysteine, C-S stretch at  $658\text{ cm}^{-1}$  [33]) that dominate the SERS spectra because they will occupy proportionally more of the available sites. The variation in band intensities due to SC ratio changes were not linear, for example, Figure 3c plotted the  $666\text{ cm}^{-1}$  band intensity (which probably includes the cysteine C-S stretch) against SC ratio. Band intensity first rapidly increased (with a logarithmic dependence) before attaining a steady-state. It was suspected that weaker bound analytes were being displaced by a smaller range of analytes which give a stronger SERS response (*i.e.* those species which have a stronger affinity to the Ag surface).

Therefore, the SC ratio was critical for the analysis of complex media and the ratio selected should be such that there were sufficient binding sites available which generated a diverse and stable surface-bound population of analytes, and thus in turn led to reproducible and informative SERS spectra. This must be balanced against the loss in SERS signal intensity, increased background from particle scatter, and decreased signal-to-noise (S/N) which is a consequence of using a small SC ratio (*e.g.*  $< \sim 1:20$ ). The lower S/N resulted from first poorer aggregation because of lower levels of intrinsic aggregating agents from the cell culture media and second the lower analyte concentrations gave weaker SERS signals. One way to overcome this second point would be to add a separate aggregating agent to the sample/colloid mixture. However, this has to be carried out with care as this addition could introduce anomalies in the SERS spectra [37]. On the basis of the experimental studies (Figure 3c) we selected an SC ratio of 1:19 for the media degradation studies as this offered the best compromise in terms of S/N and spectral quality.

**3.4 SERS monitoring of media storage induced changes:** The validity of the storage induced changes as measured by SERS was confirmed by comparison (Figure 4) of test spectra collected on freshly prepared eRDF solutions with the control sample (stored for 32 days at  $-70\text{ }^{\circ}\text{C}$ ) spectra. The plots showed that the SERS spectra did not change significantly (*e.g.* due to different colloid performance, instrument settings) over the 32 day period of the experiment. There was a small decrease in intensity for two peaks observed and this might be attributable to some variance induced during the freeze-thaw cycle. However, these were very small when compared to the differences observed due to the different storage conditions.

It was clear from the eRDF SERS spectra from the different storage conditions (Figure 5) that light exposure caused a great degree of spectral change. The largest light-induced change were manifested as an increased background which originated from riboflavin photo-products and its associated photosensitized degradation of other components [3-5, 7, 8, 10]. This resulted in changes in riboflavin and tryptophan, concentrations with the simultaneous formation of lumichrome [8, 11]. One potential source of baseline signal was the formation of a citrate-lumichrome complex [38], which has red fluorescence emission. The colloid used here had a significant citrate concentration (both surface bound and free in solution) which could bind to lumichrome, generating the red emitting fluorophore. A secondary source of background was variable aggregation dynamics caused by the changed chemical composition.

What was more interesting was the very significant changes in SERS signal for the samples dark-stored where there was no photo-degradation as shown by EEM fluorescence



analysis which showed virtually no change ([10, 11]). Since the control experiment (Figure 4) showed minimal SERS spectral change for samples stored at  $-70^{\circ}\text{C}$ , the obvious conclusion was that solution phase chemical reactions between media components had occurred. These changes in dark-stored media occurred in two principal regions: a loss of signal intensity in the  $660\text{--}680\text{ cm}^{-1}$  band and the appearance of two new bands in the  $1520\text{--}1760\text{ cm}^{-1}$  region. The interpretation of these spectral changes in terms of specific chemical constituents was not possible with a high degree of certainty because of the sample complexity and because of the convoluted interplay between selective enhancements, aggregation rates etc. In addition the presence of high aggregating agent concentrations (*e.g.* KCl, NaCl,  $\text{MgSO}_4$ , *etc.*) in the eRDF made definitive band assignment more problematical as it is well known that the SERS behaviour of some amino acids like cysteine can be strongly influenced by the aggregating agent [37]. However, cysteine which was present at relatively high concentration was easily oxidised to cystine in solution, particularly in the presence of ferric [39] or cupric ions (both present in eRDF). Furthermore, the SERS behaviour of cysteine/cystine shows good correlation with the changes observed here (*vide infra*) [28, 33, 37, 40-42]. To validate these observations we attempted to measure the cysteine concentration changes using chromatographic means [43, 44]. However, the separation of cysteine/cystine was problematical and a pre-treatment step with iodoacetic acid was required prior to the derivatisation step with PTC to obtain the S-carboxymethyl-derivative of cysteine [45] (details in *supplemental information*). This was time-consuming and in the case of the eRDF media, had a negative impact on the quality of the HPLC results. Thus it was not possible to obtain accurate cysteine concentration data from our HPLC setup.

Therefore, to better understand the source of chemical change, Principal Component Analysis (PCA) was then used to analyse the SERS spectral changes. The dark stored samples were modelled using two components (99.77 % explained spectral variance) whereas light exposed samples required three components (99.83 % explained variance). In both cases (Figure 7), the first and second PCA components were very similar (see *SI*). PC1 scores increased with storage time while PC2 decreased (Figure 6) which suggested that these components were associated with the degradation product(s) and the loss of a component respectively. A possible interpretation of the variations modelled by PC1 and PC2 could be the oxidation of cysteine (PC2) and possible production of cystine (PC1). When the PC1 and PC2 loadings were compared with published SERS data for both cystine and cysteine [28, 37, 40, 46] there was good agreement. In particular, the match between all the bands in PC2 and the SERS spectra of cysteine on citrate reduced silver colloids was very good [37, 40] (see *SI*). We note that in PC2 we did not observe any bands around  $540\text{ cm}^{-1}$  (S-S stretch) which would indicate the presence of the cystine dimer bound to the surface [46]. In PC1, there was a broad band at  $538\text{ cm}^{-1}$  which could be the  $\nu(\text{S-S})$  vibrational mode as suggested by recent SERS studies into the behaviour of both cysteine and cystine on Au and Ag substrates [40]. The most likely explanation for these observations was that, during storage the cysteine (PC2) originally present in the medium gradually oxidises to the dimer, cystine (PC1). An alternative cysteine oxidative degradation pathway involving the generation of sulfonic acids by more extreme oxidation was studied by SERS [42]. The oxidation products (tentatively identified as sulfonic/sulfonic acids) have SERS spectra

similar to PC1 (see *SI*). The third possibility is that as cysteine is removed by oxidation, binding sites on the colloid are freed up thus enabling other media components with poorer affinity for the colloid to adsorb and generate a different SERS spectrum. However, on the balance of the available evidence it is the cysteine-cystine conversion that is the most probable source of the observed changes in the SERS spectra for the dark-stored samples.

In the case of the light exposed samples, SERS is less useful as the degradation is more easily monitored using fluorescence [11]. However, it was noted that in the PCA model of the light exposed samples that the first two components were very similar to those obtained for the dark-stored samples. The third PC in the light exposed PCA model originates from baseline effects probably induced by the photo-products (see above). It was also noted from the PCA scores (Figure 6) that the rates of change were greater in the light than for dark-stored media (see *SI* for 2D scores plots, Figure S-11). In addition, for the light-stored samples there was no observable temperature effect (Figure 6d) on the PC2 scores (cysteine decrease) as was the case for the dark-stored samples (Figure 6b).

#### 4. Conclusions

SERS was able to detect changes in chemically defined cell culture media which occur under normal, cold, dark storage conditions. The SERS reproducibility issues was overcome by the implementation of rigorous quality control in the synthesis and handling of the citrate reduced Ag colloid, combined with a carefully designed sample incubation/data collection protocol. With these procedures we showed that substrate induced variation was much less than that caused by media ageing and thus the method was effective for media change analysis. Significant changes were observed both in irradiated and non-irradiated media which suggested that the phenomena observed by SERS were different to those observed by multi-dimensional fluorescence measurements [11]. A key conclusion was that large variations in the cell culture media chemical composition occur at an early stage with dark-stored media (within the first 5 to 10 days after preparation). The data suggests strongly that in the dark the most significant chemical change involved cysteine oxidation which takes place rapidly over the first 15 days, after which media composition stabilises. PCA of the SERS data allowed for qualitative monitoring of cysteine changes and this is useful since it is known that cysteine form promotes cell growth which is not the case for cystine [47]. Furthermore, to the best of our knowledge, there is no other rapid and easy to implement technique available for monitoring this cysteine-cystine change. HPLC required intensive sample handling and preparation, and was not an easy method to implement for the quantification of cysteine-cystine and thus identification of media change. In contrast, the SERS technique presented here allows for rapid monitoring of the cysteine-cystine change with minimal sample handling and preparation. Finally, since we need neither high resolution nor sensitivity, this SERS method can be implemented using relatively low-cost bench top Raman spectrometers thus reducing the overall cost of media testing.

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#### 6. Supplemental information available

383 Supporting information is available including further details on the spectral and quantitative  
384 analyses.  
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### List of Figures & Legends.

**Figure 1:** (top) Raw Raman spectra of *a*) milli-Q® water, *b*) a 17.7 g/L eRDF solution, and *c*). raw SERS spectrum of a 17.7 g/L eRDF solution with an SC ratio of 1:19. All three spectra were collected using identical collection conditions; (bottom) Plot of raw SERS spectra collected every minute (for 20 minutes) after mixing from samples with a SC ratio of 1:4 (see text). The sample-colloid mixture was re-suspended between each measurement and the average spectra of three replicates are plotted.

**Figure 2:** Change in the SERS spectra of a 17.7 g/L solution eRDF with incubation time. (a) Change in the baseline intensity of SERS spectra (average of the data points between 1802 and 2002  $\text{cm}^{-1}$ ); (b) Change in the integrated spectral area (250 to 2714  $\text{cm}^{-1}$ ) of the baseline corrected spectra. Data shown is for three replicate measurements; (c) Plot of the overlaid baselined corrected and normalised SERS spectra of all 60 spectra. The SERS were spectra collected every minute after incubation for up to 20 minutes on samples with an SC ratio of 1:4. The sample-colloid mixture was re-suspended between each measurement.

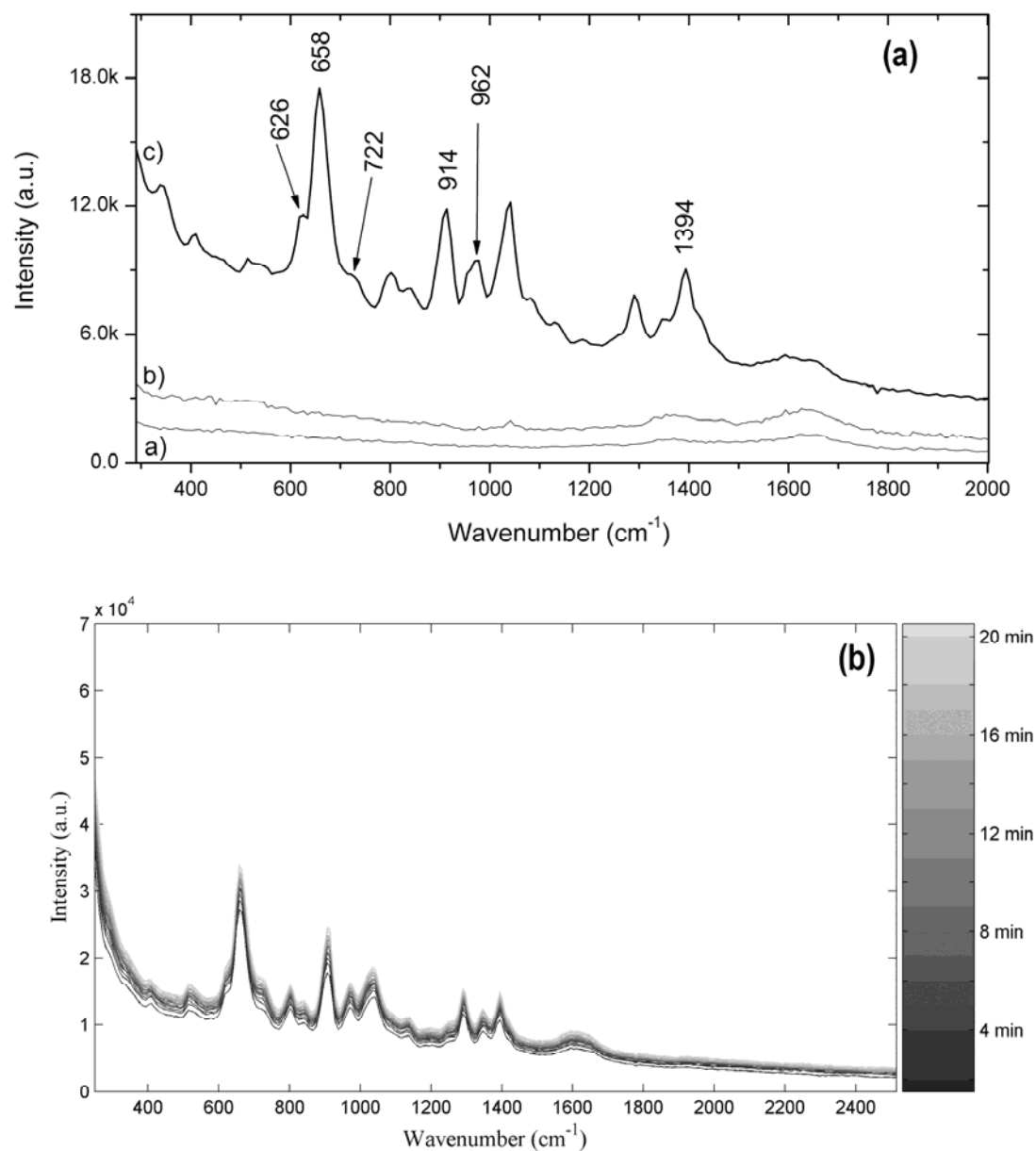
**Figure 3:** Baseline corrected (a), and baseline corrected and normalised to unit area (b) SERS spectra of eRDF (17.7 g/L) samples with 1:1 (black, batch 1 colloid), 1:9 (grey, batch 3/4 colloid) and 1:199 (grey, batch 2 colloid), ratio (v/v) of sample to colloid; (c) SERS intensity of the 666  $\text{cm}^{-1}$  peak plotted against SC ratio. The line shows the logarithmic fit:  $y = 3168.2 \times \log(x) + 16663$ ,  $R^2 = 0.981$ . The small deviations from the log fit are probably due to the use of three different colloid batches and the intrinsic synthesis variation.

**Figure 4:** Overlap of day0 (grey) and control sample (black) raw (a) and baseline corrected and normalised (b) SERS spectra collected in triplicate.

**Figure 5:** Raw SERS spectra collected from eRDF solutions (17.7 g/L) stored under various conditions: (a) C-D; (b) RT-D; (c) C-L, and (d) RT-L. The storage time increases from black to light grey and the average spectra of three replicate are plotted.

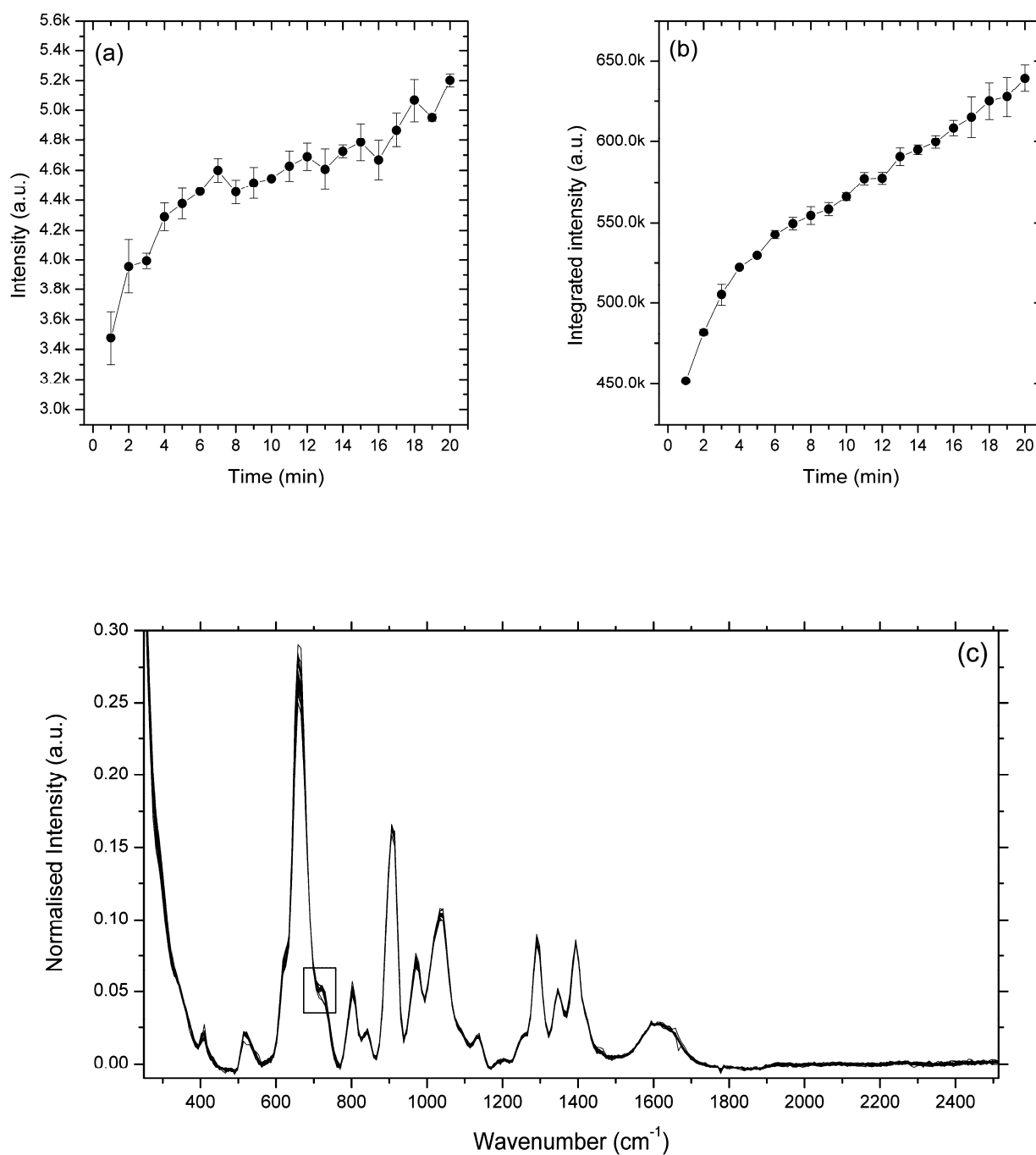
**Figure 6:** Plots of changes in the individual principal component scores versus time generated by various PCA models: (a) PC1, and (b) PC2 component scores plotted versus time at room temperature (○) and in the fridge (□) for the dark stored samples. (c) PC1, (d) PC2, and (e) PC3 component scores plotted versus time at room temperature (○) and in the fridge (□) for the light stored samples. PC1 vs. PC2 scores plots are available in the supplemental information, Figure S-11.

**Figure 7:** Loadings of the components generated by the PCA analysis of the data collected on the samples stored in the dark (a) and in the light (b) under both the cold and room temperature storage conditions.



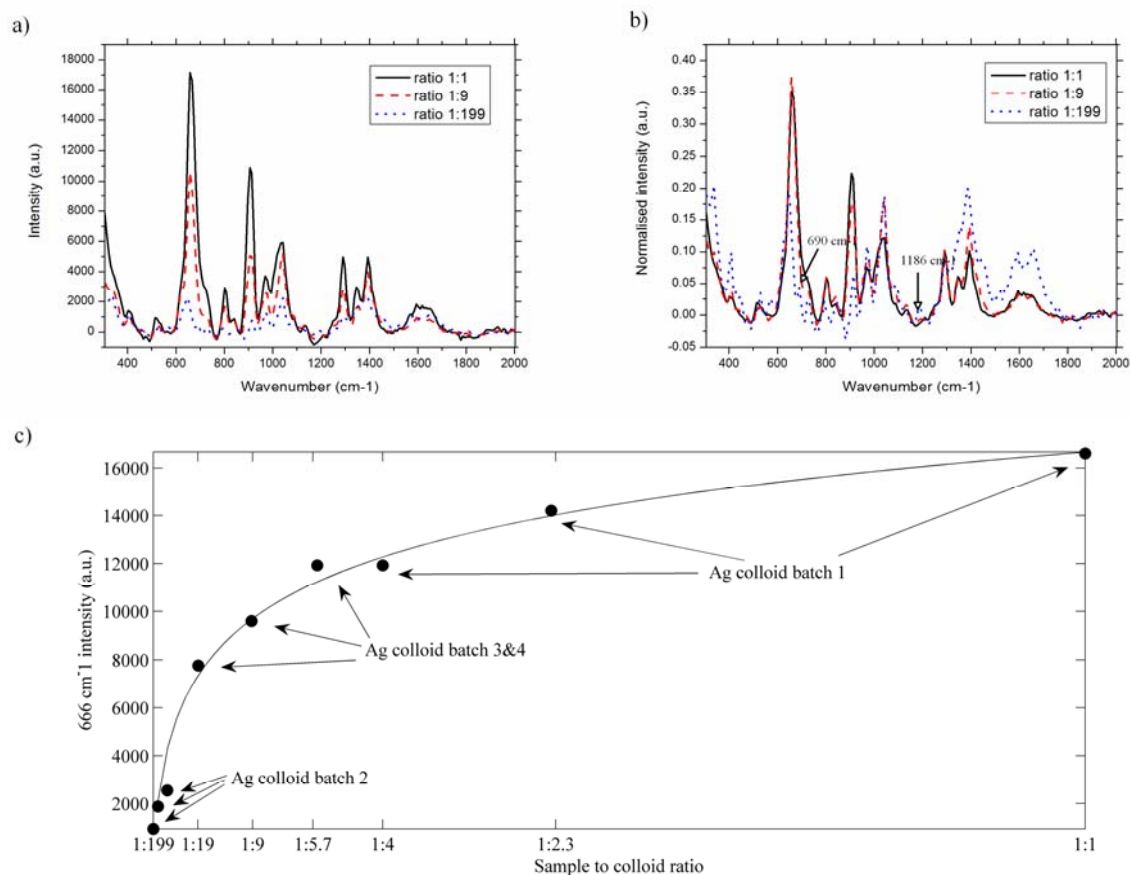
**Figure 1.**

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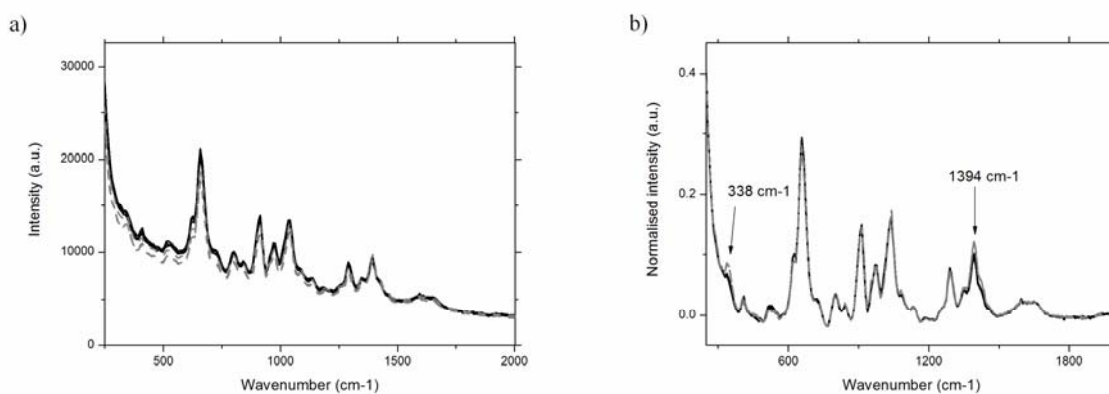


**Figure 2:**

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**Figure 3:**



**Figure 4:**



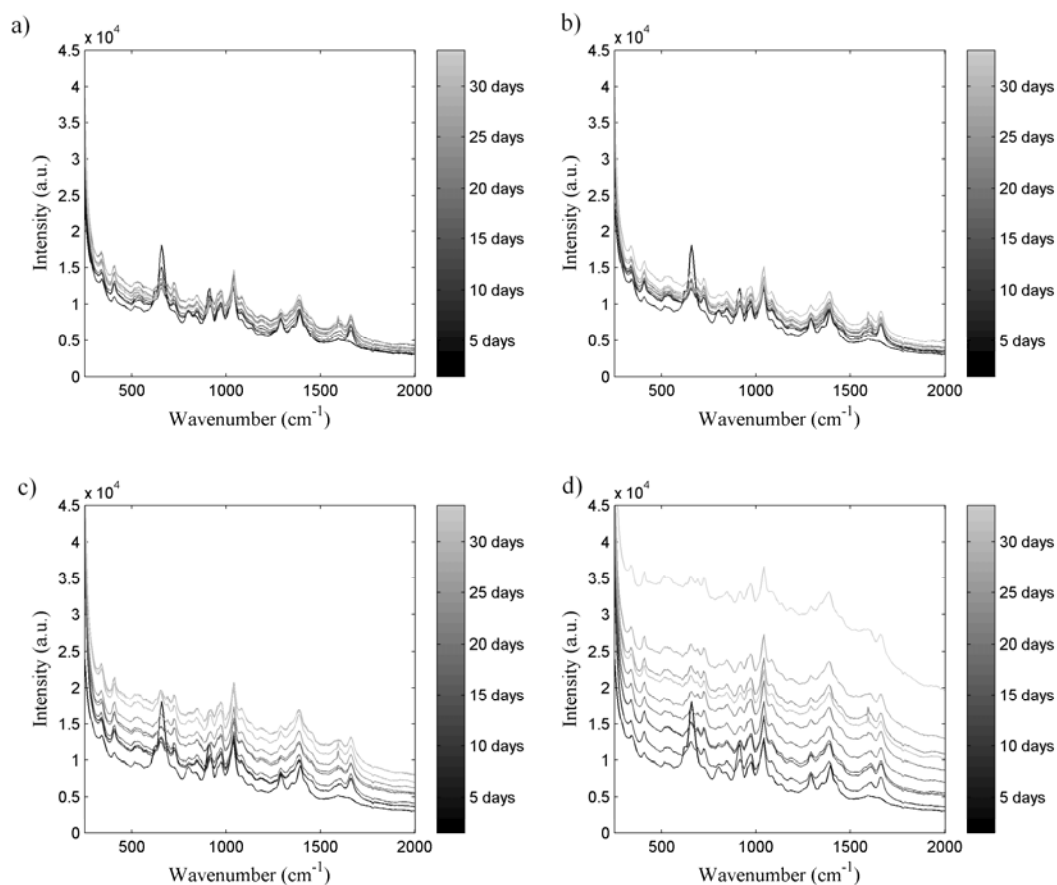


Figure 5.

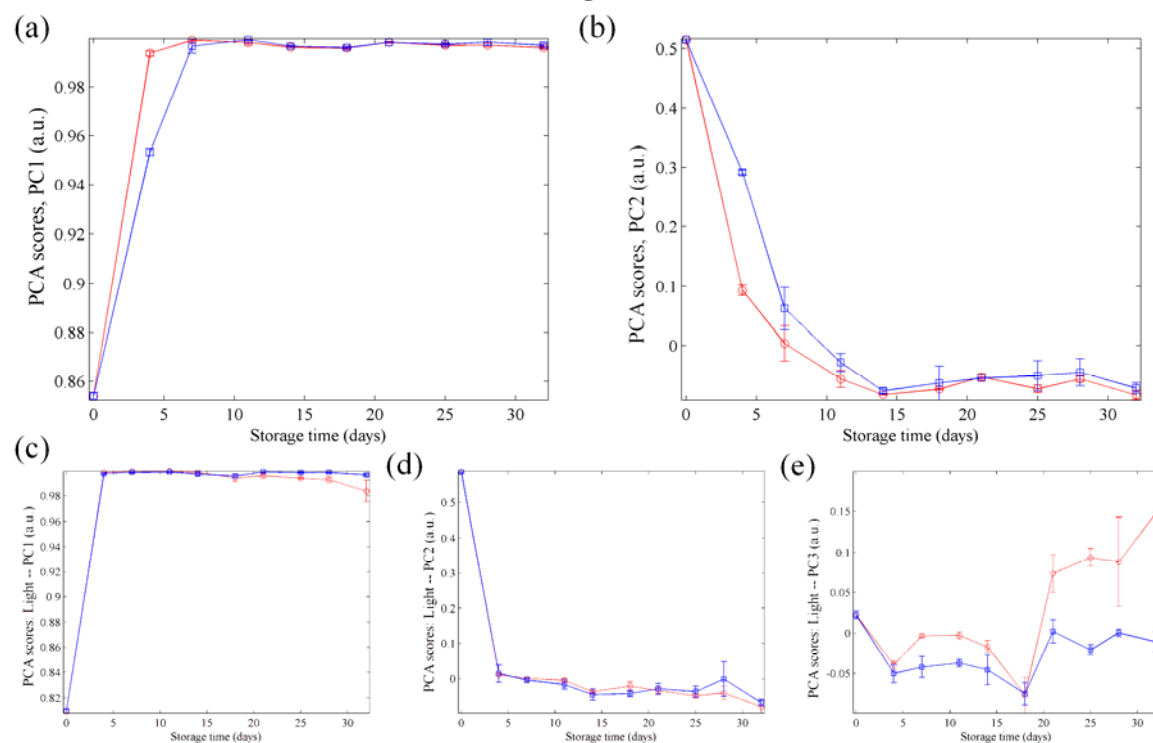
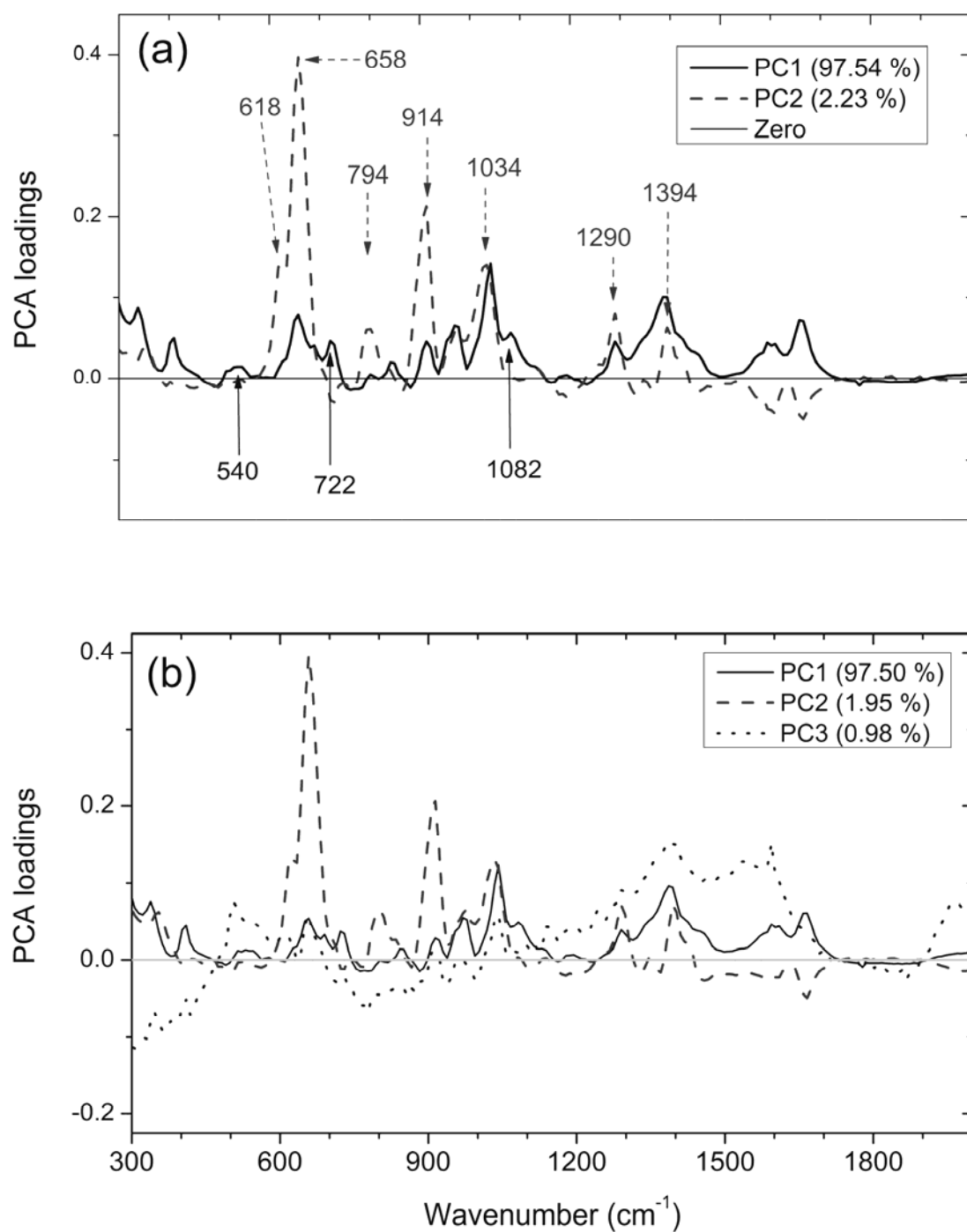


Figure 6.



**Figure 7.**