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USING SURFACE-ENHANCED RAMAN SCATTERING (SERS) AND FLUORESCENCE SPECTROSCOPY FOR SCREENING YEAST EXTRACTS, A COMPLEX COMPONENT OF CELL CULTURE MEDIA.

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Abstract:

Yeastolate or yeast extract which are hydrolysates produced by autolysis of yeast, are often employed as a raw material in the media used for industrial mammalian cell culture. The source and quality of yeastolate can significantly affect cell growth and production, however, analysis of these complex biologically-derived materials is not straightforward. The best current method, liquid chromatography-mass spectrometry (LC-MS) is time-consuming and requires extensive expertise. This study describes the use of surface-enhanced Raman scattering (SERS) and fluorescence excitation-emission matrix (EEM) spectroscopy coupled with robust principal component analysis (ROBPCA) for the rapid and facile characterization and discrimination of yeast extracts in aqueous solution. SERS using silver colloids generates time-dependant signals where adenine is the strongest contributor, and the spectra are stable and reproducible (< ~3%) at 180 minutes after mixing. Combining this spectral behavior with chemometric methods enables SERS to be used for discriminating between different yeastolate sources, for assessing lot-to-lot variability, and potentially to monitor storage-induced compositional changes. Fluorescence EEM combined with multiway ROBPCA also provides a rapid and inexpensive method for discrimination of yeastolate, yielding very similar results in terms of sample discrimination to that obtained by SERS. However, the EEM data does not provide the same level of chemical information as provided by the SERS. Thus the combination of the two methodologies has the potential to be extremely useful in biopharmaceutical manufacturing for the rapid characterization and screening of biogenic hydrolysates from animal or plant sources.

Keywords: Surface-enhanced Raman scattering (SERS) spectroscopy, fluorescence excitation-emission matrix (EEM) spectroscopy, yeastolate/yeast extract, robust principal component analysis (ROBPCA).

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INTRODUCTION

Yeastolate or yeast extract is a water soluble hydrolysate produced by autolysis of various yeast strains, normally *Saccharomyces cerevisiae*.^{1,2,3} Due to its low cost and high content of amino acids, peptides, vitamins, growth factors, trace elements, and energy sources such as carbohydrates, this complex biologically-derived material plays a key role in the preparation of the complex media for industrial fermentations particularly those involving mammalian and insect cell cultures.^{4,5,6,7,8,9,10} For efficient manufacturing, it is vital that the yeastolate quality be consistent to ensure reliable of recombinant protein production.^{11,12} Variances in yeastolate composition can have dramatic effects on product yield and this can be attributed to the use of different yeast strains, changes in yeast growth media, the autolysis method and downstream purification.⁸ The variation between different lots from the same manufacturing process can be almost 50% with respect to biomass and growth rate levels.¹³ The conventional methods for analyzing yeast extracts are either to undertake “use testing”,^{7,13} or size-exclusion chromatography (SEC) for specific component analysis.¹⁴ However, both these approaches are laborious, slow, and not particularly suited to rapid, *in-situ* measurements.⁸ The use of near-infrared (NIR) spectroscopy for correlating variation in fermenter yield with yeast exact composition has been demonstrated, however, no details as to the compositional causes are described, probably because of the poor spectral resolution of NIR.¹⁵ Therefore, there is the demand for rapid, efficient, effective, reliable, robust, and nondestructive analytical approach for yeast extract screening, which would be capable of identifying changes in the composition of this complex material.

Raman analysis of cell culture media and its components has many advantages because it has the ability to analyze materials with no prior sample preparation, in aqueous solution, gives spectra with high information content suitable for the discrimination of subtle chemical and physical effects, and it is also suited to online analysis.^{16,17} Unfortunately, the Raman effect is very weak, hindering its application to analytes at low concentrations or which are fluorescent.¹⁸ For fluorescent biogenic samples one can use excitation-emission matrix (EEM) spectroscopy which has many advantages in terms of high sensitivity, high signal-to-noise ratios, and relatively large linear ranges for quantitative analysis.¹⁹ For EEM of complex mixtures like cell culture media where there are multiple fluorophores and other photophysically active molecules, energy transfer and quenching (both static and dynamic) play a large part in determining EEM spectral contours and intensity, thus providing a unique fingerprint suitable for qualitative characterization.²⁰ There is extensive literature on the utilization of multi-dimensional fluorescence measurements for complex mixture analysis such as crude petroleum oils,²¹ industrial processes,^{22,23} and tissue.^{24,25} For effective analysis of EEM data from complex mixtures, multi-way chemometric methods are required.^{26,27,28,29,30} Our previous work has demonstrated the efficacy of conventional Raman spectroscopy^{16,31} and EEM methods³² for the identification and quality assessment of cell culture media and their components. However, fluorescence EEM spectra are generally not very informative about specific changes in composition, and a greater degree of chemical resolution is needed.

One way to circumvent these problems is to use surface-enhanced Raman spectroscopy (SERS), since this can dramatically increase measurement sensitivity (with enhancement factors of up to 10^{10})³³ and reduce background fluorescence.³⁴ To generate enhanced SERS signals

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analytes must adsorb onto, or be in very close proximity (< 10 nm) to the nano-roughened surfaces or colloidal metal particles. To adsorb onto the metal surfaces the analytes should have a sufficiently strong affinity to those surfaces, for example pyridine and chloride stick to Ag surface very strongly but species that bind via oxygen, e.g. glucose and SO_4^{2-} show little affinity.^{35,36} This makes complex materials analysis by SERS a non-trivial procedure, yet, there are many applications of SERS in the area of chemical biology,³⁷ and the life sciences.³⁸ SERS has also been used for the discrimination of different yeast species,³⁹ but there has been little interest in its use for cell culture media analysis apart from a few recent studies focused on the potential contamination of SERS spectra by growth media.^{40,41,42} It should also be noted that most media (including agars) used to grow bacteria used in SERS studies have employed complex biogenic hydrolysate type materials (yeast, meat, or soya based).^{43,44,45} Accordingly apart from the known requirement in biopharmaceutical manufacturing to characterize these materials, there is also a need to understand the SERS behavior in the context of SERS characterization of bacteria.

Here we use SERS and fluorescence EEM spectroscopies combined with robust principal component analysis (ROBPCA)⁴⁶ to characterize complex yeast extracts in aqueous solution. The results show that SERS has significant potential for discriminating different source yeastolates, monitoring storage-induced compositional changes, and assessing lot-to-lot variability. SERS provides more chemical information than fluorescence EEM, but the combination of both methods offer a rapid, inexpensive method suitable for high throughput screening of these complex biogenic materials.

MATERIALS AND METHODS

Materials: Bacto™ TC yeastolate (Product No. 255772, LOT4265361) and Difco™ TC yeastolate (Ultra-filtered, Product No. 292804, LOT5214385) were purchased from BD. Fluka yeast extract (Product No. 70161, LOT1340296), adenine (99.0%), silver nitrate (99.9999%), and sodium citrate (99.5%) were purchased from Sigma-Aldrich and used as received. Samples from 21 different batches (ten from 2006 and eleven from 2007) of an ultra-filtered yeastolate used in a current CHO-based manufacturing process were supplied by Bristol-Myers Squibb (BMS), Syracuse, NY. These samples should be consistent and were originally provided by a third party supplier. All these solid materials were stored at 2~4°C. Each yeastolate sample was prepared in sterilized Millipore water (5 g/L) under aseptic conditions. A 1 ppm adenine solution was prepared by diluting 0.5 g/L of stock solution with Millipore water. Silver colloid was prepared according to the Lee and Meisel citrate reduction method.⁴⁷ The UV-Vis absorption spectra of the Ag colloids typically had an absorption maximum (λ_{max}) at 406 nm with a full width half maximum of 80 nm but colloids with λ_{max} as long as 412 nm gave equally acceptable Raman spectra. For SERS measurements, 100 μl of yeastolate solution was pipetted into an empty well plate and then mixed with an equal volume of Ag colloid, and all spectra were collected at a set time of 3 hrs after mixing.

Reproducibility and degradation studies: A 10 ml 5 g/L stock solution of the BD-Difco yeastolate was prepared in sterilized water under aseptic conditions and then split into 18 0.5 ml aliquots in vials. These were then split into groups of six vials and stored at -70°C, 2~4°C, or room temperature (15~20°C), respectively. The remaining 1 ml stock solution was used for the

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Day 1 SERS measurements (100 μ l per measurement). Stored samples were removed for SERS measurements at six different times (Day 6, 8, 11, 15, 20, and 30) and triplicate SERS measurements were made from a single fresh vial stored at each temperature. A stock yeastolate solution (5 g/L) was prepared from BD-Difco-UF yeastolate as above, except that unsterilized Millipore water was used and no precautions were taken to control microbial contamination. This solution was split into 20 aliquots in individual vials (0.5 ml each). Batches of vials were stored at -70°C , $2\sim 4^{\circ}\text{C}$, and room temperature. SERS measurements were made on days 1, 5, 9, and 21 using a fresh vial each time.

Instrumentation and data collection: Raman measurements were performed in triplicate at room temperature using an Avalon Instruments Raman spectrometer with 785 nm excitation.¹⁶ A laser power of ~ 70 mW at the sample was used and spectra were collected with a resolution of 8 cm^{-1} and a typical exposure time of 10 s. For solution samples, stainless steel 96-well plates³¹ were used and multiple spectra were collected from a 3×3 grid (0.5 mm spot spacing) from which a single averaged spectrum was generated for data analysis. Fluorescence measurements were made at 25°C with a Cary Eclipse (Varian) fluorimeter using procedures previously described.³² Yeastolate samples were randomly removed from storage, defrosted at room temperature and allowed to reach room temperature, and handled using aseptic techniques. For each solution, 1 ml was pipetted into a cuvette and sealed before allowing to thermally equilibrate for several minutes prior to measurement.

Spectral SERS data was pre-processed to reduce the influence of baseline drift, scatter effects, and uncontrolled fluctuations. Spectra containing cosmic interference were discarded prior to averaging of the spectra. The average spectrum was then treated with a multiplicative scatter correction,⁴⁸ then an asymmetric weighted least squares algorithm⁴⁹ to remove baseline offsets before finally applying a background correction using an orthogonal projection procedure.⁵⁰ For SERS-ROBPCA analysis, the first derivative (SavGol) method⁵¹ was then implemented to further reduce measurement/instrumental effects and accentuate analyte signals. For EEM-MROBPCA analysis, Rayleigh and Raman scatter were removed from EEM data by replacing with a curve fit, connecting points either side of the bands using imputation.^{52, 53} All calculations were performed using MATLAB ver. 7.4,⁵⁴ PLS_Toolbox 4.0,⁵⁵ and in-house-written toolboxes.

RESULTS & DISCUSSION

SERS spectra: Raman spectra of yeastolate powder and aqueous solutions do not show any sharp bands (Figure 1a & b). In solid form one observes a strong fluorescent background while in aqueous solution one only observes the water bands superimposed on a sloping background which is almost identical to the spectra of water collected under these conditions.³¹ However, when the yeastolate solution was mixed with silver colloid, a very detailed SERS spectrum was obtained (Figure 1c) which arises from the adsorption of various yeastolate components onto the colloidal silver surfaces. Enhancement is significant, and one can generate good quality spectra with short accumulation times of 0.1 s. Most of the useful enhancement occurs in the fingerprint region ($480\sim 1870\text{ cm}^{-1}$) which was used for chemometric analysis. With this high concentration (5 g/L is typical of the concentrations used for cell culture media), we expect that only a small

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fraction of the yeastolate constituents will adsorb on the colloid (particularly those with sulfur or nitrogen containing groups that have a strong affinity for silver), leaving the vast bulk of the constituents unchanged in solution.

When we start analyzing the SERS signal to identify the species present we have to consider the following: 1) yeastolate is a complex mixture where many of the components are unknown, 2) competitive binding to the silver surfaces limits the total mass of adsorbed molecules to a very small fraction of the overall sample mass, 3) the components which actually give the SERS signals may be only present in very low concentrations (e.g., at ppm levels), 4) a significant fraction of the sample mass may be non-SERS active compounds such as carbohydrates, and 5) the adsorption process is dynamic and SERS signal varies with time. Thus, the identification of the exact species which give rise to the various SERS bands is not trivial. However, the combination of all these factors produces one conclusion that the SERS process generates a unique signature for these complex biogenic samples. For certain analytical applications, this is all that is required, since we are interested in rapidly checking the identity and assessing the variance of these complex materials without recourse to expensive and time-consuming chromatography or mass spectrometry-based analyses. However, despite the complexity of the SERS process for these samples, one can still expect that those species with amino or thiol groups will have the highest affinity for the silver surfaces and as such are most likely to bind strongly to the surface and thus generate strong SERS signals.

In the case of yeastolate-SERS we suspect that the greatest contribution is from adenine (Figure 1c) since it is typically present in relatively high concentration in yeast extracts (0.14~0.24%)⁸ and it is known to produce very strong SERS signals.^{36, 56, 57, 58, 59} The other major constituents of yeastolate such as trehalose and lactate are not expected to bind well to the colloid, thus they do not generate any significant SERS signals. There is a clear correlation between the SERS bands of yeastolate and adenine, and the specific vibrational modes (*Supplemental Information*) can be identified from previously published work.^{57, 59, 60} The ~655 cm⁻¹ band may be attributed to guanine,^{57, 60} which can also be present in relatively high concentration and has a high affinity for silver surfaces because of its primary and secondary amine groups (Figure S1, *Supplemental Information*). The complexity of the yeastolate and the widely varying concentrations make it nigh impossible to unambiguously identify any other species apart from adenine. The colloid spectrum also shows some bands (Figure 1d) which originate from residual citrate adsorbed on the colloidal silver, as well as the water bands. We used this colloid spectrum as the background spectrum which was subtracted from the sample spectra prior to ROBPCA analysis by way of an orthogonal projection method.⁵⁰ The reasoning for this is that the colloidal nanoparticles induce a significant background in terms of scatter artifacts.

Time-dependent SERS: Due to the complex composition of yeastolate and because of the adsorption requirement for SERS, there is always the possibility of signal variation on mixing of sample and colloid. This was indeed the case here, and we observe a very significant variation in SERS signal (overall spectral profile, intensities, and band widths) over 180 minutes (Figure 2). Directly after mixing, we got a reasonably well resolved spectrum in the fingerprint region, however, as the time increased, the intensities of some bands (e.g. 731, 1332, and 1396 cm⁻¹) continued to increase, reaching a maximum value at ~90 min., before starting to decrease. The

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731 cm^{-1} band (adenine ring breathing mode) intensity changes more significantly than any other. Looking at the relative changes in band intensities (Figure 2b) we see that the I_{1332}/I_{1396} intensity ratio remains constant from 90 minutes after mixing, but that other intensity ratios such as I_{655}/I_{731} and I_{795}/I_{731} keep increasing, indicating analyte turnover. When band intensity is referenced to the water solvent (Figure 2c), it is clear that after ~ 40 minutes the contribution of the adenine SERS signal starts to decrease while the signal from guanine (655 cm^{-1}) increases. These observations are expected in SERS spectra from complex mixtures (especially biogenic materials) since the SERS active analytes present in the mixture will compete for the available sites and these phenomena are very common even when there are only two components present.⁶¹ It is clear that in this system there are several competing processes underway: 1) Competitive binding between different analytes (as evidenced by changes in band shape/intensity), and 2) Gradual aggregation of the colloid induced by the multitude of different species present (as evidenced by the broader and weaker bands at ~ 180 minutes.). The aggregation process itself comprises of two elements that can affect the SERS spectra, first in the early stages ($< \sim 90$ minutes) there is a favourable aggregation leading to the formation of hot spots and an increase in SERS intensities. This is followed by continued aggregation leading to the formation of increasingly larger clusters which then precipitate out of solution leading to weaker and broader peaks. Based on these observations, it is essential to record the SERS spectra at a set, fixed time after mixing of the colloid and yeastolate solution. In the context of using SERS data for chemometric analysis, one needs to generate a reproducible spectral data set. For this preliminary proof of concept study we selected a long equilibration time of 3 hours. This may not be the most optimal time (from Figure 2c, ~ 40 minutes might be better), however it provided reproducible data suitable for chemometric analysis.

Reproducibility: The big issue with SERS is the reproducibility and we sought to test this by looking at SERS data collected over an extended time period using a single batch of colloid. Figures S7-S9 (*Supplemental Information*) show the SERS spectra collected from sterile solutions stored at different temperatures for 30 days and there were some small observable spectral changes which are mostly related to absolute intensity and baseline effects. These differences were however small (Figure S9, *Supplemental Information*) because their similarities in terms of correlation coefficient were all equal to or very close to 1. This indicates that sterile aqueous yeastolate solutions are stable and coincidentally that the SERS methodology is reasonably robust (see *Supplemental Information* for the raw data comparison). The reproducibility can be quantified by variance analysis (Table 1) in terms of within-group, relative within-group (RMS_w), and between-group variances.³¹ RMS_w values were between 2.8 and 3.2% for all measurements, indicating good reproducibility. The within-group variances are larger than the between-group variance which implies that there is no significant statistical difference between SERS spectra of the sterile yeastolate solution stored at different temperatures. However, we do note that the SERS spectra at the 180 minute timepoint are different when one compares Figures 2a and S8/S9. The main reason for this is that the data in Figures S8/S9 has had a baseline correction and background spectrum subtracted, whereas the Figure 2 only has a simple baseline correction. However, comparison with the raw data collected for the extended time study (see *Supplemental Information*) we still see some differences. The potential source of this small spectral variance was the use of different batches

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of colloid which can have slightly different properties in terms of analyte adsorption/aggregation dynamics. This is one of the major problems with the use of laboratory made colloid substrates where consistency of manufacture is a very significant issue affecting the reproducibility/robustness of the silver colloid based SERS.

Yeastolate Discrimination: One of the study goals is to determine the utility of SERS for the qualitative yeastolate analysis for discrimination purposes because of the significant issues with regard to material consistency. To demonstrate the utility of SERS for discriminating between different types of yeast extracts, we compared the SERS spectra (Figure 3) of yeast extracts from three different sources. The spectra are all very different in terms of SERS bands and intensities, illustrating how easy it is to discriminate different types of yeast extract. For example, the BD sourced yeastolates have very different SERS spectra, yet ostensibly they are the same material except that the BD-Difco-UF material has been ultra-filtered at a 10,000 MWCO (Molecular Weight Cut-Off). This may explain why SERS spectrum from BD-Difco-UF is much more intense than that from the less well filtered Bacto™. We speculate that the larger molecular weight species occupy more binding sites on the silver surfaces thus hindering adsorption of more SERS active smaller molecules. There is evidence that these high molecular weight species are nucleic acid fragments rather than proteins.¹⁴ The much weaker SERS spectra from the Fluka product may also be due to occlusion by high molecular weight molecules present in the less well filtered materials, or it may be a consequence of a different upstream manufacturing process, or a different downstream purification process. The exact cause of these differences is however, outside the scope of this work, as we are much more concerned with being able to rapidly discriminate different types of closely related yeastolates for the initial purpose of identification, and then subsequent quality assessment. It is likely that the basis of this discrimination may be on adenine content (which may include free monomer, peptides and possibly as higher MW nucleotides).

ROBPCA screening of yeast extracts: The next issue with the analysis of yeastolates is trying to rapidly assess their variability/consistency which is integral to good manufacturing practices. It may be feasible to use SERS to assess lot-to-lot variation because of the detailed spectra and the sensitivity to source. We analyzed in triplicate, 22 samples (21 BMS retain batches and the BD-Difco-UF) by SERS, and the pretreated spectra (Figure 4a), were analyzed by ROBPCA.⁴⁶ A three principal components (PCs) ROBPCA model explained 99.68% variance in the mean-centered data. Applying a 95% confidence level determined the threshold values for the score distance (SD) and orthogonal distance (OD) of each measurement which are used to identify anomalous measurements (Figure 4b). This outlier diagnostic map provides a quantitative evaluation of the 18 outliers which originated from eight samples. Five samples (#12/#13/#16/#17/#22) each had three outlying measurements, which suggests that the variation originates largely from compositional differences. Sample #22 is the BD-Difco-UF yeastolate and as such would be expected to be somewhat different from the other samples. Three outlying measurements (#11/#14/#21) were singular events where the remaining two of the triplicate measurements of these samples were within the 95% confidence boundaries and as such we would suggest that these outliers are most likely due to experimental events.¹⁶

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For this dataset where absolute SERS signal intensity is integral to the data, we see that the SERS spectral intensities of the samples #13 and #17 are at the same level as that of sample #22, but samples #12 and #16 have lower intensities, and spectral comparison indicates that this is due to a lower adenine SERS signal for these two samples. This might arise from either: a). a real change of adenine concentration in the sample, or b). increased competitive binding by another component onto the silver, which displaces some of the adenine. While the yeastolate materials should not contain intact proteins which can have significant effects on SERS signals,⁶² the presence of high molecular weight peptides and other charged cellular fragments are expected to have similar effects. In any event, irrespective of the specific cause, the reason for the spectral difference originates from a change in the sample composition. Thus for assessing subtle differences in composition which may be very significant from an industrial manufacturing context, the SERS method shows promise. The only alternative analytical methods to try and detect these types of changes either involve use testing (an expensive and time-consuming process) or the undertaking of an LC-MS study, which in itself is a time-consuming process.

Normalizing the spectra and repeating the analysis (*Supplemental Information*) generates an expanded, but very similar outlier set (#6/#7/#12/#13/#16/#17/# 22). All outliers have stronger SERS signals compared to the average value for the normal sample group which are located within the solid ellipses (Figure 4c & 4d). Interestingly, the ten 2006-vintage yeastolates were clearly distinguished from the remaining (eleven 2007-vintage yeastolates and #22). These 2006 samples are closely clustered in two groups (dotted ellipse) in the PC2 *versus* PC1 scores plot. The spread of the main normal sample group of also implies that there is a significant level of inherent lot-to-lot variability in these materials and this may represent a sample quality issue. To confirm these observations we use the fluorescence methods developed for the analysis of cell culture media.³²

MROBPCA of fluorescence EEM data: Figure 5a shows a typical EEM landscape plot with a fairly complex topography with many bands which originate from amino acids, peptides, and some of the water soluble vitamins (we have not definitively identified the specific chemical species). EEM data obtained from triplicate measurements of 22 yeastolate solutions was first treated by removal of both Rayleigh and Raman scattering using the interpolation method.⁵³ A MROBPCA model was generated using five robust principal components. A 95% confidence level was used to set the SD = 3.58 and OD = 47.73 parameters, then score and orthogonal distances were calculated for each measurement. The outlier diagnostic map (Figure 5b) shows 19 outliers, of which #12/#16 had 3 of 3 spectra outside the limits and #13/#17/#22/#8 had 2 of 3 spectra outside the model limits. We would be confident that these samples represent samples with a significant compositional difference to the main group samples. The other outliers (#6/#7/#10/#18/#21) are singular events where one EEM spectrum out of three is outside the model limits, and these are due to measurement errors. The major outliers (#13/#17/#12/#16) are significantly more fluorescent (*Supplemental Information*) than the main group of normal samples located in the specified ellipses (Figure 5c & 5d), and samples #22 and #8 are less fluorescent again. Repeating the analysis using the normalized data (*Supplemental Information*), generates fewer outliers indicating the overall fluorescence intensity is a significant factor that needs to be retained. The outliers identified by EEM are the same as those by SERS, except for sample #8 which largely validates the SERS methodology for outlier detection to some extent.

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We also observe in Figure 5c that we got a partial discrimination of the 2006-vintage yeastolate samples (green points within dotted ellipse). The separation, while not as clear cut as the SERS (Figure 4c) does indicate that there are subtle compositional differences between the different sample batches.

Yeastolate Degradation: One of the problems faced in the use of yeastolate for sensitive manufacturing processes like cell culture-based recombinant protein production is the potential of spoilage, degradation, or alteration due to improper/variable storage conditions. One quality assurance challenge is to find an effective, rapid, and inexpensive method for assessing storage induced variability of yeast extracts. Since SERS is sensitive to compositional changes, it may also be useful in the context of monitoring spoilage. Here we wanted to see if SERS could be used to observe subtle compositional changes that might occur with yeast extracts prepared under non-sterile conditions and stored under different temperatures. For the non-sterile samples stored at room temperature, within one week the samples had become cloudy and were obviously contaminated and thus no data was collected after 7 days. For the samples stored at 2~4°C there were very significant changes in the SERS spectra with time (Figure 6). At first there was not much change between Day1 and Day5, as indicated by the high similarity coefficient of 0.97 (*Supplemental Information*). However, by Day 9 and on to Day 21 there was a dramatic change with strong increase in band intensities at 1396, 1290, 1035, 910, and 795 cm^{-1} (Figure 6). The adenine band at 731 cm^{-1} decreased and was nearly gone by Day21 (accompanied by a shift to 722 cm^{-1}), and this is clear evidence for the removal of adenine from the yeastolate solutions. Conversely the 655 cm^{-1} band increases with time and shifts to 660 cm^{-1} (Day21), which may indicate a relative increase in the SERS signal from guanine.⁶⁰ It has also been suggested that this is a C-S vibration³⁹ which could arise from a metabolite generated by microbial growth. All of this suggests that significant changes in chemical composition (due to microbial growth) have definitely occurred in the unsterilized yeastolate solution when stored at 2~4°C for extended periods.

CONCLUSIONS

This proof of concept study shows that SERS could potentially offer a quick and reliable method for both source identification and variability assessment of complex biogenic materials such as yeastolate used in cell culture media. SERS circumvents the problem of fluorescence backgrounds and unlike fluorescence EEM spectroscopy the method provides some detail as to specific differences in sample composition. Yeastolate SERS is complex and shows considerable time dependant spectral changes so it is important to define carefully the precise sample preparation and data collection procedures. In the SERS spectra of yeastolate with silver colloid, it is clear that the main component observed is that of adenine and that during degradation induced by microbial growth the adenine signal changes dramatically. The SERS method is also sensitive to small variations in yeastolate manufacture/source and thus is suitable for implementation as rapid lot-to-lot consistency screening, particularly, since the alternative involves time-consuming LC-MS analysis. The study has also shown that this SERS method is reasonably reproducible ($\text{RMS}_w \sim 3\%$) and we expect that this can be improved as the

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manufacture of reproducible and robust SERS substrates develops. The SERS results in terms of outlier identification (e.g. sample variability) are supported by the EEM results and thus one could use a combination of these techniques for robust and rapid analysis of complex biogenic materials. In conclusion, we feel that SERS combined with chemometric analysis offers a potentially viable and robust method suitable for the rapid and inexpensive analysis of yeastolates and yeast extracts used in industrial biotechnology.

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SUPPORTING INFORMATION AVAILABLE

Supporting information may be found in the online version of this article.

REFERENCES

- [1] K. C. Chao, E. F. McCarthy, G. A. McConaghey, *Yeast autolysis process*. US Patent #4,218,481, **1980**.
- [2] C. Akin, R. M. Murphy, *Methods for accelerating autolysis of yeast*. US Patent #4,285,976, **1981**.
- [3] M. Kelly, in *Industrial Enzymology, the Application of Enzymes in Industry*, (Eds: T. Godfrey, M. Reichelt), Nature Press, New York, 1983, pp. 457-464.
- [4] J. Y. Wu, K. I. D. Lee, *Biotechnology Techniques*, **1998**, 12, 67.
- [5] S. K. Dahod, in *Manual of Industrial Microbiology and Biotechnology*, 2nd edn. (Eds: A. L. Demain, J. E. Davies), ASM Press, Washington DC, 1999, pp. 213-220.
- [6] I. Chevalot, C. Dziukala, E. Olmos, I. Marc, D. Druaux-Borreill, *ESACT proceedings*, **2010**, 4, 843.
- [7] K. Iding, H. Buntmeyer, F. Gudermann, S. M. Deutschmann, C. Kionka, J. Lehmann, *Biotechnol. Bioeng.* **2001**, 73, 442.
- [8] J. Y. Zhang, J. Reddy, B. Buckland, R. Greasham, *Biotechnol. Bioeng.* **2003**, 82, 640.
- [9] S. H. Kim, G. M. Lee, *Appl. Microbiol. Biotechnol.* **2009**, 83, 639.
- [10] M. J. Keen, N. T. Rapson, *Cytotechnology*, **1995**, 17, 153.
- [11] E. K. Read, J. T. Park, R. B. Shah, B. S. Riley, K. A. Brorson, A. S. Rathore, *Biotechnol. Bioeng.* **2010**, 105, 276.
- [12] E. K. Read, R. B. Shah, B. S. Riley, J. T. Park, K. A. Brorson, A. S. Rathore, *Biotechnol. Bioeng.* **2010**, 105, 285.
- [13] J. Potvin, E. Fonchy, J. Conway, C. P. Champagne, *J. Microbiol. Methods*, **1997**, 29, 153.

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DOI: <http://dx.doi.org/10.1002/jrs.3141>

- [14] L. W. Dick, J. A. Kakaley, D. Mahon, D. Qiu, K. C. Cheng, *Biotechnol. Prog.* **2009**, 25, 570.
- [15] R. P. Kaspro, A. J. Lange, D. J. Kirwan, *Biotechnol. Prog.* **1998**, 14, 318.
- [16] B. Li, P. W. Ryan, B. H. Ray, K. J. Leister, N. M. S. Sirimuthu, A. G. Ryder, *Biotechnol. Bioeng.* **2010**, 107, 290.
- [17] M. J. Pelletier, *Analytical Applications of Raman Spectroscopy*, Blackwell Scientific, Oxford, 1999.
- [18] R. L. McCreery, *Raman Spectroscopy for Chemical Analysis*, Wiley, New York, 2000.
- [19] J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed. Springer, New York, 2006.
- [20] B. Li, P. W. Ryan, M. Shanahan, K. J. Leister, A. G. Ryder. *Appl. Spectrosc.* **2011**, 65, 1240.
- [21] A. G. Ryder, *J. Fluoresc.* **2004**, 14, 99.
- [22] C. Lindemann, S. Marose, H. O. Nielsen, T. Scheper, *Sens. Actuat. B-Chem.* **1998**, 51, 273.
- [23] E. Sikorska, T. Górecki, I. V. Khmelinskii, M. Sikorski, D. Keukeleire, *Food Chem.* **2006**, 96, 632.
- [24] A.F. Zuluaga, U. Utzinger, A. Durkin, H. Fuchs, A. Gillenwater, R. Jacob, B. Kemp, J. Fan, R. Richards-Kortum, *Appl Spectrosc.* **1999**, 53, 302.
- [25] D. Patra, A. K. Mishra, *TrAC-Trend Anal. Chem.* **2002**, 21, 787.
- [26] P. G. Coble, *Marine Chem.* **2006**, 51, 325.
- [27] G. J. Hall, K. E. Clow, J. E. Kenny, *Environ. Sci. Technol.* **2005**, 39, 7560.
- [28] G. J. Hall, J. E. Kenny, *Anal. Chim. Acta.* **2007**, 581, 118.
- [29] J. Sadecka, J. Tothova, *Czech J. Food Sci.* **2007**, 25, 159.
- [30] A. M. de la Pena, A. E. Mansilla, N. M. Diez, D. B. Gil, A. C. Olivieri, G. M. Escandar, *Appl. Spectrosc.* **2006**, 60, 330.
- [31] A. G. Ryder, J. de Vincentis, B. Li, P. W. Ryan, N. M. S. Sirimuthu, K. J. Leister, *J. Raman Spectrosc.* **2010**, 41, 1266.
- [32] P. W. Ryan, B. Li, M. Shanahan, K. J. Leister, A. G. Ryder, *Anal. Chem.* **2010**, 82, 1311.
- [33] C. L. Haynes, A. D. McFarland, R. P. Van Duyne, *Anal. Chem.* **2005**, 77, 338A.
- [34] R. Aroca, *Surface-Enhanced Vibrational Spectroscopy*, Wiley, Chichester, 2006.
- [35] D. A. Stuart, C. R. Yonzon, X. Zhang, O. Lyandres, N. C. Shah, M. R. Glucksberg, J. T. Walsh, R. P. Van Duyne, *Anal. Chem.* **2005**, 77, 4013.
- [36] S. E. J. Bell, N. M. S. Sirimuthu, *J. Am. Chem. Soc.* **2006**, 128, 15580.
- [37] A. G. Ryder, *Curr. Opin. Chem. Biol.* **2005**, 9, 489.
- [38] R. Petry, M. Schmitt, J. Popp, *Chem. Phys. Chem.* **2003**, 4, 14.
- [39] I. Sayin, M. Kahraman, F. Sahin, D. Yurdakul, M. Culha, *Appl. Spectrosc.* **2009**, 63, 1276.
- [40] N. E. Marotta, L. A. Bottomley, *Appl. Spectrosc.* **2010**, 64, 601.
- [41] W. R. Premasiri, Y. Gebregziabher, L. D. Ziegler, *Appl. Spectrosc.* **2011**, 65, 493.

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DOI: <http://dx.doi.org/10.1002/jrs.3141>

- [42] M. Kahraman, K. Keseroglu, M. Culha, *Appl. Spectrosc.* **2011**, 65, 500.
- [43] L. Zeiri, S. Efrima, *J. Raman Spectrosc.* **2005**, 36, 667.
- [44] I. S. Patel, W. R. Premasiri, D. T. Moir, L. D. Ziegler, *J. Raman Spectrosc.* **2008**, 39, 1660.
- [45] D. Cam, K. Keseroglu, M. Kahraman, F. Sahin, M. Culha, *J. Raman Spectrosc.* **2010**, 41, 484.
- [46] M. Hubert, P. J. Rousseeuw, K. Vanden Branden, *Technometrics*, **2005**, 47, 64.
- [47] P. C. Lee, D. Meisel, *J. Phys. Chem.* **1982**, 86, 3391.
- [48] H. Martens, T. Naes, *Multivariate Calibration*, Wiley, New York, 1989.
- [49] D. M. Haaland, R. G. Easterling, *Appl. Spectrosc.* **1982**, 36, 665.
- [50] A. Lorber, *Anal. Chem.* **1986**, 58, 1167.
- [51] A. Savitzky, M. J. E. Golay, *Anal. Chem.* **1964**, 36, 1627.
- [52] A. Rinnan, C. M. Andersen, *Chemom. Intell. Lab. Syst.* **2005**, 76, 91.
- [53] M. Bahram, R. Bro, C. Stedmon, A. Afkhami, *J. Chemom.* **2006**, 20, 99.
- [54] Mathworks Inc., Cambridge, MA, 1994-2008.
- [55] Eigenvector Research Inc., 3905 West Eaglerock Drive., Wenatchee, WA.
- [56] K. Kneipp, H. Kneipp, I. Itzkan, R. R. Dasari, M. S. Feld, *J. Phys. Condens. Mat.* **2002**, 14, R597.
- [57] B. Giese, D. McNaughton, *J. Phys. Chem. B*, **2002**, 106, 101.
- [58] M. Sackmann, A. Materny, *J. Raman Spectrosc.* **2006**, 37, 305.
- [59] E. Papadopoulou, S. E. J. Bell, *Analyst*, **2010**, 135, 3034.
- [60] B. Giese, D. McNaughton, *Phys. Chem. Chem. Phys.* **2002**, 4, 5171.
- [61] S. E. J. Bell, N. M. S. Sirimuthu, *Analyst*, **2004**, 129, 1032.
- [62] D. M. Zhang, S. M. Ansar, K. Vangala, D. P. Jiang, *J. Raman Spectrosc.* **2010**, 41, 952.

Table 1: Within- and between-group variances obtained from SERS spectra of sterile BD-Difco-UF solution stored at -70°C , $2\sim 4^{\circ}\text{C}$, and room temperature for 30 days. The variance was calculated using the baseline-corrected and background-subtracted spectra.

Variance ($\times 10^7$)	-70°C	$2\sim 4^{\circ}\text{C}$	room temp
-70°C	<i>1.74</i>	0.03	0.14
$2\sim 4^{\circ}\text{C}$	0.03	<i>1.87</i>	0.15
room temp	0.14	0.15	<i>1.79</i>
RMS_w	2.97%	3.24%	2.76%

Values *in italics* correspond to within-group variance.

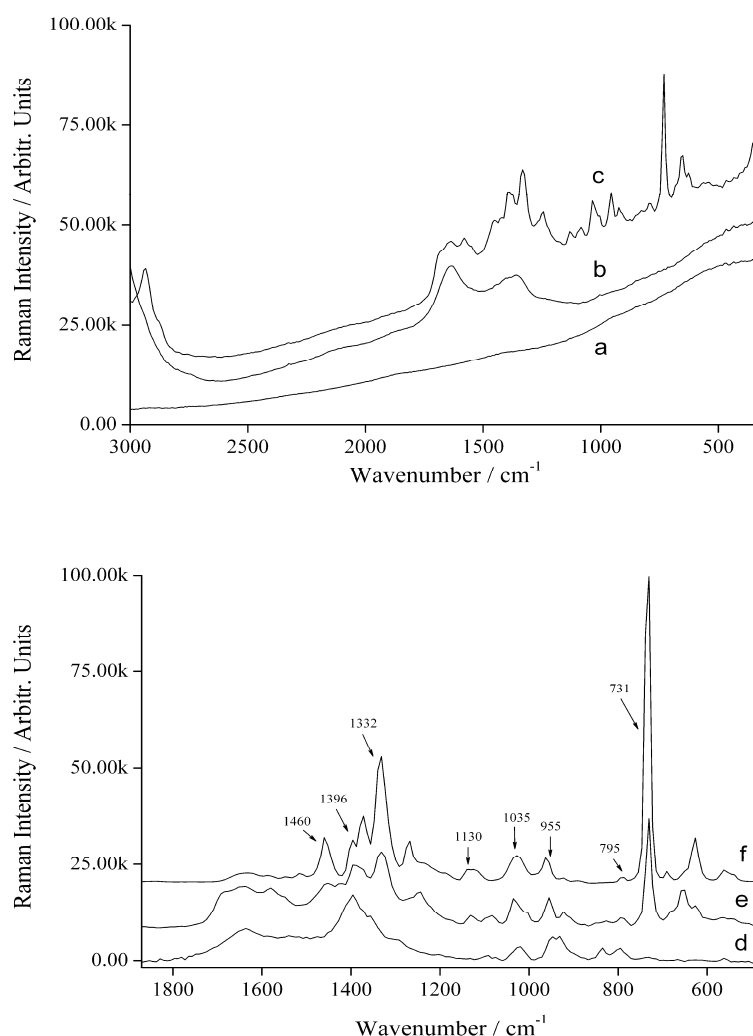


Figure 1: (Top) Raman spectra of the BD-Difco-UF yeastolate in: (a) solid form, (b) a 5 g/L solution, and (c) mixed with colloid showing SERS, taken with exposure times of 1, 10, and 10 seconds respectively. (Bottom) SERS spectra (10 second exposure) of: (d) the Ag colloid in water (100 μl :100 μl), (e) 5 g/L BD-Difco-UF solution, and (f) 1 ppm aqueous

adenine solution. All spectra measured 90 minutes after mixing with Ag colloid, and the baseline removed for clarity.

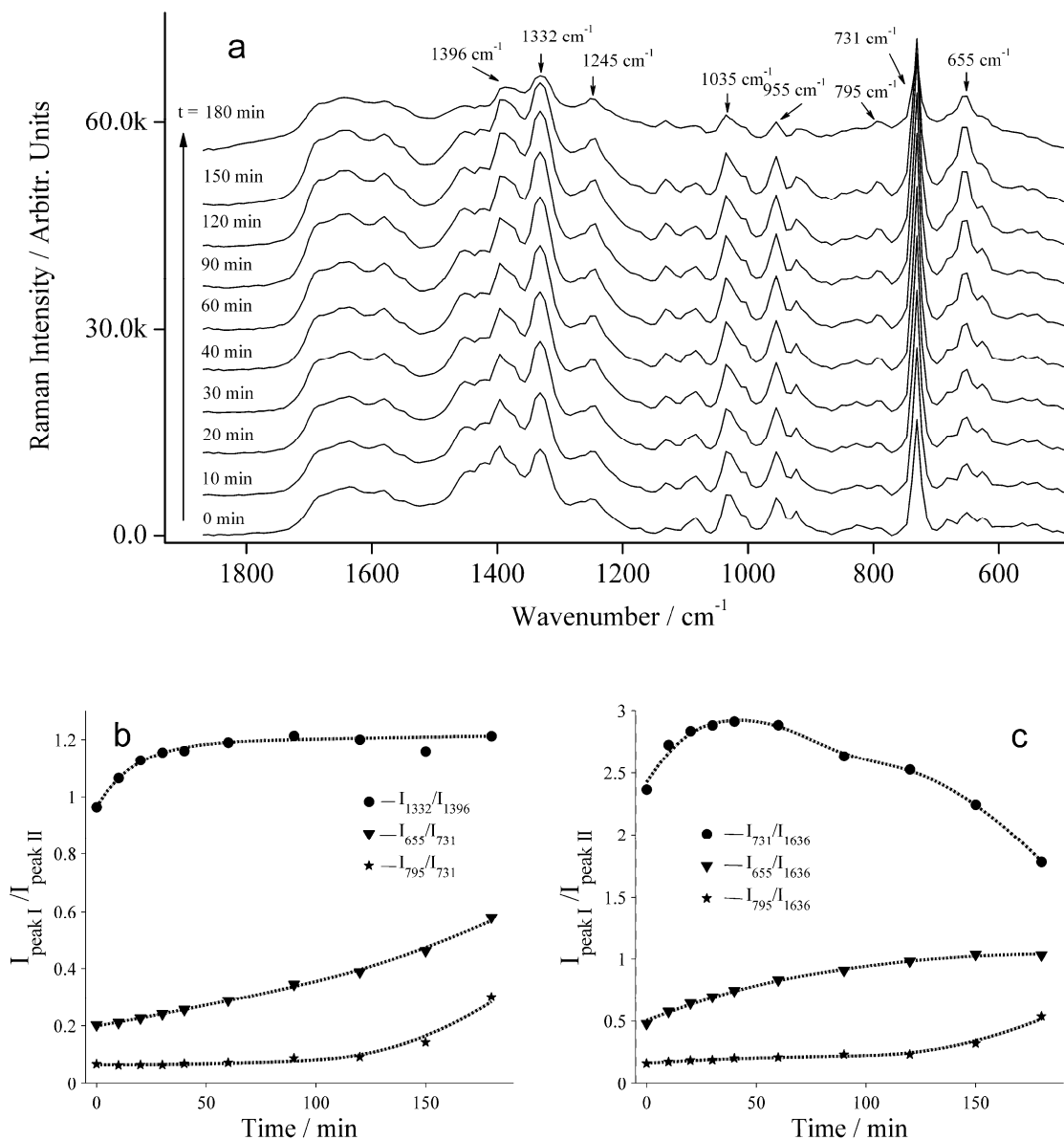


Figure 2: (a) Temporal changes of SERS spectra of a 5 g/L yeastolate (BD-Difco-UF) solution when mixed with Ag colloid over time from 0 to 180 min. Note that the baseline offsets were first removed from these SERS spectra (but no background spectrum subtraction applied). (b) SERS band ratio plot showing the relative intensity changes of SERS bands at 1332, 1396, 655, 731, and 795 cm⁻¹. (c) Band ratio referenced to the water bending vibration.

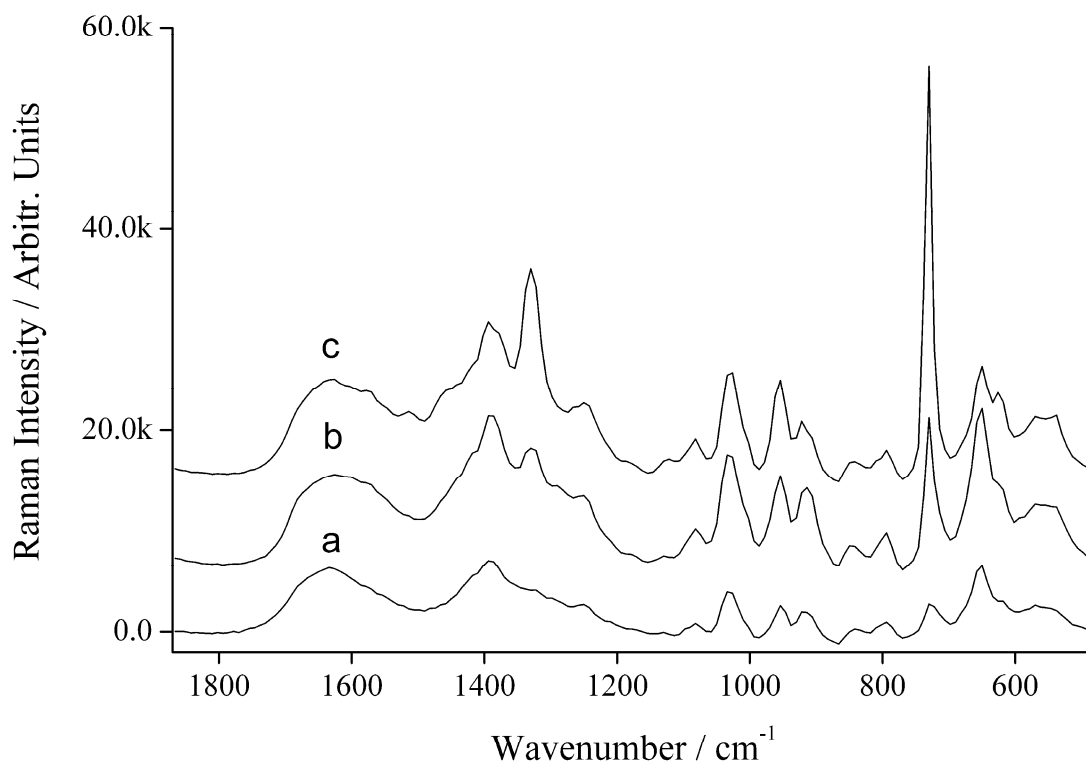


Figure 3: SERS spectra of: (a) Fluka Yeast extract, (b) BD-Bacto, and (c) BD-Difco UF, showing clear spectral differences.

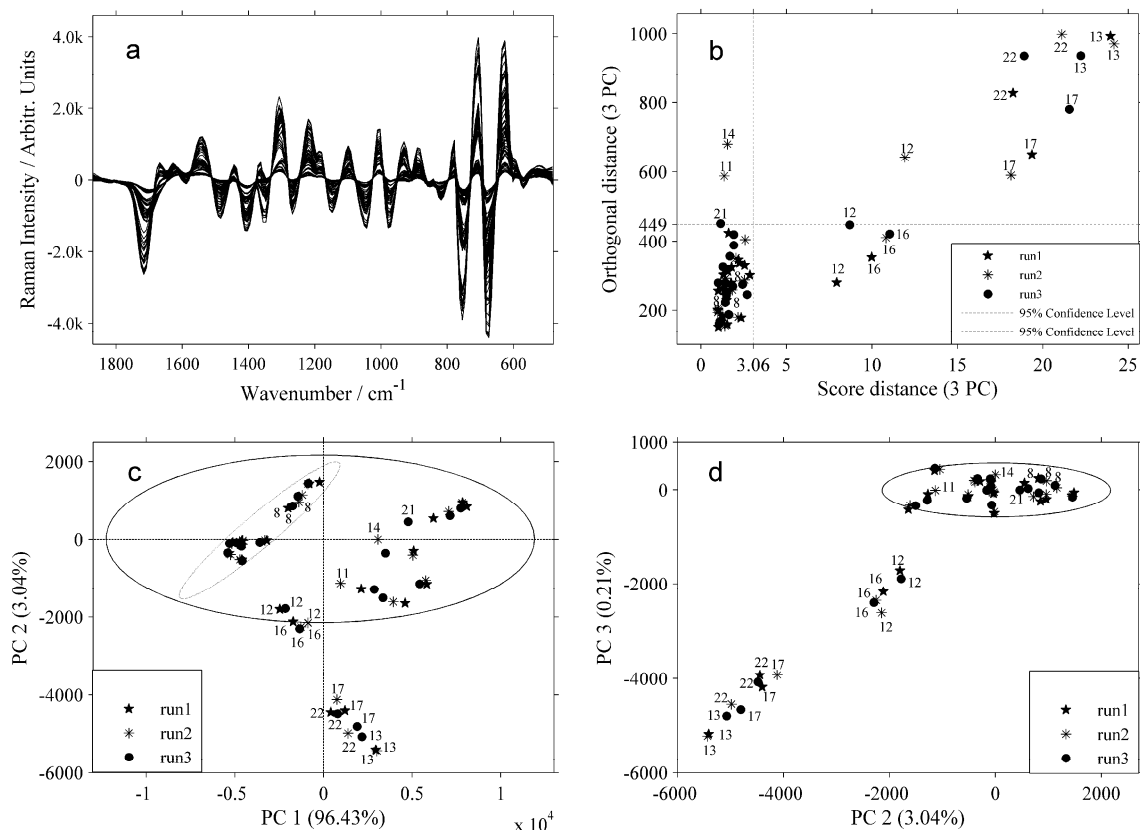


Figure 4: (a) Overlaid first-derivatized SERS spectra of 66 measurements from 22 yeastolate solutions, (b) ROBPCA outlier map plotting the outliers based on the $SD = 3.06$ and $OD = 449.42$ (straight lines) parameters, (c) and (d) PC scores plots showing the significant sample discrimination. The solid ellipse specifies a distribution of normal measurements at a 95% confidence level and the smaller dotted ellipse demarcates the 2006 yeastolate samples.

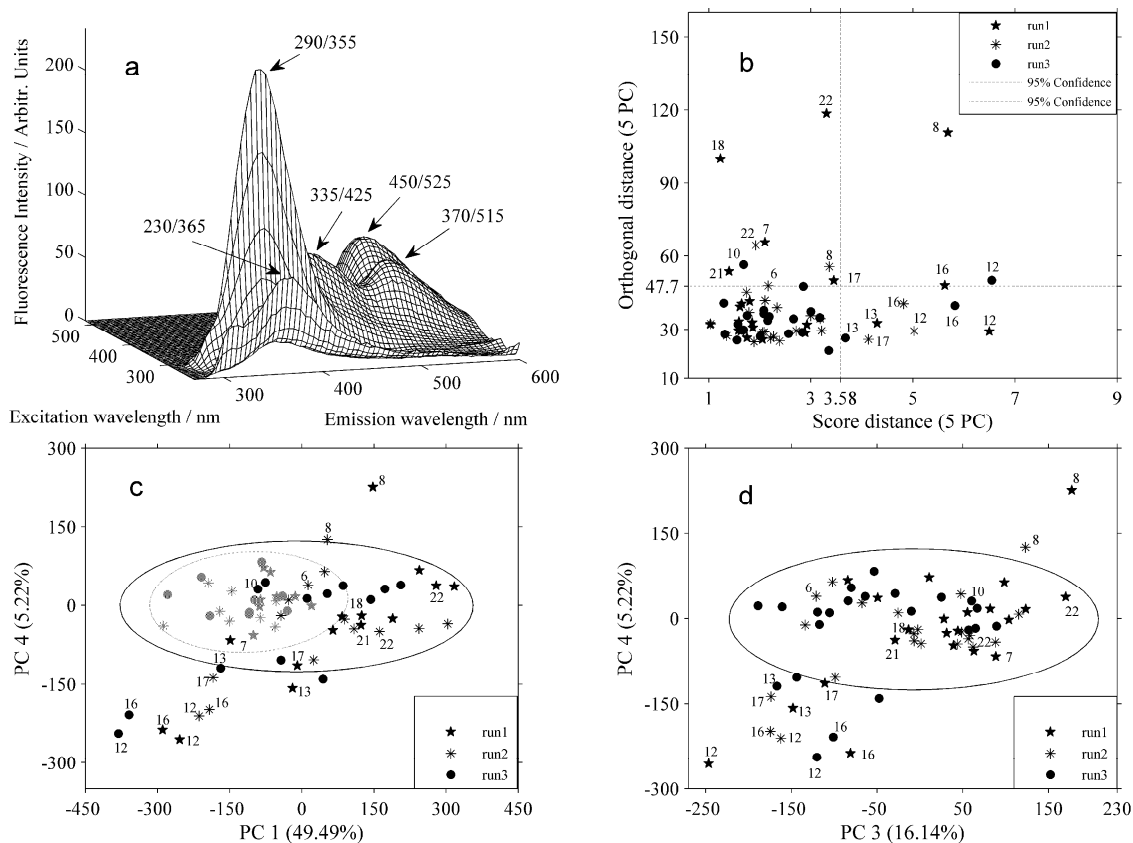


Figure 5: (a) Representative EEM landscape plot of a 5 g/L yeastolate solution after Rayleigh and Raman scattering removal. Local maxima are marked with excitation/emission values; (b) MROBPCA outlier diagnostic map (orthogonal distance *versus* score distance) for the triplicate measurement of 22 yeastolate solutions. The straight lines define two threshold values: SD = 3.58 and OD = 47.73 at a confidence level of 95% for the MROBPCA analysis with five PCs; (c) and (d) PC scores plots showing the significant sample discrimination (red markers indicate outliers). The solid ellipse signifies the 95% confidence level and the dotted ellipse locates the 2006 vintage yeastolate samples.

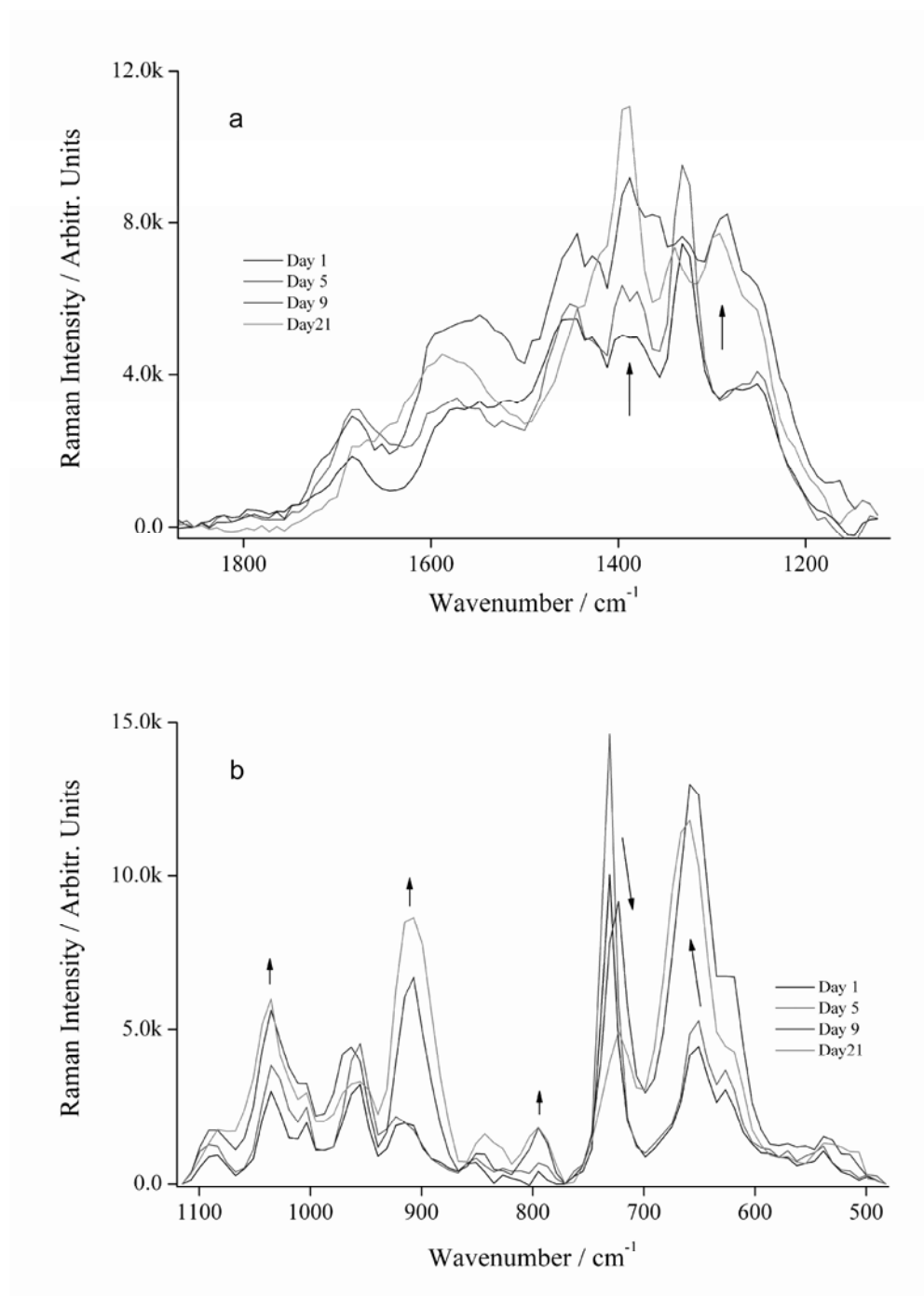


Figure 6: SERS spectra of unsterilized yeastolate (5 g/L, BD-Difco-UF) solution stored at 2~4°C recorded over 21 days. Plot shows significant changes in: (a) 1870~1115 cm⁻¹ and (b) 1115~480 cm⁻¹ regions.