



Enhancing analytical sensitivity in upstream bioprocess using time-gated Raman spectroscopy

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Received: 4 August 2025 / Accepted: 7 November 2025 / Published online: 20 December 2025
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Abstract

Upstream bioprocessing is a very complex system and requires rapid responses to process deviations. Mammalian cell culture processes are conventionally monitored for process-related and cell growth-related parameters, including pH, dissolved oxygen, viable cell density, cell viability, and key analyte concentrations that serve as primary indicators of the metabolic state of the cell culture. Raman spectroscopy (RS) has been increasingly applied as a viable inline process analytical technology (PAT) tool for cell culture monitoring and prediction of key analytes and attributes. The primary limitation to RS in these measurements is fluorescence (also referred to as sample-induced fluorescence), which interferes with the Raman signal and creates noise that makes detection of the signal from the analytes difficult. As a result, fluorescence interference decreases the signal to noise ratio (SNR) of the acquired spectra and increases the limit of detection (LOD) of analytical methods. Time-gated Raman spectroscopy (TGRS) takes advantage of the temporal delay between inelastic light scatter (Raman signal) and fluorescence emission to reduce interference from fluorescence. In this study, a pure component modeling approach and Net Analyte Signal (NAS) were applied to calculate the SNR and LOD of independent CHO cell culture samples. By reducing fluorescence interference, improving the SNR and LOD, TGRS enhanced the detectability of five key analytes in the cell culture samples, facilitating accurate monitoring and detection of analytes in a complex bioprocess system, thereby demonstrating its viability as a PAT tool for upstream bioprocess environment.

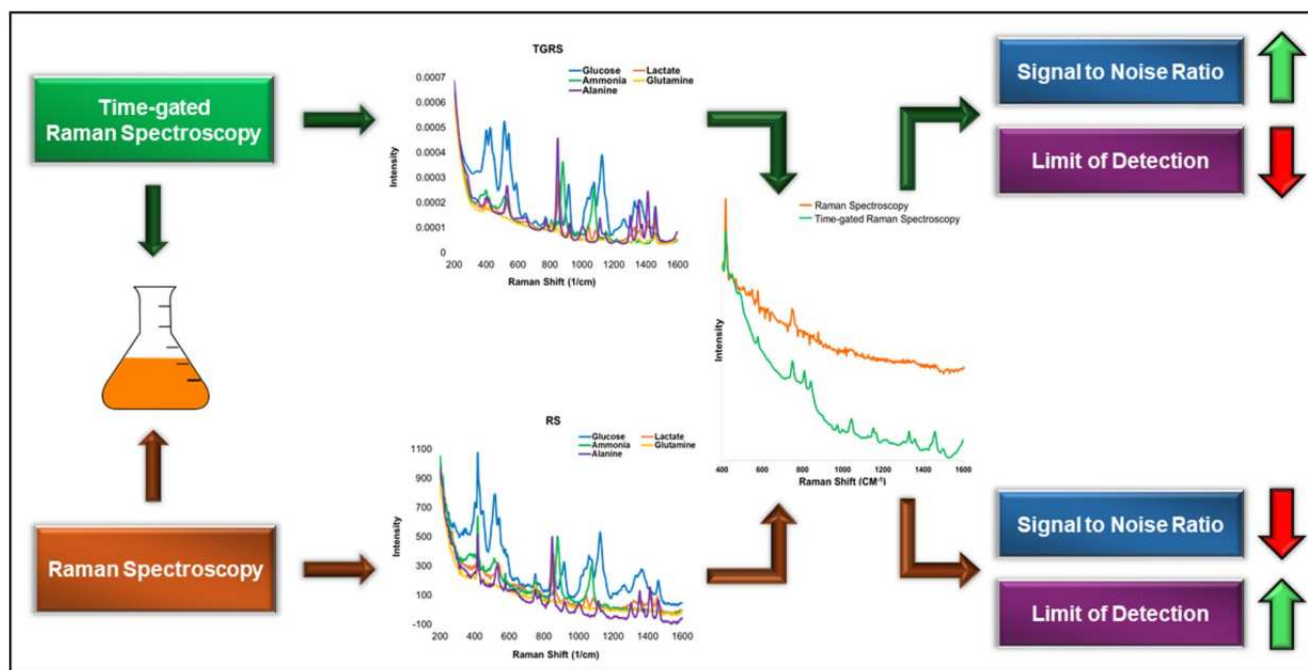
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Graphical abstract



Keywords Chinese hamster ovary cells · Analytes · Process analytical technology · Time-gated raman spectroscopy · Signal to noise ratio · Limit of detection

Introduction

Mammalian cells are widely used in the biopharmaceutical industry for the production of complex biotherapeutic proteins, and Chinese hamster ovary (CHO) cells are the predominant cell hosts used for this purpose. Owing to the complex nature of CHO cell metabolism, a high degree of control is required during upstream bioprocessing [1]. Currently, a limited set of process parameters, such as temperature, pH, agitation, and dissolved oxygen (DO), are routinely measured, monitored, and controlled in real-time. In contrast, many other parameters like analyte concentrations, ion concentrations, cell density, cell viability, product titers, and other quality attributes are typically assessed through offline sampling from bioreactors at intervals of one day or longer. While these parameters offer valuable insights into cell metabolism, process understanding, optimization, and control, however, the reliance on offline measurements presents a significant limitation [2]. The delayed availability of process data restricts real-time monitoring and control, thereby hindering timely decision-making and process adjustments [3].

In 2004, the Food and Drug Administration (FDA) introduced the guidance on process analytical technology (PAT) for process monitoring in the pharmaceutical industry. In

recent years, process analytical technology has been extensively developed for process monitoring and control within the biopharmaceutical industry [4]. Within the PAT framework, the real-time monitoring of critical process parameters and quality attributes is encouraged to ensure the consistent quality of the final product [5]. When integrated into the cell culture process, PAT tools have the potential to provide more timely and multidimensional process data, facilitating enhanced process understanding and control [6, 7].

Over the past decade, vibrational spectroscopy, particularly Raman spectroscopy, has garnered significant attention and widespread acceptance within the biopharmaceutical industry. It has emerged as one of the most promising PAT tools for the development of real-time monitoring techniques in mammalian cell culture [4]. Raman spectroscopy (RS) has emerged as a preferred spectroscopic technique in the biopharmaceutical industry due to its minimal interference from water, making it highly suitable for analyzing aqueous environments such as cell culture media [8]. Raman's applicability in real-time process monitoring has been well-documented, with numerous studies to simultaneously measure multiple process parameters including monitoring and predicting analyte concentration such as glucose, lactate, amino acids [6, 9–13], cell density [2, 13, 14], antibody titer [2, 11, 13], and glycosylation site occupancy [15]. Despite the

advantages of Raman as a PAT tool, sample-induced fluorescence emission is a notable source of interference by many constituents present in cell culture [16, 17]. Fluorescence interference causes broad spectral disturbances that obscure the weaker Raman signals, reducing the signal to noise ratio (SNR) of the spectra. The limit of detection (LOD) for RS is in the range of 0.20 to 0.46 g/L for glucose and lactate [18]. However, some analytes are present in the growth media at concentrations below this LOD, particularly toward the end of the culture. Reduction in the SNR from the fluorescence leaves RS with inadequate chemical sensitivity toward essential cell culture analytes to ensure a healthy culture, because as the cell culture time increases, the complexity of the cell culture increases. Multiple strategies have been proposed and implemented to address fluorescence interference and improve SNR for Raman spectroscopy [19–21]. In systems with predictable interference, fluorescence can be directly modeled and accounted for during spectral interpretation [22]. However, the dynamic and complex nature of the fluorescence interference in cell cultures limits the use of mathematical spectral treatments. The use of long-wavelength NIR light sources has been demonstrated to mitigate the fluorescence interference, but the overall Raman signal is weaker (as Raman scattering increases exponentially with the frequency of incident light to the fourth power) thereby capping the potential for improved SNR [23]. In order to maximize SNR and the capability of Raman spectroscopy to effectively monitor cell cultures, fluorescence interference must be mitigated at wavelengths achieving the most efficient Raman scattering. Time-gated Raman spectroscopy (TGRS) offers an instrumental solution to fluorescence interference without compromising Raman signal intensity.

Time-gated Raman Spectroscopy (TGRS) leverages the temporal distinction between Raman scattering and fluorescence emission to enhance signal detection. While Raman scattering occurs within picoseconds of excitation, fluorescence emission can persist for nanoseconds (Fig. 1). TGRS operates by synchronizing the laser and detector to capture the Raman signal while minimizing fluorescence interference by closing the detector during the fluorescence emission window. Optimizing the duration of the “time-gate” maximizes the SNR for a given excitation wavelength. Although the concept of time-gating has been known for decades, recent technological advancements have made its application in Raman spectroscopy feasible for in situ monitoring in both practical and economic terms. Initially theorized and experimentally validated in the 1970s with the advent of pulsed lasers and programmable detectors [16], TGRS demonstrated improved SNR in chemical systems containing fluorescent dyes. However, early implementations were hindered by high costs and device complexity, limiting its adoption. Despite these challenges, research efforts persisted, leading to gradual improvements in fluorescence suppression techniques through the early 2000s. A major breakthrough occurred in 2011 with the introduction of Complementary Metal-Oxide Semiconductor (CMOS) single-photon avalanche diode (SPAD) arrays, which significantly enhanced Raman detector sensitivity. These advancements reduced both the size and cost of TGRS systems, enabling their practical application in biomanufacturing, as demonstrated by TimeGate Instruments [16].

Only a limited number of studies have investigated the application of TGRS for bioprocess monitoring [23–25]. The objective of the study is to compare the analytical

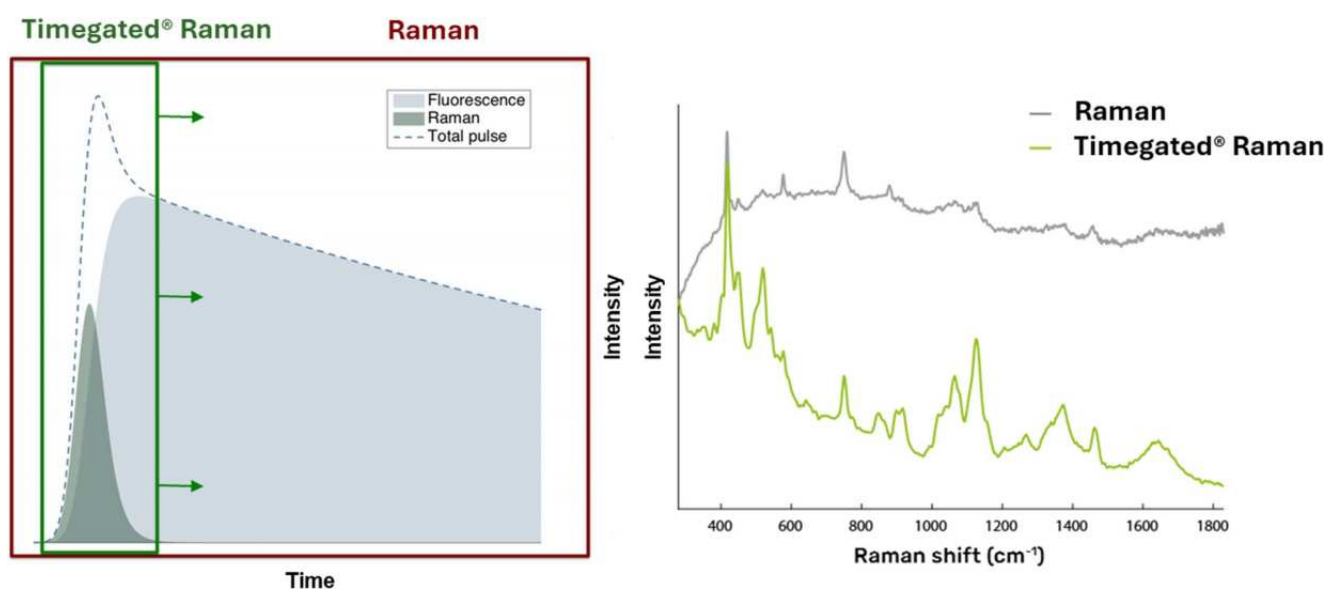


Fig. 1 Working mechanism of Time-gated Raman Spectroscopy [16]

Table 1 Six CHO cell culture samples with their cell density and cell viability values

Low Cell Density (LCD)			High Cell Density (HCD)		
Sample	Density	Cell viability (%)	Sample	Density	Cell viability (%)
LCD-N-1	4.69×10^5	96	HCD-N-1	5.50×10^6	95
LCD-N-2	5.86×10^5	91	HCD-N-2	6.32×10^6	95
LCD-N-3	1.11×10^6	92	HCD-N-3	6.34×10^6	97

sensitivity of RS and TGRS in CHO cell culture by detecting key analytes present in the CHO cell culture. In this study, the analytical sensitivity of both Raman systems was compared and evaluated by calculating the SNR and LOD from the independent CHO cell culture samples in the shake flask. Raman spectra were collected from six independent shaker flasks using both Raman instruments. Pure component modeling approach was employed to determine and calculate the net analyte signal (NAS) for five key cell culture analytes: glucose, lactate, ammonia, glutamine, and alanine. The NAS values were subsequently utilized to calculate the SNR and LOD for these analytes in both RS and TGRS. The findings of this study demonstrated that TGRS effectively reduced fluorescence interference, leading to improved SNR and LOD for the five analytes of CHO cell culture samples. These results demonstrate the improved capability of TGRS in identifying and detecting analytes in mammalian cell cultures, while highlighting its future potential to enhance quantification and prediction of these analytes and establish its role as a viable PAT tool for real-time monitoring of upstream bioprocesses.

Materials and methods

Cell culture/cultivation: low and high density

The NISTCHO cell line, a clonal CHO-K1 cell line producing cNISTmAb which is a fully humanized IgG1 κ antibody, is donated by the National Institute of Standards and Technology (NIST). The cells were cultured in a serum-free media (EX-CELL Advanced Fed Batch Medium, Sigma Aldrich) supplemented with 6mM L-Glutamine (Sigma Aldrich). Cells were inoculated at 3.0×10^5 cells/mL and cultured in 125 mL Erlenmeyer flask with a working volume of 30 mL, fed with 10% of feed media (EX-CELL Advanced Fed Batch Medium, Sigma Aldrich) supplemented with 6mM L-Glutamine (Sigma Aldrich) at every other day until the cell culture reached to our desired cell density. Cultures were carried out at 37 °C, 99% relative humidity, 5% CO₂ with an orbital shaker at 160 rpm. Total 6 cultures were performed in a fed-batch to maintain the cell density and cell viability (>90%) at optimum levels. Two

Table 2 Concentration of all analytes in the two-level of cell densities

Sample ID	Glucose (g/L)	Lactate (g/L)	Ammonia (mM)	Glutamine (mM)	Alanine (mM)
LCD-1	5.11	0.23	1.63	5.28	1.44
LCD-2	4.6	0.76	2.64	4.38	2.1
LCD-3	2.75	2.28	4.38	2.89	2.77
HCD-1	2.45	0.89	6.98	0.095	6.72
HCD-2	1.39	0.69	5.74	0.049	6.41
HCD-3	2.12	0.81	6.49	0.22	6.31

levels of cell densities were used; low cell density (LCD: 1×10^5 to 5×10^6) and high cell density (HCD: $> 5 \times 10^6$), and the analytes of interest were Glucose, Lactate, Ammonia, Glutamine, and Alanine (Table 1).

References analysis

Cell culture samples were taken daily for cell density and cell viability measurement. Cell density and viability were monitored using a Countess II automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA), using the Trypan blue dye exclusion method. After reaching the target cell viability and cell density for low and high density cell culture, Raman spectra were collected by RS and TGRS. After collection of both spectra, cell cultures were centrifuged at 220 rcf for 5 min with Centrifuge 5810 R (Eppendorf, Hamburg, Germany), then, supernatants were collected and stored at -80 °C for analytes concentration analysis. Bioprofile Flex 2 (Nova Biomedical, Waltham, MA, USA) was used to analyze glucose, lactate, and ammonia and ACQUITY UPLC H-Class PLUS System (Waters, Milford, MA, USA) was used to analyze the amino acids; glutamine and alanine. There is an inverse correlation between analytes of energy source (glucose and glutamine) and by products (lactate, ammonia and alanine) (Table 2).

Raman spectroscopy and spectra collection

Raman Spectroscopy (RS)

Raman spectra for all 6 samples were collected using Raman RXN2 system (Kaiser Optical Systems Inc., Ann Arbor, MI, USA), which contained a 785-nm laser source and a charge-coupled device (CCD) at -40 °C. The detector was connected to a MR probe, which contains a fiber optic excitation cable and fiber optic data collection cable (Kaiser Optical System Inc., Ann Arbor, MI, USA). Data was collected by the MR probe connected to a Raman BIO-Optics stainless steel immersion probe (Kaiser Optical Systems Inc., Ann Arbor, MI, USA) in a small black plastic box protected from light during spectra acquisition. All Raman spectra were acquired at 60 s of integration time and 1.5 s

of overhead time with 5 scans which resulted in a total acquisition or exposure time of 301.5 s to generate a full spectrum for a sample (Fig. 2A), and the saturation level for the detector over the 200 to 1900 cm^{-1} was around 80%. Moreover, Dark spectrum subtraction, cosmic ray removal, and intensity corrections were applied from the instrument settings to each spectrum. Before starting a new experiment, the laser wavelength and intensity were calibrated using the Calibration Accessory (HCA) from Kaiser Optical Systems, Inc. (Ann Arbor, MI, USA). The Raman spectrometer was operated by iC Raman™ software (Version: 4.1.927.0, Mettler Toledo Autochem, Columbia, MD, USA). The Raman spectra were collected over a wavelength range of 200 to 1900 cm^{-1} for each sample, however, the working region was 200 to 1600 cm^{-1} .

Time-Gated Raman Spectroscopy (TGRS)

Time-gated Raman spectra of all 6 samples were collected using the PicoRaman M3 system (Timegate Instruments, Oulu Finland), using a pulsed laser of $\lambda_{\text{exc}} = 532 \text{ nm}$ excitation. The TGRS system consists of a temperature stabilized complementary metal oxide single-photon avalanche diode (CMOS SPAD) array detector (single photon counting) with spectral resolution of 6–7 cm^{-1} . The laser emits pulses with a duration of less than 100 picoseconds and operates at a repetition rate of 100 kHz. Time-gated spectral data were collected by incrementally shifting the detection gate in 50 picosecond intervals using an electronic delay generator. Data was collected by the ProbePro Mini connected to the TGRS in a small black plastic box protected from light

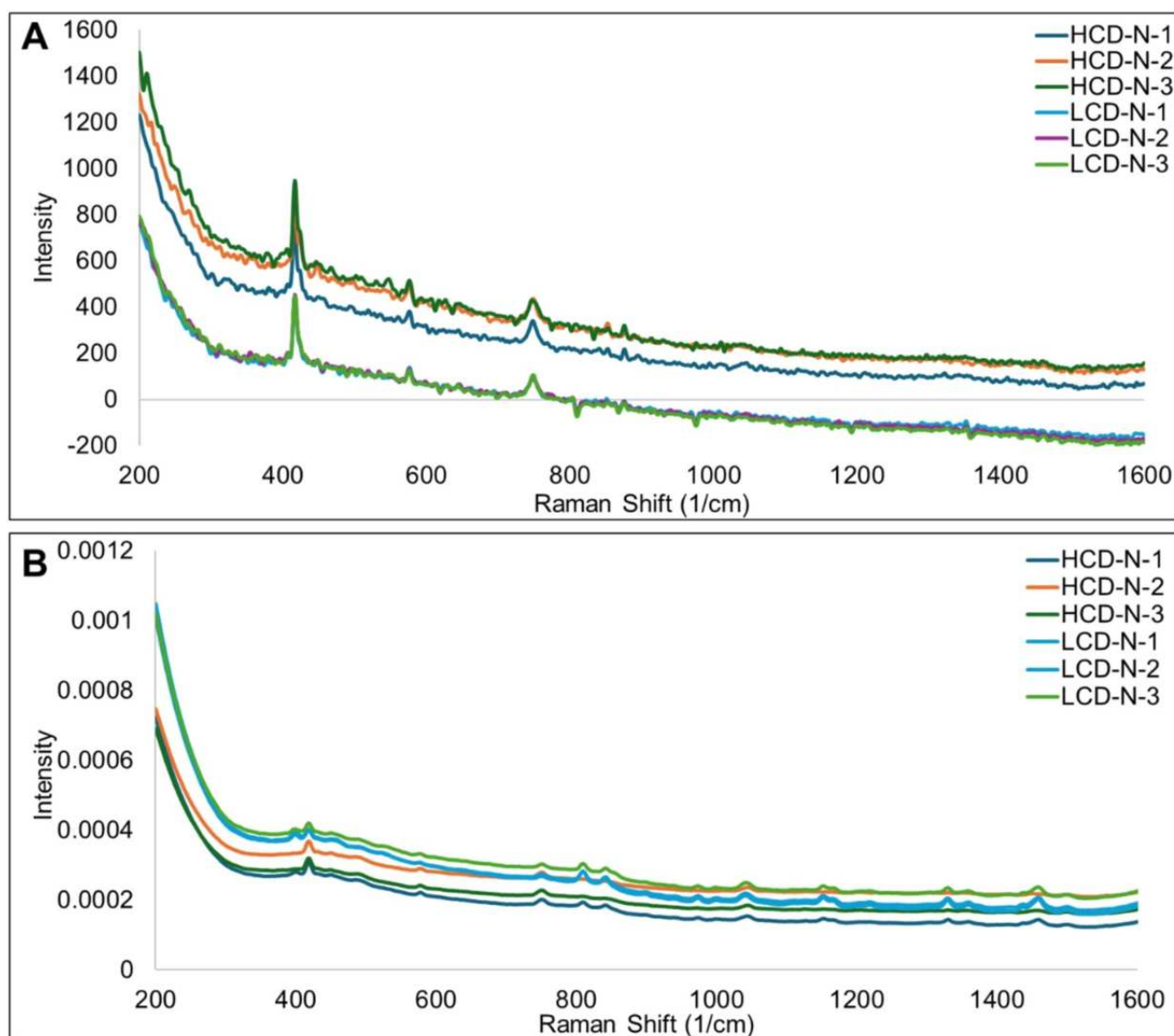


Fig. 2 Raw spectra of six samples (HCD and LCD) from, **A** RS, and **B** TGRS

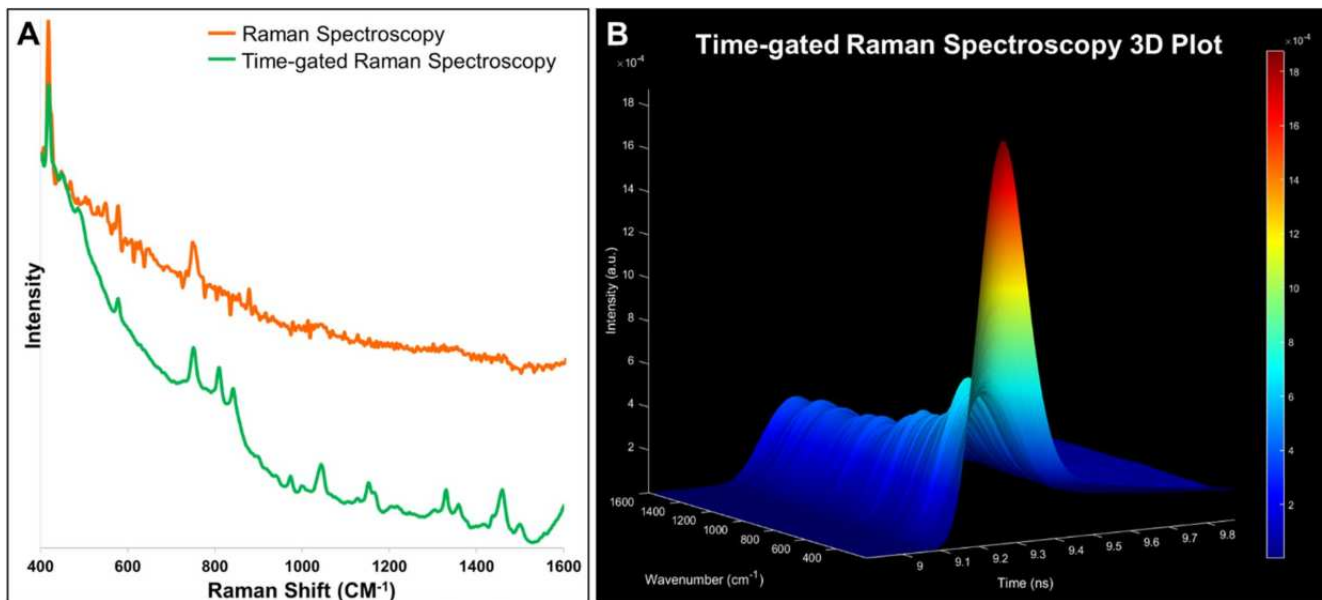


Fig. 3 **A** Average raw spectra of three HCD samples from RS (orange) and TGRS (green), showing the fluorescence suppression by TGRS in comparison with RS in high cell density (Average HCD: 6.05×10^6 cells/m). Raman shift is reduced for better visualization and

understanding, and **B** 3-dimension (intensity, wavenumber and time) plot of TGRS showing time slices of spectra in 100 picoseconds or 0.1 nanoseconds

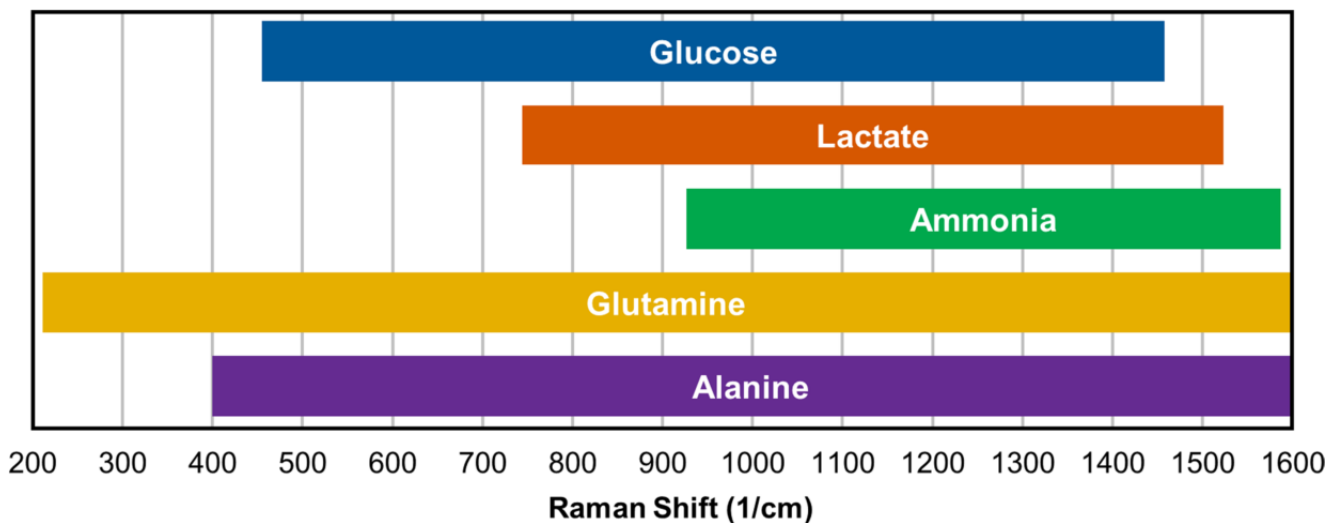


Fig. 4 Fingerprint regions of all five analytes in Raman shift (1/cm)

during spectra acquisition. All Raman spectra were acquired with an exposure time of 266 s at approximately 80% saturation level, using a single scan, 10 HSG points, and 400 sub-acquisitions within a virtual time-gate ranging from 9.00 to 9.30 nanoseconds (equivalent to 300 picoseconds). The resulting spectra represent averages of all measured data, with background signals subtracted mathematically following manual baseline fitting. The virtual time-gate ensures the collection of Raman spectra while excluding the fluorescence interference (Fig. 3A), and the spectra were collected in 3-dimensional space with intensity, Raman shift (cm^{-1})

and time (picoseconds) (Fig. 3B). TGRS spectra were collected over a wavelength range of 125 to 2500 cm^{-1} for each sample, however, the working region was 200 to 1600 cm^{-1} .

The working regions for both the Raman instrument have been selected based on the fingerprint region of all analytes (Fig. 4), and all the analytes have their most of the fingerprint regions within the working range of 200 to 1600 cm^{-1} [6, 17, 26–31].

Multivariate performance metrics

The two types of Raman spectra were evaluated by calculating various mathematical equations. These include: Net analyte signal (NAS), NAS_{noise} , Signal to noise ratio (SNR), Sensitivity (SEN), Limit of detection (LOD) and Principal component analysis (PCA).

Net analyte signal (NAS): pure component

The Net Analyte Signal (NAS) was calculated as suggested by Avraham Lorber [32]. Each chemical component within a mixture spectrum possesses a distinct net analyte signal (NAS) that remains orthogonal to the signals of other constituents. The NAS determines the detectability of a specific chemical component in relation to the presence of other components across all wavelengths [33]. The pure NAS for a given component K , ($NAS_{pure,i}$) with a pure spectrum M_{pure} can be derived from the pure component spectra matrix (K) using Eq. (1) [32]. Here, K_i represents the matrix of pure component spectra excluding M_{pure} for component K , while I denotes the identity matrix. Theoretically, if the only interference in prediction arises from the pure components, the NAS for components K in the mixture spectrum M_{mix} of sample I ($NAS_{mix,i}$), when calculated using Eq. (5), should correspond to ($NAS_{pure,i}$). Here, K_{-i} represents the matrix of pure component spectra excluding M_{pure} for component i , while I denotes the identity matrix. As a result, ($NAS_{pure,i}$) serves as the central reference against which ($NAS_{mix,i}$), is assessed. Overall, NAS was calculated to isolate the unique spectral contribution of a specific chemical component or analytes within a mixture, ensuring it remains orthogonal to signals from other analytes. This allowed accurate detection of that component or analytes even in the presence of overlapping signals from other analytes.

$$NAS_{pure,i} = (I - K'_{-i} * (K_i * K'_{-i})^{-1} * K_i) * M_{pure}' \quad (1)$$

$$NAS_{mix,i} = (I - K'_{-i} * (K_{-i} * K'_{-i})^{-1} * K_{-i}) * M_{mix}' \quad (2)$$

Net analyte signal noise (NAS_{noise})

Net analyte signal noise NAS_{noise} represents the portion of the spectrum that is not accounted for by the pure component spectra. The $NAS_{i,noise}$ quantifies the unexplained spectral contribution, including noise and unmodeled variations. The theoretical quantifies the unexplained spectral contribution, including noise and unmodeled variations. The theoretical NAS_{noise} for a given component i can

be determined using the pure component spectra matrix K as described in Eq. (3). Here, $K'K$ represents the projection of the pure component spectra onto the mixture spectrum, while I denotes the identity matrix. The M'_{media} represents the spectral data corresponding to the medium or background. If only the pure components contribute to the mixture spectrum, NAS_{noise} should primarily capture residual noise. Consequently, NAS_{noise} serves as an essential parameter in evaluating signal detectability, assessing the signal to noise ratio (SNR), and determining the robustness of spectroscopic models. Here, NAS_{noise} was calculated to quantify the portion of the spectrum not explained by pure component signals, helping assess signal detectability and improving the SNR .

$$NAS_{noise} = (I - K'K) * M'_{media} \quad (3)$$

Signal to noise ratio (SNR)

Estimating the signal to noise ratio (SNR) in spectral samples can be achieved by defining a Net Analyte Signal Noise NAS_{noise} vector [32, 34]. This method involves computing NAS_{noise} by orthogonalizing the mixture spectrum against the complete pure component matrix K , as outlined in Eq. (3). This approach operates under the assumption that all chemical components present in the mixture are comprehensively represented within the pure component matrix. The derived NAS_{noise} vector summarize the noise-related contributions within the mixture spectrum that are not accounted for by the pure component spectra. As a result, the standard deviation of NAS_{noise} across all wavelengths $\sigma(NAS_{noise})$ serve as an estimation for noise in Eq. 4.

$$SNR = \frac{\|NAS_{mix}\|}{\sigma(NAS_{noise})} \quad (4)$$

Sensitivity

Sensitivity SEN can then be determined using the following equation:

$$SEN_i = \frac{NAS_i}{y_i} \quad (5)$$

where y_i represents the measured concentration of the i^{th} sample [18]. In this study, sensitivity is reported as the average of the univariate sensitivity values across all analyzed samples. Sensitivity is particularly relevant for models developed from two spectroscopic techniques that operate

based on the same fundamental principles, as it inherently accounts for the signal unit of the instrument. In this study, SNR was calculated to assess the clarity of the analyte signal relative to background or chemical interference by dividing the NAS_{mix} by the standard deviation of NAS_{noise} across all wavelengths.

Limit of detection (LOD)

The Limit of Detection (LOD) represents the minimum concentration of an analyte that can be reliably detected using a specific analytical method within a given sample matrix [18]. It establishes the threshold at which the signal becomes distinguishable from noise but may not necessarily be quantified with high accuracy. In this study, LOD can be calculated using the following equation:

$$LOD = 3.3 NAS_{noise \text{ from media}} \frac{1}{\|SEN\|} \quad (6)$$

In this formula, $NAS_{noise \text{ from media}}$ represents the net analyte signal corresponding to the noise in the medium, while $\|SEN\|$ denotes the norm of the sensitivity vector, reflecting the instrument's response to variations in analyte concentration. The factor 3.3 is a standard statistical coefficient used to ensure that the detected signal significantly exceeds the noise level. This relationship suggests that an increase in NAS_{noise} results in a higher LOD, indicating a reduced ability to detect low concentrations, whereas greater sensitivity enhances detectability by lowering the LOD. In this study, LOD was calculated to determine the lowest detectable analyte concentration by dividing 3.3 times the NAS_{noise} from the medium by the norm of the sensitivity vector.

Principal component analysis (PCA)

The high and low cell density samples were analyzed using the MATLAB (R2024a, The Mathworks Inc., Natick, MA, USA) in combination with PLS Toolbox (Version 8.8.1) (Rigenvector Research Inc., Manson, Wa). The selection of principal components (PCs) was based on the proportion of variance they explained and their ability to differentiate between high and low cell density samples. Due to the high degree of collinearity among the original variables, a small number of PCs were sufficient to capture the underlying data structure. In the reduced-dimensional score space, samples with similar spectral profiles clustered together, enabling clear visualization of patterns and identification of outliers or distinct subgroups within the cell culture dataset [35]. The scores on the PCs vs. samples plots were used to interpret the high- and low-density cell culture samples.

Overall, these multivariate performance metrics (NAS , NAS_{noise} , SNR , SEN , LOD and PCA) was calculated to compare the analytical performance of RS and TGRS.

Raman spectra collection and preprocessing

Collection of pure component spectra and cell culture

Pure component spectra were collected for all key analytes by dissolving the solid power of analytes into water. The solubility limit of all analytes was calculated for mL10 mL of water and dissolved in 25 °C. After dissolving all solute into the solvent, spectra were collected with both TGRS and RS (Fig. 5).

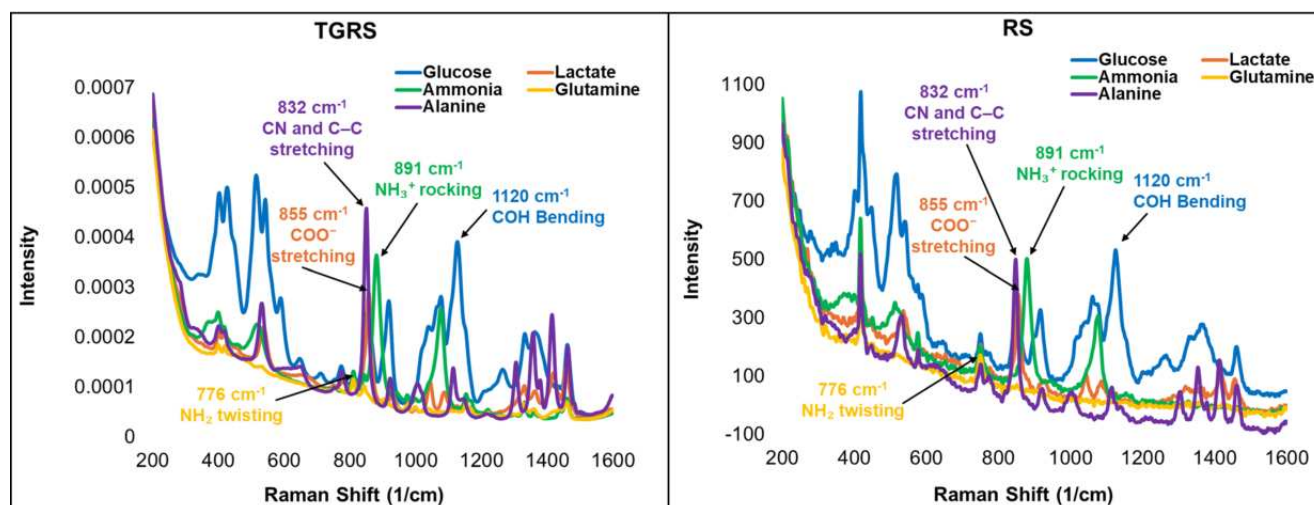


Fig. 5 These plots represent the pure component spectra, **A** is the non-processed pure component spectra of analytes from TGRS, and **B** is the non-processed pure component spectra of analytes from RS

Spectral preprocessing

Both RS and TGRS spectral data were exported and processed using the MATLAB (R2024a, The Mathworks Inc., Natick, MA, USA) in combination with PLS Toolbox (Version 8.8.1) (Rigenvector Research Inc., Manson, Wa). The spectral data were trimmed to retain only the relevant wavenumber range of 200–1600 cm^{-1} . For spectral filtering, the Savitzky-Golay (SavGol) smoothing algorithm was applied with a smoothing window size of 31 cm^{-1} or points, fitted to a second order polynomial order and first derivative. The same spectral preprocessing was applied to both of the Raman instrument to avoid the bias from preprocessing contribution. This preprocessing step was implemented to enhance key spectral regions of interest and improve the SNR, thereby optimizing data quality for subsequent analysis.

Results

Net analyte signal (NAS) of key analytes

The calculated Net Analyte Signal (NAS) spectra of five key analytes illustrated in (Fig. 6). The NAS method effectively isolates the spectral contribution of each analyte from interfering signals within the complex bioprocess matrix, allowing for improved specificity in Raman-based analyte detection. Figure 6 represents the distinct NAS-derived spectral peaks of each analyte, highlighting their signature Raman shifts. The TGRS-derived NAS spectra exhibit sharper, more well-defined and less noisy peaks compared to the RS spectra, suggesting improved spectral resolution

and reduced background or fluorescence interference in the TGRS method.

Signature Raman peaks were observed for each analyte, corresponding to their molecular vibrational modes, and calculated by zero crossing of the derivative method. For glucose, notable strong and well-defined peaks observed with TGRS are at 424, 540, 1060, and 1120 cm^{-1} , characterized by prominent intensities and sharpness (Table 3). These peaks are consistent with previously reported glucose vibrational modes, particularly those associated with C-H, C-O stretching and COH bending, as well as ring deformation [26, 27]. In contrast, RS detected a similar but slightly shifted set of peaks, including 324, 397, and 408 cm^{-1} are comparatively broader and weaker indicating potential spectral broadening and baseline distortions.

The average NAS spectra of glucose from 3 samples of HCD and LCD highlighted the advantages of TGRS over RS for glucose detection in CHO cell cultures. The average NAS spectra of glucose (Fig. 7) from 3 HCD and LCD samples highlighted the advantages of TGRS compared to RS for detecting glucose in CHO cell cultures. In TGRS (Fig. 7A), both high and low cell density samples exhibited cleaner baselines and more consistent spectral features. By contrast, RS (Fig. 7A) raw spectra were dominated by fluorescence background, especially at high cell density, which obscures analyte-specific signals in the NAS projection of those samples.

For lactate, the NAS from TGRS identified strong and distinct signature peaks at 855, 930, 1042, and 1457 cm^{-1} , which correspond to C-H bending and COO^- stretching vibrations. These vibrational assignments are in agreement with lactate spectral studies reported in the literature [26, 27, 36]. In contrast, RS displayed a broader set of peaks,

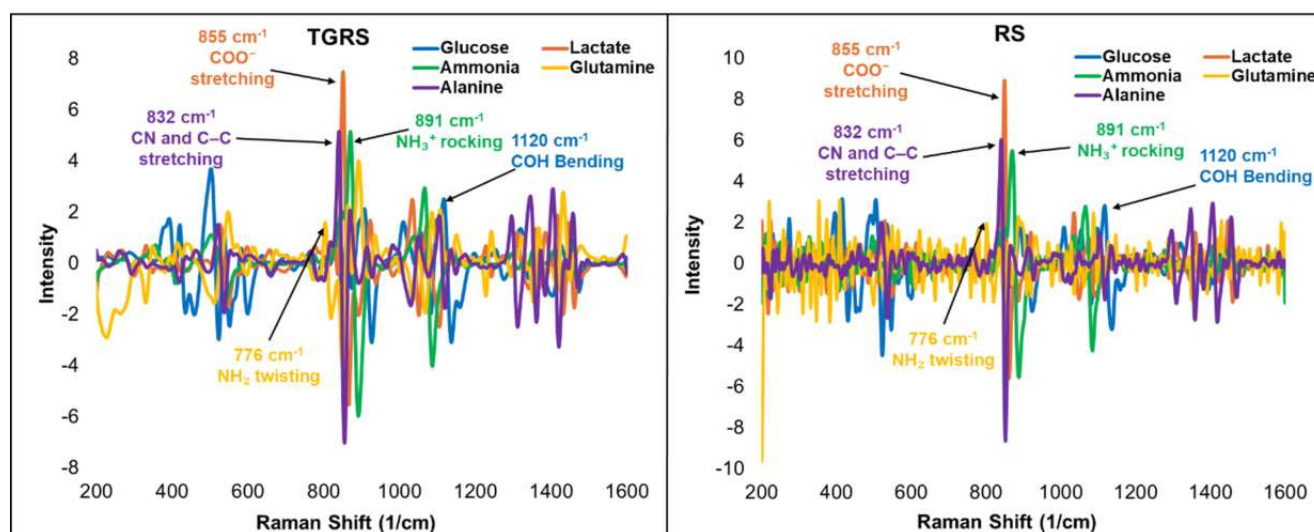


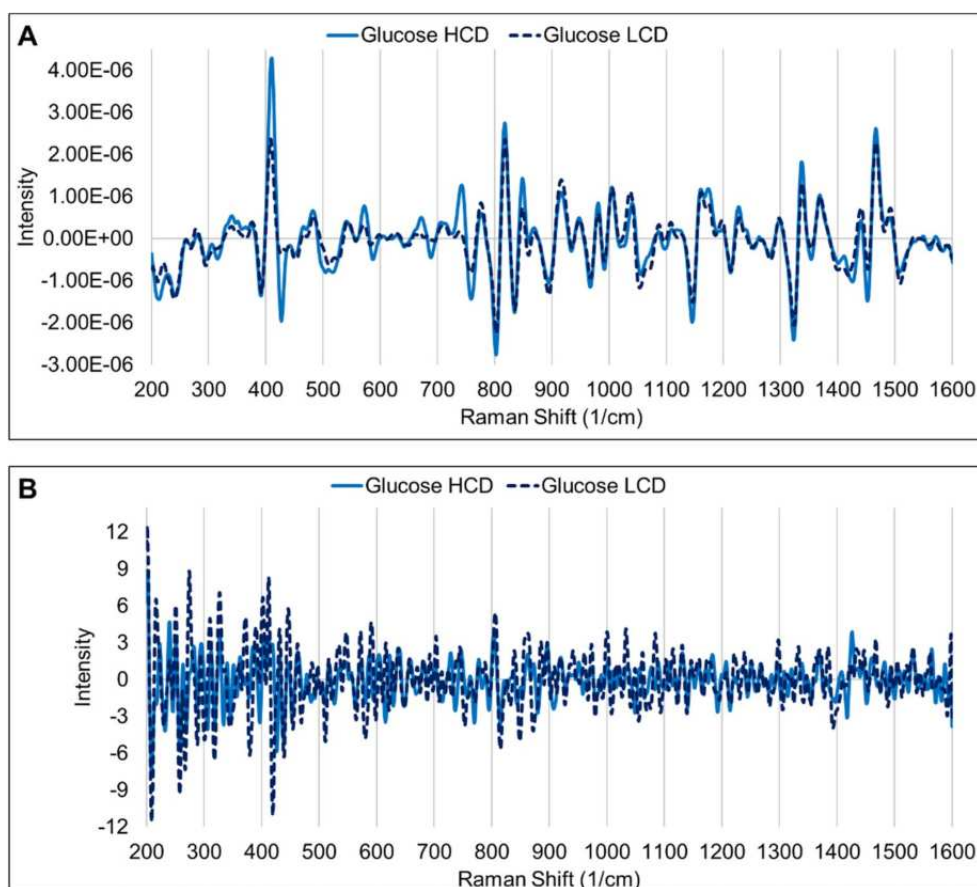
Fig. 6 These plots represent the pure component spectra Net analyte signal (NAS) of five key analytes, **A** is the preprocessed pure component NAS with signature peaks of analytes from TGRS, and **B** is the preprocessed pure component NAS with signature peaks of analytes from RS

Table 3 Signature peaks of key analytes from Net Analyte Signal (NAS)

Analytes	Signature peaks from NAS (Raman Shift cm^{-1})		
	Fingerprint Region	TGRS	RS
Glucose	C ₂ -C ₁ -O ₁ bending α (540 cm^{-1})	540	540
	C ₂ -C ₁ -O ₁ bending β (518 cm^{-1})	518	518
	CCO bending (424 cm^{-1})	424	424
	CCC bending (450 cm^{-1})		
	C-O stretching α (1035 cm^{-1})	1035	1035
	C-O stretching β (1060 cm^{-1})	1060	1060
	COH bending (1120 cm^{-1})	1120	1120
	CH ₂ - bending (1455 cm^{-1})	1455	
	CH ₂ - bending wagging α (1327 cm^{-1})	1327	
	CH ₂ - bending β (1360 cm^{-1})	1360	
	C-C stretching α (882 cm^{-1})		
	C-C stretching β (893 cm^{-1})	893	
	C - H _{β} bending (910 cm^{-1})		324*, 397*, 408*,
	[26, 30, 37]		
Lactate	Carboxyl acid (1420 cm^{-1})	1420	1420
	COO ⁻ stretching (855 cm^{-1})	855	855
	CH ₃ deformation (1457 cm^{-1})	1457	1457
	CH ₃ rocking (930 cm^{-1})	930	930
	C-CH ₃ and stretching (1042 cm^{-1})	1042	1042
	[26, 30, 37]		525*, 862*, 1034*, 1460*
Ammonia	NH ₃ ⁺ bending (1496 cm^{-1})		
	NH ₃ ⁺ rocking (891 cm^{-1} and 1133 cm^{-1})	891, 1133	891, 1133
	NH ₂ rocking (1096 cm^{-1})	1096	1096
	NH ₂ twisting (776 cm^{-1})		
	NH ₂ scissoring (1586 cm^{-1})		
	[26, 28, 38]		328*, 360*, 1067*,
Glutamine	Carboxyl acid (1420 cm^{-1})	1420	
	COO ⁻ stretching (855 cm^{-1})	855	855
	C-C stretching (842 cm^{-1})		
	NH ₃ ⁺ bending (1496 cm^{-1})	1496	1496
	NH ₃ ⁺ rocking (1133 cm^{-1})	1133	
	NH ₂ rocking (1096 cm^{-1})	1096	1096
	NH ₂ twisting (776 cm^{-1})	776	776
	NH ₂ scissoring (1586 cm^{-1})	1586	
	CH ₂ - bending (1455 cm^{-1})	1455	
	CH ₂ wagging α (1327 cm^{-1})		
	CH ₂ - bending β (1360 cm^{-1})	1360	
	CH ₂ - bending twisting (1258 cm^{-1})	1258	1258
	[17, 29, 39]		258*, 310*, 330*, 346*, 353*, 382*, 664*, 759*,
Alanine	CCN bending (skeletal) (333 cm^{-1})	333	
	CN and C-C stretching (832 cm^{-1})	832	832
	CH ₃ rocking (1114 cm^{-1})	1114	1114
	NH bending (1346 cm^{-1})	1246	1246
	CN stretching (760 cm^{-1})		
	CNH bending (995 cm^{-1})	995	
	[17, 29]	1406*, 1421*, 1458*	285*, 442*, 521*, 852*, 1296*, 1406*, 1421*, 1456*, 1471*

*Additional peaks were present besides the signature peaks

Fig. 7 **A** Average NAS of glucose from HCD and LCD in TGRS, and **B** Average NAS of glucose from HCD and LCD in RS



including additional features at 525, 862, and 1460 cm^{-1} (Table 3), but these exhibited broader profiles, suggesting less spectral purity and potential overlap with interfering signals.

Ammonia exhibited clear and strong signature peaks at 891, 1096, and 1133 cm^{-1} (Table 3) in TGRS. RS, while detecting a broader set of signals, displayed weaker and less defined peaks, particularly at lower Raman shifts such as 328 and 360 cm^{-1} . Notably, the peaks at 891 and 1133 cm^{-1} in TGRS were more defined, which are linked to N-H bending vibrations [26]. Previous Raman studies have shown that ammonia's RS spectral features are often susceptible to matrix effects, leading to signal distortion in Raman setups [18, 26].

For glutamine, the strong and clear NAS peaks in TGRS were observed at 855, 1360, 1496 and 1586 cm^{-1} , which correspond COO^- stretching, NH_3^+ bending and CH_2 - bending and β wagging [26, 27]. In RS, glutamine showed a more complex spectral pattern, with additional peaks at 258, 310, 330, 346, 353, and 382 cm^{-1} (Table 3), suggesting potential signal overlap with other cellular components. A similar pattern was observed in the CHO cell culture NAS spectra of glutamine. TGRS produced clear, more consistent and less noisy spectra for glutamine in cell culture NAS regardless of cell density level of the samples. On the other hand,

raw data of RS continued to fluoresce more in HCD samples than LCD and engulfing the Raman signals of glutamine in the NAS projection of the samples (Fig. 8).

Similarly, alanine displayed distinct and clear NAS spectral profile, with TGRS highlighting peaks at 832, 995, and 1350–1460 cm^{-1} (Table 3), corresponding to CCN bending and CH_3 rocking [26]. RS presented additional, broader, and weaker peaks particularly at 285, 442, and 574 cm^{-1} , indicating potential interference and reduced spectral resolution.

Signal to noise ratio (SNR) of analytes in low and high cell density culture

A higher SNR indicates a stronger analyte signal relative to background noise, which directly impacts the accuracy and reliability of Raman-based analyte detection and quantification. Across all analytes, TGRS consistently demonstrated a significantly higher SNR compared to RS, reinforcing its ability to enhance signal clarity by reducing background interference (Fig. 9). The most substantial improvement was observed for glucose and lactate. Glucose exhibited significantly higher SNR values with TGRS compared to RS, particularly at low cell density (154.4 ± 5.5 for TGRS vs. 12.7 ± 0.3 for RS). Although SNR values decreased at high cell density, TGRS still maintained a notable advantage

Fig. 8 **A** Average NAS of glutamine from HCD and LCD in TGRS, and **B** Average NAS of glutamine from HCD and LCD in RS

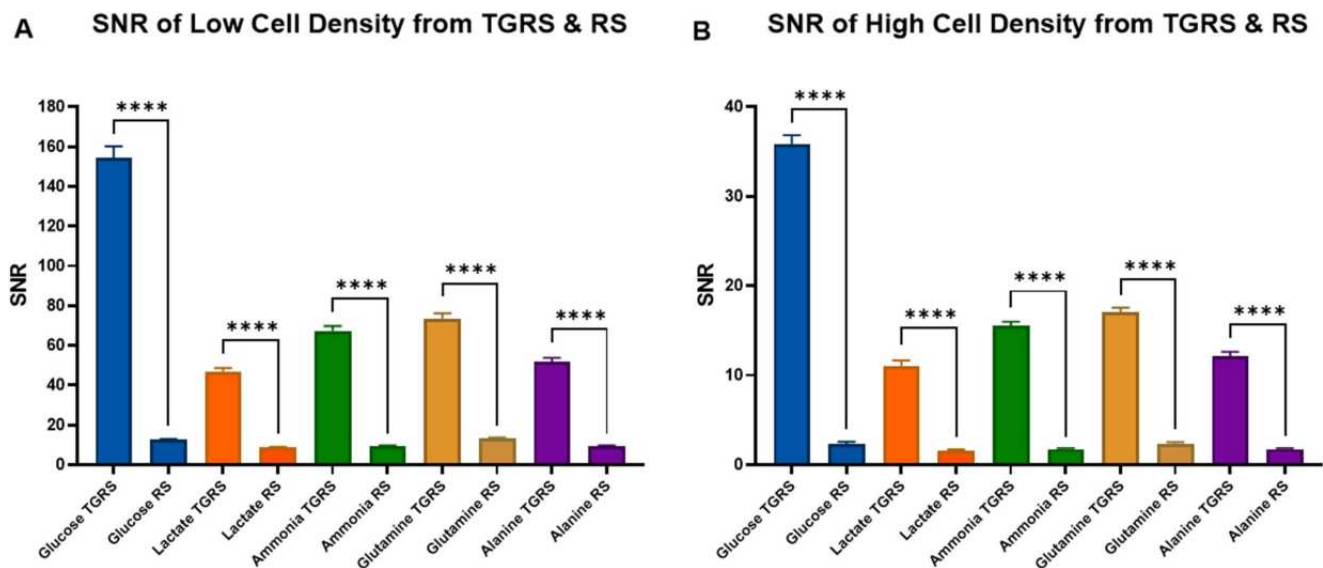
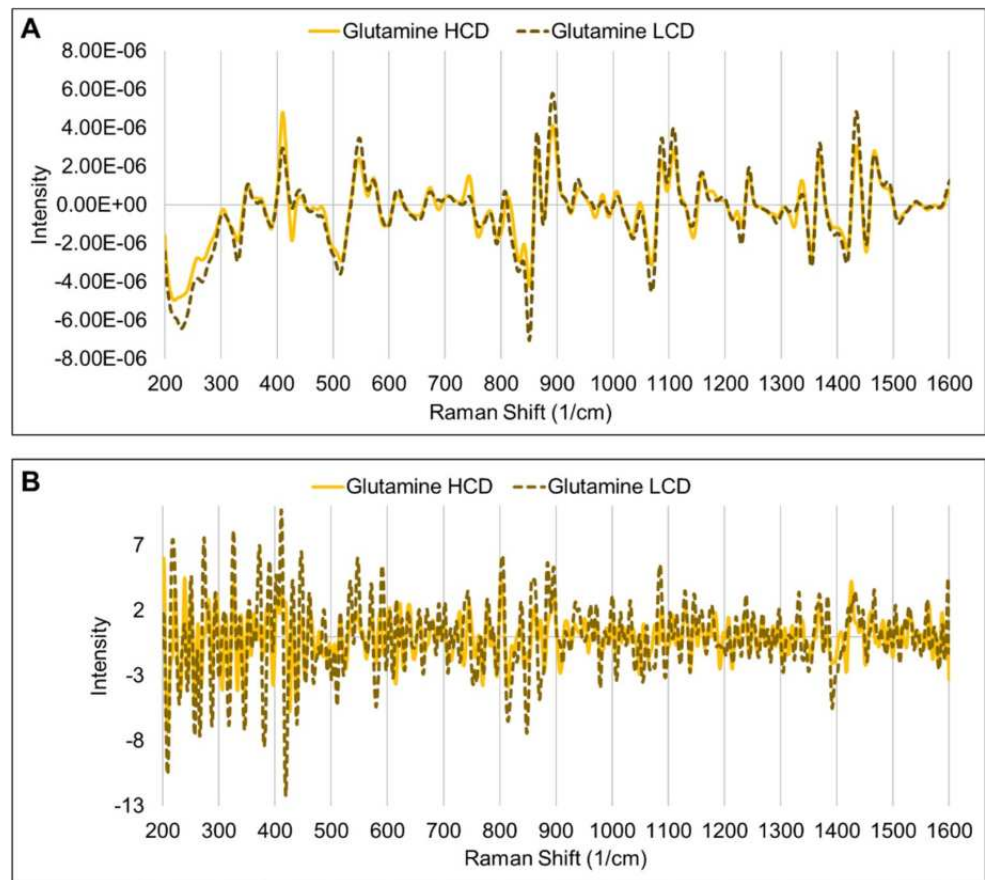


Fig. 9 Signal to Noise Ratio calculation from TGRS and RS, **A** low-density cell culture, and **B** high-density cell culture, **** = $p < 0.00005$

(35.8 ± 1.0 vs. 2.3 ± 0.2). Lactate followed a similar trend, with TGRS yielding higher SNR values at both low (46.9 ± 1.7) and high cell densities (11.04 ± 0.6), compared to RS (8.8 ± 0.15 and 1.6 ± 0.1 , respectively). Based on the SNR calculation, it is evident that TGRS produced clearer,

more intense spectral signals with less spectral broadening. In contrast, RS exhibited lower SNR values, suggesting that RS measurements are more susceptible to fluorescence interference and matrix effects.

For ammonia, glutamine, and alanine, which naturally exhibit weaker Raman signals, RS struggled to produce a reliable SNR, particularly in high-cell-density cultures. TGRS, however, significantly improved their SNR, making their detection more robust and reliable. Ammonia showed clearer signal definition with TGRS, reflected in superior SNR (67.3 ± 2.4 at low cell density and 15.56 ± 0.4 at high cell density), whereas RS yielded lower SNR (9.47 ± 0.2 and 1.73 ± 0.1 , respectively). Similarly, Glutamine detection using TGRS achieved higher SNR (73.4 ± 2.6 at low cell density and 17.03 ± 0.5 at high cell density) compared to RS (13.4 ± 0.3 and 2.37 ± 0.2). Alanine also followed similar pattern with TGRS (SNR: 51.9 ± 1.9 at low cell density and 12.12 ± 0.5 at high cell density) than with RS (9.47 ± 0.2 and 1.73 ± 0.1). This enhancement can be attributed to Time-gated technology effectively suppressing background fluorescence, allowing weak analyte signals to emerge more distinctly. The improvement in SNR with TGRS further supports the previously observed findings in NAS analysis, where TGRS provided sharper and more well-defined spectral peaks, reducing spectral overlap and enhancing analyte differentiation.

Limit of detection (LOD) of analytes in low and high cell density culture

A lower LOD value signifies a more sensitive detection capability, making it an essential metric for evaluating the suitability of Raman spectroscopy for real-time bioprocess monitoring. TGRS consistently achieved lower LOD values than RS for all analytes, indicating an enhanced ability

to detect analytes at lower concentrations. The most pronounced difference was observed in glucose (g/L) and lactate (g/L). For glucose, the LOD with TGRS was significantly reduced to 0.0255 ± 0.011 g/L at low cell density and 0.2245 ± 0.028 g/L at high cell density, compared to much higher values in RS (0.865 ± 0.278 and 7.57 ± 0.65 g/L, respectively). Lactate detection with TGRS achieved an exceptionally low LOD of 0.0049 ± 0.003 g/L at low cell density and 0.5797 ± 0.098 g/L at high cell density, while RS showed markedly poorer sensitivity (0.301 ± 0.165 and 7.63 ± 0.5 g/L, respectively). TGRS provided a notably lower detection threshold compared to RS for glucose and lactate which suggests that TGRS has the potential to detect metabolic fluctuations at earlier stages, allowing for improved process control in cell culture monitoring (Fig. 10).

For ammonia (mM), glutamine (mM), and alanine (mM), RS exhibited notably higher LOD values, reinforcing its limitations in detecting analytes with inherently weak Raman signals. For ammonia, TGRS provided LOD of 0.0177 ± 0.005 mM at low cell density and 0.7763 ± 0.226 mM at high cell density, in contrast to RS values of 0.651 ± 0.279 and 8.4 ± 1.0 mM. Glutamine's LOD with TGRS were 0.0278 ± 0.012 mM (low cell density) and 0.4225 ± 0.197 mM (high cell density), significantly better than RS (0.462 ± 0.142 and 8.03 ± 0.7 mM, respectively). Lastly, alanine showed superior detection sensitivity with TGRS (LOD: 0.0132 ± 0.002 mM at low cell density and 1.1106 ± 0.146 mM at high cell density), while RS produced much higher LODs (0.469 ± 0.124 and 10.73 ± 1.0 mM). The reduced performance of RS in these cases is likely due to

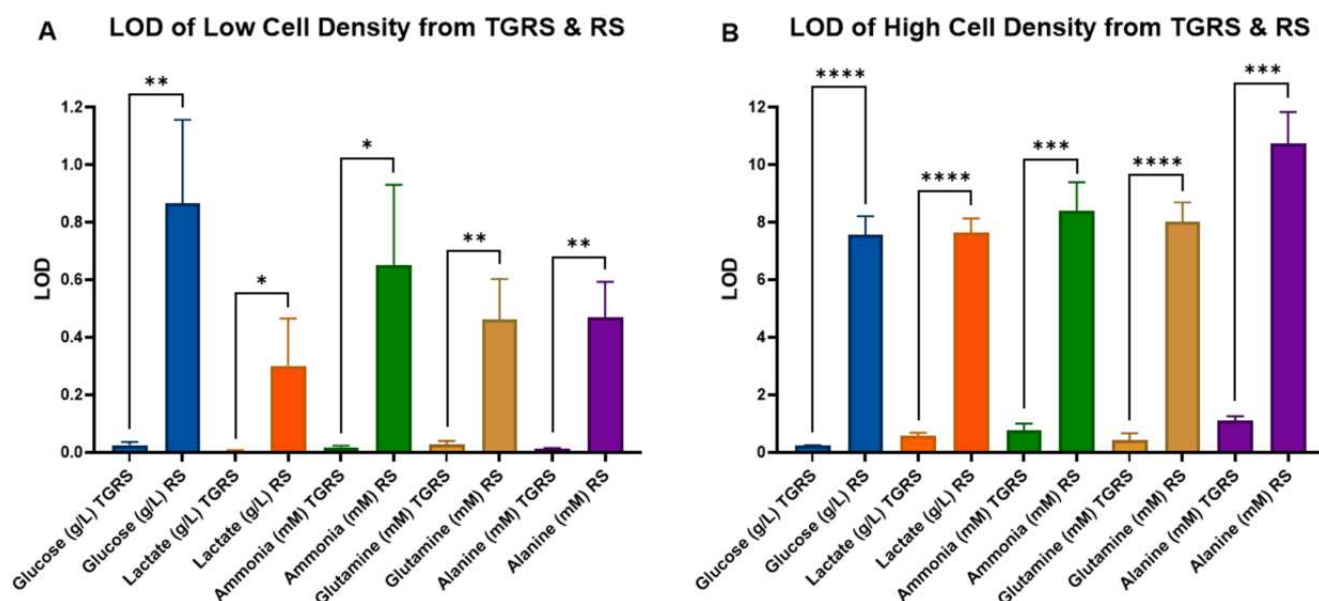


Fig. 10 Limit of detection calculation from TGRS and RS, **A** low-density cell culture, and **B** high-density cell culture, * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.0005$ & **** = $p < 0.00005$

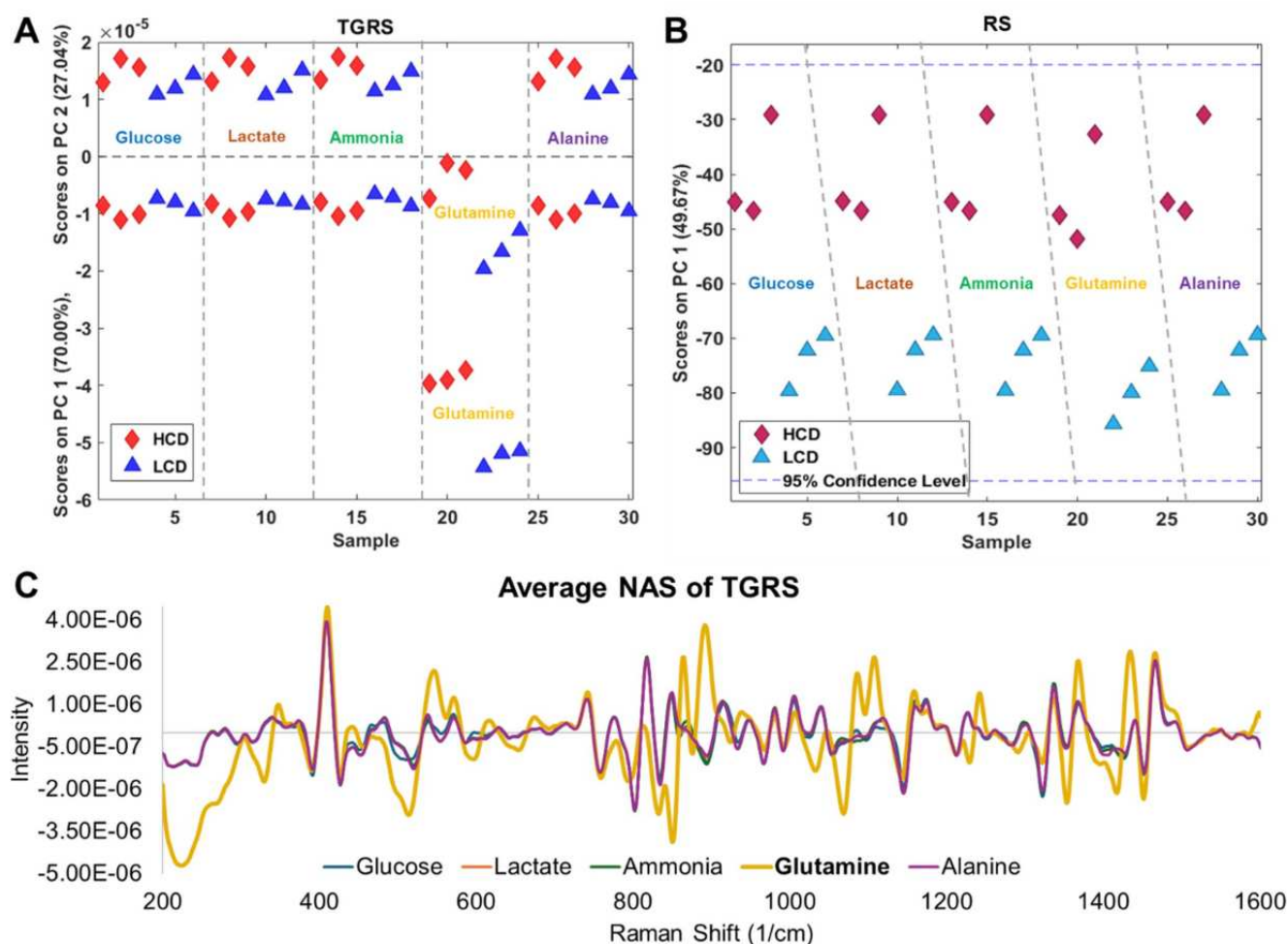


Fig. 11 A Samples vs. PC1 (70%) & PC2 (27.04%) of TGRS, B Samples vs. PC1 (49.67%) of RS, and C Averaged NAS of TGRS of all analytes

its inability to suppress interfering fluorescence and background noise, which elevates the detection limit. In contrast, TGRS successfully lowered the LOD for ammonia and glutamine, demonstrating its capability to resolve weak Raman features with greater sensitivity.

PCA analysis of HCD and LCD of TGRS and RS

In the TGRS dataset (Fig. 11A), PC1 (70.00%) and PC2 (27.04%) together explained 97.04% of the variance, indicating that the majority of spectral variation was captured within two principal components. The distribution of scores showed substantial overlap between HCD and LCD samples, suggesting that TGRS spectral variance was driven primarily by NAS shapes of analytes rather than differences in cell densities. Notably, only glutamine samples clustered separately, highlighting the sensitivity of TGRS to spectral shape-specific variations (Fig. 11A and C). In contrast, the RS dataset (Fig. 11B) showed that PC1 (49.67%) alone accounted for almost half the variance and distinctly

separated HCD from LCD samples, indicating that fluorescence contributions associated with increasing cell density strongly influenced the variance captured by the PCs.

Discussion

The primary aim of the study was to investigate the analytical sensitivity for five key analytes present in CHO cell culture using the Time-gated Raman Spectroscopy (TGRS) and Raman Spectroscopy (RS). The results demonstrate enhanced analytical performance of TGRS over RS in detecting key analytes within a complex bioprocess matrix. By employing the Net Analyte Signal (NAS) approach, TGRS was able to isolate analyte-specific spectral contributions with enhanced precision, as evidenced by the sharper and more well-defined peaks observed in its NAS spectra. These findings align with previous studies indicating that Time-gating approaches effectively minimize fluorescence

interference, a major challenge in Raman-based biochemical analysis [26, 27, 40, 41].

The comparison of NAS spectra between TGRS and RS highlights the improved spectral resolution of TGRS, particularly for glucose and lactate. The differences in peak positions and intensities between TGRS and RS suggest that Time-gating technology provided superior spectral resolution by effectively suppressing fluorescence and reducing matrix-induced interferences. For glucose and lactate, the presence of additional peaks (324, 397, 408 cm^{-1} , and 525, 862, 1460 cm^{-1} respectively) in RS suggests that Raman measurements may capture a higher level of background interference. Additionally, in Fig. 7, TGRS effectively minimizes fluorescence and enhances signal to noise ratio in HCD and LCD, allowing more accurate detection of glucose in complex CHO cultures. RS, however, was strongly affected by fluorescence at HCD, which reduced the sensitivity and masked analyte-specific signals. These findings reinforce the advantage of TGRS in providing cleaner, more defined spectral features for these analytes, which is essential for accurate bioprocess monitoring. In the case of ammonia, the increased clarity of ammonia peaks (891, 1096, and 1133 cm^{-1}) in TGRS suggests that Time-gated techniques enhance the detection of low-concentration volatile analytes by reducing spectral interference from other medium components. Similarly, glutamine exhibited well-defined peaks in TGRS, particularly in the high-wavenumber region (above 700 cm^{-1}). Moreover, Fig. 8 shows that TGRS suppresses fluorescence and improves SNR in both HCD, enabling accurate glutamine detection, whereas RS suffers from fluorescence at HCD, reducing sensitivity and obscuring analyte signals. Additionally, the improved spectral resolution of TGRS in alanine detection suggests its potential for more precise metabolic profiling in cell culture systems, indicating that Time-gated technology provides better differentiation of amino acid-related spectral features. This improvement is critical in ensuring accurate and precise analyte identification and detection, especially in dynamic cell culture environments where overlapping spectral signals are common. Raman spectroscopy appears to be more prone to baseline variations and fluorescence interference, which impact the robustness of spectral assignments. The enhanced clarity of alanine peaks in TGRS further demonstrates its ability to minimize background noise and provide more reliable metabolic profiling.

The Signal to Noise Ratio (SNR) analysis further supports the analytical advantage of Time-Gated Raman Spectroscopy (TGRS), particularly in high-cell-density cultures where analyte signals are inherently weak. TGRS consistently demonstrated significantly higher SNR values compared to Raman Spectroscopy (RS), affirming its superior detection capability. For instance, glucose exhibited SNR

values of 35.8 ± 1.0 with TGRS at high cell density, compared to just 2.3 ± 0.2 with RS. Similarly, lactate showed an SNR of 11.04 ± 0.6 with TGRS versus 1.6 ± 0.1 with RS under the same conditions. This robust performance was mirrored across other analytes such as ammonia, glutamine, and alanine, where TGRS consistently yielded higher SNR. These enhancements are primarily attributed to the suppression of background fluorescence and scattering by the time-gating mechanism, which reduces spectral overlap and improves signal clarity. In contrast, the lower SNR values observed with RS underscore its vulnerability to background or fluorescence interference, leading to compromised analyte detection in complex bioprocess environments. The PCA results (Fig. 11) further support the SNR analysis by demonstrating that TGRS spectra are dominated by chemically relevant analyte features rather than fluorescence background. In TGRS, over 97% of the spectral variance was captured by two principal components, with HCD and LCD samples largely overlapping, confirming that spectral variance was driven by analyte-specific NAS rather than cell density. By contrast, RS showed PC1 variance dominated by fluorescence, which separated HCD from LCD and masked analyte-specific information. These findings are consistent with the higher SNR observed for TGRS, where suppression of fluorescence interference allows clearer resolution of analyte signals across culture densities. These findings demonstrate that TGRS efficiently suppresses fluorescence interference and yields spectra dominated by chemically relevant analyte features, whereas RS remains confounded by sample density, thereby limiting its sensitivity to detect analyte-specific information.

The result of the Limit of Detection (LOD) analysis reinforces the superior sensitivity of Time TGRS. Lower LOD values indicate that TGRS can detect analytes at significantly lower concentrations, which is particularly advantageous for real-time bioprocess monitoring. For glucose, TGRS achieved LODs of 0.0255 ± 0.011 g/L at low cell density and 0.2245 ± 0.028 g/L at high cell density, compared to substantially higher LODs with RS, which were 0.865 ± 0.278 g/L and 7.57 ± 0.65 g/L, respectively. Similarly, for lactate, TGRS exhibited LODs of 0.0049 ± 0.003 g/L and 0.5797 ± 0.098 g/L, in stark contrast to RS values of 0.301 ± 0.165 g/L and 7.63 ± 0.5 g/L. These markedly lower LODs suggest that TGRS can capture subtle metabolic fluctuations with higher sensitivity, facilitating early-stage process interventions. Furthermore, the significantly elevated LOD values observed in RS for ammonia (up to 8.4 ± 1.0 mM) and glutamine (up to 8.03 ± 0.7 mM) highlight the limitations of Raman techniques in detecting low-concentration analytes due to fluorescence and matrix interference. In contrast, TGRS demonstrated much lower LODs for ammonia (0.0177 ± 0.005 mM at low cell density

and 0.7763 ± 0.226 mM at high cell density) and glutamine (0.0278 ± 0.012 mM and 0.4225 ± 0.197 mM), underscoring its potential in improving detection accuracy and sensitivity in complex bioprocess environments.

Collectively, these findings underscore the value of TGRS as a sensitive and reliable platform for analyte detection in real-time bioprocess monitoring.

Conclusion

Raman spectroscopy has proven to be a powerful Process Analytical Technology (PAT) tool for real-time monitoring in the biopharmaceutical industry. This study highlights the advantages of Time-gated Raman Spectroscopy (TGRS) over Raman Spectroscopy (RS) for analyte detection in mammalian cell culture processes. The superior spectral resolution, higher Signal to Noise Ratio (SNR), and lower Limit of Detection (LOD) observed in TGRS confirm its ability to provide more precise and accurate monitoring of key analytes such as glucose, lactate, ammonia, glutamine, and alanine. By effectively minimizing fluorescence interference and matrix-induced spectral distortions, TGRS enables robust in-line analyte monitoring, essential for optimizing bioprocess control. In summary, the results collectively highlight the advantages of TGRS over RS for Raman-based analyte detection. The improved spectral resolution, higher SNR, and lower LOD observed in TGRS suggest its suitability for real-time monitoring of cell culture analytes, providing a more reliable and accurate analytical tool for bioprocess applications. Our future research will focus on developing chemometric models to effectively and accurately quantify and predict analyte concentrations within the dynamic bioreactor environment.

Acknowledgements This work was performed in collaboration with Pfizer, Inc and was performed under a Project Award Agreement from the National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) and financial assistance award 70NANB21H086 from the U.S. Department of Commerce, National Institute of Standards and Technology.

Author contributions Mahdi Mubin Shaikat : Writing – original draft, review and editing, Visualization, Methodology, Investigation, Formal analysis, Data analysis, Data curation; Venkata Gayatri Dhara: Data analysis, Investigation, Writing – review and editing; James K. Drennen III: Writing – review and editing, Supervision; Guogang Dong: Investigation, Writing –review and editing; Carl A. Anderson (Corresponding Author and Email: andersonca@duq.edu): Conceptualization, Writing –review and editing, Supervision, Fund acquisition, Project administration.

Funding This work was performed in collaboration with Pfizer, Inc and was performed under a Project Award Agreement from the National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) and financial assistance award 70NANB21H086 from the

U.S. Department of Commerce, National Institute of Standards and Technology.

Data availability The data generated and analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that there are no competing interests.

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