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# INFLUENCE OF ALCOHOLIC FERMENTATION COMPONENTS ON RAMAN SPECTROSCOPY AIMING ONLINE MONITORING

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## ABSTRACT

The use of ethanol as a sustainable alternative in diversifying the global energy matrix is crucial, given the environmental and energy security pressures currently faced. Consequently, there is an increasing implementation alongside the Industry 4.0 process to obtain fermentation components' results in real-time through the use of Raman spectroscopy. This study aimed to investigate the effects of components of alcoholic fermentation on Raman spectra. Using industrial yeast cells, molasses, and sugarcane juice, experiments were conducted under controlled conditions, followed by validation by high-performance liquid chromatography (HPLC). Pretreatments including Baseline, SNV, and RMSECV were applied to enhance the calibration model. This approach allowed for the assessment of the effects of fermentation components on Raman spectra, enabling more precise and reliable analyses. The results indicated that it was possible to achieve functional calibration for sugarcane juice; however, it is not possible to read the components of molasses due to material fluorescence caused by its properties such as the dark color.

**Keywords:** RAMAN. Alcoholic fermentation, Sugarcane molasses. Spectroscopy. Fluorescence

## 1 INTRODUCTION

The global need for diversification of the energy matrix is urgent due to the challenges of climate change and energy security. In this context, ethanol stands out as a sustainable alternative, contributing to greenhouse gas emission reduction, promoting rural and regional development, and fostering technological innovation. Brazil is the second-largest ethanol producer in the world, producing more than 654.5 million liters of ethanol per year[1].

The production of ethanol from sugarcane is considered efficient due to the high yields and productivity achieved in a well-known process using the yeast *Saccharomyces cerevisiae*. Most of Brazilian sugarcane mills co-produce ethanol, sugar, and energy, which makes molasses, a byproduct of sugar production, an important raw material used as a carbon source in ethanol production. This contributes to waste reduction and the maximization of available resources. Molasses, a viscous byproduct rich in sucrose provides an alternative raw material for ethanol production without compromising food production, minimizing the impacts associated with the expansion of sugarcane cultivation areas and contributing to the integration of the plant [2].

Despite the well-established ethanol industry in Brazil, there is still room for continuous improvement. Even small improvements in the process can result in significant gains due to the large volume of production. In this sense, the implementation of Industry 4.0 concepts emerges as an essential tool to boost efficiency and productivity in the sector through the implementation of cyber-physical systems [3]. The implementation of online monitoring systems could allow real-time tracking of various key parameters in the ethanol production process, such as temperature, pressure, pH, and humidity levels [4], enhancing the quality and control of the process.

Raman spectroscopy is an analytical technique that provides detailed information about the molecular structure, chemical composition, and molecular interactions of materials. It is widely used in various fields of science and technology, such as the identification and characterization of chemical substances, quality control, and monitoring of molecular species. This technique is based on the phenomenon of inelastic light scattering, where the incident light interacts with molecular vibrations, resulting in a characteristic energy shift known as the Raman shift [5].

Raman spectroscopy is widely used in industries in homogeneous systems to detect significant variations in the medium. There have been developments in the literature to evaluate Raman in dynamic environments, such as fermentation [6]. The use of Raman to online monitoring of ethanol production could make it possible to obtain real-time responses of substrate, product, and cell concentration, offering several significant advantages as enabling the early detection of issues during the process, such as microbial contamination, substrate depletion or excessive accumulation as well as formation of undesired products, allowing for a quick response to prevent production losses [4,6].

However, numerous media and components in a fermentation process can diminish or even compromise the quality of the Raman spectral data obtained during online monitoring of fermentation. Fluorescence is an optical phenomenon that can affect spectroscopic measurements, including those performed by Raman spectroscopy, once it can overlap with the desired Raman signal, making precise interpretation difficult and hampering the analysis of the spectra. This occurs because both fluorescence

and Raman scattering involve interactions between the incident light and the sample, producing detectable signals[7]. In this context, the present work aimed to investigate the influence of the main components of alcoholic fermentation, such as the composition of the medium (sugarcane juice and molasses), on the Raman spectrum and probe interference, to apply it in the online monitoring of alcoholic fermentation.

## 2 MATERIAL & METHODS

The influence of three different fermentation media on Raman spectrum was analyzed: the first was synthetic medium using glucose (30 g/L) diluted in distilled water; the second consisted of clarified sugarcane juice (purchased in a local market) by centrifugation at 10,000 RPM for 5 minutes; and the third was composed of sugarcane molasses provided by Fermentec (Piracicaba, Brazil). Both industrial media were diluted to obtain 30 g/L of total reducing sugars (TRS). The tests were conducted in a 50 mL becker sealed against light, where the samples were inserted at a constant temperature of 25°C and magnetic stirring was kept at 100 RPM. Additional experiments aiming to access the influence of ethanol and cell metabolites in glucose concentration measurement were also carried out using pre-fermented sugarcane juice medium spiked with known concentrations of glucose ranging from 0 to 80 g/L in 250 mL Erlenmeyer flasks at 32°C and 200 RPM, with Fermel® yeast (20 g/L). In all experiments, the All-In-One Raman system from MarqMetrix (Seattle, USA) was used, consisting of a Fiber BallProbe model probe with integration time of 1000ms, average set 10, and laser power of 100mW [6].

After data acquisition, a calibration model for sugarcane juice was obtained using the acquired Raman spectra and Raman software itself, the SOLO predictor from Eigenvector, using Baseline and SNV (Standard Normal Variate) pretreatments to correct baseline issues and remove variations in light scattering that may occur in spectroscopic data. PLS (Partial Least Squares) was used to select the most relevant wavelength regions, and complexity optimization was performed based on RMSECV (Root Mean Square Error of Cross Validation), where optimization was conducted regarding latent variables and interference reduction [6,8]. For the additional experiments to evaluate glucose concentration predictions,

The validation of Raman calibration was performed during the fermentation of sugarcane juice (~150 g/L of initial TRS) using the Fermel® *Saccharomyces cerevisiae* yeast (~20 g/L) donated by Fermentec (Piracicaba, Brazil). The lyophilized yeast was rehydrated and activated in a 5 L stirred-tank bench bioreactor following the procedure adapted from Mesquita et al [9]. The experiment was conducted at 32ºC and 250 rpm in a 5 L stirred-tank bench bioreactor adapted to replicate the industrial must recirculation system by installing a peristaltic pump that maintained the recirculation flow at 17 L/h[10]. The SUPERSYS_HCDC supervision system, developed in house and programmed in LabVIEW®, was used for real-time acquisition of pH and temperature data[4]. All samples were analyzed by high performance liquid chromatograph (HPLC) with a Sugar-PakTM column (300x6.5 mm) (Waters,USA) at 80°C with ultrapure water as the mobile phase (0.6 mL/min).

## 3 RESULTS & DISCUSSION

The Raman spectrum for the three different fermentation media evaluated are presented in Figure 1. When using the synthetic medium, it was possible to identify Raman shift peaks between 1000 and 2000 and near 3000 (Figure A), which are related to, the presence of glucose. With sugarcane juice, it is still possible to visually identify the presence of those peaks, besides the influence of media components on the spectrum (Figure 1B). On the other hand, the use of sugarcane molasse influenced negatively the reading of Raman spectra due to its properties such as dark color, even when significantly diluted, as is the case of the present work (~30 g/L of TRS) it was no longer possible to identify the presence of the glucose peaks.

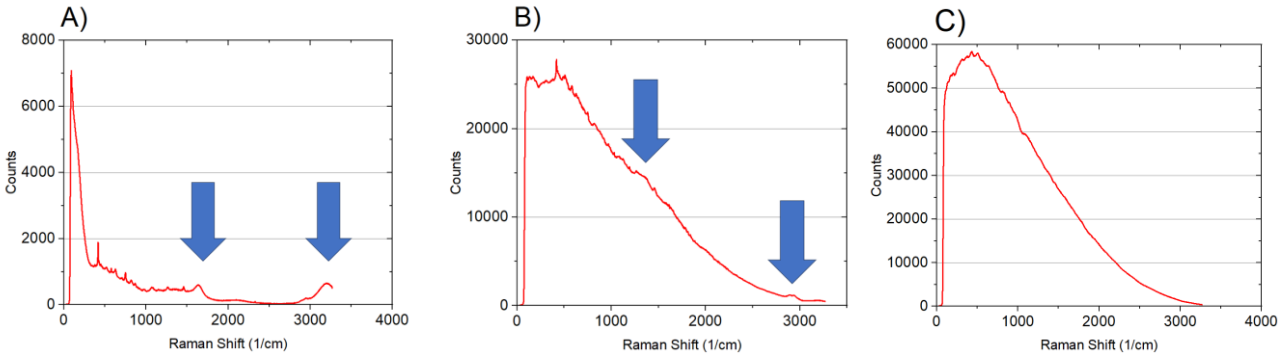


**Figure 1** Raman spectra and glucose (30 g/L) peaks (blue) for three different media (A) synthetic media, (B) sugarcane juice, and (C) molasses. Blue arrows indicate glucose peaks.

In this sense, it is not possible to use Raman to monitoring molasse fermentation once it is not possible to calibrate the equipment due to this influence on spectrum. Even when using two identical samples to calibrate and validate, the cross-validation results have a high standard deviation due to the high fluorescence relative to the medium and it was not possible to achieve a low RMSECV. Ávilla et al. [11] monitored the fermentation of synthetic media by S. *cerevisae* using Raman spectroscopy for process

control and fault detection and achieved a RMSEP of 0.53% for glucose using PLS models. These results were possible once the authors used synthetic media. Aiming to apply Raman technology in industrial media, the calibration was performed using sugarcane juice as feedstock. Figure 2 shows the results obtained for the prediction of glucose for cross-validation and test data. Cross validation data were acquire using bench tests. Test data, on the other hand were obtained from fermentation of sugarcane juice. For optimizing the model complexity, RMSECV values were used to achieve the lowest possible value without an unnecessary increase in latent variables, thus avoiding an increase in model complexity, the choice of latent variables (5 variables) was made visually with parsimony. A RMSECV of 3.10 g/L and a RMSEP value of 3.83 g/L was obtained for glucose data. These results represent a low standard deviation for cross-validation. In addition, RMSEP was very close to RMSECV which indicates that the model is robust and can be generalized for external data sets. It is worth noticing that the data are randomly distributed around the bisector which indicates a good prediction without large systematic errors.

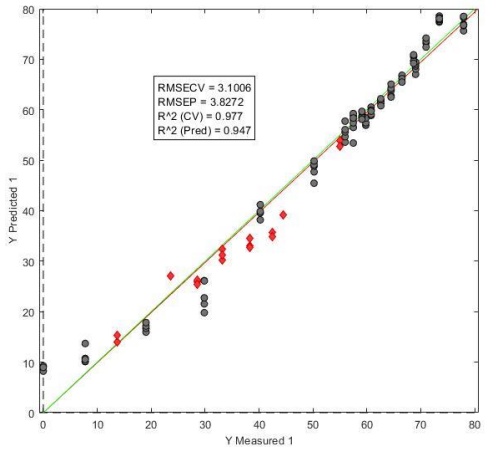


**Figure 2** Cross-validation (black) versus predicted values (red) data of glucose during sugarcane juice fermentation using RAMAN spectroscopy for online monitoring.

## 4 CONCLUSION

. The Raman spectroscopy showed applicability for the online monitoring of sugarcane juice fermentation, with an excellent predictive capability of the calibration model, evidenced by the low RMSECV of 3.1006 and the RMSEP value of 3.8272 in the external dataset. Model complexity optimization was achieved based on RMSECV values, ensuring a balance between accuracy and simplicity. However, the same evaluation for molasses was not feasible due to dark color, which interfere with the reading of Raman spectra, making it impossible to create a reliable calibration and thus rendering its prediction unfeasible. This phenomenon highlights the limitation of Raman spectroscopy for online monitoring of molasses fermentation and underscores the importance of considering the intrinsic characteristics of materials when developing calibration models.

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