Application of green analytical chemistry to a green chemistry process: Magnetic resonance and Raman spectroscopic process monitoring of continuous ethanolic fermentation

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Abstract
Compact 1H NMR and Raman spectrometers were used for real-time process monitoring of alcoholic fermentation in a continuous flow reactor. Yeast cells catalyzing the sucrose conversion were immobilized in alginate beads floating in the reactor. The spectrometers proved to be robust and could be easily attached to the reaction apparatus. As environmentally friendly analysis methods, 1H NMR and Raman spectroscopy were selected to match the resource- and energy-saving process. Analyses took only a few seconds to minutes compared to chromatographic procedures and were, therefore, suitable for real-time control realized as a feedback loop. Both compact spectrometers were successfully implemented online. Raman spectroscopy allowed for faster spectral acquisition and higher quantitative precision, NMR yielded more resolved signals thus higher specificity. By using the software Matlab for automated data loading and processing, relevant parameters such as the ethanol, glycerol, and sugar content could be easily obtained. The subsequent multivariate data analysis using partial linear least-squares regression type 2 enabled the quantitative monitoring of all reactants within a single model in real time.

KEYWORDS
bioprocess monitoring, compact spectrometer, immobilized yeast, multivariate data analysis, NMR and Raman spectroscopy, PLS2

1 | INTRODUCTION

The 12 principles of green chemistry introduced by Anastas (Anastas, 1999; Anastas & Zimmerman, 2007; Anastas, Kirchhoff, & Williamson, 2001; Sheldon, Arends, & Hanefeld, 2007) need to be supplemented by the 12 aspects of green analytical chemistry (GAC) relating process analysis to green chemistry (Eldin, Ismaiel, Hassan, & Shalaby, 2016; Farré, Pérez, Gonçalves, Alpendurada, & Barceló, 2010; Gałuszka, Migaszewski, & Namieśnik, 2013; Namieśnik, 2001; Tobiszewski, 2016; Tobiszewski, Mechlińska, & Namieśnik, 2010). The goals of GAC is resource- and energy-saving for analytical methods, too. Analyses should hence be operated in automation and at the same time be able to furnish as many parameters as possible requiring only a very little amount of sample or sample volume (Nascimento, Macedo, Santos, & Oliveira, 2017; Schalk et al., 2017; Villar et al., 2017). The use of spectroscopic methods enables the monitoring of all relevant process media in real time. The combination with multivariate methods such as partial
least-squares regression type 2 (PLS2) renders highly congested, crowded, or insufficiently resolved spectra applicable for robust concentration prediction (Kessler, 2006; Pedro & Ferreira, 2007). Further to PLS, PLS2 allows the simultaneous quantitative prediction of several y variables, such as concentrations of different chemical components in a mixture. It is advantageous to apply PLS2 when the target variables are interdependent or correlated with each other, as is often the case with chemical reactions, and when the correlated y variables possess different error ranges. The dimensions of the analyzers should follow an ecological and economical point of view (Pinto, Pereira, Ribeiro, & Farinas, 2016; Pinto, Ribeiro, & Farinas, 2018).

Compact spectrometers, that is, small-dimension analyzers, are flexible, robust, and today available for a wide range of applications (Blümich, 2016; Blümich & Singh, 2017; Danieli et al., 2014; Kern et al., 2018; Kölner, Garro Linck, Danieli, Rohwedder, & Blümich, 2015; Kreyenschulte, Paciok, Regestein, Blümich, & Büchs, 2015; Meyer, Kern, Zientek, Guthausen, & Maiwald, 2016; Singh, Danieli, & Blümich, 2017). Automated analyzes should be performed preferably without sample preparation and in real time (Maiwald, 2017; Meyer et al., 2016; Rathore, Bhushan, & Hadpe, 2011; Rosas, Blanco, Gonzalez, & Alcalá, 2012; Singh et al., 2017; Sugimoto, Fukuyama, Sumino, Takagi, & Ryu, 2009). Through an equally automated data analysis, the raw data may be received, centrally stored and converted into meaningful and applicable plots or diagrams such as concentration–time (c–t) diagrams (Jaeger & Legner, 2017; Wu, Li, & Gao, 2016). These diagrams represent the regular or optimum course of the process and might be accompanied by a confidence band. Deviations from the expected course are counter-reacted through a feedback loop (Cortés-Borda et al., 2018; Sans, Porwol, Dragone, & Cronin, 2015). Control over the whole process protects the environment, increases the safety of the operator in the workplace and ensures reliable product quality. For process monitoring, compact nuclear magnetic resonance (NMR), infrared (IR), near-infrared (NIR) and Raman spectrometers have been utilized increasingly often (Acevedo et al., 2018; Dalitz, Cudaj, Maiwald, & Guthausen, 2012; Schalk et al., 2017). They proved highly flexible and robust. The disadvantage of the somewhat lower resolution of these analyzers has been effectively compensated for by the use of multivariate data analysis (Kern et al., 2018; Šahić et al., 2016). While some processes have been conducted within a spectrometer's sample cell, vibrational spectrometers have been coupled via flow-cells or probes (Legner, Wirtz, & Jaeger, 2018; Schalk et al., 2017). Analogously, an NMR spectrometer with a flow cell has been implemented via a bypass (Ahmed-Omer, Slivinski, Cerruti, & Ley, 2016; Singh et al., 2017). This type of spectrometers often provide a sensitivity suitable for observing neat liquids or solids dissolved in neat liquids as reactants.

In this study, compact 1H NMR and handheld Raman spectrometers in combination with automated multivariate methods are used for the real-time monitoring of a continuous biotechnological process, that is, ethanol fermentation using immobilized yeast. The data will be analyzed using chemical kinetic models and PLS2 to obtain c–t diagrams and to gain process knowledge from chemical kinetic models. Reaction monitoring is shown to obey the 12 principles of Green Analytical Chemistry.

### 2 MATERIALS AND METHODS

#### 2.1 Process and analytical methods

A BIOSTAT® B fermenter I with a capacity of 2 L (Sartorius AG, Göttingen, Germany) was used for fermentation. The mounted agitator (B. Braun Biotech International GmbH, Melsungen, Germany; cf. Figure 1) was operated at a constant stirring velocity of 120 rpm. A peristaltic pump (B. Braun Biotech International GmbH) was used to flow the sucrose-containing solution from the storage vessel to the temperature-controlled reactor at a flow rate of 2.5 ml per minute during the entire process. From the reactor, the solution further passed a QS 0.5 mm quartz-flow cell (Hellma Analytics, Mühlheim, Germany) to allow for online Raman spectral recording. The implemented MultiPurposeSampler (Gerstel, Mühlheim an der Ruhr, Germany) was equipped with a stainless-steel flow cell, VT98 tray, fast wash station, and injection valve. Through this arrangement, samples were channeled through the flow cell and transferred to the NMR spectrometer and/or pipetted into high-performance liquid chromatography (HPLC) vials (Legner, Friesen, Voigt, Horst, & Jaeger, 2016). For HPLC analysis, samples of 2 ml each were collected. Spectroscopic analyses were performed as described below. Finally, the solution was conveyed towards the product vessel. For multivariate data analysis, two-thirds of the sample spectra were used as calibration set and one-third as validation set. The multivariate model was applied to all remaining recorded spectra, that is, spectra without corresponding HPLC analysis.

Fermentation was fed with a sugar solution. Sucrose (>99.5%, Merck KGaA), was used as obtained. A buffer solution containing 100 ml sodium hydroxide (1 mol·L⁻¹; Merck KGaA) and 200 ml acetic acid (1 mol·L⁻¹; Merck KGaA) in 5 L water was prepared. To this solution, 50 g of calcium chloride dihydrate and sucrose were added in the appropriate amounts. The amount of sucrose depended on the volume of the fermenter and the amount of immobilized yeast. In case of the 3 L fermenter, 17% sucrose (850 g sucrose) were added to the buffer solution.

A one-stage process was conducted by continuously conveying a 17% sucrose-containing educt solution through a 3 L bioreactor at a
temperature of 30°C. When steady state was reached, the process was terminated after 900 min. The process was transformed into a two-stage process using new immobilized yeast cells: a 15% sucrose solution was pumped until reaching steady state. Subsequently, a 20% sucrose-containing solution was conveyed until a second steady state. The temperature was again kept at 30°C but a 2 L bioreactor was used (cf. Figure 1). The process was monitored over a period of 3000 min. For the separate model development of the one-stage and two-stage fermentation, samples for calibration and validation were taken in two feasibility studies carried out previously. The separate model development requires a prior execution of the two processes to be investigated to obtain the calibration by means of the spectrometers used as well as reference analysis. For single-stage fermentation, 50 samples were taken and divided into a training set and a test set (cf. Table 1). For the two-stage process, the model was developed with 40 samples (cf. Tables S1–2).

### 2.2 Immobilization of the biocatalyst

Sodium alginate, 10 g (Technical grade, VWR International GmbH, Darmstadt, Germany) was dissolved in 350 ml of distilled water at ambient temperature. After cooling the alginate to 30°C, 42 g of Baker’s yeast (VWR International GmbH) were added under stirring. The homogeneous yeast-alginate mixture was added dropwise to a 10 g·L⁻¹ calcium chloride solution (calcium chloride dihydrate, VWR International GmbH), leading to the formation of a crosslinked solution of very uniform beads.

### 2.3 HPLC reference method

Concentration data for referencing were obtained using the HPLC system Smartline (Knauer GmbH, Berlin, Germany) equipped with a refractive index detector. A Eurokat H column (Knauer GmbH) of dimensions 300 × 8 mm, particle size 10 μm, was used for chromatographic separation. An isotropic flow was set to 0.8 ml·min⁻¹ using 0.01 mol·L⁻¹ sulfuric acid (99.999%; Merck KGaA, Darmstadt, Germany) as mobile phase. The separation of all substances involved in the process was achieved within 20 min at 60°C. The chromatograms were processed and evaluated using the software EuroChrom® 1.57 (Knauer GmbH).

### 2.4 Compact spectrometry

NMR spectra were recorded using a picoSpin80 spectrometer (Thermo Fisher Scientific, Dreieich, Germany) with a proton Larmor frequency of 82 MHz. The spectrometer was controlled by Thermo Fisher PicoSpin software 0.9.3 running on the spectrometer control board and accessed via a web interface from a laptop computer. The instrument had a flow cell with an active volume of 40 nl. The electronic chemical shift referencing corrected for magnetic field drift and allowed recording spectra of the neat liquids without the addition of deuterated solvents. A simple pulse-acquire scheme was used as an experiment. The number of accumulations was set to 16. The pulse length was set to 60 μs corresponding to a 90° pulse. A recovery delay of 500 μs followed by a repetition or relaxation delay of 6 s was applied, which proved a sufficient compromise with respect to relaxation rates, experimental conditions, sensitivity, and precision. Spectra were recorded with 4092 acquisition points and a zero filling of 9000. The spectral width amounted to 4 kHz.

Raman spectra were recorded using the handheld Raman (h-Ram) spectrometer IDRaman mini 2.0 (Ocean Optics, Dunedin, FL). Spectra were acquired by means of a point-and-shoot adapter in combination with a QS 0.5 mm quartz-flow cell (Hellma Analytics, Muellheim, Germany). The spectrum was acquired from 400 to 2300 cm⁻¹ and the spectral resolution was 13 cm⁻¹. The laser had an excitation wavelength of 785 nm ± 0.5 nm and a power of 100 mW. The software Peak 1.3.54 (Snowy Range Instruments, Laramie, WY) was used for spectral acquisition. The Multi-Mode allows any number of analyses to be carried out with a freely selectable interval. The respective raw data of the two spectroscopic methods were automatically saved into a cloud, so that data pre-processing and univariate and multivariate data analysis took place immediately afterwards.

### 2.5 Data preprocessing

Spectral processing was carried out using the software MatLab R 2016b (MathWorks, Inc., Natick, MA). A home-built Matlab script waited for new raw data, loaded them, processed the data and eventually moved the file to a designated folder. The overall analysis time required for building c-t diagrams was taken from the jdx files’ time stamp. Spectral processing, Fourier transformation, and phase correction of the NMR raw data were performed using Matlab routines. After subsequent baseline correction using a Bernstein polynomial fit 3rd order, a moving average over an interval of six data points for the reduction of file size and normalization to the largest peak were applied. Since the relevant spectral range used for multivariate data analysis laid between 0.3 and 5.5 ppm, the remaining data for the further analyses were rejected. The spectra were referenced to the water resonance at 4.7 ppm.

Raman raw data were extracted from text files very quickly. A subsequent baseline correction did not give better results due to
preset parameters in the control software, so this additional processing step was omitted. No standardization was performed, since the highest signal was specific for primary alcohols. The selected spectral range from 400 to 2300 cm\(^{-1}\) was used for multivariate analyses.

2.6 | Automated data analysis

To this purpose, the spectrometers were implemented online to the process with spectral acquisition and analysis set to a defined repetition rate. The obtained raw data were uploaded to cloud storage so that evaluations could be made independent of the laboratory. After the automated import into Matlab the data were processed, followed by the multivariate data analysis, see Figure 2.

Univariate data analysis was used to generate c-t diagrams by integrating characteristic signals. For the multivariate methods, a model was created and validated. As a result, a quantitative real-time model was obtained. An easy-to-use graphic interface allowed to obtain the relevant process data in numerical or graphical representation.

2.7 | Multivariate data analysis

For the multivariate prediction of the concentrations of the process media, a partial least-squares regression (PLS2) for more than one y variable was performed. PLS2 was performed using home-built scripts and PLS_Toolbox version 8.2.1 for MatLab (Eigenvector Research, Inc., Wenatchee, WA). As calibration or training set, 40 \(^1\)H NMR and Raman spectra out of 50, for which reference data were recorded, were used. Crossvalidation was performed applying the leave-one-out procedure. The remaining 10 spectra were used for prediction to identify the best model. The optimal number of latent variables was determined by minimizing the sum of the residual square errors of the prediction (PRESS) for the validation set (Kessler, 2006)

\[
\text{PRESS} = \sum_{i=1}^{n} (y_i^{\text{measured}} - y_i^{\text{reference}})^2,
\]

where \(y_i\) is the actual sample, \(n\) the number of samples and the indices measured and reference refer to predicted and reference data, respectively. A number of 5 latent variables was applied to PLS yielding the best agreement between experimental and predicted data. Through minimizing the root of the mean square error sums of the prediction (RMSE), the quality of the internal crossvalidation (RMSECV) and external validation (RMSEP) was determined as (Kessler, 2006)

\[
\text{RMSE} = \sqrt{\text{PRESS}} = \sqrt{\frac{\sum_{i=1}^{n} (y_i^{\text{measured}} - y_i^{\text{reference}})^2}{n}}.
\]

Goodness and robustness of the multivariate models is characterized as the smallest possible RMSE's close to zero.

3 | RESULTS AND DISCUSSION

In this study, PLS2 was used for multivariate real-time quantification of all reaction components for the one-stage and two-stage biotechnological process. To evaluate the quality of the multivariate model and its predictive power, the reaction profile was first extracted from the univariate data and then described by a mathematical model (cf. Tables S4–5). The C–OH vibration at

![Figure 2](image-url)
880 cm⁻¹ in the Raman spectrum and the methyl resonance in the range of 0.9 and 1.5 ppm in the NMR spectrum were selected to this purpose, see Figure 3. The multivariate results were compared to this method. While for process monitoring the observation of all relevant species and their concentrations is important and a goal of GAC, the univariate treatment only allowed the identification of product signals and their increase to be observed. Educt and by-product signals were difficult to assign in the recorded Raman spectra. In the NMR spectra, a superposition of the educt and product signals occurred in the range of methylene resonances between 3.3 and 4.2 ppm. This was due to signals from fructose, glucose and glycerol. Thus, a multivariate model became essential such that all process media could be interpreted and quantified.

All NMR spectra displayed the solvent water resonance at 4.7 ppm (cf. Figure 3, left). The use of a spectrometer with multiple or gradient pulse capabilities thus allowing for water suppression sequences would improve sensitivity and ultimately the quality of the data analysis (Gouilleux, Charrier, Akoka, & Giraudeau, 2017). The resonance of methyl groups at 1.2 ppm stemmed from the ethanol and could be used for univariate data analysis, as this range was not superimposed by fructose, glucose and glycerol resonances. At 2.0 ppm, the methyl resonance of the acetic acid from the buffer system was clearly recognized. In the Raman spectra, the strong signal at 880 cm⁻¹, which originates predominantly from the C–OH vibrations from ethanol slightly superimposed by the corresponding sucrose, fructose, and glycerol vibrations (Figure 3, right and Figure S2) was visible. Further signals originating from C–C–O stretching vibration of ethanol were observed at 1065 cm⁻¹ (Socrates, 2004). The O–H deformation vibration of primary alcohols appeared at 1460 cm⁻¹ (Socrates, 2004).

Although representative signals of all components are a prerequisite for reaction monitoring, signals of all process media do not need to be fully resolved, specific or even assigned. Whereas true for process monitoring data after calibration and model generation, reference data require resolved, specific, hence quantifiable signals, as was the case for the HPLC reference data used in this study. Since the sugar educts did not provide intense signals, univariate analysis of the sugar transformation was not possible, requiring hence the use of multivariate methods.

### 3.1 | Multivariate data analysis

For the direct determination of the ethanol, fructose, glucose, and glycerol concentration, a multivariate PLS2 model was devised. Forty spectra were used for the creation of the calibration set and another ten spectra for the external validation of the model, see Table 1.

For a representative selection, every fifth spectrum was used as validation, so that no distortions occurred due to the two steady states at the beginning and end of ethanol fermentation. Figure 4 shows the results of concentrations predicted by means of PLS2 from the external validation samples and plotted versus values obtained from the HPLC data as the reference method.

Very good predictions for ethanol and fructose concentrations were achieved using external validation. This is due to the fact that fructose was degraded enzymatically more slowly than glucose. Hence, higher concentrations were observed which can be detected more reliably (Cason, Reid, & Gatner, 1987). The same applied to the ethanol concentration, which was easily detectable due to its very pronounced bands, see Figure 3. Glycerol as a by-product of the incomplete anaerobic process was detectable in small amounts and thus led to variations in the prediction. The RMSEs are listed for both spectroscopic methods. Values were calculated using five latent variables for internal and external validation, see Table 2.

The prediction errors are well comparable for both spectroscopic methods. Only the RMSEP for the ethanol concentration was significantly higher in the NMR data based model with 0.029 as compared to the Raman spectra based model. However, the results were fully adequate for a direct real-time concentration estimation, so that a one-stage ethanolic fermentation could be monitored.

### 3.2 | One-stage fermentation process

The validated model was used to determine the concentration of the process constituents of unknown fermentation samples. In Figure 5, the concentration data of the calibration set (crosses) were plotted against time together with that predicted concentrations from the unknown samples (circles).
Figure 5 shows a typical sigmoidal course of the product formation of ethanol for biotechnological processes. The sugar levels increase over time but then decrease due to the increased activity of the yeast cells. Due to the yeast’s enzymatic reaction mechanism, glucose was more rapidly converted to ethanol and carbon dioxide than fructose. The data and the mathematical model for the ethanol calibration samples showed stable production after several hours of continuous fermentation.

The parameters of the mathematical model listed in Table 3 agree very well between the two spectroscopic methods. The data can be interpreted in terms of the mean maximum concentrations $M_1$, the rate constants $k_1$ and the inflection points $t_1$ of the sigmoidal curves at 17% sucrose for Raman and NMR spectroscopic data.

A detailed explanation of the mathematical description and model is given in equation (3). The model can be used to create a control chart after the steady state has been reached, making it easy-to-identify process fluctuations. This is particularly relevant for an automated feedback loop control to take or adjust countermeasures such as temperature adjustment or educt supply in the event of excessive deviations. The model could also be applied to reflect changes in the

| Table 2 | Root mean square errors (RMSE) for the validation (CV), prediction (P) and explained variance from performing partial least-squares regression type 2 with five latent variables for concentration value determination based on handheld Raman and NMR data

| Component | RMSECV* | RMSEP* | Explained variance |
|-----------|---------|--------|-------------------|
|           | h-Ram   | If-NMR | h-Ram  | If-NMR |
| Glucose   | 0.026   | 0.025  | 0.007  | 0.012  | 0.91  | 0.92 |
| Fructose  | 0.026   | 0.003  | 0.008  | 0.011  | 0.97  | 0.98 |
| Glycerol  | 0.003   | 0.002  | 0.001  | 0.002  | 0.91  | 0.93 |
| Ethanol   | 0.025   | 0.026  | 0.012  | 0.029  | 0.98  | 0.98 |

*amol·L⁻¹.

FIGURE 4 Predicted concentrations of the validation samples of the ethanolic fermentation for the one-stage process (dots), using Raman spectra. The sets consisted of 40 training and 10 test samples for the prediction of fructose, glucose, glycerol, and ethanol. Fit and theoretical bisector are shown as red and green lines, respectively [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 5 Concentration–time diagram of the one-stage ethanolic fermentation based on the handheld Raman and $^1$H NMR data. Calibration data (crosses) are plotted along with concentrations determined via partial least-squares regression type 2 (circles): Glycerol (lowest, yellow), glucose (second lowest, blue), fructose (third lowest, orange) and ethanol (top, purple); univariate fit for the ethanol concentration of the calibration data (purple line). Corresponding $^1$H NMR spectroscopic data were comparable but have been omitted for reasons of clarity with the exception of the multivariate predicted ethanol concentration (diamond, gray) [Color figure can be viewed at wileyonlinelibrary.com]
educt concentration. Hence, after successful monitoring of the one-stage process, a two-stage fermentation was monitored.

3.3 Two-stage fermentation process

The resulting Raman and NMR spectra were processed and analyzed using the established PLS2 model. The detailed list of training and test samples as well as the obtained RMSEs for both spectroscopic methods can be found in Tables S1–2. Figure S1 shows the application of the predicted concentrations from the validation samples against the concentrations determined by HPLC reference analysis. The predicted concentrations were plotted in a c-t diagram (cf. Figure 6). The predicted fructose, glucose, and glycerol concentrations were predicted.

The predicted values are consistent among both spectroscopic techniques in the first stage of the fermentation, that is, 15% sucrose with a maximum yield of 0.7 mol·L⁻¹. When after 1300 min the 20% educt solution was passed into the fermenter, an increase in the ethanol production was observed following the typical sigmoidal curve up to a maximum ethanol concentration of 1.4 mol·L⁻¹. Due to delay time and slower flow rate between the two stages, the second start concentration was 0.9 mol·L⁻¹. In this second stage, the predictions based on the NMR data varied in comparison to the predictions using Raman spectroscopy. This could be explained in terms of NMR acquisition and spectral features, reaction contributions and external laboratory influences. The latter would include fluctuating ambient temperature and its effects on the magnetic field homogeneity, since the spectrometer used proved relatively sensitive to temperature change. Another factor might stem from changes in the large water resonance, as could be recognized in the loadings plot. The signal-to-noise ratio remained comparable in the course of the spectral series. Originating from the reaction, the release of carbon dioxide may have led to gas bubbles in the tubing and the NMR cell itself. Since the amount of carbon dioxide produced increased with time and educt concentration, effects on the precision of the NMR based predicted values are expected to increase along with the reaction. Solid-state particles from the reaction vessel might have also travelled along the connecting tubes and enter the NMR flow-cell, causing similar effects on the data quality. Nevertheless, most of the predicted NMR based c-t values were found close enough to the Raman values. With increasing time, the concentrations of the educts also increased. This was attributed to the aging of

| Parameter | h-Raman | ¹H NMR |
|-----------|---------|---------|
| M₁ | 0.41 | 0.42 |
| k₁ | 0.02 | 0.02 |
| tₛ | 173 | 172 |

³mol·L⁻¹; ⁴min.

FIGURE 6 Concentration–time diagram of the two-stage ethanolic fermentation, based on the handheld Raman and ¹H NMR data. Calibration data (crosses) are plotted along with via partial least-squares regression type 2 indicated concentrations (circles): Glycerol (lowest, yellow), glucose (second lowest, blue), fructose (third lowest, orange) and ethanol (top, purple); univariate fit of the ethanol concentration of the calibration data (15% yellow line, 20% green, upper solid line and whole fit red dashed line). Corresponding ¹H NMR spectroscopic data were comparable but have been omitted for reasons of clarity with the exception of the multivariate predicted ethanol concentration (diamond, gray) [Color figure can be viewed at wileyonlinelibrary.com]
TABLE 4 Parameters from fitting the multivariate predicted ethanol concentration. Handheld Raman and NMR spectra from the two-stage fermentation were used as database

| Parameter | h-Ram | $^1$H NMR |
|-----------|-------|-----------|
| $M_1$     | 0.64  | 0.57      |
| $M_2$     | 0.81  | 0.77      |
| $k_1$     | 0.007 | 0.007     |
| $k_2$     | 0.009 | 0.008     |
| $t_1$     | 403   | 362       |
| $t_2$     | 1783  | 1649      |

$^a$mol·L$^{-1}$. 
$^b$min$^{-1}$. 
$^c$min. 

to the respective maximum concentrations of the product, where $M_2$ represented the difference between the maximum of the second stage and $M_1$. Thus, a relationship of the maximum yield with the sucrose was apparent. However, there was a maximum yield with respect to educt concentration. This could be explained by substrate inhibition at too high levels of educt concentration and by the inactivation of the biocatalyst through ethanol. The rate constants $k$ were found of the same order of magnitude for both methods. However, values varied due to somewhat lower sensitivity and robustness of the NMR instrument due to its 40 nL flow cell, see Figure 6. Further process monitoring would need more extensive isolation and shielding of the NMR instruments. Nevertheless, the parameters $k_1$ and $k_2$ were of the same order of magnitude, which may be seen indicative for the type of yeast. In terms of process optimization, the process monitoring and analysis revealed that the biocatalyst could convert more sucrose, but at the same rates.

The application of bioreactions was aligned with the Green Chemistry principles in using immobilized biocatalysts and water as a solvent. The compact spectrometers matched the size of the small-scale reactor and could be easily coupled to the continuous bioreactor. Fast monitoring techniques enabled real time and automated concentration determination. Compared to conventional chromatographic methods, sampling was unnecessary and hence solvent waste was avoided. The automated near-line process monitoring allowed to prevent frequent physical contact with the reaction vessel, being a major advantage in terms of occupational safety. Automation, waste reduction, small-scale operation, real-time monitoring, and minimum sampling are all goals of GAC.

4 | CONCLUSION

Compact spectrometers proved ideal for monitoring continuous ethanolic fermentation in real time. Disadvantages of the low resolution compared to compact analyzers were well compensated by the use of multivariate data analysis. After automated data acquisition, fast laboratory independent processing of the raw data to generate the relevant reaction profiles was performed such that the current reaction progress in the bioreactor was visualized. As a result, a maximum yield of 1.4 mol·L$^{-1}$ of ethanol was produced with the yeast cells having reached their optimum turnover frequency. Higher temperatures, other immobilization support, different yeast strains, and buffer systems may increase the yields further. During condition optimization, preliminary work indicated that the present compact spectrometers were able to monitor changes in real time as well. The developed PLS2 model may further be optimized by increasing the data set for calibration. Yet, GAC principles suggest to use, the lowest possible number of samples and that spectrometry should replace chromatography with respect to consumed solvent, energy and resources. This could be achieved with the compact spectrometers as described. Conducting the fermentation as one-stage and two-stage processes, obtained c-t fermentation profiles increased the process knowledge of all reactions components and rates, yields and induction periods. Based on the mathematical model developed therefrom, a control chart may be devised which might realize automated control via a feedback loop.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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