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Comparison of Partial Least Square, Artificial Neural Network and Support Vector Regressions for real time monitoring of CHO cell culture processes using *in situ* Near Infrared spectroscopy

Daniel Arturo Zavala-Ortiz^{1,2}, Aurélia Denner¹, Maria Guadalupe Aguilar-Uscanga², Annie Marc¹, Bruno Ebel¹, Emmanuel Guedon¹

1- Laboratoire Réactions et Génie des Procédés, Université de Lorraine, CNRS, LRGP, 2 avenue Forêt de Haye, TSA 40602, 54518 Vandœuvre-lès-Nancy, France.

2- Tecnológico Nacional de México/Instituto Tecnológico de Veracruz: Calz. M.A. de Quevedo 2779, Veracruz, Ver., México.

Corresponding author

Bruno Ebel

Laboratoire Réactions et Génie des Procédés

CNRS-Lorraine University, UMR 7274

ENSAIA, TSA 40602, 2 avenue Forêt de Haye

54518 Vandœuvre-lès-Nancy, France

Email address: bruno.ebel@univ-lorraine.fr

Telephone: +33 3 72 74 39 75

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Abstract

The biopharmaceutical industry must guarantee the efficiency and biosafety of biological medicines, which are quite sensitive to cell culture process variability. Real time monitoring procedures based on vibrational spectroscopy such as NIR spectroscopy, are then emerging to support innovative strategies for retro-control of key parameters as substrates and by-product concentration. Whereas monitoring models are mainly constructed using Partial Least Squares Regression (PLSR), spectroscopic models based on Artificial Neural Networks (ANNR) and Support Vector Regression (SVR) are emerging with promising results. Unfortunately, analysis of their performance in cell culture monitoring has been limited. This work was then focused to assess their performance and suitability for the cell culture process challenges. PLSR had inferior values of the determination coefficient (R^2) for all the monitored parameters (i.e., 0.85, 0.93, 0.98 respectively for the PLSR, SVR, and ANNR models for glucose). In general, PLSR had a limited performance while models based on ANNR and SVR have been shown superior due to better management of inter-batch heterogeneity and enhanced specificity. Overall, the use of SVR and ANNR for the generation of calibration models enhanced the potential of NIR spectroscopy as a monitoring tool.

Keywords

Process Analytical Technology, *In situ* Real-time monitoring by Near Infrared Spectroscopy, Partial Least Squares Regression-PLSR, Artificial Neural Network Regression-ANNR, Support Vector Regression-SVR.

1 Introduction

The production of biologicals remains a challenge due to the structural complexity of these molecules and their sensitivity to changes during the manufacturing process. That is why the Quality by Design (QbD) initiative is being established for building quality through the production process. Accordingly, continuous monitoring of Critical Process Parameters (CPP) affecting the Critical Quality Attributes (CQA) of biopharmaceuticals is required for the establishment of advanced retro-control systems that guarantee the final clinical effect of medicines. Among the diverse CPP affecting cell culture performance, culture media composition has a wide effect since it contains substrates for cell proliferation and product synthesis, and inside which toxic by-products could accumulate and spoil the cell physiological state and thus process performances.

Monitoring by NIR spectroscopy (NIRS) is a promising tool since it is capable of providing multicomponent information directly without sampling using *in situ* probes (Cervera, Petersen, Lantz, Larsen, & Gernaey, 2009; Li, Ebel, Courtès, Guedon, & Marc, 2016). Nevertheless, its use in aqueous matrices is challenging due to the strong absorption of water in the NIR spectrum. Thus, the use of other spectroscopies is emerging. Nowadays, Raman spectroscopy is likely the prevalent technology while NIR developments are likely decelerating for cell culture processes. Recently, a study showed a comparison between the monitoring performance of Raman and NIR spectroscopy in real-time during CHO cell cultures (Li, Ebel, Chauchard, Guédon, & Marc, 2018). Although Raman spectroscopy led to a slightly better predictions, NIR spectroscopy showed a higher signal-to-noise ratio, though in more complex spectra. The inferior capacity of the NIR model was thus mainly attributed to the lack of linear PLS regression for handling complex NIR spectra, likely containing information in non-linear ways. As far as can be ascertained, pure nonlinear calibration has not been explored yet for cell culture monitoring. Therefore, we explored the use of nonlinear regression approaches in NIR spectroscopy. NIR spectra and culture media dynamics are complex and proper multivariate calibration methods are required to extract and relate in an estimation manner, the observed spectra to desired variable properties such as nutrient and by-product concentrations.

The design of the multivariate calibration model is not a trivial matter and relies on technical and regulatory factors. Technical factors mainly concern the nature of the calibration process, while regulatory factors on proper validation (accuracy, precision, specificity, linearity, range of operation and robustness) and management of the NIRS calibration procedure lifecycle (E.M.A., 2014; U.S. F.D.A, 2015).

Concerning the technical factors, there are several challenges to address before successfully calculating compound concentration using NIRS calibration models: confused relationships between compounds, complicated relationships between spectra and compound concentration, inter-batch heterogeneity, noisy spectra, and process changes during normal operation (Li et al., 2016). Moreover, for *in situ* evaluations in chemically and physically complex matrixes such as cell culture media, extreme care must be taken due to scattering effects (Martens, Nielsen, & Engelsen, 2003).

Currently, Partial Least Squares regression (PLSR) is the most used regression method for the generation of calibration models for cell culture monitoring. PLSR maps spectral data linearly into low-dimensional space, and then low-dimensional coordinates are employed to generate the regression or calibration equations using only linear combinations (Höskuldsson, 1988). Although PLSR can address complicated linear relationships, it is unable of properly addressing natively non-linear relationships. However, it may be modified to handle them by including non-linear regression coefficients in the calibration equation or by local modelling (Centner & Massart, 1998; Zavala-Ortiz et al., 2020). Several varieties of PLSR such as Poly-PLSR, Spline-PLSR, Linear-quadratic PLSR and others have been tested for NIR calibrations, but unfortunately, such approaches have shown to be inferior in predictive capacity compared to Artificial Neural Network Regression (ANNR) and Supported Vector Regression (SVR) in spectroscopic data (Balabin & Lomakina, 2011; Balabin, Safieva, & Lomakina, 2007; Blanco, Coello, Iturriaga, MasPOCH, & Pagès, 1999; Zavala-Ortiz et al., 2020). Nevertheless, PLSR and other linear regression approaches have been proven proper methods in major cases and are broadly considered in guidelines for NIRS analytical procedures as recommended by regulatory agencies for cell culture monitoring (E.M.A., 2014; U.S. F.D.A, 2015). This can be explained by the fact that those semi-parametric methods also provide a friendly frame for interpretation issues that facilitate proper management of the NIRS procedure lifecycle.

Contrary to PLSR which maps data into lower dimensional space, SVR firstly maps data into a higher dimensional feature space which is nonlinearly related to the spectroscopic and chemical space (input vectors) (Amendolia et al., 2003). The feature space is the space where a maximal separating hyperplane is constructed. Then, the goal of SVR is to generate a regression function, or hyper plane, that has a maximum number of calibration samples input vectors at most an ε deviation from an actual concentration, and at the same time keeping the function as flat as possible (Smola & Schölkopf, 2004). However, it may not be the case that such regression function which approximates all input vectors with ε precision, actually exists. Then a soft margin of slack variables is introduced for coping with unfeasible constraints of optimization (Vapnik, 2000). The main difference of SVR from other regression methods is that its objective is not merely to reduce the fitting error but to fit the error within a particular threshold ($\pm \varepsilon$). Indeed, this method considers not only nonlinear relationships between components of the input vectors (spectroscopic and chemical data) but also inputs themselves as they can be used for definition of the hyperplane as supported vectors (SV).

ANNR is inspired in biological neural networks. At first, they were focused on learning tasks by considering patterns in examples, but now they are used in diverse applications, especially for those that require difficult-to-express algorithms. In the context of NIRS calibration, ANNR are mostly employed under the supervised learning paradigm (Næs, 2004). The core of an ANNR is its basic unit, called the artificial neuron, which constitutes the building brick of the network used for regression. The structure and function of a single neuron can be summarised as an element receiving two main inputs: inputs p (i.e. absorbance at specific wavelength) which is multiplied by a scalar weight w , and a bias value b . Both inputs are passed to a sum, and the sum output, often referred as the net input, goes into a transfer function, which produces the final neuron output value. Neurons are arranged for the construction of a particular network topology and w and b values from all neurons must be tuned by training as a whole for achieving a particular learning task as to calculate a concentration value based on spectra inputs.

Non-linear approaches such as ANNR or SVR, have several advantages, namely their flexibility to model complex and non-linear relationships. Recently, non-linear and non-parametric regression approaches based on SVR and ANNR have gained popularity in

developing NIR calibration models (Barchi et al., 2016; Beiroti, Aghasadeghi, Hosseini, & Norouzian, 2019; Dong et al., 2017; Jin, Wu, Liu, & Wu, 2013; Nadian, Rafiee, Aghbashlo, Hosseinpour, & Mohtasebi, 2015). As far as can be ascertained, management of nonlinearity in cell culture process monitoring has been addressed in only some few works through using local modelling (Tulsyan et al., 2019, 2020; Zavala-Ortiz et al., 2020) and global non-linear SVR calibration (Zavala-Ortiz et al., 2020). Most of these works are focused on the direct comparison of accuracy achieved by different regression approaches, while little attention has been paid to the nature of the process being modelled and its impact on precision, linearity, and robustness. Therefore, the main aim of this study is to provide an extensive frame to evaluate the convenience of PLSR, ANNR and SVR for monitoring CPP in CHO cell culture processes.

2. Materials and Methods

2.1 Cell culture for data acquisition

Cell cultures of CHO cells were performed in 2 L bench-top bioreactors (Pierre Guérin, France) with a 1.5 L working volume. Six bioreactor cultures were performed for the calibration set, obtaining off-line measurements and for covering bioprocess variability: Three batch cultures, two feed-harvest cultures with medium renewal and one batch culture with glucose spiking. The three batch cultures were intended to observe inter-batch heterogeneity as well as in-line and off-line values expected routinary responses. Moreover, seeking limitation of model overfitting, feed-harvest cultures were used for increasing the variance of cell cultures within the calibration process, which could enhance model prediction capability by breaking some confused relationships in spectra. These cultures were started after a first phase in batch mode, then 2/3 of cell culture was withdrawn and replaced by fresh culture medium. This procedure was repeated 2 and 4 times for these 2 feed-harvest cultures respectively. Batch culture with glucose spiking was used for increasing cell density and break the relationship between low glucose concentration with high cell density, as well as other eventual confused relationships.

The culture medium was a protein-free medium mixture consisting of a 1:1 volume ratio of PF-CHO (HyClone) and CD-CHO (Fisher Scientific) supplemented with 4 mM L-glutamine (Sigma Aldrich) and 0.1 % pluronic F-68 (Sigma Aldrich). Moreover, a totally independent-from-calibration batch culture was also performed to test the prediction power of models, using ActiPro (HyClone) as the culture medium. The genetically modified DG44 CHO (CHO M250-9) cell line was used, kindly provided by the Bioprocessing Technology Institute (Singapore). Dissolved oxygen (DO) was controlled at 50 % air saturation; agitation rate was fixed at 90 rpm throughout the culture. Temperature was maintained at 37 °C and pH was set and controlled at 7.2 using 0.5 M sodium hydroxide and CO₂.

Off-line concentrations of glucose, lactate, glutamine and mAb were determined using enzymatic kits (Roche 06681743001, 07395655001 for mAb and glutamine respectively; Thermo Scientific 981780, 984308 for glucose and lactate respectively) with an automatic spectrophotometer (Thermo Scientific GALLERY) against external standards. Viable cell density (VCD) was calculated by the trypan blue dye exclusion technique using an automatic cell counter (Beckman Coulter, Vi-CELL).

2.2 Development of calibration models (PLSR, SVR & ANNR)

In situ spectral scanning of bioprocess culture media was carried out with a NIR transfectance probe with a 1 mm pathlength (Precision Sensing Devices, MA). The sterilisable probe was connected to an Antaris II spectrometer (Thermo Scientific, USA). Each NIR spectrum corresponded to an average of 128 scans with an 8 cm⁻¹ resolution from 4,000 to 10,000 cm⁻¹ (equivalent to 1000 – 2500 nm). A main set of calibration, consisting of 168 spectra, was acquired from the six bioreactor cultures with PF-CHO (HyClone) and CD-CHO (Fisher Scientific) culture media, and divided into a calibration set (135 samples) and validation set (33 samples). A sample consisted in a NIR spectrum corresponded and an off-line concentration data. Consequently, the inputs of models were spectra while concentration values were the outputs of models.

The main calibration set, used for model development, was partitioned using the Kennard-Stone algorithm. The validation set was not used for calibration. Calibration models for VCD, glucose, lactate, and glutamine were generated while spectral pre-processing for PLSR models was as simple as possible seeking conservation of maximum information contained.

The main strategy was to normalise spectra using Multiplicative Scatter Correction (MSC), Probabilistic Quotient Normalization (PQN), Standard Normal Variate (SNV) and/or filter (derivative, Extended Multiplicative Signal Correction (EMSC)) spectra and once a promising combination of spectra pre-processing was determined, only slight tuning was performed based on model performance so that predictive power of the models was enhanced.

The construction of models was focused on low values of the Root Mean Square Error of Cross-Validation (RMSECV) using the calibration set of 135 samples, while assessment of generalization and final quantitative performance of models were evaluated through the Root Mean Square Error of Validation (RMSEP) using the validation set of 33 samples. Determination of latent variable (LV) number for PLSR models was based on the goodness of estimation (Q²_Y): the minimum number of LVs was obtained when Q²_Y ceased to improve, using a venetian-windows cross-validation approach. The particular spectral pre-processing technique used for each compound with PLSR models was also used for all other regression methods for comparison of models to be mainly based on regression methods.

For the SVR models, an epsilon-support vector regression using a Gaussian radial basis function kernel was used. Optimization for gamma and epsilon values was also performed using a venetian-windows cross-validation approach with maximal error values corresponding to deviations up to 10 % from actual values. In the case of ANN, the network size was firstly evaluated without cross-validation (100 iterations) for inferring the minimum number of neurons to be used in regressions, and then the nature of the network structure was assessed. Early stopping (maximum 20 training iterations using backpropagation with tanh as an activation function at 0.125 learn rate) was used to avoid overfitting (Hagan, Demuth, Beale, & De Jesus, 2004). The selection of the particular structures of ANN and SVR models were those with the lowest RMSECV and RMSEP. The best model of each regression method (PLSR, ANN and SVR) for each compound was retained for further evaluation.

Multivariate calibration models and statistical analysis were performed in MATLAB[®] (Statistics and Machine Learning Toolbox™, MATLAB R2016a, The MathWorks, Inc., Natick, Massachusetts, United States). Chemometrics software was also used (PLS_Toolbox[®] 8.2.1, Eigenvector Research, Inc., Manson, WA, United States).

2.3 Comparison of calibration models

Comparison of models was based on requirements proposed by regulatory agencies for validation of NIR quantitative analytical procedures, such as accuracy, precision, linearity, specificity and robustness (E.M.A., 2014; U.S. F.D.A, 2015). Because of such different structures of PLSR, ANNR and SVR models, the comparison was addressed under quantitative and qualitative approaches. Quantitative comparison of models comprised direct comparison of accuracy, precision, and linearity, using calibration data (168 samples). Qualitative approaches were undertaken to analyse the specificity of models and particularly, for evaluating the performance of models under similar conditions, as expected during real-time monitoring. Real time monitoring consisted in automatic acquisition of NIR spectra every 20 min. Thus, a single cell culture process was qualitatively analysed using some hundreds of samples. For instance, the batch with mean inter-batch heterogeneity (Figure 8) consisted of 550 spectra. Discussion of some aspects of models, such as range of operation and robustness, is undertaken for the two types of comparison, particularly during qualitative analysis. For proper comparison (Anscombe, 1973), quantitative analysis was focused on numerical calculations and qualitative analysis on graphical analysis of model performances. For quantitative comparison, accuracy was represented as the RMSEP, and statistically compared (One-way ANOVA with a post-hoc Tukey test) in terms of the absolute differences between the predicted and the real concentration values. Precision was represented as the mean of residuals (MoR) and statistically analysed in terms of homogeneity of variance of the residuals using multiple Levene's test. Linearity was represented as the correlation coefficient (R^2) of the calibration plot and statistically evaluated in terms of correlation of the residuals from the calibration plot using the Durbin-Watson test (Zavala-Ortiz et al., 2020).

For qualitative comparison, the three batch cultures were used for analysing the effect of inter-batch heterogeneity on predictive power of models. The batch culture with glucose spiking was used to evaluate the specificity of models to identify and relate glucose NIR signal within other NIR confused signals. The culture with mean inter-batch heterogeneity was used as internal validation while considered as usual operational condition. Finally, an independent-of-calibration culture with a different culture media (ActiPro) was used for

external validation through direct analysis of kinetic profiles, then some aspects of model robustness is discussed.

3. Results and Discussion

3.1 Spectra analysis

Analysis of spectra revealed noisy responses caused by optic fibre noise around to the 2500 nm range (Figure 1A) as frequently reported (Clavaud, Roggo, Von Daeniken, Liebler, & Schwabe, 2013a). Consequently, this spectral section was not used for regressions. The most common techniques to eliminate undesired spectra variations caused by light scattering (MSC, EMSC, first derivative) were evaluated (Figure 1B). The standard deviation per each wavelength was used to elucidate the effect of scattering on calibration spectra. The presence of additive, multiplicative and wavelength-dependent effects due to scattering was evaluated within the calibration set spectra. Raw spectra contained great variation within all the wavelength range, likely linked to multiplicative effect (offset of spectra). Analysis also revealed some scattering effects such as additive effect (baseline shift), and a likely wavelength-dependent effect (Rinnan, Berg, & Engelsen, 2009). All spectra pre-processing techniques reduced the standard deviation caused by scattering and some of its effects. However only EMSC was capable of limiting the wavelength-dependent effect (Martens et al., 2003), though the use of MSC plus spectra derivation also limited this late effect. Models based on PLSR are particularly sensitive to multiplicative effects (Martens et al., 2003) and proper spectra pre-treatment is essential for developing accurate calibration models. Final spectral pre-processing is shown in Table 1.

Analysis of calibration spectra by Principal Component Analysis (PCA) allowed data analysis using only few spectral variables in terms of principal components as shown in Figure 2. The first principal component (PC) explained 77 % of the spectral variance, and the second PC 9 %. This analysis revealed the evolution of the culture process in the PC space, also called as the process trajectory (Clavaud et al., 2013a; Henriques, Buziol, Stocker, Voogd, & Menezes, 2009). The main direction of the process was explained by PC1, which showed that spectra in the PC space go from right to left during the progression of the

cultures, as shown by the dark arrow in Figure 2. Inter-batch variability is mainly emerged by PC2 in the way that batch trajectories remained similar with only different offsets in the PC2 axis (grey line in Figure 2).

PCA was also used to infer the nature of the relationship between spectra in the PC space and concentration as shown in Figure 3. Non-linear relationships were observed for ammonium ions and glutamine for all the concentration ranges, with hyperbolic and exponential profiles respectively. There were compounds with a major linear relationship for a large concentration range such as viable cells and glucose, but also for monoclonal antibodies (mAb) and lactate to a lesser extent. For these compounds, nonlinearity was observed at relatively low and high concentrations, corresponding to the end of cell culture processes. For instance, when viable cell concentration surpassed $70 \times 10^5 \text{ cells.mL}^{-1}$, there was no clear linear relationship between spectra (in terms of PC 1) and concentration. The nonlinear relationship for VCD appeared in calibration samples of the stationary and dead phase of cultures, when major changes in cell physiology strongly impacted chemical and physical properties of the culture broth. For instance, the scattering profile in the stationary and dead phase is extremely different in regard of the growing phase. In the other hand, there is a release of cytoplasmatic compounds from dying cells, which changes the chemical properties of the culture broth.

This phenomenon was even more marked for lactate and mAb, where an obvious linear relationship is completely lost when concentration surpassed 10 mM and 200 mg.L⁻¹, respectively. Although there are many reports of calibration models showing good fitting for several parameters, concentration ranges are limited and not broader than those reported here (Arnold, Crowley, Woods, Harvey, & McNeil, 2003; Cervera et al., 2009; Qiu, Arnold, & Murhammer, 2014). Moreover, when lack of accuracy is reported, particularly during the cell death-phase, it is usually disregarded or not investigated (Clavaud, Roggo, Von Daeniken, Liebler, & Schwabe, 2013b). As shown in Figure 3, our analysis suggests that such lack of accuracy in broad concentration ranges can be caused due to nonlinear relationships between spectra and concentration.

The case of glucose and glutamine is perhaps the most thought-provoking, where a linear response is observed while no relationship is detected for some samples (Figure 3). In glucose profiles, there are three main obvious linear profiles, separated likely due to inter-batch heterogeneity effects. Even at low concentrations such a linear relationship is preserved.

However, there were samples that showed no relationship between PC1 and glucose concentration, shown as horizontal and vertical profiles close to the axis. Similar phenomena were observed for the mAb and glutamine concentration-spectra relationship. The parabolic and exponential profile relationship for ammonium and glutamine, respectively, may be a strong challenge for PLSR modelling. The nonlinear patterns for all other compounds also represent a major challenge for linear calibration models. Therefore, calibration based on nonlinear regression techniques are likely required for management of calibration spectra and thus proper monitoring of the complete cell culture process.

Although it is common to make general assumptions of non-linearity based on the physical properties of the analysed matrix such as scattering effects (Miller, 1993), data suggested that there may be inherent nonlinear relationships between spectra and some compound concentrations, which may be independent of the physical properties of the matrix. Such particular nonlinear relationships are mainly related to changes in the interaction of several absorbing functional groups during the culture progression, which may lead to absorption bands shifts or resonance effects such as the Fermi and Darling-Dennison resonances (Siesler, 2002), requiring particular management of nonlinearity relationships for information extraction from spectra (Agranovich & Kamchatnov, 1999). Consequently, nonlinear relationships are different depending on the compound analysed. Therefore, treatment of all variables under the same regression approach (i.e. particular nonlinear PLSR (Balabin et al., 2007)) seemed inappropriate to generate accurate calibration models. Therefore, two main regression techniques were used for addressing nonlinearity, SVR which is a sample-based method and ANN which is a variable-based method. The main focus was put on classic compounds (glucose, viable cells, glutamine and lactate) since mAb monitoring has been previously analysed elsewhere (Zavala-Ortiz et al., 2020).

3.2 Quantitative comparison of calibration models

Direct comparison of model performances is summarized in Table 2. Accuracy, precision, and linearity were measured through different perspectives. Accuracy, as the RMSEP is intended to depict the distance between actual and predicted concentration. For all compounds, nonlinear models achieved lower RMSEP than those observed with PLSR

models, which suggested the limited performance of PLSR for cell culture monitoring. For instance, the accuracy for glucose was 3.37, 2.29 and 1.32 mM for PLSR, SVR and ANNR respectively. However, statistical analysis by one-way ANOVA with Tukey test revealed no significant difference. In fact, two group means are significantly different if their intervals are disjoint and intervals overlapped for all variables using the three different regression models. This fact explained why even promising, results using nonlinear models are statistically equal to PLSR performance.

In contrast to accuracy, which describes the distance between actual and predicted values, precision describes the variation on predicted values when the same (or almost similar) sample is measured by the same model. The nature of calibrating using samples from heterogeneous cell culture processes is a great constrained to acquire data for precision analysis. Accuracy and precision are often analyzed using the same data (differences between actual and estimated values) rather than acquiring particular data for proper precision analysis (repeated measurements). Therefore, accuracy and precision are generally confused and then precision has not received proper attention in cell culture monitoring studies. Then, it was proposed to analyzed precision by homoscedasticity analysis using the variance of the residuals.

The residuals for a particular compound were calculated using a particular calibration model based on either PLSR, SRV or ANNR. Then, their distribution was computed and analyzed. If the variance of the residuals has the same distribution, then it is likely that PLSR, SVR and ANNR models have globally the same precision. Contrary, if models differ on distribution shape, it is clear that those with narrow distributions are likely more precise. The differences between those distributions were statistically analyzed by Leven's test. Analyses revealed that SVR and ANNR are likely more precise than PLSR as shown in Table 2. These results are in line with a previous report on spectroscopic data (Balabin et al., 2007). In general, SVR is more precise than ANNR. For instance, the precision for viable cells was 3.28, 0.25 and 0.54 (cells.mL⁻¹) x10⁵ for the PLSR, SVR and ANNR respectively (Table 2). A former report showed that ANNR and SVR regressions had similar confused accuracy-precision performance when analysing NIR spectra of gasoline-ethanol mixtures (Balabin & Lomakina, 2011), whereas our results clearly suggests that SVR is superior as statistically different distributions of residuals with lower MoR values are achieved (Table 2). This could

be due to samples from cell culture contained confused information related to process progression. Then the better performance of our SVR models can be explained by the fact that SVR is a sample-based regression method, which could have also managed information of the process progression (Sun, Zhao, Shi, & Yu, 2014), and thus performed better than models based on ANNR even if both models relate data in a nonlinear way.

Although R^2 is a parameter that shows how much variability is explained by the model, it is usually used as linearity term using the calibration plot data. Relative higher R^2 coefficients were achieved when both ANNR and SVR were used for generation of calibration models in contrast to PLSR. However, higher R^2 values do not guarantee that models predict concentration linearly to actual concentration. Analysis of correlation of the residuals revealed that there was correlation of the residuals for all compounds when using calibration models based on PLSR. The use of SVR and ANNR for generation of calibration models improved the performance. In general, those nonlinear regression methods lead models to linearly predict concentration values based on real concentration of compounds experimentally measured. However, this was not the case for compounds such as glutamine and lactate when ANNR was used. In general terms, SVR seemed not only as the more accurate and precise regression method for building calibration methods but was also capable to predict concentration linearly to actual concentration using spectra in major cases.

3.3 Qualitative comparison of models during real-time monitoring

After quantitative analysis, the models were evaluated qualitatively based on their performance during real-time monitoring. First, the effect of inter-batch heterogeneity for glucose monitoring was evaluated using three different batch cultures. Then, the specificity of models was analysed using the batch with glucose spiking. For analysing the performance of models during routine monitoring, special attention was focused on the batch with mean inter-batch heterogeneities since it is likely to depict most cultures under normal conditions. Finally, an independent batch culture with glucose spiking and dedicated calibration, was analysed and discussed for some robustness issues.

3.3.1 Effect of inter-batch heterogeneity

Usually, the effect of inter-batch heterogeneity is not particularly addressed since the majority of reports show only the predicted profile of a single culture (Henriques et al., 2009; Kozma et al., 2017; Li et al., 2018; Milligan, Lewin-Koh, Coleman, Arroyo, & Saucedo, 2014; Qiu et al., 2014; Rhiel, Cohen, Arnold, & Murhammer, 2004) or are limited to numerical analysis (Chung, Arnold, Rhiel, & Murhammer, 1996). Therefore, the performance of models within different batch cultures were analyzed.

The three batches shown different glucose profiles, as predicted by the PLSR-based model (Figure 4A). For instance, batch 3 has a marked offset during the whole culture while batch 1 and 2 had some frames with accurate predictions and some other frames with miss predictions, particularly at the beginning and at the end of the culture. The effect of inter-batch heterogeneity on prediction performance was then analyzed using PCA (Figure 4B). Though calibration set is composed of several cell cultures with particular trajectories (Clavaud et al., 2013b), a global trajectory may be obtained when considering all cultures within the calibration set, which is depicted by the dark arrow in Figure 4B. It is worth to note that when a particular batch trajectory agreed with the global trajectory (dark grey arrow, Figure 4B), there are accurate predictions as those for batch 1 and 2 in the 55-130 h range (Figure 4A), even if there are a strong offset in PC2 from global trajectory. On the contrary, deviations to calibration main process trajectory by particular batches explained limited prediction performance (Figure 4A). Deviations occurred in three main patterns: semi-perpendicular batch trajectory (light grey arrow 1 in B) affecting the beginning of batch 2, batch trajectory with lower slope (light grey arrow 2 in B) affecting the beginning of batch 1 and 2, and batch trajectory with higher slope (light grey arrow 3 in B) affecting the majority of batch 3. As far as can be ascertained, there is only one report showing systematic performance of NIR models through different batches, though no discussion on inter-batch heterogeneity effects is provided (Clavaud et al., 2013b). Our results suggest that this phenomenon could be explained by the fact that a trajectory is a relationship between PC1 and PC2 (likely similar to PLSR latent variables), which is then used for performing regression. If such relationship between PC1 and PC for a particular batch is not conserved compared to that of the calibration data, there will be mispredictions as those for batch 3 (Figure 4B).

Preparation of the three batches was focused on repeatability and no special change on neither inoculation, nor culture media was intended. Results have shown that PLSR is particularly vulnerable to inter-batch heterogeneity. Therefore, the implementation of nonlinear regression methods was evaluated for the generation of calibration methods. The in-line profiles for SVR and ANNR were closer to off-line concentration data (Figure 5) than those established with PLSR (Figure 4A). Major improvements were observed for batch 1 and 2 at the beginning of cultures, where glucose concentrations were properly predicted. Inter-batch heterogeneity, which strongly affected batch 3 using PLSR (Figure 4A), was more efficiently managed by calibration models based on either SVR or ANNR (Figure 5). For instance, in batch 3, while the in-line concentration profile by PLSR model (Figure 4A) was completely offset for all the culture, profiles obtained by ANNR and SVR models were properly fitted to off-line profiles during the first 90 h of the culture process. However, the effect of inter-batch heterogeneity still affected the prediction of ANNR and SVR models during the last part of the culture.

Prediction power by models is affected by inter-batch heterogeneity since models are not totally theoretical but rather partially empirical. This means that prediction is based on confused and global changes in cell culture media rather than in particular changes on glucose vibrational NIR movements. Inter-batch heterogeneity or variability can then be understood as changes in the composition of the cell culture processes that do not match with the variability pattern established by the calibration method. Enhancement of prediction power in inter-batch heterogeneity conditions would necessarily require that models be highly specific for the compound of interest and then less dependent on confused relationships (Dingari et al., 2011; Norris & Ritchie, 2008) within culture progression.

3.3.2 Specificity of models

Specificity, as defined by the International Conference on Harmonization (ICH), is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present (ICH, 2005). Specificity analysis of NIR models is a very challenging task as such analytical procedures are mainly based in correlation. Correlation between variables (i.e. spectra and concentrations) cannot be sufficiently explained by the source of such

relationships, even if calculated concentration values by models are in perfect agreement with the off-line reference data (Norris & Ritchie, 2008).

The more specific the model is to the analyte of interest, the less the model will depend on the pattern of confused relationships in the cell culture media for proper prediction. Therefore, in this work we used an alternative approach to test specificity using the batch culture with glucose spiking. This batch was operated similarly to other batches, but glucose was added at 173 h which completely interfered with the normal nature of batches and thus, the normal confused pattern of cell culture media used by calibration models to predict concentration. If a model is highly specific for glucose, then it would perform prediction more likely based on particular glucose NIR signals rather than on confused patterns of cell culture media.

The performance of models for the batch with glucose spiking is shown in figure 6. During the period operated between 0 h and 172 h, the three models performed relatively good. However, once glucose was spiked into the bioreactor, the performance of models greatly differed. Addition of a concentrated glucose solution into the bioreactor caused an increase up to 20 mM. Only the model based on SVR properly predicted such concentration value, while both models based either on ANNR or PLSR showed a limited increase in glucose concentration of 16 and 7 mM respectively. Moreover, both PLSR and ANNR predicted an increase in glucose concentration after the 200 h of the batch culture, which is totally contradictory with decrease in glucose concentration, as observed by offline analysis. These phenomena were then analyzed using PCA.

The fact that the model based on PLSR predicted an increase in glucose concentration rather than the observed decrease, can be explained by the fact that addition of glucose strongly changed the trajectory of the culture process as shown by black arrow in figure 7. PCA had previously shown (Figure 2) that the progression of the culture was mainly explained by PC1 in a right to left direction, which agree with the decrease in glucose concentration. In this context, while the trajectory of the batch agrees with the global trajectory of the calibration process, the model rightly predicted glucose concentration. Glucose spiking changed the trajectory of the culture (light arrow in Figure 7) to a right direction, as the kinetic of the culture went backward up to states similar to those observed at the beginning of cultures where high concentrations of glucose were observed. This could explain the increase in

glucose concentration as predicted by PLSR-based model. This revealed the lack of specificity of the model based on PLSR, which performed prediction using mainly global changes of culture media rather than on glucose NIR signals. The lack of specificity for the model based on ANNR may be caused by the fact that calibration samples of this condition (glucose spiked culture) comprised only a negligible fraction from the calibration sample set. Thus, generalization of glucose information was mainly based on the majority of samples. Prediction of glucose concentration by SVR was likely enhanced by the fact that it is a sample-based regression method, which first detected abnormality in those few calibration samples, separated them in the feature space and then relate them to concentration using a nonlinear relationship. This nature of SVR is likely advantageous as it could properly depicted the off-line concentration profile (Figure 6). However, noise in the form of bounce was important and thus accuracy and precision were limited, which could eventually compromise further control strategies (Burns & Ciurczak, 2007; Craven & Whelan, 2015).

3.3.3. Models for routine monitoring

The models were used for real-time monitoring using the batch culture with mean inter-batch heterogeneity. Comparison for viable cells, glutamine, lactate, and ammonium concentrations is shown in figure 8. For viable cells concentration, ANNR and PLSR did not predict properly this parameter since both models sub-estimated cells concentration at the stationary phase. Only the model based on SVR properly predicted the viable cell concentration. On the other hand, both ANNR and SVR accurately predicted glutamine concentration while the model based on PLSR misestimated concentrations at the beginning and end of cultures, due to extrapolation phenomena (data not shown).

The lactate concentration profile estimated by the three methods were likely the same though PLSR and ANNR showed more bounce. In fact, during the last part of the culture, the three models failed to accurately predict lactate concentration. Cell viability during this part of the culture was low and diverse intracellular compounds as well as cell debris were in the culture media, which could have a strong impact on the prediction and limited generalization of models as discussed elsewhere (Clavaud et al., 2013a; Henriques et al., 2009; Zavala-Ortiz et al., 2020).

For monitoring of ammonium ion concentration, the model based on PLSR completely failed at the beginning of the culture, while models based on SVR and ANNR accurately predicted concentration in this initial frame. Between 45 h and 60 h of culture, there was an abrupt concentration increase that was not properly predicted by any model. Only SVR predicted some actual concentration properly though the majority of prediction sub-estimated actual concentrations. Prediction by the three models was enhanced after the 90 h of culture, where bounce was more evident for PLSR models and limited for ANNR and particularly for SVR.

1.1.4 Robustness analysis

As calibration models are partially empirical, evaluation of robustness could then also be considered in an empirical and interpretative manner. The performance of models based on PLSR, SVR and ANNR for glucose monitoring in a cell culture process with ActiPro as the culture medium, is shown in Figure 9. The culture first consisted in a batch culture and then glucose was spiked at 120 h. The three models predicted this binomial nature though with different closeness to off-line profile. The model based on ANNR had the best performance since it accurately predicted glucose during the first part of the culture (0 h – 60 h) and accurate trends were observed until glucose depletion. Then, ANNR based model detected the increase in concentration, though this was not accurate. The model based on PLSR had a similar performance, though with a larger offset, sub-estimating experimental concentrations, this behavior was more remarkable for SVR-based model. After glucose spiking, the PLSR did not predict the abrupt increase in glucose concentration. On the contrary, predicted a decrease in concentration. On the other hand, SVR had likely the worst performance during the first part of the culture with a large offset by comparison with experimental concentration patterns. However, during the last part of the process when glucose was spiked into the culture, a concentration increase was predicted, as observed with the model based on ANNR. Former analysis of model performances suggested that PLSR was vulnerable due to lack of specificity and an inability to handle inter-batch heterogeneities. However, analysis of the batch culture performed with ActiPro culture media revealed the PLSR model was more accurate than SVR. This could be explained by the fact that PLSR was more likely based on information about cell culture progression rather than specificity. As the direction of the

culture progression using ActiPro also matched that from calibration, PLSR could predict the profile though with a strong offset. Once glucose was spiked, spectra rested outside the calibration space (data not shown) and PLSR was not able to properly perform prediction in extrapolation conditions. This could explain why it predicted a decrease in concentration whereas an increase was expected. On the other hand, SVR had been shown to have more specificity to glucose and it was less vulnerable to inter-batch heterogeneity. However, it is likely that such high specificity for glucose in the normal culture media was based on information of glucose in association to some compound(s) that was not as abundant or included in the ActiPro culture medium as in the initial culture medium used in the study, causing misestimation of glucose concentration. The model based on ANNR was likely to better generalize specificity for glucose. In contrast SVR is a sample-based regression method which only generalized using some samples rather than from all calibration samples. Thus, for robustness issues, ANNR is likely the best option if such great variations on culture media are intended during cell culture processes.

4. Conclusion

In this work, experimental evidence was provided of nonlinear relationship between *in situ* captured NIR spectra and culture media compounds during CHO cell cultures. This fact limited the performance of the widely used PLSR for the generation of monitoring procedures using spectra. Moreover, nonlinear relationship was unique for each culture media compound, which is a challenge since modifications to PLSR should be taken into account as to enhance prediction power. The novel use of SVR and ANNR for the generation of monitoring procedures was shown to overcome some of the PLSR limitations, particularly for nonlinearity issues, management of inter-batch heterogeneities and enhanced robustness. Although there are numerous reports for the monitoring of cell culture processes, the great majority is performed in academia environments and is PLSR based. This fact could explain by the fact that, this strategy treatment has not been totally established in industry. Overall, the data presented in this work highlighted the complex nature of cell culture processes and the need of novel and innovative chemometric techniques for the enhancement of monitoring procedures using vibrational spectroscopy such as NIR. This study encourages future studies to expand the capabilities of vibrational spectroscopy in the animal cell culture technology.

Real time monitoring could provide immediate information about the state of the cell culture process so that retro-control could be performed, leading to cutting edge strategies according to Process Analytical Technology and QbD initiatives.

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DATA AVAILABILITY STATEMENT

The datasets generated and analyzed in this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Spectral pre-processing for calibration models

Compounds	Spectral pre-processing
Viable cells	MSC + 1st derivative (15, 2)
Glutamine	EMSC
Glucose	MSC
Lactate	EMSC + 1st derivative (15, 2)
mAb	EMSC
MSC: Multiple Scatter Correction; EMSC: Extended Multiple Scatter Correction; Values for derivative: filter width, polynomial order.	

Table 2. Statistical analysis of PLSR, SVR and PLSR models

	Accuracy (RMSEP)	Precision (MoR)	Linearity (R^2)	Model structure
Glucose	PLSR: 3.37 ^A SVR: 2.29 ^A ANNR: 1.32 ^A	PLSR: 2.08 ^C SVR: 0.02 ^A ANNR: 0.12 ^B	PLSR: 0.85 ^{NL} SVR: 0.93 ^L ANNR: 0.98 ^L	PLSR: 5 LV SVR: 135 SV ANNR: 4 N
Lactate	PLSR: 3.54 ^A SVR: 2.93 ^A ANNR: 3.23 ^A	PLSR: 1.46 ^C SVR: 0.62 ^A ANNR: 1.00 ^B	PLSR: 0.75 ^{NL} SVR: 0.83 ^L ANNR: 0.78 ^{NL}	PLSR: 5 LV SVR: 97 SV ANNR: 4 N
Glutamine	PLSR: 0.67 ^A SVR: 0.42 ^A ANNR: 0.43 ^A	PLSR: 0.270 ^B SVR: 0.16 ^A ANNR: 0.22 ^B	PLSR: 0.68 ^{NL} SVR: 0.87 ^L ANNR: 0.87 ^{NL}	PLSR : 6 LV SVR : 131 SV ANNR : 5 N
Ammonium	PLSR: 0.82 ^B SVR: 0.53 ^A ANNR: 0.53 ^A	PLSR: 0.15 ^B SVR: 0.03 ^A ANNR: 0.18 ^C	PLSR: 0.45 ^{NL} SVR: 0.78 ^L ANNR: 0.70 ^L	PLSR : 4 LV SVR : 135 SV ANNR : 4 N
Viable cells	PLSR: 6.88 ^A SVR: 7.18 ^A ANNR: 5.29 ^A	PLSR: 3.28 ^C SVR: 0.25 ^A ANNR: 0.55 ^B	PLSR: 0.93 ^{NL} SVR: 0.93 ^L ANNR: 0.97 ^L	PLSR : 5 LV SVR : 128 SV ANNR : 2 N

Different letters of exponents indicate statistically significant differences between regression technique for accuracy and precision. Exponents for linearity indicate if models predict concentration linearly to actual concentration (L) or a non-linear deviation during prediction is detected (NL). Accuracy was measured as the RMSEP and statistically compared (One-way ANOVA with Tukey test) in terms of the differences between the predicted concentrations and the actual concentration. Precision was measured as the mean of residuals (MoR) and statistically analyzed in terms of homogeneity of variance of the predicted residuals using multi-way Levene's test. Linearity was measured as the correlation coefficient (R^2) of the calibration plot and statistically evaluated in terms of correlation of the residuals using the Durbin-Watson test.

LV: Latent variable, SV: Support vector for SVR models, N: Neurons for ANNR models;
Units for RMSEP and MoR are the same: (cells.mL⁻¹) x10⁵ for viable cells, and mM for
glucose, lactate, glutamine and ammonium.

Figure legends

Figure 1.

Nature of spectra for calibration. Process instrument raw spectra for calibration (A), effect of common spectral pre-treatments on spectra variability and scattering effects (B). MSC: Multiplicative Scatter Correction, EMSC: Extended Multiplicative Scatter Correction, SG1D: Savitzky–Golay first derivative.

Figure 2.

Principal Component Analysis of calibration spectra. Main process trajectory associated to culture progression is depicted by the dark arrow, points in the right side corresponding to early process samples while those in the left side to last process samples. Inter-batch heterogeneity is emerged as an offset of the process trajectory in principal component 2, which is depicted by the grey line.

Figure 3.

Main relationships between spectra and compounds concentration. Each plotted point represents a single process sample defined by concentration (y-axis) and spectra (x-axis; roughly 1557 NIR absorbance values compressed into a single data point using PCA).

Figure 4.

Effect of inter-batch heterogeneity on glucose model based on PLSR. Limited performance shown as profile offset and deviation to actual concentration trends (A), calibration main process trajectory (grey arrow) and trajectories of the three batch cultures (B). Deviations to calibration main process trajectory explained limited prediction power by inter-batch heterogeneity, which occurred in three main phenomena: semi-perpendicular batch trajectory (light grey arrow 1 in B) affecting the beginning of batch 2 in A, batch trajectory with lower slope (light grey arrow 2 in B) affecting the beginning of batch 1 and 2, and batch trajectory with higher slope (light grey arrow 3 in B) affecting the majority of batch 3.

Figure 5.

Limitation of inter-batch heterogeneity effects by modelling using ANNR and SVR.

Figure 6.

Performance of models for real-time monitoring of glucose concentration under abnormal condition (glucose spiking)

Figure 7.

Principal Component Analysis of the batch culture with glucose spiking

Figure 8.

Performances of models based on PLSR, SVR and ANNR during real-time monitoring of diverse compounds concentration (Batch with mean inter-batch heterogeneity)

Figure 9.

Evaluation the robustness of models using a batch culture with different-from-calibration culture media (ActiPro) and glucose spiking.

Figure 1

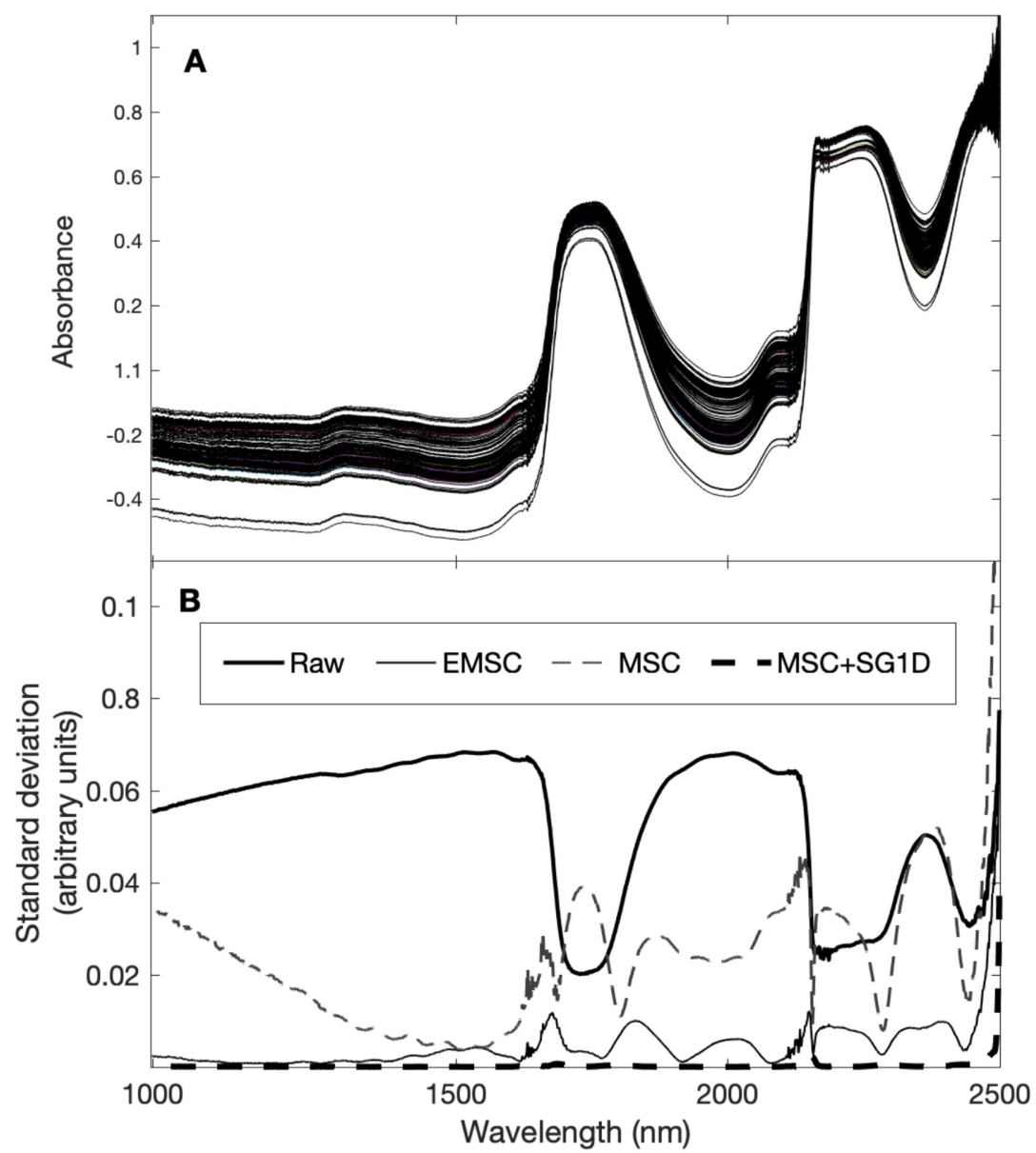


Figure 2.

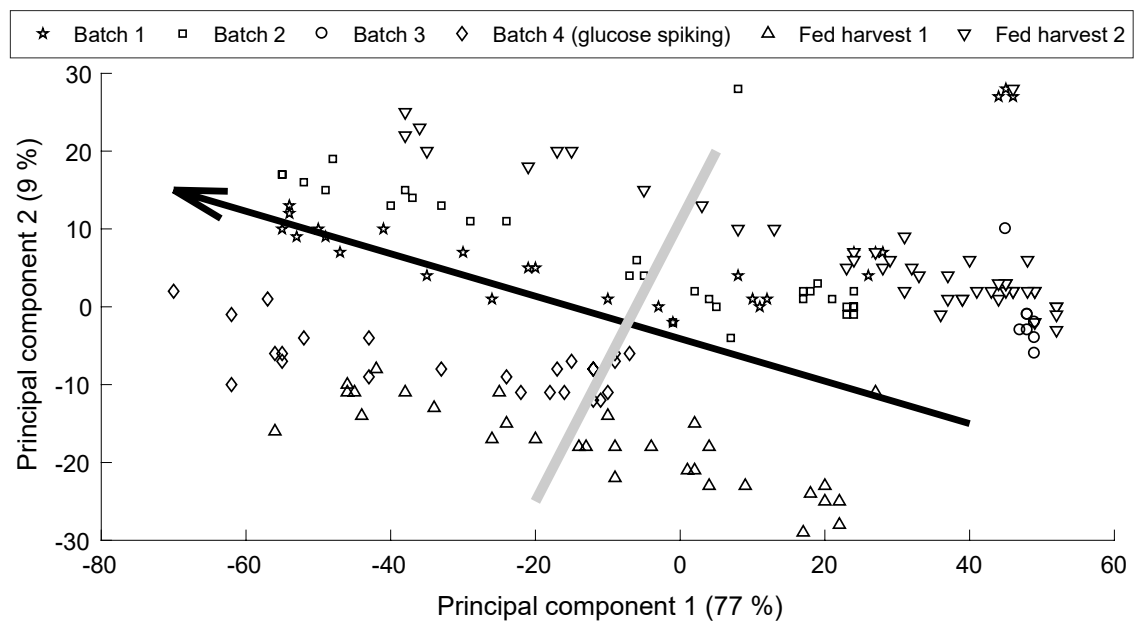


Figure 3

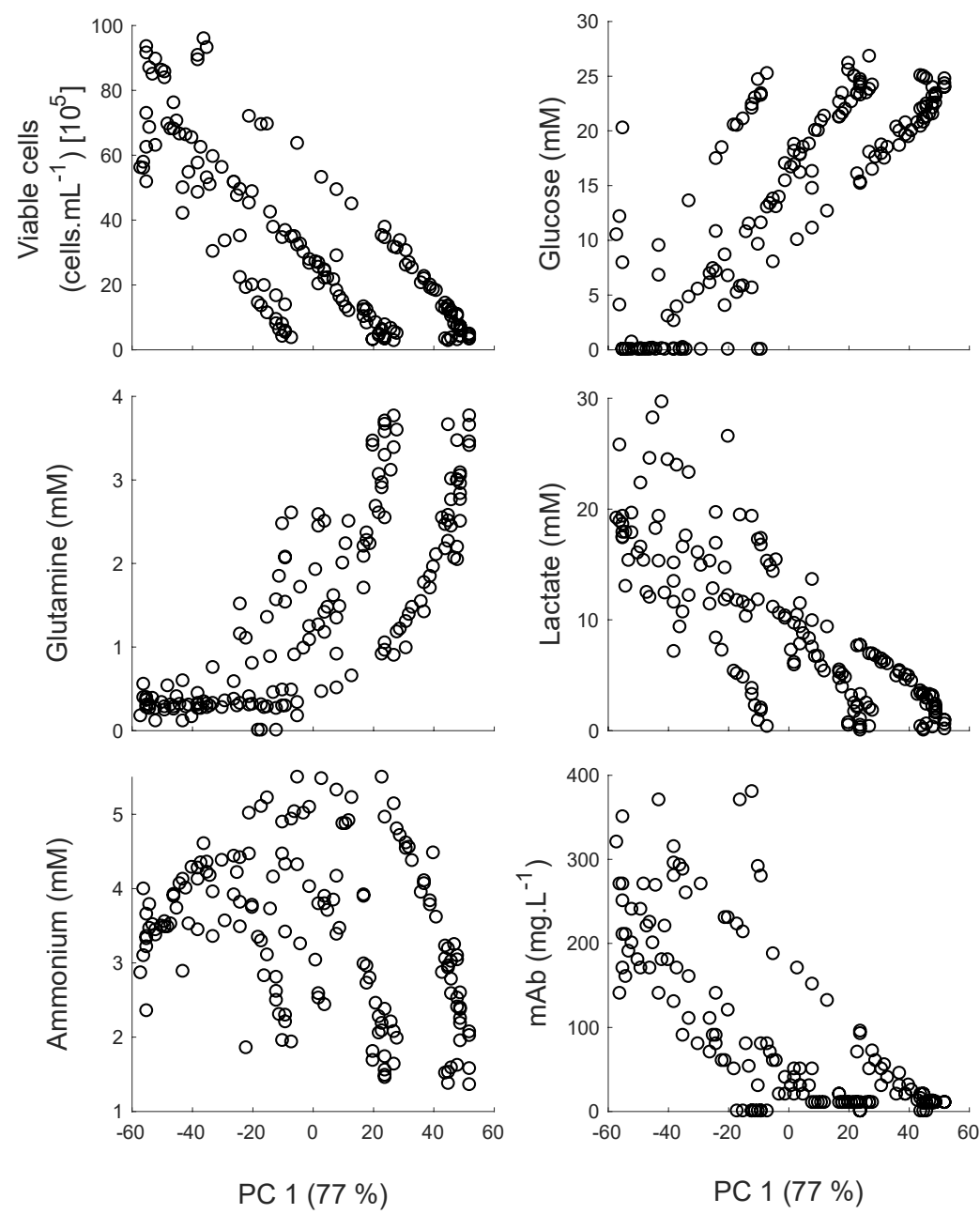


Figure 4

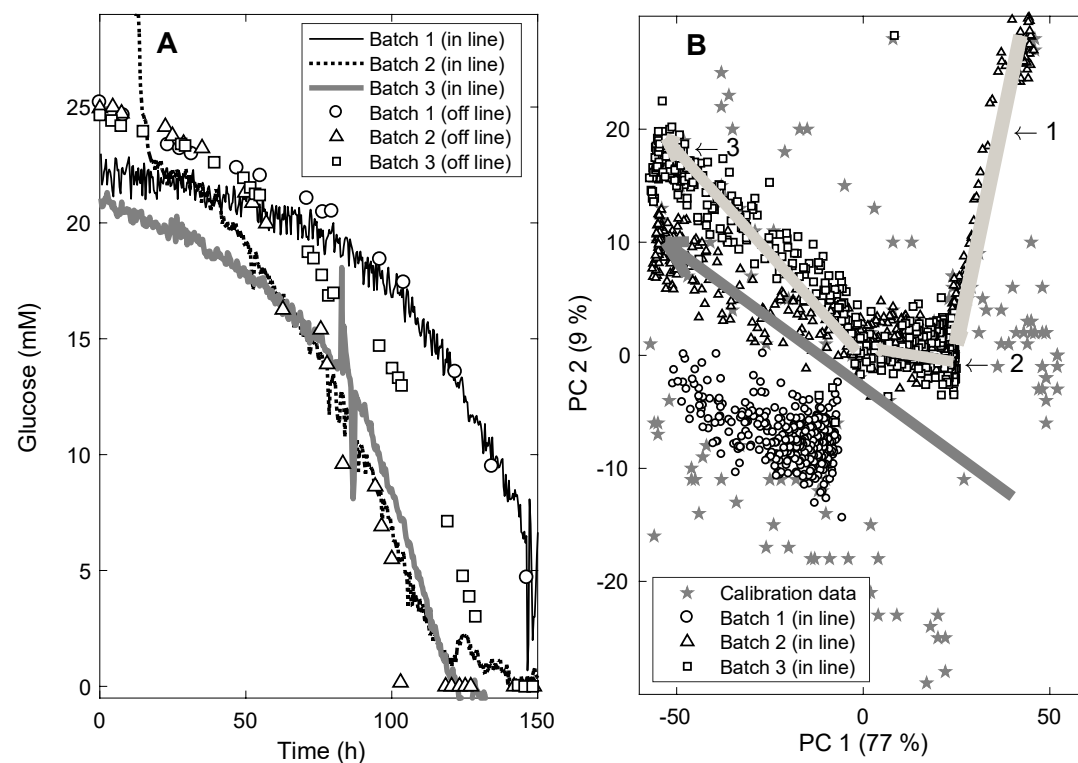


Figure 5

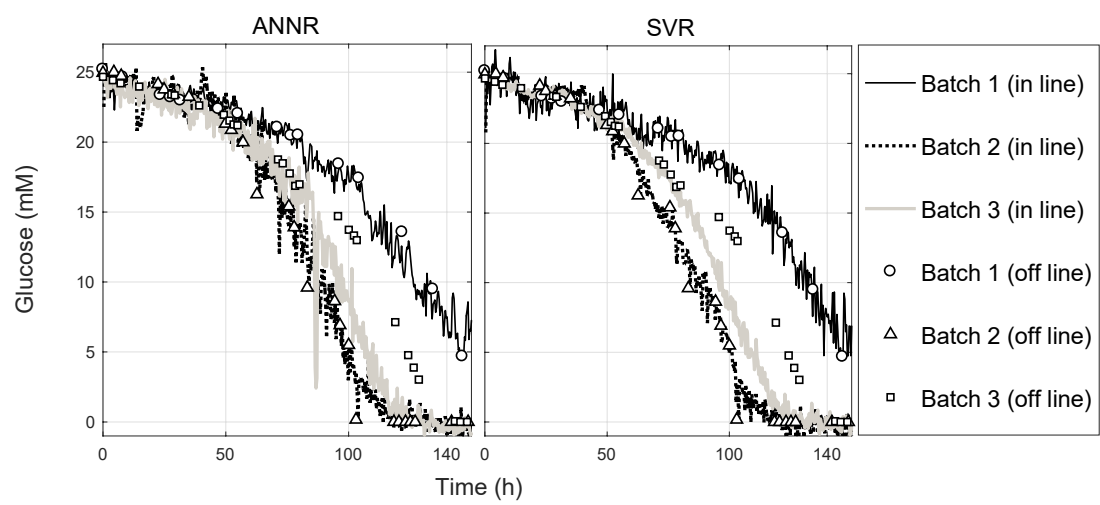


Figure 6

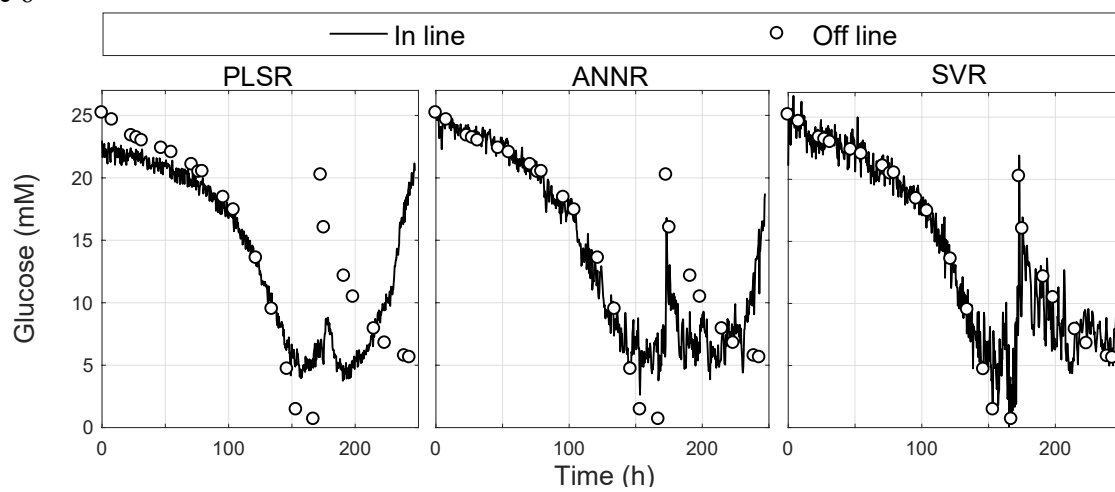


Figure 7

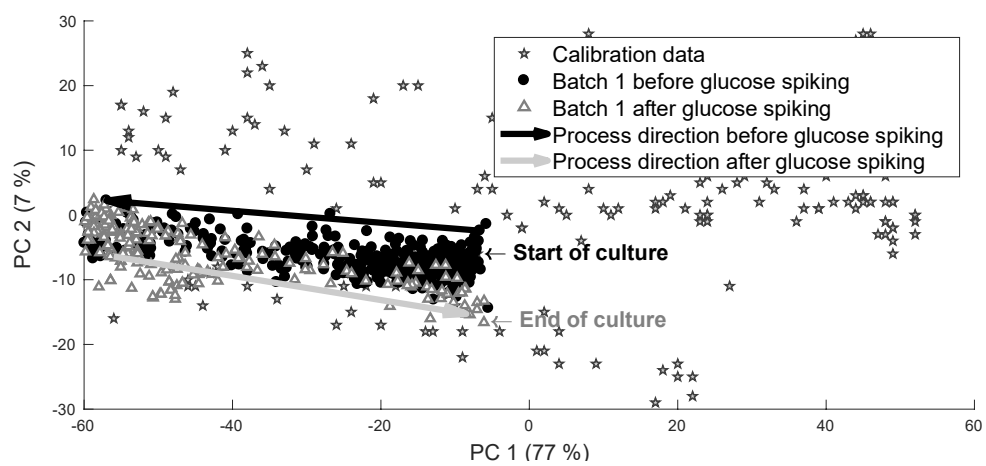


Figure 8

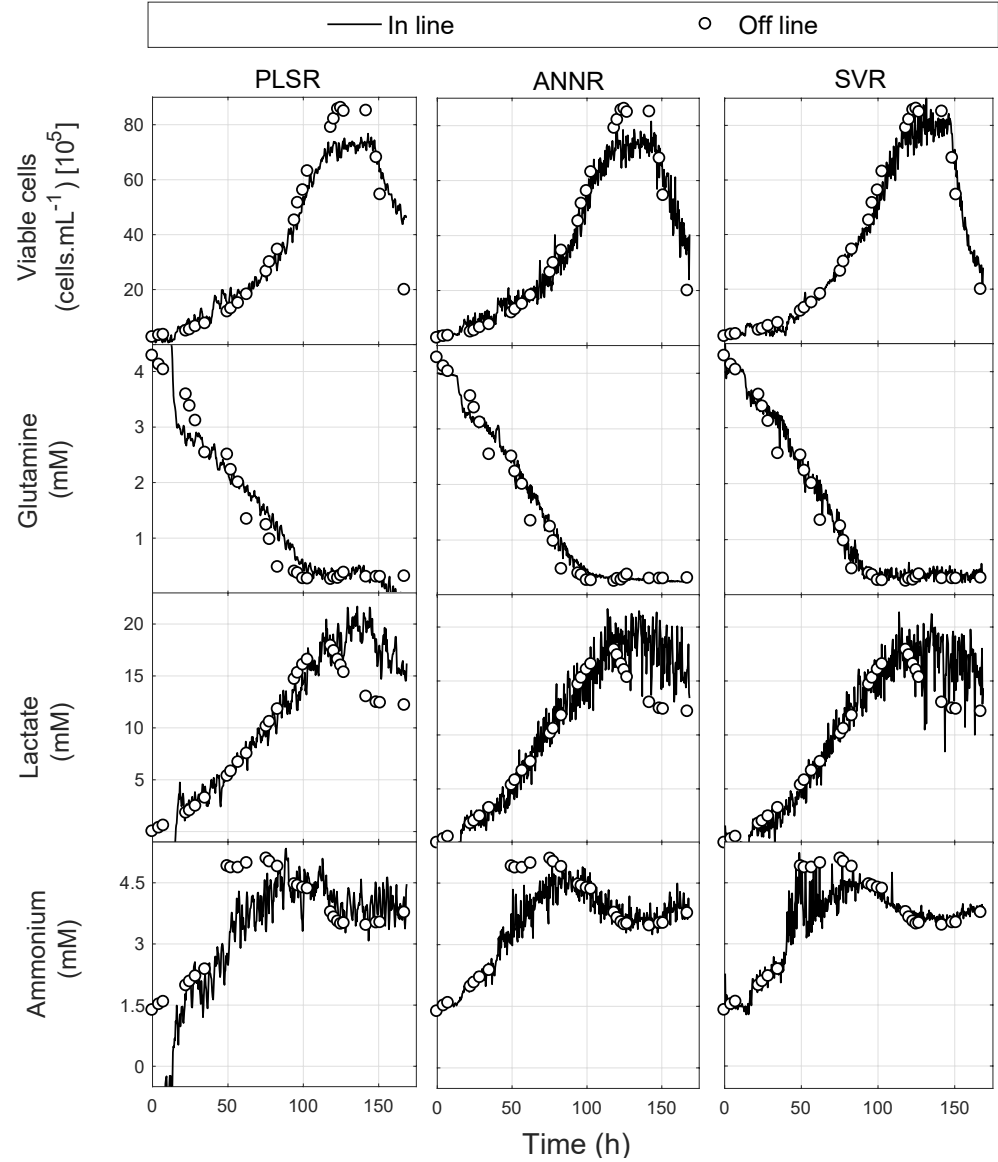


Figure 9

