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RESEARCH ARTICLE

Automized inline monitoring in perfused mammalian cell culture by MIR spectroscopy without calibration model building

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Abstract

Process Analytical Technologies (PATs) are taking a key role in the run for automatization in the biopharmaceutical industry. Spectroscopic methods such as Raman spectroscopy or mid-infrared (MIR) spectroscopy are getting more recognition in the recent years for inline monitoring of bioprocesses due to their ability to measure various molecules simultaneously. However, their dependency on laborious model calibration making them a challenge to implement. In this study, a novel one-point calibration that requires a single reference point prior to the inline monitoring of glucose and lactate in bioprocesses with MIR spectroscopy is assessed with 22 mammalian cell perfusion (PER) processes in two different scales and four different products. Concentrations are predicted over all PERs runs with a root mean square error (RMSE) of 0.29 g/L for glucose and 0.24 g/L for lactate, respectively. For comparison conventional partial least square regression (PLSR) models were used and trained with spectroscopic data from six bioreactor runs in two different scales and three products. The general accuracy of those models (RMSE of 0.41 g/L for glucose and 0.16 g/L for lactate) are in the range of the accuracy of the one-point calibration. This shows the potential of the one-point calibration as an approach making spectroscopy more accessible for bioprocess development.

KEYWORDS

analytics, CHO, MIR, PAT, perfusion, spectroscopy

Abbreviations: ATF, alternating tangential flow; ATR, attenuated total reflection; BR, bioreactor; CHO, Chinese hamster ovary; CPP, critical process parameter; FDA, Food and Drug Administrative; FTIR, Fourier-transform infrared; HCCF, harvest cell culture fluid; MIR, mid-infrared; PAT, Process Analytical Technology; PBS, phosphate-buffered saline; PCM, pure component modeling; PER, perfusion; PLSR, partial least square regression; RMSE, root mean square error; RMSECV, root mean square error of cross validation.

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1 | INTRODUCTION

The global pharmaceutical market is a fast changing and highly innovative industry. The demand for accelerating the development of products increased over the last decade. In 2004 the US Food and Drug Administrative (FDA) started the Process Analytical Technology (PAT) Initiative [1] to guarantee for robust and high-quality products. The market offers different specialized sensors for continuous monitoring of critical process parameters (CPPs) ensuring consistent control of product quality attributes during biopharmaceutical manufacturing. The focus on automation and digitalization of bioprocesses demands for sufficient PAT solutions to realize automated inline monitoring and control strategies [2].

Commonly established sensors for monitoring of CPPs are, for example pH, temperature, and dissolved oxygen. However, the measurement of further CPP like metabolite or nutrient concentration is mostly conducted as daily offline sample analysis [3]. Due to the limited amount of bioprocess information associated with this approach the execution of appropriate countermeasures and the gaining of a deeper process understanding are highly restricted. Spectroscopic methods such as Raman spectroscopy or infrared spectroscopy are promising tools for a detailed online or inline monitoring of CPPs and can therefore contribute to a better understanding and control of bioprocesses. These non-invasive methods do not require sample preparation and can be easily sterilized with the bioreactor (BR) making them an ideal choice as inline sensors. Spectroscopic technologies can predict nutrients and metabolites simultaneously. The inline monitoring of glucose and lactate of Chinese hamster ovary (CHO) cell cultures has been well demonstrated by near-infrared, mid-infrared (MIR) and Raman spectroscopy [3–7]. Control strategies based on these methods can optimize product titer, cell growth, and glycosylation patterns [8–11].

Hurdles for a widespread of spectroscopic methods are high costs, the lack of single-use components, and the dependency on laborious calibration model building, which is time-consuming and requires expertise in chemometrics for data evaluation. Calibration models often rely on the operating conditions of the calibration set-up (Tulsyan, 2019). The need for reliable calibration models is one of the biggest challenges in the widely use of spectroscopy methods in the biopharmaceutical field. To build a robust model using standard multivariate methods such as partial least square regression (PLSR), a vast data set, knowledge of spectroscopy methodology and data analysis are needed. This requires increased personal, product, and time resources. Additionally, the prediction and accuracy of most models are highly dependent on the data set and the used modalities and are therefore often not transferable [12]. An upcoming topic to reduce the effort of extensive calibration model building and data generation is the usage of generic calibration models that predict parameters for changing cell lines, media, and operation settings. However, these algorithms require a huge set of data from several batches and modalities for model building [9, 13, 14].

In this study, direct inline monitoring of glucose in a CHO perfusion (PER) process using MIR spectroscopy was developed. For two of the main components in bioprocesses, glucose and lactate, a novel one-point calibration that requires just one initial reference point was created and tested. To assess its robustness the method was evaluated with one cell line expressing four different products, two different scales and compared against regular PLSR models. Glucose and lactate were chosen as parameter for the one-point calibration due to its importance in cell culture processes. The goal of this study was to develop and present a simple ready-to-use method based on MIR spectroscopy for CHO PER processes that can be used without prior knowledge of spectroscopy.

Practical Application

Process analytical spectroscopy is an essential topic in the biopharmaceutical industry to ensure robust and high product quality. Critical process parameters for upstream processing as metabolites and nutrients are analyzed mainly through daily offline sampling. Spectroscopic methods offer a significant advantage realizing inline monitoring, but the extensive calibration effort has constrained the widespread use. This study aims to address this limitation, by introducing a novel ready-to-use method based on mid-infrared spectroscopy for CHO perfusion processes. The generic model requires merely an initial one-point calibration, that allows automated monitoring and control without the effort of calibration model building. In summary, we demonstrated a method that simplifies glucose and lactate monitoring without prior knowledge of spectroscopy making the tool suitable for its use in the biopharma industry.

2 | MATERIAL AND METHODS

2.1 | Perfusion cultures and cell lines

Four stable CHO cell lines expressing non-glycosylated bispecific constructs (molecules A, B, C, D) were used, from

both pool and clonal cell banks. Cells were thawed and expanded to generate sufficient cell mass to ultimately inoculate 2 and 10 L scale PER bioreactors (BR).

The PER process can be defined in three process steps. First phase was a 3-day batch phase for cell accumulation. In the second 9-day phase an alternating tangential flow (ATF) filtration system (Repligen, Waltham, MA, USA) connected to polysulfone filters (Cytiva, Westborough, MA, USA), and a proprietary chemically defined medium (PER-Medium) to increase cell density and accumulate product were used. The collected permeate stream is cell and product free (waste). PER rate was increased gradually to a maximum of one BR volume per day (RV/d). In the third phase product was harvested using tangential flow filtration during days 12–15 of the PER cell culture process. On day 12, the ATF filter was switched from a 30 kDa retentive membrane to a 750 kDa membrane which allows product to pass through the membrane while cells are still retained in the BR. In addition to the PER-Medium 50% glucose solution was added on demand as bolus to the BR to keep the glucose level above a critical concentration.

Temperature set point and agitation were controlled by distributed control units, while the BR pH set-point was controlled automatically by carbon dioxide or sodium carbonate addition. Culture temperature, pH, and dissolved oxygen set points were equal for each individual cell line used. A temperature-shift was performed as soon as a defined cell density was reached. To reduce foam formation antifoam was added to the PER-Medium as required.

2.2 | Analytical methods

2.2.1 | Mid-infrared spectroscopy

All measurements were performed using a multi-channel MIR Fourier-transform infrared (FTIR) spectrometer Monipa (IRUBIS GmbH, Munich, Germany). Single use flow-cells were attached to the spectrometer, each including a silicon attenuated total reflection (ATR) crystal (IRUBIS GmbH, Munich Germany). Mid-IR spectra were continuously collected in the wavelength range of 2–12.5 μm (5000–800 cm^{-1}) at a resolution of 2 cm^{-1}. A total of 150 spectra were recorded within 1 min, averaged, and used for further data analysis. An internal ATR crystal in the Monipa instrument was used for background correction.

The permeate glucose and lactate concentrations were monitored continuously inline. Integrating the flow cell in the permeate stream has the advantage to avoid any influence of air bubbles and stirrer speed [15]. Therefore, each permeate line (waste and harvest cell culture fluid [HCCF]) was equipped with an IRUBIS single use flow cell

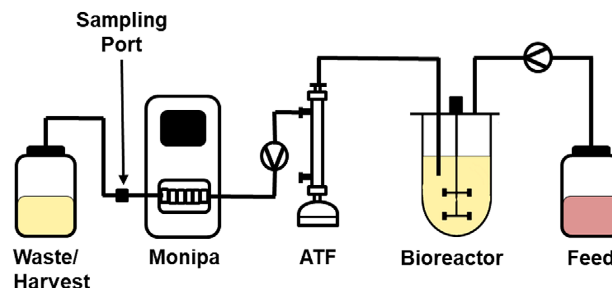


FIGURE 1 Schematic overview of the integration of Monipa into the perfusion process. The device was integrated after the alternating tangential flow (ATF) and peristaltic pump. Additionally, an extra sample port was connected directly after the Monipa.

connected to the Monipa and a sampling port downstream (Figure 1). Spectral results were compared to offline measured permeate samples using the Cedex Bio HT (Roche, Swiss).

2.2.2 | Offline analytic

BR and permeate line were sampled daily. The permeate sample was taken at a defined time point after the BR sample. The sampling time was determined by the residence time, which depends on the flow rate, line diameter, and length. For offline glucose and lactate determination the cell containing BR sample was centrifuged for 10 min at 680 × g. The glucose and lactate concentration of both samples was determined by using the Cedex Bio HT.

The measurement accuracy of the Cedex Bio HT was determined using PER-medium with predefined glucose concentrations of 2, 4, and 8 g/L. A single batch of glucose free PER-medium was used to prepare two separate batches (A and B) of each glucose concentration level. A five-fold determination of both batches from each level was performed. The device internal 1:10 dilution was conducted for the 8 g/L glucose solution.

2.3 | Data analysis

2.3.1 | Calculation of absorption spectra

The absorption spectra are calculated by the following formula, where S_0 represents the initial sample and R_0 is the initial internal reference measurement. R_t represents the reference and S_t is the sample measurement at timepoint t.

$$A\ (t) = \log_{10}\left(\frac{S_0}{R_0}\cdot\frac{R_t}{S_t}\right)$$

The internal reference spectra were taken at a fixed interval. The sampling interval depends on the number of active channels and the acquisition time. An example of a calculated absorption spectra for a glucose concentration study with the reference being water is shown in Figure S1.

2.3.2 | Pure component modeling

Pure component modeling (PCM) is based on the assumption that the shape of a specific component stays the same regardless of other components and that spectral changes can be explained almost entirely by known components. This applies, for example, to moderate concentration changes in PER processes. As reference spectra a glucose solution with a concentration of 8 g/L and of a lactate solution with a concentration of 6 g/L were measured with 2 cm^{-1} resolution for 10 min. Raw absorption spectra were pre-processed with Savitzky–Golay smoothing with a window size of 31. All predictions are based on these references.

First order derivative spectra and multilinear regression were applied to fit the measured spectra and to predict the concentrations of glucose and lactate.

2.3.3 | One-point calibration method

PCM can predict absolute concentrations if the main components of a solution are well known and available as pure spectra. Cell culture media are made up of several components, which are not always known precisely. However, the concentrations of the components of interest are known at the beginning of the process or can be measured by offline measurement tools.

The pure cell culture medium was used as initial sample S_0. The static components cancel out and only the dynamic components of interest will be visible in the absorbance spectra. The absolute concentrations of glucose and lactate are calculated by adding the initial and the predicted concentrations.

2.3.4 | Data analysis with PLS model

For comparison of the one-point calibration with well-known chemometric methods, calibration models were built for glucose and lactate using PLSR. Therefore, samples collected and stored at −80°C from previous BR runs were thawed and measured with Monipa using specialized ATR cells designed for offline sample measurement. To obtain glucose and lactate reference concentrations the samples were analyzed using a Cedex Bio HT analyzer. In total 206 samples from six different BR runs, two different scales (10 and 2 L) and three different molecules were used to create the PLSR calibration models together with five samples of pure phosphate-buffered saline (PBS) solution as reference. The concentrations covered a range from 0 to 7.95 g/L for glucose and from 0 to 2.15 g/L for lactate. Leave-one-out cross validation plots of PLSR models for glucose (A) and lactate (B) are shown in Figure S2. All spectra were referenced to previously measured spectra of PBS solutions. As pre-processing step, a wavenumber trimming was applied (950−1300 cm^{-1} for glucose, 950−1350 cm^{-1} for lactate). The calculation of the calibration models was carried out via a Python program using the "PLSRegression" function of the Scikit-learn library (Version 0.24.2).

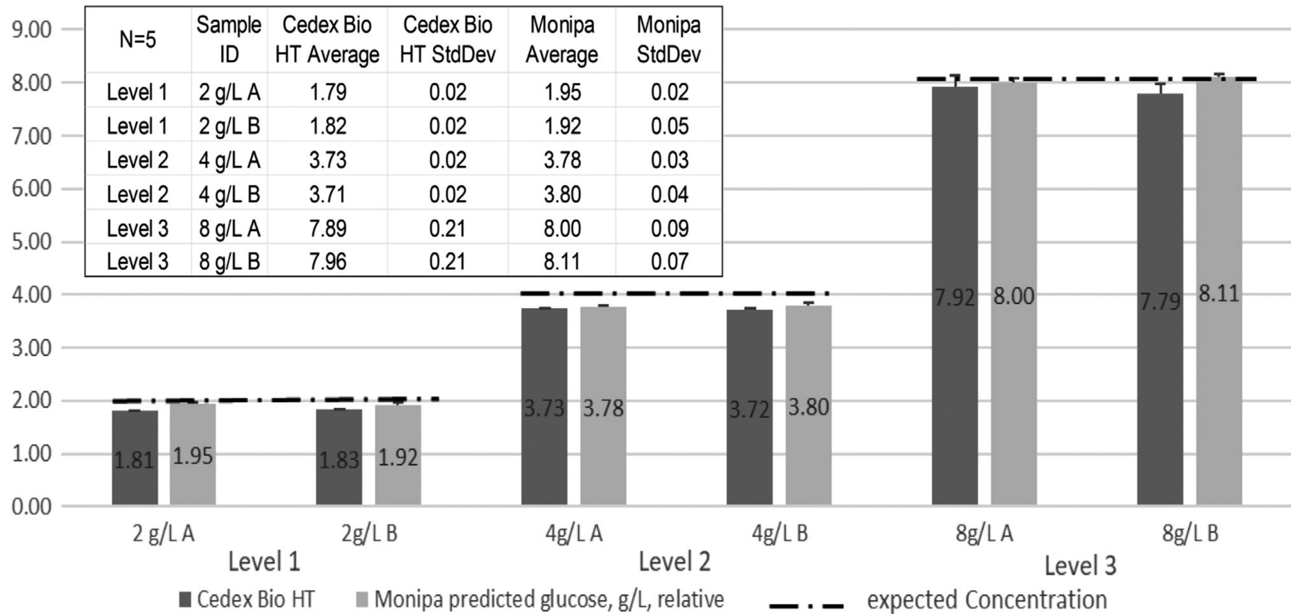
The accuracy of the PLSR models was evaluated by leave-one-out cross validation. For the glucose PLSR model a rank of 9 was used, which could explain 98.4% of the variance (R^2) and lead to a root mean square error of cross validation (RMSECV) of 0.19 g/L. For the lactate PLSR model a rank of 12 was used, giving an R^2 value of 97.7% and an RMSECV value of 0.12 g/L.

3 | RESULTS AND DISCUSSION

The focus of this study is on characterization of the one-point calibration and comparing its prediction accuracy to commonly used PSLR method for evaluating spectroscopic data. First, the set-up is discussed on its ability to produce reliable data for an evaluation of the novel one-point calibration. Secondly, the performance of the one-point calibration is analyzed followed by a discussion of potential drawbacks of this approach. Finally, the results from one-point calibration are compared to those of the PLSR model.

3.1 | Characterization of the one-point calibration

Characterizing a new analytical method relies strongly on the availability of representative and reproducible data. To ensure the collected data set is reliable, some preliminary tests were carried out. First, it was tested whether the metabolite concentration in the permeate stream was comparable to the metabolite concentration in the BR. Samples from the permeate line and the BR of two PER runs were analyzed with the CEDEX Bio HT. A mean deviation of 0.22 g/L for glucose and 0.14 g/L for lactate, respectively, was found between these samples. This deviation was deemed acceptable and therefore an integration of the MIR spectrometer system Monipa in the permeate line



N=5	Sample ID	Cedex Bio HT Average	Cedex Bio HT StdDev	Monipa Average	Monipa StdDev
Level 1	2 g/L A	1.79	0.02	1.95	0.02
Level 1	2 g/L B	1.82	0.02	1.92	0.05
Level 2	4 g/L A	3.73	0.02	3.78	0.03
Level 2	4 g/L B	3.71	0.02	3.80	0.04
Level 3	8 g/L A	7.89	0.21	8.00	0.09
Level 3	8 g/L B	7.96	0.21	8.11	0.07

FIGURE 2 Comparison of the device internal error for both systems (Monipa and Cedex Bio HT) by using glucose solutions with three different concentrations (2, 4, and 8 g/L).

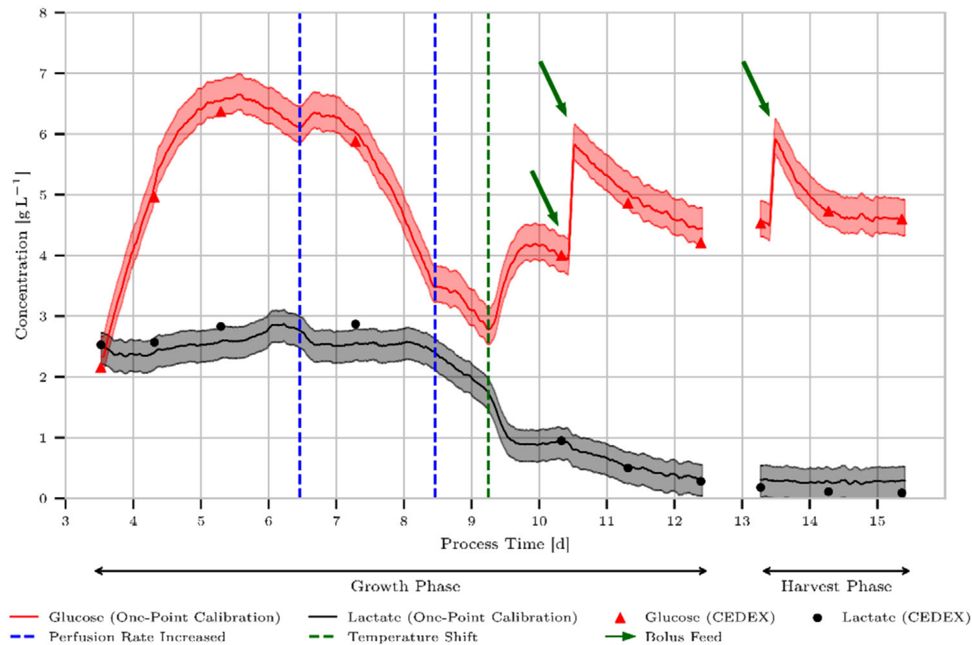


FIGURE 3 Predictions in g/L for glucose and lactate concentration over a process using the one-point calibration model for a 2 L bioreactor run (molecule C). One-point calibration predictions are represented by the red (glucose) and black line (lactate). For both lines the prediction uncertainty of the one-point calibration was included, which was calculated as the root mean square error (RMSE) from all 22 runs. Reference values glucose concentration (red triangle and lactate (black dots) were measured with the Cedex Bio HT. Changes in perfusion rate (blue dashed line), temperature shift (green dotted line), and bolus feed (green arrows) are displayed. The alternating tangential flow (ATF) switch was performed on day 12, therefore the monitoring of the process was paused, occurring in a lack of data this day. The monitoring was restarted with the morning sample on day 13.

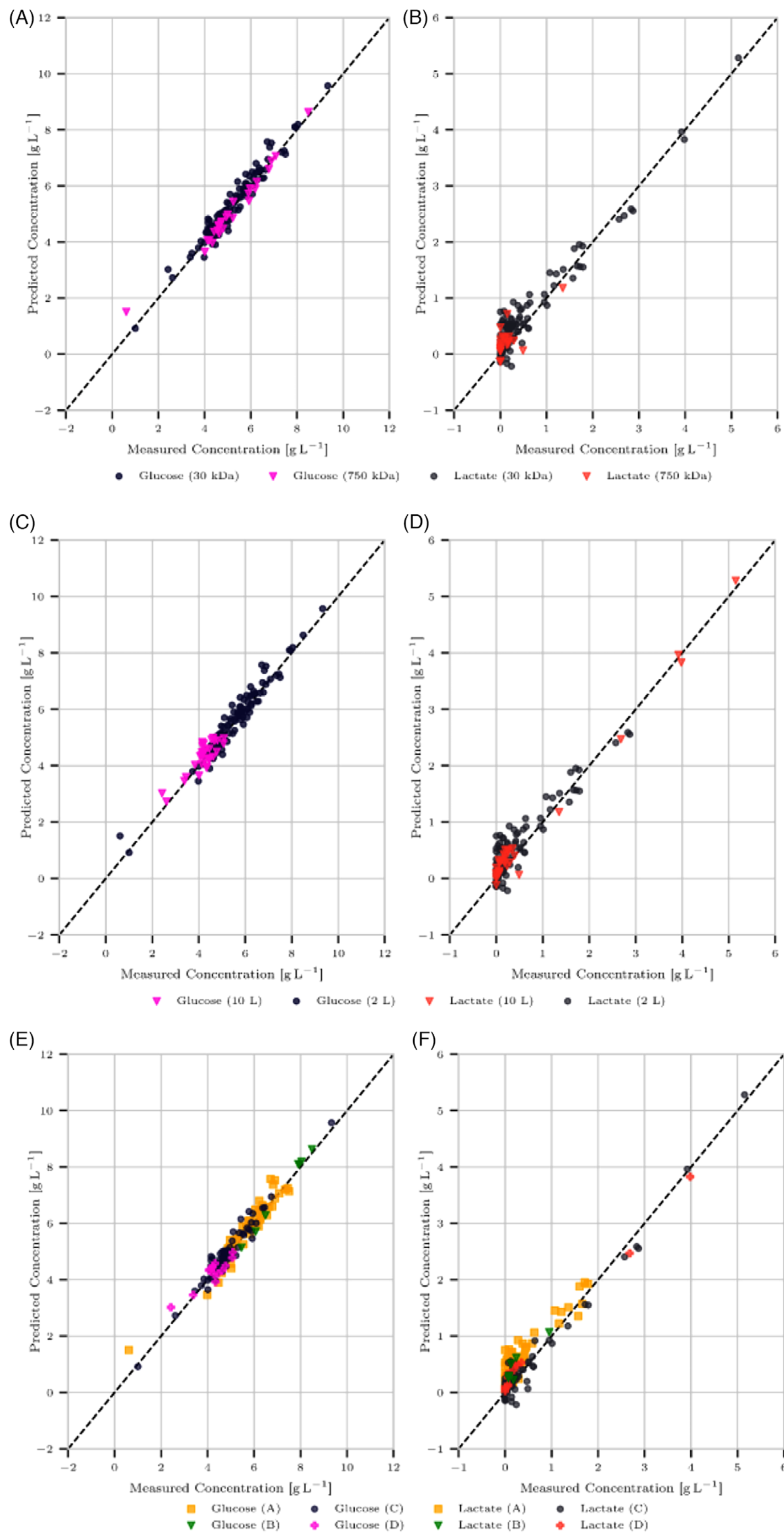


FIGURE 4 Prediction evaluation for glucose and lactate concentration for different alternating tangential flow (ATF) filter pore sizes (A, B), scales (C, D), and molecules (E, F). The dashed line illustrates an ideal correlation between predicted and measured concentration.

TABLE 1 Summary of evaluated parameter and its corresponding data amount, RMSE, and R^2 for glucose and lactate concentration.

Monitored molecule	Parameter	Amount of datapoint, *n*	RMSE (g/L)	R^2
Glucose	Product A	44	0.36	0.908
	Product B	7	0.23	0.997
	Product C	71	0.27	0.940
	Product D	14	0.29	0.829
	2 L-scale	104	0.30	0.947
	10 L-scale	32	0.32	0.728
	Pore size 30 kDa	110	0.31	0.945
	Pore size 750 kDa	26	0.27	0.968
	Deionized water	19	0.77	0.823
	PBS	44	0.62	0.767
	PER-media day 3	136	0.30	0.945
	One-point calibration	80	0.24	0.955
	PLSR	80	0.41	0.908
	Total	136	0.29	0.908
Lactate	Product A	44	0.36	0.853
	Product B	7	0.24	0.848
	Product C	71	0.18	0.962
	Product D	14	0.18	0.992
	2 L-scale	104	0.28	0.852
	10 L-scale	32	0.18	0.985
	Pore size 30 kDa	110	0.27	0.936
	Pore size 750 kDa	26	0.21	0.540
	Deionized water	19	1.27	0.764
	PBS	44	1.67	0.961
	PER-media day 3	136	0.26	0.928
	One-point calibration	80	0.16	0.979
	PLSR	80	0.23	0.96
	Total	136	0.24	0.871

PBS, phosphate-buffered saline; PER, perfusion; PLSR, partial least square regression; RMSE, root mean square error.

became feasible. However, the deviation was in the limit of quantification of the Monipa (~0.2 g/L), which might influence the accuracy of the prediction. Additionally, long permeate lines resulted in a significant time lag of around 15–60 min between changes in the BR and the changes being visible in the permeate line. Therefore, an additional sample port was integrated directly after the Monipa system to retrieve a representative permeate sample as reference.

The second pre-test was performed to evaluate the reference analytics and the reproducibility of the Monipa. Therefore, the internal measurement error for both devices (Cedex Bio HT and Monipa) was determined using stock solutions as described in Section 2.2.2. Both devices show a high accuracy with low standard deviation for glucose (Cedex Bio HT: 0.02 g/L for 2 and 4 g/L glucose, Monipa 0.02−0.08 g/L over all concentrations). Noticeable is a 10-fold higher error of 0.2 g/L of the Cedex system for 8 g/L glucose concentration. This result, however, can be explained by the device internal 1:10 dilution carried out for concentrations greater than 7.5 g/L.

In addition, the analysis shows that the CEDEX Bio HT underdetermined the target concentration by 0.2−0.3 g/L whereas the concentrations predicted by Monipa where closer to the expected concentration with ±0.15 g/L (Figure 2). As the solutions were prepared manually, it might be possible that the expected concentration does not reflect the true concentration. However, it highlights the challenge in applying reliable reference analytics for spectroscopic methods, which is a commonly known problem for the implementation of such [16].

A feasibility study with 22 BR runs in two different scales (2 and 10 L) and with four different molecules was conducted, after verifying the reference analytics

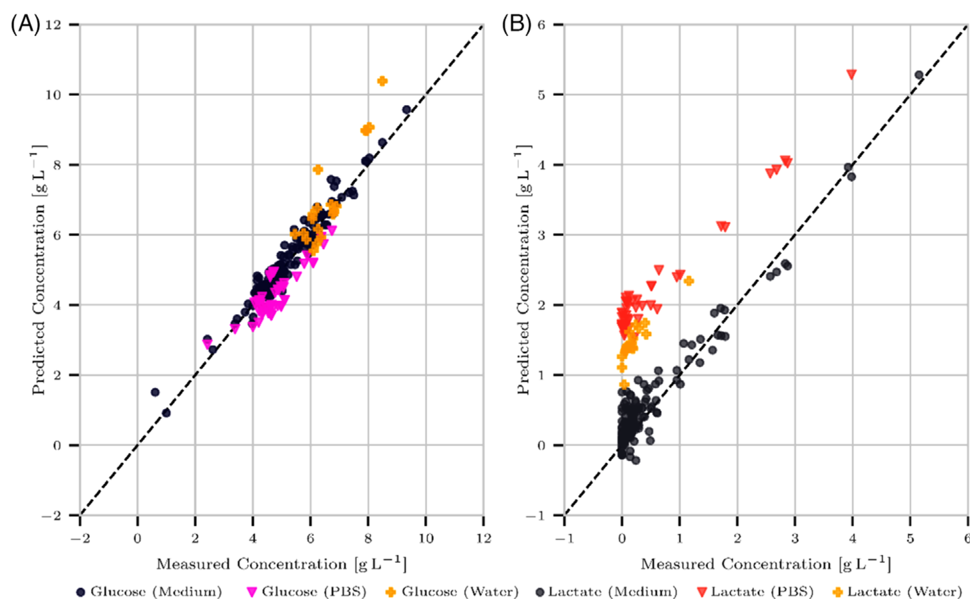


FIGURE 5 Evaluation of the predictions of the one-point calibration model for glucose (A) and lactate (B) with different reference measurements. Ideal linearity between the one-point calibration predictions and the measured concentrations by the CEDEX Bio HT is illustrated by a dashed line through the origin. Comparing the different reference measurements, the most accurate prediction has been achieved for the lactate concentration by medium as reference measurement.

reliability. The BR runs were performed as described in 2.1. The recording for glucose and lactate of an entire PER process with a cell line producing molecule C is shown in Figure 3. During the 3-days batch phase no PER is carried out, and thus no concentrations are measured. After PER start on day 3 the concentrations are monitored reliably, regardless of the permeate composition change occurring on day 12. The glucose and lactate predictions are following the results of the offline measured permeate samples closely (Figure 3). A similar behavior was observed in all 22 PER runs. Individual data can be found in the Appendix.

An evaluation of all runs is shown in Figure 4 and the corresponding root mean square error (RMSE) and R^2 values are summarized in Table 1. The results reflect the model's robustness for both glucose and lactate concentration with a prediction accuracy of 0.29 g/L for glucose concentration and 0.24 g/L for lactate concentration regardless of the produced molecule or scale. A differentiation between non-protein containing samples (until day 11) and protein-containing samples (from day 12) shown in Figure 4A,B for glucose concentration and lactate concentration shows no significant deviation from each other. The method is also able to track a wide concentration range as shown by the datapoints with a lactate concentration above the expected range of 0–2 g/L. The results are comparable to Raman spectroscopy combined with PLSR models for online monitoring of glucose and lactate [17]. MIR spectroscopy combined with the one-point calibration proofs to be a valid method for continuous monitoring. Possible effects of process changes in metabolic profiles, such as an increase in the PER rate, temperature shift, or bolus feed, are well demonstrated by the continuous monitoring.

The results highlight the model's robustness to changes in media composition, including larger molecules such as proteins. The sensibility to media changes of PLSR is well studied and to achieve a similar robustness like the one-point calibration model, an evaluation of a large number of samples would be required [18].

The accuracy of the one-point calibration model is depending on the initial media composition (PER media, deionized water, PBS) for the reference spectra. To better understand the impact of the reference spectra on the accuracy of its predictions, different reference matrices (PER media, PBS, deionized water) were investigated. The results of this evaluation are shown in Figure 5. The predictions of a measurement series using PBS or deionized water show a higher deviation from the reference values than runs using PER-media as the reference spectra (Table 1). This deviation is especially visible in the lactate measurement (Figure 5B). Glucose has its main absorption peaks at 1038 and 1080 cm^{-1}, while lactate has them at 1040, 1132, and 1550 cm^{-1}. The media shows some major peaks in areas above 1100 cm^{-1}, which might explain the larger interference of the reference point on the lactate predictions. Each value of a continuous measurement series is correlated to the reference measurement, leading to a high impact on the glucose and lactate predictions.

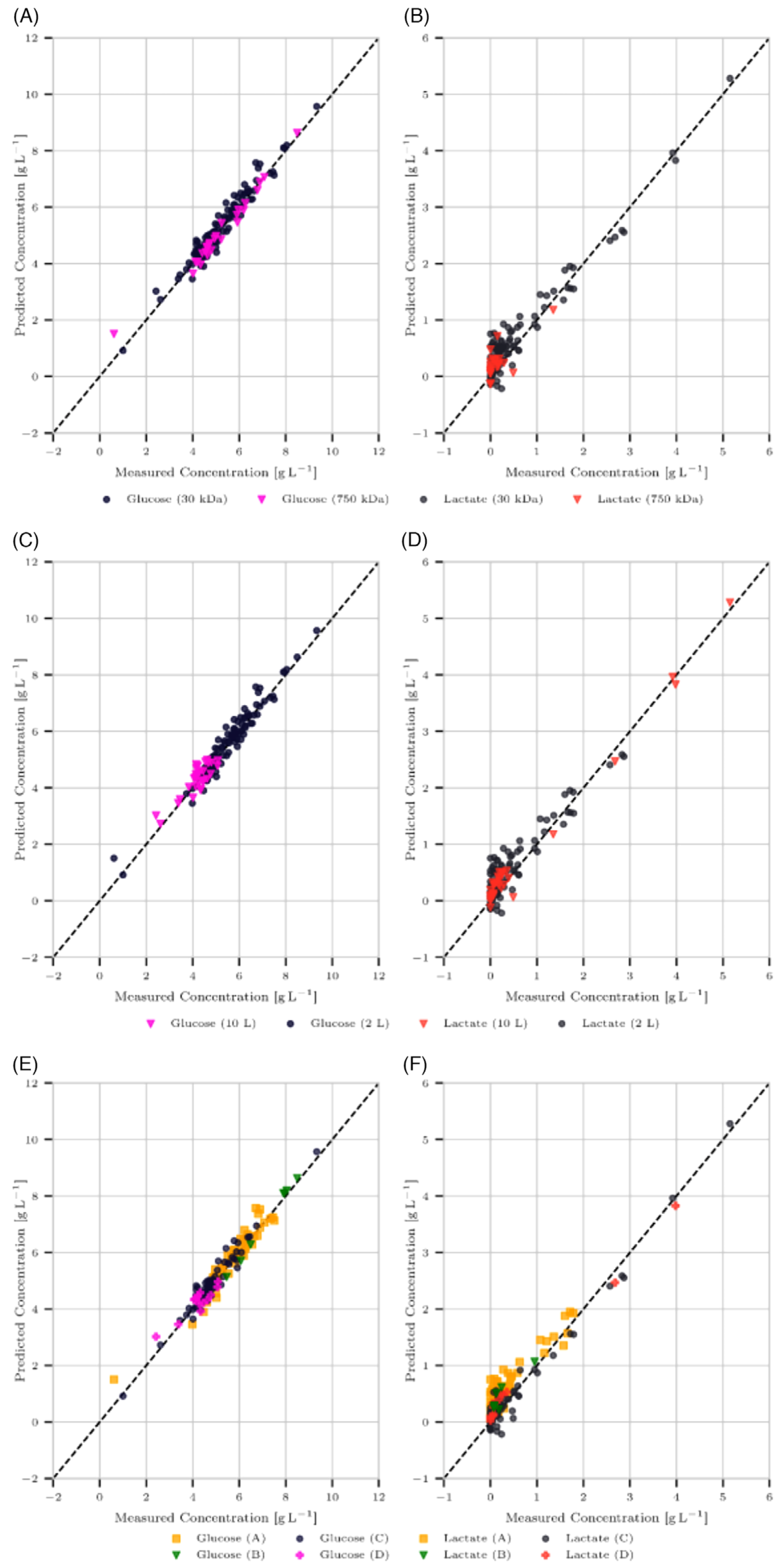


FIGURE 6 Predictions of partial least square regression (PLSR) model versus the one-point calibration for glucose concentration (A) and lactate concentration (B). The dashed line illustrates an ideal correlation between predicted and measured concentration.

3.2 | Comparison with PLSR model

For positioning the one-point calibration as a robust tool for MIR spectroscopy, a comparison with a commonly used multivariate method PLSR was performed. The PLSR model for glucose concentration and lactate concentration were built as described in Section 2.2.2. For the comparison 6 BR runs were used for model building and 13 runs for the performance comparison. Three of the 22 runs were not conducted with PBS as initial reference and therefore excluded. Figure 6 presents the results of both investigated calibration types in comparison using the same data set for both methods. The accuracy and robustness of the one-point calibration for glucose prediction was better compared to the PLSR model. For lactate concentration, however, the predictions from the PLSR model are more accurate. These results position the accuracy of the one-point calibration in the range of the PLSR models, which is also in agreement with results found in the literature [13]. In general, the accuracy of PLSR models can be enhanced by increasing the data set [3, 18]. However, this implies the impracticability in creating and fine tuning PLSR models for process development needs, as these processes tend to change more rapidly.

4 | CONCLUSION

In this study, a novel one-point calibration method using the MIR spectroscopy system Monipa was thoroughly investigated, and its performance was compared to the standard multivariate method PLSR. The concentration of glucose and lactate was inline monitored in 22 runs, varying in scale and products. The results showed a comparable performance to the PLSR method regarding its accuracy and robustness. Furthermore, no significant influence of varying conditions on the accuracy of this method was found, proving its capability to work as a generic approach for real-time monitoring of CHO PER processes. The proposed one-point calibration can help to reduce the implementation hurdle for PAT technologies in bioprocesses. The algorithm takes advantage of the properties of MIR spectroscopy to reduce the complexity of this technology. This method in combination with a robust MIR spectrometer has the potential to make spectroscopy more applicable for users with less chemometric knowledge and thus might help the technology to become more widespread in the industry. It can help cut material and labor costs as it is not dependent on prior test runs to gather data for model building. The MIR spectroscopy system Monipa can be used directly in process development, which might help to make it more useful even in early-stage development. In manufacturing processes this method can be a reliable basis for a robust glucose control. Since changes in glucose concentration will be monitored continuously in real time, this information can help to optimize feeding strategies, for example, by coupling the glucose feed to the lactate concentration in the BR [11].

However, in practice the one-point calibration depends on reliable reference analytics or known initial concentrations. Therefore, scientists should be aware of the standard deviation of the reference analytics to know the upper and lower limits for their process.

The one-point calibration promises to be a universal application for cell culture processes over all process steps—from early development to production scale.

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[Correction added on 20-Feb-2024 after first online publication: Copyright license updated.]

CONFLICT OF INTEREST STATEMENT

Authors Anja Müller and Lorenz Sykora-Mirle are shareholder of the IRUBIS GmbH.

DATA AVAILABILITY STATEMENT

Data supporting of this study are not publicly available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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