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At-line NIR spectroscopy as effective PAT monitoring technique in Mab cultivations during process development and manufacturing

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ABSTRACT

The application of at-line NIR transmittance spectroscopy on supernatant samples from Chinese Hamster Ovary Cells (CHO) based monoclonal antibody (Mab) cultivation processes spanning several scales from 2.5 L to 1000 L, cell-lines and development years is described. The collected and preprocessed spectra were used to do process state estimation and to obtain several culture parameters. Multivariate process trajectories were computed from NIR spectra acquired at-line. These were used to enhance process understanding across different scales up to industrial scale, assess batch-to-batch variability, and examine the relative importance of different sources of process variability. Many parameters of interest in industrial cell culture, like nutrient or product concentrations can be reliably estimated by NIRS with an accuracy of 15% or better, compared to reference methods General calibrations (scale and cell-line independent) are valid across a range of process conditions and different feed regimes. The proposed approach is therefore applicable throughout process development as well as to existing large-scale validated CHO bioprocesses for continuous improvement.

1. Introduction

Process analytical technology (PAT) involves the combined use of in-process monitoring techniques including chemometrics, multivariate data analysis and modeling, process and multivariate control, supervision and diagnosis [1]. The perspective taken in PAT is that of the process (not the sample's or that of a single parameter over time), while Quality by Design (QbD) is about a wider context and is product-centered. The ultimate goal of QbD requires a high-level of process knowledge and understanding, which can only be realistically obtained and dynamically updated throughout a product's life-cycle, through PAT [2].

Monitoring is at the core of PAT and is done preferably in situ or at-line on whole samples by multi-parametric methods. Many spectroscopies are multi-parametric and enable several parameters to be measured on the same sample simultaneously. That has obvious measurement benefits (e.g., lowered cost per parameter), and more significantly it can highlight correlations among different parameters of a sample (e.g., makes stoichiometries between substrates and products more evident) which can be exploited for increased process state estimation and understanding.

Several reports on bioprocessing of small and large molecules have discussed the feasibility of different spectroscopies as multiparametric monitoring techniques. The first reports on the application of near-infrared spectroscopy (NIRS) to bioprocess monitoring appeared in the 1980s by Karl Norris – the founding father of modern NIRS – who described its use in the quantitative analysis of solid-state fermentations [3]. The first accounts of NIRS in submerged cultivations for small molecules were described in the 90s, first to monitor ethanol in yeast fermentations [4], then fungi cultivations [5] followed by industrial demonstrations in bacterial systems shortly after [6,7]. However, for larger biomolecules made by mammalian cell platforms the interest in NIRS started a bit later, but has since been gaining momentum. Harthun et al. [8] described the determination of a recombinant protein in animal cell culture supernatants, while [9–11] initiated the use of NIRS in Mab cultivations as a multi-parametric monitoring technique. The use of NIRS as a process-fingerprinting technique was demonstrated in small molecule biomanufacturing by Rodrigues et al. [7] and for proteins by Henriques et al. [12]. When used to establish a process-fingerprint throughout time the intrinsic ability of NIRS to capture the effects of several chemical and physical attributes of a sample is used. Instead of developing calibrations

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to describe the trajectories of each attribute, the entire NIR spectrum is used through multivariate projection methods (e.g., PCA, principal component analysis) to obtain a batch trajectory containing the combined effect of all measurable attributes. This approach is very interesting for process supervisory control (e.g., batch-to-batch analysis), process optimization and fault detection and diagnosis.

The use of different PAT monitoring techniques has been recently reviewed in regard to Mab cultivations by Carvalhal and Saucedo [13] and by Fazenda et al. [14] in relation to the capabilities and limitations of on-line NIRS. NIR spectroscopy is a relatively insensitive technique, that is why it is so ubiquitously applied in the quality control of highly concentrated systems (e.g., the quality control of bio/pharmaceutical raw-materials). The detection limits for specific compounds may be in the range of g/L. However, the detection limits can be lowered to ca. 100 ppm by exploring the effect that such compounds have on several parts of the NIR spectra (e.g., water bands) and specially by lumping all those influences together. Such indirect calibrations can be very accurate (i.e., as typical reference analytical methods) and therefore useful as long as 'good modeling practices' are used. Those regard not only the use of an NIR calibration inside its own design-space, but equally important, the proper use of available chemometrics' techniques – i.e., adequate spectral preprocessing and wavenumber selection, a parsimonious model structure chosen not over-fitting the available data, proper statistical figures of merit used throughout, and different data sets for calibration and validation steps containing samples spanning the required design and operating spaces of the NIR calibrations [15]. The challenges and opportunities for PAT in biomanufacturing are considerable for both new and existing processes [16].

Here we report on the use of at-line NIR transmittance spectroscopy performed on supernatant samples from Mab cultivation processes at several scales, from 2.5 L to 1000 L, with different cell-lines and media, spanning several development years, with the aim to develop scale and cell-line-independent models to be applied as process supervision and process control techniques (i.e., NIRS used as a true PAT tool).

2. Materials and methods

2.1. Cell culture

In the course of this work samples from 3 different scales (2.5 L, 100 L and 1000 L) with four different Chinese Hamster Ovary (CHO) cell lines (A, B, C, D) in two different media platforms, were analysed.

Cell line A was culture in media containing soy and rice peptones. Seed train and production cultures were all fed-batch cultures lasting 3–5 or 14–18 days respectively. For the cell lines B, C and D the seed train fermentations were all batch cultures, using a chemically defined medium based on CD-CHO (Invitrogen Corp., USA). The production cultures were fed-batch cultures with a culture duration typically between 10 and 14 days, the basal media and feed media were also based on CD-CHO with various supplements (e.g., amino acids).

Filtered supernatant samples for ca. 100 fermentation batches from lab, pilot and production scales available were analysed. For each of these fermentations, up to 19 samples were collected at regularly spaced intervals along the cultivation. In total, a more than 1500 samples were collected and analysed by reference methods for several parameters, and their NIR spectra acquired. Several batches were identified as extreme outliers (with very high leverage and significantly out of a 95% Hotelling T^2 limit) and therefore not used in model development.

2.2. Reference analysis

Samples from production fermentations were analysed by standard reference analytical methods for product titer, lactate dehydrogenase activity (LDH), osmolality, cell density and viability, ammonium, glucose, glutamate, glutamine, and lactate. Standard reference analysis included BioprofileAnalyzer 300 (Nova Biomedical, Waltham, US) for metabolites, Cell Counter (Roche Innovatis AG) for cell density and viability, GonotecOsmomat 030 (Gonotec GmbH, Germany) for osmolality and ProteinA HPLC to determine antibody titer. Samples were centrifuged to remove cells and debris, and the supernatants collected and frozen until reference analysis and spectra acquisition.

2.3. NIR spectra acquisition

The spectra were collected using quartz cuvettes (2 mm optical length) in a Bruker® MPA FT-NIR system, equipped with a tungsten–halogen source and an InAs detector. Each spectrum was an average of 32 scans, with 8 cm^{-1} of spectral resolution recorded in the wavenumber range of 4999–11,003 cm^{-1}. A reference air spectrum was recorded once a day before any sample's spectra acquisition (without a cell/cuvette). These blanks captured instrument variations and were useful to correct for deviations from sample measurement.

2.4. Data analysis

Data manipulation including multivariate calibrations with NIR spectra were performed using PLS Toolbox® version 5.5 (Eigenvector Research, Inc., USA) for Matlabversion 7.2 for Mac (Mathworks, U.S.A.). Principal component analysis (PCA) and multiway-PCA (MPCA) were used for sample and batch classification, particularly to obtain process trajectories directly from spectral data. PLS modeling with contiguous block cross-validation was adopted for all quantitative regression models. Cell culture supernatant spectra were pre-processed by calculating second derivatives, using a second order Savitzky–Golay algorithm plus mean-centering as implemented in Eigenvector's toolbox [17,18]. A procedure based on i-PLS [19] also implemented on that toolbox was used to search for the spectral regions that gave the lowest cross-validation errors during calibration. The i-PLS algorithm divides the spectra into predefined intervals and then develops calibration models based on those intervals followed by validation.

In our work we have done the i-PLS search on pre-processed spectra with an automatic forward search using an interval width of 25 points for all models except glucose which required 33 points (i.e., 25×8 or 33×8 wavenumbers width). The maximum latent variables (LVs) number was set at 10. Those choices resulted from a comprehensive search of parameters done a priori for the studied system. We have also checked the i-PLS wavenumber regions selection against the VIP (variable importance plot) scores provided by the same software package, as VIP is normally more broadly available than i-PLS in chemometrics's packages. In general both selection strategies had a good agreement. In the few cases for which i-PLS selected wavenumbers gave VIP scores with an importance below the usually accepted unity threshold, an attempt to use the VIP selection regions always resulted in inferior predictive ability when compared to i-PLS.

The data set was divided in two sets: the first was used for model development and the second for external validation. The split of available samples between calibration and validation sets was performed by means of the SPXY algorithm – subset partitioning of X and Y spaces [20]. The SPXY procedure ensures that the calibration set covers all sources of variability in data and that the validation set is within the variation range for both spectral (X-space) and/or

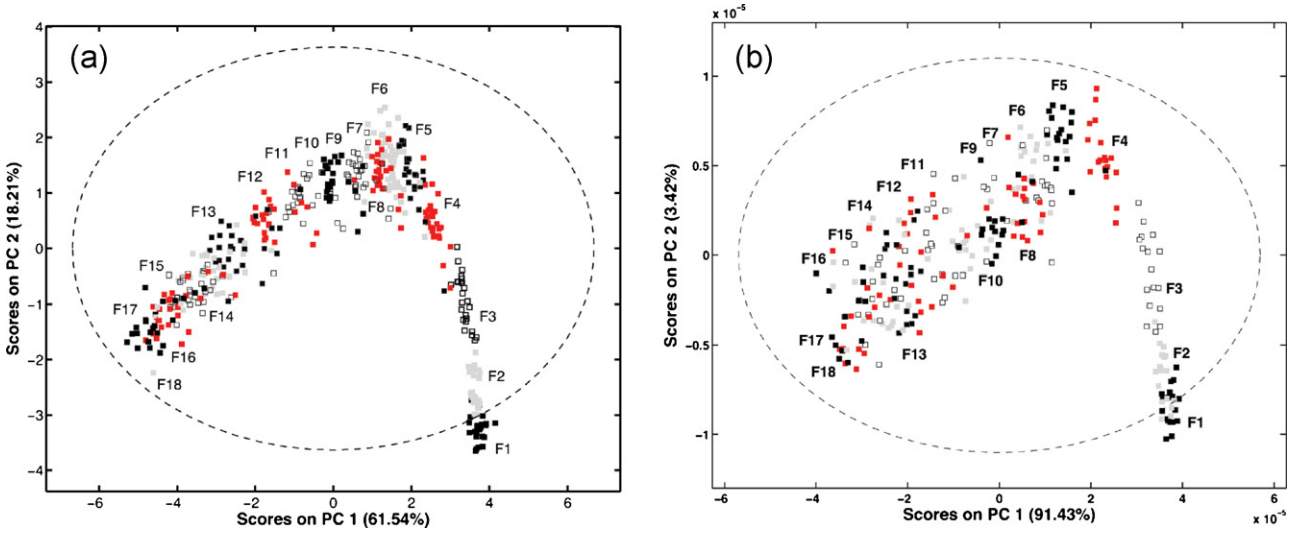

Fig. 1. Process trajectories (signatures) for 22 Mab cultivations at 1000 L, as described by PCA of (a) 8 quality attributes measured off-line by reference methods, and (b) NIR supernatant spectra (samples clustered by fermentation day: F1–F18).

analytical data (Y-space). The calibration set contained ca. 2/3 of the available number of samples. As samples and not entire batches were selected for calibration or validation, three batches were completely set aside in order to truly challenge the predictive ability of the models in not only new samples (as in validation) but on whole new batches. Performance of calibration models was assessed computing the root mean square errors of validation and prediction (i.e., RMSECV and RMSEP) and the respective correlation coefficients, R^2, between predicted and measured values.

3. Results and discussion

Three major questions are addressed in this work: (1) how much information can NIR spectroscopy capture from Mab cultivations supernatant samples, (2) how accurate are the predictions and (3) how general are these findings?

Regarding the first question we are interested not only in a multi-parametric assessment of NIRS but on being able to define process trajectories (i.e., process-signatures or fingerprints) from consecutive spectra of at-line supernatant samples. Of special interest within this work is to evaluate how general the NIRS approach is and if reasonably accurate general calibrations can be developed to explore different cell-lines, feeding-strategies, media-formulation and process scales, without the need to have specific calibrations (e.g., of scale or cell-line).

3.1. How much information can NIR spectroscopy capture?

Several process parameters (e.g., temperature or pH) and quality attributes (e.g., culture viability or metabolites) are routinely measured in cell cultivations both in development and routine manufacturing. Most process parameters are measured on-line automatically and stored into a DCS computer (distributed control system). Generally, most quality attributes are measured off-line taking samples and laboriously analyzing them with different bench instrumentation. Results are often being known only with delays of several days. Relating inputs to outputs is often done only retrospectively when several batches are available. An

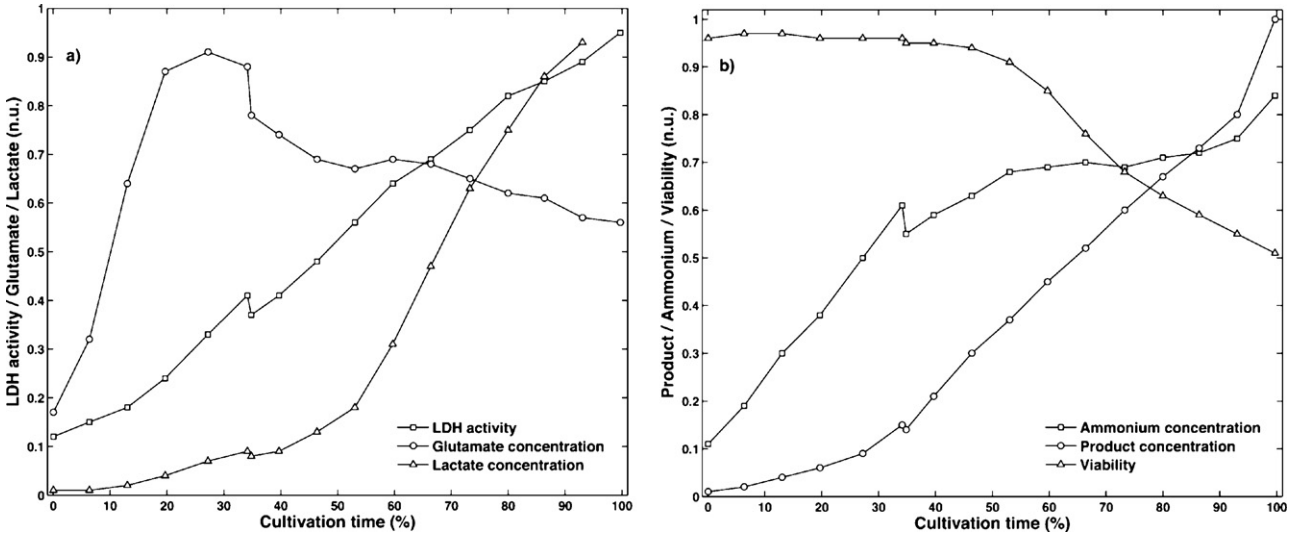

Fig. 2. Time profiles of different culture parameters, showing a high degree of auto-correlation (monotonically increasing/decreasing profiles) and cross-correlation (interrelations of different parameter profiles).

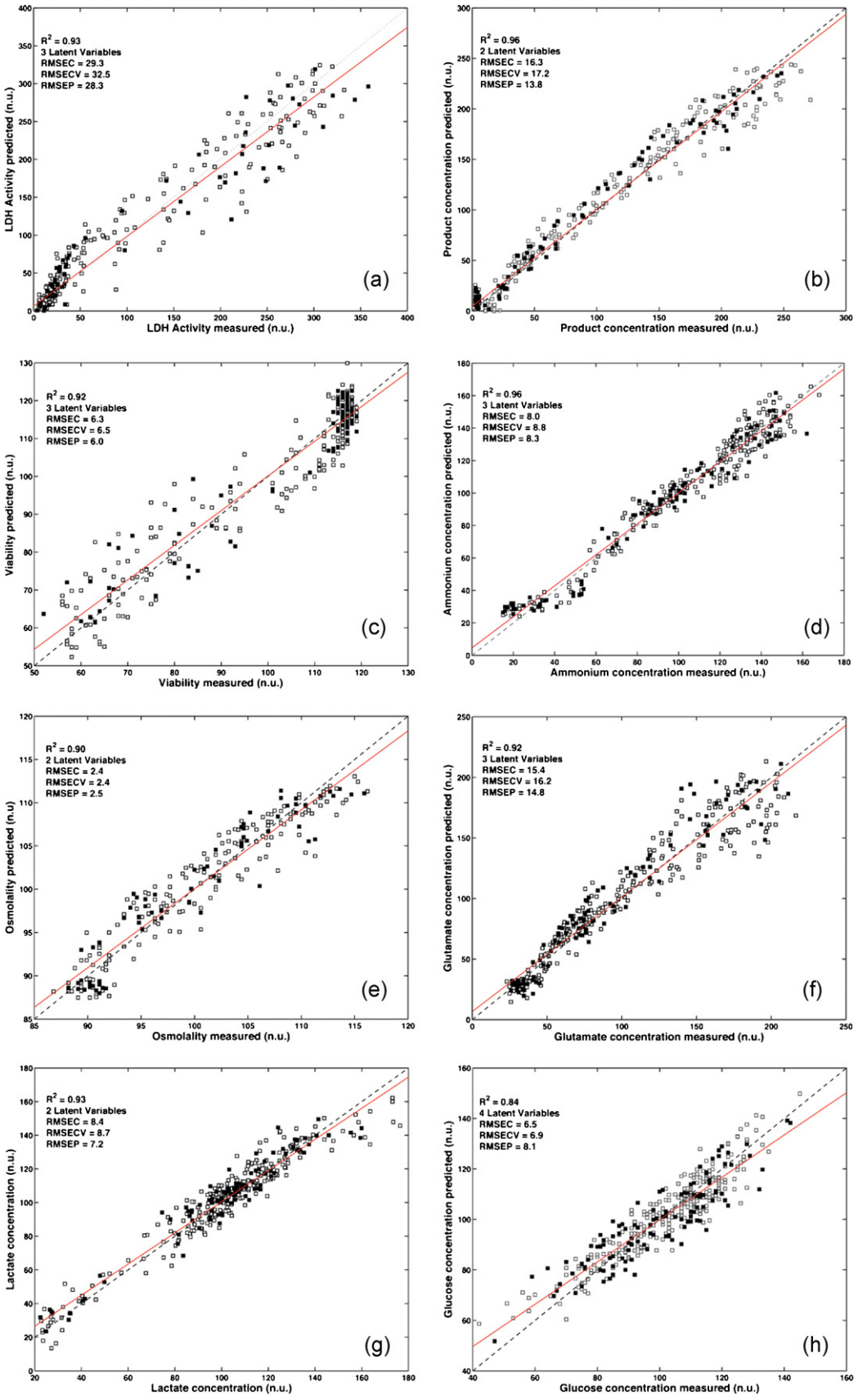

Fig. 3. NIRS calibrations for several culture parameters in supernatant samples, used to monitor Mab fed-batch cultivations at 1000 L scale and cell line A. (Open squares (□) samples used for calibration; closed squares (■) validation samples.)

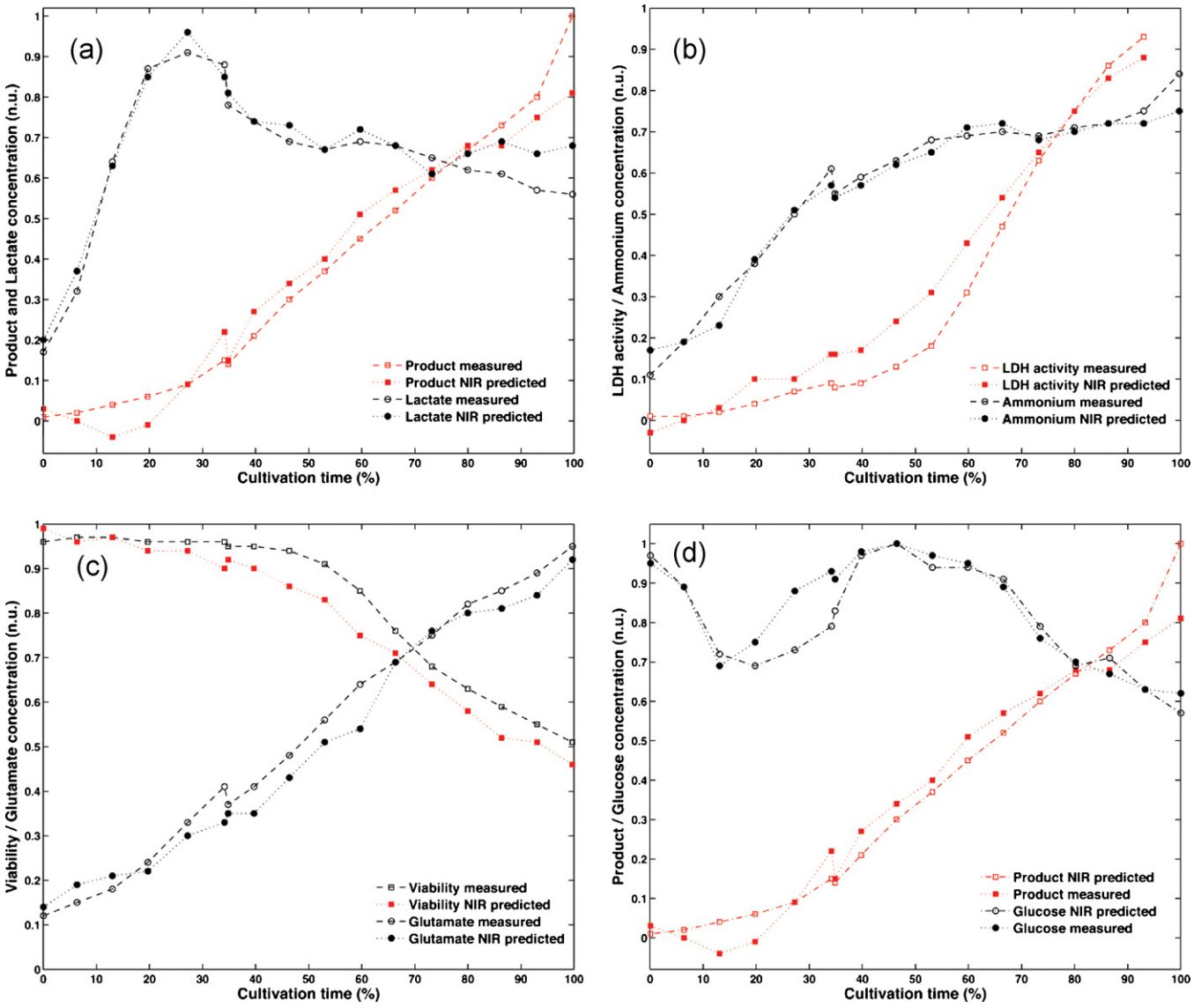

Fig. 4. NIR predicted concentration profiles (closed symbols) based on supernatant spectra collected at-line for a 1000 L scale fed-batch cultivation (cell line A) not used in calibration development, versus the off-line reference values (open symbols).

alternative would be to investigate to what extent an at-line NIRS procedure could be used to capture a sample's main characteristics and from the spectra alone without any calibration derived process-signatures. Such average trajectories and associated upper and lower control limits would establish a region of nominal operation. Process excursions away from the average trajectory and out of pre-defined warning and control limits would indicate the need for control actions to be taken (e.g., taking a sample for off-line analysis by reference methods for confirmation).

Using historical data from 22 fed-batch cultivations (cell line A) at 1000 L scale with peptone containing media under similar conditions (all except raw-material lots), NIR spectra were obtained at-line of supernatant samples taken once a day. The PCA score plot of the time-profiles for 8 of the quality attributes available (Fig. 1a) is compared with the MPCA (multi-way PCA) score plot of the NIR spectra available (Fig. 1b). The MPCA procedure unfolds a 3D array (NIR spectra × process time × cultivation run) into a 2D array (stacking the cultivation runs as they are reasonably similar to each other) and performs a PCA on the resulting 2D array.

Table 1
General NIR calibrations: multiple process scales, cell-lines and operating conditions.

Parameter	LV	Range (n.u.)	Accuracy reference assays	RMSECV (n.u.)	RMSEP (n.u.) (VAL)	R^2 (VAL)	RMSEP (n.u.) (NB)	R^2 (NB)
Product	3	9.7–426.8	±20%	25.3	22.1	0.94	24.8	0.94
Glucose	5	5.1–206.0	±10%	17.2	13.2	0.85	16.5	0.84
Glutamate	3	3.7–329.1	–	25.8	33.8	0.90	33.3	0.92
Glutamine	3	6.6–301.9	–	35.1	35.2	0.80	42.9	0.79
Lactate	3	11.2–320.7	±15%	15.4	15.7	0.96	20.8	0.97
Osmolality	3	72.4–161.1	±15%	3.7	3.9	0.96	5.2	0.95

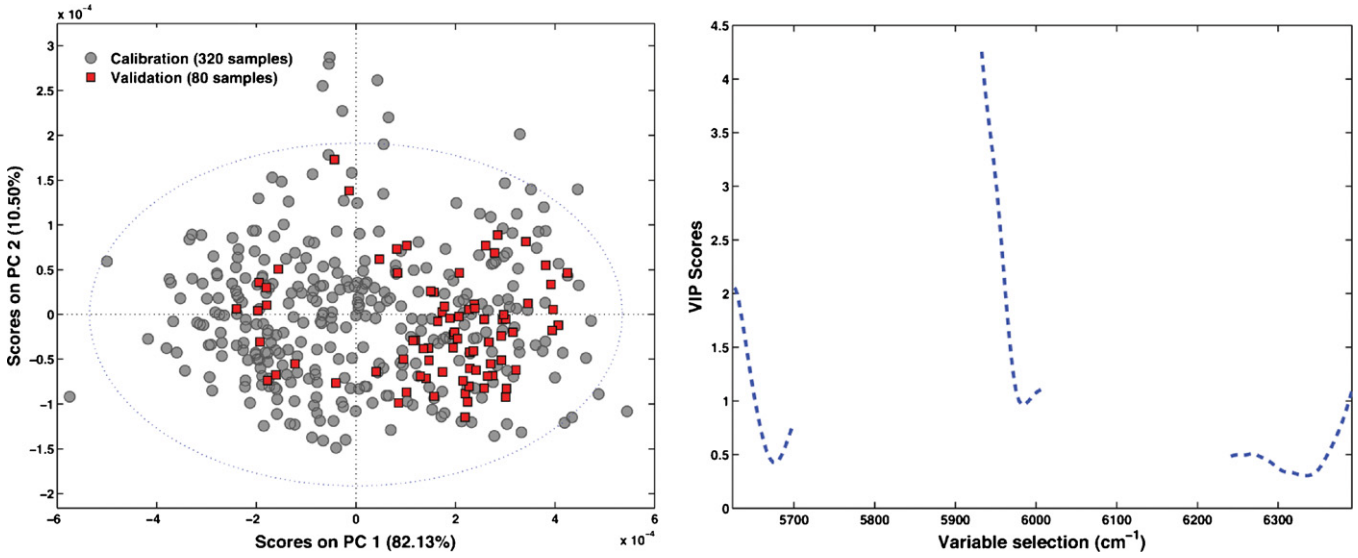

Fig. 5. Calibration design-space (2.5 L, 100 L and 1000 L scales) in the PCA space (left plot) of specific pre-process wavelength regions (right plot) indicating samples available: calibration samples (circles) and validation samples not used in model development (squares).

Fig. 1 shows that the trajectories (process-signatures) described by 8 quality attributes considered together, obtained by laborious and expensive analytical procedures, resemble well that obtained by the NIR spectra obtained in a few minutes next to the process. This is a significant result indicating that a process state estimation is done equally well through NIRS as with all other quality attributes (lactate dehydrogenase activity, osmolality, cell viability, ammonium, glucose, glutamate, glutamine and lactate). This can be done for existing processes as the at-line spectra acquisition procedure does not interfere with the GMP containment and environment.

The process pattern can be split into two distinct phases, before and after the continuous fresh media feeding is started (Fig. 1a and b). In the first phase (until about sample F4) PC2 captures the significant process dynamics in the growth phase (different samples for same batch over time) as well as batch-to-batch variability (different batches at same sampling time). In the following less dynamic fed-batch phase (Fig. 1a and b), changes in PC2 are reverted and decelerate (a smaller change in PC2 scores from F5 to F18 than from F1 to F4); the drift through PC1 over process time is unaffected (comparable change in PC1 scores from F1 to F4as from F4 to F8 or F8 to F12); and batch-to-batch variability is now compounded with the effect of different raw-material lots used in the feed media for each batch (Fig. 1b).

The effect of differences in raw-material lots used in initial media and feeding formulations are known to be the main sources of batch-to-batch variability at the same sampling time [21].

Furthermore, Fig. 1 shows that in both cases two principal components can describe almost all variance in the data, 79.8% for off-line analytics and 94.8% for at-line NIRS. Applying wavelength or variable selection based on spectral regions with a higher covariance with product concentration along the process, NIR-based process signatures resemble those based on off-line culture variables (not including the product concentration profile).

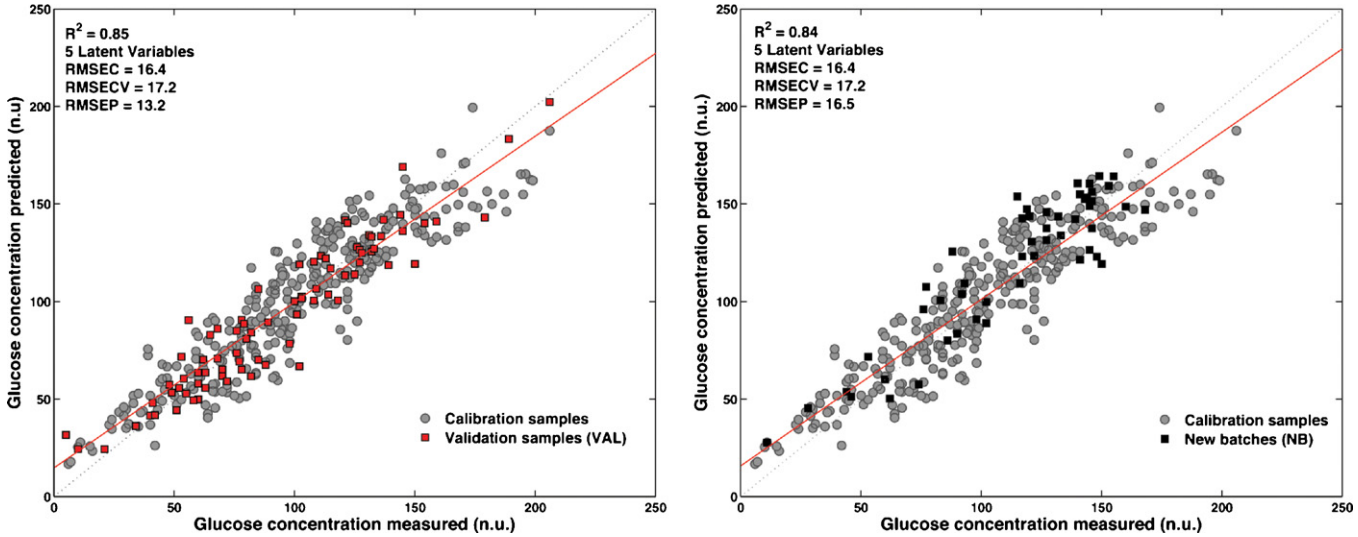

Fig. 6. NIR model for glucose monitoring valid across development and manufacturing scales and different protocols. The average accuracy (RMSEP) for validation samples not used in calibrating the model is reported (VAL), as well as the accuracy for entire cultivations set aside at different scales (NB).

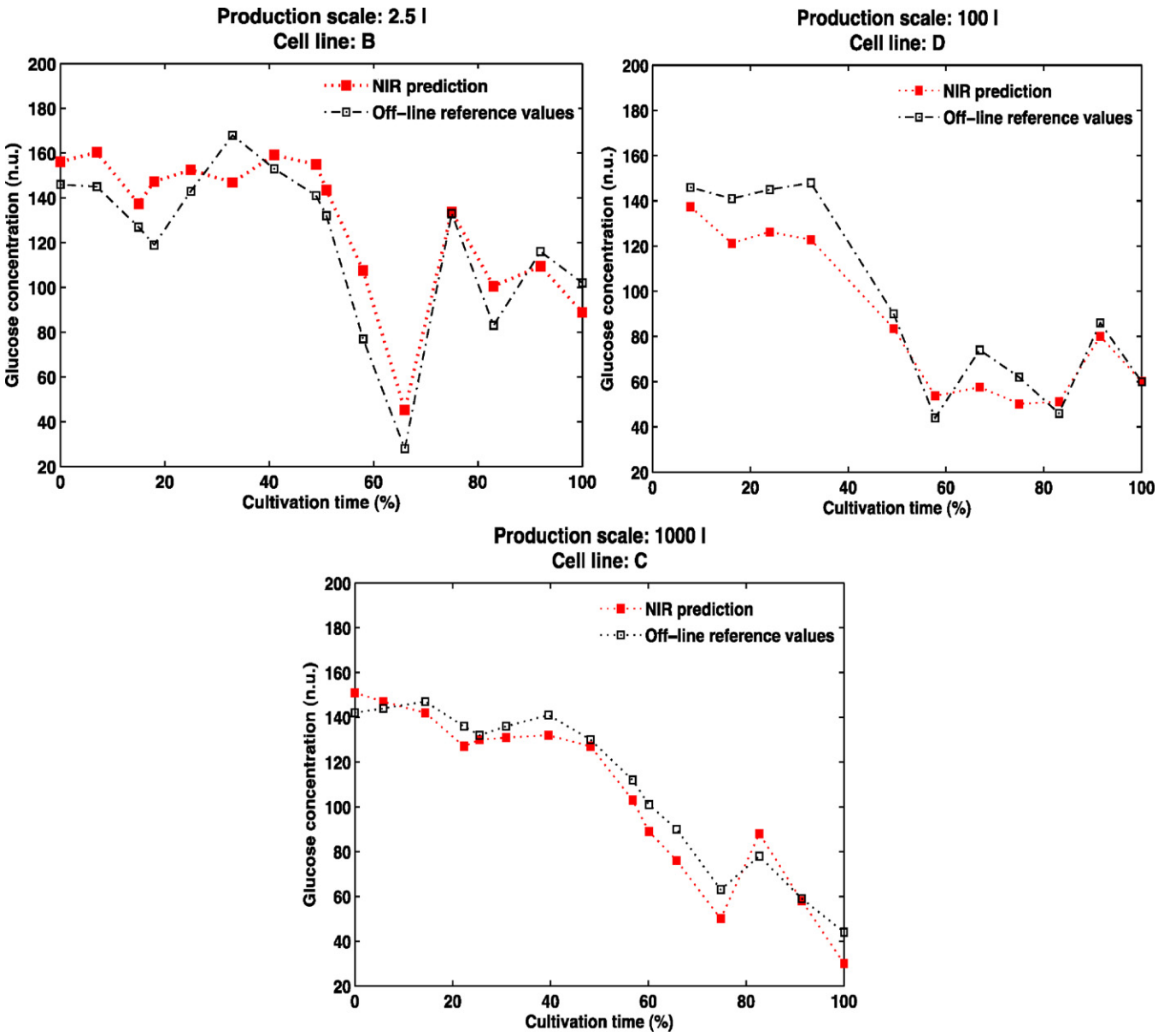

Fig. 7. NIR predicted glucose concentration profiles for each of the different process scales and cell-lines investigated, compared to reference values measured. These batches have been arbitrarily set aside for model evaluation (cf. Fig. 6 RMSEP-NB) and none of their samples have been used either for calibration or validation before.

3.2. How accurate can NIRS be?

The NIRS characteristics that make it a powerful process-fingerprinting technique in multivariate monitoring of cell cultivation systems, may cause problems when developing calibrations for specific parameters. The parameters to be monitored generally present high correlation as a result of metabolic pathways – e.g., converting substrates into metabolites with fixed stoichiometries (Fig. 2). In such circumstances, the calibrations obtained with process samples can only be used to predict samples with a similar correlation structure (i.e., samples from similar cultivations) otherwise models will probably fail. In practice this can be minimized by making the calibrations more general, using samples from batches under different conditions, or by more complex procedures involving designed-samples (e.g., spiked or synthetic samples with a composition defined by a DOE or experimental design). From a modeling point of view, a trade-off is generally obtained between generality (the model is robust and long term stable because it can describe different process regimens) and accuracy (the model is specific for a particular type of cultivation recipe). Several chemometric techniques exist that can enhance the information already present in the spectra, but no technique will be able to compensate for a severe lack of sensitivity or spectral selectivity [18]. Moreover, since NIRS is sensitive to different sample attributes, the selection of samples to include in a calibration cannot be limited to samples that only vary in the analytical parameter being calibrated for. The calibration design-space should be defined in the NIR spectra domain as they capture other sources of variability affecting the calibrations and disturbing the trade-off above. As described before, that can be done if knowledge about the process being monitored is included (e.g., different process phases should be equally represented in the calibration data set, therefore process dynamics must be taken into account in sampling) and using a sample selection algorithm such as SPXY [20].

Here we first consider calibration development with samples under similar cultivation conditions for one CHO cell line (cell line A) using peptone containing media at the 1000 L scale only to

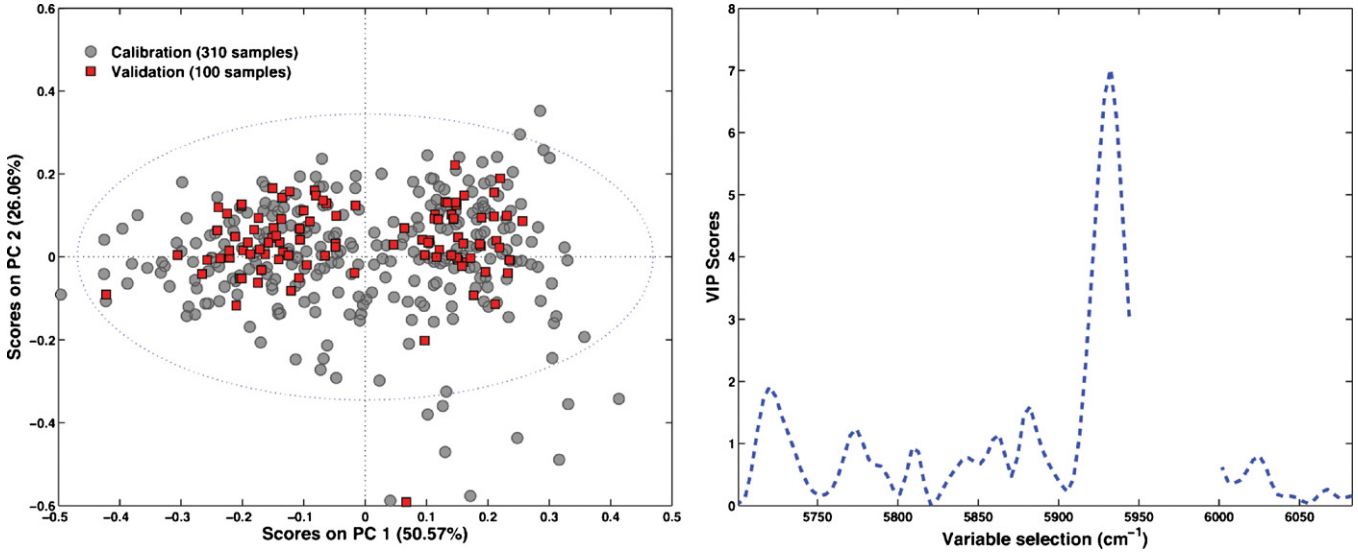

Fig. 8. Calibration design-space (2.5 L, 100 L and 1000 L scales) in the PCA space (left plot) of specific pre-process wavelength regions (right plot) indicating samples available: calibration samples (circles) and validation samples not used in model development (squares).

examine how accurate NIRS could be and evaluate fit-for-purpose. We then go on to investigate how general the approach is by considering different cultivation conditions.

Fig. 3 summarizes the results obtained for several culture parameters. Both calibration and validation results are presented. The accuracy obtained (as measured by the average prediction error in validation, RMSEP) is very close to the reference method used for each parameter. Also, all models are parsimonious with a very small number of latent variables. To prevent over-fitting, model order or rank was decreased to the minimum so that the lowest RMSECV was obtained. The calibrations developed are capable of describing well not only the trends but the values observed for all parameters from inoculation to harvest. Some models show slight curvatures and sensitivity problems at extreme values of the parameter range (e.g., ammonium and cell viability calibrations which are indirect calibrations for these parameters), but still are reasonably accurate supervisory models for capturing trends of culture parameters (Fig. 4). The differences found between predicted and measured profiles (Fig. 4) for a 1000 L scale cultivation not used in calibration development are always below 15% of the normalized scale, corresponding to the reference method accuracy.

3.3. How general is the approach?

To show that NIR monitoring on supernatant samples can be used as a general approach, 67 fermentations of three different CHO cell lines, at different scales (lab, pilot, industrial) using the same chemically defined media, based on CD-CHO, but with slight changes in formulation with supplemented components, and also using different feeding and operating strategies at each different scale, were analysed. For illustration purposes only two parameters are shown in detail – glucose and product – but results for all other parameters are summarized at the end in Table 1.

3.3.1. Glucose monitoring

To build the NIR glucose calibration model 320 samples from different scales, CHO cell-lines and cultivation conditions were used. The most important wavelength regions for a predictive glucose

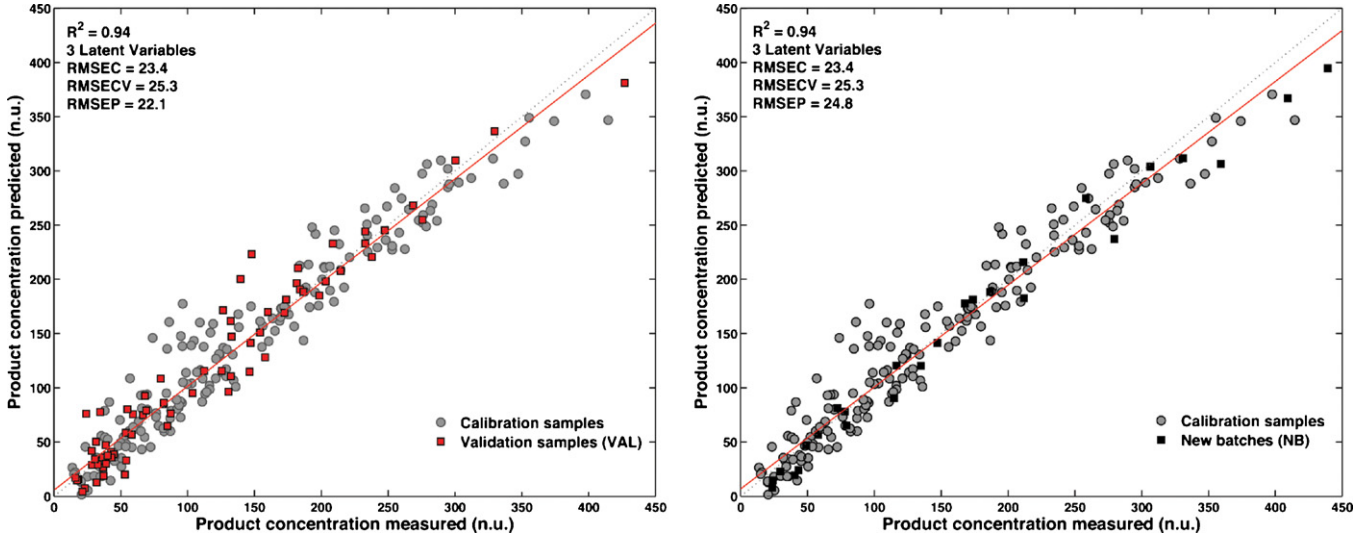

Fig. 9. NIR model for product monitoring valid across development and manufacturing scales and different protocols. The average accuracy (RMSEP) for validation samples not used in calibrating the model is reported (VAL), as well as the accuracy for entire cultivations set aside at different scales (NB).

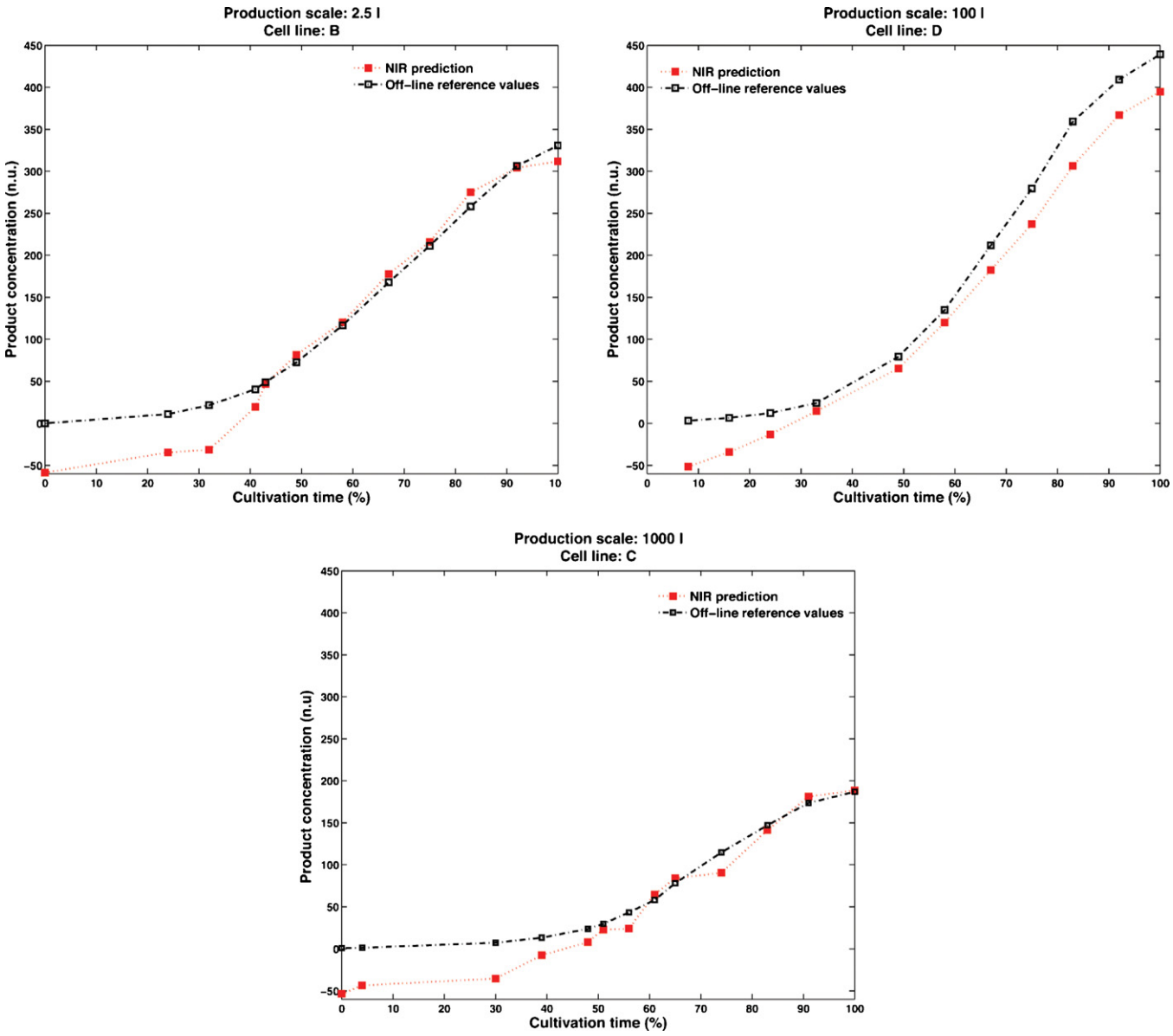

Fig. 10. NIR predicted product concentration profiles for each of the different process scales and cell-lines investigated, compared to reference values measured. These batches have been arbitrarily set aside for model evaluation (cf. Fig. 6 RMSEP-EB) and none of their samples have been used either for calibration or validation before.

calibration model were selected with the i-PLS algorithm [19] and are highlighted in Fig. 5. Five latent variables were identified as the optimal rank based on the variance captured by the model together with cross-validation errors obtained for each latent variable (Fig. 6). The NIR predicted glucose concentration profiles showed acceptable accuracy in the range investigated (Fig. 7), especially considering that it is a general calibration (i.e., scale-independent, cell-line-independent and valid with different feeds). Fig. 7 shows an off-set between predictions and measured values visible for the 100 L scale, most likely because batches from that scale are the least represented in the calibration data set. Comparable RMSEPs were obtained for validation samples and for batches set aside (EB, external batches) thus suggesting a good predictive ability of the developed model. As expected, the model obtained when several sources of variability are considered (e.g., scales, cell-lines, feeding strategies) has a rank that is higher than the model developed earlier for one scale and cell-line only (cf. Figs. 3h and 6).

3.3.2. *Product monitoring*

The same strategy was used for the product concentration as described for glucose. The available samples are different not only because glucose and product were not measured simultaneously for all samples, but also because the spectral regions selected for product and glucose are different and as such the calibration design-space for each parameter will differ. Nevertheless the calibration and validation sample-sets for product were defined as before through the SPXY algorithm, while the most informative wavelength regions were selected via the i-PLS algorithm (Fig. 8). A total of 310 samples were employed to build the NIR product calibration model. Three latent variables were identified as optimal (Fig. 9). The three product profiles predicted for batches not used before, show a good track of main trends and are reasonably accurate. As before model rank was increase by 1 when considering multiple scales, cell-lines and feeding strategies. Again for the 100 L scale, perhaps because it is not sufficiently represented in the calibration design-space, the predictions of product titers

towards harvest are less accurate than for the other two process scales (Fig. 10).

In general, a very good agreement was found between spectral regions selected by i-PLS and VIP. As VIP is more readily available in chemometrics' packages in Figs. 5 and 8 we show them instead. In the few cases in which i-PLS selected wavenumbers with a VIP below the usually accepted threshold of 1, the use of the VIP selected wavenumbers instead of the i-PLS ones always resulted in inferior predictive ability.

Table 1 summarizes the figures of merit for all parameters for which general models were developed (i.e., scale-independent, cell-line-independent, different feeding strategies). Considering the data set properties the models achieved are quite acceptable for process supervision (Table 1). It was not possible to develop accurate scale-independent models for LDH and ammonia concentration, most likely due to spectral selectivity issues. Moreover, a scale independent model for cell viability was not accomplished due to the lack of variability in the *Y* range for most batches considered for developing general models – for most samples the range of variation is within 95–100%. Nevertheless, accurate scale specific models are achievable, as shown previously. The robustness and general validity of the models achieved show that they were correctly developed and can be used in routine to supervise and control Mab cultivations. Most of the parameters of interest can be reliably estimated by NIRS, using defined wavelength regions, with an accuracy of 15% or better, compared to reference methods. Occasional fine-tuning of the models might be needed if cell-line, raw materials or process conditions change significantly (i.e., if one attempts to use the calibrations out of their own design-space). A more balanced representation of each individual process scale should be sought in order to obtain better models; that balance may not come only from having the same number of samples for each scale but in having samples from each scale of equal leverage in the design-space (i.e., each scale should contribute with a similar variance to the design-space).

4. Conclusions

The experience gathered in the application of NIR transmittance spectroscopy on supernatant samples from a Mab cultivation process spanning several scales from 2.5 L to 1000 L, CHO cell-lines and development years was described. The proposed approach demonstrates that NIR monitoring carried out at-line on samples already taken for reference methods analysis, (1) is very effective in replacing in-process monitoring of some critical process parameters by reference methods, (2) enables batch trajectories as defined by multiple culture parameters to be computed, (3) can be used to do guided sampling (i.e., near harvest or to investigate a deviation from the nominal trajectory by established bioanalytical methods) and (4) as such has potential in implementing process corrections or even control strategies (e.g., feeding). The proposed approach is applicable throughout process development as well as to existing large-scale validated CHO bioprocesses, as it does not require more complex and expensive set-ups (like in situ, on-line probes and process equipment are not needed), and is capable of supporting continuous improvement efforts on legacy non-QbD Mab manufacturing processes as well as new QbD-based processes.

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