Near-infrared spectroscopy in upstream bioprocesses

The quality of upstream processes and their products strongly depends on the control of all influencing parameters. However, several relevant parameters are not measured in standard bioreactor systems. Near-infrared spectroscopy (NIRS) is one promising technology capable of becoming the missing link in sensor technology. This review gives an overview of the technological principles and the technological progress. A broad range of possible applications is presented, forming in its entirety a valuable toolbox for process risk mitigation. Recent applications of NIRS in upstream bioprocesses are discussed. Moreover, the review includes regulatory aspects in implementation, calibration and validation of NIRS instrumentation and models.

The (bio)pharmaceutical industry is currently going through a phase of substantial changes. The reasons are complex. Major contributors are the decline in selling prices due to increasing market share of generics and biosimilars as well as expiring patents of former blockbusters. Additionally, regulatory aspects increase the costs and prolong the development of new pharmaceuticals. The resulting serious cost pressure requires highly efficient development and production processes. Optimal processes include the control of every process parameter having an impact on the product or the process itself. Why is controlling biotechnological production processes more challenging compared with other continuous production processes?

Biotechnological production processes are usually divided into two areas. The cultivation of cells starts with precultures from a cell bank. The series of cultivations until final harvest is defined as the upstream part. The downstream part includes all steps to process the resulting cell broth to meet purity and quality requirements regarding the final formulation of the product. Depending on the type of cell that is cultivated the upstream processes can be classified into microbial fermentations (bacteria, fungi, yeasts and algae) or cell cultivation (mammalian cells and insect cells).

Any biotechnological cultivation represents a complex process, including biological (e.g., uptake, conversion and degradation rates), physicochemical (e.g., pH, fractioning of inorganic carbon) and physical (e.g., concentrations, mass transfers and aggregation) variations. The complexity is further increased as cultivations are dynamic processes where all parameters are time dependent. The complete biology changes with proceeding process time, mainly due to cell age and the increase of biomass, resulting in an increased uptake, conversion and degradation of nutrients. Depending on process conditions, the same biological systems can follow different reaction pathways. Moreover, similar process settings at one stage do not necessarily result in similar process quality as biological systems memorize former process conditions [1]. For optimal process understanding real-time control of every parameter having an impact on product or process quality is crucial. In order to control a parameter, it needs to be constantly monitored. Thus, sensor technology is of particular importance when it comes to bioprocess development or bioprocess control. Spectroscopic techniques offer distinct advantages over other sensor technologies as they often provide noninvasive real-time...
measurements without the need for sampling and sample preparation.

According to the Aspen Brooks survey [2] almost every fourth bioprocess user expects spectroscopic tools to be industrial standard practice in bioprocesses within the next 5 years. However for most spectroscopic techniques the robustness of the instrumentation still hampers the implementation in production facilities. From instrumental point of view near-infrared spectroscopy (NIRS) is likely the most advanced technique with regard to robustness and reliability. NIRS does not require any laser excitation; instead a simple halogen lamp can be employed. In contrast to mid-infrared (MIR) spectroscopy, near-infrared (NIR) light can be guided via standard fused silica or polymer fibers instead of using sensitive halide fibers with high attenuation. This simplicity resulted in NIRS being the workhorse of spectroscopic techniques in process analytics for more than 20 years [3].

There are numerous terms describing the adaption of a measurement system or sensor to a process such as on-line, in-line, at-line, off-line, in situ and real-time, among others. In this publication we follow the definition of the US FDA, claiming in-line measurements when no sample is removed from the process, on-line when measuring in a bypass mode and at-line/off-line, when a sample is removed from the process and measured in close proximity/in far distance of the process stream [4]. The following chapter will give an overview on the measurement principle of NIRS, its spectral information and instrumentation. Additionally, opportunities and challenges of varying data analysis tools will be discussed. Finally, a progress overview of NIRS applications in biotechnological upstream processes is presented.

### Theory

**Near-infrared spectroscopy**

Spectroscopy in general describes the analysis of the interaction of matter with electromagnetic radiation. NIR spectroscopy belongs to the group of molecular spectroscopies. Together with MIR and Raman spectroscopy, it is part of the subgroup of vibrational spectroscopies. Two major effects contribute to a vibrational spectrum with varying percentage, depending on the excitation wavelength and the sample itself. First, scattering effects result in varying spectral backgrounds and reveal physical information of the sample. The amount of scattering and thus detected physical information decreases with increasing wavelength and therefore is the highest for dispersive Raman and the lowest for MIR spectroscopy. Second, vibrational spectra contain chemical information. Raman detects inelastic scattering of monochromatic radiation whereas MIR spectroscopy and NIRS detect the absorption of broadband light (NIR: 800–2500 nm ≅ 12,500–4000 cm⁻¹, MIR: 2500–25,000 nm ≅ 4000–400 cm⁻¹) [5].

Depending on the vibrational energy of molecular bonds in the sample, different discrete portions of energy of the provided broadband light can be absorbed. The absorbed energy of each molecular bond depends on the involved atoms, the type of bond (e.g., single or double) and its structural surrounding. Thus, the wavelength (Δ energy) of the absorbed light gives insight into the molecular structure of the molecules that contribute to a specific sample. As only molecular vibrations are detected, atomic ions species and metals do not absorb infrared light. Additionally, black samples completely absorb infrared light independent of wavelength or contained molecular bonds, thus no light reaches the detector and no spectrum is obtained.

Although the chemical information of MIR spectroscopy and NIRS is mostly equivalent, the origin differs. In MIR spectroscopy fundamental oscillations that result in narrow bands are detected and absorption bands do not necessarily overlap. This allows for simple quantification models via band height or area in some cases. On the other hand in NIRS detects overtones and combination bands. The number of overtone and combination bands exceeds the number of ground vibrations by several orders. This results in broader spectral bands originating from overlapping vibrations [5,6]. Contrary to MIR spectroscopy quantification models, extraction of the chemical information from NIR cannot be done in a univariate way using band height or area. Instead multivariate tools like principal component analysis (PCA) and partial least squares (PLS) are employed to extract the desired chemical or physical information from the spectra [7].

### Multivariate data analysis

Multivariate data analysis (MVDA) tool offers the distinct advantage of revealing hidden patterns in datasets as well as interactions between parameters. Spectra consist of hundreds, sometimes thousands of data points, called variables in MVDA. Evaluating one variable at a time (univariate analysis) results in hundreds and thousands of similar or even contradictory outcomes. This is why data reduction is one of the key achievements of MVDA. All variables containing similar information are bundled and variables without relevant information (noise) are separated from the important ones [7]. The decision of which multivariate
method has to be employed depends on the purpose of its application. This can be either classification or quantification. Application examples for classification in upstream are media classification and harvest point determination. Most methods include a PCA. The following chapter will give an excursion on data pretreatments followed by an intuitive explanation of the most commonly used mathematical methods PCA and PLS on the example of a spectral dataset.

Data pretreatments
Spectral data are often pretreated to account for varying background and/or scatter effects. However, pretreatments should only be applied with reason and not arbitrarily. The reason should be either physical or chemical motivated, such as the correction of physical scattering or a specific instrument signal. First of all the optimal pretreatment depends on the nature of the parameter. Process parameters as pH or temperature require unit variance normalization to account for the different parameter scaling. It equalizes the impact of each parameter on the model. If applied to spectroscopic data, unit variance scaling will amplify the noise of regions with low variation and negate useful spectral information. Thus for spectroscopic data, only mean centering should be used, sometimes in combination with a scatter correction when appropriate.

Several pretreatments are used to correct for baseline shifts, slopes or curvatures. They reduce the impact of particle size, scattering and other influencing factors, for example, drifts from instrument instabilities [7]. For example, varying particle sizes will cause a baseline shift in the spectra as the spectral pathlength is defined by the particle size [8]. Pretreatments should be applied in the same order as the physical effects occur in the measurement line (e.g., instrument, light pathway, sample).

Typical methods for baseline/scatter correction include first and second derivatives, multiple scatter correction standard normal variate transformation and orthogonal scatter correction [9]. Constant underlying backgrounds and other systematic effects can be reduced by derivatives. Variations in the optical pathlength due to particle size variation of solid samples, emulsions and dispersions require multiplicative scatter corrections. In principle all scatter corrections aim to separate chemical and physical information. The intuitive approach is to find a band that is affected by variations of optical pathlength but not influenced by changes of the sample composition. However, the overlapping bands in NIRS hamper this simple method. Several efficient approaches as multiple scatter correction and extended multiple scatter correction include spectra shifting and scaling to fit a target spectrum. The mean spectrum of the spectral dataset is used as target spectrum. This leads to three difficulties. First, spectral outliers contribute to the mean spectrum. Second, for quantitative analysis, the calibration set is often based on different products and the spectral mean does not correlate with any single product but is an artificial generated spectrum. Third, the mean spectrum depends on the overall dataset and thus changes whenever a new spectrum is acquired. Standard normal variate transformation overcomes these issues; the correction is done separately for each single spectrum and is not based on an overall mean spectrum. Each spectrum is first centered to 0 (subtraction of spectral mean), followed by normalization (dividing by the standard deviation of the complete spectrum). The basis of orthogonal scatter correction is the assumption that spectral information with predictive quality is orthogonal (noncorrelated) to noise or other influence factors. Both are separated with the help of PLS algorithms (see section ‘Least squares algorithms’).

The decision whether a scatter correction is required, strongly depends on the nature of the analyte and the scattering characteristics of the sample. Most solid samples require scatter correction, whereas liquid homogeneous solutions do not justify any scatter correction pretreatments. Figure 1 gives an overview of when to apply each pretreatment or scatter correction.

Principal component analysis
PCA consists of three major steps. First, the data are transformed. Second, the data are reduced including a separation between useful information and noise. Third, the visualization of the data is adapted to human beings, accounting for our poor capability of recognizing patterns in huge data tables.

In a spectrum intensities are plotted over wavelengths (Figure 2A). It consists of N data points, one data point per recorded wavelength. Figure 2A displays a dataset of simplified spectra consisting of two wavelengths $\lambda_1$ and $\lambda_2$. In a first step an N-dimensional coordinate system is created, one axis for each wavelength. Now the spectrum with originally N data points is transformed into a single point in this N-dimensional coordinate system. This is done for each spectrum of the dataset resulting in a data cloud in this N-dimensional coordinate system (two dimensions in the example in Figure 2B). Next a new axis is inserted covering the biggest variation of the dataset. This means the algorithm looks for the largest weighted distance between the data points ($\Delta$ spectra) and creates a new axis in this direction. This new axis is called principal component 1 (PC1); the number indicates that it covers the biggest variation in the dataset. The angle of the new axis will be in between the original wavelengths axes.
Therefore, the original axes contribute more or less to the new PC1 and information of different axis is combined in the new PC. Now a second axis (PC2) can be added being orthogonal to the PC1. It is set in a way that covers the second biggest variation in the dataset (Figure 2C). As the axis is orthogonal by definition, the information does not correlate at all with the information of PC1. Following PCs are again orthogonal to all preceding PCs. As a result, most variations in a dataset of hundreds or thousands of variables (wavelengths) are combined in a few PCs.

Finally, the visualization is optimized in so-called score plots where two or three PCs (often the first two or three PCs) are plotted against each other. In this plot grouping or separation patterns become very obvious as the first PCs present most of the variance in the dataset in a condensed way. An example of a score plot of a cultivation process is given in Figure 3.

This procedure automatically separates useful information and noise: Wavelengths that show the biggest variations in the dataset will dominate the direction of PC1. On the other hand wavelengths with no variations in the dataset will hardly influence the direction of the PC. This information is visualized in the loadings plot which shows the impact of each variable on the model. Here, it is implied that areas without variation in the dataset do not hold valuable information. With other words, sample properties that cause no significant variation in the spectral response cannot be modeled. The reason can be either that there are no molecular vibrations in the recorded spectral range (NIR inactive) or that the parameter variations of the sample set are too small to influence the spectral response significantly.

PCA reveals the biggest variations in the dataset, independent of its origin. No additional information but the spectra itself is used for classification. It is a powerful tool as long as arbitrary variations are not very high compared with the structured variations that shall be revealed.

When previous knowledge about class membership or quantitative attributes is available, least squares algorithms offer distinct advantages as they take this extra information into account.

Least squares algorithms
Least squares algorithms include the most common algorithm for quantification purposes, PLS and clas-
classification algorithms such as partial least squares discriminant analysis (PLS-DA) [10]. Besides the spectra, qualitative information of the measured samples is part of the dataset. Depending on the specific algorithm this can be classification information (PLS-DA) or quantitative data from reference analyses (PLS). In contrary to the normal PCA algorithm, the biggest variations do not define the orientation of the generated PCs. Instead directions are chosen that correlate best with the trend of the reference values (PLS) or that give the biggest distance between the predefined classes (PLS-DA). Thus PC1 does not necessarily cover the biggest variation in the dataset anymore. It covers the biggest variation in the dataset that correlates with the known sample information. These models when applied on real-time acquired spectra of unknown samples enable on-line prediction of analyte concentrations or class memberships.

Application examples of MVDA in upstream processes
MVDA can be applied manifold when analyzing NIR spectra in upstream processes. First qualitative models can be used for classification purposes and for process control with batch trajectories (see section ‘Batch trajectories’). Sometimes quantitative models are used to support classification models, however the primary use is monitoring and/or control of critical process parameters or critical quality attributes.

Classification
In media preparation, NIRS can be used to classify the media. Here, the spectrum of the media or media powder is used as a fingerprint of ‘good’ media, which means media of batches that delivered satisfying product quality and quantity. The classification of the powder reveals variations in raw material quality. Classification on the liquid media identifies handling errors, for example, whether all components were added in the right amount [11,12].

At the end of the upstream process a classification can be made to define the optimal harvest time. Spectra are taken around harvest times and defined as good or bad harvest time points. After model building, optimal harvest points are predicted for new batches.

Batch trajectories
NIRS is a multiparameter technique and detects multiple variations that occur during upstream processes. Figure 3 displays a typical score plot of a complete cell cultivation run, visualizing process variations over time. In this example, the first PC can be linked to the cell count whereas PC2 indicates the cell metabolism. Now for each measurement the value on any PC (so-called score values) can be plotted against process time. Usually, a PLS is applied with process maturity being the qualitative parameter instead of reference values. The resulting line is called batch or process trajectory and describes the process evolution of this specific batch. If this is done for a set of N batches that were considered as good, an average trajectory can be calculated, often referred as ‘golden batch trajectory’. Adding upper and lower limits based on the standard deviation of the N batches at each time point results in a road of process evolution as shown in Figure 4, sometimes also named as batch evolution model (BEM) or batch control chart. This method allows operators to monitor deviations from desired process behavior in

![Figure 2. Principle of principal component analysis.](image-url)
Figure 3. Example of a score plot from a cell cultivation. The axis score 1 and score 2 are the two principal components (new coordinates) that summarize the spectra variations over the cultivation. Each point represents one collected spectra. The cultivation evolves from point A to point B (increase in score 1) and time point C shows a point where the metabolism changes, for example, exhaustion of a nutrient or start of catabolism of intermediate metabolites.

real time. Process deviations are indicated by the actual batch trajectory leaving the defined road of process evolution. Even if the precise reason for the deviation or the link to a control parameter might not be obvious, guided sampling and off-line analysis can be triggered, thus reducing the time delay of counteractions significantly. This leads to a more event-based process control strategy.

In addition to BEMs, an extra step in data reduction can be taken. The so-called batch level models, rearrange the data in a new PCA model. Complete runs are classified revealing patterns or clusters in the set of batches. This allows visualizing and analyzing similarities between complete batches and the detection of general outliers, which is beneficial for the development of general process strategies.

Quantification
The quantitative evaluation of NIR spectra is likely the most common application of NIRS in upstream processes. Several parameters can be monitored simultaneously, including chemical parameters as nutrients, metabolites and product titer. Additionally, physical parameter as cell count, optical density and viability can be modeled, whereby special attention has to be paid to data pretreatment as the physical information originates from scattering (see section 'Data Pretreatments').

Model validation
Model validation is part of every model building process. There are two commonly used methods for model validation, cross-validation and external test set validation. The first divides the calibration test set into N blocks of spectra. Then a model is generated from N-1 spectral blocks and the last block is predicted. This procedure is repeated until every block was N-1 times part of the calibration test set and one time of the validation test set. An average prediction error of the N models is calculated which is called standard error of cross-validation.

External test set validations form a more demanding and realistic validation. The sample set is divided into two parts. The first part is used for model building (calibration set). The model is then applied to predict the second part of the sample set (validation test set). It is important not to use repetitive measurements in calibration and validation test set. A sample including all repetitive measurements (or repacks) should either be in the calibration or the validation test set. The range of the analyte concentration as well as the process variations for the validation set must be covered by the calibration set variables as statistical models are designed for interpolation but not for extrapolation.

Cross-validation is only to be used when the number of calibration samples do not allow for an external test set validation. The reason is that all samples are in both, the calibration and the validation test set. Thus, the validation test set is not independent from the calibration set.

For bioprocesses, complete batches should be used as external test set for model validation. Only then, the validation set is truly independent. Selecting every fourth sample of each batch as a validation test set will give too optimistic results mainly due to the reason that interpolation is one of the key features of this kind of model building. Thus, calibration and validation samples are very similar and just differ slightly by sampling time.

Methods
NIR instrumentation
NIR systems can be based on several measurement principles. One difference can be how the wavelength-dependent information is gathered. The wavelength separation can be done either on the illumination or the detector side. In the first case, the sample is illuminated with monochromatic (or narrow band) light.
demanding for a sequential detection without the need of a diffractive element. In the latter case, the sample is illuminated with broadband (or so-called white) light. A diffractive element is employed on the detector side to gain wavelength depending information. Following the most common instrument types are briefly discussed and a comparison is displayed in Table 1 [14,15].

Historically, the first NIR devices were modified UV–Vis spectrometers with a scanning grating and an NIR sensitive lead(II)sulfide (PbS) detector. The monochromator is used to select specific wavelengths for excitation. The interacted light is detected without any wavelength selection. More recent instruments employ a simple indium gallium arsenide photodiode instead of the PbS detector, however the overall principle remains the same. The advantage is the very high sensitivity as on the detection side the light is not attenuated by any diffractive element. Additionally, sample heating is minimized as no broadband light source is used. On the other hand, the spectrum is acquired sequentially, one wavelength fraction at a time. After each measurement, the grating is repositioned and the next data point is acquired. Thus, this technique is not suited for process adaptation whenever the sample might vary over time. In worst case the resulting spectrum is a mix of spectral information, the lower wavelengths originating from another sample than the higher wavelengths. Another drawback is the permanent requirement of a wavelength standard to correct for the inaccuracy and low repeatability of the positioning of the grating. The best applications for this kind of instruments are off-line measurements without sample movement, whenever highest sensitivity is crucial.

The monochromator can be replaced with a wheel of interference filters. The detector side can remain untouched. The number and selection of filters in combination with the bandwidth of the filters determines the range and resolution. The biggest advantage compared with scanning grating instruments is the increased speed as the filters are changeable hundreds of times per second. No precise positioning is required, resulting in a higher robustness. Even though the spectra are acquired sequentially, the overall measurement time for a complete spectrum is in the range of tens of ms, resulting in a quasi-simultaneous measurement. However, the number of filters is limited and must be preselected. Thus, such an instrument has to be designed for a specific application. A new application will require preliminary trials in which the set of wavelengths and therefore the set of filters are defined, followed by a change of filters. Additionally, any product variation that was not included when defining the instrument’s filter set might require new application tests and a redefinition of the filter set. In summary, filter devices are well suited for process application whenever the process and/or the product do not change over a long period of time.

Fourier transform (FT) devices employ broadband light and a (Michelson) interferometer. First, a beam splitter divides the light into two fractions. One beam is guided via a moving mirror toward the sample and the other to a reference arm with a fixed mirror. Both reflected beams are unified afterward again and interfere depending on the contained wavelengths and the mirror position on the sample side. The recorded interferograms are mathematically processed to an absorption spectrum via Fourier transformation. The scanning data acquisition of the interferograms in the time domain leads after Fourier transformation to a complete simultaneous spectrum in the frequency domain. However, several scans might be necessary to improve signal to noise ratio. The moving mirror and the positioning of the beam splitter make the FT systems with Michelson interferometer sensitive to vibrations and shocks. Thus, the optimal application for FT systems is laboratory use.

Diode array spectrometers also employ broadband excitation. After interaction with the sample, the light is guided to a grating mirror and the diffracted light is imaged on a linear plate of photodiodes (diode array). Advantageous for process application is the exceptional robustness due to the lack of moving parts. The spectral resolution is limited by the number of photodi-
odes. However, care has to be taken when employing a higher number of photodiodes with the same overall chip size as sensitivity is reduced. The optimal sensitivity is reached with lower resolution, though the broad nature of NIR bands does not demand high resolution. Spectra are acquired simultaneously, being advantageous for process applications with fast product variations. Multichannel spectrometers allow for fiber and fiber free coupling. So-called free beam optics offer the distinct advantage of large sample spots. Thus, a higher amount of product is measured simultaneously, resulting in less fluctuation of the measured signal. Additionally, light losses due to fiber coupling and fiber transport are minimized. The lack of fragile fiber optical cables increases the robustness. Furthermore spectral distortions due to fiber bending or temperature effects are avoided. In summary, multichannel spectrometers are optimal for versatile process use.

Ultimately, the decision of which instrument to use is very application dependent. While for lab equipment extra weight is given to spectra resolution and wavelength accuracy, for a production environment, measurement speed, compatibility with existing standard ports and robustness of the device have higher relevance.

### Process validation

Regarding NIRS, **process validation** in its entity comprises the instrument itself, the software, the model and the interfaces. Major steps are: installation qualification, operational qualification and performance qualification. The regulatory requirements for setting up a monitoring procedure using NIRS can be found in the US Pharmacopeia (USP), Chapter 1119 and EU Pharmacopoeia (EUP), Chapter 2.2.40. The documents encompass guidelines for the qualification of the spectroscopic hardware and for the validation of the chemometric methods [16,17]. These documents focus on laboratory applications and lack detailed descriptions for in-line process analytical technology (PAT) applications as recommended by the FDA in 2004 [4]. However, they are described in the following paragraph as they represent the base on which further industry guidelines were issued that are described afterward.

According to the pharmacopeias the hardware qualification of an NIR device should be divided into three qualification steps: installation qualification, operational qualification and performance qualification. During installation qualification, it is tested whether the device is installed according to the manufacturer specifications. Therefore, the necessary steps differ between instrument types. Operational qualification demonstrates that the device operates within its specifications. Recommended specifications can be found in the pharmacopeias and the industry standard guidelines. Properties to be tested are, for example, wavelength uncertainty, photometric linearity and spectrophotometric noise. For the performance qualification,
above-mentioned measurements are repeated on a regular base to ensure long-term stability of the device.

In addition to the hardware qualification, the chemometric method needs to be validated in order to demonstrate the suitability for its respective purpose. Guidelines for the general validation of analytical methods can be found in the USP 1225 [18]. The validation of chemometric NIRS models in particular is discussed in the USP 1119 and EUP 2.2.40 [16,17]. Key points are the investigation of:

- Model specificity: testing the correct identification of all samples, taking into account different suppliers or production lots. Moreover, samples that are not included in the classification library must fail identification;
- Model linearity: testing if the NIR response is correlated throughout the defined range of the calibration model;
- Model range: testing if values outside the calibration range are correctly marked as outliers;
- Model accuracy: the standard error of prediction (SEP) is determined and compared with the standard error of the reference method;
- Model precision: calculating the standard deviation of replicate measurements with and without changing the sample position and stacking. Intermediate precision is determined by calculating the standard deviation of replicates made from different analysts on different days;
- Model robustness: challenging the model with expectable process variations. These can encompass but are not limited to variations of environmental conditions (temperature and humidity, among others), sample temperature, sample handling (positioning and material compression, among others) and instrument hardware.

As mentioned before the described Pharmacopoeias were designed for laboratory measurements and lack guidance for in-line processes. This gap was closed when the American Society for Testing and Materials (ASTM International, PA, USA) issued their general guide applicable on all PAT measurement procedures in 2011 [19]. In 2012, the EMA published a more specific guideline on the PAT application of NIRS [20]. The guideline stresses the iterative nature of the development and implementation of NIRS procedures which is roughly organized in five stages. During the initial development stage a clear scope of the NIRS procedure has to be set up. Feasibility studies should be carried out to demonstrate the suitability of the method for the defined scope. The second stage consists of data collection and interpretation. This includes proper sample preparation and presentation as well as reference analysis and spectral library creation. During the third stage the calibration model is set up and validated in the fourth stage with similar key parameters as previously discussed for USP and EUP. During the fifth stage maintenance procedure of the NIRS method is initiated. The guideline recommends updating the calibration models in case of spectral variations which are not included in the actual model (e.g., new production batches or change in raw material). The interval in which the procedure is updated is defined as lifecycle. It might be necessary to change the NIR procedure which may involve its redevelopment. The guideline defines which changes are to be considered out of scope resulting in the necessity of redevelopment. For clarification purposes, an addendum to the EMA guideline has been issued giving examples of how different changes would have to be managed according to the guideline [21].

**NIRS applications in bioprocesses**

Lourenço et al. [22] present an overview on different spectroscopic techniques for bioprocess monitoring. In their review, a summary of in-line NIRS applications is given. It is interesting to note that the majority of applications stick to small-scale reactors and microbial fermentations with just a few examples of industrial fermentations and cell cultivations. In our review we try to cover advancements made since that publication. In Tables 2 & 3, a summary of the reviewed applications is given with the main analytes modeled and validation errors for microbial applications and cell cultivations, respectively.

**Microbial fermentations**

Fermentations with microbial cells are characterized by high metabolic rates and rapid growth. They can be performed in small scale for research and process development but production facilities range up to 100-m³ process volume. Often these processes require vigorous stirring and aeration and especially in large fermenters the heat exchange becomes challenging. Process analyzers have to cope with these environments. This means they need to have a robust design which allows them to function even on vibrating reactors and under elevated temperatures. Table 2 gives an overview about NIRS applications in microbial fermentations.

**At-line**

Morita et al. [34] used NIRS as a high-throughput screening technique of recombinant *Saccharomyces*
Table 2. Summary of near-infrared spectroscopy applications in microbial processes.

<table>
<thead>
<tr>
<th>Application</th>
<th>What was measured?</th>
<th>Calibration models</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch <em>Escherichia coli</em> DH5α fermentation for plasmid production</td>
<td>Biomass (OD&lt;sub&gt;600&lt;/sub&gt;)</td>
<td>Constant offset elimination 0.2 g/l 0–18 g/l</td>
<td>Test set validation for biomass and cross-validation for glycerol</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>Biomass (dry weight)</td>
<td>Constant offset elimination 0.3 g/l 0–6 g/l</td>
<td>–</td>
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</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>Constant offset elimination</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Lab scale (3 and 4 l); <em>Streptomyces coelicolor</em>; batch antibiotic production</td>
<td>Glucose</td>
<td>Second derivative 1.1 g/l 0–32 g/l</td>
<td>Cross-validation</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Ammonium</td>
<td>SNV, first derivative 11 mM 39–111 mM</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lab scale (7 l); batch fermentation; <em>Bordetella pertussis</em></td>
<td>OD&lt;sub&gt;90&lt;/sub&gt; (lactate, glutamate)</td>
<td>Data centering –</td>
<td>Cross-validation</td>
<td>[25]</td>
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<tr>
<td></td>
<td></td>
<td>–</td>
<td>Semisynthetic used for prediction model</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td>–</td>
<td>Near infrared was part of a full design space investigation involving DoE and batch evolution models</td>
<td>–</td>
</tr>
<tr>
<td>Lab scale (1, 6 and 3 l) fed-batch <em>Pichia pastoris</em> cultivation</td>
<td>Glycerol</td>
<td>Not disclosed (proprietary) 4.1 g/l 0–50 g/l</td>
<td>One batch used to improve factory calibrations; five batches for validation</td>
<td>[26]</td>
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<tr>
<td></td>
<td>Methanol biomass</td>
<td>–</td>
<td>Models come preloaded in device</td>
<td>–</td>
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<tr>
<td></td>
<td></td>
<td>0, 8 g/l 0–25 g/l</td>
<td>Feedback control of methanol implemented</td>
<td>–</td>
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<tr>
<td>Lab scale (5 l), batch <em>Lactococcus lactis</em> fermentation</td>
<td>Nisin titer</td>
<td>FFT, SG, first or seconf derivative 3320.22 IU/ml 3000–18,000 IU/ml</td>
<td>Optimized calibration with Monte Carlo simulations</td>
<td>–</td>
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<tr>
<td></td>
<td>Reducing sugar concentration</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>Cell concentration</td>
<td>–</td>
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<td></td>
<td>pH</td>
<td>–</td>
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DoE: Design of experiment; DOSC: Direct orthogonal signal correction; FFT: Fast Fourier transform; MC: Mean centering; MSC: Multiplicative scatter correction; OD: Optical density; PLS: Partial least squares; SG: Savitzky–Golay smoothing; SNV: Standard normal variate; SUM: Sum parameter of all metabolites (AnalyteSUM) or all sugars (SugarSUM).
Table 2. Summary of near-infrared spectroscopy applications in microbial processes (cont.).

<table>
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<tbody>
<tr>
<td>Lab scale (30 l) fed-batch <em>Corynebacterium glutamicum</em> temperature triggered fermentation</td>
<td>Glutamate</td>
<td>MSC</td>
<td>2.56 g/l</td>
<td>15–165 g/l</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>SNV</td>
<td>1.29 g/l</td>
<td>0–45 g/l</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>First derivative, straight line subtraction</td>
<td>0.0062 g/l</td>
<td>0.14–1.93 g/l</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>First derivative, SNV</td>
<td>0.243 g/l</td>
<td>0.66–5.50 g/l</td>
</tr>
<tr>
<td>Lab scale (100 ml) for high-throughput screening, <em>Saccharomyces cerevisiae</em> batch fermentations</td>
<td>Glucose</td>
<td>MC</td>
<td>1.08 g/l</td>
<td>0–65 g/l</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>DOSC</td>
<td>1.19 g/l</td>
<td>0–40 g/l</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>–</td>
<td>1.47 g/l</td>
<td>0–45 g/l</td>
</tr>
<tr>
<td></td>
<td>Xylitol</td>
<td>–</td>
<td>0.34 g/l</td>
<td>0–5 g/l</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>–</td>
<td>0.42 g/l</td>
<td>0–5 g/l</td>
</tr>
<tr>
<td>Industrial scale (50 m³) sporulating <em>Bacillus</em> fermentation</td>
<td>AnalyteSUM</td>
<td>–</td>
<td>0.81 g/l</td>
<td>0.5–43 g/l</td>
</tr>
<tr>
<td></td>
<td>SugarSUM</td>
<td>–</td>
<td>1.33 g/l</td>
<td>0–35 g/l</td>
</tr>
<tr>
<td></td>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>–</td>
<td>2.88 OD</td>
<td>3.6–50</td>
</tr>
<tr>
<td></td>
<td>Dry mass</td>
<td>–</td>
<td>0.09%</td>
<td>0.4–1.7%</td>
</tr>
<tr>
<td></td>
<td>Acetoin</td>
<td>–</td>
<td>0.94 g/l</td>
<td>0–11 g/l</td>
</tr>
</tbody>
</table>

DoE: Design of experiment; DOSC: Direct orthogonal signal correction; FFT: Fast Fourier transform; MC: Mean centering; MSC: Multiplicative scatter correction; OD: Optical density; PLS: Partial least squares; SG: Savitzky–Golay smoothing; SNV: Standard normal variate; SUM: Sum parameter of all metabolites (AnalyteSUM) or all sugars (SugarSUM).
cerevisiae strains. Recombinant strains were cultivated in 100-ml bottles. Samples were collected from the fermentation, and their supernatants were analyzed with NIRS. The samples were analyzed in transmission mode with a dispersive device in a cuvette with 1-mm pathlength. The spectra were collected in the 1100–2498-nm range. Quantitative calibration models for glucose, xylose, ethanol, glycerol and xylitol were developed with PLS. Furthermore, different strains were classified. It is concluded that the NIR spectral data contain information about the genotypic and phenotypic differences between the strains. The quantification models had high prediction accuracy. Models made with samples from an individual strain performed worse when predicting samples for other strains. A global model incorporating samples of all strains had good performance but slightly worse than each individual model.

Guo et al. [35] used at-line NIRS to monitor the production of nisin – a bacteriocin with 34 amino acids – in a Lactococcus lactis fermentation. The fermentations took place in 5-l reactors and the fermentation conditions were set up by applying design of experiment (DoE). Initial pH, temperature and work volume were the variables changed in the experimental design. The spectra were acquired in the range of 800–1850 nm. The analytes modeled were the nisin titer, the concentration of reducing sugars, cell concentration and pH. The calibration models were built with PLS and Monte Carlo simulations were used to optimize the calibration parameters (number of PCs, wavelength selection and outlier identification). However, different pretreatments were applied without taking into account the nature of the signal. For example, background or scatter corrections should not be applied for physical parameters as cell count. Otherwise model validity must be questioned. Validation metrics were also estimated with Monte Carlo simulations, but no independent cultivation was used for final validation.

Liang et al. [36] presented an application of at-line NIRS in the monitoring of the glutamate-producing Corynebacterium glutamicum. The bacteria were cultivated in 30-l fermenter and eight fed-batch runs were performed to build the calibration models. The spectra were acquired in the 833–2500-nm range with a FT-NIR spectrometer. Calibration models were built with PLS for glutamate, glucose, lactate and alanine. The external validation set was a fermentation run in batch mode in contrast to the fed-batch runs used to build the model.

On-line
Goldfeld et al. [26] have recently evaluated the use of an NIR device for real-time monitoring and control of a Pichia pastoris bioprocess. Six fermentations took place in 1.6- and 3-l reactors. The spectroscopy system used an Acousto Optic Tunable Filter (AOTF) spectrometer with an extended indium gallium arsenide detector. The system configuration involved the circulation of the cell culture broth continuously from the reactor through a sample module in the monitor system and back to the reactor. Spectra were collected in transmission mode with a pathlength of 1 mm in the range of 2000–2500 nm. The spectroscopic system provided factory calibrations from the manufacturer for glycerol, methanol and relative cell density. The employed chemometric methods are not disclosed. A first calibration trial was performed to adjust the offset between preinstalled calibrations and actual process. The robustness of the models is shown over a period of 274 days after calibration. A feedback control was developed to keep methanol concentrations constant.

In-line
Streefland et al. [25] investigated the design space of bacterial vaccine cultivation process using several PAT tools, including NIRS. A DoE was used to investigate the impact of different process parameters on product quality. The cultivations were performed in a 7-l bench-top reactor. Spectra were collected in the range of 833–2500 nm using a FT-NIR device with a transmission probe. PLS calibration models were built for optical density (OD) of lactate and glutamate. Given the high degree of correlation between the modeled analytes, semisynthetic samples were prepared and measured in an NIR cuvette bench. The compositions of the prepared samples did not follow the usual correlations between analytes. Additionally, comprehensive BEMs were generated based on the merged data from NIR scores and process variables. The NIR data provide a ‘fingerprint’ of the process evolution without the need of building a calibration model with reference samples. By integrating all the data sources, a model able to monitor the process evolution on-line and able to predict the expected final product quantity was obtained.

Petersen et al. [24] monitored the fermentation of the filamentous bacterium Streptomyces coelicolor with in-line NIRS. The cultivations took place in 3-l reactors and additional parallel runs were carried out to generate in-line semisynthetic samples. Spectra were collected both, in-line with a FT-NIR device equipped with a transfectance probe as well as off-line in a cuvette configuration. The covered spectral range was from 633 to 2500 nm. They built models for glucose and ammonium using in-line and off-line samples separately. Validation was done with an independent batch run. The model for in-line ammonium prediction was not
Table 3. Summary of near-infrared spectroscopy applications in cell cultivations.

<table>
<thead>
<tr>
<th>Application</th>
<th>What was measured?</th>
<th>Calibration models</th>
<th>Validation error</th>
<th>Range</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed-batch production scale (12,500 l); CHO cells for mAb production with temperature shift</td>
<td>Osmolality</td>
<td>SNV, deresolving function</td>
<td>22.24 mOsm</td>
<td>288–414 mOsm</td>
<td>Cross-validation</td>
<td>Model accuracy by ratio of performance deviation and range error ratio</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>MC</td>
<td>2.22 g/l</td>
<td>0.5–15.86 g/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Product titer</td>
<td>MC</td>
<td>0.2 g/l</td>
<td>0.04–1.71 g/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCV</td>
<td></td>
<td>0.54%</td>
<td>0.22–5.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ivPCV</td>
<td></td>
<td>68.25 ml/dl</td>
<td>0.84–457.12 ml/dl</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VCD</td>
<td></td>
<td>15.27 × 10^5 cells per ml</td>
<td>12.98–145.126 × 10^5 cells per ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ivCC</td>
<td></td>
<td>176.86 × 10^5 cells per dml</td>
<td>5.43–1345.95 × 10^5 cells per dml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory scale (10 l); mammalian cells form Ab production</td>
<td>Glucose</td>
<td>First derivative</td>
<td>190</td>
<td>331 mg/l</td>
<td>0–1 (normalized)</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>SNV, first derivative</td>
<td>196</td>
<td>276 mg/l</td>
<td>0–1 (normalized)</td>
<td>Cross-validation</td>
</tr>
<tr>
<td></td>
<td>Cell density</td>
<td>–</td>
<td>3.5 × 10^6 cells per ml (both phases)</td>
<td>0–1 (normalized)</td>
<td></td>
<td>Qualitative models: process trajectories</td>
</tr>
<tr>
<td></td>
<td>Ammonia</td>
<td>SNV</td>
<td>5.7</td>
<td>14.6 mg/l</td>
<td>0–1 (normalized)</td>
<td></td>
</tr>
<tr>
<td>Lab scale (3 l); attachment cell line in microcarriers followed by virus infection</td>
<td>pH</td>
<td>SNV, second derivative</td>
<td>0.0134</td>
<td>7.02–7.16</td>
<td></td>
<td>No details about number of batches and selection of validation set are given</td>
</tr>
<tr>
<td></td>
<td>Osmolality</td>
<td>SNV, second derivative, normalized</td>
<td>1.8825 mOsm/kg</td>
<td>370–430 mOsm/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Labscale (2 l); vero cells on microcarriers</td>
<td>Glucose</td>
<td></td>
<td>0.36 g/l</td>
<td>0–35 g/l</td>
<td>Cross-validation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td></td>
<td>0.29 g/l</td>
<td>0–2 g/l</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CHO: Chinese hamster ovary; DOSC: Direct orthogonal signal correction; ivCC: Integrated viable cell count; ivPCV: Integrated viable packed cell volume; mAb: Monoclonal antibody; MC: Mean centering; mOsm: Molar osmolarity; MSC: Multiplicative scatter correction; n.u.: Normalized units; PCV: Packed cell volume; SG: Savitzky–Golay smoothing; SNV: Standard normal variate; TCC: Total cell count; VCD: Viable cell density.
**Review**  Hoehse, Alves-Rausch, Prediger, Roch & Grimm

<table>
<thead>
<tr>
<th>Application</th>
<th>What was measured?</th>
<th>Calibration models</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab scale (10 l); batch and fed-batch CHO cultivations</td>
<td>TCC</td>
<td>None</td>
<td>0.48 x 10^6 cells per ml</td>
<td>External</td>
</tr>
<tr>
<td></td>
<td>Viability</td>
<td>None</td>
<td>4.18%</td>
<td>30–100%</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>None</td>
<td>0.478 g/l</td>
<td>0–9 g/l</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>None</td>
<td>0.444 g/l</td>
<td>0–4 g/l</td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>SNV</td>
<td>0.071 g/l</td>
<td>0–1000 mg/l</td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>SNV</td>
<td>0.019 g/l</td>
<td>75–350 mg/l</td>
</tr>
<tr>
<td></td>
<td>Ammonium</td>
<td>None</td>
<td>0.010 g/l</td>
<td>0–100 mg/l</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lab scale (3 l) insect cells cultivation (<em>Trichoplusia ni</em>)</th>
<th>Glucose</th>
<th>None</th>
<th>1.54 mM</th>
<th>0.3–53.3 mM</th>
<th>External</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lactate</td>
<td>0.83 mM</td>
<td>0.4–18.3 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biomass</td>
<td>0.38 x 106 cells per ml</td>
<td>0–7 x 10^6 cells per ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lab scale (2.5, 100, 1000 l), fed-batch CHO cell cultivations; four cell lines</th>
<th>Product</th>
<th>Second derivative using SG, MC</th>
<th>24.8 n.u.</th>
<th>9.7–426.8 n.u.</th>
<th>Qualitative models: process trajectories</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>16.5 n.u.</td>
<td>5.1–206.0 n.u.</td>
<td>Cross-validation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamate</td>
<td>33.3 n.u.</td>
<td>3.7–329.1 n.u.</td>
<td>External</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutamine</td>
<td>42.9 n.u.</td>
<td>6.6–301.9 n.u.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>20.8 n.u.</td>
<td>11.2–320.7 n.u.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Osmolality</td>
<td>5.2 n.u.</td>
<td>72.4–161.1 n.u.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**CHO:** Chinese hamster ovary; **DOSC:** Direct orthogonal signal correction; **ivCC:** Integrated viable cell count; **ivPCV:** Integrated viable packed cell volume; **mAb:** Monoclonal antibody; **MC:** Mean centering; **mOsm:** Molar osmolarity; **MSC:** Multiplicative scatter correction; n.u.: Normalized units; **PCV:** Packed cell volume; **SG:** Savitzky–Golay smoothing; **SNV:** Standard normal variate; **TCC:** Total cell count; **VCD:** Viable cell density.
considered satisfactory. They also reported problems with the optical fibers that connect the probe to the NIR instrument: differences in bending and connection of the optical cables resulted in significant changes between the spectra.

Lopes et al. [23] implemented a kinetic model for the plasmid production in *Escherichia coli* and used off-line FT-IR as a high-throughput technique to characterize the plasmid expression profile under different media compositions and conditions. Finally, in-line NIRS was used for real-time monitoring of the cultures. The spectra were captured by a FT-NIR spectrometer with a fiber optic probe in the range of 800–1852 nm. PLS models were built for biomass (OD$_{600}$ and dry cell weight) and glycerol.

Alves-Rausch et al. [37] used NIRS to monitor sporulating *Bacillus* fermentations in an industrial environment. An NIR sensor with a fiber free design was attached directly to 50 m$^3$ reactors. Spectra were collected in-line in the range of 1050–1650 nm and quantitative models were built for total sugars, total analytes, acetoin, OD$_{600}$ and dry mass. Validation was made with an independent batch run. Additionally, media classification was made with spectra collected in the reactor before the inoculation and the possibility of identifying media formulation errors based on the spectra was shown. Furthermore, qualitative BEMs showed the use of NIRS to compare the evolution of batches.

**Cell cultivations**
Most biotechnologically produced proteins that are intended for human therapy are produced in mammalian cell cultures as they are able to perform the necessary posttranslational modifications which lower eukaryotes and microbial organisms are not capable of. On the downside mammalian cells have a much more complex metabolism and with their low proliferation rates they are more susceptible to contaminations than bacteria or yeasts. Thus, methods to monitor cell cultivations noninvasively are highly desirable. However, the low nutrients and metabolite concentrations that occur in cell cultivations can be challenging for spectroscopic techniques. Table 3 summarizes the literature on NIRS in cell cultivations.

**At-line**
Supernatant samples from Chinese hamster ovary (CHO) cultivations were subject to at-line NIRS measurements performed by Hakemeyer et al. [33]. The samples were analyzed with regard to their product concentration (monoclonal antibodies), nutrient and metabolite concentration (glucose, lactate, glutamate, glutamine) as well as cell viability. The experiments were carried out at different scales ranging from 2.5- to 1000-l production scale. The spectra were acquired using a FT-NIR system in transmission mode in the wavelength range from 909 to 2000 nm. For the development of quantitative models the spectra were preprocessed using second derivatives with a second order Savitzky–Golay filter and mean centering. PLS algorithms were used for modeling and variable selection. Moreover, qualitative models were developed to calculate process trajectories that were used for qualitative process monitoring. Hakemeyer et al. conclude that at-line NIRS is a suitable technique to replace reference methods for monitoring of critical process parameters and that it has further potential for the implementation of guided sampling and process control strategies.

**On-line**
NIRS was used by Qiu et al. [32] for monitoring of glucose and lactate concentrations as well as cell density in insect cell cultivations. The cultivations were carried out in 1.6-l bioreactors. The FT-NIR device was operated in bypass mode employing a transmission flow cell (pathlength 1.5 mm). Spectra were acquired in the range from 2000 to 2500 nm. The use of a bypass required cleaning steps with sterile water and air between measurements. For the generation of calibration models no preprocessing of the data was performed. Off-line data from glucose and lactate spikings were included to break the metabolism-induced correlations between glucose and lactate concentrations. The spectral ranges used for the calibration models were optimized for each analyte. The calibration models resulted in a SEP of 0.15 g/l for glucose and a SEP of 0.14 g/l for lactate. The high number (7, respectively, 8 factors) might be an indicator for overfitting. For the estimation of the cell density a univariate model was created on the base of the mean absorbance of the cell broth in the spectral range from 2105 to 2210 nm. The calibration test set was limited to samples from the lag and exponential phase and a SEP of $0.8 \times 10^6$ cell per ml was achieved.

**In-line**
Mattes et al. [29] used NIRS to monitor the osmolality and pH in cell cultures. An adherent cell line was grown in a 3-l bioreactor with microcarriers. The NIR probe, attached to the reactor before autoclaving, was connected with a 3-m microbundle of optical fibers to the spectrophotometer. The spectra were collected in the 800–2200 nm range in transfection mode. A spectral scan was taken every 15 min over the duration of the 12-day cell culture. PLS models were built for osmolality and pH value. They discuss the fact that ionic analytes should not have a signature in the
NIR; but that the perturbation of the water absorbance bands by the change in ionic concentrations makes it possible to track these analytes. No details are given about the number of batches used or how the validation set was selected. For the authors of this review, both pH and osmolality are not undoubtedly detectable with NIR. Therefore, the presented models are very likely based on indirect correlations. Furthermore, employing NIRS for pH measurements seems inappropriate as reliable and cost-effective pH probes are available for bioprocesses.

Henriques et al. [28] present an industrial pilot-plant mammalian cell cultivation to illustrate the use of NIRS for bioprocess monitoring. Five 10-l scale fermentations were monitored with an NIR transreflectance probe, with an optical pathlength of 1 mm connected to a 10-m fiber optic cable. Quantitative PLS models were developed to predict concentrations of glucose, ammonia, lactate, and total cell density. They carefully explain the different steps of model development, including outlier detection, analysis, spectral preprocessing, and variable selection. In addition, qualitative process trajectories based on the NIR spectra are shown. They claim two main advantages of the qualitative approach. First, differences between historical and actual batches can be analyzed. Second, new batches are monitored to stay inside the multivariate design space. A process monitoring experiment was made in which one of the batches was contaminated in an early process stage. The use of the multivariate batch trajectories allowed for the early detection of the contamination.

The monitoring of glucose and lactate concentrations in cultivations of adherent Vero cells on microcarriers is presented by Petiot et al. [30]. An FT-NIR device, measuring in the spectral range from 1110 to 2500 nm, was used with a transreflectance probe (1-mm pathlength) on 21 lab-scale bioreactors. The presence of microcarriers contributed to the complexity of the measurement matrix. Different agitation rates, fed strategies, and bead concentrations introduce a variability that needs to be included into the calibration models. Therefore in situ calibrations on samples from the bioreactor were carried out. Additionally, spiking of glucose and lactate with feed media was performed to break the correlation between the analytes. Quantitative calibration models for glucose and lactate were achieved with SEPs of 0.36 and 0.29 g/l, respectively.

Sandor et al. [31] carried out several CHO fermentations in 75-l scale to evaluate the potential of NIRS and MIR spectroscopy for bioprocess monitoring. To introduce process variability different cultivation strategies were used and additional spiking with substrates (glucose, glutamine) was performed. The NIR spectra were acquired using a free beam diode array spectrometer. The transreflectance probe (pathlength 5 mm) was connected to the bioreactor via a 25-mm standard side port (Sartorius Stedim Biotech GmbH, Germany). The spectra were acquired in the range from 950 to 1650 nm. Monitored key parameters were cell density, viability, and glucose concentration and SEPs of $0.48 \times 10^6$ cell per ml, 4.18% and 0.48 g/l, respectively, were achieved. The authors conclude that MIR spectroscopy offers higher accuracy for glucose and lactate monitoring due to higher absorption coefficients and narrower absorption bands in the MIR region. However, they point out that NIRS is better suited for bioprocess monitoring as additional scattering information is available in the NIR region. This allows prediction of cell density and viability which is not possible with MIR spectroscopy. Moreover, the robustness of the NIR device is seen as an advantage for process monitoring whereas MIR technology has to rely on attenuated total reflection fiber optics which are still a fragile component.

Another example of monitoring CHO cell cultivations with NIRS is presented by Clavaud et al. [27]. A FT-NIR device fiber optic transreflectance probe (1 mm pathlength) was connected to the bioreactor. A total of 10 batches with a scale of 12,500 l were monitored. It was observed that, due to the strong absorption of water, the absorption values were saturated in the region around 1950 nm and consequently only the wavelength region between 1000 and 1785 nm was used for analysis. It was shown that NIRS is useful for monitoring of process evolution via trajectories. This approach allows identification of abnormal process behavior without further reference analysis. Moreover, calibration models for several parameters were generated and root mean square error of prediction (RMSEPs) of $1.52 \times 10^6$ cell per ml for viable cell density, 2.2 g/l for glucose and 0.2 g/l for protein were achieved.

**Challenges**

One of the best-known challenges of NIR in bioprocesses is the water band issue. Water is a strong absorber of NIR radiation, leading to saturation effects of the detector. However, this impact can be minimized by two different approaches. First, the pathlength can be set constant all over the trial. Even though some areas of the spectra might be in saturation, the nonsaturated spectral parts are valid and contain useful information. Second, the pathlength can be reduced to minimize saturation effects. A pathlength of 1 mm showed good results, even in microbial fermentations [37].

Even though modern NIR sensors have increased robustness with designs able to withstand the harsh
conditions of industrial production environments, it is still necessary to pay attention to the robustness of the models. Changes in temperature, pH, use of a different cell line, changes in raw material providers, use of a different reactor geometry/scale or even differences in the optics of different sensors can have an impact in the collected spectra. There has been research showing how these process variations affect the models and describing strategies to overcome these limitations. Roychoudhury et al. [38] identified that optical differences between probes influence the quality of the NIR signal. Hageman et al. [39] give an overview of methods to deal with temperature influences on NIR spectra. When referring to PLS they mention that it is only required to have samples measured across the temperature range of interest and the algorithm will just require more PLS factors to give accurate predictions.

Another point of care should be on choosing a validation set for the method. In bioprocesses there will always be differences between batches that may come from the inoculum, the raw materials or process conditions; these differences cannot be totally reduced. A proper chemometric model will need to incorporate samples with different sources of variation and the model performance must be validated with a truly independent set. It is not enough to randomly separate samples into calibration and validation sets. A separate batch, not used in the development of the model must be used as the external validation set. Additionally, the model must be maintained throughout the lifecycle of the process to ensure it can cope with future changes.

Model robustness and accuracy sometimes have to be weighed against each other. On the one hand high model accuracy is desired. Optimal conditions for high model accuracy would be a process without any variations but the change of target parameter. This high accuracy model would lack of robustness as no process variation is known to the model. On the other hand a global model has to cope for different process scales, organism strains, NIR instruments and process controls, among others. This model would be of the highest robustness however model accuracy would suffer. Thus, a balance between model robustness and accuracy has to be found for optimal results.

The same principle is valid for process trajectories. If too many variations are included in the golden batch trajectory, the road of evolution is broad and even lower performing batches might never leave the accepted limits. If only the best of the best batches are used for generating the golden batch trajectory, any small deviation will lead to an alarm. Thus, batch trajectories are valuable tools to control well established processes. For process development, the benefit of batch trajectories might be lower as the data basis does not consist of a sufficient number of similar good batches. Additionally too many variations, necessary to find optimal process settings, will decrease model accuracy. Here, statistical test planning (DoE) will help to find optimal settings with a low number of experiments. The acquired NIR data of these DoE trials are very valuable for quantitative model building as correlations might be broken.

Furthermore, PLS methods rely on finding directions of most covariation between spectra variables and reference values. Given the overlapping nature of NIR bands, there is always the risk of calibrating on signals of other analytes that change always in a correlated way with the modeled analyte. By nature all upstream processes are highly correlated processes: nutrients are converted to metabolites, cell growth depends on nutrients and metabolites, titer on cell count and so on. Thus, correlations in bioprocesses are often causal and given by the process itself. However, some strategies are useful to break these correlations and make sure that the NIR calibration method is based on the NIR signal of the corresponding analyte. Spiking experiments and interpretation of regression coefficients are the most straightforward approaches. Spiking refers to spike a sample with known amounts of the analyte being modeled in order to break the correlation with the concentration of other analytes. This can either be made off-line, by spiking a sample removed from the reactor, or in-line, by spiking the reaction vessel. In-line spiking is hard to implement in industrial environments. Alternatively spiking can be performed on a smaller scale, but then the models may lack robustness on the production scale. The interpretation of regression coefficients requires good knowledge of the pure spectra of the components being analyzed. This is often complicated by the interference of other parameters, like temperature and pH shifts. Additionally, some parameters like viscosity or cell density do not have a defined spectrum. However, in bioprocesses the concentrations of substrates and products will be inexorably correlated through the natural reaction stoichiometry. It is therefore essential to look at the NIR data from a broader angle. The focus must not necessarily be on the accuracy of the quantitative models. Additionally, attention should be paid on the overall process fingerprint that NIRS can provide. This is in alignment with a more holistic approach that should be implemented all across the process stream.

**Conclusion & future perspective**

From the use of NIRS to perform high throughput screening in small 100-ml bottles, to the industrial monitoring of 50 m³ Bacillus fermentation, the adoption of NIRS for upstream process monitoring seems to be gaining traction in the industry. In all the applications NIRS is used to monitor multiple analyte con-
centrations simultaneously and some authors report the use of the NIRS signal as a fingerprint to make classification (organisms, media or harvest point) and build process trajectories. The quantitative models provide real-time concentrations of analytes and can be used to develop advanced control strategies. The qualitative approach allows for process state estimation, which may be used for event-based control or guided sampling.

Even though NIRS instrumentation has become capable of working under harsh industrial process conditions, it is equally important to generate and validate reliable and robust calibration models. Due to the natural correlations between the concentrations of substrates, metabolites and products this can be challenging. Moreover objective assessment of model quality is difficult as there is no standardized way of building and validating chemometric models.

Future trends in NIR include microelectromechanical systems technology, which are already available for NIR region. The production of these miniaturized spectrometers makes multiplexing redundant as sensor price allows for a separate sensor for each measurement point. Besides the miniaturization, this technique offers the possibility to concentrate on a selection of wavelengths and to customize each sensor for a specific target analyte. In theory, these devices should have a very high sensitivity as optics are kept to a minimum and throughput is maximized (no entrance slit, photodiodes with large sensitive area). However, first tests with prototypes in our lab still showed a reduced sensitivity compared with a diode array spectrometer. Thus some development work still has to be done to match the high requirements on spectrometer performance in upstream bioprocesses. Besides the NIRS region, this technique will be of particular importance in the MIR region as the length of sensitive halide fibers can be minimized to a few centimeters. The short distance between fiber and spectrometer (few cm) will allow a complete housing of the fiber and will increase process MIR robustness.

The broad capabilities of NIRS include more than simple quantitative prediction of analyte concentrations like glucose, lactate, cell count and cell viability. An even bigger impact originates from the NIRS fingerprint of the bioprocess state and its visualization in process trajectories. However, NIRS is not the only useful analytic technique in bioprocesses. It is one valuable tool of the toolbox. Why not expanding the multivariate approach to other analytics or even to any data source? Why not evaluating all data sources together? In Upstream processes, this includes reactor parameters, sensors and other spectroscopic techniques. In the next years, we

**Executive summary**

**Near-infrared instrumentation**
- Instrument selection depends on its application!
- For process applications, the performance of an instrument comes second. The system’s robustness and its acceptance in production environment are the major criteria.
- Spectral resolution is not of highest importance, but noise and sensitivity are crucial for detection limits.

**Multivariate data analysis**
- Low validation errors do not always result in a successful model – model robustness is the key!
- Avoid correlations for model building if causal relationship is obligatory (e.g., glucose level for feed control).
- Correlation models are only valid as long as the all process parameters stay in the defined limits. This is indicated also by the process trajectory not leaving the road of process evolution.
- Achievable limits of detection are a result of the physical characteristics of NIR; any working model with analyte concentrations far beyond 1 g/l is likely based on correlations.
- A valid calibration requires samples in a much broader range than the accuracy of the reference method (range > ten times the reference accuracy).
- Test set validation with independent batches is obligatory! The validation result must be independent of the validation batch selection as long as the validation batch is covered by the model space.

**Application examples of multivariate data analysis in upstream processes**
- Employing near-infrared spectroscopy (NIRS) only for quantitative analysis is a waste of its capabilities!
- Quantitative: NIRS is a multiparameter analyzer for chemical (e.g., nutrients and metabolites) and physical parameters (e.g., cell count, viability and contaminations). Predictions can be employed for parameter controls, for example, feed controls.
- Qualitative: NIRS gives a fingerprint of each process state. The resulting process trajectories allow for event-based control and guided sampling. Classification algorithms can be used for optimal harvest point determination, media classification and metabolic state monitoring.

**Future perspective**
- A holistic approach is not limited to near-infrared!
- A combined data evaluation of all sensor and bioreactor data will result in a comprehensive picture of your bioprocess and allow for optimal risk mitigation.
expect spectroscopy in general to gain importance in bioprocess applications. In medium term spectroscopy will not only be employed in R&D environment or process development but will find its way into production processes. Besides infrared (NIR, MIR) and Raman spectroscopy, UV–Vis and fluorescence spectroscopy are expected to have the biggest potential for process use. Combined data evaluation of all data sources will improve real-time process monitoring and allow for the early detection of potential process deviations.

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- Extensive guide through the steps of NIR model building and its possibilities.


- Extensive comparison of NIR and mid-IR for cell cultivation monitoring.


- Excellent example of NIR in rough industrial environment with a solid data base and good modeling practice with external test set validation.
