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Short communication

# *In situ* quantification of microcarrier animal cell cultures using near-infrared spectroscopy☆

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ABSTRACT

In-line monitoring tools are still required to understand and control animal cell processes, particularly in the case of vaccine production. Here, *in situ* near-infrared spectroscopy (NIRS) quantification of components in culture media was performed using microcarrier-based cultivations of adherent Vero cells. Because microcarriers were found to interfere with NIRS spectra acquisition, a suitable and innovative *in situ* calibration was developed for bioreactor cultures. A reliable and accurate NIRS technique for the quantification of glucose and lactate was established, with a calibration standard error of 0.30 and 0.21 g $l^{-1}$, respectively. The robustness of this method was evaluated by performing NIRS calibration with operating conditions similar to those of industrial processes, including parameters such as microcarrier concentrations, cell seeding states and changes in analyte concentration due to feed and harvest strategies. Based on this calibration procedure, the predicted analyte concentrations in unknown samples was measured by NIRS analyses with an accuracy of 0.36 g $l^{-1}$ for glucose and 0.29 g $l^{-1}$ for lactate.


## 1. Introduction

A large number of human and veterinary viral vaccines are currently produced by large-scale cultures of adherent Vero cells cultivated on microcarriers [1–4]. Despite this important use, a lack in the understanding of Vero cell metabolism in serum-free media remains a hindrance for either the development of new industrial processes, or the optimization of already established ones [5]. Moreover, regulatory agencies have recently encouraged the development of the Process Analytical Technology (PAT) approach for biological production using animal cells, mainly to improve the *in situ* process monitoring through timely measurements [6]. To date, only a few process-related variables, including pH, temperature, $pO_2$, biovolume and cell density, can be monitored using *in situ* probes that can be sterilized [7,8]. Therefore, to access to the real-time evolution of cell metabolism and physiology, the development of new PAT methods for on-line measurement of other key parameters, including nutrients, products or metabolic byproducts, is needed and could support large-scale production by cell culture.

NIR spectroscopy (NIRS) is a promising alternative *in situ* PAT tool. NIRS is an absorption spectroscopy technique, providing a spectrum representative of the "signature" of all components present in the analyzed solution. It possesses numerous advantages when compared to classical analytical methods: rapid simultaneous detection of a large number of molecules, no requirement for chemical reagents or disposable materials, and the availability of a probe that can be sterilized. To date, in the field of biological processing NIRS has been mainly used for bacterial [9–12], microalgae [13] and fungal [14] cultures. In the case of animal cell culture processes, NIRS is currently not routinely used, with only few reports describing NIRS analysis of soluble components in cell culture supernatants [15–18]. All these studies were performed with CHO and insect cells cultivated in suspension mode, and similar work has not been reported for adherent cells. Only two publications report results using NIRS-implemented *in situ* in bioreactors [16,19], whereas the remaining reports were performed off-line using quartz cuvettes and were mainly focused on the calibration procedure [15,18,20–22]. The quality and robustness of calibration are highly dependent on the standard matrix composition and the evolution of the cell culture supernatant composition during the

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culture process. Therefore, the off-line calibration and the generation of a standard formulation are often a challenge.

In the present work, we evaluated NIRS as an *in situ* PAT tool to monitor glucose and lactate concentrations during bioreactor culture processes of Vero cells cultivated on microcarriers. A suitable and innovative *in situ* NIRS calibration procedure dedicated to adherent cell culture processes was developed. This method addresses matrix variations introduced by routine procedures used for adherent cell culture processes, such as different feeding strategies, cell trypsinization as well as microcarrier and analyte concentration variations. Therefore, this method is useful for the monitoring of industrial Vero cell culture processes.

## 2. Materials and methods

### 2.1. Vero cell cultures

The Vero cell line used in this study was provided by Sanofi Pasteur (Marcy L'Etoile, France) and was adapted in a homemade, serum-free culture medium (the composition was described in a previous work) [5]. Cultures were performed using two concentrations of Cytodex 1 microcarriers (GE Healthcare Bioscience-AB, Sweden) in a 2 l bioreactor (Pierre Guérin, France) kept at pH 7.2, 37 °C, with an agitation rate of 90 rpm. Oxygen concentration was set at 25% of air saturation. Seeding cells for bioreactor cultures ($2.75 \times 10^5$ cell ml$^{-1}$) were previously expanded either from a static culture in 1720 cm$^2$ Hyperflasks® (Corning incorporated, USA) seeded with $3.15 \times 10^5$ cell ml$^{-1}$, from 500 ml spinner flasks, or from a bioreactor culture. Cultures in Hyperflasks® or spinner flasks were performed in an incubator controlled at 37 °C and 5% $CO_2$.

### 2.2. Reference analyses

For the quantification of viable cells attached to microcarriers, samples were washed twice with phosphate buffer saline (PBS) after microcarrier settling and then treated with Crystal Violet (Sigma, France) for at least 1 h at 37 °C. The quantification of released nuclei was performed on a Fuchs-Rosenthal haemocytometer (Preciss, France) with 15% accuracy. Glucose and lactate concentrations in filtered cell culture supernatants were quantified by using the Vitalab selectra E enzymatic analyzer (Vital Scientific, The Netherlands). Analyses were carried out in triplicate with a standard error of about 0.07 g l$^{-1}$.

### 2.3. Near-infrared spectroscopy

#### 2.3.1. NIR spectra acquisition

Spectra acquisition was performed with a Fourier transform near-infrared (FT-NIR) analyzer (Antaris II spectrometer; Thermofisher Scientific, France) for both off-line and *in situ* analyses. Spectra were collected in triplicate with an 8 cm$^{-1}$ optical resolution, a spectral point spacing of 3.856 cm$^{-1}$, and with each spectra representing 128 co-added scans. A single air spectrum acquired prior to cell culture seeding was used as a reference spectrum. Spectra were obtained in a spectral range from 4000 to 9000 cm$^{-1}$. Off-line NIRS analyses were measured in quartz cuvettes with a 2-mm optical path. *In situ* measurements in bioreactor cultures were performed using a single optic fiber transflectance probe with a variable optical path (Series 625 transflectance probe from Precisions Sensing Devices, USA). The maximal path length of the probe was 20 mm, and NIRS radiations reflected by a mirror covered twice the fixed optical path. The numerical aperture was 0.22 and the collection efficiency was about 30%. The probe path length was fixed at 1 mm, leading to an effective optical path of 2 mm.

#### 2.3.2. Development of NIRS calibration models

Spectra processing was performed using the TQ Analyst software (Thermofisher Scientific, France). This software allows the user to perform both principal component analysis (PCA) and partial least square (PLS) regression, to optimize the wavelength range and to calculate the root mean square (RMS) noise values [23]. The spectra RMS noise was calculated using the following equation in a spectral region:

$$\mathrm{RMS}_{\mathrm{noise}} = \sqrt{\frac{\sum (y_i - \bar{y})^2 - \left( \left[ \sum (x_i - \bar{x})^2 (y_i - \bar{y})^2 \right]^2 / \sum (x_i - \bar{x})^2 \right)}{n - 2}}$$

$y_i$: absorbance intensity at data point $i$; $x_i$: value of the data point $i$; $\bar{y}$: average of $y$ data values within the spectral range; $\bar{x}$: average of $x$ data values within the spectral range; $n$: number of data points within the spectral range.

Spectra were processed as absorbance spectra and no mathematical treatment was needed, except for the baseline correction. NIRS calibration models were established for glucose and lactate by using a spectral range and a number of partial least square (PLS) factors specific and optimal for each analyte. Two data processing steps were performed to establish the calibration models. For the first step the data set was composed of all calibration samples, while for the cross-validation step all samples were used as standards, except for two samples randomly chosen by the data treatment software. The second procedure was repeated to determine the standard error average of all the cross-validation correlations. The quality of the calibration models was then evaluated by several parameters: the correlation coefficients ($r^2$) between concentrations obtained from NIRS analyses and from enzymatic analyses, calculated for both calibration and cross-validation data processing, and their respective standard errors (calibration (SEC) and cross-validation (SECV)). The glucose and lactate concentrations were also directly evaluated in samples not previously integrated in the calibration procedure. The prediction quality of NIRS analyses was then quantified by the standard error of prediction (SEP).

## 3. Results and discussion

### 3.1. Influence of microcarriers on NIRS analyses of Vero cell culture supernatants

Previously the calibration procedure of NIRS has been mainly performed off-line using standards from various chemical formulations, including samples with non-correlated concentrations of the quantified molecules [18–21]. In some cases, these off-line calibrations were then used for *in situ* monitoring of animal cell culture suspensions [19]. However, the analyzed matrix is composed of both culture medium and beads when cultivating adherent cells growing on microcarriers. The presence of microcarriers in the NIRS probe sensing space could then modify the real optical path length and interfere with the spectra quality and intensity. To evaluate this effect, microcarriers were added to culture supernatants before off-line NIR spectra acquisition in quartz cuvettes. Comparison of the spectra recorded with or without the addition of the microcarriers showed a variation in absorbance intensity, even though they had similar profiles (Fig. 1: spectra A and B). By comparing the path length in filtrated sample ($P_N$), it could be hypothesized that the optical path length in the quartz cuvette was increased due to the presence of microcarriers ($P_M$). This hypothesis could explain the higher spectra intensity observed in sample containing beads.

For an accurate *in situ* quantification of analyte concentrations, it is recommended to calibrate using samples that best represent the matrix of interest, i.e., the supernatant during cell culture. During off-line analyses in quartz cuvettes, the rapid settling of microcarriers resulted in an increased bead concentration in the NIRS sensing space. This bead concentration was significantly higher than the bead concentration in agitated cultures. Indeed, agitation condition is an important element contributing to variations in matrix composition, especially in the case of a heterogeneous system such as microcarrier cultures. To evaluate the effect of sample agitation, three spectra acquired in presence of microcarriers were compared (Fig. 1). The spectra acquired, either off-line in static cuvettes and in a stirred 10 ml sample tube, or *in situ* in a stirred bioreactor culture, showed different intensities. First, these results demonstrated that off-line calibration in static conditions did not allow for a correct calibration procedure using samples containing beads which have to be maintained in a homogeneous suspension. Secondly, the use of different agitation systems provoked various trajectories and bead movement speeds within the optical field. This variation led to an observed gap in intensity between the two spectra acquired with the fiber optic probe in stirred conditions (spectra C and D). Thus, an off-line calibration procedure, either in static or stirred conditions, is not ideal for cultures performed with cells attached to microcarriers. Therefore, it was necessary to develop an *in situ* calibration procedure which can be performed directly in the bioreactor vessel, to ensure that calibration reflects the medium-bead matrix during cultivation.