Simultaneous Measurement of Glucose and Glutamine in Insect Cell Culture Media by Near Infrared Spectroscopy

Mark R. Riley,[1]* Martin Rhiel,[1] Xiangji Zhou,[2] Mark A. Arnold,[2] David W. Murhammer[1]

[1]Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, Iowa 52242
[2]Department of Chemistry, The University of Iowa, Iowa City, Iowa 52242; telephone: 319-335-1368; fax: 319-353-1115; e-mail: arnold@mairlab1.chem.iowa.edu

Received 23 April 1996; accepted 15 August 1996

Abstract: The purpose of this study was to develop noninvasive techniques to monitor the composition of cell culture media in insect cell bioreactors. Such a monitor could be used in conjunction with a fed-batch feeding scheme to ensure that cells are maintained in an optimal environment for growth and protein production. Glucose and glutamine concentrations in an insect cell culture bioreactor were determined off-line with near-infrared (NIR) absorption spectroscopy. Spectra were collected from 5000 to 4000 cm^{-1} with a 1.5-mm optical path length. Partial least squares (PLS) regression was applied to correlate the collected spectra with the concentration of the desired analytes. Under the culture conditions evaluated here, glucose and glutamine concentrations ranged from 38 to 55 m*M* and from 3 to 13 m*M*, respectively. Accurate measurements of glucose and glutamine in insect cell culture samples were possible over these entire ranges. The standard error of prediction (SEP) and mean percent error (MPE) for glutamine were 0.52 m*M* and 5.3%, respectively. Glucose could be measured with an SEP of 1.30 m*M* and an MPE of 2.3%. These levels of error are quite low considering the changing complexity of the growth media due to the shifting levels of amino acids, carbohydrates, yeastolate, proteins, and cell debris. This study represents an important step in the development of noninvasive on-line monitoring devices for cell culture bioreactors. © 1997 John Wiley & Sons, Inc.
Biotechnol Bioeng **55:** 11–15, 1997.
Keywords: NIR spectroscopy; bioreactor monitoring; insect cell culture

INTRODUCTION

The controlled cultivation of cells in a bioreactor depends on the continual supply of nutrients such as glucose and glutamine. Glacken et al. (1989) showed that some types of cells may perform optimally when fed nutrients at a slow but well-controlled rate. Due to heterogeneities between different culture batches, the optimal feeding rate to a reactor must be measured for each run. Conditions such as pH and dissolved oxygen concentration are commonly measured with electrodes placed in the reactor. There are difficulties, however, in reliably measuring the concentration of cellular nutrients and waste products. *In situ* glucose enzymatic biosensors are temperature sensitive and typical sterilization techniques may degrade the enzymes. Additionally, such sensors require repeated calibration, may drift during operation, and can measure the concentration of only one chemical species (White et al., 1995). Off-line measurement methods such as gas chromatography can circumvent these problems, but they add the complication of repeatedly taking samples out of the reactor, which may introduce microbial contamination.

Near infrared (NIR) spectroscopy in conjunction with chemometric techniques offer an attractive alternative to the aforementioned techniques. A beam of NIR light is introduced to a sample through a quartz window. The amount of light absorbed by the sample can be correlated to the concentration of the constituent species. The method is noninvasive, and so does not increase the chance of reactor contamination. No sample preparation is required, and the method is generally stable. Additionally, the concentration of many species may be determined from one spectroscopic measurement, as long as their concentration is 1 m*M* or greater. These advantages make NIR spectroscopy and chemometrics particularly attractive for monitoring cell culture processes.

Reports in the literature have discussed the use of NIR spectroscopy to monitor nutrients and waste products in bacterial fermentations (Hall et al., 1996; Vaccari et al., 1994). Vaccari and co-workers (1994) measured the concentration of lactate, glucose, and biomass in bacterial cell fermentations. Hall et al. (1996) monitored the concentration of acetate and ammonia and the cell density in *Escherichia coli* fermentations. Monitoring errors over large concentration ranges were approximately 7 m*M* for each analyte with a mean percent error (MPE) of 1–3%. The growth medium used in this bacterial study contains few components at high concentrations, compared with the com-

* *Present address:* Department of Agricultural and Biosystems Engineering, University of Arizona, Tucson, AZ 85721.
Correspondence to: M. A. Arnold
Contract grant sponsors: NASA; American Cyanamid
Contract grant number: WAG 9-824 (for NASA)

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plex growth media used for insect and mammalian cell culture. Sf-900 II medium (a common, commercially available insect cell culture medium) contains 20 amino acids, three carbohydrates, several cofactors, and yeastolate (Weiss et al., 1981). Many compounds found at millimole concentrations in the growth medium present possible interferences that may complicate the discrimination of desired analytes by spectroscopic methods. In a bioreactor, the concentrations of many of these species change due to cell metabolism.

In this study, we took a new approach to account for the complicated nature of the growth media. Sf-9 insect cells were grown in a batch bioreactor with Sf-900 II serum-free media. Twenty-four cell-free samples and five cell-containing samples were removed at different times over the course of operation. The bioreactor run was stopped after 5 days when the cells entered the stationary phase. At this time, the glutamine and glucose levels in the media were much lower than their initial levels. The spent media in the reactor at the end of the run was collected and divided into 50 aliquots. Random and known amounts of glutamine and glucose were added to each aliquot to form ''spiked'' samples. Spectra of these spiked samples were collected and the actual levels of glutamine and glucose were determined by standard methods. Partial least-squares (PLS) regression analysis was used to build calibration models for glucose and glutamine. Spectra were also collected for the 24 cell-free samples taken during the bioreactor run. These reactor samples constituted a prediction data set that was used to evaluate the calibration models. Calibration models generated in this manner could be used to predict the concentrations of glutamine and glucose in samples taken during the bioreactor run.

MATERIALS AND METHODS

SF-9 insect cells were cultivated in a 3-L bioreactor (Applikon, Foster City, CA) with SF-900 II serum free growth medium (Gibco, Grand Island, NY) at 27°C for 5 days. Details of the culture methods are described elsewhere (Rhiel and Murhammer, 1995; Rhiel et al., 1995). For this study, the initial cell density seeded in the reactor was 47×10^5 cells/mL. The dissolved oxygen concentration was maintained at 50% air saturation by adjusting the flow rate of pure oxygen into the reactor. The pH was controlled at 6.3 by addition of 1*M* KOH. Twenty four cell-free and five cell-containing samples of 20 mL each were removed at various times throughout the run. After 5 days (118 h) of operation the cell density had reached 6.5×10^6 cells/mL. At this time, the cell-free spent medium was removed. Spectra were collected on a Nicolet 740 Fourier transform infrared spectrometer (Nicolet Analytical Instruments, Madison, WI). This spectrometer was equipped with a tungsten-halogen lamp, a calcium fluoride beam splitter, and a liquid nitrogen cooled indium antinomide detector. The optical sample cell was maintained at 27°C, as in the bioreactor. Spectra were collected as 256 co-added scans from 5000 to 4000 cm^{-1} with a 1.5-mm optical path length. Three spectra were collected for each sample. A background spectrum of deionized water and/or spent medium was collected after every fourth sample. Absorbance spectra were calculated by taking the negative log of the single beam spectra of the sample divided by the single beam spectra of the background. Additional details of the experimental setup may be found elsewhere (Chung et al., 1995, 1996).

The concentrations of glutamine and glucose in the spent media and in the samples taken during the bioreactor run were quantified by standard off-line methods. The concentration of glucose was measured with a YSI glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). This method has an accuracy of approximately ±0.28 m*M* at the concentration range used (Burmeister and Arnold, 1995). Glutamine was measured by HPLC by using a C-18 reversed phase column (Supelco, Bellefonte, PA) in conjunction with an OPA derivatization based fluorescence detection scheme (Burbach et al., 1982). This method has an accuracy of approximately ±0.8 m*M* (data not shown). These reference measurements were used to verify the spectroscopic measurements.

The calibration data set of spiked samples was generated by dividing the spent bioreactor media into 50 aliquots. The spent media was found to contain 38 m*M* glucose and 3 m*M* glutamine. To each of these aliquots was added a random known amount of glucose (0–40 m*M*) and a random known amount of glutamine (0–18 m*M*). Therefore, spiked samples had a range of glucose from 38 to 78 m*M* and a range of glutamine from 3 to 21 m*M*.

Unique calibration models were established for glutamine and glucose. In both cases, collected spectra of the spiked media aliquots were used as a calibration data set, and the 24 reactor samples were used as a prediction or monitoring set. Calibration models were developed and evaluated while varying spectral range, number of PLS factors, and type of background spectrum. Spectral range is the most critical of these parameters. As shown in Figure 1, glutamine exhibits three absorption bands centered around 4700, 4580, and 4390 cm^{-1} while glucose has three bands centered at 4710, 4400, and 4300 cm^{-1} (Chung et al., 1995). Because these absorption bands contain the analyte concentration information, calibration models built with spectral ranges encompassing these absorption bands should provide reliable analyte predictions. A C-shell computer script was written to systematically develop calibration models with varying numbers of PLS factors and with varying spectral ranges within the 5000–4000 cm^{-1} region. Models were evaluated for a low standard error of calibration (SEC) and a low standard error of prediction (SEP). These are the errors obtained when the PLS model is used to predict the analyte concentrations in samples from the calibration set and from the prediction set, respectively. The SEP is commonly used to evaluate the expected error in an NIR measurement using PLS regression analysis.

RESULTS AND DISCUSSION

PLS is a powerful multivariate analytical technique for extracting analyte concentration information from complex spectra containing spectral features of multiple analytes. A univariate method, such as commonly used to calibrate ultraviolet-visual spectroscopic measurements, correlates light absorption at a single wavelength (e.g., 280 nm) with the concentration of a chemical species. A multivariate technique, such as PLS, correlates light absorption over many wavelengths or wavenumbers (e.g., 4800–4200 cm^{-1}) with the concentrations of chemical species. By incorporating spectral information from many wavelengths, PLS can discriminate information due to different analytes present in a sample. For example, glucose and glutamine both have absorbance maxima around 4400 cm^{-1}. By including spectral information from 4500 to 4300 cm^{-1}, PLS can discriminate which information is due to glucose and which is due to glutamine. This increases the specificity of measurements and allows quantitation of multiple species with a single instrument.

When developing a calibration model for PLS analysis, it is critical that the concentrations of individual analytes and interferences in the samples be random. If a correlation exists between two species, PLS will incorporate this dependency into the calibration models. Such a model will result in systematic prediction errors when applied to samples that do not have such a correlation. Development of a PLS model requires two data sets: one for calibration, or training, and one for prediction. The calibration set contains standards with known concentrations, while the prediction set contains samples of unknown concentrations. Ideally, the calibration set should include all of the sample variation observed in the prediction set. Typically this is accomplished by preparing synthetic samples with varying concentrations of the desired analyte (Chung et al., 1995, 1996). When building models applicable to cell growth and metabolism in a bioreactor, it is difficult to incorporate all of the analyte variations because of the large number of species that are changing (i.e., amino acids, carbohydrates, proteins, cell mass, cell debris, etc.). An alternative approach is to collect many reactor samples and use these to build a calibration model. Such models could be applied to monitor the concentration of nutrients in similar bioreactor runs. However, such a method will introduce correlations between all the species in the growth media, making it difficult to determine if one is actually predicting the analyte of interest or some other species that correlates with the analyte concentration. This study was motivated by a desire to avoid such analyte correlations.

PLS regression was applied to analyze the collected spectra and to develop calibration models for glucose and glutamine. These models were used to predict concentrations of glutamine and glucose in the 29 bioreactor samples collected during this bioreactor run. Parameters for the calibration models that yield the lowest SEPs for glutamine and glucose are shown in Table I and Table II, respectively. These tables contain the parameters that were altered for different models: the spectral range incorporated into the model, the type of background spectrum used, and the number of PLS factors. Also included in these tables is a summary of the model results: the SEC, SEP, and the MPE in predicting the analyte concentration. This MPE is the average percent deviation between the actual analyte concentration and the concentration predicted by the PLS model.

Optimal glutamine models (Table I) have a spectral range centered around the 4580 cm^{-1} glutamine absorbance band. Models using backgrounds of water or media yield generally similar levels of prediction error. SEPs obtained for glutamine were generally below 0.57 m*M,* which represents an MPE of about 6%. This level of prediction is excellent considering previous studies of glutamine in pure water had SEPs of around 0.80 m*M* (Chung et al., 1995). In general, reference HPLC methods for off-line glutamine analysis can have errors equal to or greater than 0.80 m*M* (Burbach et al., 1982).

For glucose predictions, optimal models (Table II) contain the 4400 cm^{-1} glucose absorbance band. Models using backgrounds of water and media again yield similar levels of prediction errors. SEPs obtained for glucose were generally between 1.4 and 1.8 m*M,* which corresponds to MPEs of 2–3% given the high glucose concentrations involved (38–55 m*M*). Previous studies of glucose in pure water yielded lower SEPs of around 0.40 m*M* (Chung et al., 1995). However, the insect cell media used here contains several carbohydrates whose absorbance bands interfere with the glucose information. In addition, the concentration of many species in the growth media change due to cell metabolism. This changing matrix presents an added degree of difficulty for accurate measurements.

The accuracy of a PLS model is judged by how well it predicts the actual concentrations as measured by a reference method (YSI analyzer for glucose and HPLC for glutamine). In addition, there should be no systematic bias of over- or underpredicting concentrations. As an illustration, glutamine concentrations predicted using one of the PLS models noted in Table I are presented as a function of the actual concentration in the samples (Fig. 2). In this concentration correlation plot, calibration and prediction data points closely followed the unity line, as expected for a good model. There was no apparent systematic bias. Glucose models yielded similar agreement with the unity line (data not shown).

Table I. Glutamine PLS models.

Spectral range (cm^{-1})	Background	PLS factors	SEC (m*M*)	SEP (m*M*)	MPE (%)
4600–4450	Media	8	0.25	0.51	5.2
4630–4530	Water	5	0.28	0.53	5.2
4630–4530	Media	6	0.23	0.53	5.8
4650–4500	Media	4	0.30	0.55	6.0
4650–4500	Water	5	0.30	0.56	5.8

Table II. Glucose PLS models.

Spectral range (cm^{-1})	Background	PLS factors	SEC (m*M*)	SEP (m*M*)	MPE (%)
4450–4300	Media	4	1.45	1.46	2.3
4500–4300	Media	4	1.40	1.47	2.5
4400–4300	Water	4	1.53	1.73	2.9
4470–4250	Media	4	1.35	1.79	2.8
4470–4250	Water	4	1.58	1.83	3.1

For bioreactor monitoring, one must know the metabolite concentrations at various times during reactor operation. Figure 3 displays the glutamine and glucose concentrations in the insect cell bioreactor as a function of time. The PLS predictions were independent of the reference measurements; the reference method was used here for validation purposes only. Glutamine predictions were very consistent and had less scatter than the reference measurements (Fig. 3a). The predictions for samples taken from the bioreactor at later times were more consistent than the predictions for samples taken at earlier times. This effect was due to differences between the culture media and the material used to generate the calibration model. Cell-free spent media from the end of the bioreactor run was spiked with glucose and glutamine and used to generate the calibration model. However, fresh media differed from spent media by more than just the glutamine and glucose levels. The concentrations of other species in the media such as amino acids, carbohydrates, proteins, and cell debris had changed. Each of these constituents could potentially affect the NIR absorbance of the sample, thereby degrading the prediction capability of the model.

Glucose predictions followed the reference method measurements reasonably well (Fig. 3b) for samples collected throughout the bioreactor run. The increase in glucose at the beginning of the run was most likely due to the decomposition of maltose to two molecules of glucose.

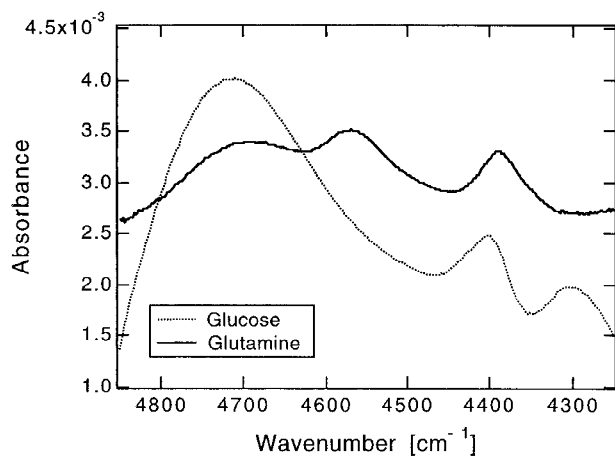

Figure 1. Absorbance spectra of pure glutamine in water and of pure glucose in water. Note the distinct absorption bands for glutamine centered at 4700, 4580, and 4390 cm^{-1} and for glucose centered at 4710, 4400, and 4300 cm^{-1}.

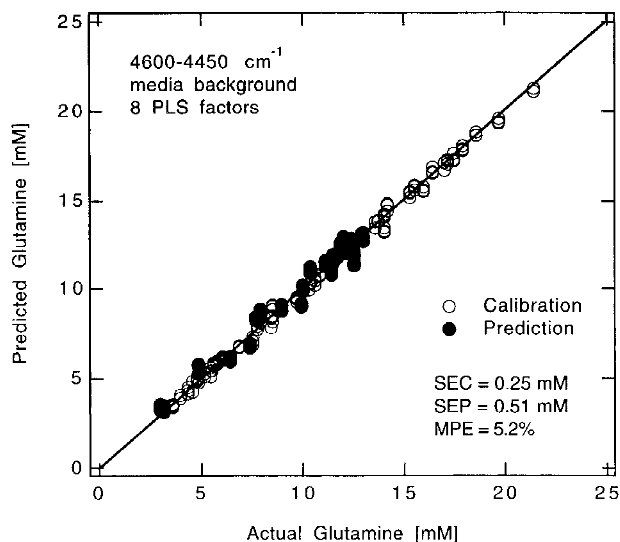

Figure 2. Glutamine concentration correlation plot. Open symbols represent spiked media samples that were used to develop the calibration model. Closed symbols represent bioreactor samples predicted by this calibration model.

The effect of the presence of cells in the samples was also investigated. Cells in the media will scatter some of the incident light, reducing the intensity of light reaching the detector. Spectra were collected for five bioreactor samples containing cells (with cell densities ranging from 4.7×10^5 to 6.5×10^6 cells/mL). Glutamine and glucose predictions for these samples are displayed in Figure 3a (glutamine) and 3b (glucose). These predictions are in good agreement with the reference measurements, so it appears that the spectroscopic measurements were not greatly affected by the presence of cells.

CONCLUSIONS

The purpose of this study was to develop noninvasive techniques to monitor the composition of cell culture media in insect cell bioreactors. Such techniques could be used in conjunction with fed-batch methods to maintain the cells in an optimal environment for growth and product generation. We developed a method to build calibration models for NIR spectroscopic measurements of glucose and glutamine. The method comprised adding random analyte spikes to spent reactor media, collecting spectra, and applying PLS regression analysis. We evaluated, off-line, Sf-900 II medium used for Sf-9 insect cells. Glutamine and glucose in bioreactor samples were predicted with standard errors of 0.51 and 1.46 m*M*, respectively. These levels of error were quite low considering the changing complexity of the growth media. This approach of spiking spent reactor media with the analyte of interest proved quite successful for developing calibration models and may lead to a feasible method for monitoring metabolites on-line during bioreactor operation.

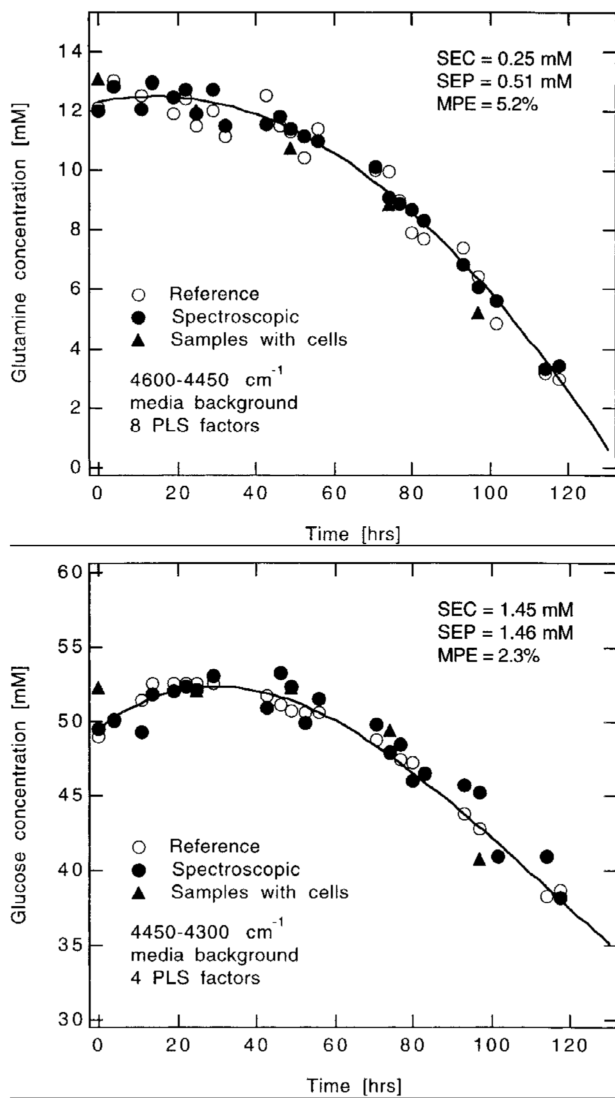

Figure 3. (a) Glutamine concentrations at various times in the bioreactor. Closed symbols represent spectroscopic measurements, while open symbols represent the reference, HPLC measurements. Predictions for samples containing cells are represented by the closed triangles. (b) Glucose concentrations at various times in the bioreactor. Closed symbols represent spectroscopic measurements, while open symbols represent the reference, YSI analyzer measurements. Predictions for samples containing cells are represented by the closed triangles.

Under the conditions described here (high glucose and glutamine concentrations), such a spectroscopic approach in conjunction with a fed-batch control mechanism would be adequate to maintain high nutrient levels as one observes with many commercially available insect cell culture media. However, based on the SEP values, these calibration models could not be directly applied to situations where the glucose and glutamine concentrations must be maintained at low levels, such as less than 1.0 m*M* for glucose and less than 0.5 m*M* for glutamine. Most likely if similar techniques were used to develop calibration models focusing on lower (and smaller) concentration ranges, the SEP would decrease while the MPE would remain relatively constant. Currently we are evaluating the feasibility of applying such methods to various mammalian cell culture media that have lower nutrient concentrations. We are also investigating methods to improve the prediction capabilities of the models for glucose and glutamine, and we are evaluating the feasibility of this approach for other analytes such as cysteine and lactate. In addition, we are developing methods to interface an FTIR spectrometer with the bioreactor.

The authors would like to thank Dr. John Wiencek for use of his HPLC.

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