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Matrix-Enhanced Calibration Procedure for Multivariate Calibration Models with Near-Infrared Spectra

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A novel method is introduced for developing calibration models for the spectroscopic measurement of chemical concentrations in an aqueous environment. To demonstrate this matrix-enhanced calibration procedure, we developed calibration models to quantitate glucose and glutamine concentrations in an insect cell culture medium that is a complex mixture of more than 20 components, with three components that manifest significant concentration changes. Accurate calibration models were generated for glucose and glutamine by using a calibration data set composed of 60 samples containing the analytes dissolved in an aqueous buffer along with as few as two samples of the analytes dissolved in culture medium. Standard errors of prediction were 1.0 mM for glucose and 0.35 mM for glutamine. The matrix-enhanced method was also applied to culture medium samples collected during the course of a second bioreactor run. Addition of three culture medium samples to a buffer calibration reduced glucose prediction errors from 3.8 mM to 1.0 mM; addition of two culture medium samples reduced glutamine prediction errors from 1.6 mM to 0.76 mM. Results from this study suggest that spectroscopic calibration models can be developed from a relatively simple set of samples provided that some account for variations in the sample matrix.

Index Headings: **NIR spectroscopy; Bioreactor monitoring; PLS calibration; Insect cell culture medium.**

INTRODUCTION

A number of industrially relevant processes utilize complex samples for which the monitored chemical species are greatly outnumbered by interfering components present at similar concentrations. The development of calibration models for spectroscopic analyses of these complex samples can be a time-consuming process, as accurate calibration models for partial least-squares (PLS) regression analysis of near-infrared data must incorporate all the chemical variation to be encountered in the sample population.[1] Calibration standards must span the full concentration range for all analytes and all spectroscopically relevant matrix components. In addition, covariance cannot be tolerated between concentrations of analytes or between analytes and other chemical component within the sample matrix. If a correlation exists between two chemical species, the PLS regression analysis will include this covariance, which could result in systematic prediction errors when the model is applied to samples that do not contain such a correlation. Such correlations can readily appear when the composition of samples used in a calibration data set is not properly controlled,[2] and this problem is particularly onerous for monitoring biological systems that naturally yield analyte correlations within a single bioreactor due to the cell metabolism.

A difficulty in developing robust spectroscopic calibration models for industrial processes with complex samples arises in properly accounting for the variations that exist between separate production runs. Such variations are typical of biological reactors for which small changes in initial conditions may affect the rate of change of chemical species during reactor operation. Calibration models for such processes, which display strong analyte correlations within a single run but large variations between separate bioreactor runs, are often developed by generating an extensive database of samples covering a wide range of operating conditions. These samples can be collected from multiple batch runs or produced through a purely synthetic approach. Either scheme for developing calibrations is time consuming, and a more efficient approach for building robust calibration models is desirable.

In this paper, we introduce a novel approach for developing multivariate calibration models with near-infrared spectra for the quantification of multiple analytes in a complex matrix. The method is based upon a simple calibration set used to train a PLS algorithm to recognize analyte-dependent information. Samples in this initial calibration set consist of the analytes of interest dissolved in a buffer. This initial calibration set alone cannot reliably correlate near-infrared spectra to the concentrations of analytes in complex solutions because of significant differences between the background matrices. By adding a small number of samples containing this complex background matrix, one can incorporate these variations into the PLS model and thus reliably predict analyte concentrations in the complex samples. This matrix-enhanced calibration method is demonstrated with calibration models for the measurement of multiple analytes in two sets of samples of an insect cell culture medium. The first set of samples is comprised of synthetic variations of fresh and spent culture medium from the beginning and end of a bioreactor run, respectively. The second set includes samples collected throughout the course of a separate bioreactor run with the use of a different culture medium.

Insect cells are commonly cultivated in bioreactors to generate proteins and viral insecticides.[3] To permit cell growth and product generation, one adds a culture medium containing high levels of nutrients to the bioreactor. Insect cell culture media generally contain three carbohydrates and the 20 common amino acids, all at concentrations of 1 mM or greater.[4] The insect cells consume many of these chemical species for energy, for cellular

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building materials, and for protein production; the cells also produce metabolic waste products such as alanine.[5,6] The composition of the culture medium continually evolves during reactor operation, and so there is a need for the development of noninvasive monitoring schemes for such insect cell bioreactors, as the insect cells often exhaust the available supply of glucose and glutamine.[6,7] Such limitations subsequently decrease the production of desired products and halt cell growth. Some types of cultivated cells have been reported to perform optimally when fed nutrients at a slow, but well-controlled, rate.[8] To maintain these tightly controlled conditions, frequent measurements of the nutrient concentrations must be available. Spectroscopic methods are ideal for making such measurements in bioreactors,[9–13] since concentration information for multiple analytes can be supplied in a noninvasive and nondestructive manner without introducing microbial contamination. The constraint of uncorrelated species concentrations substantially complicates the development of valid calibration models for a cellular culture medium. The concentrations of many species in a bioreactor (e.g., amino acids, carbohydrates, proteins, cell mass, and cell debris) are inherently correlated through cellular metabolism. For example, the correlation between glucose and alanine concentrations suggests that alanine derives from glucose metabolism.[7]

One approach for building a calibration data set is to operate a bioreactor, collect multiple samples, and directly employ these samples as a calibration data set. However, such a method will introduce correlations between all the species in the culture medium, restricting the application of this calibration to subsequent bioreactor runs. Invariably, significant differences exist between subsequent bioreactor runs because of the complex nature of the cellular, biochemical, and hydrodynamic processes involved. If samples with different variations or background compositions are presented to a correlated calibration data set, the resultant concentration predictions ultimately will be flawed. A better approach would be to collect samples from multiple bioreactor runs; however, this approach can be both time consuming and expensive.

Alternatively, working calibration models can be developed by preparing synthetic samples with varying concentrations of the desired analytes and of interfering compounds.[14] However, culture medium commonly contains an undefined fraction of cell debris, cell waste products, and variable factors added to enhance cell growth such as blood serum of yeastolate. These components cannot be accurately reproduced by addition of known chemical species.

The proposed matrix-enhanced calibration method circumvents such difficulties through use of two types of samples. The first set consists of simple, well-characterized standard solutions that provide analyte-specific information. The second set of samples provides spectral information on changes in the background matrix. This matrix-enhanced calibration method is demonstrated for the development of calibration models to quantify glucose and glutamine in an insect cell culture medium. Such calibration models are based on simple binary mixtures of glucose and glutamine dissolved in an aqueous buffer which are used to train a PLS algorithm for glucose concentrations of 0–60 mM and glutamine concentrations of 0–35 mM. By themselves, such calibration models yield poor analyte concentration predictions in culture medium. Model performance is greatly enhanced by incorporating spectra from a few culture medium samples into the calibration set. With the addition of as few as two samples of culture medium to the calibration data set, standard errors of prediction for glucose and glutamine fall by factors of ~5 and 3, respectively. The type and number of culture medium samples added to the calibration set significantly impact analyte prediction errors.

The matrix-enhanced calibration method has also been applied to develop calibration models to predict glucose and glutamine concentrations in samples removed at varying times from an insect cell bioreactor. Addition of three culture medium samples to a buffer calibration reduced glucose prediction errors by a factor of ~3; while addition of two culture medium samples reduced glutamine prediction errors by a factor of ~2.

TABLE I. Distribution of samples into calibration, monitoring, and prediction data sets.

Matrix	Total samples	Calibration set	Monitoring set	Prediction set
Buffer	55	40	15	0
Spent culture medium	25	0–6	6	13
Fresh culture medium	22	0–6	6	10
AC2	20	0–3	5	12

MATERIALS AND METHODS

Samples for the first set of experiments were prepared in either an aqueous buffer, fresh insect cell culture medium, or spent insect cell culture medium. Fresh culture medium is that placed in a bioreactor at the beginning of a run, and spent culture medium is that which remains at the end of the run. Samples from these three matrices were divided into small aliquots to which random, known amounts of glucose and glutamine were added. Samples were further subdivided into separate calibration, monitoring, and prediction data sets (see Table I). Calibration samples were used to train the PLS regression algorithm. Monitoring samples were used to select optimal calibration model parameters including the spectral range and number of PLS factors. Prediction samples were withheld from the parameter selection process and used to evaluate the models.

Samples were prepared by weighing random, known amounts of glucose and glutamine into one of the starting solutions. The aqueous buffer solution contained 1.05 g $NaHCO_3$ and 3.039 g NaH_2PO_4 in 3L deionized water, adjusted to pH = 6.35. These conditions are similar to the buffer conditions of Sf-900 II culture medium, commonly used to cultivate Sf-9 insect cells. The fresh Sf-9 insect cell culture medium used here was obtained from American Cyanamid (San Leandro, CA); the composition is generally similar to that of other commercial Sf-9 culture media. Spent culture medium was collected from an Sf-9 insect cell culture bioreactor operated in batch mode with continuous sparging of gases and pH control. The concentrations of glucose, glutamine, and other amino acids decreased significantly during the bioreactor run, and so the compositions of the fresh and spent media are substantially different.

The concentrations of glucose and glutamine in the fresh and spent culture medium were quantified by standard off-line methods. Glucose concentrations were measured by using a YSI glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). This method has a precision among replicates of approximately ±0.28 mM at the concentration range used.[15] Glutamine was measured by HPLC with a C-18 reverse phase column (Supelco, Bellefonte, PA) in conjunction with an OPA derivatization-based fluorescence detection scheme.[16] Standard deviations among replicates are approximately 0.6 mM (data not shown).

Samples with culture medium were prepared by adding random and uncorrelated amounts of dry glucose and glutamine to individual aliquots of the pooled fresh or spent culture medium. The resulting total concentrations of glucose and glutamine ranged from 0 to 50 mM and 0 to 25 mM, respectively. These concentration ranges are wider than the changing nutrient concentrations observed over the course of a typical bioreactor run.[6,13] With the addition of various amounts of glucose and glutamine to aliquots of the fresh and spent media, a substantial number of samples were generated with *uncorrelated* analyte concentrations. The correlation coefficient between the glucose concentration and the glutamine concentration in the samples from the fresh culture medium is 0.201 and between the glucose concentration and the glutamine concentration in the samples from the spent culture medium is 0.269. These samples are termed the AC1 set.

Twenty samples of a second type of American Cyanamid insect cell culture medium were also collected from a second insect cell culture bioreactor operated for one week with pH and dissolved gas control. These samples, termed the AC2 samples, provide a more rigorous test of the matrix-enhanced calibration method than the synthetic samples because the AC2 samples not only have variations in the components to be quantified but also have alterations in the concentrations of background constituents including amino acids, carbohydrates, proteins, and cell debris. The composition of these samples had not been modified, and so this sample set reasonably represents a common trajectory of concentrations obtained during an insect cell cultivation.

Spectra were collected on a Nicolet 550 Fourier transform infrared (FT-IR) spectrometer (Nicolet Analytical Instruments, Madison, WI). This spectrometer was equipped with a 50 W tungsten–halogen lamp, calcium fluoride beamsplitter, and liquid nitrogen-cooled indium antinomide (InSb) detector. The optical sample cell was maintained at 27 °C, as in the bioreactor. Single-beam spectra were collected with a resolution of 2 cm^{-1} for each sample as 128 coadded scans from 5000 to 4000 cm^{-1} with a 1.5 mm optical pathlength. An interference filter (Barr and Associates, Cambridge, MA) was used to isolate this spectral region. Three spectra were collected for each sample. A background spectrum of pure buffer was collected after every fourth sample. Additional details of the experimental setup may be found elsewhere.[13,14]

Triplicate spectra of 122 samples were collected (55 aqueous buffer, 22 fresh culture medium for AC1, 25 spent culture medium for AC1, and 20 for AC2). These samples were divided into calibration, monitoring, and prediction data sets (as detailed in Table I). Replicate spectra were always incorporated into the same set. PLS regression was applied to develop unique calibration models for glucose and glutamine using samples in the calibration data sets. A large number of calibration models were developed by varying spectral range, number of PLS factors, and number and type of culture medium samples incorporated into the calibration model. 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} (Fig. 1). Calibration models that encompass these absorption bands should provide reliable analyte predictions. Independent monitoring data sets were generated from a subset of the calibration data sets and were used to select optimal calibration parameters independent of the prediction data set. Models that yielded good predictions for the monitoring set were applied to determine analyte concentrations in the prediction data set. Prediction errors for these different data sets were computed as standard error of calibration (SEC), standard error of monitoring (SEM), and standard error of prediction (SEP).

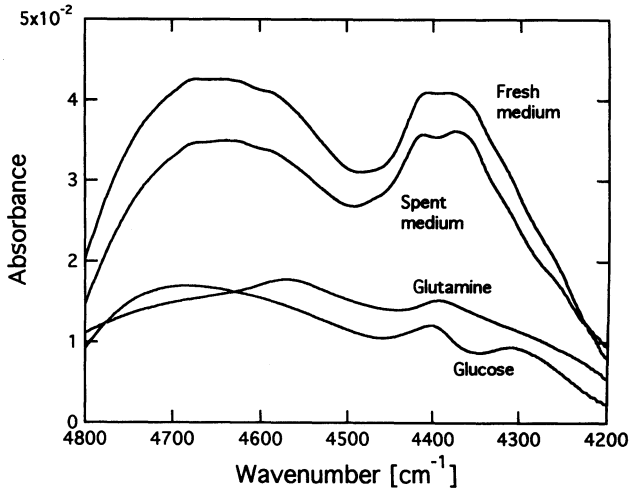

FIG. 1. Individual absorbance spectra of glucose in buffer, glutamine in buffer, fresh culture medium, and spent culture medium.

The AC2 samples were divided into three sets (Table I). The first set contains the first, middle, and last samples collected in time (samples numbered 1, 10, and 20), which are employed as additions to the previously described buffer calibration set. The second set contains 5 randomly selected samples which, in addition to 15 buffer samples, were used as a monitoring set to optimize the spectral range and number of PLS factors used. The third set applies the remaining 12 samples as a prediction set. The prediction and monitoring samples were randomly mixed three times, and models were optimized each time to remove artifacts caused by sample heterogeneity. Prediction results are reported as averages of these three rounds of model evaluation.

To allow a thorough investigation of the many possible combinations of calibration parameters, 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. The script followed a modified grid search, which provided the unbiased evaluation of many calibration

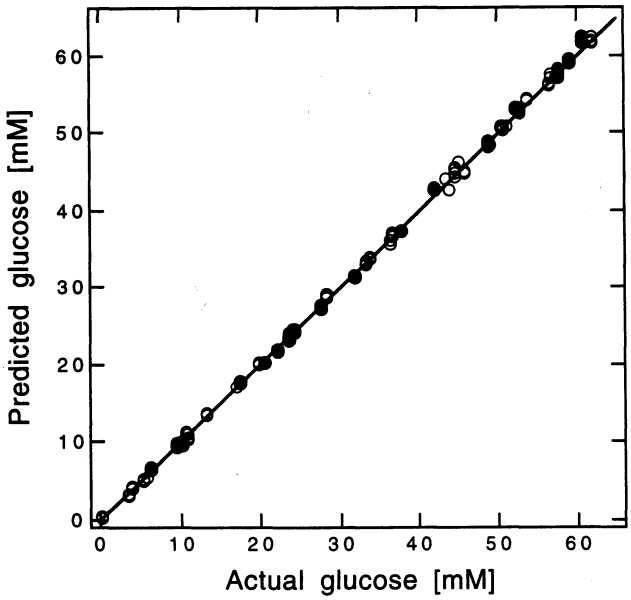

FIG. 2. Concentration correlation plots for glucose in an aqueous buffer matrix showing points for calibration (open circles) and prediction (filled circles). Solid line indicates perfect correlation.

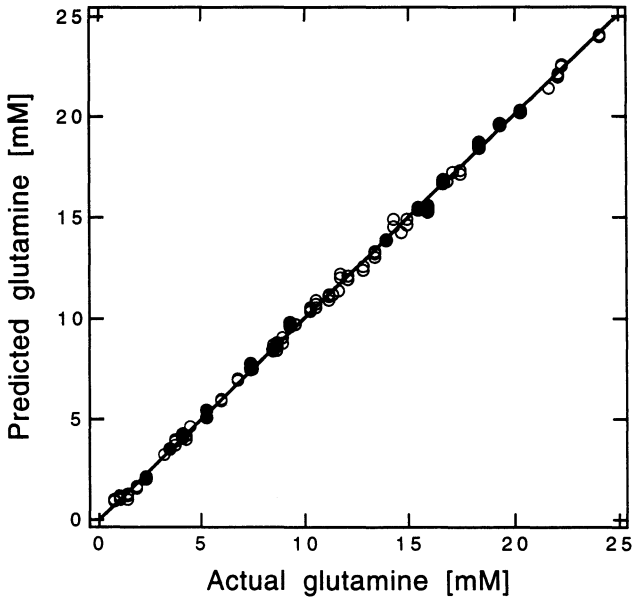

FIG. 3. Concentration correlation plots for glutamine in an aqueous buffer matrix showing points for calibration (open circles) and prediction (filled circles). Solid line indicates perfect correlation.

data sets in a short period of time. For each model size (as defined by the total number of PLS factors), the script searched for spectral ranges within the 5000–4000 cm^{-1} region that contained significant analyte information, as judged by a low SEM. Initially, SEM values were calculated for 100 cm^{-1} wide regions at 100 cm^{-1} intervals beginning with 4100–4000 cm^{-1} and progressing to 5000–4900 cm^{-1}. The region that yielded the minimum SEM was judged to contain significant analytical information. The upper and lower values of this range were increased and decreased by a predetermined amount, and the corresponding SEM values were calculated. The lowest SEM identified the optimum spectral range. This process was repeated four times; each subsequent iteration had a more narrow interval in the spectral range. The number of PLS factors was then incremented, and another spectral range search was implemented.

RESULTS AND DISCUSSION

Buffer Calibration for Buffer Samples. Initially, calibration models were built to predict analyte concentrations within a homogeneous set of samples that would provide a best-case scenario. Calibration models consisting of 40 aqueous buffer samples were used to predict glucose and glutamine concentrations in 15 independent buffer samples. These models yield accurate predictions with a small degree of error because of the similarity between the calibration and prediction data sets. Glucose models with a spectral range of 4700–4200 cm^{-1} and eight PLS factors yield an SEP of 0.37 mM and a mean percent error (MPE) of 1.65%. Glutamine models with a range of 4800–4250 cm^{-1} and eight PLS factors yield SEP and MPE values of 0.33 mM and 2.9%, respectively. Concentration correlation plots are displayed for glucose in Fig. 2 and for glutamine in Fig. 3. Predicted and actual analyte concentrations have excellent agreement, and both calibration and prediction data sets closely follow the unity line with no indication of a systematic bias.

While it does appear that a large number of factors were employed to model relatively simple samples, several previous works from the literature have employed similar numbers of factors to model variations in samples with similar numbers of varying components. In particular, Zhou et al.[17] used between 7 and 12 factors to model samples of glutamine and asparagine dissolved in buffer. Chung et al.[14] employed as many as 12 factors to model components in samples containing 6 varying components. Most likely the large number of factors incorporate variations in baseline shifts, temperature effects, and alignment variations, particularly for samples that contain low analyte concentrations. For the present study, the loading vectors for calibrations were examined and found to display nonrandom spectral features.

Buffer Calibration for Culture Medium Samples. Next, several types of calibration models were investigated for their ability to predict glucose and glutamine in fresh and spent insect cell culture media. Our goal was to use the simplest calibration models that yield good predictions with errors less than 5%. The cell culture medium contains considerably more components than an aqueous buffer, and so analyte measurements would be expected to have more error for the culture medium samples than for the buffer samples.

The simplest type of calibration would include only the aqueous buffer samples. Such samples are completely independent of the culture medium composition, cell type, and bioreactor operating conditions; calibration models using these samples would focus entirely on the analyte spectral information. When applied to the measurement of glucose and glutamine in culture medium, the calibration set with only buffer samples yields poor analyte predictions, as displayed in Fig. 4. Summaries of glucose and glutamine models are presented in Tables II and III, respectively. SEPs for glucose are approximately

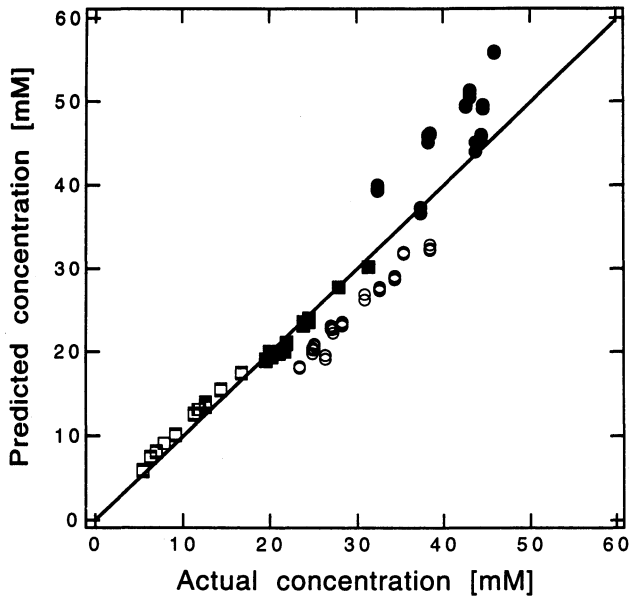

FIG. 4. Concentration correlation plot for glucose (circles) and glutamine (squares) predictions in fresh (filled symbols) and spent (open symbols) culture medium with the use of calibration models constructed from buffer-only samples.

5.6 mM with MPEs of 15% (Model #1 in Table II). Predictions for glutamine are more accurate with an SEP of 1.0 mM and an MPE of 7.8% (Model #1 in Table III). These models would not be acceptable for monitoring a cell culture bioreactor primarily because of the systematic bias apparent in the predictions. For glucose, the fresh culture medium sample concentrations are over-predicted, while the spent culture medium samples are under-predicted (see circles in Fig. 5). Alternatively, glutamine concentration predictions in fresh culture medium samples are under-predicted, while spent culture medium samples are over-predicted (squares in Fig. 6). Clearly, buffer samples alone are not sufficient to build accurate calibration models for the culture medium samples, because the calibration and prediction data sets are improperly matched.

Matrix-Enhanced Calibration for Culture Medium Samples. To systematically increase the complexity of the calibration models, we added several culture medium samples to the original set of buffer samples. Calibration models include the same 40 buffer samples used previously, in addition to three fresh culture medium samples and three spent culture medium samples. Culture medium samples with high, moderate, and low analyte concentrations were incorporated into the calibrations.

The resulting matrix-enhanced calibration models were much more successful at predicting glucose and glutamine concentrations in culture medium samples (Model #3, Tables II and III). The best glucose model has an SEP of 1.0 mM with an MPE of 2.4%, and that for glutamine yields an SEP of 0.37 mM and an MPE of 2.6%. These errors are greatly reduced from those with the buffer-only calibration models. Such levels of error are generally similar to the measurement error for quantification of the analytes in a buffer and would most likely be acceptable for monitoring insect cell culture bioreactors. Concentration correlation plots presented in Fig. 7 show good agreement between the calibration and prediction data sets. Predicted concentrations have a small degree of scatter about the unity line, similar to the predictions of the buffer samples shown graphically in Fig. 2. Prediction residuals for both glucose and glutamine are evenly distributed and have no systematic bias (open symbols in Figs. 5 and 6, respectively).

Clearly, adding six culture medium samples to the calibration data sets greatly improves concentration predictions in the culture medium samples. Calibration models with several culture medium samples are able to yield good analyte predictions in a set of complex samples, because the models contain two important types of information: pure analyte spectral features and sample background variation similar to that encountered in the prediction samples. This same information could be incorporated into the calibration models by using a large number of samples taken directly from a bioreactor; however, these samples must have independent analyte variations so as to avoid incorporating unwanted correlations. The necessary variation may be achieved by adding random amounts of the individual analytes to the culture medium samples. By carefully selecting samples that contain significant matrix and analyte variations, one may minimize the number of culture medium samples required to generate good models. For comparison, calibration models composed of only six culture medium samples, without the buffer samples, are unable to accurately predict analyte concentrations (data not shown), because insufficient amounts of analyte concentration information are provided.

The applicability of the calibration models to predict glutamine concentrations in the culture medium is depen-

TABLE II. Optimum models for glucose measurements in samples of fresh and spent media spiked with glucose and glutamine.

Model #	Fresh culture medium samples added to calibration	Spent culture medium samples added to calibration	Spectral range (cm^{-1})[a]	# Factors[a]	SEC (mM)	SEM (mM)	SEP (mM)	MPE
1	0	0	4490–1420	9	0.35	4.3	5.6	15%
2	1	1	4630–4230	9	0.44	0.86	1.0	2.6%
3	3	3	4750–4250	9	0.52	0.63	1.0	2.4%
4	6	6	4660–4240	11	0.40	0.57	1.0	2.3%
5	6	0	4400–4200	9	0.92	1.6	2.6	6.4%
6	0	6	4790–4570	11	0.86	3.0	3.7	8.0%

[a] Optimized value.

TABLE III. Optimum models for glutamine measurements in samples of fresh and spent media spiked with glucose and glutamine.

Model #	Fresh culture medium samples added to calibration	Spent culture medium samples added to calibration	Spectral range (cm^{-1})[a]	# Factors[a]	SEC (mM)	SEM (mM)	SEP (mM)	MPE
1	0	0	4740–4380	8	0.28	0.82	1.0	7.8%
2	1	1	4450–4300	7	0.43	0.52	0.59	3.9%
3	3	3	4480–4300	7	0.42	0.52	0.37	2.6%
4	6	6	4480–4300	7	0.42	0.54	0.35	2.5%
5	6	0	4680–4490	8	0.29	0.71	0.56	3.2%
6	0	6	4600–4300	7	0.39	0.54	0.44	2.3%

[a] Optimized value.

dent on the number of culture medium samples incorporated into the calibration set. With a reduction in the number of culture medium samples to one fresh and one spent (Model #2 in Table III), the prediction error for glutamine increases nearly twofold. Incorporating a large number of culture medium samples into the model might sound appealing; however, models with 6 culture medium samples and models with 12 culture medium samples display only small differences. Increasing the number of fresh and spent culture medium samples to six each (Model #4) only slightly reduces prediction errors. In this case, three fresh and three spent culture medium samples added to the buffer samples are sufficient to yield accurate glutamine predictions.

Comparatively, calibration models for glucose are less sensitive to the number of culture medium samples added to the calibration set (Table II). In this case, 2, 6, or 12 total culture medium samples (Models #2, 3, and 4, respectively) added to the calibration yield SEPs of about 1.0 mM and MPEs around 2.4%. Note that, with the addition of only two culture medium samples to the buffer calibration set, the SEP for glucose falls substantially, from 5.6 mM to 1.0 mM, and the MPE drops from 15% to 2.6% (Models #1 and 2).

More glutamine culture medium samples are required here to build good calibrations because the culture medium contains many other amino acids that have spectral features, concentrations, and dynamic variations similar to those of glutamine. The PLS algorithm requires more information to discriminate glutamine from such a complex background. Glucose, however, is the primary carbohydrate present in the culture medium, and the glucose spectral features are more readily distinguishable from the background. Therefore, fewer culture medium samples are required for the glucose calibrations to yield good predictions.

The *types* of samples added to the calibration set can have a significant effect on the prediction errors. When only six fresh culture medium samples (Model #5) or only six spent culture medium samples (Model #6) are added to the glucose models (Table II), errors are only slightly lower than those for the original model (Model #1). The errors for Models #5 and #6 are two to three times greater than those obtained when a single fresh *and* a single spent culture medium sample are incorporated into the calibration set (Model #2). The fresh and spent media have significantly different compositions, and these differences appear to have a large impact on glucose predictions. A well-balanced calibration model must incorporate this range of variation.

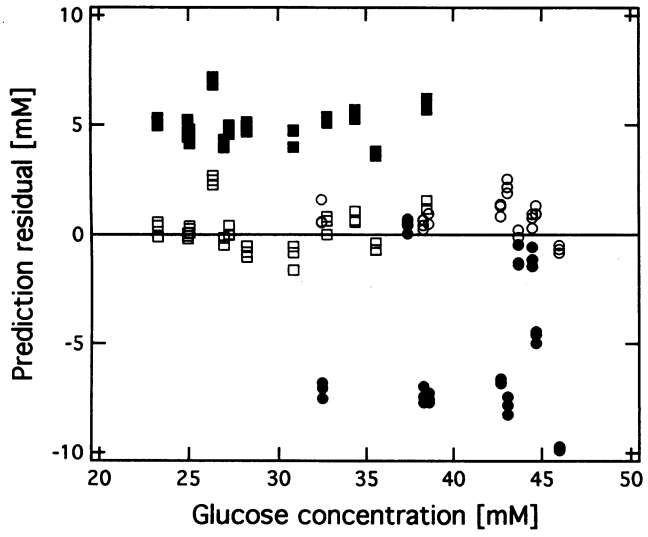

FIG. 5. Prediction residuals for glucose measurements in fresh culture medium (circles) and spent culture medium (squares) with buffer-only calibration (filled symbols) and buffer plus three spent and three fresh culture medium samples (open symbols).

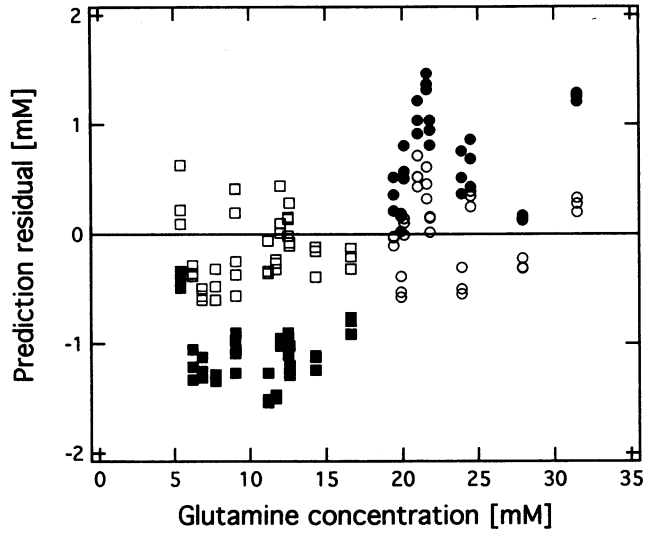

FIG. 6. Prediction residuals for glutamine measurements in fresh culture medium (circles) and spent culture medium (squares) with buffer-only calibration (filled symbols) and buffer plus three spent and three fresh culture medium samples (open symbols).

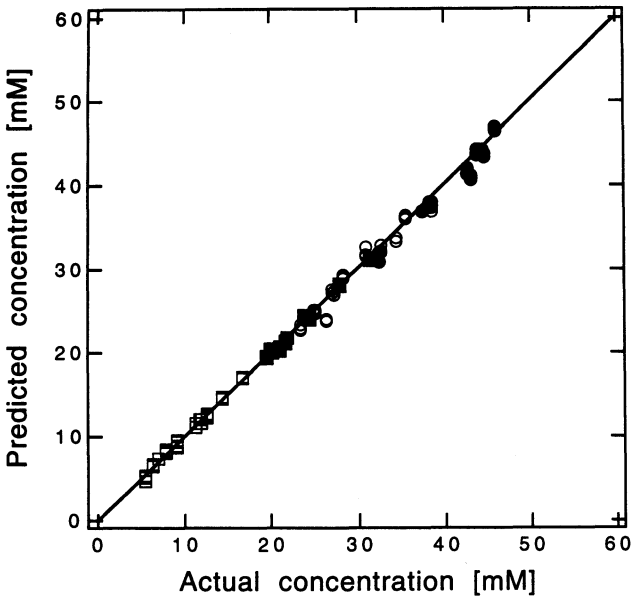

FIG. 7. Concentration correlation plot for glucose (circles) and glutamine (squares) predictions in fresh (filled symbols) and spent (open symbols) culture medium with calibration models constructed from buffer samples plus three fresh and three spent culture medium samples.

Glutamine predictions are less sensitive to the type of samples incorporated into the calibration. When only six fresh culture medium samples (Model #5) or only six spent culture medium samples (Model #6) are added to the glutamine models, errors are somewhat lower than those obtained for one of each type of culture medium sample (Model #2).

Matrix-Enhanced Calibration for AC2 Culture Medium Samples. A major limitation of the example described above is that the matrix for samples in the prediction data set contains either fresh or spent culture medium, and this binary nature of the sample matrix in both calibration and prediction samples allows for an exact match in the matrix-enhanced calibration models. A much more demanding experiment would include prediction samples collected intermittently during a bioreactor run where the matrix composition is not exactly incorporated into the calibration model. Insect cell culture medium samples were collected from a subsequent bioreactor run (AC2) to assess model performance under such conditions.

In this experiment, 20 samples were collected during the bioreactor run and subsequently analyzed for glucose and glutamine concentrations. Five of these AC2 samples along with 15 of the previous buffer samples were employed as a monitoring set for selection of PLS model parameters. Spectra for the first, middle, and last samples (denoted 1, 10, and 20 in Tables IV and V) were added to the buffer calibration spectra, and the resulting PLS models were evaluated on the basis of prediction accuracy for a prediction data set corresponding to the remaining 12 samples. The 5 monitoring samples and 12 prediction samples were randomly selected. Under this protocol, none of the sample matrices within the prediction data set is exactly matched within the calibration set. The effect of different combinations of these three matrix enhancement samples is assessed. Results reported below represent averages for concentration predictions for three independent sets of sample selections.

TABLE IV. Optimum models for glucose measurements in the AC2 samples.

Calibration samples[a]	Spectral range (cm^{-1})[b]	# Factors[b]	SEC (mM)	SEM (mM)	SEP (mM)	MPE
Buffer	4540–4200	8	0.42	2.0	3.8	18%
B + 1	4760–4200	10	0.38	1.6	2.7	12%
B + 10	4720–4250	8	0.63	1.4	3.4	13%
B + 20	4690–4200	8	0.44	3.0	5.7	16%
B + 1, 10	4510–4200	7	0.76	1.0	2.5	10%
B + 1, 20	4570–4270	8	0.43	0.59	1.5	5.2%
B + 10, 20	4600–4240	7	0.76	0.92	1.6	6.1%
B + 1, 10, 20	4580–4200	7	0.81	0.62	1.1	4.4%

[a] "B" indicates buffer sample spectra, and 1, 10, and 20 correspond to the first, middle, and last samples collected during the AC2 bioreactor run, respectively.
[b] Optimized value.

Initially, the 40 buffer samples were applied as a calibration data set to predict the concentrations of glucose and glutamine in the AC2 samples. Prediction errors are substantial, with SEPs of 3.8 mM for glucose and 1.7 mM for glutamine. Results are summarized in Tables IV and V for glucose and glutamine, respectively. Predictions of glucose and glutamine concentrations with the use of the buffer calibrations are presented as open symbols in Figs. 8 and 9, respectively. Predictions are provided as normalized concentration (relative to their maximum value) because of the proprietary nature of this insect cell culture medium.

The effect of addition of one matrix-containing sample to the calibration data was assessed for glucose, and the results are tabulated as entries B + 1 (buffer samples plus sample number one), B + 10, and B + 20 in Table IV. Addition of the first and middle samples, individually, improves model performance, with SEP values of 2.7 and 3.4 mM, respectively. Addition of the last sample, however, degrades model performance and yields an SEP of 5.7 mM. The average SEP (4.0 mM, as presented in Table VI) for these three conditions is slightly higher than that obtained for calibrations constructed with only buffer samples. The addition of two culture medium samples to the buffer calibration substantially reduces the prediction error, with an average SEP of 1.9 mM for the three available permutations. This amount of error is less than half

TABLE V. Optimum models for glutamine measurements in the AC2 samples.

Calibration samples	Spectral range (cm^{-1})[b]	# Factors[b]	SEC (mM)	SEM (mM)	SEP (mM)	MPE
Buffer	4700–4360	7	0.40	1.1	1.7	56%
B + 1	4650–4200	12	0.22	0.70	1.3	39%
B + 10	4740–4300	9	0.28	0.45	0.60	20%
B + 20	4590–4290	9	0.28	0.56	1.1	21%
B + 1, 10	4610–4300	9	0.29	0.42	0.77	17%
B + 1, 20	4570–4220	8	0.32	0.55	0.87	18%
B + 10, 20	4660–4200	10	0.21	0.50	0.70	14%
B + 1, 10, 20	4590–4200	8	0.33	0.54	0.86	25%

[a] "B" indicates buffer sample spectra and 1, 10, and 20 correspond to the first, middle, and last samples collected during the AC2 bioreactor run, respectively.
[b] Optimized value.

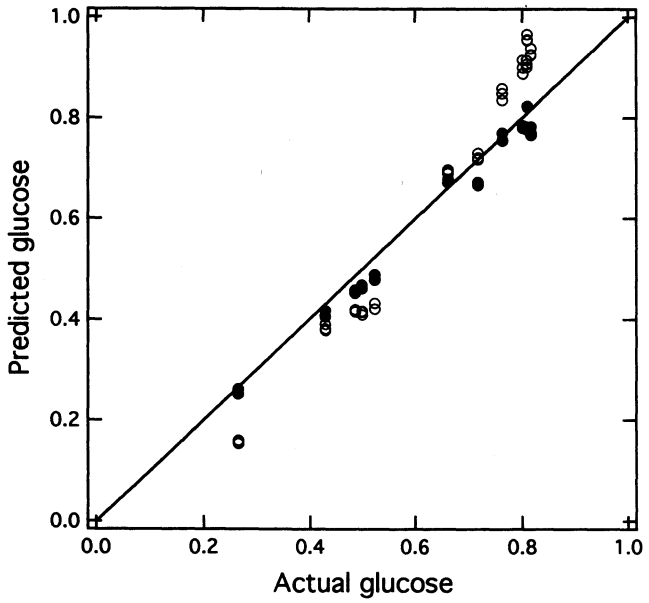

FIG. 8. Concentration correlation plot for glucose measurement in the AC2 samples with application of calibration models of buffer-only (open circles) and buffer plus three culture medium samples (closed circles). All concentrations are normalized to the maximum values.

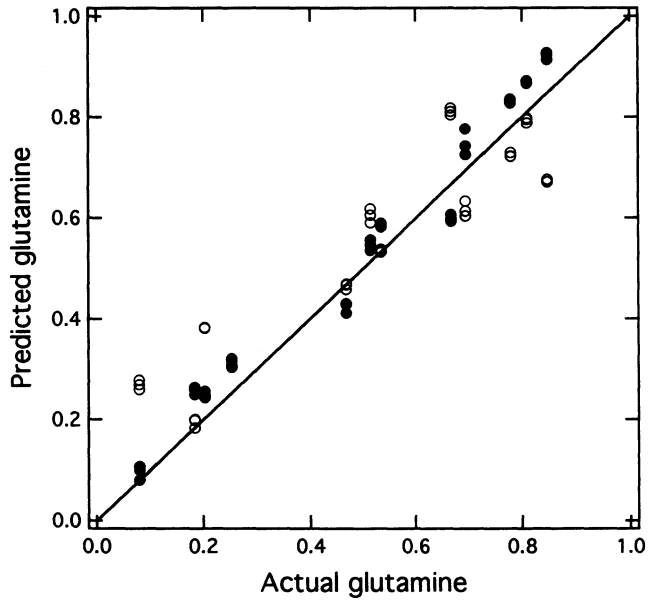

FIG. 9. Concentration correlation plot for glutamine measurement in the AC2 samples with application of calibration models of buffer-only (open circles) and buffer plus three culture medium samples (closed circles). All concentrations are normalized to the maximum values.

that obtained for the buffer calibration. Combining all three culture medium samples with the buffer calibration yields an even greater reduction in the glucose prediction error, with an SEP of 1.1 mM. Glucose predictions with the use of calibrations of buffer samples plus three culture medium samples are presented as the closed symbols in Fig. 8. The enhanced-buffer calibration predictions are in good agreement with the unity line, whereas the buffer-only predictions over-predict at high concentrations and under-predict at low concentrations.

The addition of culture medium samples to the buffer calibrations has a substantial effect on glutamine predictions (Table V). Incorporation of the first and last samples reduces prediction error to an SEP of 1.3 and 1.1 mM, respectively. Addition of the middle sample to the buffer calibration reduces prediction error by an even greater amount to an SEP of 0.60 mM. The average SEP for these three permutations is 1.0 mM, almost half that for calibrations from only the buffer samples. The addition of two or three culture medium samples produces similar results with average errors of 0.78 and 0.86 mM, respectively (Table VI). Glutamine predictions with the use of calibrations of buffer samples plus three culture medium samples are presented as the closed symbols in Fig. 9. The enhanced-buffer calibration predictions are in good agreement with the unity line, whereas the buffer-only predictions are more scattered.

Measurement errors reported here for glucose and glutamine compare favorably with previous reports of spectroscopic measurements of nutrients in cell culture media. Hall et al.[12] monitored the concentrations of acetate and ammonia and the cell density in *Escherichia coli* fermentations. Monitoring errors over large concentration ranges were approximately 7 mM for each analyte with an MPE of 1–3%. Vaccari et al.[11] measured the concentrations of lactate, glucose, and biomass in bacterial cell fermentations with similar levels of error. We have previously used a more time-intensive method to generate calibration models for insect cell culture media and obtained errors of SEP of 1.5 mM/MPE of 2.3% for glucose and SEP of 0.51 mM/MPE of 5.2% for glutamine.[13]

The extent of matrix variation is a major factor in our ability to model the sample matrix with only a few samples. Although the insect cell culture medium contains over 23 individual components at relatively high concentrations, only three of these components exhibit substantial concentration changes during cultivation. An analysis of the culture medium at the beginning and end of a bioreactor run reveals that, on a molar basis, the absolute sum of the change in the culture medium composition is 72 mM. Three components—glucose, glutamine, and alanine—account for an absolute change of 58 mM, which represents 81% of the total composition change in the culture medium. The remaining components provide a relatively constant background composition. Minor changes in the background are supported by comparing spectra for the fresh and spent media in Fig. 1. Although some differences are apparent, these spectra are reasonably similar and do not indicate major chemical variations between these limiting cases. Of course, greater matrix variation will likely demand more matrix-containing samples to model matrix variation adequately and produce robust calibration models.

TABLE VI. Summary of processing results for the AC2 samples.

Samples added to calibration	Glucose SEP (mM)	Glucose MPE	Glutamine SEP (mM)	Glutamine MPE
0	3.77	18%	1.7	56%
1	3.96	14%	0.99	27%
2	1.89	7.1%	0.78	16%
3	1.06	4.4%	0.86	25%

For most models displayed in Tables II, III, IV, and V, the number of PLS factors increases slightly with addition of more complex samples to the calibration set. Compared with the initial calibrations, which contained no additional samples, the number of required PLS factors increases for 10 models, decreases for 7 models, and remains the same for 7 models. In the situations where the number of factors decreases, such as for glutamine models listed in Table III and several glucose models listed in Table IV, the decrease is quite small, from 8 to 7 factors. The number of factors was selected through use of a computerized optimization scheme, and the model parameters that yielded the lowest error for a distinct monitoring set were selected as optimal. This approach removes all human bias in selecting model parameters but led to some variation in both the number of factors and the spectral range reported as optimal. A possible cause for these variations is that the monitoring set consists of samples with matrices both of buffer and of culture medium. Optimal models were selected on the basis of the minimum standard error in predicting analyte concentrations in the entire set of monitoring samples. Certain conditions yield models that most accurately predict the concentrations of the buffer samples in the monitoring set, while other conditions most accurately predict the concentrations of the medium samples in the monitoring set.

The results of this study have implications for the development of spectroscopic calibration models that are to be applied to complex situations, such as those encountered in cell culture bioreactors. Calibration models composed entirely of samples of the analytes in an aqueous buffer yield poor concentration predictions when applied to culture medium samples. However, with the addition of as few as two culture medium samples (one of fresh culture medium and one of spent culture medium), prediction errors decrease significantly. The advantage of this approach is that it relies on only a small number of samples taken from a bioreactor, and most of the calibration samples can be produced synthetically. This approach could be applied to spectroscopic studies of other complex situations where it is difficult to generate representative and uncorrelated calibration samples. To date, the matrix-enhanced calibration method has not been applied to other such complex situations so it is unknown how well the method would perform for samples in which a much larger number of components vary substantially in concentration.

The method presented here to generate calibration models with many buffer samples and a small number of culture medium samples has similarities to approaches used for the transfer of calibrations among multiple spectrometers. Difficulties often arise when a calibration performed on a primary spectrometer is applied to a secondary instrument that yields a different response.[18] These differences may be due to variations between the primary and secondary instruments or due to changes in the prediction samples, perhaps from dissimilar production batches.[19] In an effort to circumvent these problems, standardization methods have been developed by using a transfer set of samples that are measured on both the primary and secondary instruments, and computational algorithms adjust the response of the secondary instrument to that of the primary instrument.[19–21] Often a small number of transfer samples are required to properly account for instrument variations; for example, Wang et al. used as few as three samples,[19] while Bouveresse et al. used five samples[21] to obtain transferable calibrations.

These transfer spectra are somewhat analogous to the culture medium samples added in the matrix-enhanced calibration method. The buffer samples originally used in the calibration set are similar to samples measured on one instrument; the culture medium samples can be considered as samples with a different baseline due to the additional components present in the culture medium. With the addition of culture medium samples to the calibration set, the PLS algorithm incorporates a greater extent of the sample variations and thus is able to correct for differences between these two data sets.

When using the matrix-enhanced calibration method, one must evaluate the dependence of analyte predictions on the number and type of enhancements, or in this case culture medium samples, incorporated into the calibration set. In the present study, glucose and glutamine measurements follow different trends in their sensitivities to the types of samples added to the calibration sets. Most likely, similar variations would be observed in the development of calibration models for other complex materials.

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