Continuous Bioprocessing: Technology for Next-Generation Biopharmaceutical Manufacturing

Development of a Python-coded Bench-scale Raman-based Continuous Bioprocess Platform

By

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A Dissertation submitted to the Faculty of Keck Graduate Institute of Applied Life Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Applied Life Sciences

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ABSTRACT OF THE DISSERTATION

Continuous Bioprocessing: Technology for Next-Generation Biopharmaceutical Manufacturing

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Keck Graduate Institute of Applied Life Sciences: 2023

Current industrial practices for producing biopharmaceuticals include fed-batch production with batch isolation and purification. Nonproductive hold-up steps and manual offline measurements are common in batch processing which increases processing time and contributes to a high cost of production. Current market trends and cost pressures in the biopharmaceutical industry are creating a push to innovate bioprocessing platforms. Continuous bioprocessing has been considered a solution to the current limitations of batch production of biopharmaceuticals. Continuous bioprocessing involves intensifying individual processing steps by eliminating hold-up steps through a continuous operation to increase productivity, which results in advantages such as lower capital and production costs, higher equipment utilization efficiencies, smaller facility footprints, and increased manufacturing flexibility. Current bottlenecks of implementing continuous bioprocessing include technologies for real-time monitoring and control of critical/key process parameters and versatile scale-down models for process understanding and development. Commercially available platforms

for implementing continuous bioprocessing are often expensive and inflexible. Technologies including Raman spectroscopy, perfusion cell culture, and continuous chromatography are explored in this Ph.D. study to develop a proof-of-concept, versatile bench-scale continuous platform driven by open-source software. Real-time, at-line monitoring of critical nutrients for cell culture via Raman spectroscopy allows for providing feedback control to nutrient pumps to maintain a continuous supply of these nutrients to cells for the production of biopharmaceuticals, and the products are continuously harvested in a perfusion process to a two-column platform for protein A capture. The preliminary data supports that the bench-scale platform is readily maneuverable to customized requirements, adaptable for the production of different modalities, and much cheaper for implementation.

DEDICATION

In loving memory of my dear parents,

This dissertation is dedicated to the unconditional love, support, and sacrifices of my beloved parents, Maria and Juan Urrea. Though they are no longer with me, I am grateful that they were able to see the beginning of my Ph.D. journey.

To my parents, this dissertation is a testament to your legacy and the values you instilled in me, I hope it serves as a small tribute to the remarkable individuals you were.

Seran extrañados para siempre.

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TABLE OF CONTENTS

ABBREVIATIONS

ATF	Alternating Tangential Flow Filtration
CAGR	Compound Annual Growth Rate
CHO	Chinese Hamster Ovarian Cells
CPPs	Critical Process Parameters
CQAs	Critical Quality Attributes
CSPR	cell specific perfusion rate
GUI	Graphical User Interface
mAbs	Monoclonal Antibodies
MAPE	Mean Absolute Percentage Error
MIR	Mid-Infrared Spectroscopy
NIR	Near-Infrared Spectroscopy
PAT	Process Analytical Technology
PCA	Principal Component Analysis
PCC	Periodic Counter Current Chromatography
PLS	Partial Least Squared Regression
QbD	Quality by Design
R^2C	Coefficients of Determination for Calibration
R^2CV	Coefficients of Determination for Cross Validation
RMSE	Root Mean Square Error
RMSEC	Root Mean Square Error of Calibration
RMSECV	Root Mean Square Error of Cross Validation
SMB	Simulated Moving Bed Chromatography
TFF	Tangential Flow Filtration
TMP	Transmembrane Pressure
UV-Vis	Ultraviolet-Visible Spectroscopy
VCD	Viable Cell Density
VVD	Vessel Volumes per Day

1 INTRODUCTION

1.1 BIOPHARMACEUTICAL PROCESSING MARKET AND TRENDS

The biopharmaceutical industry is involved in researching, developing, and commercializing drugs derived from living organisms such as bacteria, yeasts, and animal cells. These drugs, known as biologics, treat various diseases, including cancer, autoimmune disorders, infectious diseases, genetic disorders, and rare diseases. Biologic products include molecules such as proteins, nucleic acids, and cells. This market is rapidly growing and highly competitive. Companies invest heavily in research and development to bring new therapies to market. The biopharmaceutical sector is also heavily regulated to meet stringent safety and efficacy testing requirements, manufacturing practices, and quality control. (U.S. FDA, 2022)

The global market for biopharmaceuticals in 2021 was estimated at $389.3 billion, and it is expected to grow at a compound annual growth rate (CAGR) of 7.1% between 2021 and 2030, leading to an expected value of $730.8 billion by 2030(P & S Intelligence, 2023a). Several aspects lead to the growth of this industry, including an increasing demand for biological drugs due to increasing occurrences of chronic diseases worldwide, the growing geriatric population, and a growing number of clinical trials and research and development activities on novel biologics and biosimilars. Although novel biologics and biosimilars are contributing to the growth of the biopharmaceutical market, monoclonal antibodies (mAbs) constitute the largest share of 36% at a $140 billion valuation, and they are expected to hold the largest share of the market in the foreseeable future. As

of June 2022, monoclinal antibodies account for the most significant portion of approved biologics, with 162 approved by major drug regulatory agencies worldwide. Among them, 122 have been approved in the United States(Lyu et al., 2022).

There are currently several trends in the biopharmaceutical space(BioPhorum Operations Group, n.d.). First, from a market perspective, novel biologics and biosimilars are continuously introduced. Their market was $6 billion in 2018, and it was expected to grow at a CAGR of 30% from 2019-2024 (P & S Intelligence, 2023b). The second trend is increased efforts to improve the global reach of these biologics so that more patients can benefit from these drugs and improve their life quality. There is also a desire for in-region manufacturing to mitigate supply chain issues, which could accelerate the time-to-market by complying with the regulations of local agencies. The cost pressures stem from payers, biosimilar competition, and high capital and operation costs for biologics. There are also pressures from the uncertainty of product success, market demand, and investment. Diversification of products is seen within biologics due to the advances in human genetics, cell biology, and gene and cell therapies(El-Kadiry et al., 2021; Sinclair et al., 2018). Diverse mAbs products have emerged, including bispecific mAbs, radio-labeled mAbs, and antibody-drug conjugates (Khongorzul et al., 2020; Pettinato, 2021). Manufacturing flexibility has been pursued to allow for the adaption of these trends in the biopharmaceutical market, leading to a push to innovate biopharmaceutical manufacturing platforms.

1.2 CONTINUOUS BIOPROCESSING

Current industrial practices for producing biopharmaceuticals include manual ten-to-fourteen-day fed-batch production and batch isolation and purification, which are often involved with nonproductive hold-up steps and manual offline measurements, leading to an increase in the processing time and a high cost of production. Operating at a large volume (5-25 kL) is also typical, which contributes to a high capital cost and an inflexible manufacturing facility (Kelley, 2009). These practices are not favorable for unstable proteins and may not apply to the new modalities discussed previously. Current market trends and cost pressures in the biopharmaceutical industry are creating a push to innovate bioprocessing platforms. Continuous bioprocessing has been considered a solution to the current challenges of batch production of biopharmaceuticals. It has advantages such as lower investment and production costs, increased volumetric productivities, more consistent and improved product quality, reduced residence times, minimal scale-up requirements, higher equipment utilization rates, and small facility footprints, all of which contribute to increased manufacturing flexibility(Pollock et al., 2017; Warikoo et al., 2012). Process analytical technology (PAT), perfusion bioprocessing, and continuous chromatography are considered to be the primary enablers for continuous bioprocessing.

1.2.1 Process Analytical Technology

PAT is a system for designing, analyzing, and controlling manufacturing through timely measurements with the goal of ensuring final product quality(FDA et al., 2004). PAT is developed for real-time and online/at-line measurements of critical quality attributes

(CQAs) and/or critical/key process parameters (CPPs) while designing, analyzing, and controlling a manufacturing process (Krull et al., 2012). Spectroscopic techniques have become the most popular for process monitoring. The most commonly explored spectroscopic techniques for bioprocess monitoring are ultraviolet-visible spectroscopy (UV-Vis), near-infrared spectroscopy (NIR), mid-infrared spectroscopy (MIR), fluorescence, and Raman spectroscopy. High-performance liquid chromatography (HPLC)(Tiwari et al., 2018) and capacitance(Konakovsky et al., 2015) have been used for monitoring products and biomass, respectively. Commercial Raman spectroscopy technology has gained increasing interest in monitoring cell culture metabolites during process development. However, a few commercial software packages are required for spectrum acquisition, pre-processing, calibration model construction, and prediction. It is time-consuming to develop, implement and validate models. More often, its use focuses on monitoring cell culture metabolites rather than for control purposes.

1.2.2 Perfusion Cell Culture

Perfusion cell culture involves continuously supplying fresh media to a bioreactor while removing spent media containing the product. Perfusion utilizes a cell retention device to retain or recirculate the cells within the culture vessel. The device may be a filter (depth, cross-flow, hollow fiber), centrifuge, gravity settler, or acoustic wave separator. Perfusion is considered a primary solution for continuous cell culture. It has advantages over batch bioprocessing, such as higher cell densities, longer culture periods, lower product residence times, and increased volumetric productivities. (Bielser et al., 2018) (Suttle et al., 2019) (Voisard et al., 2003) The application of external cross-flow filtration

using hollow fiber modules for cell retention is the mainstream method. It offers a direct harvest of cell-free permeates for downstream processing. Two different modes of operation of cross-flow filtration have been established: tangential flow (TFF) filtration and alternating tangential flow (ATF) filtration. (Karst et al., 2016) During TFF, the retentate stream flows tangentially over the surface of hollow-fiber filters, and the permeate penetrates through the pore of the filters and enters into a collection tank. ATF uses the same principle of tangential flow, but the direction of the flow is cyclically reversible to minimize fouling and reduce shear forces on the cells(Suttle et al., 2019). Commercial systems for TFF and ATF are available at several scales and allow monitoring and controlling perfusion parameters such as recirculation flow rates and pressures over the feed stream to the filter, the retentate stream, and the permeate stream. To maintain the balance of the working volume in the bioreactor, the same volumetric flow rate of the permeate stream is applied to the feed cell culture media stream. However, currently there is no control over nutrient conditions in the bioreactor.

1.2.3 Multi-Column (Continuous) Chromatography

Challenges with batch chromatography include an under-utilized rate of the resin binding capacity, a large volume of resins and buffers, a high equipment cost, and product quality variability due to a long process time (Zydney, 2016). Multi-column chromatography breaks up the loading zone of one column into a few smaller columns and places them in series. The smaller columns are cycled using the same process steps in a batch process, resulting in parallel processing of multiple columns. (Arnold, 2018) This technology allows continuous loading of the stream containing the product,

maximizing the resin utilization rate, reducing resin and buffer requirements, and increasing yield and productivity. Periodic counter current (PCC) chromatography and simulated moving bed (SMB) chromatography are two continuous chromatography techniques used to purify biopharmaceuticals. The control of these systems is generally accomplished in a time-based or dynamic control mode. In the time-based control mode, the loading step for a column is executed as a function of time, and the variability in the performance between columns and changes in the feed concentrations are not considered. This method is considered simple, but it often leads to under or overloading the columns, which can affect productivity and/or yield. In a dynamic control mode, the difference in the ultraviolet signal between the column inlet and outlet is continuously monitored. The breakthrough in each column is used to control the loading step. This method allows the loading onto a column to a pre-defined breakthrough level independently of the variability in the column performance or the feed stream. However, the flow rates for different operation steps must be elegantly manipulated to maintain continuity in each column. Very few commercial instruments for PCC and SMB are available, but they are expensive and challenging for customization. They are often designed for pilot-scale, aiming to manufacture clinical trial products. (Cytiva, 2016) Process development and understanding of continuous chromatography are pressing for small-scale, flexible, configurable instruments for PCC and SMB.

1.3 INTEGRATED PLATFORMS

Several lab-scale integrated continuous platforms have been reported in the literature for the production of biologics. Warikoo et al. (2012) reported the first demonstration of integrating a perfusion bioreactor (ATF with a 0.2 µm polyethersulfone filter, 12 L working volume) and a four-column PCC chromatography system. Two molecules were produced using this system: a monoclonal antibody, a model of a stable protein, and a recombinant human enzyme, a model of a complex, less stable protein. High cell densities of 50-60 $x10^6$ cells/mL were reached in both cases. A bleed was implemented, and the extended culture periods were over 60 days. The PCC system, modified from an AKTA system, ran continuously for 30 days with no signs of reduced performance or fouling. The peak volumetric productivity for the mAb was five-fold greater than that in a fed-batch process using the same cell line, and a decline in productivity was observed after day 40. More impactfully, the volumetric productivity for the enzyme was 40-fold higher when compared to the legacy process. Apart from a higher cell density in the perfusion cell culture, the continuous capture step significantly reduced the residence time of unstable proteins, thus improving the product quality. The chromatography media capacity utilization rate was increased by 20 and 50%, buffer usage was reduced by 25 and 46%, and the individual column size was reduced by 75 and 50 folds when compared to a batch mode for the capture of the mAb and the enzyme, respectively.

Godawat et al. (2015) reported an integrated continuous process with an ATF-based perfusion process at a 12 L working volume and two PCC chromatography systems for producing mAbs. In this platform, the clarified harvest from the bioreactor was pumped

into a 2 L surge vessel, and the harvest stream was then subjected to a three-column capture step. The eluent from each capture column was pumped into a glass vessel for viral inactivation. After viral inactivation, the stream was introduced to the second PCC system for further purification, followed by a polishing step. These steps were performed continuously in a fully automated manner. The results were normalized to a batch process, and an 1100-fold increase in productivity was achieved for the upstream process and a 600-fold increase for the downstream process. Advantages in equipment use, resin capacity utilization, buffer consumption, and hold-up steps elimination were demonstrated. Simplifying the process train was achieved by eliminating multiple hold-up steps and fully integrating multiple unit operations. Full automation of the continuous downstream process was realized over an extended period (31 days), leading to constant process flows and consistent product quality.(Godawat et al., 2015)

Steinebach et al. (2017) also used mAbs as the model molecule for their integrated continuous process at a bench scale. The process included continuous harvest of the product stream from a bioreactor with a 12 L working volume via a filter-based cell-retention system, a continuous two-column protein A capture step, a virus inactivation step, a semi-continuous polishing step, and a batch-wise flow-through polishing step. The process was operated for 17 cycles (3.5 days) and consistent product quality was achieved in terms of product-related impurities including different glycoforms, charge isoforms, and process-related impurities such as host cell DNA, host cell proteins, and leached protein A. In addition to benefits observed in previous platforms, they also

reported a 92% yield in the downstream operation and an overall 80% yield after considering the loss to cell bleeds, comparable to other integrated processes.

More recently Gomis-Fons et al. (2020) reported a proof-of-concept of a continuous end-to-end monoclonal antibody production platform. A major difference from the previously reported platforms was that the working volume for this platform was 200 mL compared to a working volume of over 10 L in other platforms. This platform included a perfusion process equipped with an ATF filtration device and a purification process with model-based design and control. Downstream processing consisted of periodic twin-column protein A capture, virus inactivation, cation exchange chromatography, and anion exchange chromatography. The entire downstream process was operated on a single chromatography system. This process produced mAbs for 17 days at a high cell density between 70 and 90×10^6 cells/ml. Mechanistic models were built for the downstream process, and the models were implemented as a control strategy to automatize and optimize the operation of the process. The maximum recovery yield observed for this process was 60%. Such a small working volume is appealing for the early process development of continuous processing. The models can be developed and validated at a small cost, and the process parameters can be optimized for high yield and better product quality.

1.4 AIM AND SCOPE

Previously reported platforms and commercially available technologies have demonstrated the great potential of continuous bioprocessing. However, the vast majority of them are very expensive, inflexible, and challenging for their configurations

for different processes/products. In addition, a high working volume in a bench-scale bioreactor (>10 L) is often required for those available continuous chromatographic systems. Meanwhile, in-line monitoring of key cell culture metabolites and control of key nutrients during perfusion is still in the early development stage. This Ph.D. study aims to develop a versatile, Python-code-driven, lab-scale continuous platform for an integrated perfusion and capture process (mAb as a model molecule) in a cost-effective way, which could be employed for process development and process understanding. The platform incorporates a Raman-based PAT system for monitoring critical cell culture metabolites such as glucose, glutamine, glutamate, lactate, and ammonium and feedback control of the glucose and glutamine concentrations in the bioreactor.

1.5 ORGANIZATION OF THE DISSERTATION

Biopharmaceutical market trends are pushing the development of continuous bioprocessing platforms. Enablers for continuous bioprocessing are briefly reviewed, and their gaps are identified in Chapter 1. Chapter 1 also includes the overall research aim of this Ph.D. study and the organization of the dissertation.

Chapter 2 focuses on the development of methodology and software for building Raman-based calibration models for cell culture metabolites. Pre-processing methods are screened and evaluated for processing raw Raman spectra. Then, partial least squared (PLS) regression models are built in Python and applied to predict these metabolites' concentrations.

In Chapter 3, the Raman-based system is applied to monitor cell culture metabolites and control glucose and glutamine concentrations in a fed-batch process. Through open-source software, the Raman-based models are seamlessly integrated into a control system. The concentrations predicted from Raman spectroscopy are used to trigger the pump operations for the control of glucose and glutamine concentrations to their pre-determined setpoints.

In Chapter 4, the Raman-based models are applied to a perfusion process. Apart from continuous monitoring of critical metabolites, the glucose and glutamine concentrations are maintained at a constant level, leading to a reduction in feed media consumption and an increase in the product stream at a relatively low exchange rate.

The development of a two-column chromatography system and its integration into a perfusion/capture process is detailed in Chapter 5. Continuous loading of the permeate stream directly from the bioreactor onto two columns are realized. The resin utilization capacity of 100% is achievable in the two-column system.

Conclusions are drawn in Chapter 6. Future directions from the Ph.D. study are projected.

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2 RAMAN-BASED CALIBRATION MODEL DEVELOPMENT

2.1 ABSTRACT

Process analytical technology (PAT) is a system for designing, analyzing, and controlling manufacturing processes through real-time measurements of critical quality attributes (CQAs) and critical/key process parameters (CPPs). Raman spectroscopy with chemometric analysis has been demonstrated to be a viable and promising PAT for manufacturing biopharmaceutical processes. It has successfully been used for monitoring media components, cell culture metabolites including amino acids, biomasses, and protein-based products. This chapter is devoted to developing Raman-based calibration models using the concentration of cell culture metabolites obtained from a Bioanalyzer such as glucose, glutamine, lactate, glutamate, and ammonium. The spectra are processed via the partial least squares (PLS) regression method. The established models are then used for predicting metabolite concentrations during a cell culture process.

2.2 BACKGROUND AND INTRODUCTION

2.2.1 Process Analytical Technology (PAT) In Biopharmaceutical Processing

Process analytical technology (PAT) is developed for real-time and online/at-line measurements of CQAs and CPPs during the designing, analyzing, and controlling of a manufacturing process (Krull et al., 2012). PAT, a framework that uses a combination of measurement, analysis, and control, can ensure that a product is manufactured consistently and its quality attributes are within the release acceptance criteria. The application of PAT to biopharmaceutical processing is broad, including process development, drug product manufacturing, real-time product release of commercial products, and raw material testing. It is often associated with other initiatives such as quality by design (QbD) and continuous manufacturing (FDA et al., 2004). There are many benefits to implementing PAT in biopharmaceutical processing. Implementation of PAT has the capability of acquiring real-time, potentially actionable data that can be integrated directly into a process control system. By contrast, in traditional approaches, the process is sampled at pre-determined intervals, and the test result is generated using an offline method after sampling. The offline data may not be able to be used for real-time tuning of the manufacturing process since the data may be out-of-date for a dynamically changing process. Another benefit is the potential to improve process understanding through real-time process monitoring. The real-time process information can help reveal the cause-to-effect relationship between CPPs or KPPs and CQAs. PAT may also help identify inefficiencies in the manufacturing process that, when corrected, could lead to cost and waste reductions.

Traditional biopharmaceutical manufacturing practices include manual batch processes with offline analysis of collected samples to monitor CPPs and evaluate CQAs. Despite many opportunities for improving biopharmaceutical development and manufacturing through innovation in process development, process analysis, and process control(FDA et al., 2004), the biopharmaceutical industry hesitates to introduce innovative systems for several reasons, including regulatory uncertainties and technical challenges. To encourage the introduction of innovation to improve and modernize pharmaceutical process development and manufacturing, the Food and Drug Administration (FDA) released a new initiative called “Pharmaceutical CGMPs for the 21st Century: A Risk-Based Approach” in 2002. In 2004, a guidance named “Guidance for Industry PAT — A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance” was released. This guidance describes a framework based on scientific and engineering principles as a tool to mitigate risks related to poor product and process quality. The PAT framework (Section IV) defines PAT as “a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality”. This guidance aims to move towards real-time monitoring and control of CPPs that influence CQAs of biopharmaceuticals(FDA et al., 2004) (Claßen et al., 2017). PAT implementation can reduce regulatory risk and improve compliance with regulatory requirements by providing a detailed understanding of the manufacturing process and the factors affecting product quality.

PAT tools can be multivariate tools for the design, analysis, and control of processes, or a combination of these. They can also be used for both in-line and at-line measurements. In-line measurements are performed directly on the production line. In contrast, at-line measurements are performed on samples collected from the production line and analyzed within a small proximity of the production line and a short period after collection. Both methods have advantages and limitations, and the choice depends on the specific needs of the process. Univariate process analyzers have been used extensively in biopharmaceutical manufacturing, specifically mammalian cell-based processes, such as sensors for pH, temperature, dissolved oxygen (DO), and pressure. Chemical and biochemical properties such as nutrient and by-product concentrations, cell counts, and cell viability are often measured via laboratory or offline testing. Current analyzers for metabolite concentrations and cell counts are not applicable for in-line continuous monitoring of mammalian cell-based process-related metabolites, products, or cells for real-time decision-making. Even though new analyzers as PAT tools have emerged, the lack of reliable and robust in situ sensors with great sensitivity has hampered the application of PAT in the biopharmaceutical industry for advanced process controls.

Spectroscopic techniques have become the most popular for process monitoring. The most commonly explored spectroscopic techniques for bioprocess monitoring are ultraviolet-visible spectroscopy (UV-Vis), near-infrared spectroscopy (NIR), mid-infrared spectroscopy (MIR), fluorescence, and Raman spectroscopy. These techniques are based on the interaction of light with the molecules of interest. UV-Vis, NIR, and MIR

spectroscopy techniques detect the intensity of light absorbed and scattered by molecules. In contrast, fluorescence spectroscopy measures the intensity of light emitted by excited fluorophores, and Raman spectroscopy differentiates the inelastic scattering intensity of light by molecules (Claßen et al., 2017). These spectroscopic methods have the advantages of being non-invasive and non-destructive. They can monitor multiple analytes simultaneously in real-time without reagent or sampling requirements and do not interfere with cellular metabolism, making them suitable for monitoring bioprocesses. However, spectroscopic techniques encounter challenges such as the requirement of chemometric analysis through spectra interpretation, a low ratio of signal-to-noise, and measurement interference from cells and cell debris (Whelan et al., 2012). Raman has a weak water signal compared to other spectroscopic techniques such as NIR and MIR, and it has become a promising candidate for monitoring mammalian cell culture processes in media whose main component is water.

2.2.2 Raman Spectroscopy

The Raman spectroscopic technique is used to reveal characteristic vibrational, rotational, and other low-frequency movements in a molecule by filtering the scattered signal from a sample. The scattered Raman signal is generated when a sample is irradiated by a high-intensity monochromatic laser in the UV-visible region. Raman (inelastic) scattering occurs when a photon interacts with a molecule to induce a change in its vibrational energy. The scattered photon has a different energy level from the incident photon, the change in the energy level can be detected and assigned to a specific molecule. There are two types of light scattering: Rayleigh scattering (the

majority of the scattered light has the same frequency as the incidence light (ν_o)), and Raman scattering (a small portion of the scattered light has the frequencies of $\nu_o \pm \nu_m$, where ν_m is the vibrational or rotational frequency of a molecule). Raman scattering can be divided into Stokes and anti-Stokes types. Stokes Raman scattering occurs when the frequency of the Raman scattering signal is lower than that of the incidence light, which is typically acquired for molecular analysis, while anti-Stokes scattering occurs when the frequency of the Raman scattering signal is higher than that of the incident light, and the intensity of the anti-Stokes signal is generally much weaker than that of the Stokes signal(John R. Ferraro, 1994). Raman spectra are generated by plotting the intensity of the scattered signal at different wavelengths that correspond to unique chemical "fingerprints" of molecules in the sample. The fingerprint information includes the vibrational or rotational frequencies and intensities of the molecular bonds in the sample, and a linear correlation is expected between the intensity of the Raman signal and the concentration of the molecule in the sample.

2.2.3 Raman-Based PAT in Bioprocessing

Raman spectroscopy has been applied in biopharmaceutical processing, applications range from raw material testing for cell culture media, monitoring metabolites and amino acids, measuring cell density, and quantifying monoclonal antibody titer. Raman spectroscopy has been employed to identify, characterize, and quality test cell culture media components, allowing rapid in-house sample testing, tracking, and quality control. In-house testing of these raw materials reduces assay costs and their rapid release shortens the batch duration(Li et al., 2010). Real-time monitoring of cell culture

processes would eliminate the need for daily, offline, and manual measurements, leading to better process understanding and the development of more robust and consistent processes. Bhatia et al. assessed Raman-based calibration models to measure four amino acids including tyrosine, tryptophan, phenylalanine, and methionine, in cell culture media, which are essential in cell growth and product formation. They demonstrated the potential for quantifying these amino acids in cell culture media with the exception of methionine, which has Raman peaks masked by water (Bhatia et al., 2018). In-line Raman probes have been explored to monitor multiple parameters simultaneously (Abu-Absi et al., 2011), including the concentration of glutamine, glutamate, glucose, lactate, and ammonium, viable cell density, and total cell density during mammalian cell culture. Raman calibration models were built for each parameter and tested in a 500 L bioreactor. Generally, the predicted parameters from these calibration models followed the trend of offline reference values, and 10-30% errors were observed. Mehdizadeh et al. built generic calibration models for glucose, lactate, and viable cell density. The generic models could be applied to multiple products, different Chinese Hamster Ovarian (CHO) media, a variety of CHO cell lines, and scales ranging from laboratory to manufacturing. They used a broad data set from several mammalian cell culture batches and tested the resulting models in varying manufacturing conditions, processes, and scales to validate the robustness and scalability of the developed models. The models were demonstrated to perform well at multiple scales and conditions (Mehdizadeh et al., 2015). Raman spectroscopy has also been used to measure the monoclonal antibody concentration produced from CHO cells

(Yilmaz et al., 2020). The model built was able to estimate the concentrations of different monoclonal antibody isotypes with prediction errors of 0.2 (g/L). Therefore, Raman spectroscopy and chemometric analysis could be a viable and promising method as a PAT tool for multiple steps in biopharmaceutical processing.

2.2.4 Aim

This chapter aims to develop a methodology for building Raman-based calibration models for cell culture metabolites such as glucose, glutamine, lactate, glutamate, and ammonium. The established methodology is based on Partial Least Squares (PLS) Regression models for predicting metabolite concentrations via in-house developed Python software.

2.3 MATERIALS AND METHODS

2.3.1 Mammalian Cell Culture and Sampling for Model Calibration

A CHO S cell line was cultured in a 3L Applikon bioreactor (Applikon Inc., Delft, Netherlands). The Applikon's ez-Control was used to control pH, DO, temperature, and agitation at 7.0, 30%, 37 °C, and 140 rpm, respectively. All runs were conducted with a 1.5 L working volume and the ProCHO^{TM} 5 media was supplemented with 4.0 mM glutamine. Samples were withdrawn from the bioreactor at pre-determined time points throughout the culture period and spun down to prepare cell-free supernatant. The supernatant was frozen at -80 °C and stored for processing after the run was completed. The cell density and viability were measured once per day offline to monitor the health of the culture using a Vi-CELL XR (Beckman Coulter, IN, USA). Three runs were used to develop the calibration models for glucose, glutamine, glutamate, lactate and

ammonium: the first bioreactor run in a batch mode, while the second and third in a fed-batch mode with a concentrated glucose and glutamine feed.

2.3.2 Raman Spectra Acquisition

Samples were thawed and analyzed offline using a Roche Cedex Bio Analyzer (Roche CustomBiotech, Penzberg, Germany) to measure the concentration of glucose, glutamine, lactate, glutamate, and ammonium. These values were used as input to calibrate the Raman models. Spectral data was collected offline using 700 µL of the same sample as that analyzed by the Cedex Bio Analyzer. The Supsense Raman Bioanalyzer, coupled with a fiber-optic Raman probe with an optical excitation of 785 nm, was used to generate the Raman spectra. Each spectrum was acquired from five scans after 14 seconds of exposure. Raw spectra processing, including water spectra subtraction, was accomplished using ENLIGHTEN™ spectroscopy software (Wasatch Photonics, Logan, UT).

2.3.3 Spectra Preprocessing

Spectra were pre-processed using Principal Component Analysis (PCA) to determine the fingerprint region and identify outliers. Outliers were identified from the PCA models using Hotelling T^2 plots with a 95% confidence interval. Smoothing and baseline preprocessing methods were screened to reduce noises in the signal and remove baseline drifting. PCA modeling, fingerprint region identification, and pretreatment method screening were accomplished using Umetrics SIMCA (Ver. 15.0.2).

2.3.4 PLS Model Calibration

Once a preprocessing method was established, Python software was developed in-house to preprocess and build PLS calibration models. The complete dataset in a CSV format was imported. Each dataset only included the established fingerprint region of the spectra. A preprocessing method was compared and selected. The optimal number of components for the PLS model was determined by minimizing the mean squared errors. The optimal number of components was implemented in the PLS model using 80% of the preprocessed data for a given metabolite. The model was then cross-validated with the remaining 20% of the dataset, coefficients of determination(R^2) for calibration (R^2C), cross-validation (R^2CV), root mean square error of calibration (RMSEC), and root mean square error of cross-validation (RMSECV) were calculated before the model was completed.

2.4 RESULTS & DISCUSSION

2.4.1 Spectra Pre-processing

The fingerprint region for the calibration spectra is identified to be between 350 and 1750 cm^{-1} (Figure 2A), as no significant differences between samples are observed outside this region. Each data set includes the concentration of each metabolite obtained from the Cedex Bio Analyzer and its corresponding spectrum data. Datasets for each metabolite (glucose, glutamine, lactate, glutamate, and ammonium) are used to generate the PCA models for them. PCA, a dimension-reduction method, reduces datasets with a broad range of wavelengths to a dataset with a lower dimension. Dimensions are reduced while a high variability is maintained, resulting in a compressed

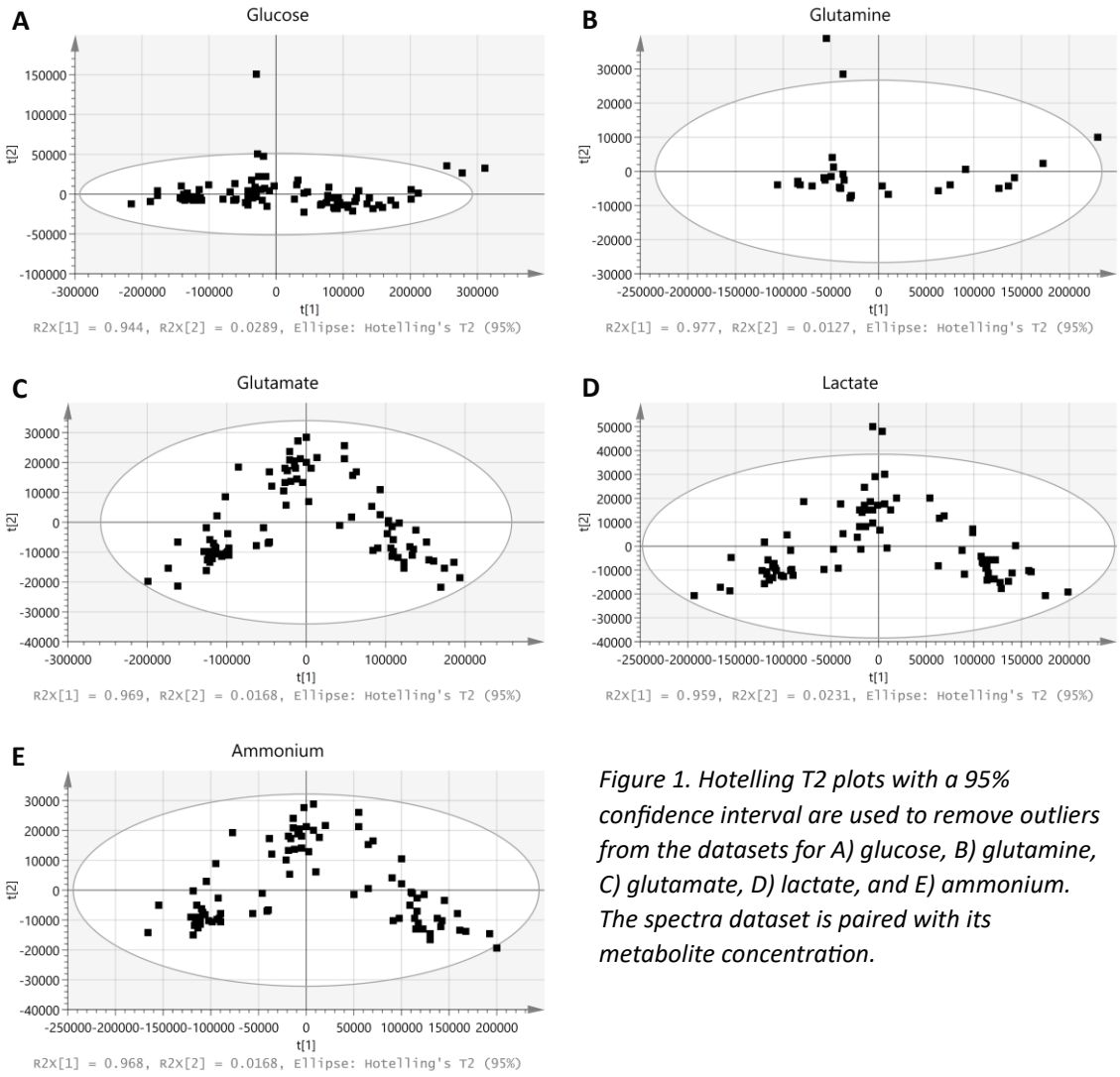

Figure 1. Hotelling T2 plots with a 95% confidence interval are used to remove outliers from the datasets for A) glucose, B) glutamine, C) glutamate, D) lactate, and E) ammonium. The spectra dataset is paired with its metabolite concentration.

version of the original dataset for visualization and processing. The resulting PCA models were used to identify outliers in each dataset. The resulting Hotelling T^2 plots with a 95% confidence interval for each dataset are shown in Figure 1. Any data points outside of the 95% confidence interval are removed. The new spectra are then subjected to additional preprocessing methods (Figure 2B). Several smoothing preprocessing methods are screened to reduce noises in the Raman signal and remove the baseline drifting. It is found that the Savitzky-Golay smoothing method by applying a quadratic

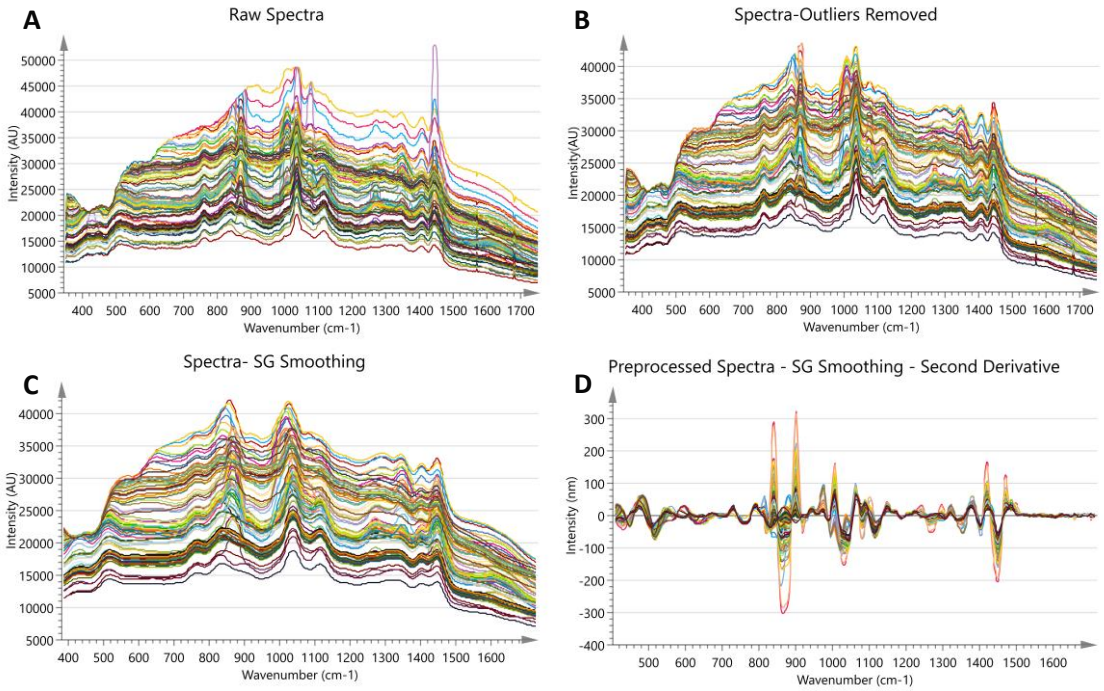

Figure 2. Spectra preprocessing before applying the PLS method. A) The fingerprint region of the spectra is identified between 350 and 1750 cm^{-1}. B) Spectra after removal of outliers from each metabolite data set. C) Spectra after applying the Savitzky-Golay smoothing method to reduce the signal-to-noise ratio. D) Spectra after implementing a second derivative to remove the baseline drift and improve the peak resolution. .Raw spectra were generated from samples withdrawn from bench-scale bioreactor runs in a batch/fed-batch mode. Samples were taken at pre-determined time points during culture periods ranging from 0 to 150 hours.

polynomial fitting over 25 points (Figure 2C) is the best to reduce the signal-to-noise ratio. Methods such as offset correction, row-center correction, moving average and derivatives application are screened for adjusting the baseline. A second derivative (Figure 2D) is selected for baseline correction, and the other methods do not result in drift removal for the entire dataset. The second derivative of each spectrum allows retaining the spectral information of signal peaks, while helping normalize the data and remove drifts in the baseline.

2.4.2 PLS Model Construction

PLS-based predictive models for the cell culture metabolites, including glucose, glutamine, lactate, glutamate, and ammonium, are built from pre-processed spectra and their corresponding metabolite concentrations. PLS calibration models are developed using the steps outlined in the flowchart in Figure 3. The dataset is imported and preprocessed before building the PLS predictive models. As described in the flow diagram, the optimal number of principal components for the PLS model is then determined for the pre-processed dataset. This is obtained by minimizing the MSE, and a minimal MSE is often correlated with better predicting ability of the PLS model. Up to 25 principal components are tested and graphed to display the MSEs against each number of principal components. Figure 4 shows the number of principal components versus the MSE for glucose, glutamine, lactate, glutamate, and ammonium. The number of components increases, and a broad level of variability in the spectra covers, therefore, the MSE is often reduced. However, when more components are included in the model, the chance of overfitting the model is elevated. For example, similar MSE is seen for 3,4 and 5 components for glutamine. Sacrificing a slight reduction in the MSE and building a model with three components may lead to a more general model with better predictability. The red cross on each graph indicates the lowest MSE for each data set

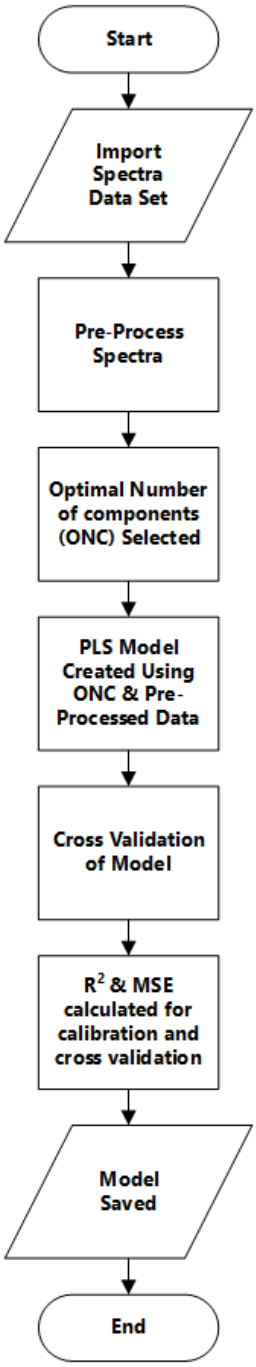

Figure 3. Flow chart for building Raman based PLS calibration models.

and the number of principal components is obtained for the models for these metabolites. 80% of the preprocessed calibration data is then used to generate the PLS model for each metabolite, while the remaining portion of the calibration data is used to test the model. Cross-validation plots of the measured values versus the model-predicted values are shown in Figure 5. The coefficients of determination(R^2) for calibration (R^2C) and cross-validation (R^2CV), the root mean square errors of calibration (RMSEC), and the root mean square errors of cross-validation (RMSECV) are calculated for each model, and they are shown in Table 1.

R^2 measures the level of variability between the measured values and the predicted values by a model. This value is one or less, and a value of one means the predicted values are completely in alignment with the measured values. R^2C and R^2CV are high for all models for these metabolites, indicating each model could accurately predict the metabolite concentration using the acquired Raman spectra. RMSE measures the total error between the predicted values and the actual measured values. A lower (closer to zero) RMSE indicates a lower error. RMSEC and RMSECV calculated for each model are comparable or better when compared to the reported values for similar models(Abu-Absi et al., 2011). R^2 and RMSE values for cross-validation are lower than those for building

the model, which is expected because cross-validation introduces new data to the model. Higher RMSECV values are observed for glutamine and ammonium, which could result from overfitting. A small calibration dataset or clustered data may also lead to a less general model. Nevertheless, the Raman-based PLS model for each metabolite

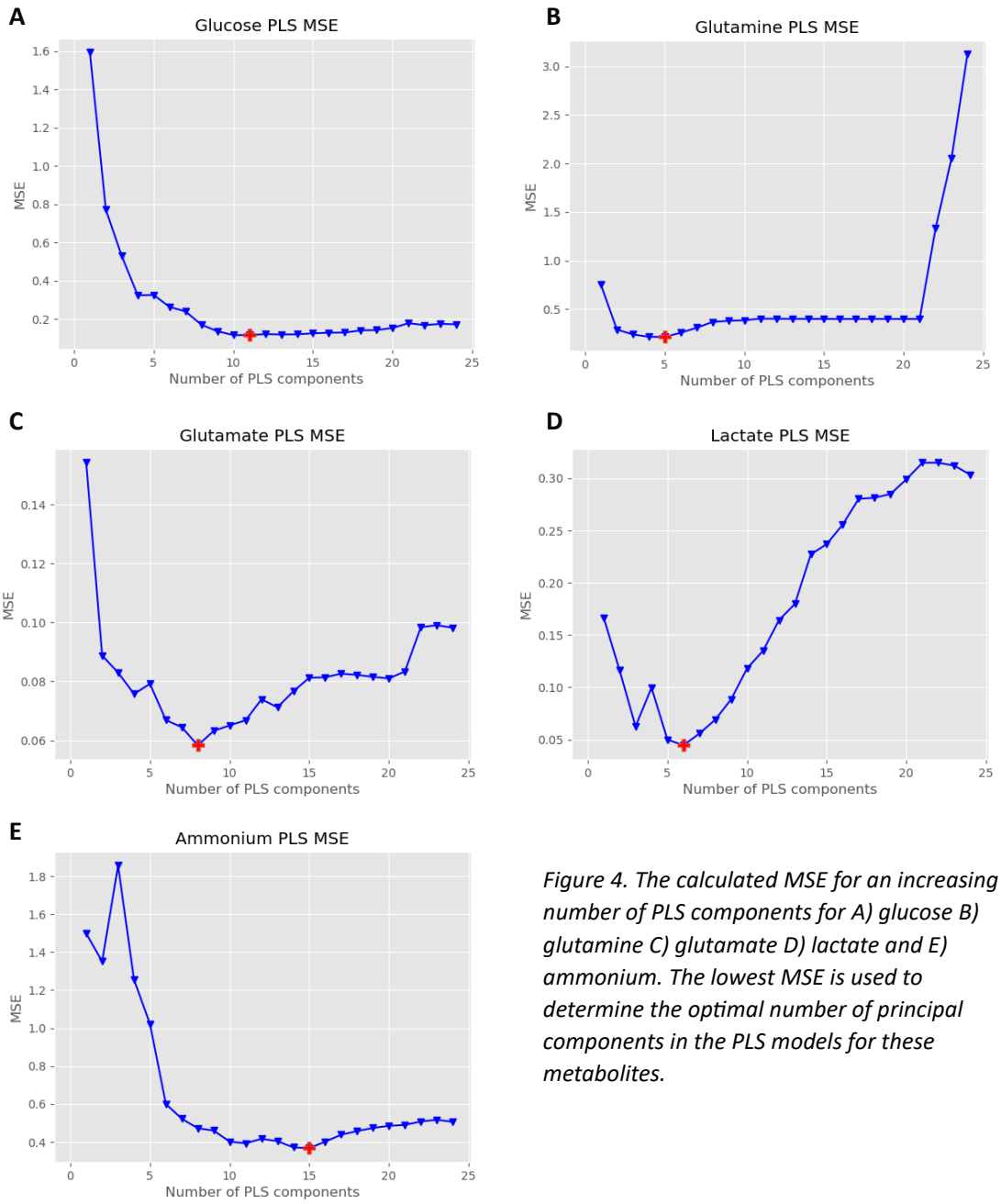

Figure 4. The calculated MSE for an increasing number of PLS components for A) glucose B) glutamine C) glutamate D) lactate and E) ammonium. The lowest MSE is used to determine the optimal number of principal components in the PLS models for these metabolites.

could be applied to predict the metabolite concentration during the cell culture process within a high R^2 value and a low RMSE value.

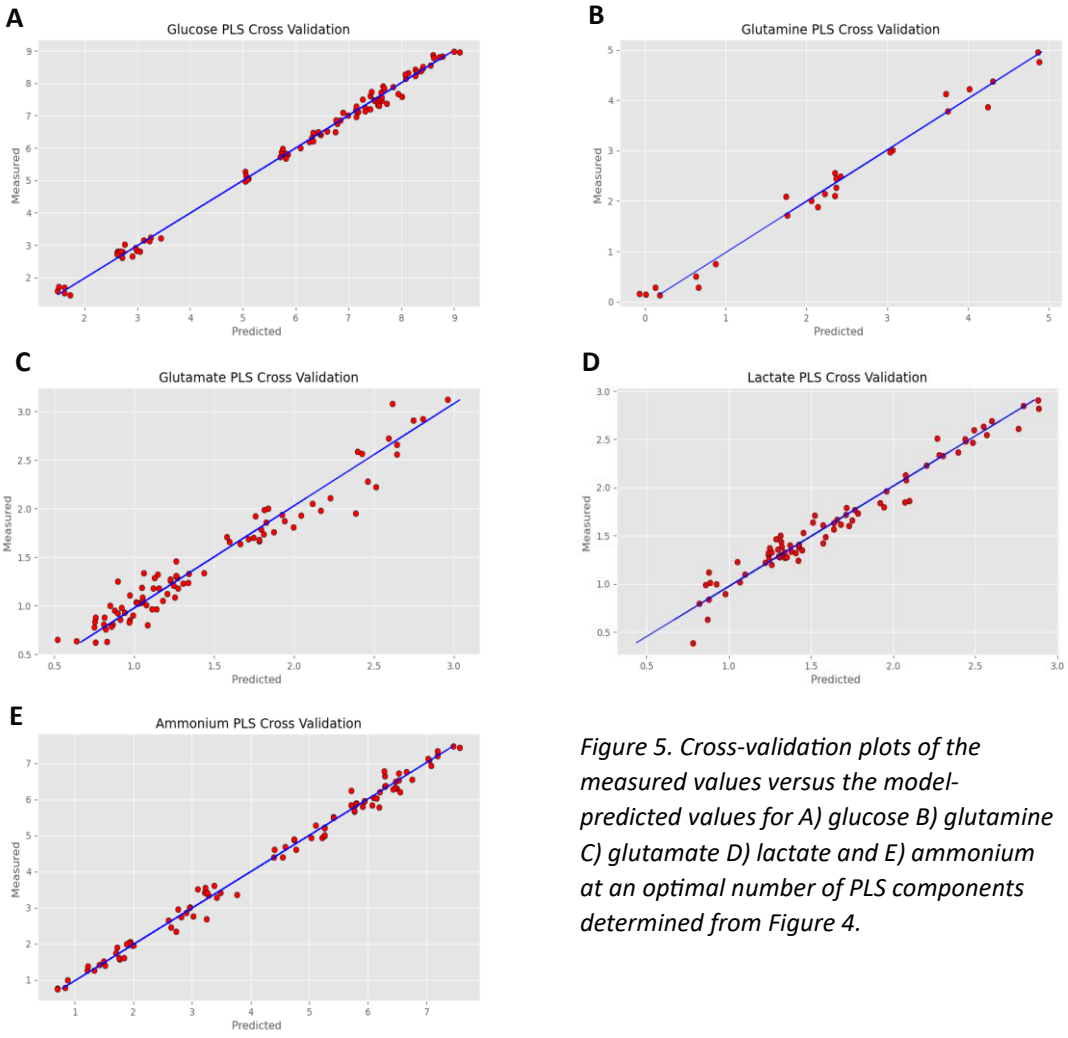

Figure 5. Cross-validation plots of the measured values versus the model-predicted values for A) glucose B) glutamine C) glutamate D) lactate and E) ammonium at an optimal number of PLS components determined from Figure 4.

Table 1. Summary results of Raman-based PLS calibration models

Metabolite	Range	N	Components	R^2C	R^2CV	RMSEC	RMSECV
Glucose (g/L)	1.3-9.0	93	11	0.995	0.975	0.023	0.115
Glutamine (mmol/L)	0.2-5.0	30	5	0.983	0.905	0.038	0.213
Glutamate (mmol/L)	0.6-3.0	88	8	0.949	0.848	0.019	0.058
Lactate (g/L)	0.4-3.0	80	6	0.961	0.858	0.012	0.045
Ammonium (mmol/L)	0.7-7.5	96	15	0.991	0.909	0.036	0.367

2.5 CONCLUSION

Raman-based PAT has the potential to be used in the pharmaceutical industry to ensure that the manufacturing processes for biological or small-molecular drugs are efficient, reliable, and reproducible. By monitoring the process in real-time, PAT can help identify and correct potential issues before they impact product quality, reducing the risk of product recalls and improving patient safety. A methodology for building Raman-based calibration models for cell culture metabolites has been developed in this chapter. The preprocessing methods for the calibration dataset include reducing the acquired wavelength range of each raw spectrum to the fingerprint region, applying Savitzky-Golay smoothing to improve the signal-to-noise ratio, and selecting a second derivative

for baseline correction. The Python-based in-house software is capable of generating PLS calibration models for each metabolite, including glucose, glutamine, glutamate, lactate, and ammonium, performing cross-validation for these models, determining the calibration performance by calculating the coefficient of determination(R^2) for calibration (R^2C), the coefficient of determination for cross-validation (R^2CV), the root mean square error of calibration (RMSEC), and the root mean square error of cross-validation (RMSECV). The input for the models is a comma-separated-value file with the reference metabolite concentrations and the spectra at the corresponding concentrations. The high R^2 and low RMSE values for the models suggest that they could be applied to predict the metabolite concentration from real-time spectra. In-house Python-based software for these models offers the flexibility of its full integration into the feedback control software for driving external pumps or other instruments, thus providing real-time monitoring/control of cell culture metabolites during the cell culture process.

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3 Raman-Based Control System Applied to a Fed-Batch Process

3.1 ABSTRACT

Fed-Batch is the most common mode of operation in the biopharmaceutical processing industry. Benefits of this cultivation mode include a high cell density, an extended culture period, and enhanced productivity compared to a batch process while maintaining relatively low operational cost and a low level of operational complexity compared to a perfusion process. Bolus feeding, supplying a great amount of concentrated feed at a single time point, is the most used strategy. Challenges for bolus feeding include nutrient imbalance, sudden pH changes, and heterogeneous cell culture conditions that can affect cell viability, productivity, and product quality. An excessive feed of glucose and glutamine can lead to an increased level of toxic byproducts such as lactate and ammonia, while underfeeding or depletion of critical nutrients for a period may lead to decreased cell viability, lower productivity, and reduced product quality. Control of glucose and glutamine at a constant concentration has been demonstrated to reduce the production of toxic byproducts and improve product quality. A Raman-based monitoring and control system is developed in-house for monitoring the concentration of metabolites in a fed-batch process, including glucose, glutamine, glutamate, lactate, and ammonium. The predicted concentrations of glucose and glutamine are input into the control system to control the feed of two nutrients. Glucose and glutamine are maintained within 0.5 g/L and 0.35 mmol/L of their setpoints, respectively. The proof-of-concept study has demonstrated the potential of this Python-

driven system for real-time monitoring of the metabolites and automated control of critical nutrient concentrations in a fed-batch process.

3.2 BACKGROUND & INTRODUCTION

3.2.1 Fed-Batch Processing

Generally, there are three cultivation modes of operation in bioprocessing: batch, fed-batch (semi-batch), and continuous. A batch operation introduces all necessary components, such as media and inoculum, at the beginning of the run, and no additional components are introduced throughout the culture period. In a batch process, the product produced is only harvested at the end of the run. Advantages of this mode of operation include a lower and fixed cost of operation and operation simplicity. Typical optimization in this mode focuses on the initial media composition and running process parameters such as pH, temperature, and dissolved oxygen, limiting optimizing opportunities. A fed-batch operation starts with a batch phase, then at least one feed stream containing nutrients, precursors, inducers, or minerals is introduced periodically or continuously to the cell culture vessel. This mode of operation has the advantages of reaching a higher cell density and extending the culture time, leading to an increase in the product yield. Similar to a batch process, the product is harvested at the end of the run. A continuous process, a perfusion process, allows the continuous addition of nutrients or other components and removal of wastes and products throughout the culture period, while cells are retained within the culture vessel. Continuous cultivation can significantly increase volumetric productivity with challenges of run complexity and a high operational cost. (Henry C. Lim & Hwa Sung Shin, 2013)(Kadic & Heindel, 2014)

The goal of fed-batch operation is to regulate the feed rate of nutrient components to prevent nutrient depletion and extend the culture period to prolong product generation. A higher cell density, a longer culture period, and increased productivity are often achieved from a fed-batch operation compared to batch operation, while there is a smaller operational cost and a lower level of run complexity compared to a perfusion process, consolidating that fed-batch is the most common mode of operation in the current biopharmaceutical industry (Yang & Sha, n.d.). Bolus feeding is the most used strategy, where a large amount of the feed media is added at a single time point. Typically, bolus feeding happens daily after offline metabolite sampling. The advantages of bolus feeding are an infrequent feeding schedule and a simple feeding process, which can lead to time and resource savings and reduce the chance of contamination. However, bolus feeding of highly concentrated components into the culture vessel may lead to nutrient imbalances, pH changes, and other stress-causing conditions that can affect cell performance and product quality. The nutrient and metabolite imbalance has been one of the main challenges in fed-batch bioprocessing. Excessive feeding of glucose and/or glutamine can produce an increased level of toxic byproducts such as lactate and ammonia while underfeeding of them or a long period for a low nutrient concentration can lead to decreased cell viability, low productivity, and a severe impact on the product quality.

The concentration of critical nutrients (e.g. glucose and glutamine) and by-products (e.g. lactate and ammonia) have been known to have an impact on cell culture longevity, product quality (Berry et al., 2016), and titer production. For example, glucose and

ammonia affect the glycosylation patterns of proteins of interest (Whelan et al., 2012), and high glucose increases the glycation of produced proteins (Yuk et al., 2011). Therefore, it has been explored in biopharmaceutical operations to monitor and control the concentration of critical nutrients and by-products in the cell culture media. Improvements in the product quality in CHO cell culture were realized through glucose control at 2.5 g/L to reduce the glycation of produced proteins (Berry et al., 2016). Other benefits of controlling the feeding rate of critical nutrients include improving cell production performance, extending the culture period, and maintaining high viability by reducing the excessive buildup of toxic byproducts and maintaining consistent nutritional conditions throughout the process. Controlled feeding may also lead to an improvement in productivity and a reduction in operation cost as these critical nutrients are added as needed and not at a pre-set frequency. Controlled feeding is achieved by monitoring the critical nutrient concentration and providing feedback control on the feeding pump rate of the nutrient throughout the culture period. Therefore, online sensors for real-time measurements of critical nutrient concentration are needed for a controlled feeding process.

3.2.2 Controlled Fed-Batch

Controlling key metabolites such as glucose and glutamine through optimized feeding strategies in mammalian cell culture has shown benefits such as increased cell growth and productivity (Mehdizadeh et al., 2015), reduced glycation (Berry et al., 2016), and more consistent product quality. A few PAT tools coupled with process control software have been developed to enable maintaining the critical nutrient concentration at a pre-

determined setpoint. The PAT tools can be used to measure the concentration of nutrients directly or provide measurable values which could be directly or indirectly correlated with the nutrient concentration. The in-situ glucose sensor from CITSens Bio, for example, is developed for mammalian cell culture for real-time monitoring of glucose and lactate concentrations, and a micro pump is activated when the glucose is below the setpoint. At-line PAT tools, such as an HPLC system, can be installed within close proximity of the culture vessel to measure the concentration of critical nutrients, and the values are then used to control the feed pumps of these nutrients. Kurokawa et al. used an at-line HPLC system to maintain lower concentrations of glucose and glutamine at 0.2 g/L and 0.1 g/L, respectively, for a hybridoma cell culture process, resulting in a 2-fold increase in viable cell density and monoclonal antibody titer compared to that at a higher glucose and glutamine concentration of 2.0 g/L and 0.6 g/L, respectively(Kurokawa et al., 1994). An online predictor for glucose concentration was developed and implemented based on a relationship between the oxygen transfer rate and the glucose consumption rate, the fluctuation of the glucose concentration could be mitigated during the CHO cell culture process (Zhou et al., 1995) (Goldrick et al., 2018). A pH-based method was explored to control the glucose concentration and reduce the effect of accumulated lactate in the cell culture media. An increase in pH indicates lactic acid is consumed by cells since the glucose supply is below the threshold in the media. An increment in the pH triggered the addition of a small amount of glucose to the bioreactor, thus preventing bolus feed of highly concentrated glucose (Gagnon et al., 2011). Another commercially available dissolved oxygen biosensor was applied to

develop a model for online monitoring of glucose concentration(Tric et al., 2017). The readings from this sensor were used to control the feed of glucose to the bioreactor(Lederle et al., 2021). Raman spectroscopy-based calibration models can be used to real-time predict the concentration of critical nutrients. Raman sensors were employed to be integrated into a control system for maintaining a constant glucose concentration in the bioreactor. Berry et al. developed a Raman-based automated process enabling control of glucose concentration at a setpoint of 0.24 g/L. A commercially available Raman instrument was used, and the prediction model was built from the built-in software package for the instrument. The product quality, including glycation, was found to be significantly improved compared to that of bolus glucose feeding. (Berry et al., 2016)

3.2.3 Aim

Previous studies have demonstrated the benefits of controlling cell culture feeds such as glucose and glutamine. While one or two metabolites are controlled during the fed-batch process, other metabolites are still monitored via offline sampling. pH or DO sensors are often used to maintain a constant setpoint of pH (e.g., 7.0) and DO (e.g., 40%), respectively. While these sensors are used to monitor the glucose concentration, the key process parameters such as pH and DO cannot be controlled. In this chapter, a Raman-based monitoring and control system was developed for monitoring the concentration of glucose, glutamine, glutamate, lactate, and ammonium and controlling the glucose and glutamine concentrations in a fed-batch process. The Raman-based calibration models were coded in Python. The code for monitoring metabolites via

analyzing their Raman spectra was seamlessly integrated into the Python code for controlling the pump speed to feed glucose and glutamine. The in-house developed system is very flexible, which can be tuned for bench-scale instruments to realize a constant nutrient concentration in a fed-batch process.

3.3 MATERIALS & METHODS

3.3.1 Raman-Spectroscopic Calibration Models

Cell culture samples were withdrawn from a bioreactor using a CHO-S cell line with ProCHO^{TM} 5 media (Sartorius Stedim Biotech, Göttingen, Germany). Samples were centrifuged to remove cells and cell debris. 0.7 mL of the cell-free supernatant was transferred to a 3.0 mL quartz cuvette for Raman analysis. A Supsense Raman Bioanalyzer, coupled with a fiber-optic Raman probe at an optical excitation wavelength of 785 nm, was employed to irradiate the sample and collect the scattered Raman signal. Each spectrum was acquired by co-adding five scans after 14 seconds of exposure. Water spectra subtraction, co-addition, and acquisition were accomplished using ENLIGHTEN™ spectroscopy software (Wasatch Photonics, Logan, UT). Meanwhile, the samples were analyzed via a Roche Cedex Bio Analyzer (Roche CustomBiotech, Penzberg, Germany) to obtain the reference concentrations of glucose, glutamine, glutamate, lactate, and ammonium. Both the Raman spectra and reference values were used to build separate PLS calibration models for glucose, glutamine, glutamate, lactate, and ammonium, and the detailed procedure was described in Chapter 2.

3.3.2 Development of a Raman-based Monitoring and Control System

A Raman spectrum was generated from a cell culture sample in a similar procedure to that in Section 3.3.1. The Raman spectrum was fed into the PLS calibration models to predict the concentrations of glucose, glutamine, glutamate, lactate, and ammonium. A Mettler Toledo SB8001 scale (Mettler Toledo, Columbus, OH) was used to monitor the bioreactor weight. The bioreactor weight, the concentrations of glucose and glutamine from the PSL models, and the feed concentrations of glucose and glutamine were used to estimate the volumes of concentrated glucose and glutamine feed needed to reach the pre-set setpoints for glucose and glutamine in the bioreactor. The volumes of concentrated glucose and glutamine were delivered by two New Era NE-9000G peristaltic pumps (New Era, Farmingdale, NY) to the bioreactor within a specified duration.

3.3.3 Fed-Batch Operation

The Raman-based monitoring and control system was tested in a fed-batch process using a 3.0 L glass Applikon bioreactor with a working volume of 1.5 L (Applikon Inc., Delft, Netherlands). The Applikon ez-Control was used to control pH, dissolved oxygen, temperature, and agitation at 7, 30%, 37 °C, and 140 rpm, respectively. A CHO-S cell line was cultured in the ProCHO^{TM} 5 media. The glucose and glutamine setpoints in the bioreactor were set at 5 g/L and 1 mmol/L, respectively. The concentrations of glucose and glutamine in the feed bottles were 400 g/L and 200 mmol/L, respectively. The Roche Cedex Bio Analyzer was used for offline measurements of glucose, glutamine, glutamate, lactate, and ammonium. In addition, viable cell density (VCD) and viability of

cell culture samples were measured offline to monitor the health of the cells using a Vi-CELL XR (Beckman Coulter, IN, USA). The bioreactor was sampled every four hours starting at 24 h post-inoculation. The Raman-based monitoring and control system was applied to control the glucose and glutamine concentrations at their setpoints. The bioreactor run lasted 76 h.

3.4 RESULTS & DISCUSSION

3.4.1 Design and Implementation of the Raman-Based Monitoring and Control System

The Raman-based calibration models were combined with an in-house developed system to monitor the concentrations of glucose, glutamine, glutamate, lactate, and ammonium in the cell culture media and control the glucose and glutamine concentration in a fed-batch process. The flowchart in Figure 1 describes the software (in Python) used for monitoring and controlling cell culture metabolites. The software is initiated with inputs, including the starting working volume of the bioreactor, the file name for the predicted concentrations of metabolites, the file name for feed volumes dispensed and the bioreactor weight, and the glucose and glutamine feed concentrations and their pre-determined setpoints. Once the software is initiated, the software monitors an “active” folder where spectra are saved after they are generated from the ENLIGHTEN™ spectroscopy software. Once a file is detected in the “active” folder, the software imports the raw spectra as a CSV file. The spectrum is pre-processed by removing any signal outside the fingerprint region (350-1750 cm^{-1}), smoothing to reduce the signal-to-noise ratio by implementing the Savitzky-Golay smoothing

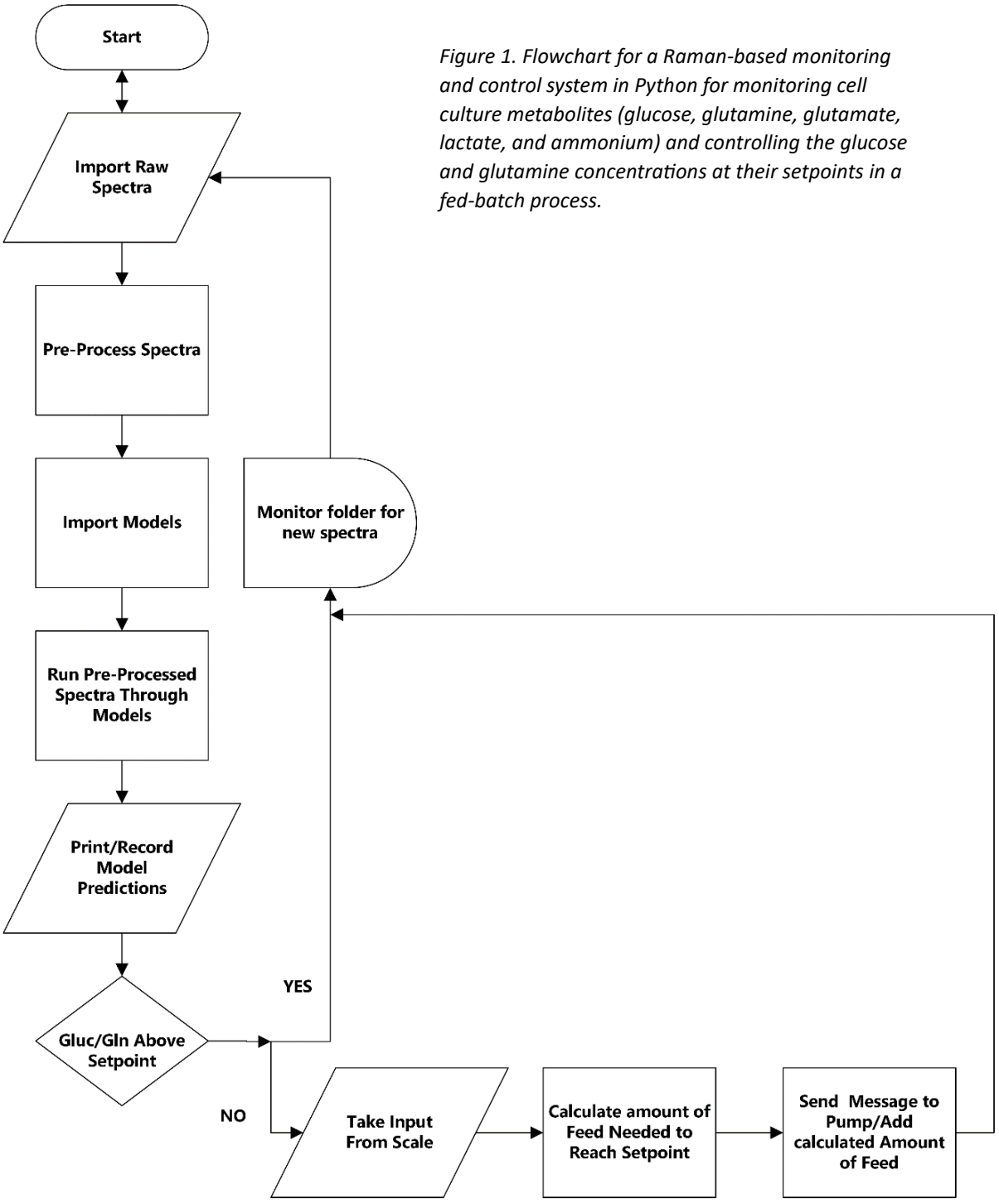

Figure 1. Flowchart for a Raman-based monitoring and control system in Python for monitoring cell culture metabolites (glucose, glutamine, glutamate, lactate, and ammonium) and controlling the glucose and glutamine concentrations at their setpoints in a fed-batch process.

technique and applying a second- derivative for drift removal. The Raman-based calibration models for glucose, glutamine, glutamate, lactate, and ammonium imported are activated. The pre-processed spectrum is fed into each model to predict the

concentrations of these metabolites. Predicted values for each metabolite are printed on the screen and saved to a file with a timestamp. The predicted glucose and glutamine values are checked against the preset setpoints. If the concentration is above the setpoint, a message is printed on the screen stating that the metabolite is above the setpoint and no pump action is initiated. The software monitors the "staging" folder to receive the next saved spectrum. If the metabolite concentration is below the setpoint, the volume of concentrated glucose or glutamine to reach the setpoint in the bioreactor is calculated from the process information, including the current volume in the bioreactor, the glucose and glutamine feed concentrations, and the setpoints of glucose and glutamine. The software reads the input from a scale that

monitors the bioreactor weight to determine the updated working volume in the bioreactor. The weight of the bioreactor, the calculated bioreactor volume, the calculated feed volume for glucose and/or glutamine, and a timestamp are saved to a file. The arrival of the new file triggers the activation of the pump for glucose or glutamine or both pumps, and the calculated feed volume is added to the bioreactor within a few seconds. The raw spectra file is moved from the "active" folder to an "archive" folder, and the software continues monitoring the "active" folder. The hardware required for this system and layout is described in Figure 2. The system is comprised of two New Era NE-9000G peristaltic pumps, a Mettler Toledo SB8001 balance, the Supsense Raman Bioanalyzer, and a computer.

3.4.2 Application of The Raman-based System to a Fed-Batch Process

The Raman-based calibration models for the fed-batch process are built in a similar way to those in Chapter 2, while the reference values are obtained from the Cedex. The model parameters are shown in Table 1. The model performance parameters, including R^2C, R^2CV, RMSEC, and RMSECV suggest these models could have a strong predictive power for the spectra acquired during the fed-batch process.

Table 2. Summary of Raman-Based Calibration Models

Metabolite	Range	#Components	R^2C	R^2CV	RMSEC	RMSECV
Glucose (g/L)	0.5-8	7	0.997	0.952	0.014	0.265
Glutamine (mmol/L)	0.2-5.0	3	0.965	0.88	0.092	0.316
Glutamate (mmol/L)	0.6-1.3	5	0.984	0.88	0.001	0.008
Lactate (g/L)	0.2-2.5	5	0.992	0.926	0.006	0.052
Ammonium (mmol/L)	0.8-6	3	0.967	0.904	0.15	0.436

The VCD and the viability were monitored during the 76-hour bioreactor run, and they are shown in Figure 3. The VCD and viability for a batch operation mode as a control are also included in Figure 3. Both VCD and percent viability are very similar between the batch and fed-batch operations. Both runs result in a final 5.2-fold increase in the cell density at the end of 76 culture hours. The initial cell growth rate for both runs is equivalent since the nutrients are adequate for cell growth, maintenance, and product formation. When nutrients in the batch operation become depleted more significantly than those for fed-batch operation, cells start to grow much slower. It is noted that the fed-batch process was inoculated at a higher density so that the set points for glucose and glutamine could be reached earlier in the run.

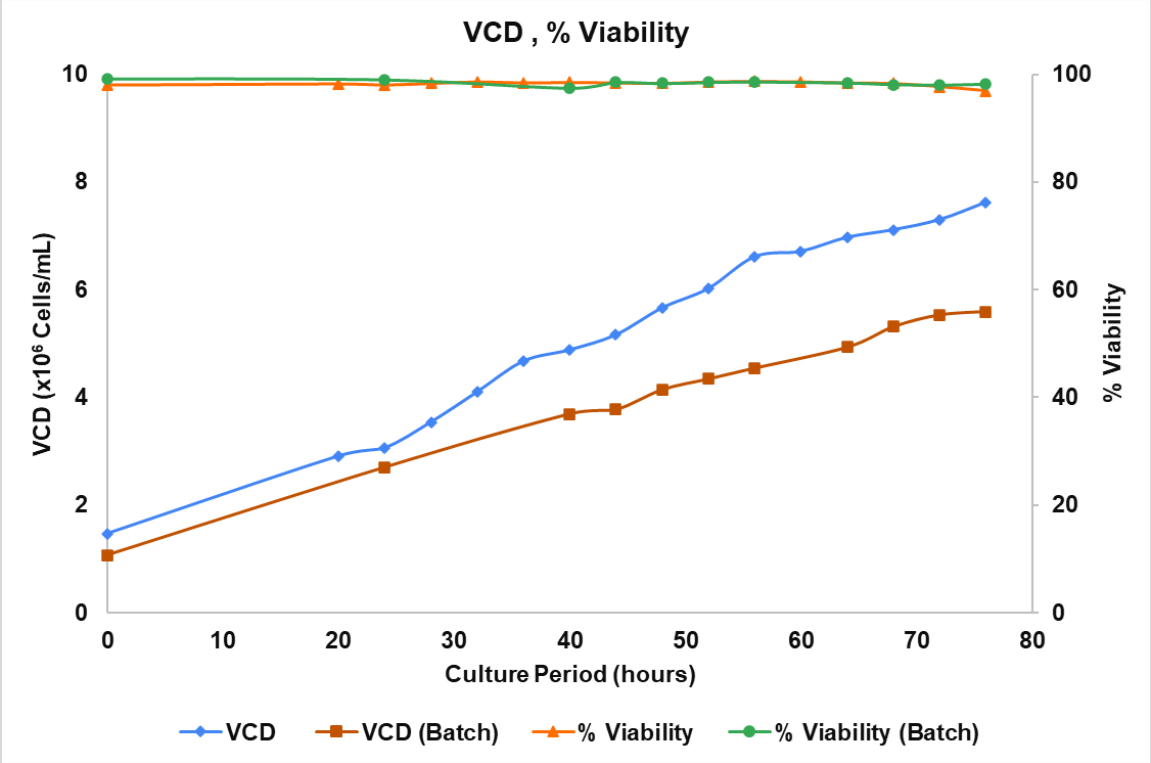

Figure 3. VCD and percent viability for a fed-batch bioreactor run with a Raman-based monitoring and control system. A batch bioreactor run and

The Raman-based system was used to monitor the glucose and glutamine concentrations and feedback-control them at 5.0 g/L and 1.0 mmol/L, respectively. The Cedex Bio Analyzer reference values and predicted values from the Raman spectra via the calibration models are shown in Figure 4 for glucose (A) and glutamine (B). The glucose and glutamine concentrations for the batch operation measured via the Cedex are also included in Figure 4. In the batch process, the glucose concentration falls below 5.0 g/L after 56 h. However, the Raman-based control system could maintain the glucose concentration within 0.5 g/L of the setpoint (5.0 g/L) in the fed-batch process from the culture hour of 44 h through the end of the run. A correction factor of multiplying by 0.6 was applied to the glutamine prediction model to compensate for glutamine

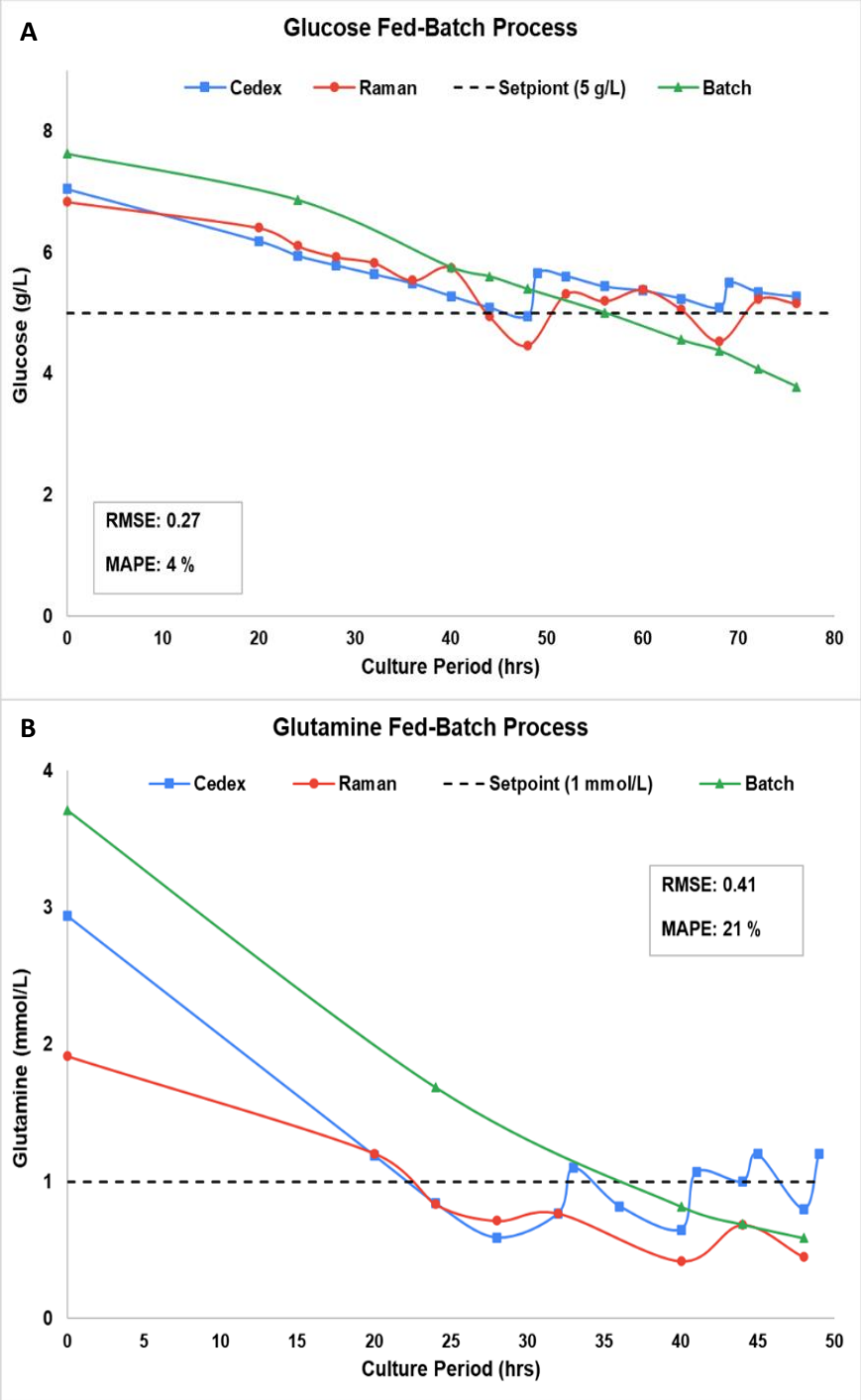

Figure 4. A Raman-based monitoring and control system to monitor glucose (A) and glutamine(B) concentrations throughout the culture period and control glucose at 5.0 g/L from the cell culture hour of 20 to 76 and glutamine at 1.0 mmol/L from the cell culture hour of 20 to 59. Samples were withdrawn from a 3L Applikon bioreactor every four hours in fed-batch mode. A decline in the glucose or glutamine concentration below the setpoint triggers the feed pump to supplement concentrated glucose or glutamine.

over predictions observed from a small dataset obtained from shake flask samples (data not shown). In the batch process, the glutamine concentration drops below 1.0 mmol/L after 36 h, which is slightly later than that in the fed-batch process, since the initial glutamine concentration is higher and the inoculation cell density is lower for the batch process. Control of the glutamine concentration was implemented between the culture hour of 32 and 49 h. The glutamine concentration is controlled within 0.35 mmol/L of the setpoint (1.0 mmol/L). The glutamine control is terminated at 49 h to reduce the glutamine concentration in the bioreactor to strengthen the glucose consumption so that the glucose concentration can reach the setpoint and the glucose control can be realized.

The Cedex Bio Analyzer was used for obtaining reference values for glucose and glutamine. These values were used to determine the predictive accuracy of the Raman-based calibration models. Both the root mean square error (RMSE) and the mean absolute percentage error (MAPE) were calculated between the reference values from the Cedex Bio Analyzer and the predictive values from Raman calibration models. The RMSE measures the total error between the model-predicted values and the reference values; a lower RMSE indicates a lower error, and an RMSE of zero means a perfect prediction power of the calibration model. The MAPE, the absolute percentage error between the model-predicted values and the reference measured values, reveals the average deviation between two sets of values. The RMSE and MAPE calculated for glucose are 0.27 g/L and 4%, respectively, indicating that the predicted values agree with the reference values obtained from offline measurements. An RMSE of 0.27 g/L suggests

the model is not overfitted, and no clustering data contributes to the predicting bias as the RMSE for cross-validation of this calibration model is 0.265 g/L, very close to the value of 0.27 g/L for the fed-batch control process. The RMSE of prediction has been reported to be around 0.28 ±0.09 g/L, which is estimated from the reported RMSE values in Table 3 of the review paper by Esmonde-White et al. (the value from reference 80 in that table is not included since the RMSE is up to 0.936 g/L). The RMSE from the model built for glucose in this study is very close to the mean value from the literature, suggesting that the model could accurately forecast the glucose concentration from the acquired Raman spectra of the samples in a fed-batch process. A MAPE of 4% further supports that the predicted values are within 4% of the measured values. Therefore, the calibration model built has great predictive power for the concentration of glucose in a cell culture vessel.

The calculated RMSE and MAPE calculated for glutamine are 0.41 mmol/L and 21%. The RMSE of prediction is very close to 0.42-0.44 mmol/L reported by Li et al. (2018). However, a MAPE of 21% indicates that there is great variability between the predicted values and the measured values for glutamine. This could be due to variations from the reference values measured by Cedex as well as interference signals in the Raman spectra. The accuracy specification for the analyzer is around 15%, which may contribute to a high variation of MAPE of the calibration model. Since glutamine shares similar carboxyl and amino groups with other amino acids and proteins, the vibrations in the molecular bonds of glutamine may be interfered with those from other amino acids and

proteins, resulting in a high prediction error. The interference could be diminished by increasing the data points for the calibration model.

Raman-based calibration models were also built and tested for monitoring glutamate, lactate, and ammonia. Figure 5 shows the comparison of the model-predicted values for glutamate (A), lactate (B), and ammonia (C) with the reference values obtained offline (Cedex). The Cedex measurement values for these metabolites for a control batch operation are also shown in Figure 5, and. Both batch and fed-batch operations result in a similar trend for glutamate and ammonia production because the batch operation starts at a higher glutamine concentration, while the fed-batch operation receives glutamine feed during the glutamine control period. The calculated RMSE and MAPE

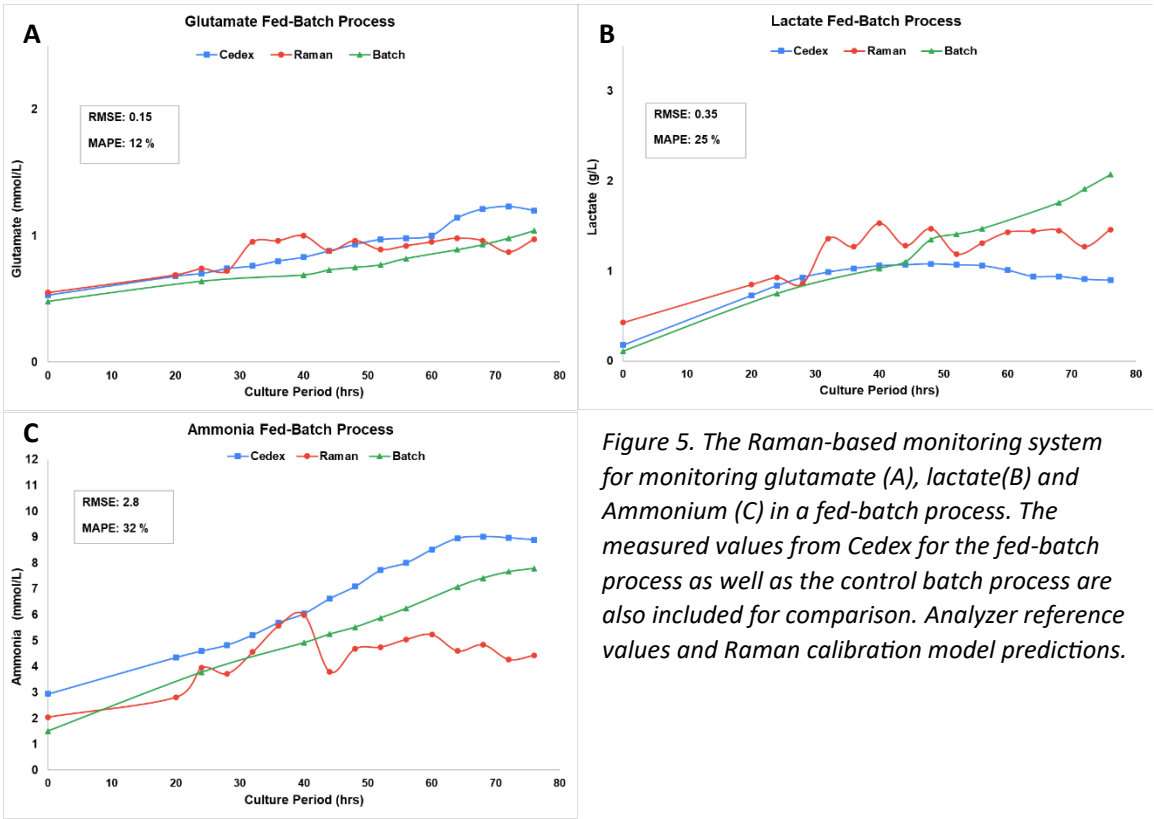

Figure 5. The Raman-based monitoring system for monitoring glutamate (A), lactate(B) and Ammonium (C) in a fed-batch process. The measured values from Cedex for the fed-batch process as well as the control batch process are also included for comparison. Analyzer reference values and Raman calibration model predictions.

values for glutamate are 0.41 mmol/L and 12%, respectively. The glutamate calibration model underpredicts the glutamate concentration after the culture hour of 60 because the accumulated glutamate concentration is at or above the upper limit of this model (1.3 mmol/L). Generally, the model predicts the glutamate concentration within its range (Table 1). The calculated RMSE and MAPE values for ammonium are 2.8 mmol/L and 32%, respectively, in line with errors observed in reported models(Abu-Absi et al., 2011). The ammonium calibration model underpredicts the ammonium concentration, especially when the reference values are above 6.0 mmol/L after the culture hour of 40, which is the upper limit for this model. However, considering the samples taken before 40 h, the calculated RMSE and MAPE values are reduced to 0.87 mmol/L and 17%. The RMSE range is very similar to 0.819 mmol/L, and 0.76-0.77 mmol/L reported by Rafferty et al. (2020) and Li et al. (2018), respectively. Another contributing factor for high RMSE and MAPE values is the smallest datasets available for the ammonia model, and more valid data points for ammonium could be collected to improve its calibration model

One of the benefits of controlling the glucose concentration in a fed-batch process is to maintain a low lactate concentration in the bioreactor since only a small amount of glucose feed is added at a time for cellular consumption. Lactate accumulation in cell culture has been associated with a lower cell growth rate and reduced culture longevity. The lactate concentration in the batch operation becomes higher than that in the fed-batch operation since the culture hour of 45. It is continuously elevated as the culture prolongs, while the concentration of lactate in the fed-batch operation fluctuates at around 1.0 g/L (Cedex) The calculated RMSE and MAPE values for lactate are 0.35 g/L

and 25%, respectively. The RMSE for the lactate prediction is close to the reported values of 0.18 g/L and 0.21-0.23 g/L reported by Rafferty et al. (2020) and Li et al. (2018), respectively, but it is much lower than 1.16 g/L reported by Goldrick et al. (2020).

The prediction power of the current calibration models for the metabolites in Table 1 could be improved by (1) increasing the number of data points used to calibrate the models; (2) screening and removing clustering data points; (3) optimizing the number of principal components for calibrating each PLS model to reduce the probability of overfitting each model, and (4) expanding the concentration range for each metabolite. These models were built from the data points obtained from a batch process, either from shake flasks or bench-scale bioreactors. These data points have a narrow concentration range, depending on the concentration of nutrients in the media, the cell growth rate, and the culture days. The datasets obtained from the fed-batch operation could be included in the calibration models to build reinforcement learning models for these metabolites, which could increase the number of samples for the calibration model, and expand the range of the metabolite concentrations, ultimately enhancing the prediction capability of these calibration models. Fluctuations around the setpoint for glucose and glutamine could be diminished by reducing the sample intervals, especially during the rapid consumption period, such as the exponential phase. This can be achieved by incorporating an in-line Raman emersion probe, allowing for real-time continuous monitoring of these metabolites and offering simultaneous feedback to control the concentration of glucose and glutamine.

3.5 CONCLUSION

An in-house Raman-based monitoring and control system is successfully developed for a fed-batch process. Separate PLS calibration models are built for glucose, glutamine, glutamate, lactate, and ammonium from the datasets obtained from batch and fed-batch processes. The concentrations of glucose, glutamine, glutamate, lactate, and ammonium are continuously monitored throughout the culture period. All PLS models are able to predict the metabolite concentrations within the model concentration ranges. The concentrations of glucose and glutamine are controlled within 0.35 g/L of the setpoint (5.0 g/L) and 0.35 mmol/L of the setpoint (1.0 mmol/L), respectively, in a fed-batch operation. The monitoring system could be improved by increasing the calibration sample size, optimizing the number of components, and increasing the concentration range, while the control system by implementing an immersion probe to reduce the sample interval and smoothen the fluctuations around the setpoints. Impressively, the system is developed using in-house software without expensive commercial software packages to enable the construction of calibration models, pre-processing of raw Raman spectra acquired from the process, prediction of metabolites concentration, and instant feedback to control the feed of critical nutrients.

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4 Raman-Based Control System Applied to a Perfusion Process

4.1 ABSTRACT

Perfusion cell culture, a leading continuous bioprocessing technology, has been explored and adopted to address issues for batch and fed-batch processing. A higher volumetric productivity in a perfusion process suggests that smaller bioreactors could be used to achieve the same product throughput, resulting in a lower capital cost, a smaller facility footprint, and fewer scale-up steps from clinical to commercial production. Real-time monitoring of nutrients and metabolites in the bioreactor in a perfusion process could allow the feeding of critical nutrients to the bioreactor in addition to a continuous supply of the perfusion media, potentially reducing media consumption and culturing cells in a homogeneous cell culture environment for product formation. A Raman-based monitoring and control system is developed and applied to a perfusion process. The PLS calibration models for glucose, glutamine, glutamate, lactate, and ammonium are built and calibrated from the batch/fed-batch datasets in Chapter 3. The model predictions for these metabolites are well aligned with the reference values from Cedex for the culture period between 0 and 100 h, while these models generate inaccurate predictions for these metabolites beyond 100 h because a high cell density of 20x106 cells/mL could interfere with the molecular vibrations of these metabolites upon exposure to laser irradiation. The Raman-based system is also employed to control glucose and glutamine concentrations at 4.0 g/L and 0.5 mmol/L, respectively, by activating the feeding of concentrated glucose and glutamine solutions when they are below the setpoints, while a constant perfusion

rate of 0.5 VVD is maintained during the perfusion period. A constant monoclonal antibody (mAb) concentration is obtained under this perfusion condition, suggesting improved productivity could be realized at a low perfusion rate and at a constant concentration of glucose and glutamine through the Raman-based monitoring and control system, thus reducing the media consumption and the operation cost for the perfusion process.

4.2 BACKGROUND & INTRODUCTION

4.2.1 Perfusion

Increasing demand for a lower cost and a higher level of quality for biopharmaceuticals has led to a push to innovate current production methods. (Henry et al., 2008) Batch, fed-batch, and continuous operation are the three cultivation modes in biopharmaceutical processing. The simplest mode of operation is batch, which involves introducing all necessary components at the beginning of the run. Batch bioprocessing has the benefits of a low cost, simple operation, and a closed system, which reduces the chance of contamination. The finite nutrient availability after a few culture days in a batch process prevents cell growth and product production, leading to lower productivity. Fed-batch processing begins with a batch period. A concentrated fed is then introduced to extend the period for the availability of nutrients to cells. This leads to an increased cell density and higher productivity when compared to batch processing. However, this mode of operation can result in an increased level of waste which can affect cell viability and product quality. Fed-batch processing is a currently dominant mode of operation in the biopharmaceutical industry. (Wlaschin & Hu, 2006) In a continuous operation, nutrients from a feed stream are constantly introduced while cells, products, and wastes are continuously removed at an equal volumetric flow rate of the feed stream, thus maintaining a constant concentration for the biomass, nutrients, products, and wastes. (Henry C. Lim & Hwa Sung Shin, 2013) Continuous culture has been explored as a mode of operation since the 1980s. In the 1990s, the industry shifted from this method of operation back to fed-batch processing due to the lack of

technology and equipment, the complexity of implementing continuous operations, and high failure rates due partially to contamination and volume imbalances. Meanwhile, advances in expression systems, culture media, and feed supplements contribute to significant breakthroughs in batch and fed-batch processing. These advances lead to a doubling of the yield every five years, directing the focus to fed-batch processing.(Langer & Rader, 2014)

Instead of maintaining a constant concentration for all components during the continuous operation, the perfusion process is developed to become the leading continuous bioprocessing technology. During the perfusion process, a cell retention device is employed to retain the cells within the culture vessel, and the spent media, including products and wastes, are constantly removed at the same flow rate as the feed media. A cell bleed may also be implemented to maintain a constant cell density after the cell density reaches a pre-determined target value. Several commercial products produced from perfusion culture systems have been approved since the 1990s (Pollock et al., 2013). The cell retention device clarifies the harvest stream. It may take the form of a filter (depth, cross-flow, hollow fiber), a centrifuge, a gravity settler, or an acoustic wave separator(Bielser et al., 2018) (Suttle et al., 2019) (Voisard et al., 2003). The selection of a cell retention device depends on the type of cells, the type, and size of products, and the scale of the process (Willard et al., 2017). Two primary cell retention methods, settling and filtration, are often used for suspension cell culture. Settling methods include passive settling, acoustic separation, and centrifugation. The advantages of settling methods include a lower level of fouling risk and a reduced cost,

while the disadvantages of these methods consist of a lower separation efficiency and a longer operation time than filtration methods. Examples of filtration methods include tangential flow filtration (TFF), tangential flow depth filtration (TFDF), and alternating tangential flow filtration (ATF). The benefits of the filtration method are ascribed to its scalability and a high cell retention efficiency, but it may have a high risk of fouling. The clarified material from filters may be fed directly to a downstream operation. (Gibco, 2020) Advances in bioprocessing development, perfusion instrumentation, cell retention devices, single-use technologies, and process automation, as well as regulatory supports have fueled an interest in perfusion processing in the biopharmaceutical industry.

A perfusion process is often accompanied with a higher cell density and an extended culture period (up to 90 days) (Bonham-Carter & Shevitz, 2011)(Willard et al., 2017), resulting in a higher volumetric productivity when compared to batch and fed-batch processing. A higher volumetric productivity indicates the same product throughput could be accomplished in a smaller bioreactor, which leads to a lower capital cost, a smaller facility footprint, and fewest scale gaps from clinical to commercial production(Bielser et al., 2018). The product of interest during the perfusion process often has a short residence time during the perfusion process, which is preferable for unstable products in the cell culture environment. In this context, the perfusion process could help improve the product quality (Warikoo et al., 2012). This method of operation can be applicable to multiple biotherapeutic modalities and it is flexible for manufacturing. Challenges of a perfusion process include an increased chance of

contamination, equipment failure due to a long operation duration, and an increased cost of materials such as cell culture media.

As the viable cell density in a perfusion process becomes high, the nutrient supply should increase. The perfusion media is often fed into the bioreactor to ensure an adequate amount of nutrients in the bioreactor. To maintain a constant volume in the bioreactor, the feed rate of the perfusion media is matched by the rate at which material is removed from the bioreactor in the form of cell bleed and spent media. The flow rate is typically expressed as vessel volumes per day (VVD). An increase in the VVD is applied when the cell density is increased to keep a constant cell-specific perfusion rate (CSPR, pL/cell/day), for example, a CSPR of 30-50 pL/cell/day is suggested for CHO cell culture perfusion process. However, an increase in the VVD leads to an increased cost for the consumed media and dilution of the product. Real-time monitoring of cell culture media components or metabolites in the bioreactor can map the nutrient/metabolite landscape in the bioreactor. Preventing depletion of critical nutrients in the bioreactor can be achieved by either increasing the VVD for the perfusion media stream, alternatively, by adding concentrated critical nutrient solutions at a low VVD. The latter operation could potentially reduce media consumption, leading to a saving on the operation cost.

4.2.2 Aim

In this chapter, A Raman-based system is developed in-house and applied to monitor the concentrations of glucose, glutamine, glutamate, lactate, and ammonium in the bioreactor during a perfusion process. It is integrated with a feedback control system to

maintain a constant concentration for both glucose and glutamine in the retentate, while a constant and relatively low VVD is used for the feed media stream.

4.3 MATERIALS & METHODS

4.3.1 Raman-Based Control System

An in-house Raman-based monitoring and feed control system was developed using separate PLS calibration models built for glucose, glutamine, glutamate, lactate, and ammonium. The system was based in Python. It was designed to monitor glucose, glutamine, glutamate, lactate, and ammonium via a Raman spectroscopic method and use the concentration of both glucose and glutamine from the Raman method to provide a feedback control on the feed rate of glucose and glutamine during the perfusion process.

Cell culture samples withdrawn from the retentate in the bioreactor were analyzed at-line via a Supsense Raman Bioanalyzer coupled with a fiber-optic Raman probe with an optical excitation wavelength of 785 nm. Samples were spun down and 0.7 mL of the cell-free supernatant was transferred to a 3.0 mL quartz cuvette for analysis. Each spectrum was acquired by co-adding five scans after 14 seconds of exposure. Water spectra subtraction, co-addition, and acquisition were accomplished using ENLIGHTEN™ spectroscopy software (Wasatch Photonics, Logan, UT). The pre-processed spectrum was read into the PLS calibration models and the concentration for glucose, glutamine, glutamate, lactate, and ammonium was predicted.

A Mettler Toledo SB8001 scale (Mettler Toledo, Columbus, OH) was employed to monitor the weight of the bioreactor. The bioreactor weight, the predicted glucose and glutamine concentrations and the feed concentrations of glucose and glutamine were used to calculate the volume of glucose and glutamine required to reach their pre-determined setpoints. Two NE-9000G peristaltic pumps (New Era, Farmingdale, NY) were used to feed the calculated amount of glucose and glutamine to the bioreactor, respectively.

4.3.2 Perfusion Operation

The models and the control system developed were tested in a perfusion process using a 3L Applikon bioreactor with a working volume of 2 L (Applikon Inc., Delft, Netherlands). The Applikon ez-Control system was used to control pH, dissolved oxygen, temperature, and agitation at 7, 30%, 37°C, and 140 rpm, respectively. A monoclonal antibody-producing CHO-S cell line was used with ProCHO^{TM} 5 media (Sartorius Stedim Biotech, Göttingen, Germany). The seeding cell density was 2.5 x10^6 cells/mL. Perfusion was initiated at 26 h post-inoculation at a rate of 0.5 VVD (1 L/day), and the ProCHO^{TM} 5 media was used for the perfusion process. This perfusion rate at 0.5VVD was maintained throughout the run. Instead of increasing the perfusion rate to meet the requirements for critical nutrients including glucose and glutamine for an incremental cell density, the Raman-based control system was used to control the glucose and glutamine concentration at a set point of 4 g/L and 0.5 mmol/L, respectively. The feed concentration of glucose and glutamine were 400 g/L and 200 mmol/L, respectively. Cell retention was realized via a Meissner SepraPor 0.2 µm hollow fiber filter. The Levitronix

LCO-i100 console system was used to control the recirculation rate at 0.3 L/min and monitor the transmembrane pressure (TMP).

4.3.3 Offline Measurements

The Roche Cedex Bio Analyzer (Roche CustomBiotech, Penzberg, Germany) was used for offline measurements of the concentration of glucose, glutamine, glutamate, lactate, and ammonium. The viable cell density (VCD) and viability were measured to monitor the health of the culture via Vi-CELL XR (Beckman Coulter, IN, USA). The forteBIO BLItz system with protein A biosensors was used to measure the monoclonal antibody concentration in the bioreactor.

4.4 RESULTS & DISCUSSION

4.4.1 Model Calibration Summary for Perfusion Process

Separate Raman-based calibration models for the CHO-S cell line and the ProCHO^{TM} 5 media were built to monitor the concentration of glucose, glutamine, glutamate, lactate, and ammonium during the cell culture process and they were applied to a 150-hour perfusion run. The summary of these models is shown in Table 1, including the calibration range, the number of components used to build the PLS model, and the model performance for glucose, glutamine, glutamate, lactate, and ammonium. The model performance parameters, including coefficients of determination(R^2) for calibration (R^2C) and ross-validation (R^2CV), root mean square error of calibration (RMSEC), and root mean square error of cross-validation (RMSECV) suggest that these models could be used to predict these metabolites in the calibration range.

Table 3. Summary of Raman-Based Calibration Models

Metabolite	Range	#Components	R^2C	R^2CV	RMSEC	RMSECV
Glucose (g/L)	0.8-9.0	9	0.987	0.907	0.067	0.488
Glutamine (mmol/L)	0.2-5.0	8	0.911	0.677	0.245	0.89
Glutamate (mmol/L)	0.6-3.0	5	0.984	0.88	0.001	0.008
Lactate (g/L)	0.4-3.0	9	0.997	0.953	0.003	0.039
Ammonium (mmol/L)	0.7-7	9	0.986	0.857	0.084	0.859

4.4.2 Application of the Raman-Based System to a Perfusion Process

A diagram for the setup for this perfusion process and the developed Raman-based control system is described in Figure 1. The perfusion process at a constant VVD of 0.5 is controlled via a Levitronix LCO-i100 console system and two Watson Marlow 120U pumps. A Meissner SepraPor 0.2 µm hollow fiber capsule filter is employed to continuously harvest the monoclonal antibody product stream and recirculate the cells back to the bioreactor. A Raman Bioanalyzer at a wavelength of 785 nm is used to acquire the Raman spectrum, which is fed into the PLS calibration model to predict the concentrations of metabolites, including glucose and glutamine. The control system triggers the pumps to feed the amount of glucose and glutamine to the bioreactor estimated from the inputs, including the bioreactor weight, the glucose and glutamine concentration in the retentate, and the feed concentrations of glutamine and glucose.

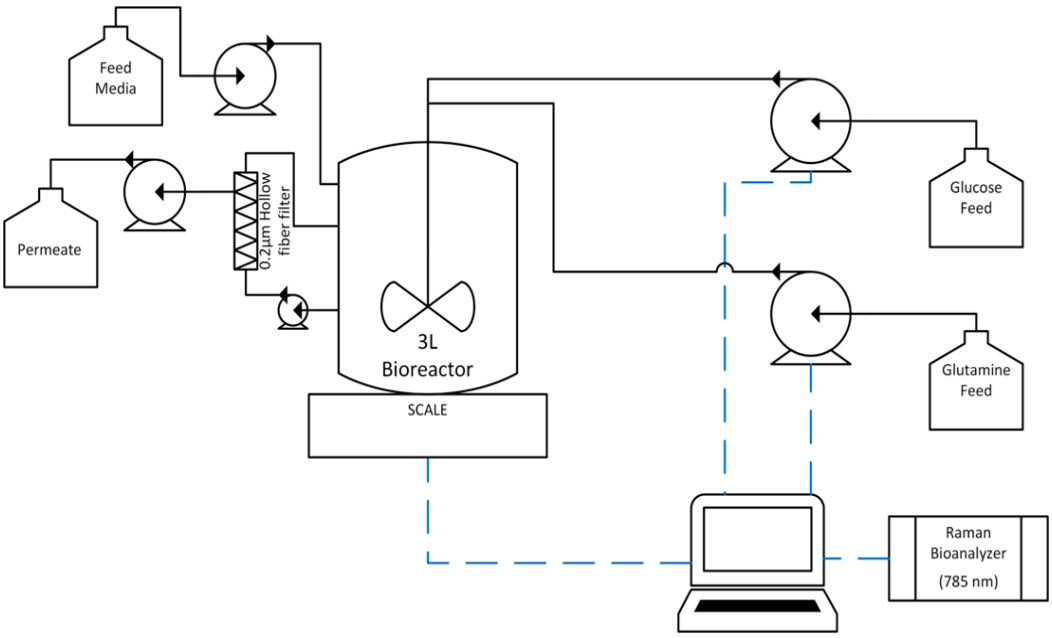

Figure 1. Diagram of a Raman-based monitoring and control system and its application to a perfusion process. The system is composed of two peristaltic pumps to dispense the glucose and glutamine feeds, a balance to monitor the volume in the bioreactor, a Supsense Raman Bioanalyzer to generate Raman spectra, and an in-house software to predict the metabolite concentrations and control the pump rates . Two additional peristatic pumps are used for feeding the media and collecting the permeate, respectively. A Levitronix LCO-i100 console system is used to control the recirculation rate and monitor the TMP, and a Meissner SepraPor 0.2 µm hollow fiber capsule filter for cell retention. Blue dashed lines indicate RS-232 serial connections to the computer.

Since the control system could maintain the glucose and glutamine concentrations at their setpoints during a perfusion process at a constant VVD, the consumption of the feed media could be significantly reduced, while the viable cell density could be increased and high cell viability maintained, thus reducing the operation cost and enhancing the productivity.

4.4.3 Perfusion Process at a Constant VVD

The viable cell density and viability for the 150-hour perfusion process are shown in Figure 2. The bioreactor was inoculated at 2.4 x10^6 cells/mL to shorten the duration before the start of the perfusion process. Perfusion at a rate of 0.5 VVD started at 26 h post-inoculation. Exponential cell growth is observed for up to 100 h; after this time point, a viable cell density of about 20 x10^6 cells/mL is maintained at a perfusion rate of 0.5 VVD and with the feed of concentrated glucose and glutamine. The percent viability is above 95% for the entire run. A total of five days of perfusion were achieved.

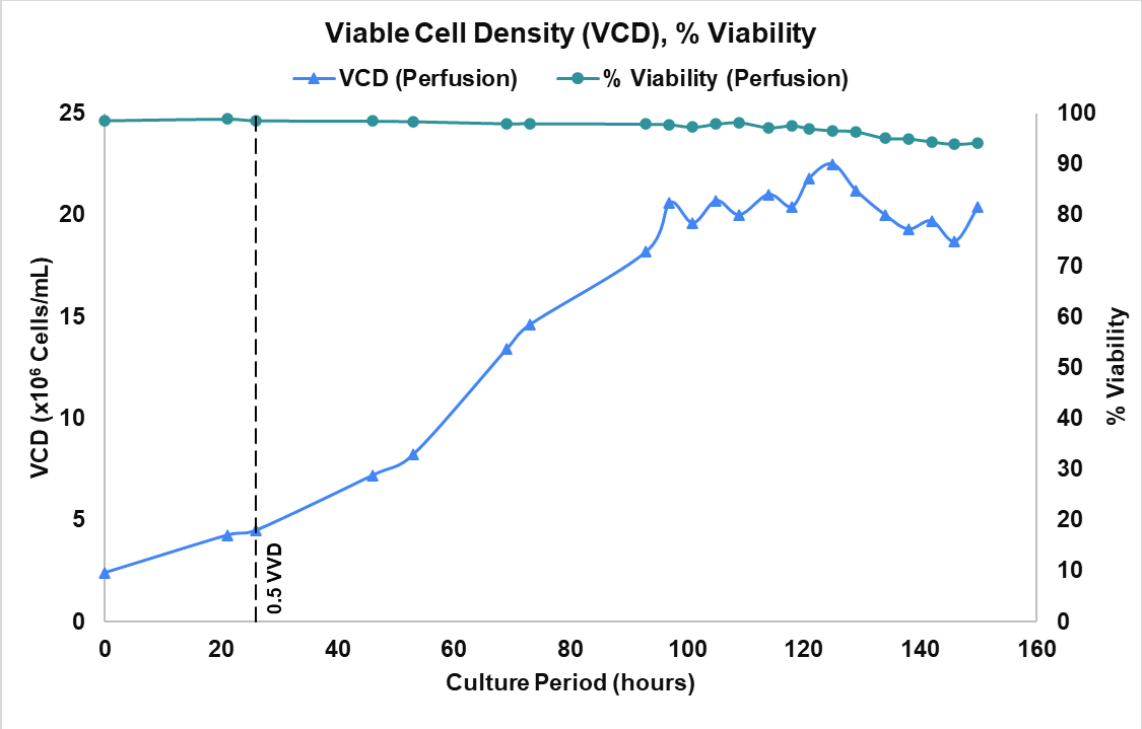

Figure 2. VCD and percent viability for perfusion mode bioreactor run with controlled glucose and glutamine. Perfusion started at 26 hours with a perfusion rate of 0.5VVD. The control setpoints for glucose and glutamine were 4 g/L and 0.5 mmol/L, respectively. Percent Viability was maintained above 95%, and the peak VCD achieved was 23x106 cells/mL.

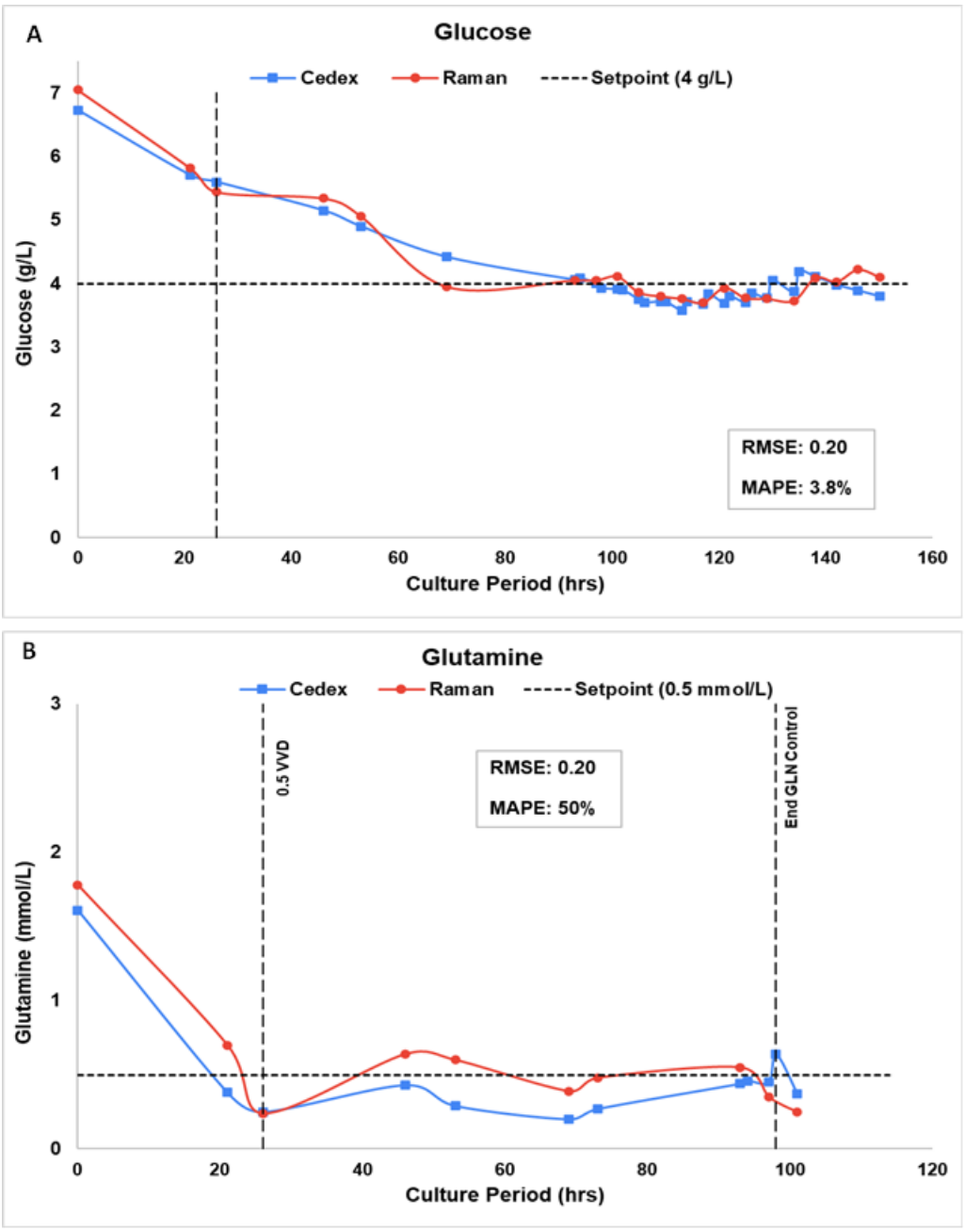

Figure 3. A Raman-based control system was used to monitor and control glucose (A) and glutamine(B) concentrations at 4 g/L and 0.5 mmol/L, respectively. Samples were taken from a 3L Applikon bioreactor process in perfusion mode. The working volume was 2L. Perfusion began 26 hours post-inoculation at a rate of 0.5VVD. Glutamine control was terminated after 98 hours to allow glucose to fall below the setpoint. The Raman predicted values (squares) and offline reference Cedex Bio Analyzer values (circles) were used to determine the accuracy of the Raman models, a RMSE of 0.2 and a MAPE of 3.8 % were calculated for glucose, and an RMSE of 0.20 and MAPE of 50 % for glutamine.

The Raman-based monitoring and control system was applied to estimate the glucose and glutamine concentrations, and their concentrations are fed into the control system to control the concentration of glucose and glutamine at 4.0 g/L and 0.5 mmol/L, respectively. The Cedex Bio Analyzer reference values measured offline are used to evaluate the prediction power of Raman-based calibration models and assess the accuracy of the control system. Figure 3 displayed the measured Cedex values and the Raman model predicted values. The concentration setpoints for glucose and glutamine are also added in Figure 3. An RMSE of 0.2 g/L and a MAPE of 3.8 is estimated between the reference values and the model prediction values. The RMSE measures the aggregated error between experimental measurements and predictions; a lower RMSE indicates a better prediction power of the model. The MAPE, the absolute percentage error in a dataset, is an indicator for the average deviation between the model-predicted values and the reference measured values. Such a low RMSE for the glucose model indicates it has a relatively low error. The RMSE is less than 0.28 ±0.09 g/L, the reported RMSE values for glucose in Table 3 of the review paper by Esmonde-White et al. A MAPE of 3.8% suggests that the glucose model, on average, is able to predict the glucose concentration with an error of 3.8%, which falls within the error range from all commercially available offline bioanalyzers. From the culture hour of 94 to 150, the glucose concentration is controlled through the Raman model-based control system. When the glucose concentration predicted from the Raman model is below the setpoint, the pump is activated to supply the concentrated glucose concentration to the bioreactor to increase the glucose concentration. During the glucose concentration

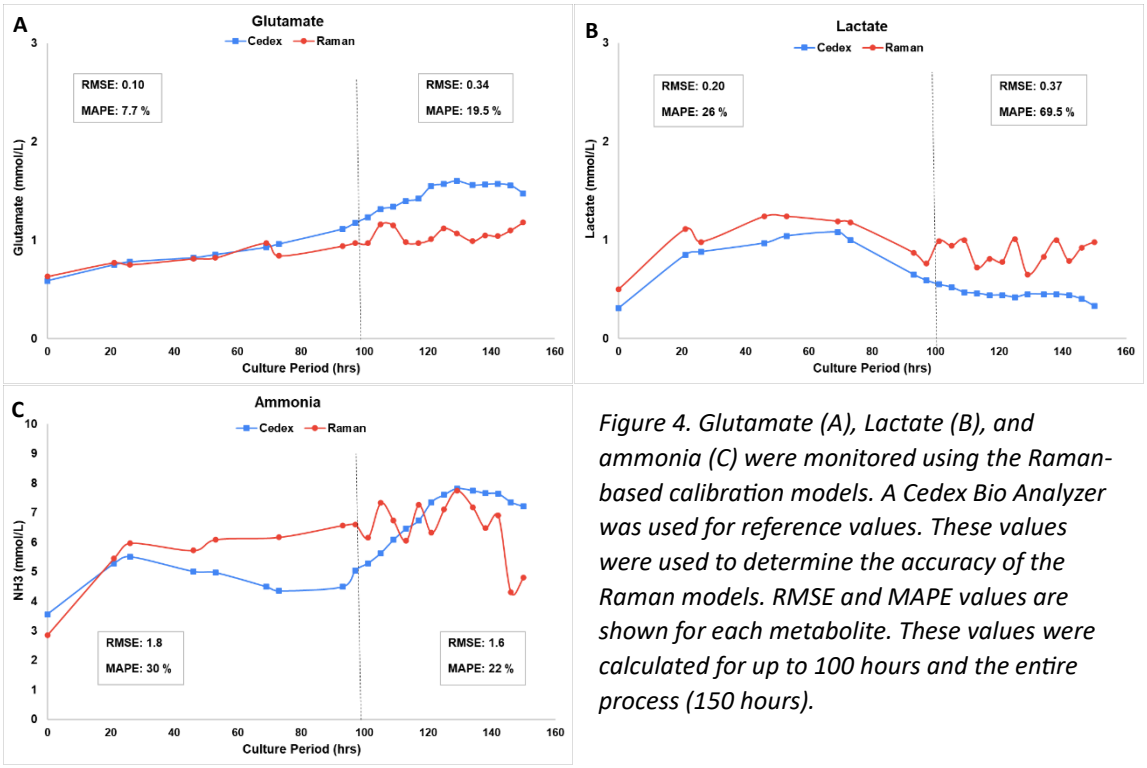

Figure 4. Glutamate (A), Lactate (B), and ammonia (C) were monitored using the Raman-based calibration models. A Cedex Bio Analyzer was used for reference values. These values were used to determine the accuracy of the Raman models. RMSE and MAPE values are shown for each metabolite. These values were calculated for up to 100 hours and the entire process (150 hours).

control period, the glucose concentration in the bioreactor is maintained within 0.3 g/L of the setpoint (4.0 g/L). Fluctuations from the setpoint could be smoothened by increasing the sampling frequency and improving the accuracy of the pump feeding rate. However, an RMSE of 0.2 mmol/L and a MAPE of 50% are estimated from the glutamine calibration model. The RMSE of glutamine prediction is much lower than 0.42-0.44 mmol/L reported by Li et al. (2018) and that for the fed-batch operation in Chapter 3, which could be the fed-batch dataset included in the glutamine calibration model. However, the model has a much higher MAPE for the perfusion process than that for the fed-batch operation. Since the glutamine setpoint is quite low (below 1.0 mmol/L), a slight difference between the predicted and measured values could result in a large

MAPE. The predicted glutamine concentration from the model is able to provide quite accurate feedback to the control system, enabling the glutamine concentration within 0.3 mmol/L of the setpoint.

Raman-based calibration models were also built for monitoring the concentrations of glutamate, lactate, and ammonia. Figure 4 compares the reference values obtained offline (Cedex) and the Raman model-predicted values for glutamate, lactate, and ammonia. The RMSE and MAPE between reference values and predictive values were calculated for each metabolite. It can be clearly seen that pronounced differences between the offline reference values and the Raman model predicted values for these three metabolites become distinct at the culture hour of 100 in this perfusion process. At around 100 h, the cell density in the bioreactor reaches $20x10^6$ cells/mL (Figure 2). Therefore, the culture process could be differentiated into two distinct phases: a batch and fed-batch process up to 100-hour, and a perfusion process after 100 hours to the end of the process. It is noted that the calibration models for these metabolites are built from the datasets obtained from batch and fed-batch operations from previous chapters, and they are applied to the perfusion process. A much higher cell density in the perfusion process than that in a batch or fed-batch process could compromise the prediction capability of the calibration models built from the batch/fed-batch datasets. In this context, two sets of RMSE and MAPE values were calculated: one set for the metabolites at the culture hour of 0-100 h and another for the entire culture period.

An RMSE of 0.01 mmol/L and a MAPE of 7.7% are found for glutamate at the culture hour of 0-100, while an RMSE of 0.12 mmol/L and a MAPE of 19.5% for the entire run.

The glutamate calibration model has a remarkably low RMSE before 100 h, much lower than that for the fed-batch process in Chapter 3. Incorporation of the data points for both batch and fed-batch datasets in Chapter 3 into the calibration model significantly improves the accuracy of the predicted glutamate concentration, suggesting that a reinforcement learning algorithm could be implemented in these metabolite models. However, the RMSE and MAPE values increase after the glutamate concentrations for the entire culture period are applied to the glutamate calibration model. A similar pattern is observed with lactate, with an RMSE of 0.04 g/L and a MAPE of 26% between the culture hour of 0 and 100 h, and an RMSE of 0.13 g/L and a MAPE of 69.5% for the entirety of the bioreactor run. Interestingly, an RMSE of 3.3 mmol/L and a MAPE of 30%

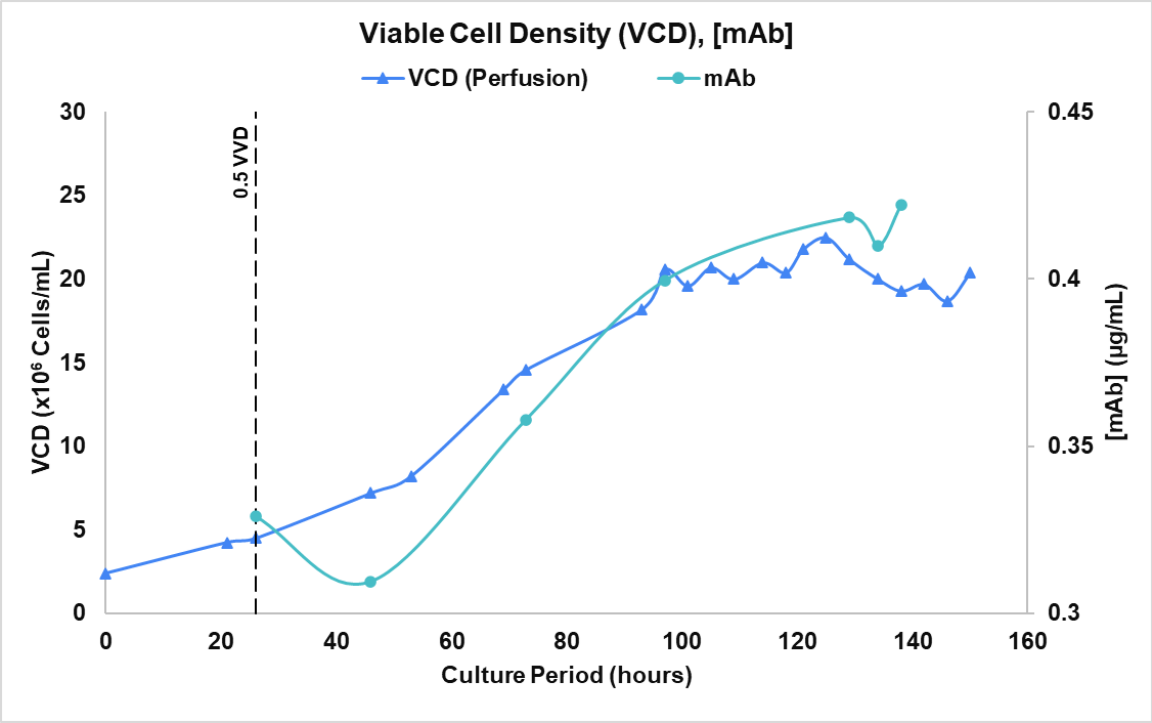

Figure 5. A CHO-S, mAb-producing cell line was used in this perfusion run. The forteBIO BLItz system was used to measure mAb concentration in the bioreactor using protein A biosensors. Measurements were taken periodically from the start of perfusion at 26 hours to hour 129. The mAb concentration generally increased and followed a similar trend as VCD. mAb concentration was maintained with a perfusion rate of 0.5 VVD and glucose and glutamine control.

are found between the measured ammonium concentrations and the model-predicted values for up to the culture hour of 100, while an RMSE of 2.54 mmol/L and a MAPE of 22% for the entirety of the bioreactor run. It is observed that the ammonium concentration after the culture hour of 120 exceeds the upper limit (7.0 mmol/L) for the calibration model. The model accuracy in predicting the ammonia concentration could be significantly impacted after 120 h, thus the RMSE and MAPE values may not be able to precisely capture the errors between the measured ammonium concentrations and the predictions for the entire bioreactor run.

The monoclonal antibody (mAb) concentration in the bioreactor was measured periodically. Figure 5 shows the VCD and the mAb concentration over the culture period. It can be seen that the mAb concentration increases as the VCD increases, and it appears to stabilize as the VCD is maintained at about $20x10^6$ cells/mL. Therefore, the mAb concentration could be maintained at a constant perfusion rate and a constant concentration for glucose and glutamine by feeding their concentrated solutions. Therefore, productivity could be improved without the need to increase the perfusion rate, thus reducing a significant amount of perfusion media.

4.5 CONCLUSION

A Raman-based monitoring and control system was developed and applied to a perfusion process. The PLS calibration models are built from the datasets from both batch and fed-batch processes in Chapter 3. These models have a great performance in predicting the metabolite concentrations in the initial culture period, during which the cell culture process could be considered as a batch/fed-batch process, while less

accurate predictions are found from the models at a high cell density of $20x10^6$ cells/mL. The model performance could be improved by incorporating this perfusion dataset into the calibration models and expanding the range of the metabolite concentrations. By feeding the predicted glucose and glutamine concentrations to the control system, the pump is activated to maintain the glucose and glutamine concentration at their setpoints with a range of 0.3 g/L and 0.3 mmol/L, respectively, leading to a low perfusion rate and a reduction in the consumption of the perfusion media. Our experimental results suggest that the Raman-based monitoring and control system could be applied to improve productivity in a perfusion process with a low operation cost.

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5 MULTI-COLUMN CHROMATOGRAPHY

5.1 ABSTRACT

Application of multicolumn chromatography to processes such as protein A capture has shown significant improvements in the volumetric productivity, the resin utilization capacity, the buffer consumption amount, and the frequency of equipment occupation, as well as reduction in the facility footprint. Challenges in applying this technology include the high cost of equipment and its associated control software, equipment customization requirements, and availability of multi-scale equipment. The aim of this chapter is to develop a bench-scale flexible two-column chromatography system free of commercial software and apply it to an integrated perfusion and capture process. The system was tested with three operations: a single-column operation (equivalent to a traditional batch operation), a two-column operation at a loading capacity of 80%, and a two-column operation at a loading capacity of 100%. The permeate from the permeate line was directly applied to the columns, and a residence time of 1.4 min was applied to the feed stream and equilibration, wash, and elution buffers. The offline generated chromatograms strongly support of this two-column system for continuous capture of products from the permeate during a perfusion process.

5.2 BACKGROUND & INTRODUCTION

5.2.1 Multi-Column Chromatography (MCC)

The advantages of continuous manufacturing include consistent operation conditions, a small equipment size, high volumetric productivity, a streamlined process flow, and a low cycle time, leading to reduced capital cost and a small facility footprint (Warikoo et al., 2012). These advantages have been a drive to explore continuous bioprocessing methods for downstream operations. A packed chromatography column is often used for initial product capture in the production of biopharmaceuticals in a batch-operation mode. Challenges with batch operation include an under-utilized binding capacity of resin, a large amount of resin and buffers, a high equipment cost, a high level of product quality variability due to long process times, and non-productive hold-up steps(Zydney, 2016). Batch chromatography is also challenged by a continuous perfusion process. Since the perfusion operation generally produces a large volume of permeate containing the product, and the culture period can be significantly extended, the volume of the permeate collected from the perfusion process requires a large column and/or multiple batch operations, resulting in long hold periods for the clarified permeate and varied product quality after the capture step.

Multi-column chromatography has been demonstrated to be a promising technology for continuous capture and purification of biopharmaceuticals. Multi-column chromatography breaks up the loading zone of the column into smaller columns and assembles these smaller columns in series. The smaller columns are cycled with the same process steps used in a batch process, resulting in the parallel processing of

multiple columns. (Arnold, 2018) This technology allows continuous feed of the product into the column, and maximization of the resin utilization capacity, leading to reduced consumption of resins and buffers, and increased yields and productivities.

5.2.2 Application of MCC

Applications of continuous chromatography have been seen in the capture and purification of biopharmaceuticals (Bisschops et al., 2009) (Pagkaliwangan et al., 2019) (Zydney, 2016). Warikoo et al. (2012) described the integration of a perfusion bioreactor and a four-column periodic counter-current chromatography (PCC) system for the continuous capture of proteins. Continuous capture of proteins from cell culture media could help reduce the residence time of unstable molecules in the cell culture media. In this study, two model molecules were explored: a monoclonal antibody, a model of a stable protein, and a recombinant human enzyme, a model of a complex, less stable protein. A custom-modified AKTA system capable of running up to four columns was employed for protein capture. The system was operated using a UV-based dynamic control strategy. The difference in the UV absorbance between the feed inlet and the column outlet triggers the column switching. The automated PCC system ran uninterruptedly for 30 days, and the product quality for both molecules was comparable to a batch column. Godawat et al. (2015) applied two PCC operations to the downstream processing of monoclonal antibodies. The first was a protein A capture process followed by cation exchange chromatography as a polishing step. The PCC system used in this study was the customized ÄKTA system which can run four columns. The productivity, resin utilization, buffer requirements, and equipment utilization were compared

between continuous chromatography and batch processing. The productivity was enhanced by over 1100-fold, the resins capacity was increased 120-fold, buffer usage reduced by 80-fold, and equipment use increased significantly after two PCC operations over their corresponding batch operations, eventually leading to a small facility footprint. Other two-column systems have been applied to the capture step with improved performance(Steinebach et al., 2017). Mechanistic models were implemented in multicolumn systems to predict the performance in terms of yield, productivity, and capacity utilization, which could allow continuous capture processes to be well controlled and become more robust (Steinebach et al., 2016).

5.2.3 Affinity Chromatography – Protein A

Affinity chromatography is a type of chromatography used in biopharmaceutical processing to purify biomolecules due to their specific binding to ligands on chromatographic resins. This method is based on reversible and highly specific interactions between two molecules, such as interactions between enzyme and substrate or antibody and antigen (Urh et al., 2009). A ligand with specific functional groups and/or structural conformation is developed to display specific interactions with the target molecule of interest. Affinity ligands are separated into two groups: specific ligands that bind to a specific solute, and general ligands that bind to specific groups on molecules (Walters, 1985). Affinity chromatography is a widely used technique used in bioprocessing, and it is often a primary purification step for the purification of biomolecules, such as monoclonal antibodies (mAbs) because of its specificity, high yield, and its ability to reduce the virus burden (Magdeldin & Moser, 2012). Protein A is a

bacterial cell wall protein that specifically binds to the Fc region of mAbs. In protein A affinity chromatography, the protein A ligand is covalently attached to a chromatography resin, and the resin is packed into a column.

A typical chromatogram for protein A purification of mAbs is shown in Figure 1. The chromatogram in Figure 1 shows the A280 absorbance over column volumes (CV) or process volumes of the operation. The column is first equilibrated in preparation for the binding of mAbs. The crude mAbs are then loaded onto the column. The mAbs specifically bind to the protein A ligand, and all other impurities including host cell proteins, host cell DNA, and viruses in the media pass through the column. After loading, the column is washed to remove non-specifically bound impurities. The mAbs are then

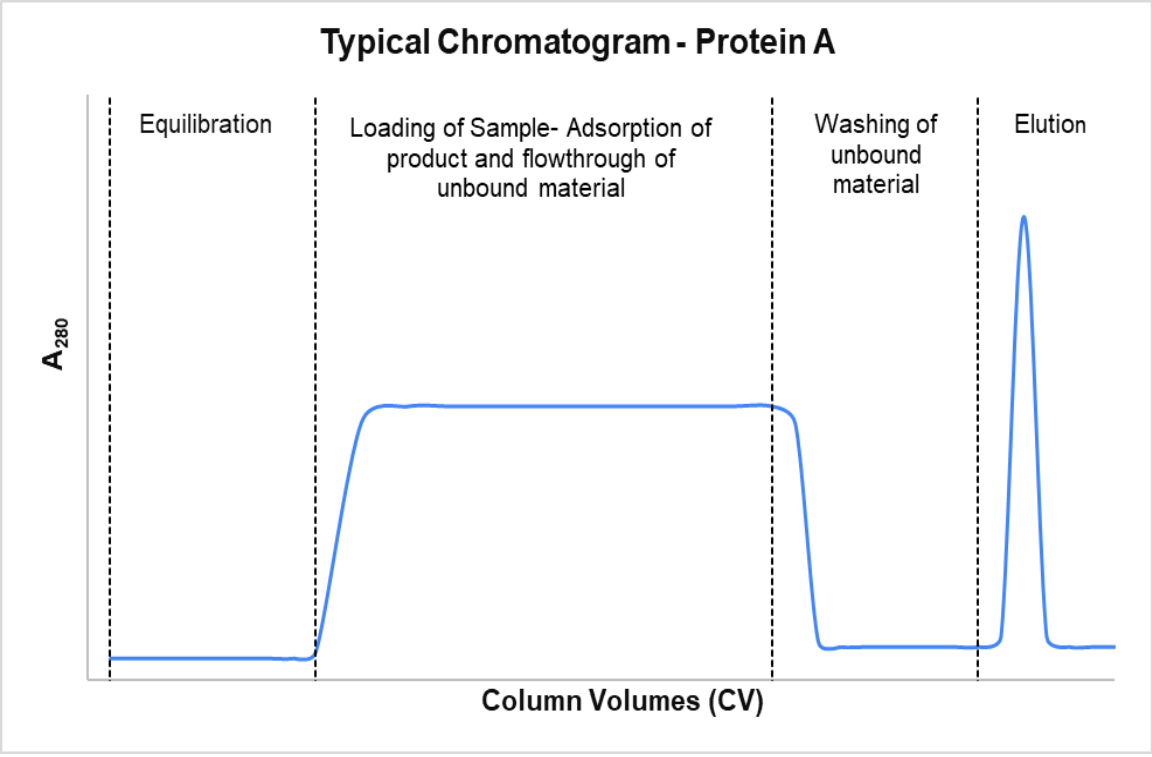

Figure 1. Typical chromatogram for protein A capture. Four distinguished steps: equilibration, loading, washing and elution.

eluted from the column using a low pH buffer that disrupts the interaction between the protein A ligand and the mAbs. Protein A affinity chromatography is a reliable and efficient technique for the capture of mAbs in bioprocessing, and it is one of the most widely used techniques for the purification of mAbs in the biopharmaceutical industry.

Protein A purification of mAbs has many advantages, while it is also one of the most expensive operations in mAb production. Protein A resin can account for up to 30% of the total cost of producing mAbs in a batch process. With the implementation of perfusion cell culture, the purification of mAbs in the cell culture media becomes very challenging. A great amount of resin is required to process a large volume of the permeate harvested from the perfusion process; a large column is needed for these resins, or multiple cycles of a smaller column could be operated; a long holdup time for the permeate during multiple cycles could compromise the product quality of mAbs. Application of multi-column chromatography to protein A purification has shown significant improvement in the yield and reductions in the cost over batch operation.

Table 4. Comparison of protein A Capture- Batch vs Continuous

	Batch	*4 -Column MCC*	*3- Column MCC*
Resin (L)	57	28	9
Cycles	6	8	25
Buffer (L)	6,100	4,000	4,200
Productivity (g/L_{resin}/h)	20	60	55
Cost (resin)	$648,000	$336,000	$108,000
Process Time (h)	8	6	20

Arnold explored the MCC as an alternative to batch chromatography for monoclonal antibody capture using protein A. A typical batch capture step utilizes 60-80% of the resin's binding capacity, and loading of products over 80% of the resin capacity often results in product loss due to breakthrough. A batch column can typically achieve a binding capacity of 35 g mAbs/L of resin while operating in a continuous manner with a MCC operation is shown to increase the binding capture to greater than 50 g/L of resin. Three scenarios were compared to show the impact of switching from a batch operation to a continuous operation. In this comparison, a volume of 2,000 L spent media with a titer of 5 g mAb/L was processed in a batch mode (one 57 L column), a continuous mode with four columns (four 7 L columns), and a continuous mode with three columns (three 3 L columns). Table 1 displays the total resin used per process, the amount of buffer required, productivity, the cost for the resin assuming $12,000 /L of resin, and the total

process time. The continuous operations are found to have a significantly higher productivity and reduce the buffer and resin requirements. The cost of resin is notably reduced from batch operation to continuous processes by 2-6X. The four-column operation has the benefits of reduced buffer requirements, a small volume of resins, increased productivity, and reduced process time. The processing time for the three-column system is significantly increased from 8 hours in the batch process to 20 hours. This may seem disadvantageous, but this process is applied to a perfusion process which often lasts many days longer than a batch cell culture process, in this case the process time is no longer an issue. There is an additional advantage of resin cost reduction over the four-column process.(Arnold, 2018) Continuous chromatography, especially protein A capture chromatography with the most expensive resins, outperforms batch chromatography in terms of resources, productivity, cost, and process time. More importantly, it can be integrated with the perfusion process, thus laying the foundation for establishing an end-to-end continuous bioprocess.

5.2.4 Aim

Although there are a very few commercially available continuous chromatographic systems including Resolute® BioSMB (Sartorius) and ÄKTA periodic countercurrent chromatography (PCC) (Cytiva), they are very expensive and most of them are applied to a pilot-scale and manufacture-scale operation. There is a drive to increase the availability of a variety of affordable, off-the-shelf continuous chromatographic systems, especially at the bench-scale so that biotechnology companies can consider this technology from their early process development. Meanwhile, the commercial software

is not modifiable thus the customizations of these systems to different processes, especially those for new modalities, are often very challenging. The aim of this chapter is to develop a bench-scale flexible two-column chromatography system free of commercial software and apply it to an integrated perfusion and capture process.

5.3 MATERIALS & METHODS

5.3.1 Development of a Two-Column Chromatography System

The two-column chromatography system was assembled and controlled by an in-house developed software. It consisted of four VICI Cheminert 6 position valves (VICI Valco Instruments, Houston, TX) with actuators and two Watson Marlow 120U peristaltic pumps (Watson Marlow, Devens, MA). Poly(etheretherketone) (PEEK) polymer tubing was used for all valve inlets and outlets. Cole-Parmer EW-06424-13 tubing was used to replace the PEEK tubing to which the peristaltic pumps were attached to.

The control software was developed in Python to control the valves using serial connections for three different methods. The first method was for a single-column operation. The second method was for a two-column operation with a capacity utilization efficiency of 80%. The loading and non-loading steps (washing, elution, and equilibration) cycled between two columns. The third method was for a two-column operation with a capacity utilization efficiency of 100%. The first column was overloaded to reach its maximum capacity, and the un-bounded product was redirected as a flowthrough to the second column. Non-loading steps were then conducted on the first column, and the loading step was on the second column.

5.3.2 Application of the Two-Column Chromatography System

The chromatography system developed was applied to a perfusion process described in Chapter 4. The cell line was a monoclonal antibody-producing cell line with a low titer between 0.3-0.45 µg/mL. The permeate flow rate for this perfusion process was 0.7 mL/min. Two 1 mL HiTrap® Protein A High-Performance columns from Cytiva were used to capture the monoclonal antibody produced in the permeate. A residence time of 1.4 min in the column was estimated from the permeate flow rate and the column size. A 20 mM sodium phosphate buffer at a pH of 7.4 was used for equilibration. A 0.1 M citric acid buffer at a pH of 3 was used for elution. A neutralization buffer composed of 1 M Tris-HCL at a pH of 9 was used to increase the pH of the elution material for maintaining the protein stability.

To generate purification chromatograms offline, 1-2 CV fractions were collected throughout each chromatography run. The fractions were loaded onto a 96-well plate, and the absorbance was measured at 280 nm on a Tecan Infinite plate reader. The absorbance values were then graphed against the column volumes to generate the offline chromatograms.

5.4 RESULTS & DISCUSSION

5.4.1 Design of A Multi-Column Chromatography System

A two-column chromatography system was designed and assembled from commercially available components. Four VICI Cheminert 6 position valves with actuators and two Watson Marlow 120U peristaltic pumps were used for directing the feed stream, and buffers for equilibration, elution and stripping. 2 x 1 mL HiTrap® Protein A High-

Performance columns were employed for the capture step. The diagram in Figure 2 shows the layout for valves, pumps, and chromatography columns. Valves A and B are used to direct the flow streams in and out of column one, while valves C and D for column two. The operation procedures for valves A and B are identical to those for valves C and D; therefore, the inlets and outlets for one set (valves A and B) are described in the context of protein A purification. Protein A capture is carried out in four steps: equilibration, loading, washing, and elution. Positions one to six on valve A are inlets for the feed stream and buffers. The position one is designated for an equilibration buffer, position two for feed stream loading, position three for an elution buffer, positive four for a stripping buffer, position five as a standby, and position six for receiving the flow of the overloaded feeds to the second column. At the center of valve A is an outlet for all inlet streams from positions one to six. This outlet is connected to the inlet of the first column, and a pump is placed between the valve and the column. The outlet of the first column is connected to the inlet at the center position of valve B. Positions one through six on valve B are used as outlets to direct the flow out of the first column to different collection bottles. Position one is designated for the waste stream, position two for the eluent, position three for unbound materials during the loading step, position four for the stream during the washing step, and position five for redirecting the overloaded material of the first column to the second column. Position six is a standby. All valves and pumps are controlled via software developed in Python in a time-based mode. This two-column system was applied to the perfusion process detailed in Chapter 4, and the setup is shown in Figure 3.

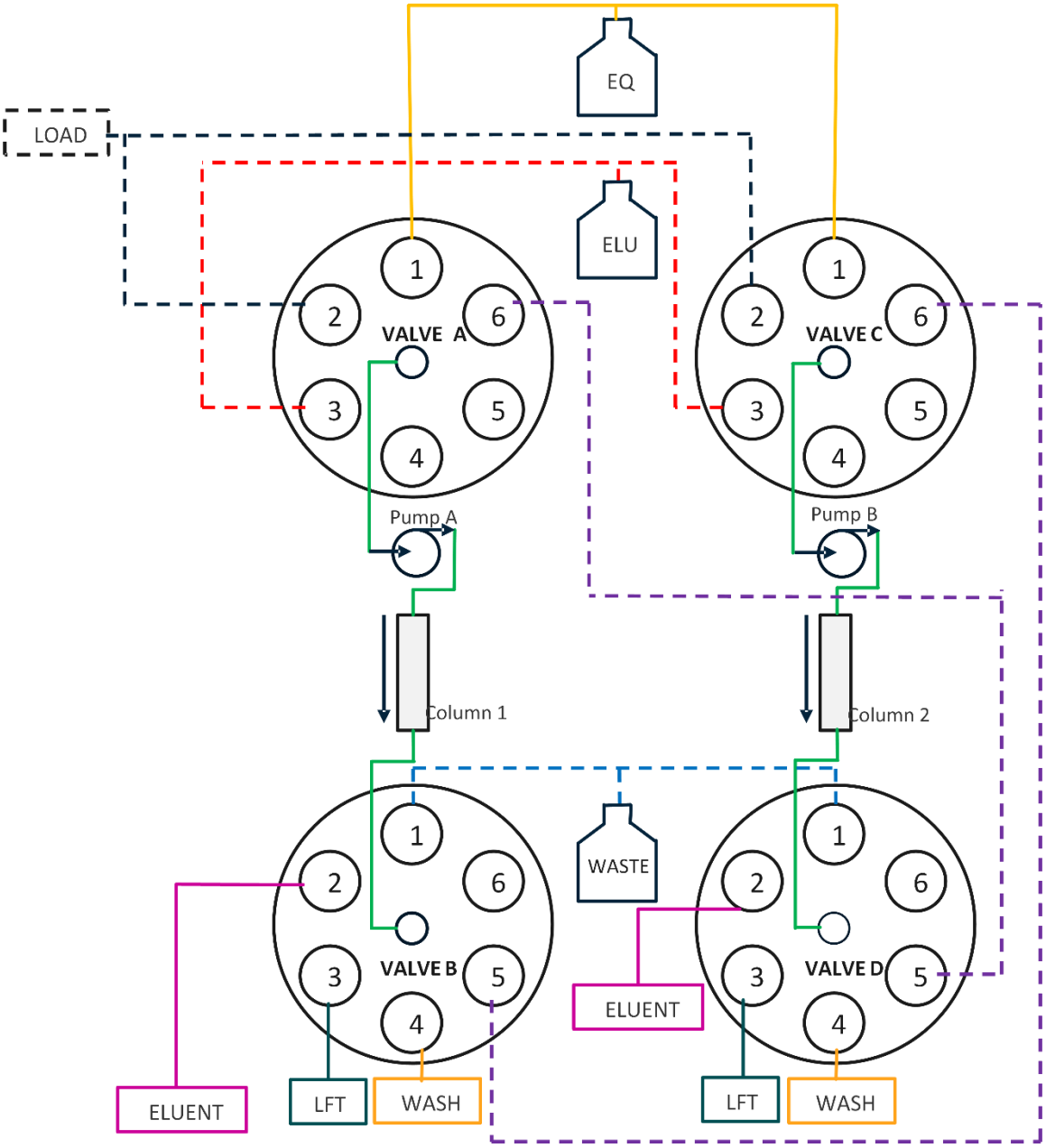

Figure 2. Diagram of the two-column chromatography system developed. The system consists of four VICI Cheminert 6 position valves with actuators and two Watson Marlow 120U peristaltic pumps. The system is controlled using software developed in Python. EQ: equilibration buffer; Elu: Elution buffer; LFT: load flow through.

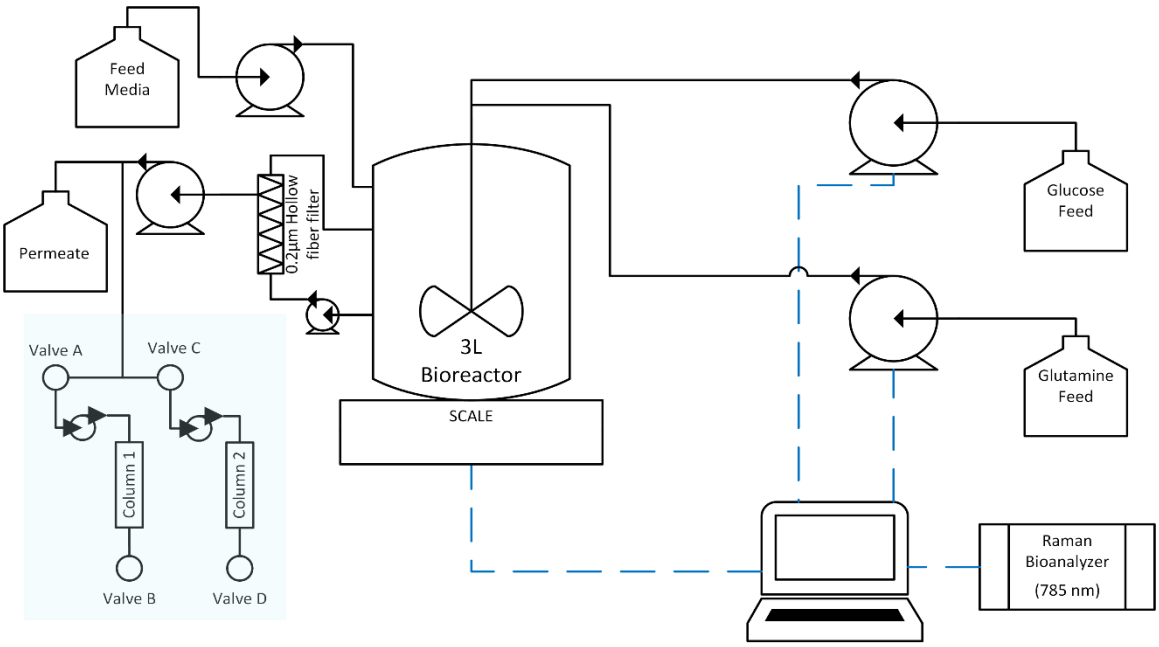

Figure 3. Diagram of the two-column chromatography system applied to a perfusion process described in Chapter 4.

5.4.2 Single Column Application (Batch)

A single-column method was developed to test the software and hardware of this system. The feed stream was loaded directly from the perfusion permeate line at a flow rate of 0.7 mL/min, resulting in a residence time of 1.4 min for the product. The operation steps, valve positions, column volumes, and residence times for each step are shown in Table 2. The 1 mL protein A column was loaded with 20 CV of the permeate, a chromatogram was generated offline by collecting one to two CV fractions throughout the run.

Table 5.Single Column Chromatography Method

Step	Valve	Position	Stream	CV	Time (min)
Equilibration	A	1	Equilibration Buffer	10	14.3
	B	1	Waste		
Loading	A	2	Load	20	28.6
	B	3	Flowthrough		
Washing	A	1	Wash Buffer	10	14.3
	B	4	Wash stream		
Elution	A	3	Elution Buffer	5	7.1
	B	2	Eluent		

Figure 4 shows the chromatogram for this method. This chromatogram shows typical features similar to those in Figure 1, indicating the system is successful in performing protein A capture. During the first 10 CV, the column was equilibrated with the equilibration buffer, and a relatively low absorbance was read during this step, indicating no protein is present in the equilibration stream out of the first column. During the flow volume of 10-30 CV, an increase in the absorbance value is seen, and this correlates with an increase in the loading volume of the permeate. A plateau of the absorbance value is reached after the permeate is continuously loaded onto the column. After finishing the loading, 10 CV of a wash buffer was applied. A gradual reduction in the absorbance value indicates unbound proteins are washed out of the column. At a flow volume of 40

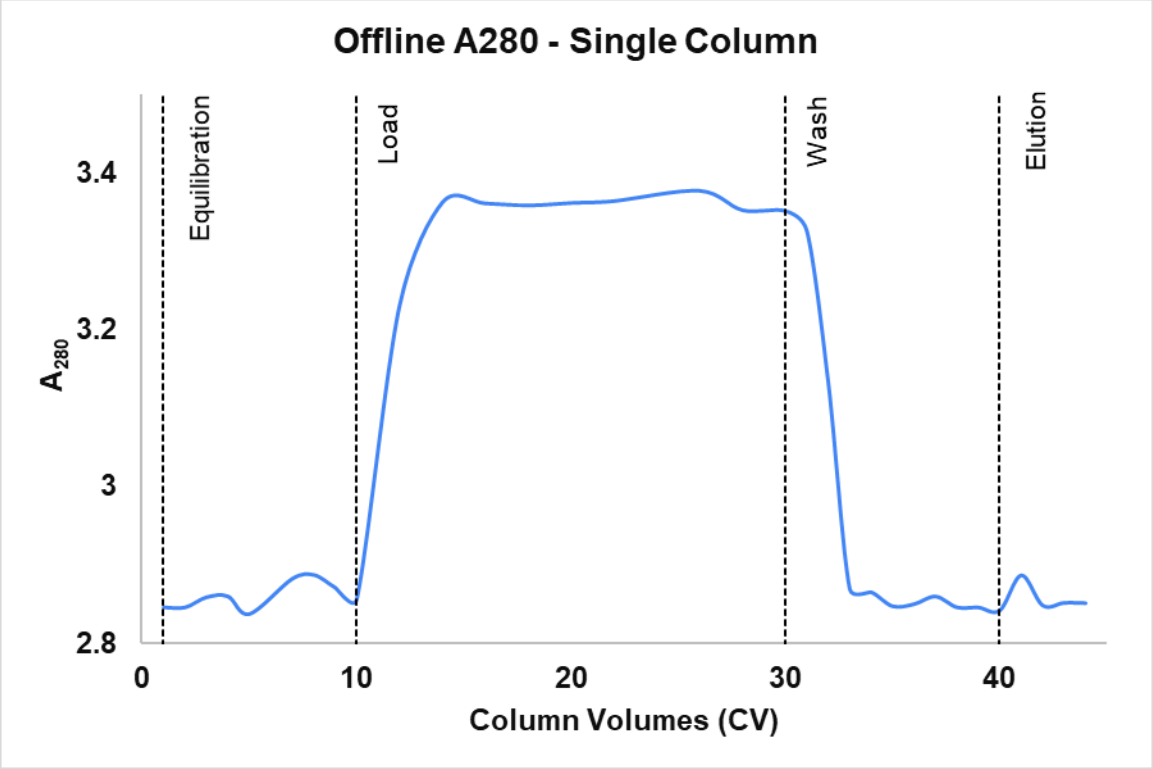

Figure 4. Chromatogram for a protein A single column run with a 20 CV load. Equilibration, loading, washing, and elution steps are distinguishable in the chromatogram generated from this run.

CV, the elution of the column started. An elution peak is seen between 40-43 CV. Since the cell line used in the perfusion process has a very low titer, the elution peak is quite small.

5.4.3 Double Column Application at 80% Capacity

The first double-column operation was conducted to mimic the load of the permeate at a capacity utilization of 80 % for both columns. In this operation, each column was underloaded to prevent product loss due to the breakthrough of the product during the loading step. The loading step with 25 CV and the washing, elution, and equilibration steps with a total of 25 CV alternated in each column. The process volume, the valve position, and the residence time in each step are described in Table 3. The process information is programmed to control the operation of valves and pumps. Before initiating the two-column operation, an equilibration step is operated in column one. After this process step, the loading of the permeate from the perfusion process onto two columns is continuously operated. The product stream was loaded directly from the perfusion permeate line at a flow rate of 0.7 mL/min, resulting in a residence time of 1.4 min. Our two-column system allows the product to be captured immediately into the resin after it is pumped out of the bioreactor, which could be essential for unstable products. Two 1 mL protein A columns were used, and a chromatogram was generated offline by collecting one to two CV fractions throughout the run.

Table 6. Double Column Chromatography Method - 80% Capacity (EQ: equilibration)

Column 1						Column 2					
Step	Valve	Position	Stream	CV	Block Time (min)	Step	Valve	Position	Stream	CV	Block Time (min)
Equilibration	A	1	EQ Buffer	10	14.3						
	B	1	Waste								
Load	A	2	Load	25	35.8	Wash	C	1	EQ/Wash Buffer	10	14.3
							D	4	Wash Collect		
						Elute	C	3	Elution Buffer	5	7.2
							D	2	Eluent		
	B	3	Flow through			Equilibrate	C	1	EQ/Wash Buffer	10	14.3
							D	1	Waste		
Wash	A	1	EQ/Wash Buffer	10	14.3	Load	C	2	Load	25	35.8
	B	4	Wash Collect								
Elute	A	3	Elution Buffer	5	7.2						
	B	2	Eluent								
Equilibrate	A	1	EQ/Wash Buffer	10	14.3		D	3	Flow through		
	B	1	Waste								

The chromatogram for one cycle is shown in Figure 5. The cycle begins with a 10 CV equilibration in column one, followed by a 25 CV load of the permeate. An increase in the absorbance is observed as the sample is loaded, indicating the permeate from the bioreactor is loaded onto column one. Upon the completion of the loading of 25 CV to column one, the equilibration of the second column with 10 CV of an equilibration buffer is finished and the second column is ready for loading. The loading of the permeate was switched to column two with a loading volume of 25 CV, meanwhile, the first column entered the stages of washing, elution, and equilibration. At 45 CV in column one, elution started, and an elution peak can be observed between 45-48 CV. At a total flow volume of 60 CV, column two started a 10 CV wash after completion of 25 CV loading, and this step helps remove the unbound proteins, which is indicated by a decrease in the absorbance. Elution in column two started at 70 CV, and an elution peak is observed between 70-73 CV. This chromatogram in Figure 5 supports that the valves, pumps, and

columns are operated successfully to capture and elute the product from two columns, importantly, the system developed is capable of operating continuously and directly from the permeate stream of a perfusion operation.

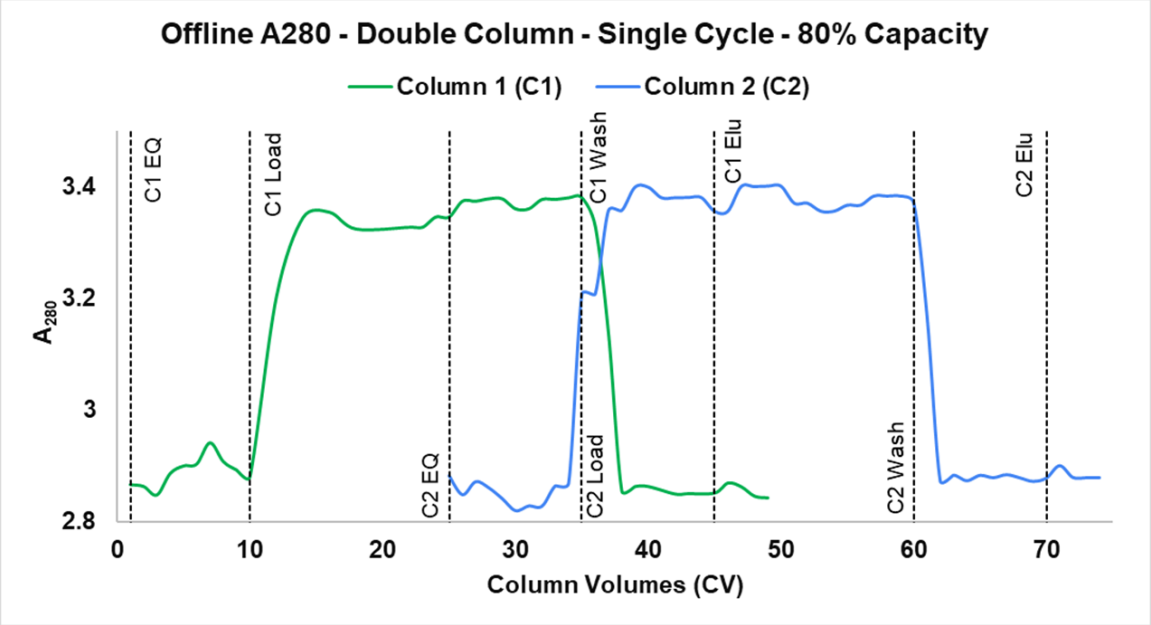

Figure 5. Chromatogram for protein A capture at 80% capacity using the double-column system developed in-house. A_{280} was measured over 75 CVs (column volumes), which is corresponding to one cycle of the method described in Table 3.

5.4.4 Double Column Application at 100% Capacity

Although continuous capture of the permeate directly from the permeate line through the two-column system is realized with the 80% capacity method, 20% of the resin capacity in each column is not utilized. To achieve 100% utilization capacity and prevent product losses in each column, each column is overloaded with the permeate. One column is initially loaded with the permeate and the product is bound to the Protein A resin. When the resin in the column is saturated with the product, excess products are

unable to bind to the resin in the column, and they flow through the column. Rather than directing the flowthrough stream containing the product to the waste collection, the

Table 7. Double Column Chromatography Method at 100% Capacity

Column 1						Column 2					
Step	**Valve**	**Position**	**Stream**	**CV**	**Time (min)**	**Step**	**Valve**	**Position**	**Stream**	**CV**	**Time (min)**
Equilibration	A B	1 1	EQ Buffer Waste	10	14.3						
Load	A	2	Load	25	21.4	**Wash**	C D	1 4	EQ/Wash Buffer Wash Collect	10	14.3
	B	3	Flowthrough Collection			**Elute**	C D	3 2	Elution Buffer Eluent	5	7.2
	B	5	Redirect to Column 2	10	14.3	**Equilibrate**	C D	1 1	EQ/Wash Buffer Waste	10	14.3
Wash	A B	1 4	EQ/Wash Buffer Wash Collect	10	14.3	**LFT from C1**	C	6	Flowthrough Column 1	10	14.3
Elute	A B	3 2	Elution Buffer Eluent	5	7.2	**Load**	C	2	Load	25	21.4
Equilibrate	A B	1 1	EQ/Wash Buffer Waste	10	14.3		D	3	LFT		
LFT from C2	A	6	Flowthrough Column 2	10	14.3		D	5	Redirect to Column 1	10	14.3

stream from this column is redirected to load onto the other column. In this way, the utilization capacity of the resin in the first column reaches 100%, and excess unbound products are loaded onto the second column to avoid product loss. By alternating this operation, both columns could have a capacity utilization of 100%. Table 4 describes detailed process information to perform this operation. The feed stream was loaded onto two columns directly from the perfusion permeate line at a residence time of 1.4 min. Figure 6 shows the resulting chromatogram from one to two CV fractions collected throughout the run. Typical chromatograms are seen for each column. Distinct operation steps are differentiated from the absorbance values. An elution peak is observed in each column, indicating the product is successfully captured and eluted via

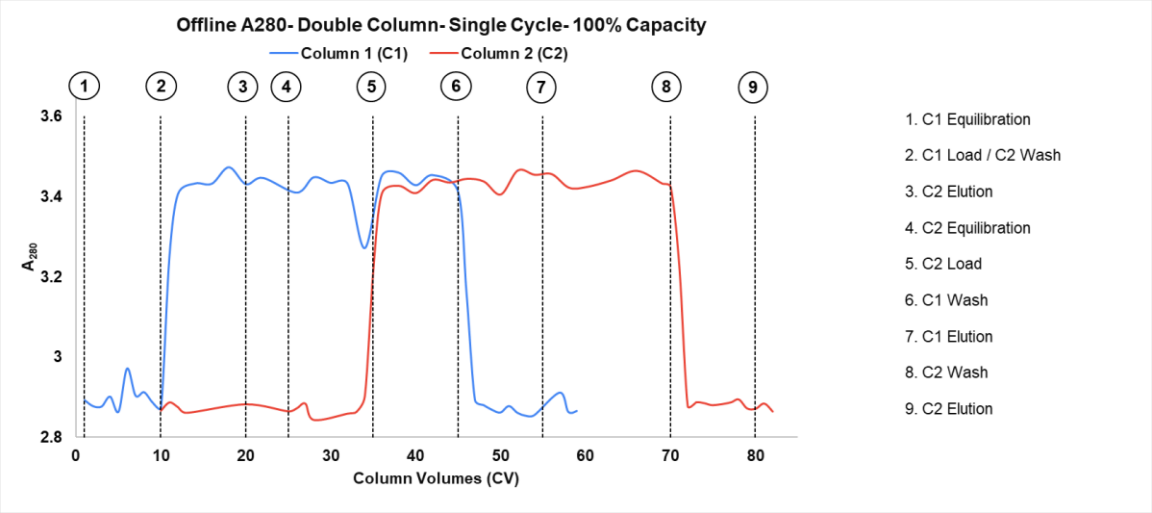

Figure 6. Chromatogram for protein A purification using the double-column system developed in-house. A_{280} was measured over 85 column volumes which is equivalent to one cycle of the method described in Table 3.

this two-column system. It is noted that there is an overlapping region from 35 to 45 CV for both columns with a high absorbance value. The overlapping occurs when the flowthrough from the column one is redirected and loaded onto the column two. In this way, products in the permeate stream can be fully captured, while the column resin can reach a 100% utilization capacity.

This proof-of-concept study has demonstrated the potential of the two-column system for the application in monoclonal antibody capture via the Protein A resin. The system hardware can be improved through (1) the implementation of on-line detectors including UV-280, pH and conductivity sensors and air sensors; (2) replacement of two peristatic pumps with two piston pumps (metering type); and (3) installation of mixers for gradient elution and prefilters. Currently the switch between two pumps is based on the equal column volume (or block time) for the loading step in one column and

other steps in the other column. The control system could be refined to allow flexibility in varied feed stream compositions, flow rates, different residence times for equilibration, wash, and elution buffers. Since the current control system is programmed in Python, physical/mechanistic models in combination with artificial intelligence models could be integrated into the control system for better automated control of the system and a higher resin utilization capacity, leading to a higher production yield, and a better efficiency in impurity removal. Ultimately, this multi-functional two-column system could be used for continuous capture/purification of products, especially emerging modalities, directly from the permeate from a bioreactor.

5.5 CONCLUSION

A flexible two-column chromatography system was assembled from valves, pumps and two 1 mL Protein A columns with the control by a Python-based in-house software. This system was applied to a bench-scale process for capturing a mAb in the permeate during a perfusion process. Three operations were successfully realized in this two-column system, including a single-column operation, a two-column operation with the loading of the mAb product to 80% of the resin capacity in each column, and a two-column operation with the loading of the product to 100% of the resin capacity in each column. The permeate from the permeate line was directly loaded onto the columns without any holdup tanks. A residence time of 1.4 min was applied to the feed stream and buffers. The offline-generated chromatograms display distinct stages for equilibration, loading, washing, and elution. Alternating operations in two columns between loading

and non-loading steps are successfully conducted. This proof-of-concept study demonstrates the feasibility of using an in-house lab-scale two-column system for continuous capture of products in the permeate during a perfusion process, laying the foundation for an in-house integrated perfusion and capture system.

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6 CONCLUSION & FUTURE DIRECTION

Current bottlenecks of implementing continuous bioprocessing include technologies for real-time monitoring and control of critical/key process parameters and versatile scale-down models for process understanding and development. Commercially available platforms for continuous bioprocessing are often expensive and inflexible. Technologies such as Raman spectroscopy, perfusion cell culture, and continuous chromatography have been explored in my PhD project to develop a versatile bench-scale continuous platform driven by open-source software as a proof-of-concept.

Methodology and software for building Raman-based calibration models for cell culture metabolites have been developed. The preprocessing methods for the calibration dataset include reducing each spectrum to a fingerprint region (350 to 1750 cm^{-1}), applying the Savitzky-Golay smoothing technique to improve the signal-to-noise ratio, and selecting a second derivative for baseline correction. The Python-based software is capable of generating PLS calibration models, performing cross-validation, determining calibration performance based on R^2C, R^2CV, RMSEC, and RMSECV, and predicting the metabolite concentrations from pre-processed Raman spectra. No commercial software requirements endow this methodology with flexibility and inexpensiveness. Separate Raman-based calibration models have been built for glucose, glutamine, glutamate, lactate, and ammonium. These models consistently have high R^2 and low RMSE values, indicating that these models have great predicting capabilities.

An in-house Raman-based monitoring and feed control system was developed using the Raman calibration models with R^2CV values above 0.7. The system is successful in monitoring the concentrations of glucose, glutamine, glutamate, lactate, and ammonium during the cell culture process, and controlling the glucose and glutamine concentrations at their pre-specified setpoints in a fed-batch and perfusion operation. The predicted metabolite concentrations from the PLS models are in alignment with the offline measurement values within their model ranges in the fed-batch operation, and their RMSE values are 0.27,0.41,0.15, 0.35, and 2.8 for glucose, glutamine, glutamate, ammonia, and lactate, respectively. These RMSE values are better than or similar to those reported in the literature. These RMSE values could be further improved by increasing the calibration sample size, optimizing the number of components, and expanding the model range. The glucose and glutamine concentrations are controlled within 0.5 g/L and 0.3 mmol/L of their setpoints of 5 g/L and 1.0 mmol/L, respectively. The fluctuations could be mitigated via the implementation of an immersion Raman probe. Two regions with distinguished model performance are seen in the perfusion process. The models perform well in the initial culture period in the perfusion process since the media composition and VCD in this period are similar to those in a batch or fed-batch process. Deviations of metabolite concentration predictions from the offline measurements are found when the VCD of $20x10^6$ cells/mL is reached. This deviation could be ascribed to the calibration models developed from the batch and fed-batch data at a VCD of less than $9 x10^6$ cells/mL. However, these models can be improved by feeding the perfusion data back to the models to increase the model range and enhance their predicting capability. Meanwhile, the

glucose and glutamine concentrations in the bioreactor at a constant VVD of 0.5 are controlled within 0.3 g/L and 0.3 mmol/L of their setpoints of 4 g/L and 0.5 mmol/L, respectively, leading to a reduction in the media consumption.

A flexible two-column chromatography system has been developed along with Python-based software and applied to a bench-scale integrated perfusion and capture process for mAb production. Three different methods are used to test the system, including a single-column operation, a two-column operation at a resin loading capacity of 80%, and a two-column operation at a resin loading capacity of 100%. Chromatograms from three runs display four distinct operation steps for each column including equilibration, loading, washing, and elution. Continuous loading of the permeate directly from the bioreactor without holdup tanks onto the two-column system is achieved via a time-controlled mode. Overloading into two columns to reach the loading capacity of 100% could significantly reduce the use of resins and buffers.

The preliminary data support that this bench-scale platform is readily maneuverable to customized requirements, adaptable for the production of different modalities, and much cheaper for implementation.

This bench-scale system could be significantly improved by the implementation of a Raman immersion probe, which would allow more data points to be generated from batch, fed-batch and perfusion processes for building the calibration models and real-time in-line monitoring of cell culture metabolites. Increasing the number of data points is expected to improve the model predicting capability and the concentration range, while

real-time inline monitoring enables frequent sampling, thus realizing automatic control of the glucose and glutamine concentrations and reducing their fluctuations.

The next improvement would be the implementation of UV, pH and conductivity sensors to the multi-column chromatography system. With the aid of these sensors, real-time chromatograms could be produced to monitor different process steps, meanwhile of the two-column system could be operated in a dynamic control mode through which the column switch is based on breakthrough instead of time.

The last improvement is to develop a graphical user interface (GUI) for the Raman-based control system and the multi-column chromatography system. For the control system, a GUI allows real-time visualization of the predicted values, the weight of the bioreactor, and the pumping rates over the culture period. The current system prints the predicted values and the amount of feed dispensed on the screen and writes these values to a file. This GUI could be more user-friendly when interacting with the system, such as selecting different pre-processing methods and setting/changing the concentrated feed concentrations and the glucose and glutamine setpoints. The GUI for the chromatography system would also be more user-friendly for inputting start-up chromatography values, such as the load volume per column. In combination with the UV modules, the GUI would allow real-time chromatograms to be visualized.

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