CAPILLARY ELECTROPHORESIS IN PROCESS ANALYTICAL TECHNOLOGY

MONITORING OF BIOPHARMACEUTICAL CULTIVATION PROCESSES

Debbie van der Burg

Capillary electrophoresis in process analytical technology

Monitoring of biopharmaceutical cultivation processes

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ABBREVIATIONS

2-AA	2-aminobenzoic acid
2-AB	2-aminobenzamide
2-PB	2-picoline borane complex
AA	Amino acid
APTS	8-aminopyrene-1,3,6-trisulfonic acid
AQbD	Analytical Quality by Design
ATP	Analytical target profile
BFS	Bare fused silica
BGE	Background electrolyte
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CHO	Chinese hamster ovary
cIEF	Capillary isoelectric focussing
CPP	Critical Process Parameter
CQA	Critical Quality Attribute
CZE	Capillary Zone Electrophoresis
DoE	Design of Experiments
DS	Drug substance
DSP	Downstream processing
DXS	Dextran sulfate
EOF	Electro-osmotic flow
FASS	Field-amplified sample stacking
HC	Heavy chain
HPMS	High-pressure mass spectroscopy
IA	Immunoassay
icIEF	Imaging capillary isoelectric focussing
IEC	Ion exchange chromatography
ITP	Isotachophoresis

LC	Light chain
LE	Leading electrolyte
LED	Light emitting diode
LWM	Low molecular weight
mAb	Monoclonal antibody
MCE	Microchip electrophoresis
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
PAT	Process analytical Technology
PB	Polybrene
pI	Isoelectric point
PL	Pyridoxal
PM	Pyridoxamine
PN	Pyridoxine
PTM	Post translational modification
PVA	Polyvinyl alcohol
QbD	Quality by Design
SDS	Sodium dodecyl sulfate
SMIL	Successive multiple ionic-polymer layers
TE	Terminating electrolyte
TEA	Triethylamine
T-EthA	Triethanolamine
Tris	Tris(hydroxymethyl)aminomethane
USP	Upstream processing

SUMMARY

Biopharmaceuticals are drugs that are produced in living organisms, such as fungus, bacteria, or mammalian cells, and they include a wide variety of products among which proteins, antibodies, nucleic acids, and vaccines. They are important products in the pharmaceutical industry, used to treat a wide range of diseases, including cancer, autoimmune disorders, and infectious diseases. These drugs are typically larger and much more complex than traditional small molecule drugs. This makes their production more challenging. Biopharmaceuticals also require more rigorous testing and regulatory approval processes than traditional drugs, which can add to the cost and time required to bring them to market.

Biopharmaceutical process monitoring is thus of utmost importance. Process Analytical Technology (PAT) is a system for designing, analysing, and controlling manufacturing processes by real-time monitoring of the process. Many PAT tools include probes, sensors, and spectroscopic techniques, and recently more complex analytical techniques such as capillary electrophoresis (CE), liquid chromatography, and mass spectrometry are being used.

The upstream process comprises the expression of the drugs in cells in a bioreactor filled with a medium consisting of all nutrients the cells require. This process can take up to days to weeks and needs to be closely monitored to ensure an ideal environment for the cells to grow and produce the therapeutic drug. Process monitoring includes process parameters such as temperature, pH, oxygen levels, nutrients such as amino acids, saccharides, and vitamins, and by-products produced by the cells that could inhibit growth. The European project iConsensus was initiated to develop a fully automated, integrated monitoring platform for the real-time monitoring of the upstream biopharmaceutical process. Part of this platform are CE applications to monitor various nutrients, and the production, or concentration, of monoclonal antibodies (mAbs). In order to fit in the automated, integrated platform, and to allow for automation, these applications should be transferred to microchip electrophoresis (MCE).

This thesis provides an overview of capillary and microchip electrophoresis applications in the biopharmaceutical industry and focuses on the development of (M)CE applications for upstream process monitoring. Four methods were successfully developed for the monitoring of nutrients (mono- and disaccharides and vitamins) and for mAb concentration. The methods were developed to enable automation; they are robust and facilitate rapid analysis with minimal sample preparation requirements. The vitamins and mAb methods require no sample pretreatment or only a simple (automated) dilution. The saccharide method requires fluorescent

derivatisation, which was developed to allow for automation in a closed system. Method optimisation with Design of Experiments optimised all significant factors simultaneously, reducing the number of experiments required. It provided information on the relationship between the tested factors and the response over the design space, including information on the influence of or the interaction between factors. To allow transfer from conventional CE to microchip CE (MCE), the CE methods were developed on capillaries with short effective lengths, similar to the separation length on chip. Additionally, fused silica chips were selected, as their surface chemistry is similar to fused silica capillaries commonly used for conventional CE. The method for saccharides was successfully transferred to MCE and the method for mAb concentration monitoring is ready for transfer to MCE. All developed methods show good potential for integration in the automated monitoring platform as well as for use as stand-alone PAT tools.

Chapter

Introduction and scope

1.1 BIOPHARMACEUTICALS

Biopharmaceuticals are important products in the pharmaceutical industry and the market is rapidly increasing. By 2018 a total of 374 individual biopharmaceutical products have been approved in the European Union and/or in the United States [1]. They include a wide variety of products such as antibodies, Fc-fusion proteins, hormones, enzymes, and vaccines, and have proven to be effective in treating a wide range of diseases, including treatments of chronical diseases, infections, cancer, and autoimmune diseases [2,3]. Biopharmaceuticals are produced by living organisms such as fungus (yeast), bacteria (*Escherichia coli*), or mammalian cells (often Chinese hamster ovary (CHO) cells). The latter are predominantly used for therapeutic proteins like monoclonal antibodies (mAb), due to their ability to incorporate post translational modifications (PTMs) and biosynthetic complexity into the targeted protein [4]. The biopharmaceutical process is complex. Many factors, such as the host cell line, cell culture medium, and process conditions, affect the quality of the product [5].

The production can be divided into two main processes; upstream processing (USP) which consist of cell growth and the expression of the drug by the cells, and the downstream processing (DSP) where the biopharmaceutical is purified from process-derived impurities and formulated in an appropriate solution. In both UPS and DSP, process analysis is an essential part. In USP, the cells are cultivated in bioreactors containing cell culture medium. This is a complex system where the conditions have to be optimal for cell growth and the production of the therapeutic protein. The cell culture medium has a large impact on cell growth as well as on product concentration and quality. Accumulation of metabolic by-products and depletion of nutrients are limiting factors for cell growth and production [6,7]. This issue is addressed by using fed-batch mode, the standard mode of operation, where the cells are provided with sufficient nutrients during the process by administrating a feed solution. More recently, perfusion modes are being implemented, where the culture conditions are maintained stable by both continuously administrating nutrients and removing metabolic waste from the culture [8]. Both strategies improve cell growth and production yield by prolonging the phase where viable cells are present at high density in a productive state [9].

As biopharmaceuticals are typically administered by injection, it is essential that they are sterile and non-pyrogenic [10,11]. Therefore, the DSP process focusses on purifying the drug from process-derived impurities such as lipids, antifoaming agents, antibiotics, and host cell proteins, and extrinsic impurities such as viruses, bacteria, or fungi, as well as product formulation into a stable, safe and efficacious drug

product. Often chromatographic purification methods such as gel filtration, ion exchange, hydrophobic interaction and affinity chromatography are used [11].

1.1.1 MONOCLONAL ANTIBODIES

The most rapidly growing class of biopharmaceuticals are mAbs [12,13]. The first therapeutic mAb was approved in 1986, it took eight years for the second mAb to be approved. Currently, more than 100 mAbs have been approved, of which more than 50 approved mAbs during the last six years [14], indicating the rapid increase in new therapeutic mAbs.

Antibodies are complex glycoproteins produced by the body as part of the immune response to bind to specific antigens. The high specificity of antibodies makes them suitable as therapeutic proteins. Five different classes of immunoglobulins are present in humans: IgA, IgD, IgE, IgG, and IgM. All of the currently marketed recombinant mAbs are IgG antibodies [15].

mAbs are composed of four polypeptide chains; two identical heavy chains (HC) and two identical light chains (LC), connected to each other by disulfide bonds, forming the typical Y-shape (Fig 1). Each chain is composed of several constant and one

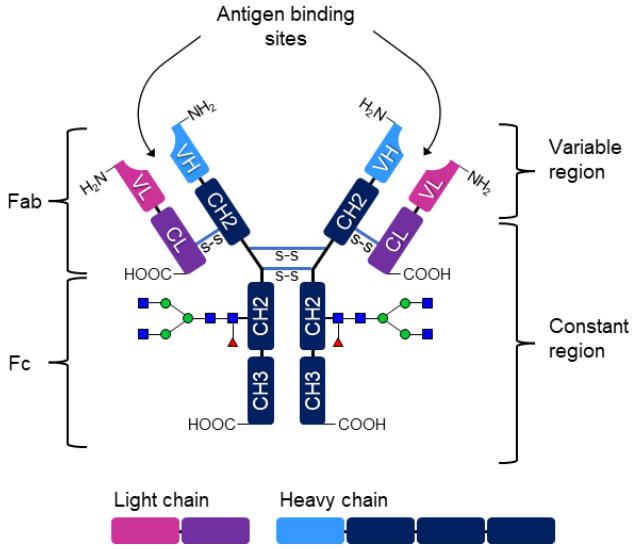

Figure 1. The schematic structure of an antibody. Heavy and light chains bound by disulfide bonds, comprised of constant and variable domains (CH: heavy chain constant domain, CL: light chain constant domain, VH: heavy chain variable domain, VL: light chain variable domain). N-glycan attached to the CH2 domains of the mAb.

variable domain. The variable domains can bind to specific antigens and are positioned at the top of the heavy and light chain. The arms containing the variable domains are called the “antigen binding fragments” or Fabs. The stem of the Y-structure is called the “fragment crystallizable region” or Fc. The Fc region determines the class of the antibody and its functional properties [16]. mAbs are large molecules (~150 kDa) and although the primary structure is homogeneous, the large variation of modifications mAbs can undergo introduces heterogeneity.

1.2 PROCESS MONITORING

1.2.1 CRITICAL QUALITY ATTRIBUTES

Every drug product should adhere to a certain product quality. According to the ICH Q8 (R2) guideline, pharmaceutical development should include the determination of critical quality attributes (CQAs) [17]. A CQA is a product property that should be kept within a certain range to ensure product quality, typically a property affecting safety or efficacy of the drug product [18].

One factor affecting safety and efficacy is the immunogenicity of biopharmaceuticals, by potentially causing reduced drug half-life, neutralization, or even anaphylaxis, a severe, potentially fatal allergic reaction [19]. An immune response occurs when the human immune system categorizes the therapeutic protein as foreign. For mAbs, the risk of immunogenic responses could therefore be reduced by using human-like antibodies [19].

Proteins are known to undergo a wide variety of PTMs including aggregation, fragmentation, C- and N-terminal modifications, oxidation, deamidation, glycosylation, and disulfide bond modifications. As PTMs can affect safety and efficacy, many are marked as CQAs [18]. The efficacy of therapeutic proteins is highly dependent on its structure. Aggregation of therapeutic proteins can cause loss in function and increase the risk of immunogenic responses, causing adverse effects ranging from protein neutralisation to anaphylaxis [20]. N-glycosylation, the most common form of glycosylation, influences many factors such as biological activity, stability against proteolysis, pharmacokinetics, serum half-life, immunogenicity, and antibody effector functions [21]. These effector functions are important for the efficacy of antibodies for cancer treatments. Many PTMs influence the charge heterogeneity of therapeutic proteins. PTMs could result in more acidic species, for example by sialyation, deamidation, or glycation, or in more basic species, by for example succinimide formation or incomplete removal of C-terminal lysine. Depending on the position of the PTM, it could affect antigen binding affinity,

potency, secondary structure, and aggregate formation [22]. It is essential to determine what the CQAs are and to control them early on in the process. This aids the production of well-defined therapeutic proteins.

1.2.2 PROCESS ANALYTICAL TECHNOLOGY

Process parameters affecting CQAs are called critical process parameters (CPPs) and should be tightly controlled. Quality by Design (QbD) is a concept where acceptable ranges of CQAs and CPPs are defined for the biopharmaceutical process so that the process can be controlled to stay within these ranges [23]. These ranges can only be defined with a deep understanding of the effect of CPPs on CQAs. To increase this understanding, and to build in quality into the pharmaceutical manufacturing process, all factors should be monitored.

The Food and Drug Administration started the Process Analytical Technology (PAT) initiative to gain a deeper understanding of the production process, and with that, more predictable and efficient manufacturing [24]. PAT is a system to aid real-time monitoring to design, analyse, and control the biopharmaceutical process. Ideally, the conditions and quality of the process and product should be known during the entire process. Currently, this knowledge is often lacking. This knowledge gap could be filled by PAT solutions that monitor the process in real-time [25]. The information obtained with PAT could then be used to correct the process based on the control space determined by QbD [23]. PAT includes in-line and at-line analysis performed on-site in (near) real-time. In-line analyses are performed directly in the bioreactor without sampling, with for example sensors. At-line analyses require bioreactor sampling before analysis. This could either be online, with automated sampling and analysis, or offline, requiring manual steps. Offline analysis where samples are transferred to different laboratories for analysis, or that are not directly performed after sampling, are not considered PAT.

Many PAT tools include in-line probes and at-line sensors and spectroscopic techniques [26]. Recently, the PAT toolbox is further increased by using more complex analytical techniques like liquid chromatography, capillary electrophoresis (CE), and mass spectrometry (MS) [27]. CE is a suitable technique for biopharmaceutical process monitoring. The simple setup, its capacity to handle complex matrices, the limited sample clean-up required, low sample volumes (nL range), fast analysis, and the possibility for miniaturisation and automation provide great possibilities for implementation in PAT. CE is especially useful for monitoring micro-bioreactors or analysis during clone selection where only a limited sample volumes are available.

1.3 THEORETICAL BACKGROUND

1.3.1 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is a separation technique which separates analytes based on their charge and size [28,29]. A capillary with an inner diameter typically of 25 – 75 µm is filled with a conducting buffering solution, the background electrolyte (BGE). Sample is introduced either hydrodynamically or electrophoretically and a high voltage is applied over the capillary, resulting in a constant electric field strength. A schematic illustration of a CE system is depicted in Figure 2. In the electric field, charged molecules have an electrophoretic mobility in the direction of the oppositely charged electrode. Molecules with a higher net charge are affected more by the electric field and, consequently, have a higher electrophoretic mobility. Larger molecules experience more friction, reducing their electrophoretic mobility. Separations in CE are based on charge-to-size ratio differences.

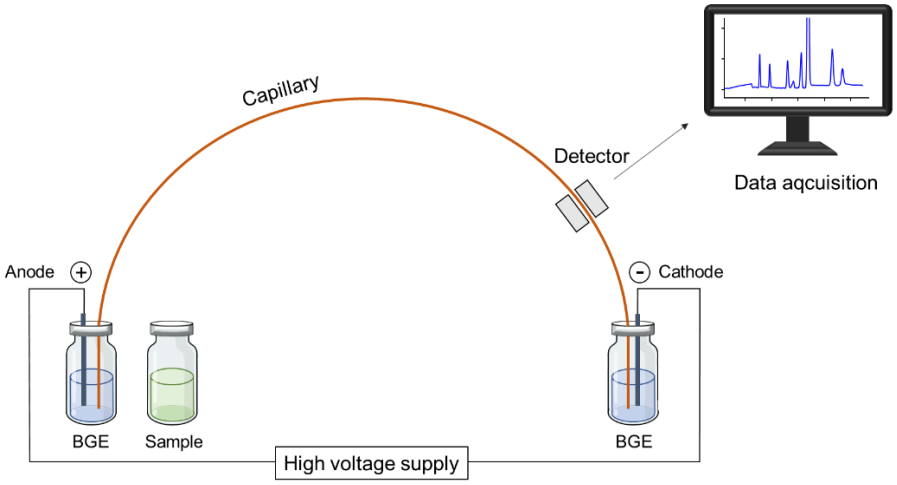

Figure 2. Schematic illustration of a capillary electrophoresis system.

CE is often performed in fused silica capillaries, where free silanol groups at the capillary wall could be deprotonated resulting in a negative charged at the capillary wall. When the capillary wall is charged, an electric double layer is formed by ions in the buffer to balance the charge on the capillary wall. The electric double layer consists of a static Stern layer and a mobile diffuse layer. When an electric field is applied over the capillary, ions in the diffuse part of the electric double layer move and drag the bulk liquid along, creating a plug flow. This flow is called the electro-osmotic flow (EOF). The EOF is affected by several factors, such as the ionic strength

of the buffer and the charge on the capillary wall [30,31]. The apparent mobility of an analyte in the capillary is the sum of the mobility of the analyte (electrophoretic mobility based on its charge-to-size ratio) and the mobility of the EOF (electro-osmotic mobility, constant for all analytes in a certain system) (Figure 3). The mobility of the EOF is dependent on a large number of factors, including pH, ionic strength, capillary coatings, and BGE additives. With a slow or negligible EOF, anions migrate in the direction of the anode, cations migrate in the direction of the cathode, and neutral molecules have no mobility. When a strong EOF is present, typically in the direction of the cathode, cations, anions, and neutral analytes can be detected in one run at the cathodic side of the capillary. Note that neutral molecules will all migrate with the velocity of the EOF and, therefore, will not be separated. As the separation is determined by the electrophoretic and electro-osmotic mobilities, factors influencing these mobilities must be firmly controlled (1.5).

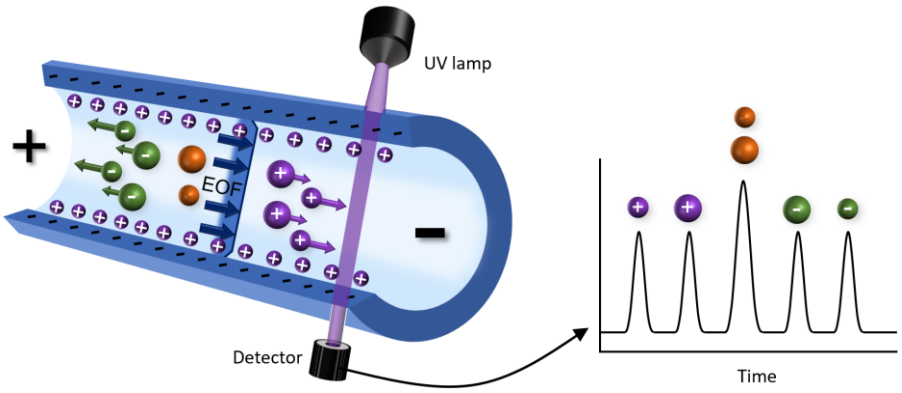

Figure 3. Schematic illustration of the electrophoretic mobilities of anionic, neutral, and cationic species, and the EOF in a CE capillary. Anions have an electrophoretic mobility in the direction of the anode, cations in the direction of the cathode, and neutrals do not have an electrophoretic mobility. In this illustration, the mobility of the EOF inside the capillary is stronger than the electrophoretic mobilities, dragging along all analytes in the direction of the cathode, where the detector is placed.

Different modes of separation can be achieved by adding certain additives to the BGE. Capillary zone electrophoresis (CZE) is the simplest mode of CE and most widely used [28,32]. The BGE consist of buffers and salts and the separation mechanism is as described above. The selectivity can be easily influenced by varying the BGE pH or by using additives. CZE can be applied to a large variety of analytes, products, impurities, nutrients, metabolites etc., from small (e.g., ions) to large (e.g., intact mAb, intact virus) analytes.

For the separation of neutral molecules, or additional selectivity, micellar electrokinetic chromatography (MEKC) could be used. This CE mode combines electrophoretic and chromatographic principles by the addition of micelles to the BGE [28,31,33–35]. Surfactants are added to the BGE in a concentration above their critical micelle concentration to create micelles. These micelles are often spherical, with the hydrophobic tail directed towards the centre of the micelles and the charged heads to the surface. The micelles act as a pseudo-stationary phase, adding the chromatographic aspect by differential partitioning of the analytes between the micelles and the aqueous BGE. Micelles are usually charged and thus have their own mobility. The mobility of analytes that interact with the micelles will be between their own mobility and the mobility of the micelles, hence neutral molecules migrate between the migration time of the micelles and the migration time of the EOF. This is called the separation window, which can be increased by reducing the mobility of the EOF or by using micelles with high mobility in opposite direction of the EOF. Selectivity can be enhanced by using different surfactants or by using several surfactants at once. Surfactants can be anionic, cationic, non-ionic, or zwitterionic, with varying alkyl chain lengths or structures. The most commonly used surfactant is sodium dodecyl sulfate (SDS). Factors such as buffer concentration, pH, temperature, additives, and organic modifiers can all influence the analyte/micelle interaction.
Size heterogeneity could be determined using capillary gel electrophoresis (CGE) [36–38]. A gel or polymeric network is added in low concentration to the BGE. Proteins are denatured with SDS, which gives them a uniform charge-to-size ratio, allowing for a size-based separation through the gel sieving-matrix in the capillary.
For charge heterogeneity, capillary isoelectric focusing (cIEF) or imaging cIEF (icIEF) could be used to determine the apparent p*I*. The capillary is filled with ampholytes within a specific pH range to create a pH gradient in the capillary [37,39,40]. This approach provides information on the levels of acidic, main, and basic variants.

1.3.2 MICROCHIP CAPILLARY ELECTROPHORESIS

The relatively simple setup of CE allows for miniaturisation to microchip CE (MCE) [41–43]. Chips with dimensions in the mm to cm scale are used with channel dimensions in the order of 10 – 100 µm. The sample and BGE consumption are even lower than for CE, with sample volumes down to the pL range. The small volumes improve heat transfer, allowing for the use of high voltages without Joule heating. The short separation channels increase the analysis speed and throughput, which could be even further increased by the use of parallel channels. The chip design could be made in such a way that allows for on-chip sample preparation, mixing, dilution etc. Generally, glass (*e.g.*, quartz, borosilicate) or polymeric (*e.g.*,

polydimethylsiloxane or polymethylmethacrylate) are used as substrate [44]. Polymeric chips are often cheaper than glass, and easier to fabricate. However, the EOF is more difficult to control. Controlling the EOF is even more important for MCE analyses than for CE analyses, because it is also used for sample injection, as explained later in the text. Glass chips, especially fused silica chips, are more similar to CE capillaries, and their surface chemistry is better understood. BGE/sample reservoirs and sample and separation channels are etched in the chip. Electrodes coupled to a high voltage supply are placed in the BGE/sample reservoirs and detection is performed directly on the chip, see Figure 4 for a schematic illustration.

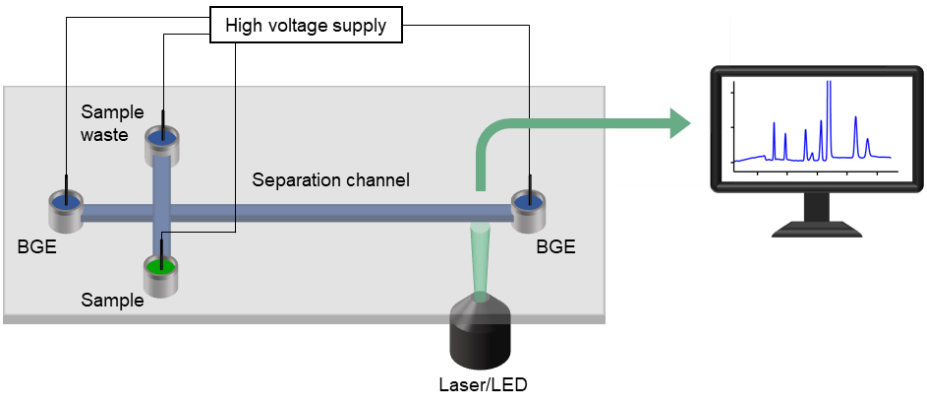

Figure 4. Schematic illustration of a microchip electrophoresis system.

1.3.2.1 SAMPLE LOADING

In conventional CE, sample is injected by placing a sample vial at the capillary end and applying pressure or voltage. As this physical movement of the channel to various reservoirs is not possible in MCE, the injection is more complex. In MCE, the sample and BGE solutions are all on the same chip, and additional channels are required to inject sample. Frequently used channel geometrics contain two channels perpendicular to the separation channel, either at an equal height crossing the separation channel as a T (injection cross) as in Figure 4, or with a slight offset as a double-T (injection offset). Hydrodynamic injection is complicated due to the need of an external pump and the high backpressure in narrow channels. The most common injection in MCE is therefore by pumping with EOF; voltages are applied to the reservoirs in such a way that the EOF, and thus the bulk liquid, is directed from the sample reservoir towards the intersection with the separation channel [45] (Fig. 5). This could either be a gated injection [46] (Fig. 5, top), or cross injection [47] (Fig. 5, bottom). In a gated injection, sample is continuously loaded by EOF pumping from the sample reservoir to the sample waste reservoir. Diffusion of sample into the

separation channel is prevented by also applying a potential from the BGE inlet reservoir to the sample waste and the BGE outlet reservoirs. Injection of the sample is achieved by switching off the voltage on the sample and sample waste reservoirs, resulting in an electrokinetic injection of the sample from the sample channel to the separation channel. A gated injection allows for injection of variable plug lengths, which is uncommon for other MCE injection modes, and for continuous sampling. This injection mode does have a bias towards faster migrating analytes. In a cross injection, the sample is loaded by applying a potential from the sample reservoir to the sample waste reservoir to fill the intersection with the separation channel with sample. If only a voltage is applied to the sample and sample waste reservoirs, a so called “floated” injection, sample can diffuse into the separation channel during sample loading (Fig. 5D). This can be prevented by applying a voltage on the BGE inlet and outlet reservoirs to create a BGE flow towards the intersection and the sample waste reservoir, shaping the injection plug. This is called a “pinched” injection (Fig. 5E). When the sample channel is filled with sample, the voltage scheme is changed such that the EOF is directed from BGE inlet towards BGE outlet, transferring the sample in the intersection into the separation channel (Fig. 5F). To prevent sample leaking into the separation channel during separation, small “pull back” voltages are applied to the sample and sample waste channels. The cross injection minimises the bias toward faster migrating analytes by mobilising a larger volume of sample so that even the slowest migrating analyte is loaded in the intersection. The injected sample volume is defined by the volume of the channel intersection, and cannot be varied. If a larger sample volume is required, the plug length could be increased by using a variant to the cross injection, the offset injection (double-T). Here, there is a slight offset in the sample channel and sample waste channel, such that the sample is loaded on the separation channel into the offset between the sample and sample waste channel.

1.3.2.2 DETECTION

Although UV detection is the most widely used detection technique for conventional CE, it is often insufficiently sensitive for MCE as the dimensions of the microchip result in very small sample volumes and optical pathlengths [43,48]. Instead, the most common detection technique for MCE is fluorescence detection, mainly due to its high sensitivity and selectivity [43,44,48]. High sensitivity is especially important on MCE as the detected volume is very small. Lasers are often used as excitation source, as this is easy to implement on MCE dimensions and the focused laser beam allows the detection of a small detection volume. Lasers are generally expensive, a low-cost alternative for lasers are light-emitting diodes (LED). LEDs have lower power consumption and simpler electronics, making them very suitable for implementation

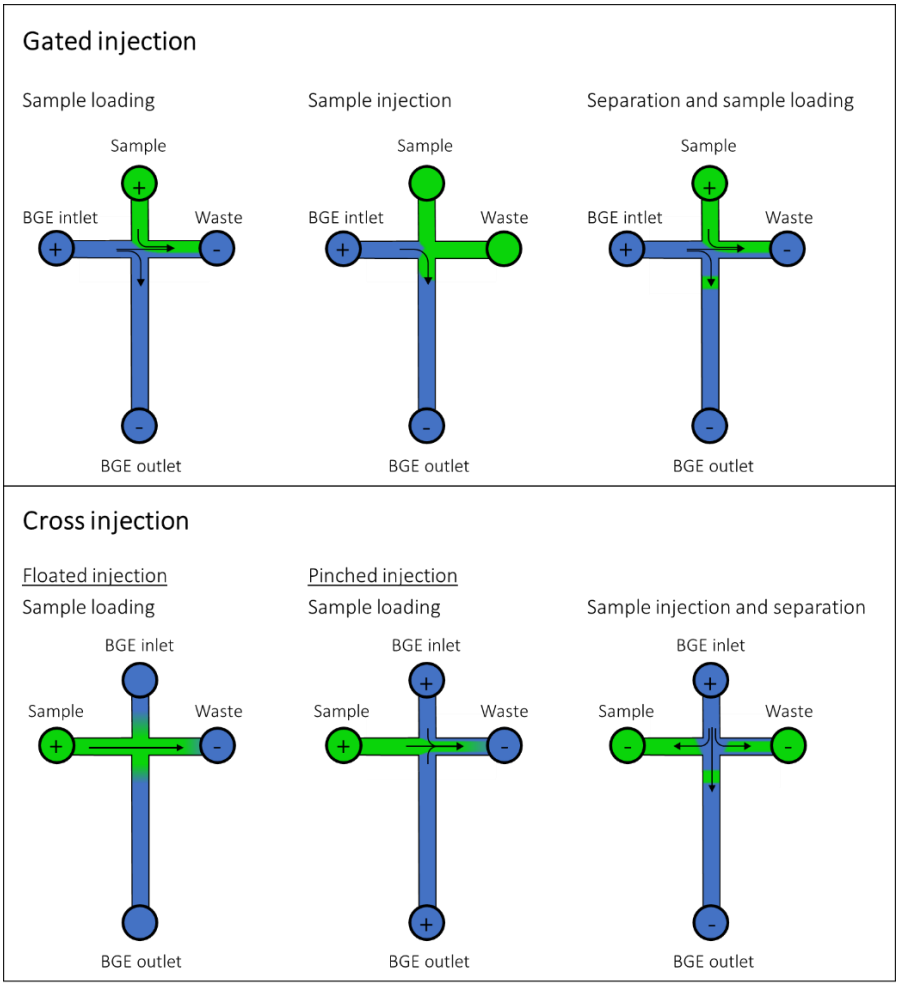

Figure 5. Schematic illustration of injection on MCE, the flow directions are indicated with arrows in the channels. In a gated injection, the sample is injected by shortly switching off the voltage at the sample and waste reservoirs. Returning to the initial voltage scheme starts the separation and continues sample loading. The cross injection is either floated or pinched. During pinched injection sample loading, the sample plug is shaped by applying a voltage to the BGE inlet and outlet reservoirs. Switching the voltage scheme starts the separation of the sample present in the intersection.

on MCE [49]. As their light beam is not as focused as a laser beam, more filters and focusing are required. The high selectivity of fluorescence detection is due to the fact that not many compounds have native fluorescence. This means that derivatisation of the analytes is often required. Another frequently used detection technique is electrochemical detection (*e.g.*, amperometric, potentiometric, conductometric)

[43,44]. Electrochemical detection can be easily implemented on MCE, is low cost and portable, does not require derivatization, and additionally, maintains its performance upon miniaturisation. Coupling of MS to MCE is also possible. This combination provides high sensitivity and high separation efficiency [44,50]. Electrospray ionisation is most frequently used. Additional channels to introduce a sheath liquid can easily be implemented on the chip. A drawback is the expensive and large instrument setup of MS. The large instrument setup is mainly due to the heavy vacuum systems required. The instrument size could be reduced by using high pressure MS [51,52].

1.4 AIM AND CONCEPT OF THE THESIS

The work in this thesis was part of a larger European project, the iConsensus project. This project focuses on developing an integrated control and sensing platform for biopharmaceutical cultivation processes, with the goal to achieve a safer, faster and more cost-effective process for the production of a wide range of biopharmaceuticals. The monitoring of USP is critical for process understanding and development, as well as for manufacturing efficacy and robustness. The vision of this monitoring platform is an automated platform employing several on-/at-line PAT tools for the (near) real-time detection of physical factors, reagents, and IgG quality attributes in the bioreactor. The data should be transferred to a monitoring information database and immediately processed by modelling tools, allowing for efficient feedback control of the cultivation process. A schematic overview of the platform is depicted in Figure 6. Many factors can be monitored using sensors and affinity-based techniques, but some analytes require separation prior to detection.

CE has proven to be a valuable technique for biopharmaceutical analysis, and has a number of benefits for the analysis of upstream and downstream process-intermediates. CE can handle highly complex matrices such as USP samples, has high resolving power, requires little sample preparation and low solvent and sample consumption, and provides rapid analysis. Additionally, the simple setup provides the possibility for miniaturisation and automation on microchip CE. CE was therefore selected as the separation technique for the monitoring of monosaccharides, vitamins, amino acids, lipids, and mAbs.

The intended purpose of the methods in the monitoring platform is unattended analysis for the duration of the upstream cell process campaign (generally 7–14 days [8]) for monitoring of complex upstream process samples, operated by process operators rather than analytical chemists. This sets more stringent requirements on the methods, especially on robustness, repeatability and reproducibility. Additionally,

the methods must be developed as integrated sample preparation, separation, and detection. This sets more stringent requirements on sample preparation and compatibility of all solvents and chemicals with the materials of the integrated platform.

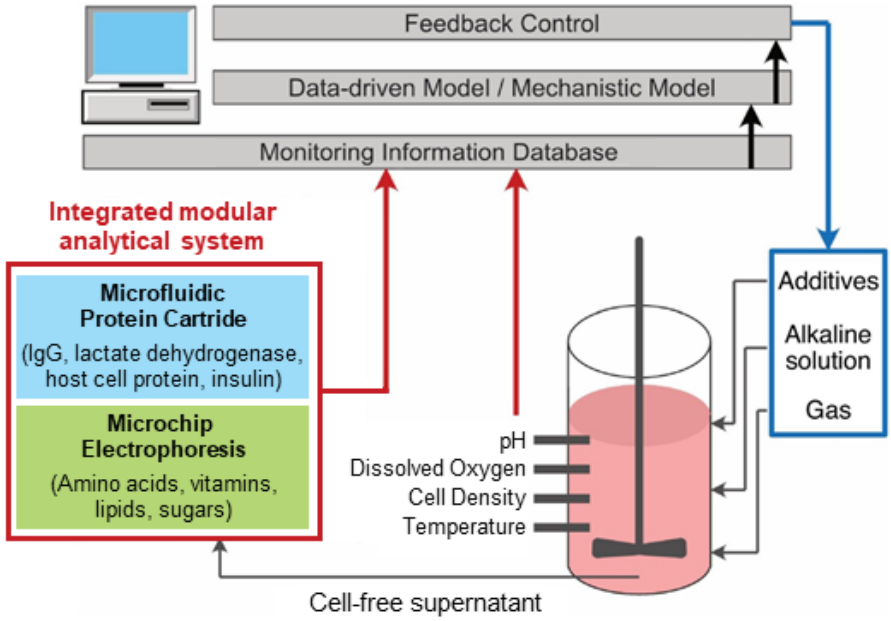

Figure 6. Integrated modular analytical system, the modules contain one or several multiplexed miniaturised quantification techniques. Adapted from ref. [53] under the Creative Commons CC BY licence (Elsevier).

The project involved the simultaneous development of the analytical methods, the microchip instrumentation, and the instrumentation for the integration of all different modules. As the microchip instrumentation was developed in parallel to the CE methods, initial method development was performed on conventional CE instrumentation. To simplify the transfer from conventional CE methods to MCE methods, silica was selected as chip material. Currently, the iConsensus project is still in the exploratory phase, hence, the developed capillary methods could be valuable as stand-alone at-line PAT solutions as well.

Method development for amino acids [54,55] or lipids monitoring was performed by Saara Mikkonen and Leila Josefsson and is not a part of this thesis. This thesis focuses on the development of CE and MCE methods for the monitoring of mono- and disaccharides, vitamins, and mAb concentration, with the intention to be integrated in this automated monitoring platform.

1.5 CE METHOD DEVELOPMENT

Any analytical method has to be fit for its intended purpose. All compounds of interest must be separated and the method has to be sufficiently accurate, precise, sensitive, and robust. For integration into an automated, integrated monitoring platform, requirements on the method are higher than for offline analysis. Unattended analysis in combination with integrated calibration and data analysis places more stringent requirements on method performance, especially on robustness, repeatability, and reproducibility. Furthermore, the integration of the method into the monitoring platform imposes certain limitations. One such limitation is that miniaturisation to MCE restricts the use of UV detection and hydrodynamic injection. Additionally, choices for sample preparation in a closed system are limited, as certain procedures such as drying steps or high temperature incubation steps become unfeasible.

Key factors in CE method development are the selection of the appropriate BGE, capillary dimensions, and capillary coatings. The BGE provides the electrical current in the capillary, which is required for separation. This means the BGE must contain charged species, at least one cationic and one anionic species. Ionic species with like charge as the analytes are called co-ions, and with opposite charge are called counterions. Analytes have to be stable and soluble in the selected BGE. The composition and the concentration of the BGE determines its pH, ionic strength, and conductivity. All factors can influence the method performance and appropriate choices should be made to develop a method that is fit for purpose. The main aspects of method development in CE are outlined below.

1.5.1 ANALYTICAL QUALITY BY DESIGN

Pharmaceutical development widely implemented QbD. These principles are nowadays also applied to analytical method development and called Analytical Quality by Design (AQbD). The concept is that quality should be built in by design rather than tested afterwards. A science-based understanding of the analytical technique and the analytes aid decision making to achieve a reliable, robust, fit-for-purpose analytical method.

At the start of method development, information on the analyte, sample type, and purpose of the analytical result are translated into method performance requirement. This information is called the analytical target profile (ATP). The method performance requirements in the APT should be built in the method by design. This includes the selection of an appropriate technique. Due to the possibility of miniaturisation, low sample volumes required, high separation power, capability to

handle complex samples, and fast analysis times, CE (with ideally transfer to MCE) was selected as technique. To develop a CE method that is sufficiently accurate, precise, sensitive, and robust to monitor upstream process samples in an automated, integrated platform, several factors should be taken into account, which are described in the following paragraphs.

1.5.2 ROBUSTNESS

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal use. For days or weeks of automated and unattended process monitoring, robustness is extra important. Robustness is built in to the method by making conscious decisions during development. The most important factors to keep in mind during method development are to keep the mobility of the analytes and the mobility of the EOF constant and thus selecting parameters that control all aspects influencing the mobility of the analytes and the EOF. Robustness is achieved by many aspects, which are described in the following subchapters, including controlling the pH by selecting high buffering capacity BGEs, controlling the ionic strength and conductivity, avoiding mobility changes by selecting a pH far from the pKa of the analytes, avoiding adsorption, controlling the EOF, etc. Controlling the EOF is one of the most important factors for robustness and continuous reproducible use.

1.5.3 SELECTIVITY

For accurate and precise analysis, all analytes of interest should be optimally separated. Optimal separation is achieved by enhancing mobility differences between analytes of interest and matrix components. The mobility is determined by the charge-to-size ratio. The pH of the BGE determines the charge on the analytes. First of all, for CZE separations, analytes must be charged. The pKa value of the ionizable groups determine at which pH they are charged. At 2 pH units below the pKa the group is fully protonated, at 2 pH units above the pKa the group is fully deprotonated, and at equal pH and pKa, the ionizable group is 50% protonated and 50% deprotonated [56]. For a robust method, the pH should be sufficiently far from the pKa of the analytes so that small changes do not significantly affect the charge of the analytes [57]. If two analytes however have similar mass and number of ionisable groups, a pH closer to the pKa is required to enhance selectivity. The net charge on proteins is determined by the isoelectric point (pI). If the pH of the BGE is close to the pI of the protein, its electrophoretic mobility will be very low, causing slow analysis

and broad peaks. The hydrodynamic size of an analyte correlates to its molar mass and its structure. The hydrodynamic size can be influenced by interactions of analytes with BGE components. An example of this is the complexation of borate with hydroxyl groups. By using borate in the BGE, isomeric monosaccharides can be separated due to the difference in degree of complexation with borate.

1.5.4 PRECISION

Precision is a measure of the degree of scatter in multiple analysis of the same sample and comprises repeatability (precision within assay), intermediate precision (precision within laboratory), and reproducibility (precision between laboratories). For automated, integrated data analysis, good migration time repeatability is extremely important. Since the pH affects both the mobility of the EOF and the analyte charge and thus its mobility, the pH of the BGE should firmly controlled. Buffer depletion could be reduced by selecting a BGE with a high buffering capacity [57,58]. Several factors influence the buffering capacity, such as the concentration and the pH of the BGE; a higher buffering capacity is obtained with a higher buffer concentration, and when the buffering components has a pKa close to that of the desired pH. The increase in buffer concentration is however limited by the resulting increased Joule heating. Excessive Joule heating results in temperature gradients in the capillary which cause band broadening. Joule heating could be minimised by using buffers with low conductivity, such as ampholytic buffers [35].

The separation in CE is not only influenced by the BGE components, concentration, and pH, also the ionic strength and the conductivity influence the separation. The ionic strength affects the EOF, and the conductivity the amount of Joule heating. For good reproducibility, all these factors should be controlled, and the composition of the BGE should be consistent. Mathematical models exist to calculate the ionic strength, conductivity, and pH for given BGE components (co-ions and counterions) and concentrations, and predict the migration behaviour of analytes. An example of this is PeakMaster [59,60]. For reproducibility, the pH of the BGE should be set by using calculated concentrations of buffer components, rather than setting the pH afterwards by the addition of acids or bases. This approach controls the ionic strength and conductivity of the BGE [57]. Preparing larger BGE volumes provide more precise concentrations.

Adsorption of sample components to the capillary wall could cause an irreproducible EOF and must be minimised. Coulombic attraction could be reduced when the capillary wall is neutral, or has the opposite charge of that of the analytes. The charge of bare fused silica capillaries is pH depended, the silanol groups ($pKa > 5$) are negatively charged at high pH, while at low pH the charge is negligible. Operating at

low pH thus reduces interactions. Adsorption of acidic analytes can be reduced by operating at a high pH, providing a negative charge on the analyte as well as on the capillary wall, resulting in coulombic repulsion.
Silanol groups are reactive molecules and adsorption cannot be fully avoided, especially not for complex cell culture medium samples. BGE additives or capillary coatings could further reduce adsorption [61–63]. The capillary coating should reduce attractive electrostatic interactions with the analytes; neutral or oppositely charged coatings must be used.
Coatings can be categorised as covalently linked or non-covalently linked. Covalently linked coatings involve chemically bonding a polymer to the capillary wall, resulting in a permanent wall modification. An example of such coatings is the neutral polyvinyl alcohol (PVA) coating, which supresses the EOF over a broad pH range [64]. Non-covalent coatings are not permanently bound and are achieved by flushing coating material over the capillary to be adsorbed to the wall. They can be either static or dynamic. An example of a static, non-covalent coating is successive multiple ionic-polymer (SMIL) [65,66]. The coating is applied before an experiment by successively flushing with cationic and anionic polymer solutions, often polybrene and dextran sulfate. The charge of the coating depends on the outer layer. Dynamic non-covalent coatings can be achieved by using BGE additives, such as amines, which form a positively charged layer on the capillary wall [67–69]. BGE additives could increase the conductivity of the BGE, limiting buffer concentration and electric field to prevent excessive Joule heating. Zwitterions [70] or neutral surfactants [71] also reduce the interactions with the capillary wall, without significantly increasing the conductivity.
Other good working practises that improve precision are capillary preconditioning and temperature control. Capillary preconditioning is important for high migration time repeatabilities since it returns the capillary to the same consistent conditions [72,73]. The capillary should first be rinsed with a solution to remove potentially adsorbed components from the capillary wall, usually sodium hydroxide or strong acids in the range of 0.1 to 1.0 M are used. Then the capillary should be rinsed with the BGE to re-equilibrate the capillary surface. Good capillary preconditioning can provide a well-defined, reproducible state of surface hydroxylation on the capillary wall. Temperature control of the capillary can improve precision by avoiding viscosity fluctuations. A change of 1 °C can result in a 3 % fluctuation in mobility [74]. Temperature control could however be more challenging to implement in MCE.

1.5.5 CONTROLLING THE EOF

The EOF affects the mobilities of the analytes, and thus the precision, resolution, and robustness of the method. The EOF is affected by several factors, such as the

temperature, electric field strength, ionic strength, viscosity of the BGE, and the charge on the capillary wall [30]. The EOF mobility (μ_{EOF}) is controlled by the zeta potential at the capillary wall (ζ), the dielectric constant (ε), and the viscosity of the BGE (η) according to the following equation:

$$\mu_{EOF} = \frac{\varepsilon\zeta}{\eta}$$

The zeta potential is the electric potential at the electrical double layer and is therefore influenced by the charge of the capillary wall and the ionic strength of the BGE. In bare fused silica capillaries, the charge on the wall is pH depended. At high pH the silanol groups are deprotonated, creating a negative charge and a strong EOF, and at low pH the silanol groups are protonated, resulting in low EOF mobility. Controlling the EOF by selecting the appropriate pH is limited by the analytes, as the pH determines their charge and thus strongly influences the selectivity. The EOF should not be significantly impacted by small pH variations. At a pH < 2.5 or in the pH range 8 – 10, the EOF is least affected by variations [35]. Selecting a BGE with a low pH significantly reduces, and thus controls, the EOF. A low EOF could be advantageous due to improved resolution and less significant migration time variations upon EOF fluctuation. In addition, the minimised charge on the capillary wall at low pH reduces adsorption of sample components to the capillary wall.

Adsorption of analyte or matrix components could affect the magnitude of the EOF by altering the zeta potential. Changes to the wall from run-to-run result in an irreproducible EOF. Adsorption cannot be fully avoided by selecting a low pH, especially for complex cell culture medium samples. In in order to further reduce adsorption of components from the complex cell culture medium samples, BGE additives or capillary coatings could be used. Capillary coatings also control the charge on the capillary wall, and thus influence the EOF. As described in section 1.5.4 *Precision*, coatings can be covalently linked or non-covalently linked, and neutral, positively charged, or negatively charged. Positively charged coatings reverse the direction of the EOF in the direction of the anode, and in neutral coated capillaries the EOF is negligible. For some coatings, such as the neutral PVA or charged SMIL coatings, the EOF is independent of the pH.

The zeta potential could further be influenced by the ionic strength. A higher buffer concentration increases the ionic strength, reducing the zeta potential and the EOF. Additionally, high buffer concentrations decrease interactions of analytes with the capillary wall by competitive adsorption of co-ions and thus improves resolution, and increase the buffering capacity of the BGE. The latter reduces buffer depletion caused

by electrolysis and thus keeps the pH stable [58]. As described in section 1.5.4 *Precision*, the buffer concentration is limited by excessive Joule heating.
Besides the zeta potential, the EOF is influenced by the viscosity and the dielectric constant of the BGE. Increasing viscosity of the BGE decreases the EOF, and could be influenced with temperature [74], buffer concentration, and certain additives. An increased dielectric constant increases the EOF mobility. The dielectric constant generally decreases upon increasing organic modifier concentration. The dielectric constant is also inversely proportional to temperature. As many factors influence both the dielectric constant and the viscosity, the ratio ε/η should be considered rather than the separate variables. And lastly, the EOF increases proportionally to increasing applied voltage.

1.5.6 SENSITIVITY

1.5.6.1 SAMPLE STACKING

A frequently heard disadvantage of CE is low sensitivity. The narrow capillary provides a small optical pathlength for detection. This is however counteracted with the possibility for on-capillary sample concentration, or stacking [35,56]. Whereas liquid chromatography is per definition a diluting technique, CE can be used to stack and thus concentrate the sample. Sample stacking can be achieved in different ways. The simplest way is by a conductivity difference between the sample and the BGE. The relation between the current (I), conductivity (κ), and electric field strength (E) is $E = I / \kappa$ and the velocity of an analyte is the product of the electrophoretic mobility and the local electric field strength. Because the current is equal over the length of the capillary, the electric field strength increases with decreasing conductivity. When the sample has a significantly lower conductivity than the BGE, the local electric field strength in the sample zone is significantly higher. In the low conducting sample zone, analytes have a higher velocity, when they reach the BGE, the local electric field strength reduces as does their velocity. The analytes are thus stacked and concentrated in a narrow zone at the boundary between the sample zone and the BGE. To exploit this characteristic of CE, the conductivity of the sample should be lower than the conductivity of the BGE. High buffer concentrations therefore aid stacking, as well as diluting samples in low conducting solvents such as water.
When this conductivity difference is not feasible, for example for samples with high salt concentrations, isotachophoresis (ITP) principles can be used for sample stacking. ITP makes use of a leading (LE) and a terminating (TE) electrolyte sandwiching the sample zone. The LE contains a co-ion that has a higher mobility than any of the analyte ions, and the TE contains a co-ion that is slower than any of the analyte ions.

Once a voltage is applied, the ions in the LE migrate fastest, followed by the analyte ion with the next highest mobility, and the next, and eventually the ions in the TE. All individual ions migrate in separated zones, all with the same velocity. For ions with a lower mobility than the LE ions to migrate with the same speed, the electric field strength in the zones must be higher, hence the local electric field strength increases per zone from LE to TE ($E_{LE} < E_{Sample} < E_{TE}$). This prevents band broadening, as analyte ions diffusing in a zone ahead will slow down and be caught up by their zone and analyte ions diffusing into a zone behind will speed up, catching up on their own zone. Sample concentration occurs because the current is constant over the capillary, and thus a constant ratio must exist between the concentration and the mobility of the ions in each zone, as dictated by the Kohlrausch regulation function. Zones that are less concentrated than the LE are therefore sharpened [28]. Pure ITP employs discontinuous buffer systems. The principles of ITP can however also be employed using the BGE as LE and adding a low mobility co-ion in the sample to act as TE or vice versa. Sample stacking techniques have been applied to increase sample volumes up to 50% of the total capillary length [75,76].

1.5.6.2 PEAK SHAPE

Sensitivity is increased by ensuring sharp peaks. Due to the open capillary tube, the only term of the Van Deemter equation contributing to peak broadening in CE is diffusion. Band broadening due to diffusion is especially low for large molecules. Due to the flat flow profile of the EOF, radial diffusion is very small and longitudinal diffusion is dominant. Diffusion is minimised by reducing the time spent in the capillary. The speed of analysis is proportional to capillary length and the electric field strength. The electric field strength affects Joule heating, which causes band broadening due to temperature gradients in the capillary. Another reason low conducting buffers are preferred, as they allow for higher electric field strengths, and thus faster analysis [56].

Diffusion being the only factor contributing to peak broadening only holds if there is no interaction or adsorption of analytes to the capillary wall. These kinds of wall interaction can often be observed as tailing in the electropherogram. Adsorption should also be avoided for precision and control of the EOF and could be prevented by using capillary coatings and BGE additives described in sections 1.5.4 *Precision* and 1.5.5 *Controlling the EOF*. Additionally, high buffer concentrations decrease interactions of analytes with the capillary wall by competitive adsorption of co-ions and thus improves resolution. Buffer concentrations are limited, as described previously, by Joule heating.

A larger contribution to band broadening could arise from electromigration dispersion [35,56,57,77]. Where differences in local electric field strength can be used for on-capillary concentration by sample stacking, differences in local electric field strength could also result in the opposite effect, namely band broadening. The presence of analytes in the sample zone will affect the conductivity and thus the local field strength. When the analyte has a higher mobility than the co-ion in the BGE, the electric field strength in the sample zone is lower than in the BGE. Analytes at the front boundary of the sample zone will accelerate and leave the sample zone, while analytes at the back boundary will accelerate and catch up with the sample zone, causing asymmetric fronting triangled peaks. If the analyte mobility is lower than that of the co-ion, analyte molecules reduce in speed when they leave the sample zone, resulting in asymmetric tailing triangled peaks. In order to ensure sharp peaks, electromigration dispersion should be suppressed by selecting a co-ion with a mobility close to that of the analytes. In addition, electromigration is reduced when the analyte concentration is much lower than the concentration of the co-ion. Higher buffer concentrations are thus favourable to prevent electromigration dispersion.

1.5.6.3 DETECTION

The sensitivity is also largely dependent on the detection technique. For the selection of appropriate detection techniques, the properties of the analytes and the compatibility with the separation technique should be taken into account. UV/diode array detection is most commonly used for conventional CE, for MCE it is often insufficiently sensitive. As described in section 1.3.2 *Microchip capillary electrophoresis*, fluorescence detection, electrochemical detection, or mass spectrometry could be used as detection techniques. For the automated, integrated monitoring system, mass spectrometry was out of scope due to the large instrument size and high costs. Fluorescence detection was the detection technique of choice because of its high sensitivity and selectivity. Besides the detection technique, the sampling rate could influence the peak shape and thus sensitivity. As a general rule of thumb, at least 20 data points per peak are required for reliable integration.

1.5.7 SAMPLE PREPARATION

The sample preparation has a large effect on the precision and could also affect the sensitivity of the method. The sample preparation is dependent on the other analytical requirements. CE can handle complex sample matrices with reduced sample preparation because of its high separation power and simple set-up. Crude or clarified harvest samples can under certain conditions be directly injected in CE

methods [54,76,78,79], but generally, cleaning up samples reduces adsorption issues. Also, appropriate sample pretreatment and injection techniques can enhance on-capillary preconcentration. For integration into an automated monitoring platform, sample preparation should comprise simple steps that are easily integrated in the system. Sample preparation is therefore ideally minimised as much as possible. Fluorescence detection is frequently used on CE-chip systems and often requires derivatisation with a fluorophore during sample preparation. High temperature incubations, sample drying steps, complex excess dye removal, or complex sample concentration protocols are not feasible in the integrated system and should be avoided. Samples could be instable under various pH, light, agitation, or temperature conditions. Sample preparation should not degrade the sample and the sample must be stable in the final sample matrix. Loss of analytes due to adsorption on glassware, pipette tips, filters, etc. should be prevented by selecting appropriate materials. For example, proteins could adsorb on glassware or plastic. As this is more pronounced on glass, plastic tubes and pipettes are recommended for protein sample preparation. Adsorption of protein could in some cases be further minimised by the addition of surfactants, such as SDS, to the sample matrix [80]. As discussed in section 1.5.6.1 *Sample stacking*, on-capillary sample stacking occurs when the conductivity of the sample is lower than that of the BGE. It is therefore recommended to use low conducting solvents for dilution, e.g., water. All these aspects should be taken into account during method development. For quantitative determinations, the addition of an internal standard or calibration standard could be beneficial.

Generally, better precision is achieved by applying fewer sample preparation steps and handling larger volumes. The errors on handling small volumes are typically higher, and due to error propagation, each step adds on to the total error. When possible, solutions should be premixed using larger volumes. Precision could be increased when using reversed pipetting. Automation of the sample preparation improves the precision, also the automated protocol should use large volumes where possible and use reverse pipetting to increase precision.

1.6 CONTENT AND CONTRIBUTIONS

The content of each paper included in this thesis is briefly outlined below, including the contributions of all authors.

Chapter 2. Recent capillary electrophoresis applications for upstream and downstream biopharmaceutical process monitoring

Debbie van der Burg, Leila Josefsson, Åsa Emmer, Cari E. Sänger - van de Griend. Trends in Analytical Chemistry 160: 116975 (2023)

Chapter 2 provides an overview of CE applications for biopharmaceutical process monitoring. This review paper focuses in particular on applications for the analysis of upstream and downstream process-intermediates and shows the potential for CE in PAT solutions. CE application of the last year for product concentration determination and characterization of critical quality attributes (glycosylation, charge heterogeneity, and size heterogeneity) were described, as well as applications for nutrients and metabolite monitoring in the upstream process and applications to support process development were outlined. The potential of implementing CE for near real-time and on-site in PAT was described.

Leila Josefsson wrote the introduction. Debbie van der Burg performed the literature research, wrote the main parts (Discussion and Concluding remarks) of the manuscript, and reviewed and edited the introduction to match the main chapters. Åsa Emmer and Cari Sänger - van de Griend critically read and commented on drafts and the final version of the manuscript.

Chapter 3. Method development for quantitative monitoring of monoclonal antibodies in upstream cell culture process samples with limited sample preparation – evaluation of various capillary coatings

Debbie van der Burg, Hermann Wätzig, Cari E. Sänger - van de Griend. Electrophoresis 44: 96-106 (2023)

In chapter 3, a platform CZE method for mAb concentration monitoring during cultivation was developed. To allow for implementation in the automated, integrated monitoring platform, robustness, simple sample preparation, and possibility for transfer to microchip are required. The method should be applicable to a broad range of mAbs to allow for its use for various processes. Sample were analysed without sample preparation other than a dilution for highly concentrated samples. For robustness, adsorption of the mAb or matrix components must be avoided, as well as the EOF controlled. For this purpose, various capillary coatings were investigated.

This included the neutral covalent PVA coating, a dynamic successive multiple ionic-polymer (SMIL) coating, and dynamic coatings using the BGE additives triethanolamine (T-EthA) and triethylamine (TEA). Best results were obtained with a dynamic T-EthA coating. The use of a dynamic coating simplifies microchip coating, as the additive can simply be added to the BGE. As most mAbs have high isoelectric points, using a low pH BGE allows the analysis of a wide range of mAbs. The mAb was analysed in samples from different upstream processes after different days of cultivation without interference of matrix components. The method was validated over the range 0.1 – 10 mg/mL. Samples were injected on the short end of the capillary, with similar separation lengths as the microchip. Transferring the method to MCE employing silica chips should therefore be straightforward.

Debbie van der Burg designed, performed, and evaluated the experiments, and wrote the manuscript. Cari Sänger - van de Griend and Hermann Wätzig supervised and critically evaluated drafts and the final version of the manuscript.

Chapter 4. Method development for mono- and disaccharides monitoring in cell culture medium by capillary and microchip electrophoresis

Debbie van der Burg, Leila Josefsson, Saara Mikkonen, Véronique Chotteau, Åsa Emmer, Hermann Wätzig, Cari E. Sänger - van de Griend. Electrophoresis 43: 922-929 (2022)

Chapter 4 describes the development of an MEKC method for mono- and disaccharide monitoring with fluorescence detection. Analysis was performed after fluorescent derivatization with 8-aminopyrene-1,3,6-trisulfonic acid (APTS). As all methods in this thesis are intended for use in the automated, integrated monitoring platform, sample preparation protocols were developed to allow for integration in a closed system. This includes, for example, the avoidance of common drying steps before derivatisation and the replacement of a toxic reducing agent to a non-toxic alternative, and the transfer of the method to MCE. The BGE and derivatisation reaction were optimised using multivariate Design of Experiments (DoE). This allowed the optimisation of multiple factors simultaneously with minimal experiments and provided information on the relationship between the optimised factors and the response, ensuring to find a real optimum. Since the microchip instrumentation was being developed in parallel with the CE methods, method development was started on conventional CE. The developed conventional CE method for mono- and disaccharide monitoring was transferred to MCE. The transfer was simplified by selecting silica microchips, which have a similar separation chemistry to capillaries.

Mono- and disaccharides could be analysed in USP samples on MCE within six minutes without interference of matrix components.

Debbie van der Burg designed, performed, and evaluated the experiments. This included method development on conventional CE as well as some preliminary experiments on MCE. The manuscript was also written by Debbie van der Burg. Leila Josefsson and Saara Mikkonen contributed equally and tested the method on MCE. The group of Véronique Chotteau prepared the FMX-8 MOD and saccharide-free cell culture media. Cari Sänger - van de Griend supervised the project. Cari Sänger - van de Griend, Åsa Emmer, and Hermann Wätzig critically evaluated drafts and the final version of the manuscript.

Chapter 5. Analysis of cationic vitamins in cell culture medium samples by capillary zone electrophoresis

Debbie van der Burg, Hermann Wätzig, Cari E. Sänger - van de Griend. Journal of Analytical Methods in Chemistry 2022: 1-7 (2022)

Vitamins play essential roles in cellular processes, cell growth, cell death, or productivity. Their monitoring during the USP process is crucial for process optimisation and control. Although vitamins are classified as one nutrient group, the diversity in terms of chemical structure and properties is large. Since robustness is of upmost importance for an automated, integrated monitoring platform, vitamins were divided into two groups based on their physical chemical properties and multiple robust methods were developed. The separation mechanism in CE is based on charge and size, hence the vitamins were divided based on their charge.

Chapter 5 describes the development of a CZE method for the monitoring of the cationic B-vitamins thiamine, nicotinamide, pyridoxine, pyridoxal, and pyridoxamine in untreated cell culture medium samples. In this paper, the effects of buffering capacity, co-ion mobility, and preconditioning solution on robustness were evaluated. Phosphate buffers with the co-ions Tris, glycine, or triethanolamine at different concentrations were evaluated. Glycine in the BGE distorted the peaks and with Tris, not all vitamins were baseline resolved. Using T-EthA, the vitamins were baseline resolved. Additionally, T-EthA forms a dynamic coating, protecting the capillary wall from adsorption, and thus increasing robustness for in-process monitoring. Capillary preconditioning was performed with either 0.1 M phosphoric acid or 1 M NaOH. Migration time and peak area repeatabilities were better when using NaOH as preconditioning solvent. An NaOH flush was also expected to aid the formation of a more stable T-EthA coating. In the final method using a 100 mM phosphoric acid and 55 mM T-EthA BGE at pH 2.3, the vitamins thiamine,

nicotinamide, pyridoxine, pyridoxal, and pyridoxamine could be separated with good resolution in cell culture medium samples.

Debbie van der Burg designed, performed, and evaluated the experiments, and wrote the manuscript. Cari Sänger - van de Griend and Hermann Wätzig supervised and critically evaluated drafts and the final version of the manuscript.

Chapter 6. Design of experiments for micellar electrokinetic chromatography method development for the monitoring of water-soluble vitamins in cell culture medium

Debbie van der Burg, Hermann Wätzig, Cari E. Sänger - van de Griend. Electrophoresis 44: 1548-1558 (2023)

In Chapter 6 Design of Experiments (DoE) was employed for the development of a micellar electrokinetic chromatography (MEKC) method for the analysis of the anionic and neutral vitamins in cell culture medium samples. This chapter describes the principles of DoE and DoE modelling in more detail. Some preliminary experiments provided information to select appropriate factors for BGE optimisation. The BGE could be optimised by only testing 11 BGE conditions, and this led to the separation of ten B-vitamins and vitamin C in clean sample matrix. This included the cationic vitamins described in Chapter 5. Separation of some vitamins was lost with cell culture medium as matrix, however, the combination of both methods allows for the analysis of most water-soluble vitamins in cell culture medium.

Debbie van der Burg designed, performed, and evaluated the experiments, and wrote the manuscript. Cari Sänger - van de Griend and Hermann Wätzig supervised and critically evaluated drafts and the final version of the manuscript.

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Chapter

Recent capillary electrophoresis applications for upstream and downstream biopharmaceutical process monitoring

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ABSTRACT

The biopharmaceutical market is one of the fastest growing biotechnology markets. In order to ensure affordable and reliable therapeutics, the biopharmaceutical process has to be closely monitored. Capillary electrophoresis (CE) has proven to be a valuable technique for the analysis of product concentration, critical quality attributes, product and process-related impurities, and nutrients and metabolites in the cell culture medium. Capillary zone electrophoresis, capillary gel electrophoresis, and capillary isoelectric focusing are extensively used for product concentration, and size or charge heterogeneity determination. CE has a number of benefits for the analysis of upstream and downstream process-intermediates, including the ability to handle highly complex matrices found in process-intermediates, high resolving power, little sample preparation requirements, rapid analysis, and low solvent and sample consumption. The small sample volumes (nL range) are especially beneficial for microbioreactor analysis or clone selection experiments. The simple setup and the possibility for miniaturisation and automation using microchip CE provides great opportunities for on-site, real-time monitoring of the process. This review discusses CE applications in upstream and downstream processing of the last decade.

2.1 INTRODUCTION

The biopharmaceutical market is one of the fastest growing biotechnology markets, and has matured over the recent years. It includes a wide variety of products e.g., therapeutic proteins, hormones, growth and coagulation factors, and, maybe one of the more appreciated during recent years, vaccines. Biopharmaceutical therapies include treatments of chronical diseases, infections, cancer, and autoimmune diseases. Biopharmaceuticals are produced by living organisms such as fungus (yeast), bacteria (Escherichia coli), or mammalian cells (often Chinese hamster ovary (CHO) cells). The latter are predominantly used for therapeutic proteins like monoclonal antibodies (mAb), due to their ability to incorporate post translational modifications (PTMs) and biosynthetic complexity into the targeted protein [1]. Bioprocesses are complicated systems where the environment must be optimal for cell growth and the production of the product of interest (PoI). The mammalian cell culture is a intricate solution that contains both components added to the bioreactor as culture media and additives, and components produced or leaked by the cells. The biopharmaceutical process can be divided into two major areas: upstream and downstream processing. Upstream processing (USP) includes the development and optimisation of all parameters regarding the production of the PoI, like cell engineering, cell culture medium, and type of operation (e.g., batch, fed-batch, continuous) and bioreactors. Downstream processing (DSP) focuses on the purification of the PoI from other components in the cell culture and the PoI formulation into a stable, safe and efficacious drug product. All process steps have to be closely monitored concerning product yield and quality, by analysing cell culture medium (CCM) and cell culture harvest (CCH) samples from USP and samples from all purification steps up to the clean drug substance in DSP.

The PoI is evaluated with regards to its physical, chemical, and biological properties or characteristics, that need to be within an appropriate limit, range, or distribution to ensure the desired product safety, efficacy, and quality. Each of those properties is referred to as a critical quality attribute (CQA) for the PoI [2], and the CQAs of therapeutic proteins could for example include the glycosylation pattern, charge variants, or aggregation and fragmentation [3], and are often defined through in vitro testing of the PoI. To ensure affordable and reliable treatments, it is critical that the biopharmaceutical production is cost and time effective with minimal variation between batches. For effectivization, quality by design (QbD) can be implemented, which is “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management” (ICH Q8(R2), 2009) [2]. QbD

increases the manufacturers as well as the regulatory agencies understanding of the impact of raw material and process parameters on the CQAs and helps with identification and control of sources of variability [4]. QbD helps to identify the critical process parameters, which are parameters whose variability has a significant impact on CQAs. Critical process parameters should be monitored and controlled to maintain the quality of the PoI, and this can be achieved by incorporating process analytical technology (PAT) into the bioprocesses.

PAT includes online, in-line, at-line, and offline analysis performed on-site in (near) real-time, and can be incorporated into the whole bioprocess stream. Online analyses (online, in-line, at-line) include automated sampling and analysis, while for offline analysis (offline, at-line), samples are taken manually before analysis. Traditional process monitoring techniques include in-line analysis using probes for e.g. pH, pO2, pCO2, and cell density by impedance, and at-line analysis where samples drawn from the process stream are analysed directly on the process floor using spectroscopic or other dedicated sensors i.e. glucose, lactate, glutamine and ammonia [5]. Since the PAT initiative was launched by the FDA [6], the number of PAT platforms for the monitoring of the bioprocess using more complex analytical techniques like liquid chromatography (LC), capillary electrophoresis (CE), and mass spectrometry (MS) has increased [7].

CE is an excellent technique to implement in PAT since it uses small sample volumes (nL range), which makes it a great technique to use for microbioreactors with limited volumes or for process monitoring. CE has short analysis times with high resolutions, negligible waste, the possibility of automatization, and minimal sample preparation requirements compared to other liquid-based separation techniques. In CE, analytes are separated due to their charge-to-size ratio under the influence of an electric field within a narrow capillary. In the electric field, charged molecules have a mobility which is the sum of their electrophoretic mobility and the mobility of the electroosmotic flow (EOF). The EOF can be controlled by adjusting parameters of the background electrolyte (BGE), such as the pH, ionic strength, and viscosity, as well as by the temperature, magnitude of the electric field, and dynamic or permanent coatings of the capillary wall. Controlling the EOF is important to obtain robust CE separation methods. By adding certain additives to the BGE, different modes of separation can be achieved, which can be used for analysis of therapeutic protein CQAs. Capillary zone electrophoresis (CZE), the simplest CE mode, where a buffering BGE solution with or without additives is used, can be applied directly for the analysis of intact therapeutic proteins or with any top-down, middle-up or bottom-up approaches. CZE can provide information about glycosylation patterns and charge heterogeneity at the protein level. Capillary gel electrophoresis (CGE) is created by

adding a gel or a polymeric network at low concentration to the BGE. For SDS-CGE, often referred to as CE-SDS, proteins are denatured with SDS, giving them a uniform charge-to-size ratio, allowing for size-based separation through the gel buffer. SDS-CGE can be used to determine protein size heterogeneities during process development and quality control, stability and purity as well as glycosylation patterns and assessments of host cell protein (HCP) impurities. For SDS-CGE, the sample can be both reduced and non-reduced providing different information. For apparent pI determination of a protein and levels of acidic, main, and basic variants as well as impurities, capillary isoelectric focusing (cIEF) or imaging cIEF (icIEF) mode can be used. In (i)cIEF, ampholytes within a specific pH range are used to create a pH-gradient in the capillary to separate proteins based on pI differences [8]. Another advantage of CE is the possibility to transfer the separation methods to microchip devices (microchip capillary electrophoresis, MCE), which are miniaturised versions of CE where the capillary is replaced with a chip capillary device. The microchip device can be fabricated using various materials, e.g. glass, quartz, polymers, or even paper, which facilitates its broad area of applications [9,10]. MCE uses less volume than CE, often has shorter analysis times, is more portable, can be designed to have several channels for higher throughput, or multiple analytical separations occurring at the same time, and could have integrated sample preparation on the MCE device [11,12]. The application of CE in the biopharmaceutical industry with the focus on final product characterisation has been reviewed extensively (e.g. Refs. [13,14]. This review focuses on the application of CE and MCE for analysis of USP and DSP process-intermediates during the last decade.

2.2 DISCUSSION

2.2.1 DETERMINATION OF PRODUCT CONCENTRATION IN UPSTREAM AND DOWNSTREAM PROCESSING

The final product yield of a biopharmaceutical process is limited by the protein expression in the host cells. This expression is affected by a range of cell culture conditions. To optimise the production yield, and to understand the effect of cell culture conditions on protein expression, the protein expression should be monitored. After protein expression in USP, the PoI is purified in DSP. Loss of product during purification should be avoided and therefore, the product concentration should be analysed in every step of the USP and DSP processes.

For mAb titer determination in USP directly in cell culture medium (CCM) supernatant, Wang et al. [15] developed an MCE-MS method. The limited sample

preparation consisted of only a desalting and a reduction step. A stable isotope-labelled mAb was added as internal calibrant prior to sample reduction. Heavy chain (HC) and light chain (LC) were separated with the CE method, and as no N-glycosylation variants were expected on the LC, the mAb concentration was determined using the relative signal intensity of the LC versus the isotope-labelled LC. The same method was further applied for glycosylation characterisation (section 2.2.2.3). Van der Burg et al. showed the monitoring of mAb concentration in USP in CCM supernatant without the need for any sample preparation using a simple CZE platform method [16]. By using UV detection rather than MS detection, desalting the sample was not needed. And by analysing intact mAb, using an external mAb calibration for quantification, no reduction was required either. Samples could be analysed directly in CCM supernatant without interference of matrix components. This shows the great capabilities of CE in handling complex sample matrices. A dynamic triethanolamine coating prevented adsorption from matrix components or mAb to the capillary wall, and the low pH BGE employed in this method allowed the analysis of a large variety of mAbs.

Another example of the capability of CE to handle various complex matrices is the adenovirus type 26 (Ad26) particle concentration determination with CZE developed by Van Tricht et al. Applying analytical quality by design (AQbD), they developed and implemented this CZE method for routine intact Ad26 particle (150 MDa) quantification throughout USP and DSP [17,18]. Sample matrices varied and could contain DNA, proteins, surfactants, adjuvants, salts, and/or cell debris. No sample preparation was required, except for samples containing DNA, which required a simple sample pretreatment with benzonase. Adsorption of matrix components or viral particles was prevented by using a polyvinyl alcohol coated capillary, and by the addition of the neutral surfactant polysorbate-20 to the BGE. The Ad26 particles were detected without interference of matrix components in all process-intermediates with a total run time of 3.5 min (Fig. 1). This method was applied for a large variety of applications. It was applied in USP for determining seed Ad26 concentration, in DSP to study lysing agent effects on Ad26 yield and HCP and host cell (HC) DNA release, to study HC-DNA clearing agent effects on Ad26 particles, and to investigate the root-cause of high anion-exchange chromatography filtration pressure. It was also applied for chloride content determination, Ad26 content release testing, and for Ad26 stability testing [19].

Protein analysis in biopharmaceutical samples is often performed using traditional Western Blot. However, the electrophoresis, electrical blotting, antibody probing, and signal development are labour-intensive. In 2006, O'Neill et al. [20] used the principles of western blotting performed in a capillary; they developed an cIEF

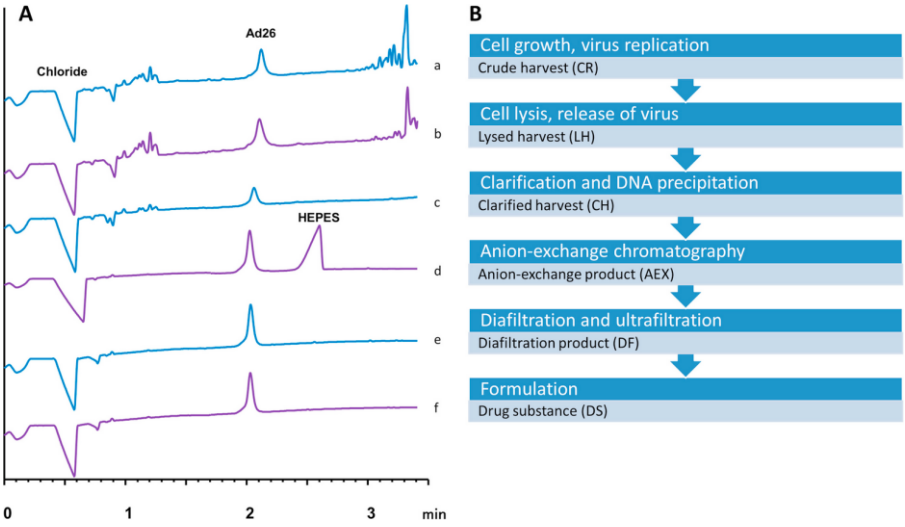

Figure 1. A) Electropherograms of adenovirus (Ad26) samples from USP and DSP (a) benzonase-treated crude harvest, (b) benzonase-treated lysed harvest, (c) clarified harvest, (d) anion exchange chromatography, (e) diafiltration, and (f) drug substance. B) Flowchart of the USP and DSP manufacturing process steps. Reprinted from Ref. [17] with permission from Elsevier.

immunoassay (IA) method employing photochemical immobilisation and antibody probing of proteins in the capillary, followed by chemiluminescent detection. Now there are commercially available capillary western instruments and analysis kits for size-based (SDS-CGE-IA) or charge-based (cIEF-IA) separations. SDS-CGE-IA is used for protein concentration determination in USP and DSP. As an example, this technique has been employed for the monitoring of pertactin concentration in vaccine samples throughout DSP [21]. Pertactin was quantified in different USP and DSP process-intermediates from CCM to drug product, with good accuracy and no matrix interference. SDS-CGE-IA has also been applied for vaccine protein monitoring throughout DSP in Ebola vaccines [22]. Ebola viruses infect host cells through interactions mediated by viral glycoprotein. SDS-CGE-IA was used to monitor the types and quantities of glycoprotein variants generated throughout the process: after viral harvest, depth filtration, enzymatic digestion, ultra-filtration, addition of Tris and rHSA (drug substance), and final dilution in Tris and rHSA (drug product). Only the sample taken after viral harvest contained an unknown peak migrating between the glycoproteins, which was filtered out after depth filtration. The method resulted in similar glycoprotein profiles as obtained with manual Western blot, but with better reproducibility, more accurate quantitation, and improved ease of use.

Xu et al. used SDS-CGE-IA for biopharmaceutical protein concentration determination directly in cell culture harvest (CCH) and drug substance samples [23].

The therapeutic protein was analysed as three peptide peaks of 100, 130, and 210 kDa. The total peak area of the three peaks was used to determine the titer with good precision and accuracy. This method was also applied for high molecular weight (HMW) species determination (section 2.2.2.1). They also applied SDS-CGE-IA for the quantification of Fc-fusion protein in DSP intermediates [24]. The Fc-fusion protein consists of two polypeptides: fusion-Fc and single chain Fc. The peak from fusion-Fc polypeptide was used for quantification against a calibration curve from purified drug substance in the wide linearity range of 24 µg/mL to 6 mg/mL. This method was also used for quantification of product related impurities in DSP intermediates (section 2.2.4).

Conventional SDS-CGE is also a valuable technique for protein concentration determination. SDS-CGE was employed to investigate the effect of process parameters on production and quality of recombinant human β-glucuronidase [25]. CCH was concentrated and buffer exchanged, and bovine serum albumin (BSA) was added as internal calibration standard for molecular weight and concentration determination. The effect of different process parameters on production was then determined by analysing the concentration upon harvest using SDS-CGE under reducing conditions. Commercially available SDS-CGE applications were generally developed for mAb analysis, but are also frequently used for non-mAb proteins; however, they cannot always be readily applied. Adjustment and optimisation of the SDS-CGE application is often required. Geurink et al. [26] described a general four-step approach for SDS-CGE method development for the quantification of viral proteins. First, factors most influencing method performance, critical method parameters (CMPs), were identified. Sample preparation could be affected by incubation time and temperature, pH, and reagent concentrations, and separation could be affected by the gel buffer and capillary effective length. For SDS-CGE, often a commercialised proprietary gel buffer is used. The composition of the gel buffer impacts the pore size, viscosity, and ionic strength, which in turn impact the amount of sample injected, sample stacking, separation, sensitivity, and analysis time. The magnitude of these effects depends on capillary temperature. The selected CMPs for method development/optimisation therefore included incubation time and temperature, pH, and reagent concentrations for sample preparation, and capillary effective length, gel buffer dilution, and capillary temperature for separation. A four-step approach was set up for quick method development. The approach included 1) assessment of feasibility of the default conditions and need for sample preparation and separation, 2) multivariate design of experiments (DoE) optimisation of sample preparation CMPs and 3) multivariate DoE optimisation of separation CMPs, and 4) method validation. This approach was applied for the development of SDS-CGE

methods for the determination of influenza group 1 mini-haemagglutinin glycoprotein, and the determination of polio virus particle proteins from an inactivated polio vaccine. Using the same development approach, two different methods were developed that met the analytical target profile requirements within week(s), considerably reducing method development time.

In SDS-CGE, sample injection is commonly performed electrokinetically. The conductivity of the sample therefore affects the amount injected; at higher conductivity, less sample is injected. Desalting to concentrations <50 mM is recommended [27]. As process-intermediates often have high salt concentrations, desalting is often required. Sample preparation of process-intermediates include the removal of impurities such as host cell proteins and cell debris, and buffer exchange. Also, to accommodate for a wide range of sample concentrations from different in-process steps, concentration normalisation is required. This results in labour-intensive sample preparation. To overcome this, Le et al. developed an automated robotic platform for sample preparation for both the monitoring of LMW species by reduced SDS-CGE and the monitoring of partial reduction by non-reduced (NR-SDS-CGE) in DSP-intermediates [28]. The platform normalises sample concentrations, removes salts and other contaminants, and adds the required SDS-CGE reagents. The use of a protein A purification purifies the sample, but also allows for buffer exchange, and, as the Pro-A columns have a finite number of protein binding sites which will be saturated above a specific sample concentration, allows for normalisation of the protein concentration. After elution, sample buffer was added. Samples were then manually aliquoted and incubated before CE analysis. An alternative to Le's approach is to use hydrodynamic injection, which is less prone to sample matrix effects so extensive sample preparation can be avoided [29].

The utility for monitoring LMW species by rCE-SDS in purification intermediates was demonstrated across a wide range of sample matrices. Normalisation of sample concentrations was achieved without biasing the distribution of species. Reproducibility studies show that the precision is similar to that with manual preparation. Application of this sample preparation technology to NR CE-SDS was demonstrated with samples from the harvest operation to monitor partial reduction.

2.2.2 CRITICAL QUALITY ATTRIBUTE MONITORING IN UPSTREAM AND DOWNSTREAM PROCESSING

All product characteristics influencing the safety, efficacy, and quality of the product are considered CQAs. In order to control CQAs early on, and to optimise the biopharmaceutical process to produce well defined therapeutic proteins, characterisation of CQAs during production is essential. This chapter describes CE

applications for the characterisation of the most common CQAs: size heterogeneity, charge heterogeneity, and glycosylation.

2.2.2.1 SIZE HETEROGENEITY

Size heterogeneity comprises product related aggregates (HMW species), and fragments (low molecular weight (LMW) species). The efficacy of therapeutic proteins is highly dependent on their structure. Aggregation of therapeutic proteins can cause loss in function and increase the risk of immunogenic responses, causing adverse effects ranging from protein neutralisation to anaphylaxis [30]. To minimise HMW and LMW size variants, the USP and DSP steps must be controlled, and size variants should be characterised throughout the process. The characterisation of size variants is frequently performed using SDS-CGE.

In one study, the effect of USP parameters on process-induced antibody disulfide-bond reduction was investigated using NR-SDS-CGE [31]. CCH samples were directly loaded on the robotic automated platform for SDS-CGE sample preparation developed by Le et al. [28] as described in section 2.2.1. After sample preparation, the main antibody was separated from LMW species and the relative area of the LMW species peaks and the main peak were used to monitor interchain disulfide reduction in various harvest experiments. The data showed that the type of mAb and the type of cell line have a large impact on reduction. This increased understanding allows for screening of cell lines and cell culture conditions to minimise process-induced reduction.

The SDS-CGE-IA method from Xu et al. [23] (section 2.2.1) was also employed for the determination of HMW species in CCH and drug substance samples. The largest of the three peptides detected, the 210 kDa peptide peak, is from the HMW species. The ratio of the HMW peak to the total peak area was used to determine the HMW percentage and could be determined with a precision of <5% RSD.

CE was employed for the optimisation of the DSP purification process. Due to the similarity in biochemical properties of HMW and LMW species to the target protein, their clearance in DSP can be challenging. It is therefore beneficial if these species could be removed early on. Depth filtration is commonly used during cell culture harvesting. Depth filtration purifies both by filtration and by adsorption of soluble impurities onto the filter surfaces. To minimise the burden on chromatographic purification steps further in DSP, the adsorption of HMW and LMW species on depth filters was studied [32]. Size exclusion chromatography was employed for HMW and LMW quantification, and NR-SDS-CGE for LMW quantification, which gave higher resolution and more accurate LMW determination. Employing these techniques increased the understanding of the depth filtration step; the results indicated

formation of LMW species during filtration, which could be reduced by using lower temperatures. This allowed the design of a control strategy, which significantly reduced HMW and LMW levels.

2.2.2.2 CHARGE HETEROGENEITY

Many PTMs influence the charge heterogeneity of therapeutic proteins. PTMs could result in more acidic species, for example by sialyation, deamidation, or glycation, or in more basic species, by for example succinimide formation or incomplete removal of C-terminal lysine. Depending on the position of the PTM, it could affect antigen binding affinity, potency, secondary structure, and aggregate formation [33]. Charge variant analysis is therefore important.

Charge heterogeneity is commonly determined using ion exchange chromatography (IEC), cIEF, icIEF, or CZE. A CZE method developed by He et al. [34], using a dynamic triethylenetetramine coating and high ε-amino-caproic acid concentration to prevent protein adsorption, is widely used for mAb charge variant analysis and has proven to be robust [35]. The robustness of cIEF [36] and icIEF [37] was also shown by intercompany studies. These studies, however, were employed on relatively clean samples of drug product.

For charge heterogeneity determination in USP and DSP process-intermediates, Michels et al. [38] developed a cIEF-IA method. They optimised the ampholyte solution to obtain a broad linear range of pH 5.5–9.8 in order to use the method as a platform method. Platform methods are desired for process development support, as they save a lot of time in reoptimizing method parameters for each product. In contrast to conventional cIEF, in cIEF-IA the ampholytes were removed from the capillary before detection, resulting in less background noise. The cIEF-IA required only 0.2 µg/mL protein, 1000-fold less than conventional cIEF. The method was evaluated for matrix interference of CCH. Three mAbs with considerably different pIs were spiked into CCH. No interference of matrix components was observed, and the relative distribution of charge variants determined was similar to those determined in samples in formulation buffer. The ability to characterize mAbs with diverse pI values without sample purification reduces analysis time and hands-on labour significantly, especially for clone screening where hundreds to thousands of samples are generated. The method could be employed for determination of charge variants or titer in USP samples and for the determination of charge variants to support DSP formulation development.

2.2.2.3 GLYCOSYLATION

N-glycosylation and O-glycosylation are the most common forms of protein glycosylation. As the vast majority of therapeutic proteins contain N-glycosylation, that is the focus of this review. N-linked glycans consist of a biantennary structure containing a pentasaccharide core of two N-acetylglucosamines (GlcNAc) and three mannoses, and a variable addition of monosaccharides. The latter results in large heterogeneity (Fig. 2). The glycosylation pattern considerably influences many factors such as biological activity, stability against proteolysis, pharmacokinetics, serum half-life, immunogenicity, and the antibody effector functions: complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cell-mediated phagocytosis (ADCP) [39]. These effector functions are important for the efficacy of antibodies for cancer treatments. While most N-glycans are core fucosylated [40], afucosylation greatly enhances ADCC. The presence of bisecting GlcNAc also enhances ADCC to a lesser extent. CDC is affected by terminal galactose, GlcNAc, and mannose residues. Sialic acid or galactose at the terminus of N-glycans enhance anti-inflammatory functions, which is important for autoimmune disease treatment [39,40]. As the glycosylation pattern has such a large impact on the antibody function, it is a CQA that must be controlled and monitored. Glycosylation should be monitored early in the process for better process understanding and the possibility for glycosylation optimisation. N-glycosylation analysis of therapeutic proteins can be performed at different levels; at the intact protein level, subunit protein level, as glycopeptides, or as released glycans. Each level provides different information, with the intact level providing information on the most abundant glycoform pairs, the subunit level on individual glycoforms, including low abundant glycoforms not detected on the intact level, glycopeptides provide information on the location of glycosylation, and released glycans on each individual glycan [41].

Released glycans are derivatised to enable detection, commonly with the fluorescent label 8-aminopyrene-1,3,6-trisulfonic acid (APTS). An example of this is the CGE-LIF method from Reusch et al. [42] for the monitoring of the N-glycosylation of mAbs in USP CCM samples for bioprocess development and characterization. A small-scale purification was performed by filtering the samples and applying them on a protein A column. The purified mAb was thereafter transferred automatically using a robot on the ultrafiltration plate for desalting and deglycosylation in ultrafiltration 96-well plates. CGE-LIF analysis was performed on a DNA analyser equipped with a 48-capillary array, allowing for high throughput analysis. All expected glycans could be detected; the high abundant G0F, G1F, and G2F and the lower abundant G0, G2, G0F–N, and Man5. Separation of the isomeric structures of G1 and G1F was also

achieved. The glycans Man5 and G0F–N co-migrated, which should be considered when, for example, afucosylation has to be determined, as this peak contains both the core-fucosylated G0F–N and the non-core-fucosylated Man5.

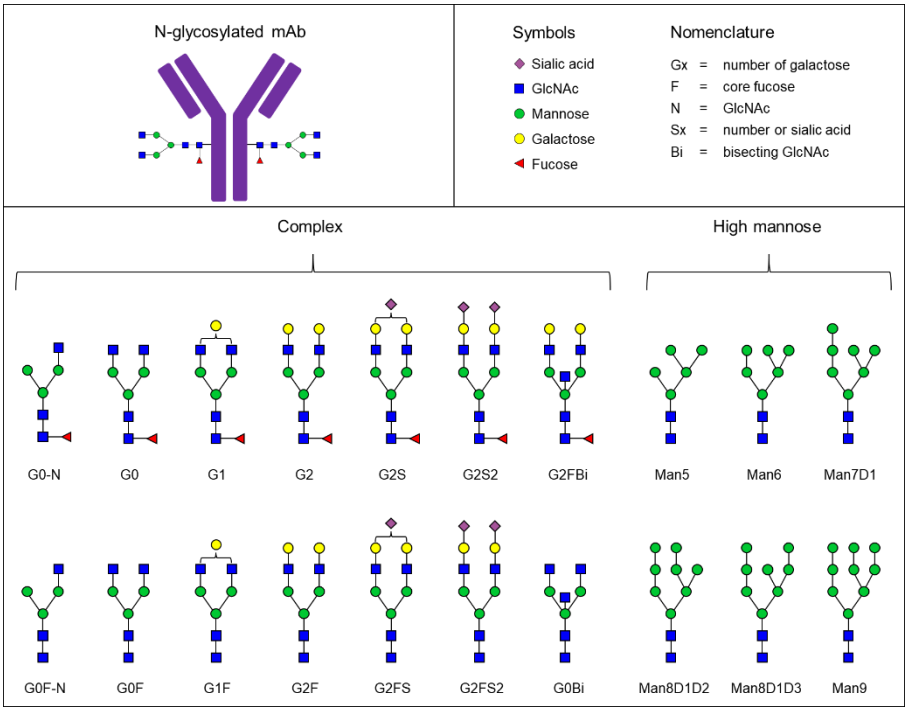

Figure 2. Some common N-glycan structures and their nomenclature.

During process development, often large datasets have to be analysed. An automated approach to data analysis could save a lot of time. Walsh et al. [43] developed a semi-automated approach for large data set analysis and applied this for the monitoring of the glycosylation of antibodies produced under 11 different USP culturing conditions with 3 replicates over 12 days. Sampling each day resulted in 391 samples (5 sampling errors). APTS-labelled released glycans were analysed by CGE-LIF. A triple internal standard was used instead of an external maltooligosaccharide ladder to calculate the glucose unit (GU). Glycans were identified through GU glycan databases. GU glycan databases for APTS-labelled glycans include GUcal [44] and GlycoStore [45]. For quantifying the glycan peaks, HappyTools [46] was employed. However, fluctuating migration times due to day-to-day variability of the method and the different culture conditions and non-Gaussian peaks limited automated quantitation. By grouping electropherograms with similar migration times using a clustering algorithm before applying automated non-Gaussian area calculation in

HappyTools, manual data analysis time was reduced from 2-3 days to 1–2 h for first-time analysis, and down to minutes for repeated analysis.

Both released-glycan methods described above allow high-throughput analysis, which is beneficial for large amounts of samples in, for example, USP bioreactor optimisation or clone selection experiments. The sample preparation protocols for released-glycan analysis are, however, labour- and time-consuming. For real-time monitoring, short analysis times through limited sample preparation are required. Sample preparation is reduced when analysing glycans at the subunit or intact protein level where deglycosylation and derivatisation steps are avoided. The MCE-MS method from Wang et al. [15] (section 2.2.1) was also applied for monitoring glycosylation directly in CCM supernatant at the subunit level. Sample preparation was limited to a desalting and a reduction step to reduce mAb to HC and LC. After a charge-based separation of HC and LC, the deconvoluted MS spectra of the HC were used to determine glycosylation patterns. The MCE method was capable of separating minor charge variants of the HC and LC, such as aglycosylated HC and glycated LC components, potentially allowing for improved detection of low abundant species [15]. The method was applied to follow changes in glycosylation over the time course of the cell culture process by quantifying the glycoforms Man5, G0F–N, G0F, G1F, and G2F, and the level of aglycosylation in CCM supernatant samples after various days of cultivation. For afucosylation determination, a deglycosylation step was added using GlycINATOR enzyme. This enzyme removes N-linked carbohydrates after the first GlcNAc, leaving only core-fucosylated or afucosylated GlcNAc intact. Using one CE-MS method and two sample preparation strategies, mAb glycoforms, the level of afucosylation, and mAb concentration could be monitored in USP. Sample preparation was completely avoided by analysing glycosylation on the intact mAb level [47]. CE-MS analysis was performed directly on CCM supernatant samples. Two MS systems were compared in this study. Although the sensitivity was better using an Orbitrap, the five most abundant glycoforms pairs (G0/G0F, G0F/G0F, G1F/G0F, G0F/G2F or G1F/G1F, and G1F/G2F) could be detected with both a QTOF and the Orbitrap with high sensitivities of 50 µg/mL and 25 µg/mL, respectively. The better sensitivity of the Orbitrap is partly attributed to the in-house two-capillary CE-MS interface used, which functioned as a valve and prevented separated matrix components and salt from entering the ESI source. The possibility to inject sample to 26% of the capillary volume adds to the high sensitivity. The reason for maintaining efficient separation with such large injection volumes is probably transient isotachophoretic stacking because of the high salt concentration in CCM supernatant, which illustrates the possibilities for on-capillary concentration with CE. The method was compared to an HPLC-MS method, showing sharper and

narrower peaks using CE. The desalting by CE led to better spectra quality and the glycoform G1F/G2F was better resolved using CE-MS.

2.2.3 METABOLITES AND NUTRIENTS MONITORING IN UPSTREAM PROCESSING

Cell cultivation processes for the expression of therapeutic proteins require CCMs that support growth and production. The composition of the CCM is important as it affects cell growth, viability, productivity, and CQAs. All CCMs require similar basic nutrients, such as sources of carbon, nitrogen, phosphate, amino acids, fatty acids, vitamins, trace elements, and salts. The concentrations of these nutrients in the CCM provide important information on cell metabolism and thus the state of the cell culture. To optimise cell culture conditions to form well defined therapeutic proteins, information on titer, CQAs, and nutrients and metabolites concentrations should be combined. The concentration of nutrients and metabolites should be closely monitored.

2.2.3.1 AMINO ACIDS

Amino acids (AAs) are essential nutrients for the cultivation of cells. They are the building blocks of proteins, and are therefore the primary constituents of all proteinaceous material of the cells, including the cytoskeleton, enzymes, receptors, and signalling molecules. Furthermore, AAs are required for cell growth and maintenance. Both a lack and an excess of AAs could lead to undesired effects [48]. Monitoring of AAs provides information on the state of the culture, and is important to identify optimal AA concentrations and to be able to adjust their concentrations to these optima.

As most AAs lack UV absorbance or fluorescent groups, other detection strategies should be employed, such as LIF detection after derivatisation, contactless conductivity detection (C4D), or MS. Mikkonen et al. [49] developed a CE-C4D method for the monitoring of free AAs directly in CCM supernatant samples. The advantage of C4D detection is that no derivatisation step is required, simplifying sample preparation. The EOF was suppressed, and adsorption from matrix components was prevented by adding 0.1% 2-hydroxyethyl cellulose to the BGE, contributing to robustness. Cyclodextrins (CDs) were employed to optimise separation. Two CDs, α-CD and β-CD, were investigated. These CDs are bucket-shaped molecules with a hydrophilic outer surface and a hydrophobic cavity. Based on the hydrophobicity of the amino acids, they can undergo different degrees of complexation by the CD cavities. As the neutral CDs have no electrophoretic mobility, CD-complexed AAs migrate slower. This additional separation mechanism enhanced

the selectivity. As baseline resolution of 20 AAs in one single method was not achieved due to the difference in physical chemical properties, two methods using different CDs at different concentrations were combined to separate all 20 AAs. The two methods were applied for the analysis of AAs in CCM supernatant samples after different days of cultivation. No interference of matrix components was observed. By combining the results of the two CE-C4D methods, 17 AAs could be detected in the CCM supernatant samples, with no other sample preparation than dilution. Arginine and histidine overlapped due to the high concentration of arginine, and cysteine was not detected in CCM samples, likely due to the low concentration and its conversion to oxidized dimer.

For reduced analysis time and minute sample volumes, and for the possibility of automation, MCE becomes increasingly popular. An MCE-LIF method for the monitoring of AAs in USP was developed employing fluorescent derivatisation with NDA [50]. NDA derivatises primary amines, present in all AAs except proline, which could therefore not be detected using this method. Due to the differences in physical chemical properties of the AAs, two methods were used for the separation of all 19 AAs; one micellar electrokinetic chromatography (MEKC) method for the more polar AAs, and one solvent modified MEKC method containing 4% 1-butanol for the less polar AAs, both using SDS as micellar surfactant. MCE separation showed to be similar to capillary separation, although resolution between a few AAs was lost. The methods were applied to spent CCM supernatant after different days of cultivation. Limited matrix component peaks were observed, and by combining the results from the two methods, 17 AAs could be quantified. In another study, derivatisation was avoided by employing MCE-MS [51]. Using isotope-labelled AA standards for internal calibration, 16 AAs could be quantified in less than 2 min. Due to the mass-specific detection of CE-MS, AAs that comigrated in CE (methionine and threonine, and phenylalanine and proline), could be resolved with MS. The method was applied on spent CCM samples after various days of a CHO cultivation process, without AAs supplementation, to determine the AAs consumption rate. The correlation of the reduction level of certain AAs with reduced cell viability was demonstrated. This information could be used for the optimisation of feed strategies. Although MCE-MS is rapid, MS requires heavy vacuum systems using turbomolecular pumps, making MS instrumentation large, and thus more difficult to implement in rapid, on-site analysis. To overcome this, the group of Ramsey developed a miniature cylindrical ion trap-based mass spectrometer operating at high pressure (≥1 Torr), eliminating the need for turbo pumps, and coupled this high-pressure MS (HPMS) to MCE [52]. The MCE-HPMS system was employed for the monitoring of AAs consumption rate in an Escherichia coli cell cultivation process [53]. 18 of the 20 AAs were detected with

limited sample preparation in less than 3 min. The smaller AAs glycine and alanine were not detected, likely because these AAs are scattered more easily by collisions with the buffer gas due to their small size. The comigrating AAs methionine and threonine, asparagine and proline, tryptophan and glutamine, and glutamic acid and cysteine were easily resolved with MS. Relative consumption rates were determined based on the peak areas normalised to the internal standard of isotope-labelled valine. Elliott et al. [54] employed this MCE-HPMS for the monitoring of AAs, vitamins, and dipeptides in microbioreactors stressed with varying levels of ammonia (unstressed, 10, or 30 mM NH3). No sample preparation other than diluting the CCM supernatant samples was required. The data were correlated with cell viability data from the viability analyser and glucose, lactate, IgG, and NH3 concentrations measured using a Cedex Bioanalyzer. A large difference in mAb production for the different NH3 stresses was found. A difference in cell viability, lactate, and glucose levels was observed for the 30 mM NH3-stressed bioreactor after approximately 132 h into the process, while AA changes could be observed even earlier in the process. To fully understand the mechanism of AA changes and therapeutic protein production, more frequent sampling is required. These rapid MCE methods allow for at-line, real-time monitoring of AAs in cell cultivation processes.

2.2.3.2 SACCHARIDES

Commonly, glucose is the primary energy and carbon source in CCMs. Glucose consumption usually leads to pyruvate accumulation and increased lactate concentration. The latter can inhibit cell growth in mammalian cell cultures. Strategies to prevent this involve using low glucose concentrations or employing alternative energy sources, such as galactose, mannose, and fructose. The replacement of glucose, or the addition of saccharides to glucose-containing CCMs, influences lactate production and consumption, and influences glycosylation of the protein [55]. To fully understand the effect of different saccharides and their concentrations on cell viability and glycosylation, a CE and MCE-LIF method was developed for the monitoring of glucose, mannose, galactose, fructose, and lactose directly in CCM supernatant [56]. As these saccharides do not possess chromophores or fluorophores, their detection was ensured by fluorescent derivatisation with APTS. Sample preparation was developed to aid automation; no sample clean-up was required and common sample drying steps were avoided. The derivatisation reaction and BGE composition were optimised using AQbD to result in a robust method. The CE method could readily be transferred to MCE. CCM supernatant after different days of cultivation could be analysed directly without any interference of matrix components in less than 6 min.

2.2.3.3 NUCLEOTIDES

Another factor in affecting glycosylation is the intracellular level of nucleotide and sugar nucleotides. Sugar nucleotides are monosaccharides bound to a nucleotide, and are precursors for glycosylation. Nucleotides play important roles in nucleic acid synthesis, cellular growth, and energy metabolism. Bucsella et al. [57] developed a CE method for the quantification of nucleotides and sugar nucleotides in CHO cell extracts to optimise the CCM conditions. For robust routine testing, the EOF should be controlled and adsorption avoided. This was achieved by employing a dynamic successive multiple ionic layers using the ready-to-use polycation and polyanion buffers from CEofix. When applying the coating, the migration time repeatability improved from 6% RSD to 0.3% RSD compared to no capillary coating. The separation was further improved by optimizing the pH, BGE concentration, capillary temperature, and applied voltage. Nucleotides and sugar nucleotides were extracted from CHO cells using cold acetonitrile, no concentration or derivatisation steps were required. Eleven nucleotides and sugar nucleotides were separated and quantified.

2.2.3.4 VITAMINS

Vitamins are essential nutrients and play important roles in cell growth, cell viability, and productivity [58,59]. Vitamins are instable molecules, sensitive to a range of external factors, such as light, oxygen, low or high pH, and temperature, as well as to interactions with other cell culture medium components [60], challenging accurate analysis.

Although vitamins are essential nutrients which should be monitored, there is a lack of methods for in-process vitamin analysis. One CZE method was developed for the analysis of five B-vitamins (thiamine, nicotinamide, pyridoxine, pyridoxal, and pyridoxamine) directly in CCM samples [61]. To prevent adsorption of matrix components to the capillary wall, a dynamic triethanolamine coating was employed. The vitamins could be analysed in CCM with good precision and accuracy. Later, van der Burg et al. developed an MEKC method employing SDS for the analysis of ten B-vitamins and vitamin C in CCM [62]. There is a need for more methods analysing vitamins in process-intermediates.

2.2.4 IMPURITIES ANALYSIS IN DOWNSTREAM PROCESSING

After expression of biopharmaceuticals in host cells, the PoI must be purified from components such as cell debris, HCPs, HC-DNA, residual proteins, etc. During the purification in DSP, other components can be added, such as lysing agents, HC-DNA clearing agents, and eluents for chromatographic purification steps, which also have

to be removed. The removal of all these impurities must be followed throughout DSP, both to improve the process and to prove clearance.

The SDS-CGE-IA method for Fc-fusion protein concentration determination developed by Xu et al. (section 2.2.1) was also employed for the evaluation of the DSP process [24]. The Fc-fusion protein consists of two polypeptides: fusion-Fc and single chain Fc. The CCH contained an excess of single chain Fc polypeptide, which had to be removed by the purification process. Also, HCP and co-expressed enzyme had to be removed. The clearance of single chain Fc polypeptide, co-expressed enzyme, and a specific HCP, heat shock protein 70 (HSP70), was quantified throughout the DSP process in CCH, eluate of first column process, and drug substance samples (Fig. 3). Different primary antibodies were used for Fc polypeptide, HSP70, and enzyme antibody probing.

For vaccine production, fetal bovine serum could be used as CCM. BSA is a major component of fetal bovine serum, and could cause allergic reactions in humans. Therefore, the WHO requires a limit of ≤50 ng per vaccine dose. To monitor these low levels in vaccine samples, sensitive analytical tools are required. For this purpose, a SDS-CGE-IA method was developed [63]. Quantification was performed using an external BSA calibration curve. BSA protein loss due to adsorption was avoided by dilution in sample buffers containing SDS. Analysing BSA resulted in monomer, dimer, and trimer peaks. A significantly higher amount of dimer was detected with SDS-CGE-IA compared to SDS-PAGE or size exclusion chromatography. It is suggested that this is because the primary antibody used preferentially recognises dimer BSA over monomer BSA. Since BSA aggregates were typically not observed in the tested vaccine samples, monomeric BSA was used for quantitating residual BSA. The method was applied to various drug product samples as well as to different DSP-intermediates to monitor the clearance of BSA with a lower limit of quantification of 5.2 ng/mL.

HCPs present a safety risk because they could cause immunogenicity, adjuvant activity, or proteolytic activity. Therefore, it is essential to confirm HCP clearance during purification in DSP. Challenges in HCP quantification include the low concentration, the wide dynamic range, and their detection in the presence of large excess of therapeutic protein. Removing the therapeutic protein before HCP analysis

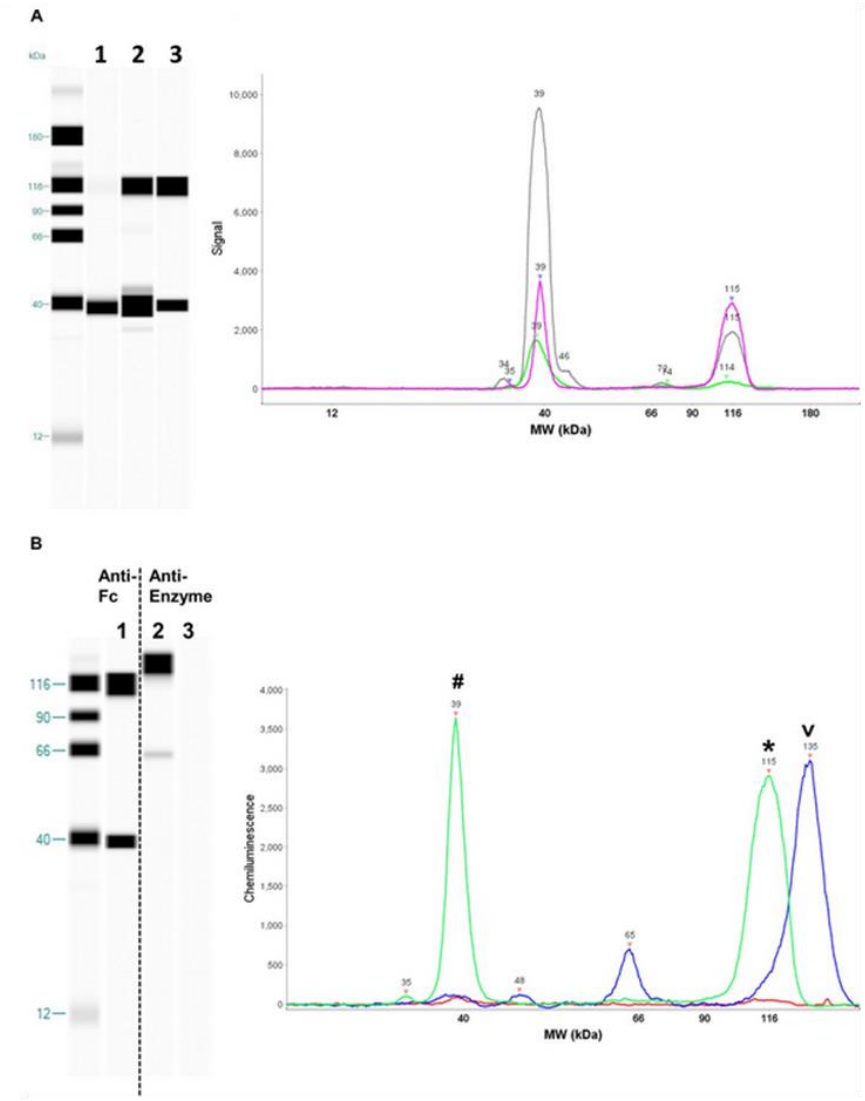

Figure 3. (A) Removal of excess single chain Fc. Left: gel-like image and right: electropherograms of fusion-Fc and Fc polypeptides detection in (1, green) CCH, (2, silver) first column elute, and (3, pink) drug substance using HRP-conjugated anti-Fc antibody. (B) Removal of co-expressed enzyme. Left: gel-like image and right: electropherograms for the first column eluate (2, blue) and drug substance (3, red) probed by anti-enzyme antibody, and drug substance probed with HRP-conjugated anti-Fc antibody as control (1, green). The three polypeptides single chain Fc, fusion-Fc and enzyme are assigned with #, ∗, and ˅, respectively. Reprinted from Ref. [24]. with permission from Wiley.

reduces the background signal from the therapeutic protein itself, however, it increases the number of sample preparation steps and time, and it induces the risk to lose HCP during sample preparation. Analysing HCP without therapeutic protein removal simplifies the workflow and eliminates loss of HCP during purification. Zhang et al. [64] evaluated the effect of mAb depletion before HCP analysis and showed that less proteins and peptides were found in a nondepleted sample, due to suppressed identification of low abundance HCP by high abundance mAb peptides. However, the non-depleted sample included 24 proteins that were not present in the depleted sample. Most of these proteins are expected to be "hitchhiker" protein that are removed along with the mAb by purification. The peptides showed a broad pI distribution, therefore, an online pH gradient fractionation using a strong cation exchange SPE monolith prepared at the tip of the capillary was introduced. Five successive elutions using buffers at various pHs were injected into the capillary. The complexity of the injected sample was decreased which improved the detection of low abundance peptides, and increased the number of proteins and peptides identified.

The removal of HC-DNA could be performed using domiphen bromide. As bromide is an anticonvulsant and sedative, it has a set limit of 4 µg bromide per dose. A CZE method was developed for the detection of bromide in DSP process-intermediates [19]. The high concentration of chloride present in some samples caused electromigration dispersion of the bromide peak. However, a simple water dilution of the sample was sufficient to improve peak efficiency and reduce electromigration dispersion, and bromide could be quantified accurately with an LOQ of 0.2 µg/mL.

2.2.5 DOWNSTREAM PROCESS SUPPORT

An MCE application for the determination of the physicochemical properties required for chromatographic purification is used to aid DSP process development. MAbs can be purified using affinity interactions with protein A, but for other therapeutic proteins, such an affinity purification step is not at hand. For these therapeutics, often chromatography is used for purification. To aid faster chromatographic process development, mechanistic models for process simulation can be used. This allows for large numbers of experiments to be carried out in silico, having the advantage of low developmental time and costs, and increased process understanding. In order to apply these mechanistic models, the physicochemical properties of the therapeutic proteins have to be known. For IEC purification, the steric-mass action (SMA) parameters such as equilibrium constant, protein characteristic charge, and steric factor are important. These factors can be determined by the retention volumes of single proteins in linear salt gradient IEC of different gradient lengths. Due to the high

complexity of DSP process-intermediates, retention volumes for single proteins cannot be readily determined from IEC alone. To determine the SMA parameters of a single protein in a cell lysate, Kröner et al. analysed IEC fractions with microchip CGE. Per IEC run, 83 fractions were collected, desalted, and concentrated [65]. Similar to SDS-CGE, proteins were given a uniform charge-to-size ratio using the surfactant lithium dodecyl sulfate (LDS). Microchip LDS-CGE analyses were performed under reducing conditions on a LabChip GX-II device using the HT Protein Express 200 assay (~45 s/sample), allowing high throughput analysis. Quantification of single proteins in each fraction was performed using an internal calibration standard. The single proteins were assigned to the retention volume belonging to the fraction number, and the exact retention volume of the protein was determined by Gaussian peak fitting of the concentrations and the retention volumes. The SMA parameters could be determined using the retention volumes of single proteins for all gradient lengths. By using IEC and microchip LDS-CGE, relevant factors for in silico prediction of chromatographic behaviour for DSP purification of proteins in complex cell lysate were characterised.

2.2.6 TOWARDS COMPLETE UPSTREAM PROCESS MONITORING

The rapid expansion of the use of biopharmaceuticals requires efficient bioprocessing. In-dept understanding of the UPS and DSP process is essential to optimise the process in terms of production yield, CQAs, and production robustness. To understand how various process variables affect the CQAs or yield of therapeutic proteins, these different variables (e.g., metabolites, nutrients, pH), together with cell viability, CQAs, and titer have to be monitored continuously during the USP process. On-line sensors for pH, pO2, pCO2, or cell density monitoring are frequently used. However, monitoring of important nutrients and metabolites is limited to only a few, including glucose, lactate, glutamine, and ammonia. The monitoring should be expanded to a broader range of nutrients and metabolites. Real-time, continuous monitoring requires on-line sampling, analysis, and data processing. CE has proven to be a valuable tool for the monitoring of titer, CQAs, and nutrients and metabolites in process-intermediates, and the possibility for miniaturisation makes it suitable for PAT.

Alhusban et al. [66] developed an on-line, near-real time analysis setup for the monitoring of USP. In this setup, employing sequential injection-CE, samples were automatically taken from a bioreactor, led through a microscope for cell counting, and directed to an injection valve through an H-filter to prevent clogging. Valves were used to direct sample and BGE solutions to the capillary or the waste and allowed for hydrodynamic sample injection and flushing of the capillary. The setup is depicted in

Fig. 4. The CE method was developed for the analysis of glucose, glutamine, leucine/isoleucine and lactate. For electrophoretic separation of glucose, a strongly alkaline BGE (pH > 12) was required. To allow for the use BGEs with such high pHs, polymethylmethacrylate (PMMA) capillaries were used. The capillary was coated with a polyelectrolyte coating. All target analytes could be separated without interference of matrix components. Over the course of 4 days, measurements were performed every 30 min. Only 8.1 mL sample was used for the total of 200 measurements, which could potentially be further reduced by optimizing dead volume and recycling of the medium solution back into the bioreactor after the H-filter.

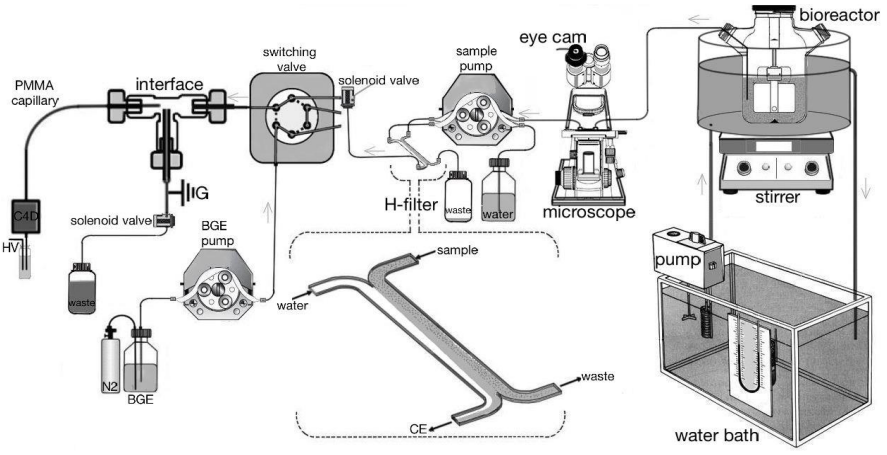

Figure 4. Schematic overview of experimental setup. Sample is withdrawn from the bioreactor and cell density is measured using automated image analysis of images captured by the digital microscope before sample enters the H-filter. The cell-free H-filter effluent is analysed by sequential injection-CE-C4D. Reprinted from Ref. [66] with permission from Elsevier.

The MCE-HPMS developed by Ramsey's group [[52], [53], [54]] was commercialised as CCM analyser “REBEL” by 908devices. The “REBEL” can analyse over 30 nutrients and metabolites in near-real-time. Although lacking the possibility for automated sampling or sample preparation, the analysis of this large number of nutrients in only minutes is a great step towards complete process monitoring. The European Union financed Horizon2020 IMI2 project iConsensus works to take it a step further, by developing a completely automated analytical control and sensing platform for continuous monitoring of the upstream process, including automated sampling, sample preparation, analysis, data processing, and data transfer to a monitoring information database and models for automatic feedback control

(https://www.iconsensus.eu/). This platform aims to correlate data from on-line sensors and at-line miniaturised quantification techniques, including MCE applications [50,56] (Fig. 5).

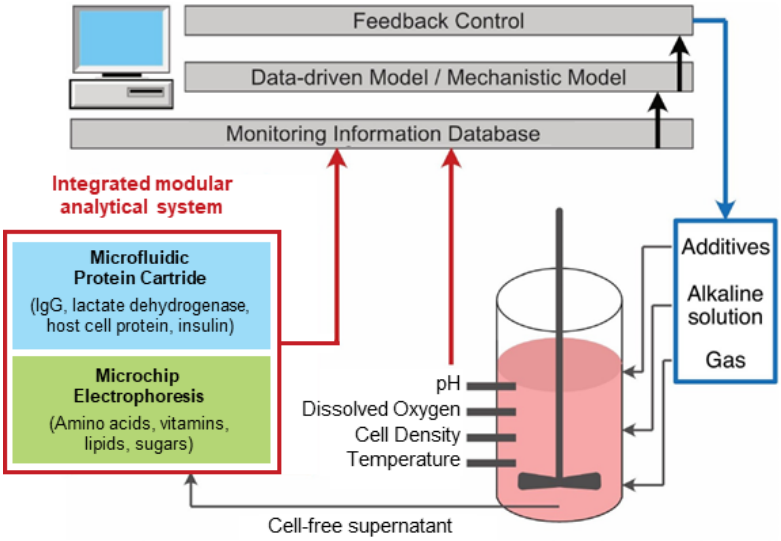

Figure 5. Integrated modular analytical system, the modules contain one or several multiplexed miniaturised quantification techniques. Adapted from Ref. [67] under the Creative Commons CC BY licence (Elsevier).

2.3 CONCLUDING REMARKS

In the past decade, CE has been extensively used in biopharmaceutical upstream and downstream processing. In particular SDS-CGE and CZE showed powerful for size and concentration determination in process-intermediates. Especially CZE has proven to be very tolerable against samples with complex matrices such as CCM samples. Although in particular cIEF, icIEF, and CZE, are valuable tools for protein charge determination, only a few applications are published for charge determination in process-intermediates. For increased process understanding, analysing charge variants in process-intermediates is essential. Considering the success of CE for process-intermediate analysis, the area of CE for charge variant analysis in process-intermediates should be further explored.

The capability to handle various complex matrices, limited sample clean-up requirements, low sample volumes, rapid analysis, and possibilities for miniaturisation and automation makes CE suitable for offline process monitoring as

well as for PAT on-site, real-time monitoring of the process. The small sample volumes required is especially advantage for analysis of micro-bioreactors or during clone selection. CE applications, using MCE in particular, will be further explored and developed for the continuous monitoring of USP.

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Chapter

Method development for quantitative monitoring of monoclonal antibodies in upstream cell culture process samples with limited sample preparation – evaluation of various capillary coatings

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ABSTRACT

Monoclonal antibodies (mAbs) have become an important class of biopharmaceuticals used for the treatment of various diseases. Their quantification during the manufacturing process is important. In this work, a capillary zone electrophoresis (CZE) method was developed for the monitoring of the mAb concentration during cell-culture processes. CZE method development rules are outlined, particularly discussing various capillary coatings, such as a neutral covalent polyvinyl alcohol coating, a dynamic successive multiple ionic-polymer coating, and dynamic coatings using background electrolyte additives such as triethanolamine (T-EthA) and triethylamine. The dynamic T-EthA coating resulted in most stable electro-osmotic flows and most efficient peak shapes. The method is validated over the range 0.1–10 mg/ml, with a linear range of 0.08–1.3 mg/ml and an extended range of 1–10 mg/ml by diluting samples in the latter concentration range 10-fold in water. The intraday precision and accuracy were 2%–12% and 88%–107%, respectively, and inter-day precision and accuracy were 4%–9% and 93%–104%, respectively. The precision and accuracy of the lowest concentration level (0.08 mg/ml) were slightly worse and still well in scope for monitoring purposes. The presented method proved applicable for analysing in-process cell-culture samples from different cell-culture processes and is possibly well suited as platform method.

3.1 INTRODUCTION

3.1.1 PROCESS MONITORING

Biopharmaceuticals have become important products in the pharmaceutical industry, with monoclonal antibodies (mAbs) as the most rapidly growing class [1, 2]. Currently, over a 100 different mAbs have been approved in the EU or the United States, and more are under review [3]. mAbs are highly effective in treating various diseases. The cell-culture process to produce mAbs is complex. Many factors affect the quality of the final product. Process monitoring is therefore important to understand the state of the process and actively manipulate it to maintain a desired state. Process analytical technology (PAT) can aid real-time monitoring to design, analyse and control the biopharmaceutical process [4]. Ideally, the conditions and quality of the process and product should be known during the entire process. Currently, this knowledge is often lacking. This knowledge gap could be filled by PAT solutions that monitor the process in real time [5]. One of the factors to be monitored during upstream processing (USP) is the concentration of mAbs. The quantification of mAbs in real time can provide insight into the state of the cell-culture. Common techniques for mAb concentration determination include protein A affinity chromatography [6-8] and bio-layer interferometry with Octet platforms [9, 10], the latter of which uses a fibre-optic biosensor for mAb concentration determination. Capillary zone electrophoresis (CZE) is a valuable addition to these techniques, with the potential to be used as PAT solution for at-line and real-time monitoring. CZE can analyse mAbs in a wide concentration range and handle complex matrices, requires little sample preparation and provides excellent separations. An additional advantage of CZE is the potential for miniaturisation and automation using microchip-CE, which could even allow for on-line monitoring of mAb concentrations. Previous experiments on mono- and disaccharide determinations in cell-culture media showed that the CZE method could be easily translated into a microchip-CE method [11]. The low diffusion coefficients of mAbs result in high separation efficiencies in CZE, and due to the open capillary tube, CZE is less prone to clogging. Therefore, no extensive sample clean-up is required [12]. Generally, CZE sample preparation and separation are fast, allowing for high-throughput analysis.

CZE already proved to be very suitable for mAb analysis. In 1998, Dai et al. successfully employed CZE for the quantification of mAbs in cell-culture medium [13]. They, however, used an uncoated capillary and high pH background electrolyte (BGE). Under these conditions, the negatively charged free silanol groups on the capillary wall can cause the adsorption of both the mAbs and cell-culture medium components. Over the years, improvements have been made, however, mainly for

the analysis of mAb charge heterogeneity, for example using 6-aminocaproic acid–based BGEs with triethylenetetramine and hydroxypropyl methylcellulose to prevent adsorption [14-16]. An intercompany study with 11 participants showed the robustness of this type of method [17].

3.1.2 METHOD DEVELOPMENT AND CAPILLARY COATING

For process monitoring, robust methods are required. Robustness is built in to the method by making conscious decisions during development. For example, the adsorption of analytes or matrix components to the capillary wall should be prevented, and the electro-osmotic flow (EOF) should be controlled. Both can be achieved by using capillary coatings. Attractive electrostatic interactions should be avoided by selecting a capillary coating with the appropriate charge. The charge of the mAb is determined by the pH of the BGE. Due to the large variation in post-translational modifications, mAbs are very heterogenous with regard to the isoelectric point and resulting charge properties. Small molecular changes such as deamidation result in a change in the mAbs charge. If the pH of the BGE is close to the p*I* of the mAb, the mobility difference due to the charge heterogeneity will be larger; moreover, the electrophoretic mobility will be very low, causing broad peaks. Selecting a BGE pH sufficiently different from the p*I* of the mAb results in the mAbs being detected as one single peak, which is beneficial for quantification and reduces band broadening. As mAbs typically have high p*I*s ($p > 6$, generally 8–9) [18, 19], a low pH BGE should be selected, which furthermore allows the application of the method to a broad variety of mAbs. To avoid attractive electrostatic interactions with the positively charged mAbs, positively charged and neutral capillary coatings were investigated.

In neutral capillary coatings, ideally, there is no EOF, and in positively charged capillary coatings, the EOF is reversed. The latter will slow down the mAbs and other positively charged compounds or even reverse their net migration direction. The charge of bare fused silica (BFS) capillaries is pH depended; the silanol groups ($pK_a > 5$) are negatively charged at high pH, whereas at low pH, the charge, and therefore the EOF, is reduced. Hence, a low pH reduces adsorption and controls the EOF. Because silanol groups are reactive molecules, adsorption cannot be fully avoided, for example by hydrogen bonding. To further reduce adsorption, capillary coatings can be applied. For neutral coatings, covalently coated polyvinyl alcohol (PVA) capillaries could be used that have proven to be stable at low pHs [20]. For positively charged coatings, dynamic coatings such as successive multiple ionic-polymer (SMIL) coatings could be used [21, 22]. A positive SMIL coating can be prepared using the cationic polymer polybrene (PB). A single layer of PB does not fully

cover the silica surface, whereas three polymer layers do provide full coverage [23]. A stable layer of PB could be formed by applying three polymer layers (SMIL-3), two layers of PB with a layer of the anionic polymer dextran sulphate (DXS) in between (PB–DXS–PB) [24-26]. The charges of both PVA and SMIL-3 coatings are ideally independent of the pH of the BGE. Other options for dynamic coatings include the use of BGE additives, for example amines, such as triethanolamine (T-EthA) or triethylamine (TEA). These amines can form a positively charged layer on the capillary wall, masking free silanol groups [27-29], thus protecting against adsorption.
In this work, a CZE method for the monitoring of the mAb concentration in various upstream biopharmaceutical processes was developed and validated while investigating the effect of different capillary coatings on the performance of the method. The aim was to obtain a method that is simple to use with no or limited sample preparation, robust, can be used as a platform method for different mAbs, and that has the potential to be miniaturised to a microchip-CE method. The method should cover a concentration range of 0.1–10 mg/ml of mAb in cell-culture medium with precision and accuracy of 15% RSD and 90%–110% recovery, respectively, and for the lower range limit 20% RSD and 80%–120% recovery, respectively.

3.2 MATERIALS AND METHODS

3.2.1 CHEMICALS

Phosphoric acid 85% solution, tris(hydroxymethyl) aminomethane (Tris), T-EthA, TEA, poloxamer 188 10% solution, PB, DXS, dimethylsulfoxide (DMSO) and Protein A agarose were obtained from Merck/Sigma-Aldrich (Darmstadt, Germany). HyClone ActiPro medium was purchased from Cytiva (Marlborough, USA). Three commercially available mAbs were purchased, NIST mAb reference material (NIST) (10 mg/ml in 12.5 mM l-histidine, 12 mM l-histidine HCl) and SILuLite SigmaMAb Rituximab mAb were from Sigma-Aldrich (Darmstadt, Germany), and Intact mAb Mass Check Standard was from Waters (Milford, USA), hereafter referred to as NIST mAb, Rituximab and Waters mAb. For method validation, adalimumab (10 mg/ml in 12.5 mM histidine buffer) was purchased from Merck/Sigma-Aldrich (Darmstadt, Germany). Spent cell-culture medium samples of process A after 6, 10 or 14 days of cultivation and purified drug substance (DS) samples were provided by Byondis BV, Nijmegen, the Netherlands. Spent cell-culture medium samples of process B after 0, 5 or 8 days of cultivation and process C after 0 or 7 days of cultivation were provided by the Department of Industrial Biotechnology, KTH Royal Institute of Technology,

Stockholm, Sweden. All chemicals were of analytical grade, water was of Milli-Q grade (18.2 MΩ cm).

3.2.2 INSTRUMENTAL CONDITIONS

Experiments were conducted on an Agilent 7100 or Agilent G1600 AX capillary electrophoresis system with a Diode Array UV detector (Waldbronn, Germany). The OpenLAB CDS ChemStation (Rev. C.01.09) software was used for instrument control, data acquisition and data analysis. BFS capillaries with 50 µm id were either from Agilent Technologies or Polymicro Technologies, and PVA-coated capillaries with 50 µm id were purchased from Agilent Technologies. Capillaries had a total length of 33 cm with an effective length of 24.5 cm for initial method development and 8.5 cm for method validation and the final method. The separation voltage was 16 kV and was ramped over 0.5 min. For the SMIL-3 coated capillary, the separation voltage was reversed to −16 kV. This resulted in a current of ~45 µA in both cases. Samples were introduced hydrodynamically at 10 mbar for 5 s, followed by the injection of a BGE plug using the same conditions. Separations were carried out at 20°C. The detector signal was recorded at 210 nm.

3.2.2.1 CAPILLARY PRECONDITIONING

Bare fused silica and dynamic T-EthA and TEA coatings

Before first use, the capillary was flushed successively with 1 M NaOH, water and BGE at 1 bar for 20 min each. At the beginning of each working day, the capillary was flushed successively with 1 M NaOH, water, and BGE at 1 bar for 10 min each. Prior to each injection, the capillary was preconditioned with 0.1 M NaOH at 1 bar for 1 min and BGE at 1 bar for 2 min.

PVA coating

The PVA capillary was flushed successively with 10 mM phosphoric acid, water and BGE at 1 bar for 20 min each before first used, and for 10 min each at beginning of each working day. Before injection, the capillary was flushed with 10 mM phosphoric acid for 1 min and BGE for 2 min, each at 1 bar.

SMIL-3 coating

For the SMIL-3 coating, a BFS capillary was flushed with 1 M NaOH for 30 min and then with water for 10 min to clean the capillary. After preconditioning, the capillary was flushed with a 10% PB solution for 10 min for the first cationic layer, then with water for 5 min, followed by a 0.5% DXS flush for 10 min to form an anionic layer, followed by a water flush of 5 min, and finally, a last 10% PB flush for 10 min for a second cationic layer. This procedure was performed before first use, and at the

beginning of each working day. In between runs, the capillary was flushed with BGE at 1 bar for 2 min.

3.2.3 BGE PREPARATION

Three different BGEs were tested: BGE 1: 100 mM phosphoric acid, 70 mM Tris, and 0.1% poloxamer; BGE 2: 100 mM phosphoric acid, 70 mM T-EthA, and 0.1% poloxamer; and BGE 3: 100 mM phosphoric acid, 70 mM TEA, and 0.1% poloxamer. These BGEs were prepared by mixing appropriate amounts of 1 M phosphoric acid, 1 M Tris, 1 M T-EthA, 0.2 M TEA, and 10% poloxamer 188 solutions. After preparation, the pH of the BGE was checked. All BGEs had a pH of 2.5.

3.2.4 SAMPLE PREPARATION

3.2.4.1 STANDARDS

The NIST mAb (10 mg/ml) was aliquoted and stored at −80°C. The Rituximab and the Waters mAb were dissolved in water to a concentration of 5 mg/ml, aliquots were stored at −20°C. For analysis, the mAbs were diluted in either water or ActiPro medium to a concentration of 1.5 mg/ml. Spent cell-culture medium samples were analysed untreated.

3.2.4.2 PROTEIN A PURIFICATION

Protein A (24 mg) was mixed with 120 µl binding buffer (10 mM phosphate buffer pH 8.0) in a 1.5 ml Eppendorf cup for 30 min before use, all mixing steps were carried out on an IKA MS 3 digital shaker at 1200 rpm. Protein A was then conditioned by mixing with an additional 240 µl binding buffer for 5 min; the supernatant was discarded after centrifugation using a Biofuge Pico microcentrifuge; all centrifugation steps were carried out at 3000 *g* for 3 min. The spent cell-culture medium sample from process C after 7 days of cultivation (4 ml) was diluted with 30 mM phosphate buffer pH 8 (1.5 ml). The diluted medium sample of 1 ml was mixed with the protein A for 30 min, and after centrifugation, the supernatant was collected as 'mAb-free spent cell-culture medium'. The last step was repeated until the entire volume of the diluted medium sample was purified. If the supernatant still contained mAb, the supernatant was purified again using a freshly prepared protein A mixture.

3.2.4.3 METHOD VALIDATION SAMPLE PREPARATION

The 10 mg/ml adalimumab solution was spiked into the protein A–purified mAb-free spent cell-culture medium at six concentrations levels, each prepared in triplicate.

The concentration levels were 0.08, 0.4, 0.7, 1.0 and 1.3 mg/ml adalimumab, respectively, and an additional triplicate of 1.0 mg/ml, which was thereafter diluted 10-fold in water to simulate the lower concentration of the extended range (1–10 mg/ml) of the method. The adalimumab standard was diluted to 1 mg/ml in water as a calibration sample. For migration time repeatability, adalimumab was spiked to spent cell-culture medium at day 0 (mAb-free) to a concentration of 1 mg/ml.

3.2.5 EOF DETERMINATION

As the EOF is very low for the coated capillaries, the EOF was determined using the method developed by Williams and Vigh [30]. In short, three bands of a neutral marker (0.02% DSMO in BGE) were injected into the capillary at 50 mbar for 5 s. The first two bands were both transferred into the capillary by applying 50 mbar for 30 s, then a voltage of −16 kV was applied over the capillary for 5 min, where both DMSO bands migrate with the velocity of the EOF. The third band was injected, and all three bands were transferred past the detector using a pressure of 50 mbar, where they were detected at 200 nm, see Figure S1. Because the EOF in the SMIL-3 coated capillary was faster and the DMSO bands passed the detector during the electrophoresis step, the electrophoresis step was adjusted to applying −10 kV for 2 min.

3.3 RESULTS AND DISCUSSION

3.3.1 CAPILLARY COATING SCREENING

During method development, different capillary coatings were investigated and assessed based on their ability to prevent adsorption and to provide a stable EOF. To prevent adsorption of the positively charged mAbs, a neutral covalent PVA coating and positive dynamic coatings, either by applying a SMIL-3 coating before analysis, or by using T-EthA or TEA as BGE additives, were selected. Additionally, an uncoated BFS capillary was used for comparison. In order to detect the mAbs as single peaks and reduce band broadening, a low pH BGE was selected: 100 mM phosphoric acid, 70 mM Tris BGE atd pH 2.5. For the dynamic coatings using BGE additives, Tris was replaced by either T-EthA or TEA in the same concentration. Interchanging Tris, T-EthA or TEA resulted in BGEs with equal pH, ionic strength and buffering capacity. Besides capillary coatings, neutral surfactants could further prevent adsorption [20]. A neutral surfactant often used in cell-culture processes as antifoaming agent is poloxamer 188, this surfactant was therefore chosen as additive to all BGEs.

Figure 1 shows the electropherograms of different mAbs obtained with the different capillary coatings. The high p*I*s of the mAbs in combination with the low pH of the BGEs resulted in single peaks for mAbs on each capillary coating. On the uncoated capillary, the mAb peaks were slightly tailing, which could indicate some interaction with the capillary wall, showing the importance of capillary coatings. Most efficient peak shapes were obtained using positively charged coatings. With positively charged coatings, the direction of the EOF reversed (Table 1). The T-EthA coating resulted in a very low EOF where the mAbs still migrated in the direction of the negative electrode, whereas the SMIL-3 coating resulted in an EOF mobility higher than the mobility of the mAbs; therefore, the separation was performed in negative polarity mode, and the migration order of the mAbs was reversed.

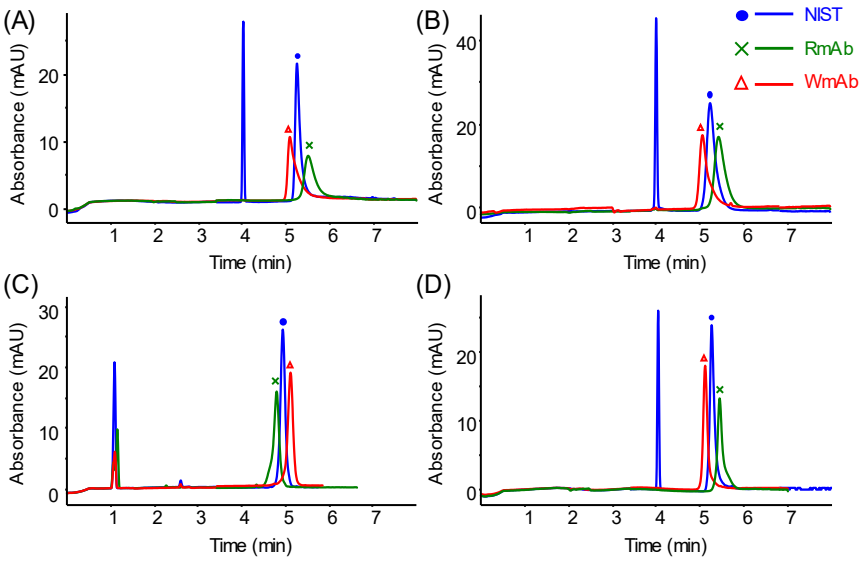

Figure 1. Rituximab, Waters monoclonal antibody (mAb) and NIST mAb analysed on (A) an uncoated capillary, (B) a polyvinyl alcohol (PVA)-coated capillary, (C) a SMIL-3 coated capillary with background electrolyte (BGE): 100 mM phosphate, 70 mM Tris and 0.1% poloxamer (no poloxamer present in BGE [C]), or (D) a dynamic triethanolamine (T-EthA)-coated capillary with BGE: 100 mM phosphate, 70 mM T-EthA, 0.1% poloxamer. Effective separation length was 24.5 cm.

Promising results were obtained with all four capillaries. As the concentration of the mAbs should be determined in USP samples, separation from medium components is required. The three different mAbs were spiked into ActiPro cell-culture medium and analysed with the different coatings. In addition to the coatings already tested, a dynamic TEA coating was added, as the positively charged coatings resulted in

sharper peaks and as dynamic coatings are simple and cheap. No or a low EOF resulted in a large separation window, increasing the separation of the mAbs from the medium components. For the SMIL-3 coating with a stronger EOF, the separation window was smaller, and the matrix components migrate closer to the mAb (Figure 2). Not only is the separation window larger on the T-EthA and TEA coatings, moreover, most matrix components migrated after the mAbs and are therefore less likely to interfere and easily flushed from the capillary after separation.

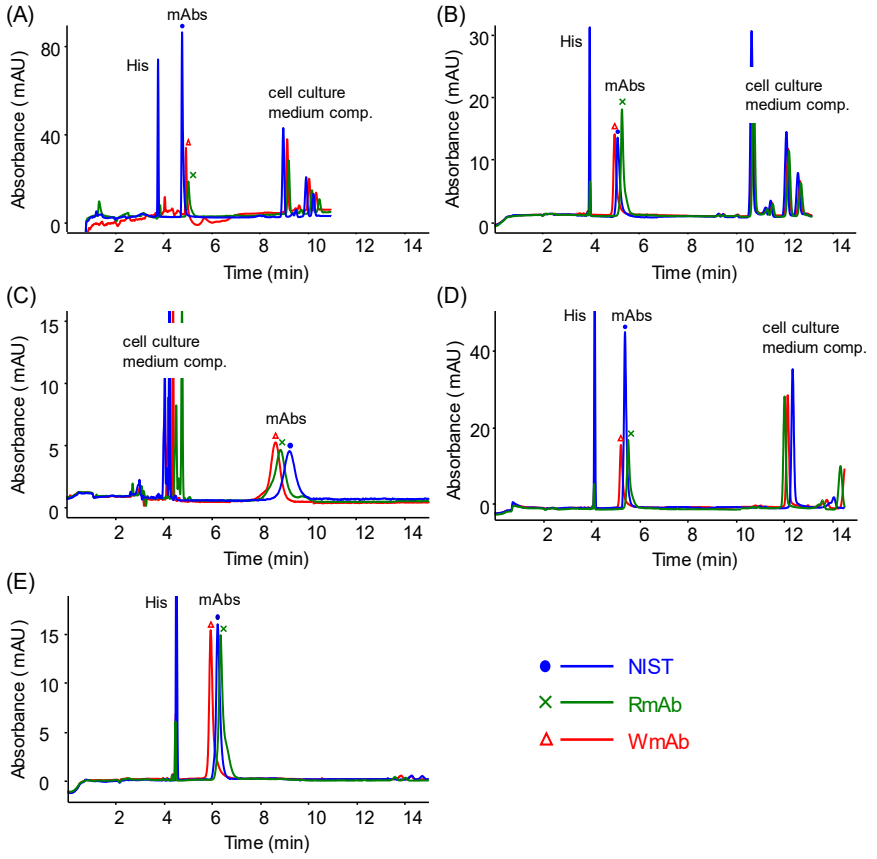

Figure 2. Rituximab, Waters monoclonal antibody (mAb) and NIST mAb analysed on (A) an uncoated capillary; (B) a polyvinyl alcohol (PVA)-coated capillary; (C) a SMIL-3 coated capillary with background electrolyte (BGE): 100 mM phosphate, 70 mM Tris and 0.1% poloxamer; (D) a dynamic triethanolamine (T-EthA)-coated capillary with BGE: 100 mM phosphate, 70 mM T-EthA and 0.1% poloxamer or (E) a dynamic TEA-coated capillary with BGE: 100 mM phosphate, 70 mM TEA and 0.1% poloxamer. Effective separation length was 24.5 cm.

The results of the mAbs spiked in ActiPro medium obtained with the SMIL-3 coating (Figure 2C) differ from the SMIL-3 results obtained previously (Figure 1C). The migration times are longer, indicating a slower EOF, and the migration time fluctuated due to a fluctuating EOF, which was indicated by the large fluctuation in migration time of the earlier migrating medium peaks. The effect of the EOF on the migration time repeatability was significantly higher in the SMIL-3 coated-capillary than for the other capillary coatings (Table 2). As the other coatings result in sharper peaks, better separation, and more stable EOFs, method development was discontinued on SMIL-3 coating.

The EOF stability was determined using the Williams and Vigh [30] method. For each coating, this determination was performed six times; three times before mAb analysis and three times after mAb analysis (Table 1). The neutral and amine coatings resulted in very low EOF values, the SMIL-3 coated capillary resulted in a significantly higher EOF mobility. Fluctuations in a low EOF mobility only have a minor effect on the migration times, which is beneficial for a robust method. As low EOF mobilities have a minor effect on migration time repeatability, the EOF variability is not a good measure to evaluate the capillary coating; instead, the effect of these EOF variations on the migration repeatability of the mAbs should be considered. This was calculated according to the scheme in Figure S3. This approach gave an estimation of the variation of the migration times caused by the fluctuations in EOF for each coating (Table 2). The third EOF measurement of the SMIL-3 coating seemed to be an outlier, see Figure S2. Because it is difficult to determine if a value is an outlier in a small sample set, the RSD of the theoretical migration times was calculated both with and without the suspected outlier. The results confirmed that the higher EOF on the SMIL-3 coating resulted in lower migration time repeatabilities, even when corrected for the suspected outlier. The fluctuations in EOF in the T-EthA-coated capillary had the lowest effect on the migration time repeatability. Besides EOF variability, Table 1 shows that the EOF mobilities were slightly different after mAb analysis than before mAb analysis. This is especially profound for the uncoated capillary. The decreased EOF mobility after mAb analysis could be caused by a shielding of the limited number of negatively charged silanol groups on the capillary wall, which indicates adsorption of cell-culture medium components to the capillary wall. Adsorption of sample components to the wall for uncoated capillaries is expected, emphasising the need for capillary coatings. The peak efficiencies achieved using the different capillary coatings were inspected visually for tailing or electromigration dispersion as well as calculated (Table S1). The highest efficiencies were obtained with the uncoated capillary; however, also more tailing was observed, indicating adsorption, which

would likely result in a non-robust method. The T-EthA coating resulted in peak shape efficiencies close to those obtained with the uncoated capillary. The peak shapes in terms of tailing or electromigration dispersion, however, were significantly better. This coating is more likely to result in a robust method due to the better capillary wall protection. In addition, the T-EthA-coated capillary is expected to result in significantly better migration time repeatabilities. Therefore, method development was continued with the T-EthA-coated capillary.

Table 1. Determined electro-osmotic flow (EOF) ($\times 10^{-9}$ m^2/V s) on the different capillary coatings

	Measure-ment #	BFS	PVA	T-EthA	TEA	SMIL-3
Before mAb analysis	1	0.96	0.12	-1.14	-4.05	-25.38
	2	0.75	0.10	-1.25	-4.04	-24.79
	3	0.57	0.10	-1.26	-4.05	-21.28[a]
	Average	**0.76**	**0.11**	**-1.22**	**-4.05**	**-23.8[b] / 25.1[c]**
After mAb analysis	4	-0.01	-0.15	-1.05	-3.65	-26.47
	5	-0.06	-0.16	-1.04	-3.73	-26.53
	6	-0.09	-0.17	-1.19	-3.76	-26.73
	Average	**-0.05**	**-0.16**	**-1.09**	**-3.72**	**-26.6**
	SD	0.46	0.15	0.10	0.18	2.06[b] / 0.85[c]

Note: A positive value represents an EOF directed towards the cathode, and a negative value represents an EOF directed towards the anode.

Abbreviations: BFS, bare fused silica; mAb, monoclonal antibody; PVA, polyvinyl alcohol; TEA, triethylamine; T-EthA, triethanolamine.

[a] Outlier, see Figure S2.
[b] Outlier included.
[c] Outlier excluded.

Table 2. Calculated theoretical migration time RSD% based on the measured EOF mobilities (Table 1)

mAb	BFS (%)	PVA (%)	T-EthA (%)	TEA (%)	SMIL-3 (%)	
					outlier included	*outlier excluded*
NIST mAb	2.7	0.9	0.6	1.4	36.6	9.8
Waters mAb	2.7	0.9	0.6	1.4	37.1	10.0
Rituximab	2.8	0.9	0.6	1.5	33.5	9.3

Abbreviations: BFS, bare fused silica; mAb, monoclonal antibody; PVA, polyvinyl alcohol; TEA, triethylamine; T-EthA, triethanolamine.

3.3.2 APPLICATION ON UPSTREAM PROCESS SAMPLES

The spent cell-culture medium of the USP process contains numerous components. In addition to the medium components already present in the medium, after cultivation, it also contains cell debris, host cell proteins, host cell DNA, and so on. To assess the suitability of the developed T-EthA method for these complex samples, spent cell-culture medium samples from USP process A along with a purified DS sample were analysed. Figure 3A shows the electropherograms of these samples clearly showing a separation of the mAb from other cell-culture medium components with good resolution. The migration time repeatability of 8 DS runs was 0.45%, confirming a stable EOF. To reduce the analysis time, spent cell-culture medium samples of USP processes B and C were analysed on the short end of the capillary, decreasing the effective separation length from 24.5 to 8.5 cm. This shortened the migration time from 6 min (Figure 3A) to 2.7 min (Figure 3B,C) while maintaining good resolution. A short effective length is also easier to convert to a microchip-CE method. Therefore, the effective separation length of 8.5 cm was selected.

3.3.3 METHOD VALIDATION

The USP process samples provided for validation were from the production of adalimumab, illustrating the general applicability of the method. A sample with 1 mg/ml adalimumab spiked into spent cell-culture medium of day 0 (mAb-free) was analysed in six consecutive runs, which resulted in migration time and peak area injection repeatabilities of 0.1% and 1.0%, respectively. Further method validation was performed with protein A–purified mAb-free spent cell-culture medium spiked with known concentrations of adalimumab. Analysis of the mAb-free medium confirmed that no mAb peak was present (Figure S4). The method was validated over the concentration range 0.1–10 mg/ml. Samples of 0.1–1 mg/ml mAb are to be

analysed undiluted. Samples of 1–10 mg/ml mAb are to be diluted 10-fold with water. Six concentration levels, each in triplicate, within 0.08–1.3 mg/ml were prepared, of which one 1.0 mg/ml sample which was thereafter diluted 10-fold with water to simulate the lowest concentration of the 1–10 mg/ml range to represent the worst case for diluted samples.

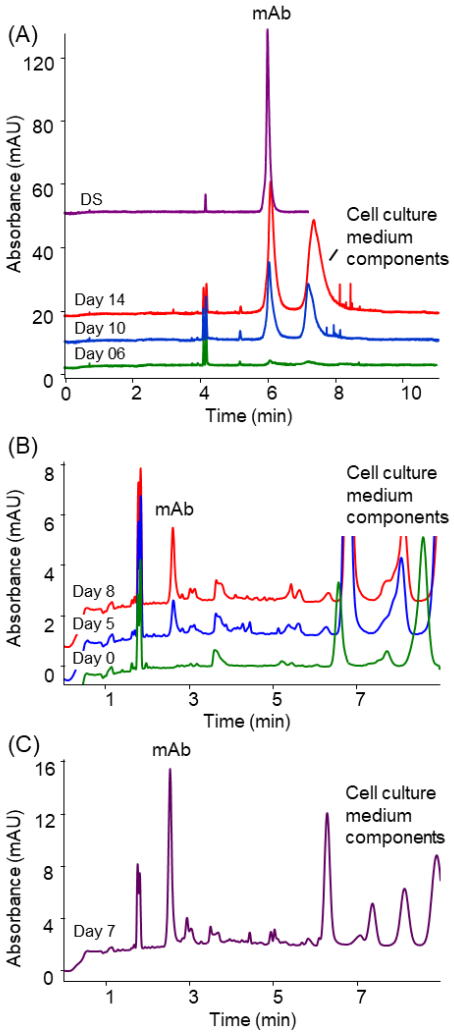

Figure 3. Spent cell-culture medium samples from different processes and different days of cultivation: (A) upstream process (USP) A after 6, 10 or 14 days of cultivation and a purified drug substance sample, separation length 24.5 cm; (B) USP process B after 0, 5 or 8 days of cultivation, separation length 8.5 cm and (C) USP process C after 7 days of cultivation, separation length 8.5 cm. Analysed with background electrolyte (BGE): 100 mM phosphoric acid, 70 mM triethanolamine (T-EthA) and 0.1% poloxamer 188

The method was linear over the range 0.08–1.3 mg/ml (Table 3, Figure S5). Total method repeatabilities (intraday) are calculated as RSD% of the determined concentrations of the specific day (n = 3), and intermediate precisions (inter-day) are calculated as RSD% of the determined concentrations from all 3 days (n = 9). The accuracy was determined as the recovery at the different concentration levels using the mean of the determined concentrations per day (n = 3) or of all 3 days (n = 9) and the actual spiked concentration (Table 3). The precision and accuracy are lower for the lower range limit (0.08 mg/ml), the repeatability and accuracy were 12%–21% and 79%–82%, respectively, and the intermediate precision and overall accuracy were 14% and 81%, respectively. As this low concentration will only be present at the start of the USP, where monitoring the increase in concentration is more important than quantifying the concentration, the lower precision and accuracy are acceptable. For the other concentration levels in the undiluted range, the repeatability and accuracy were 3%–12% and 99%–107%, and the intermediate precision and overall accuracy were 4%–7% and 102%–104%, respectively. These all fall within the set criteria of 15% precision and 90%–110% accuracy. For the lowest concentration level of the diluted range (1.0–10 mg/ml), the precision and accuracy were lower; the repeatability and accuracy were 2%–12% and 88%–98%, respectively, and the intermediate precision and overall accuracy were 9% and 93%, respectively. As the precision and accuracy were better on the higher concentration levels, samples in the concentration range 1.0–1.3 mg/ml should be analysed undiluted. Method validation was performed using a one-point calibration of adalimumab in water. The linearity data show a linear response with a slope of 1.01 and a non-significant intercept, supporting the use of a one-point calibration model.

Table 3. Validation data: linearity data over the range 0.08–1.3 mg/ml at six concentration levels, each n = 3, and the precision and accuracy data for the concentration determination of monoclonal antibodies (mAbs) in spent cell-culture medium.

	Linearity (n = 9 per level)[a]								
y-intercept	-0.009 ± 0.019								
slope	1.013 ± 0.022								
R^2	0.976								
R	0.988								
	Repeatability and accuracy (n = 3)								
	Day 1			Day 2			Day 3		
Conc. level (mg/mL)	Measured (mg/mL)	RSD (%)	Acc. (%)	Measured (mg/mL)	RSD (%)	Acc. (%)	Measured (mg/mL)	RSD (%)	Acc. (%)
1.3	1.34	12	103	1.31	5	101	1.35	4	104
1.0	1.02	5	102	1.01	3	101	1.06	4	106
0.7	0.75	5	107	0.72	5	102	0.71	8	102
0.4	0.41	6	101	0.39	6	99	0.42	8	106
0.08	0.06	12	81	0.06	13	79	0.07	21	82
1.0[b]	0.98	2	98	0.88	9	88	0.92	12	92
	Intermediate precision and overall accuracy (n = 9)								
	Day 1 – 3								
Conc. level (mg/mL)	Measured (mg/mL)			RSD (%)			Overall accuracy (%)		
1.3	1.33			7			103		
1.0	1.03			4			103		
0.7	0.73			6			104		
0.4	0.41			6			102		
0.08	0.06			14			81		
1.0[b]	0.93			9			93		

[a] Linearity plot is shown in Figure S5.

[b] 1.0 mg/ml sample diluted 10-fold in water to simulate the lowest concentration of the 1–10 mg/ml range.

3.4 CONCLUDING REMARKS

Four different capillary coatings were tested for the quantification of mAbs in cell-culture medium samples. The purpose of the capillary coating is to supress and control the EOF in order to obtain good migration time repeatabilities, and to protect the capillary wall from adsorption. The SMIL-3 coating resulted in the strongest EOF, and the fluctuation in the EOF would result in very poor migration time repeatability. In addition, medium components migrated before the mAbs, which could potentially lead to interference. The uncoated capillary in combination with the low pH of the BGE had a supressed EOF; however, fluctuations in the EOF were present, which would contribute to fluctuations in migration times. Moreover, tailing of the mAb peaks was observed for the uncoated capillary, indicating adsorption. This emphasises the need for a capillary coating. Best results were obtained with the neutral PVA and the positively T-EthA- and TEA-coated capillaries. As the best migration time repeatabilities were obtained with T-EthA-coated capillaries and this coating resulted in most efficient peak shapes, this coating was selected for mAb quantification in cell-culture medium samples. The method was linear over the range 0.08–1.3 mg/ml of mAb in undiluted cell-culture medium samples, which translates to 0.08–10 mg/ml including 10-fold diluted samples. Repeatabilities and accuracies for undiluted samples were 3%–12% and 99%–107%, respectively, and intermediate precision and overall accuracies were 4%–7% and 102%–104%, respectively. This is excluding the lower range limit, where the precision and accuracy was slightly lower. The lower precision and accuracy on this level, however, are acceptable for the intended purpose of the method. The lowest level of the 1–10 mg/ml can be determined with repeatability and accuracy of 2%–12% and 88%–98%, and an intermediate precision and overall accuracy of 9% and 93%. This could be further improved by analysing samples in the 1–1.3 mg/ml ra undiluted. By selecting a low pH BGE, with a pH sufficiently different than the p*I* of mAbs, a strong positive charge on the mAbs is obtained. As the p*I* of most mAbs is typically high (p*I* > 6, generally 8–9), the selected BGE pH of 2.5 is significantly lower than most mAbs p*I*s, ensuring a strong positive charge on a large variety of mAbs. Consequently, a large variety of mAbs will migrate in a similar way, making this method suitable as platform method. The method showed to be applicable to USP samples with no or limited sample pre-treatment from different cell-culture processes, and with good separation from the complex cell-culture medium matrix.

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SUPPLEMENTARY INFORMATION

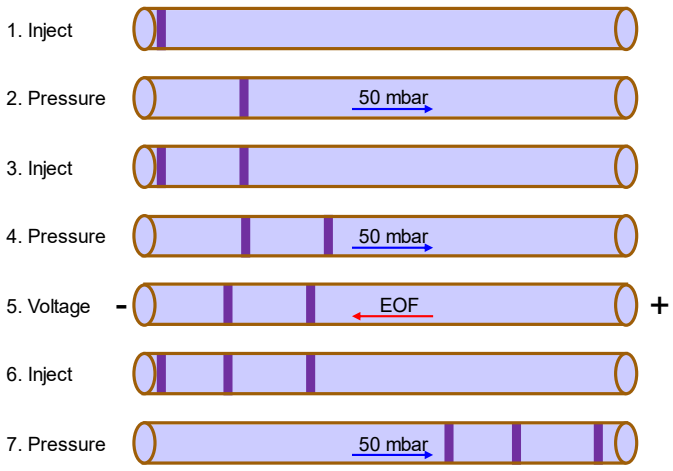

Figure S1. Scheme for EOF determination using DMSO as neutral marker. Step 1: band 1 is injected, Step 2: band 1 is transferred in capillary using pressure, Step 3: band 2 is injected, Step 4: band 1 and 2 are transferred in capillary using pressure, Step 5: a voltage is applied to move the band 1 and 2 with the EOF, Step 6: band 3 is injected, Step 7: all three bands are transferred in the capillary, past the detection window, using pressure.

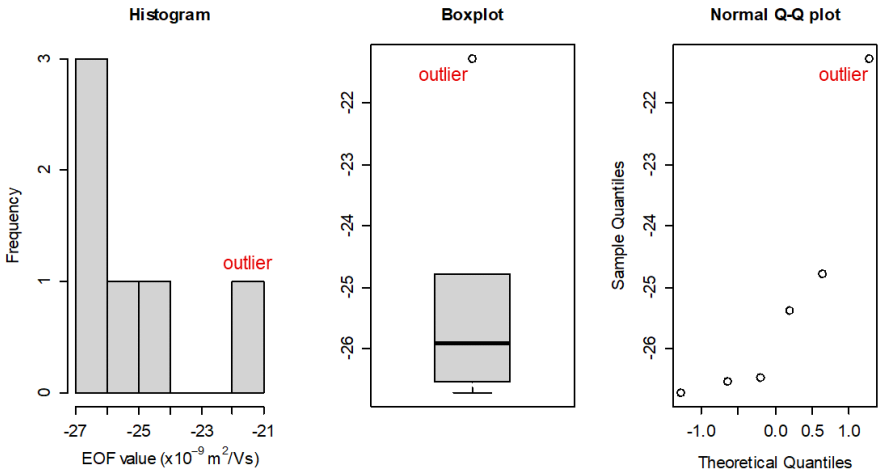

Figure S2. Histogram, Boxplot, and Normal Q-Q plot[1] of the values from the EOF determinations of the SMIL-3 coating. Value -21.28·10^{-9} m^2/Vs is an outlier.

[1] RStudio (version 4.1.2). Available from: http://www.rstudio.com/

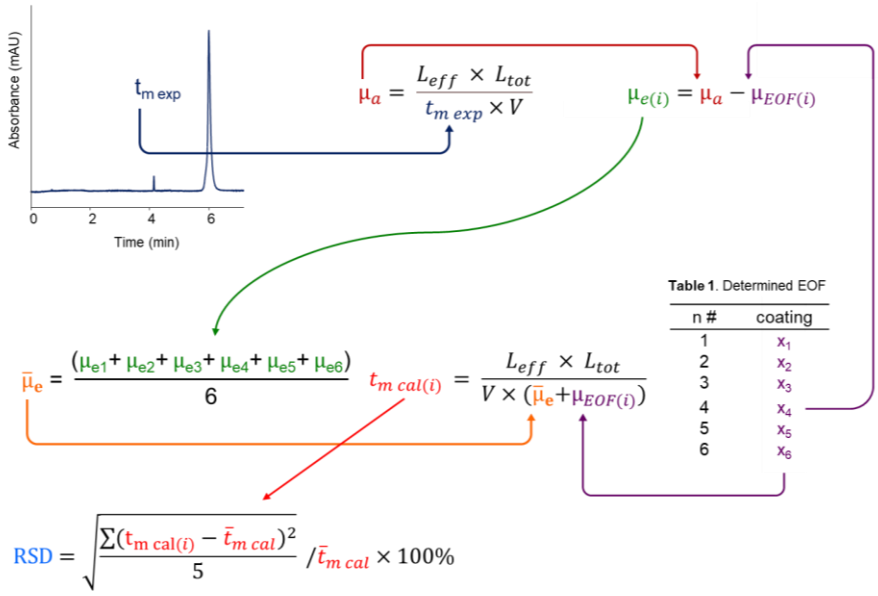

Figure S3. Calculation scheme for the theoretical variation of the migration times caused by the fluctuations in EOF. The measured migration time (t_m) was used to calculate the apparent mobility (μ_a) of the mAbs. This μ_a was used to calculate the electrophoretic mobility for each determined EOF mobility ($\mu_{e\ EOF}$) from Table 1, resulting in six electrophoretic mobilities. The average of the electrophoretic mobilities ($\bar{\mu}_e$) was then used to calculate the theoretical migration time for each determined EOF mobility ($t_{m\ EOF}$) from Table 1. The migration time RSD% of these theoretical migration times indicate the variation caused by the EOF fluctuations.

Table S1. Peak efficiency calculated as $5.54 \times (t_m / W_{0.5})^2$ of the different mAbs analysed with the different capillary coatings.

mAb	BFS	PVA	T-EthA	TEA	SMIL-3
NIST mAb	$3.6 \cdot 10^4$	$7.1 \cdot 10^3$	$1.4 \cdot 10^4$	$7.8 \cdot 10^3$	$1.8 \cdot 10^3$
Waters mAb	$2.9 \cdot 10^4$	$8.4 \cdot 10^3$	$9.4 \cdot 10^3$	$8.1 \cdot 10^3$	$2.3 \cdot 10^3$
Rituximab	$1.5 \cdot 10^4$	$1.2 \cdot 10^4$	$1.2 \cdot 10^4$	$7.5 \cdot 10^3$	$2.2 \cdot 10^3$

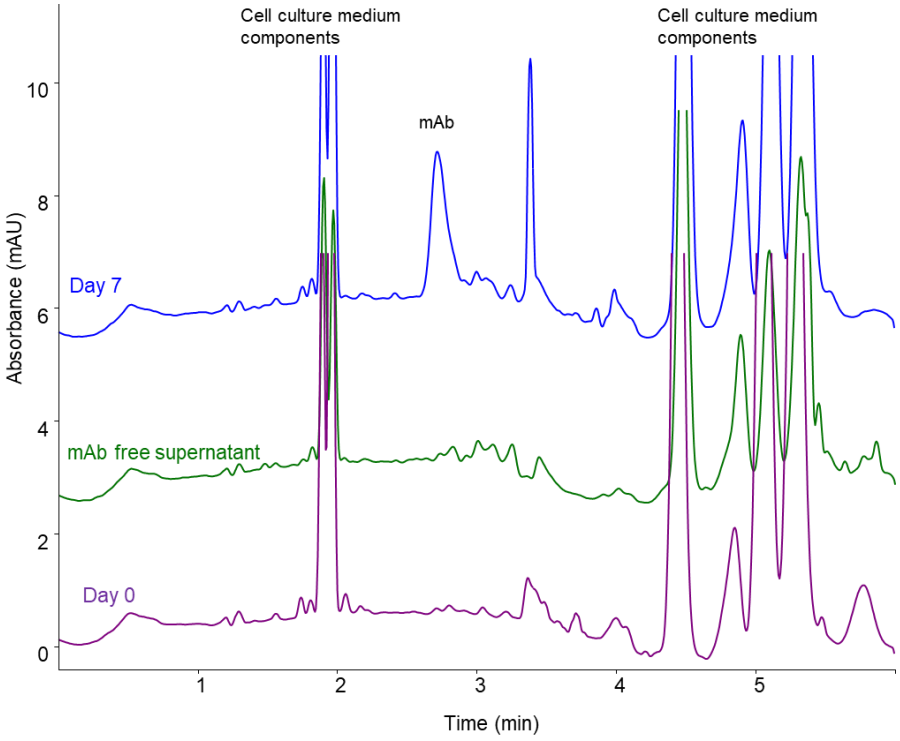

Figure S4. Electropherograms of spent cell culture medium samples from process C after 0 or 7 days of cultivation compared to the mAb-free supernatant analyzed with BGE: 100 mM phosphoric acid, 70 mM T-EthA, 0.1% poloxamer and an effective separation length of 8.5 cm

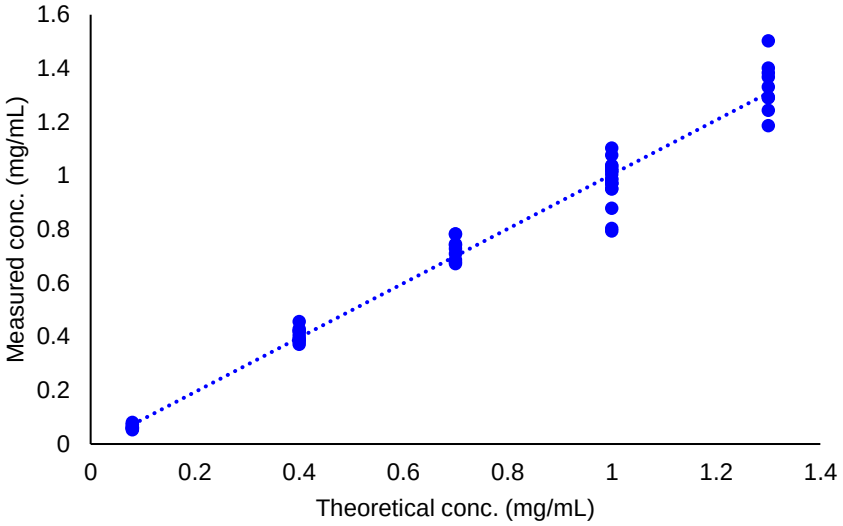

Figure S5. Linearity plot of the measured concentrations determined on the three days (n=9 for each level) against the theoretical concentration.

Chapter

Method development for mono- and disaccharides monitoring in cell culture medium by capillary and microchip electrophoresis

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ABSTRACT

The rapidly growing, competitive biopharmaceutical market requires tight bioprocess monitoring. An integrated, automated platform for the routine online/at-line monitoring of key factors in the cell culture medium could greatly improve process monitoring. Mono- and disaccharides, as the main energy and carbon source, are one of these key factors. A CE-LIF method was developed for the analysis of several mono- and disaccharides, considering requirements and restrictions for analysis in an integrated, automated monitoring platform, such as the possibility for miniaturization to microchip electrophoresis. Analysis was performed after fluorescent derivatization with 8-aminopyrene-1,3,6-trisulfonic acid. The derivatisation reaction and the separation BGE were optimized using design of experiments. The developed method is applicable to the complex matrix of cell culture medium and proved transferable to microchip electrophoresis.

4.1 INTRODUCTION

Biopharmaceuticals such as proteins, antibodies, hormones, and enzymes have become important products in the pharmaceutical industry, with mAbs as the most rapidly growing class [1, 2]. Biopharmaceuticals are produced using cell culture bioprocesses, where the cell culture medium has a large impact on the growth of cells as well as on product concentration and quality [3, 4]. To increase process understanding and control critical process parameters, cell culture monitoring is essential. Process understanding can be further increased when monitoring is also performed in microbioreactors in early stage of development. It is most beneficial if the factors of the cell culture medium are monitored continuously. An automated analytical monitoring platform with online and at-line sensors and detection methods measuring multiple factors in the cell culture together with integrated calibration and data analysis could make continuous monitoring feasible. The analytical platform will be integrated in bioreactors, and combined with advanced data management, feedback control will be applied [5]. In the context of Process Analytical Technology, although in-line or online analyses are preferred, automated at-line methods can also advantageously support real-time decision or feedback control if the measurement time is much smaller than the system dynamics. Mammalian cells have a doubling time around 24 h or more, and usually the time for decision can potentially be a couple of hours for medium or feed adjustments, 10 h according to Rathore et al. [6]. The present work, focusing on saccharides, is part of the analytical platform development.

Mono- and disaccharides are the simplest form of carbohydrates. In the cell culture medium, they are important as energy source [7], and for glycosylation of the mAbs [7-9], and are therefore one of the component groups that should be monitored. Glucose is typically used as the primary energy and carbon source in cell culture media. As the type of carbon source impacts the glycosylation pattern of the mAb, a critical quality attribute, and the consumption of glucose often leads to increased lactate concentrations, which could inhibit cell growth, the use of alternative carbon sources is being investigated [7, 10, 11]. Hence, the monitoring of other saccharides simultaneously with glucose is important.

Challenges of saccharide monitoring are that some monosaccharides are isomeric (Fig. 1) and that they have to be monitored in the complex matrix of cell culture medium. Therefore, high separation efficiency is required, which can be obtained with CE. Furthermore, CE separation takes place in an open capillary tube, making it less prone to clogging, and therefore reduces extensive sample clean-up as compared to other chromatographic techniques. This is beneficial for analysis in an automated

monitoring platform. Additional advantages of CE are the short analysis time, and the possibility of miniaturization using chip-based separations. Due to the small instrument setup of microchip electrophoresis (ME), it is more easily integrated into an automated monitoring platform. In addition to that, ME only requires small amounts of sample, which makes it more feasible for the monitoring of microbioreactors. ME is, therefore, selected as the technique most suitable for continuous monitoring of the saccharides.

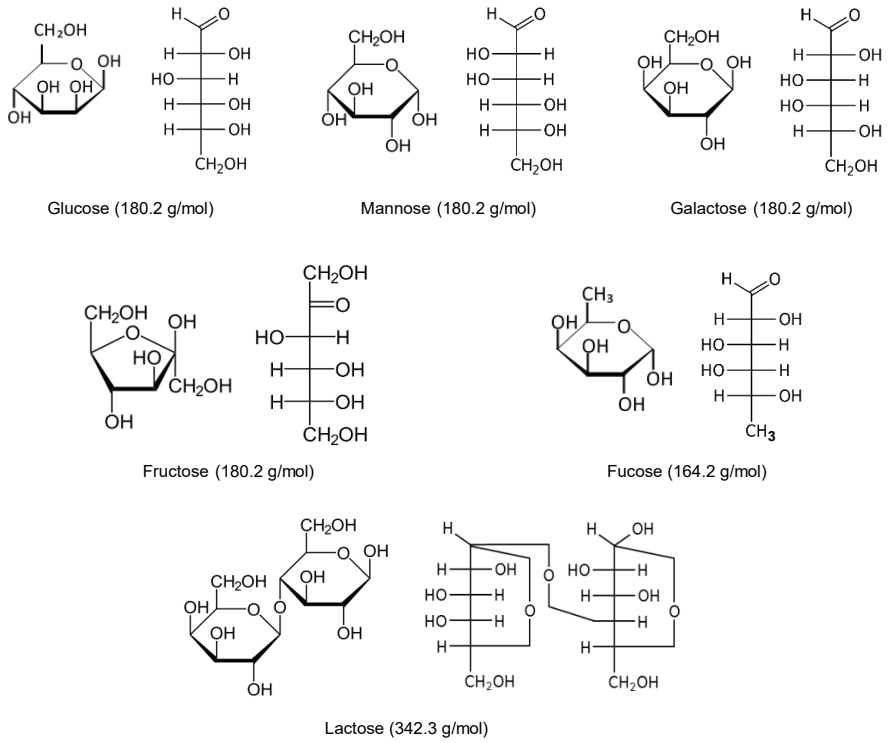

Figure 1. Mono- and disaccharide structures.

The mono- and disaccharides glucose, mannose, galactose, fucose, lactose, and, if feasible, fructose should be quantified in their concentration range in cell culture medium (0.5–50 mM). These saccharides are uncharged and lack chromophore or fluorophore groups (Fig. 1). Therefore, traditional ME/CE analysis of saccharides are based on (i) contactless conductivity detection (C4D) or UV-detection at high pH (≥12), where carbohydrates undergo a series of reactions resulting in chromophoric anions, enediolates [12-15], (ii) UV or LIF detection after derivatization with a (charged) chromophore or fluorophore [16-30], or (iii) CE coupled to MS [31-33]. To fit in an automated monitoring platform, the configuration of the detection

techniques should be compact, and due to the small sample volumes in ME, the detection sensitivity should be high. MS is not easily miniaturized and the sensitivity of UV detection is dependent on the path length, which is small in ME. Common ways to increase the path length for conventional CE, such as a bubble cell or Z-cell, are not easily achieved on chip [34]. Hence, MS and UV detection are out of scope here. Preliminary experiments showed that the resolution for mannose and fructose, and the sensitivity of the saccharides obtained with C4D after separation at alkaline conditions were insufficient (data not shown). Therefore, LIF detection after fluorescent derivatization was used for this work.

The method is supposed to be applied for unattended analysis, in a closed system, for the duration of the upstream cell process campaign. This means that the method must adhere to high-quality standards, which includes the search for less toxic and stable options for chemicals being used and the method being extremely robust and rugged. For automated analysis and reprocessing of the data, repeatability and reproducibility are extra important. These considerations for method requirements will narrow the options for method development. In this research, a method was developed for the quantification of mono- and disaccharides in cell culture medium samples using the principles of analytical quality by design to adhere to these high-quality standards.

4.2 MATERIALS AND METHODS

4.2.1 CHEMICALS

d-(+)-Glucose monohydrate, d-(+)-mannose, d-(+)-galactose, d-(-)-fructose, l-(-)-fucose, 1 M sodium cyanoborohydride ($NaBH_3CN$) in THF, 2-picoline borane complex (2-PB), boric acid, citric acid, lactose monohydrate, THF, DMSO, SDS, and poloxamer 188 10% solution were obtained from Merck/Sigma Aldrich (Darmstadt, Germany). Methanol (MeOH) absolute was obtained from Biosolve (Valkenswaard, the Netherlands). 8-Aminopyrene-1,3,6-trisulfonic acid (APTS) (≥96.0%), 2-aminobenzoic acid (2-AA), and 2-aminobenzamide (2-AB) were purchased from Sigma Aldrich. 2-AA and 2-AB were dissolved in DMSO to a concentration of 1 M. APTS-M (extra purified for monosaccharide analysis) was obtained from BeckmanCoulter (Woerden, the Netherlands). Note that 20 mg APTS-M was dissolved in 400 µL water, as described by Sciex [35]. Cell culture medium based on FMX-8 medium with certain modifications (FMX-8 MOD) (Table S1), saccharide-free cell culture medium, and spent cell culture medium were provided by the Department of Industrial Biotechnology, KTH Royal Institute of Technology, Stockholm, Sweden. The spent cell

culture medium sample was taken at day 4 during cultivation of Chinese hamster ovary cells (TurboCell(TM), Rentschler Biopharma, Laupheim, Germany) in a 4 L reactor (Belach Bioteknik, Stockholm, Sweden). Water was of MilliQ-grade (18.2 MΩ·cm), all other chemicals were of analytical grade.

4.2.2 INSTRUMENTAL CONDITIONS

4.2.2.1 CAPILLARY ELECTROPHORESIS

Preliminary experiments investigating fluorescence derivatization and separation were performed on an Agilent 7100 capillary electrophoresis system with a Diode Array UV detector (Waldbronn, Germany). Data processing for this instrument was done with Chemstation software. Bare fused silica capillaries with 50 µm id, 33.0 cm total length, and 24.5 cm effective length were purchased from Agilent Technologies. Samples were introduced hydrodynamically with 10 mbar for 3 s, followed by the injection of a BGE plug with 10 mbar for 3 s. The applied voltage ranged from 13 to 20 kV depending on the conductivity of the used BGE, and the voltage was applied in a ramp of 0.5 min. The detector signal was recorded at 200 nm. Optimization of the derivatization and the BGE was performed on a Beckman PA 800 plus with LIF detector (Woerden, the Netherlands). With this instrument, data processing was performed with 32Karat. Bare fused silica capillaries with 50 µm id were obtained from Sciex and cut to a total length of 30 cm with an effective length of 20 cm. Samples were introduced hydrodynamically with 0.5 psi for 5 s, followed by the injection of a BGE plug with 0.5 psi for 5 s. The applied voltage was 15 or 11.8 kV, depending on the BGE, and applied in a ramp of 0.5 min. The excitation wavelength was 488 nm and the emission wavelength was 520 nm.
For all experiments, positive polarity mode was used. Separations were carried out at 20°C. Before first use, the capillary was flushed successively with 1 M NaOH, water, and BGE at 1 bar for 20 min each. At the beginning of each working day, the capillary was flushed successively with 0.1 M NaOH, water, and BGE at 1 bar for 10 min each. Prior to each injection, the capillary was preconditioned with 0.1 M NaOH for 1 min, water for 1 min, and BGE for 2 min at 1 bar.

4.2.2.2 MICROCHIP ELECTROPHORESIS

ME separations were performed on a prototype ME system from Micronit B.V. (Enschede, the Netherlands) equipped with a laser diode module (Thorlabs, CPS450) and photosensor module (Hamamatsu, H7827-012) for LIF detection. Double T fused-silica CE microchips with channel dimensions of 50 × 20 µm and separation length of 7.9 cm were obtained from Micronit. Before use, the microchips were manually

flushed with 1 M NaOH, 0.1 M NaOH, and twice with water using approximately 3.5 µL of solution. The separation channels were filled with BGE before the microchip was mounted in the microchip holder (Micronit, EOF KIT 9015). The microchip holder was connected to a four-channel output high-voltage sequencer (eDAQ, Denistone East, Australia). Note that 50 µL of BGE was added to all wells and the microchip was electrokinetically flushed for 10 min using 2500 V on the sample and waste compartments, 3000 V to the BGE inlet, and –1000 V to the BGE outlet. The BGE was replaced with new BGE and sample was added with a micropipette. A pinched injection was performed by applying 1000 V to the sample compartment, grounding the waste compartment, and applying 500 V and 1000 V to the inlet and outlet BGE compartments, respectively, for 30 s. The separation voltages were the same as for the electrokinetic flushing step. LIF detection was performed with excitation wavelength at 450 nm and emission wavelength at 500 ± 25 nm. After each analysis, the sample well was rinsed with 50 µL water. The electrokinetic flushing step was repeated with new BGE in all reservoirs, and after each set of analysis, the microchip was manually flushed as described previously.

4.2.3 BGE PREPARATION

BGEs containing boric acid at alkaline conditions were tested. The BGEs had varying boric acid concentrations and different additives such as polysorbate 20, polysorbate 80, poloxamer 188, and/or SDS. The pH was adjusted with NaOH and ranged from 8.5 to 10. The BGE was optimized using design of experiments (DoE). The design was created and the data were processed using the DoE software MODDE (version 12.1, Sartorius, Göttingen, Germany). The parameters were optimized in a Central Composite Orthogonal design, and the center point was prepared in triplicate. The final BGE consisted of 160 mM boric acid, 100 mM SDS, 0.1% poloxamer 188, and 54 mM NaOH and had a pH of 8.8.

4.2.4 APTS DERIVATIZATION

Method development was performed with an equimolar (16.7 mM) standard saccharide mixture. For preliminary experiments, to 5 µL of standard saccharide mixture (either after drying in a vacuum centrifuge or directly), 2 µL derivatization agent and 2 µL reducing agent were added. The derivatization agent was either APTS-M, 2-AA, or 2-AB, and the reducing agent was either 1 M $NaBH_3CN$ in THF or 1 M 2-PB in DMSO. Samples were then incubated at 60°C for 60 or 90 min. The reaction was quenched by adding 46 µL water.

For the optimization of the reaction, the concentrations of APTS, 2-PB, and citric acid were varied in a Central Composite Orthogonal DoE, and the center point was analyzed in triplicate. Samples were prepared by mixing 3 µL saccharide mixture containing citric acid with 3 µL APTS in water and 3 µL 2-PB in MeOH. The concentrations of the stock solutions varied to obtain the appropriate end concentrations in the reaction mixture. The reaction mixtures were incubated at 60°C for 90 min and subsequently, 24 µL water was added to the reaction mixture.
The final sample preparation was performed by mixing 5 µL sample with 5 µL 2-PB (1.24 M in MeOH) and 5 µL APTS (0.26 M in 0.7 M citric acid). The reaction mixture was incubated at 60°C for 60 min and subsequently 40 µL water was added. All samples were diluted 10× with water for CE-UV analysis, 60× with water for CE-LIF analysis, or 200× with BGE for ME-LIF analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 OPTIMIZATION OF APTS DERIVATIZATION

Frequently used fluorescent derivatization agents for monosaccharides include APTS [16-24], 2-AA [25-28, 36], and 2-AB [29, 37]. Preliminary experiments were performed with these derivatization agents. Even though these experiments were performed with UV-detection instead of LIF-detection, the sensitivity was sufficient for the intended purpose. Separation of the epimers (derivatized) glucose and mannose proved challenging. Only APTS-derivatized glucose and mannose were separated. Therefore, method development was continued on APTS derivatization.
Derivatization of reducing saccharides with APTS is based on reductive amination. The first step is the acid-catalyzed opening of the ring structure. The reducing end of this open-chain structure reacts with the primary amine of the APTS, yielding a Schiff base. The Schiff base is then reduced with a reducing agent to form a stable secondary amine [38, 39]. The reducing agents used for the derivatization reaction are prone to hydrolysis. Hence, saccharide samples are often dried before derivatization, which is not feasible for sample preparation in a closed system. Experiments showed that, although aqueous derivatization was slightly less efficient, drying the sample was not required (data not shown). Furthermore, the most commonly used reducing agent for reductive amination, $NaBH_3CN$, is toxic and produces the even more toxic hydrogen cyanide gas upon hydrolysis. A safer alternative is the nontoxic 2-PB [40, 41]. When comparing the reaction efficiency with either $NaBH_3CN$ or 2-PB as reducing agent, derivatization using 2-PB was slightly less efficient; however, the reaction yield was still sufficient for analysis.

The derivatization efficiency was maximized by optimizing the reaction conditions. First, the effect of the reaction solvent was investigated. The derivatization reaction was performed by mixing a saccharide sample with aqueous APTS containing citric acid and a 2-PB solution. DMSO, MeOH, and THF were tested as solvent for 2-PB. When using THF as reaction solvent, an artifact peak appeared in the electropherogram. The derivatization efficiency increased when using MeOH instead of DMSO. This could be explained by the effect of these solvents on the pKa of citric acid. The pKas of acids are often higher in DSMO than in MeOH; hence, in DMSO fewer protons are provided for the opening of the saccharide ring structures [26, 42]. Second, the concentrations of APTS, 2-PB, and citric acid in the reaction mixture were optimized using DoE. A standard saccharide sample was derivatized with varying concentrations of APTS, 2-PB, and citric acid in the ranges 40–95 mM, 250–600 mM, and 200–500 mM, respectively. The peak areas of the derivatized saccharide peaks were modeled with the DoE software MODDE, and the optimal conditions to maximize the saccharide peak areas were calculated. The optimal reaction concentrations were 88 mM APTS, 0.4 M 2-PB, and 0.23 mM citric acid. Figure 2 shows the effect of the APTS and citric acid concentration (A), and the APTS and 2-PB concentration (B) on the total peak area of the saccharides. The peak areas do not drastically change over the design; at all tested concentrations, the reaction yield was sufficient for analysis, demonstrating the robustness of the derivatization reaction.

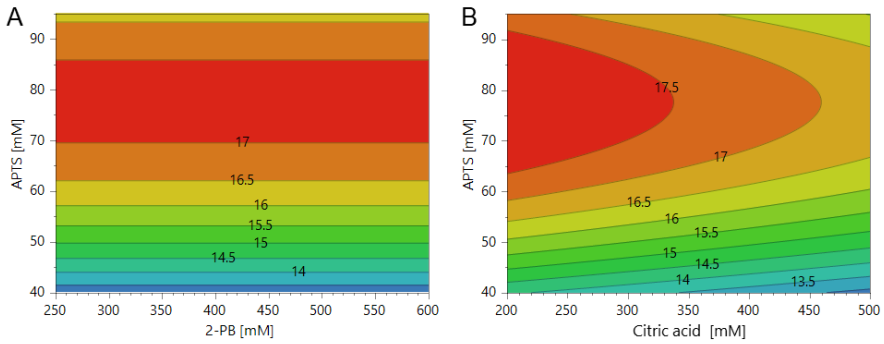

Figure 2. Response contour plot of the total peak area for the factors (A) APTS and 2-PB with the citric acid concentration set to 0.23 M and (B) APTS and citric acid with the 2-PB concentration set to 0.4 M.

Lastly, the incubation time and temperature were evaluated. Fructose, as a ketose sugar, is more difficult to derivatize [39, 43] and was not detected at any of the previous reaction conditions. It was reported that fructose could be derivatized with APTS when using a longer incubation time [23]. When derivatizing a high concentration fructose standard at 75°C for 2.5 h, fructose was indeed derivatized.

When the derivatized fructose sample was spiked into the derivatized standard saccharide mixture (5:1), all six saccharides could be detected and separated (see Fig. S1). Unfortunately, derivatizing a standard saccharide mixture at 75°C for 2.5 h did not result in a quantifiable fructose peak. This is possibly due to competition in the derivatization reaction.

In order to find the optimal derivatization conditions for the aldose sugars, a DoE with the factors incubation temperature and incubation time was conducted in the range of 60–90°C and 1–2 h, respectively. The parameters were varied in a Central Composite Orthogonal design, and the center point was prepared in duplicate. Figure 3 shows an overlay of the electropherograms obtained when performing the DoE. This figure shows the robustness of the derivatization reaction; the difference in total peak area obtained with the different conditions is minimal. This indicates that the reaction is sufficiently completed at each time point. Since the tested incubation conditions do not have a major effect on the derivatization efficiency, the lowest temperature for the shortest time, 60°C for 60 min, was selected. It was further investigated whether shorter times than 60 min at 60°C would be feasible. However, shortening the reaction time below 60 min gave reduced labeling efficiency. Analyzing the saccharide concentration each hour is sufficient for process monitoring.

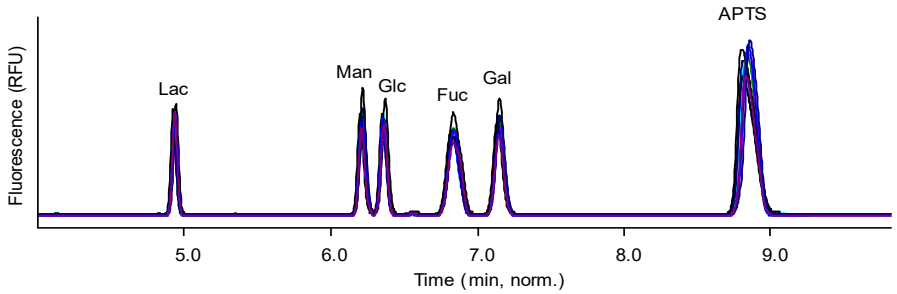

Figure 3. CE-LIF analysis of APTS-derivatized standard saccharide mixtures incubated at different temperatures (60–90°C) and for different times (1–2 h) analyzed with BGE: 100 mM borate, 0.1% poloxamer 188, pH 9.6. Applied voltage 15 kV, resulting current 46 µA.

4.3.2 BGE SELECTION AND OPTIMIZATION

Due to the charge added by the APTS fluorophore to the otherwise neutral mono- and disaccharides, separation of APTS-derivatized saccharides can be studied below the enediolate forming pH range (>12). Each saccharide reacts 1:1 with APTS, resulting in an equal charge on each saccharide. Consequently, the separation is purely based on size. Indeed, when analysis was performed in a glycine/triethylamine BGE at pH 9.3, no resolution was observed between the isomeric monosaccharides,

confirming the size-based separation (data not shown). Borate is known to form complexes with saccharides by interacting with vicinal hydroxyl groups of the saccharides [24, 28, 44], which could enhance separation of isomers. When the separation was performed with a borate BGE at pH 9.6, the isomeric monosaccharides were separated (Fig. 3).

When the APTS-derivatized saccharides were analyzed repeatedly in only borate, an increase in migration times was observed. This could indicate adsorption to the capillary wall, which would be a problem, even more so for the dirtier cell culture medium samples. Neutral surfactants such as poloxamer 188, polysorbate 20, and polysorbate 80 could prevent adsorption [45]. The addition of these three surfactants in different concentrations to the BGE was tested. All three surfactants seemed to have a similar effect on the separation. Polysorbate 20 and 80 are viscous solutions, and therefore, more difficult to work with. Poloxamer 188 does not have these issues and since poloxamer 188 is already used as antifoam in cultivation processes, no compatibility issues are expected with the samples. Hence, poloxamer 188 was selected as additive. Best results were obtained with a concentration of 0.1% poloxamer 188.

The BGE was optimized using DoE. Using micellar electrokinetic chromatography could enhance separation due to the extra separation mechanism introduced by the micelles, therefore, SDS was included as a factor in the design. In addition, compatibility with other methods developed for the integrated platform such as amino acid analysis [46] would improve. The boric acid concentration, the SDS concentration, and the pH were varied in the ranges 80–160 mM, 0–100 mM, and 8.5–9.5, respectively. For all BGEs, the poloxamer 188 concentration was kept constant at 0.1%. Next to a standard saccharide mixture, a cell culture medium sample spiked with the standard saccharide mixture was used for this experiment in order to verify that no medium components interfered with the saccharide peaks. The resolutions of the saccharide peaks were modeled in MODDE. The addition of SDS to the BGE increased resolutions. The optimal BGE conditions were calculated to be 160 mM boric acid, 100 mM SDS, and pH 8.8, which was achieved by adding 54 mM NaOH. No interfering medium peaks were present in the electropherogram (Fig. 4). The optimal concentrations for both boric acid and SDS are at the edge of the design space; this suggest that the optimal BGE concentrations could be outside of the design space. A BGE with 200 mM boric acid was tested; however, resolutions did not significantly increase. Since the resolutions obtained with the concentration at the edge of the design space were sufficient and higher BGE concentrations result in higher currents, the design space was not expanded and the BGE containing 160 mM boric acid, 100 mM SDS, 0.1% poloxamer 188, 54 mM NaOH, pH 8.8 was selected.

Migration times and resolutions were similar when a mono- and disaccharide mixture was analyzed at these final conditions in the KTH lab.

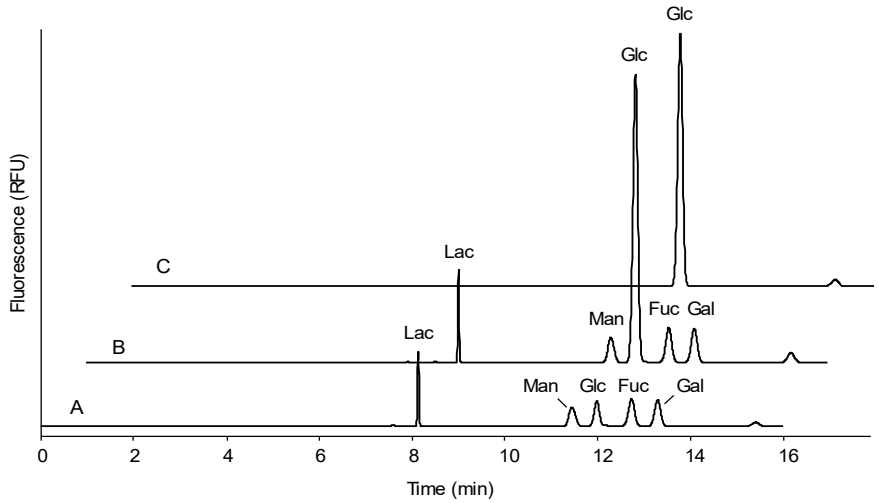

Figure 4. CE-LIF analysis of APTS-derivatized (A) standard saccharide mixture, (B) FMX-8 MOD cell culture medium spiked with a standard saccharide mixture, and (C) FMX-8 MOD cell culture medium analyzed with BGE: 160 mM boric acid, 100 mM SDS, 54 mM NaOH, 0.1% poloxamer 188. Applied voltage 11.8 kV, resulting current 48 µA.

4.3.3 METHOD VALIDATION

The migration time and corrected peak area repeatabilities, based on six consecutive runs of a standard saccharide sample prepared in triplicate, were all within 0.8% RSD and 4.5% RSD, respectively (Table S2), which is within the 10% precision required for the method. Linearity was investigated over the range of which the saccharides can be present in the cell culture medium, 0.50–60 mM. Calibration plots were linear over this range with correlation coefficients of 0.99 or higher for all saccharides (Table S2 and Fig. S2). For recovery determination, known glucose concentrations were spiked into saccharide-free cell culture medium (four samples, concentration range: 14.5–36.4 mM). The samples were analyzed in triplicate and their concentration was determined with a one-point calibration using 50 mM glucose standards. The calibration standards were prepared in water or in saccharide-free cell culture medium. Quantifying with either calibration standard, recoveries were all within 90–110% (Table S3), indicating that calibration can be performed using calibration standards in water.

4.3.4 MICROCHIP ELECTROPHORESIS RESULTS

A standard saccharide mixture, FMX-8 MOD medium, and spent cell culture medium were analyzed with the prototype ME setup. The method developed on capillary proved transferable to a silica chip. All five saccharides were separated within 5 min (Fig. 5). The medium and the spent medium samples contained only glucose, and no interfering peaks from the cell culture medium were observed. The samples were analyzed in triplicate to compare precision with the capillary method. Full validation on chip will be performed when the integrated monitoring platform will be available. For the standard saccharide sample, the migration time repeatabilities of all peaks were within 0.3% RSD and the peak area repeatabilities were all within 15%. The migration time and peak area repeatabilities of the glucose peak in the cell culture medium and the spent cell culture medium sample were slightly higher with 0.8% and 2.9% RSD on migration time and 11% and 31% RSD on peak area, respectively. The precision of the method can be improved by adding an internal standard, which can also be used as internal calibration standard when added before derivatization. These results are a good proof-of-concept for the analysis of mono- and disaccharides with ME in complex matrixes such as the cell culture samples.

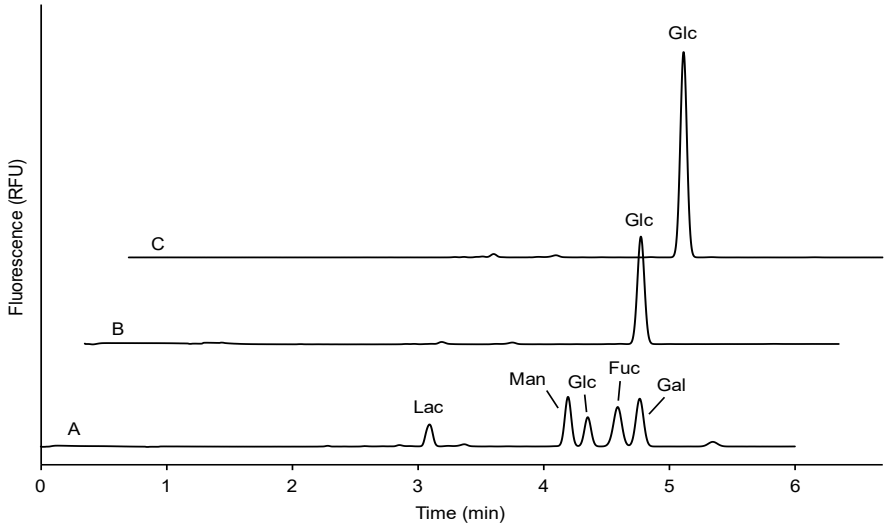

Figure 5. ME analysis of APTS-derivatized (A) standard saccharide mixture, (B) spent cell culture medium after 4 days, and (C) cell culture medium (FMX-8 MOD) analyzed with BGE: 160 mM boric acid, 100 mM SDS, 54 mM NaOH, 0.1% poloxamer 188. Applied field strength 456 V/cm, resulting current 15 µA.

4.4 CONCLUDING REMARKS

In this work, the APTS-derivatization reaction to derivatize the mono- and disaccharides glucose, mannose, galactose, fucose, and lactose together with the BGE for their separation by CE and ME were optimized. Using a BGE containing both borate and SDS, all saccharides were separated with good resolution. The method was linear over the range in which the saccharides are present in the cell culture medium (0.5–50 mM), and good correlation was found between the calculated and the measured data when one-point calibration was applied. The developed method was tested with ME, and these results show a proof-of-concept for the simultaneous analysis of the saccharides in cell culture medium samples with ME. The results also showed that method development for the integrated monitoring platform can be performed on a conventional automated CE instrument with fused silica capillaries, and then be transferred to fused silica chips. With additional testing on ME and online APTS derivatization, it is expected that the method can be adapted in such a way that it can be integrated in an automated monitoring platform with integrated calibration and data analysis. Due to the robustness of the derivatization reaction, no complications are expected for the transfer to automated derivatization. In order to adhere to the high requirements on precision, the addition of an internal standard to improve injection precision on chip should be investigated.

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SUPPLEMENTARY INFORMATION

Table S1. Composition of modified FMX-8 prepared by KTH Royal Institute of Technology

FMX-8 MOD components	FMX-8 MOD concentration (mg/L)
Amino acids	
L-Alanine	9.56[a]
L-Arginine HCl	188.33
L-Asparagine H2O	127.42
L-Aspartic Acid	9.90[a]
L-Cysteine HCl H2O	44.00
L-Glutamic Acid	23.24[a]
Glycine	14.61
L-Histidine HCl H2O	46.29
L-Isoleucine	22.35
L-Leucine	57.75
L-Lysine HCl	70.42
L-Methionine	18.11
L-Phenyalanine	18.71
L-Proline	37.08
L-Serine	13.13
L-Threonine	44.84
L-Tryptophan	25.11
L-Tyrosine	23.72
L-Valine	61.74
Na-Pyruvate	91.67
Organic compounds	
Putrescine	0.27
Linoleic acid	0.07
α-Lipoic acid	0.17
Pluronic-F68	1000[a]
D(+)-Glucose	3000[b]
Thymidine	2.00
Na-Phenol red	2.67
Hypoxanthine	10.00
Vitamins	
Biotin	0.01
D-Ca-Pantothenate	2.83
Choline chloride	45.00
Folic acid	2.33
myo-Inositol	15.17
Niacinamide	1.98
Pyridoxine HCl	2.18
Riboflavin	0.40
Thiamine HCl	1.46
Vitamin B12	0.88
Salts and traces	
CaCl2. 2H2O	56.66
KCl	199.10
Na2HPO4. 2H2O	171.00
NaCl	6964
MgSO4. 7H2O	158.38
FeSO4. 7H2O	3.11
NaHCO3	2100
ZnSO4. 7H2O	0.74

FMX-8 MOD was FMX-8 medium (Cell Culture Technologies LLC, Gravesano, Switzerland), where several components very commonly present in culture medium were added as follows:

[a] L-Alanine, L-Aspartic Acid, L-Glutamic Acid and Pluronic-F68, all absent in FMX-8, were added

[b] The concentration of D(+)-Glucose, 1700 mg/L in FMX-8, was increased to 3000 mg/L

Notice that glutamine was not added because its fast degradation

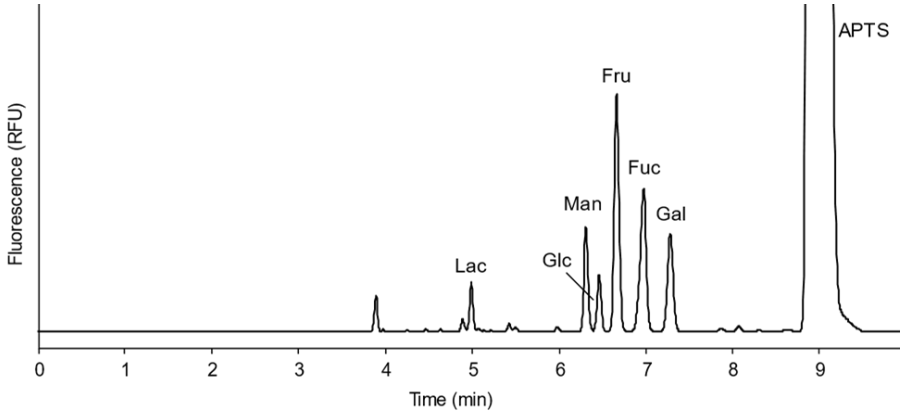

Figure S1. CE-LIF analysis of the APTS-derivatised standard saccharide mixture (16.7 mM) incubated at 60 °C for 1.5 h spiked with the APTS-derivatised fructose (100 mM) incubated at 75 °C for 2.5 h (1:5), analysed with BGE: 100 mM borate, 0.1 % poloxamer 188, pH 9.6. Applied voltage 15 kV, resulting current 46 μA.

Table S2. Validation data. Linearity data obtained from samples at concentrations 0.50 mM (n=2), 15 mM, 30 mM, 45 mM, and 60 mM (n=2) analysed in triplicate and precision data obtained from samples at 16.7 mM (n=3) analysed 6x each.

	Slope	Intercept	Correlation coefficient (r)	Migration time RSD% (n=18)	Peak area RSD% (n=18)
Lactose	3.31	1.29	0.994	0.3%	4.5%
Mannose	5.38	-0.64	0.998	0.6%	4.2%
Glucose	3.43	-0.88	0.995	0.6%	4.5%
Fucose	5.90	-0.38	0.997	0.8%	3.9%
Galactose	6.75	-2.79	0.998	0.8%	3.9%

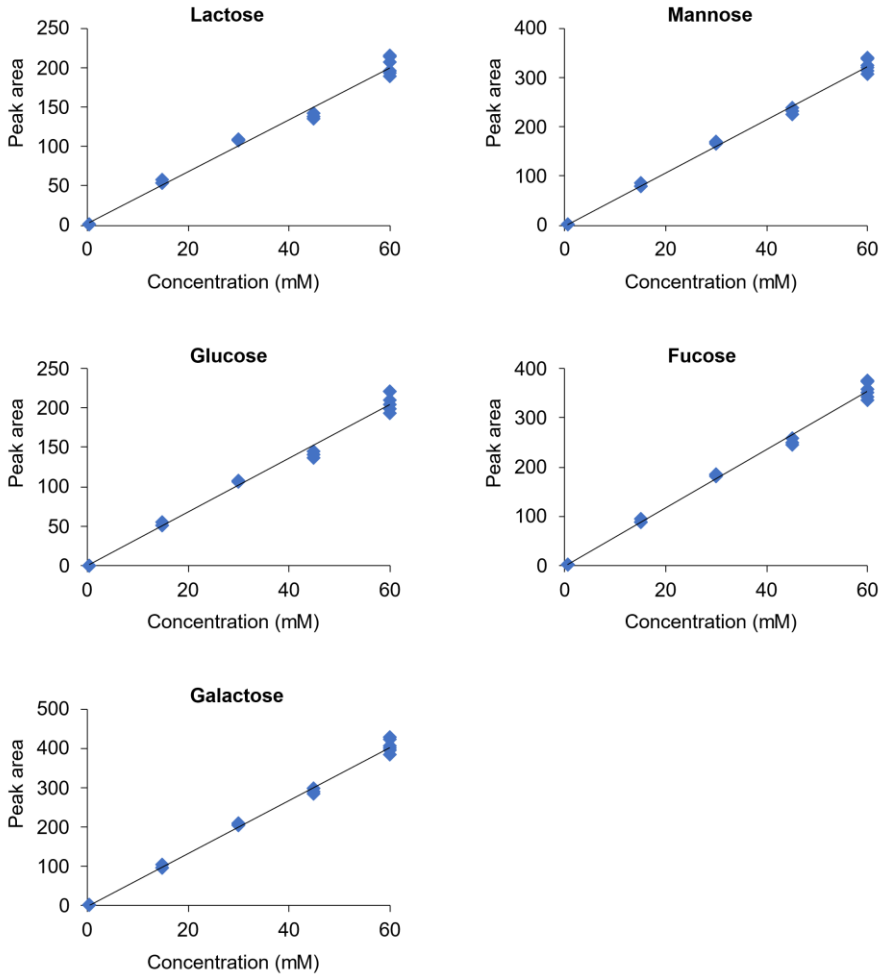

Figure S2. Calibration plots of the linearity data obtained from samples at concentrations 0.50 mM (n=2), 15 mM, 30 mM, 45 mM, and 60 mM (n=2) analysed in triplicate.

Table S3. Recovery data: calculated concentrations of four samples with different concentrations (n=3) using one-point calibration with a 50 mM glucose standard in water or using a 50 mM glucose standard in saccharide-free cell culture medium and their respective recoveries.

Sample	Calibration with standard in water		Calibration with standard in medium	
	Conc. (mM)	Recovery	Conc. (mM)	Recovery
14.5 mM	14.6	101%	14.4	99%
21.8 mM	20.8	95%	20.5	94%
29.1 mM	31.6	109%	31.2	107%
36.4 mM	38.1	105%	37.6	103%

Chapter

Analysis of cationic vitamins in cell culture medium samples by capillary zone electrophoresis

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ABSTRACT

This paper describes a capillary electrophoresis method for the determination of the cationic B-vitamins thiamine, nicotinamide, pyridoxine, pyridoxal, and pyridoxamine in untreated cell culture medium samples. The effects of the buffering capacity, the mobility of the co-ion, and the preconditioning solution on the robustness of the method were investigated. Using a 100 mM phosphoric acid and 55 mM triethanolamine background electrolyte at pH 2.3 and a capillary preconditioning with 1 M NaOH, all five vitamins could be separated with good resolution. Preliminary method validation data over the range 10 – 110 µM for undiluted samples, with 10 µM being the lower range limit of quantification QL, showed accuracy recoveries of 94 – 104%, and migration time and peak area repeatabilities within 0.4% RSD and 2.6% RSD, respectively.

5.1 INTRODUCTION

Biopharmaceuticals have become important products in the pharmaceutical industry [1,2]. Biopharmaceuticals are produced using cell culture bioprocesses. The cell culture medium quality is critical for bioprocess performance and the quality of the final product [3]. Vitamins are present in cell culture medium in small amounts as essential nutrients. They have shown to play important roles in cell growth, cell death, and productivity [4,5], as well as for increase in mAb yield [6]. Vitamins can also affect the color of the drug substance, which is a product quality attribute [6,7]. For a better understanding of the effect of vitamins on factors such as cell growth, cell viability, and productivity, the monitoring of the biopharmaceutical process is essential. The degradation of vitamins in aqueous solutions can make accurate analysis challenging. B vitamins are sensitive to a range of external factors, such as light, oxygen, low or high pH, and temperature, as well as to interactions with other cell culture medium components [8]. Vitamin monitoring is nowadays often performed offline, where samples are taken at different time points of the biopharmaceutical process and stored for analysis at a later time point. Due to the instable character of the vitamins in solutions, there is a need for a fast, robust method for cell culture medium samples that can be used for at-line analysis directly after sampling. A separation technique that can handle the complex cell culture medium, consisting of the medium components including carbohydrates, amino acids, vitamins, lipids, salts, trace elements, growth factor, polyamines, buffers, surfactants, and antifoams [9], as well as components produced or leaked by the cells, such as metabolites, proteins, nucleic acids, lipids, and membrane debris, should be selected. In addition to the complex sample matrix, vitamins, even though they are all essential nutrients, show few close chemical or functional similarities [10]. Due to the different physical chemical properties, a one-size-fits-all derivatisation or sample clean-up is difficult. Capillary electrophoresis (CE) can handle complex sample matrices with reduced sample preparation because of its high separation power and simple set-up. In addition, with CE, rapid analyses can be obtained with minimal sample volumes. For at-line monitoring of the biopharmaceutical process, a robust method should be developed. Several factors impact the migration time and peak area repeatability. Controlling the electro-osmotic flow (EOF) is one of the most important factors for robustness and continuous reproducible use. The EOF is the result of the electric double layer formed by ions in the buffer to balance the charge on the capillary wall. When an electric field is applied over the capillary, ions in the diffuse part of the electric double layer move and drag the bulk liquid along, creating a plug flow. The EOF is affected by several factors, such as the ionic strength of the

buffer and the charge on the capillary wall [11]. The charge of bare fused silica capillary walls is pH depended. The charge on the capillary wall can be minimised by selecting a background electrolyte (BGE) with a low pH, this significantly reduces, and thus controls, the EOF. In addition, the minimised charge reduces adsorption of matrix components to the capillary wall. Adsorption of matrix components to the capillary wall could affect the charge on the wall and thus the EOF. Adsorption of cell culture medium components cannot be fully avoided, so in order to further reduce adsorption of components from the complex cell culture medium samples, capillary coatings could be applied. Next to preventing adsorption, capillary coatings also control the EOF. One option is dynamically coating the capillary by adding an additive to the BGE, such as amine modifiers like triethanolamine (T EthA). T EthA could be used to supress adsorption because of its electrostatic interaction with silanol, masking free silanol groups [12–14]. These BGE additives can form a positively charged layer on the capillary wall, protecting it from adsorption of cell culture medium components, and slowing down or even reversing the EOF. The applied voltage during separation can cause electrolysis of the BGE, altering its pH. Since the pH both affects the EOF and the analyte charge, the pH of the BGE should be kept constant. Buffer depletion could be reduced by selecting a BGE with a high buffering capacity [15]. Several factors influence the buffering capacity, such as the concentration and the pH of the BGE; a higher buffering capacity is obtained with a higher buffer concentration and when a buffer pH close to the pKa of the buffering component is selected. By selecting the BGE pH, it is also important to take the pKas of the analytes into account, for a robust method, the charge on the analytes should not be significantly affected by small pH changes, and thus a pH significantly far from the analytes pKas should be selected. Lastly, capillary preconditioning is important for high migration time repeatabilities [16,17]. Rinsing the capillary is important since it returns the capillary to the same consistent conditions. The capillary should first be rinsed with a solution to remove potentially adsorbed components from the capillary wall, usually sodium hydroxide or strong acids in the range of 0.1 to 1.0 M are used. Then the capillary should be rinsed with the BGE to re-equilibrate the capillary surface. Good capillary preconditioning can provide a well-defined, reproducible state of surface hydroxylation on the capillary wall. Although several papers describing CE for the analysis of water-soluble vitamins in pharmaceutical preparations such as tablets were published [18–22], the monitoring of vitamins with CE in biopharmaceutical cultivation processes remained unexplored. Some interesting work has been shown for vitamin analysis in bacterial growth medium [23]. However, the BGE in this work has a high conductivity combined with a low buffering capacity. The presence of sodium dodecyl sulphate in the BGE can

potentially prevent adsorption of medium components to the capillary wall, but seeing the complex nature of upstream cell medium samples, this is likely not sufficient. Whereas for pharmaceutical tablets, often only pyridoxine is used as B6 vitamin, in cell culture cultivation processes, a variation of all three B6 vitamins, pyridoxine, pyridoxal, and pyridoxamine, is used. In this work, the focus is on developing a method for the detection of the cationic B vitamins thiamine, nicotinamide, pyridoxine, pyridoxal, and pyridoxamine in upstream processing cell culture medium samples with CE. During method development, factors affecting the robustness, such as capillary preconditioning, buffering capacity, buffer pH, and co-ion mobility were investigated.

5.2 EXPERIMENTAL

5.2.1 CHEMICALS

Thiamine HCl, nicotinamide, pyridoxine HCl, pyridoxal HCl, pyridoxamine 2HCl, Tris(hydroxymethyl)aminomethane (Tris), phosphoric acid 85 95%, glycine, and triethanolamine (T EthA) were obtained from Merck/Sigma Aldrich (Darmstadt, Germany). HyClone ActiPro medium and HyClone ActiSM medium were purchased from Cytiva (Marlborough, USA). Modified FMX-8 cell culture medium (FMX 8 MOD) with the composition as described in [24] was provided by the Department of Industrial Biotechnology, KTH Royal Institute of Technology, Stockholm, Sweden. Water was of MilliQ grade quality (18.2 MΩ.cm).

5.2.2 INSTRUMENTAL CONDITIONS

Experiments were conducted on an Agilent 7100 capillary electrophoresis system with Diode Array UV detector (Waldbronn, Germany). Chemstation software was used for instrument control, data acquisition, and data analysis. Bare fused silica capillaries with 50 µm id were purchased from Agilent Technologies. Capillaries had a total length of 33 cm with an effective length of 24.5 cm. The separation voltage ranged from 13 kV to 20 kV depending on the conductivity of the used background electrolyte (BGE), and was ramped over 0.5 min. Samples were introduced hydrodynamically at 30 mbar for 5 s, followed by the injection of a BGE plug using the same conditions. Separations were carried out at 20 ˚C. The detector signal was recorded at 210 nm. Before first use, the capillary was flushed successively with 1 M NaOH, water, and BGE at 1 bar for 20 min each. At the beginning of each working day, the capillary was flushed successively with 0.1 M NaOH, water, and BGE at 1 bar

for 10 min each. Prior to each injection, the capillary was preconditioned with BGE at 1 bar for two minutes.

5.2.3 BGE AND SAMPLE PREPARATION

BGEs containing phosphoric acid at acidic conditions were tested with different co-ions; Tris, glycine, and T EthA. The final BGE consisted of 100 mM phosphoric acid and 55 mM T-EthA, which had a pH of 2.3. No pH-adjustment was done. Vitamin stock solutions of 20 mM in water were prepared and stored at 20 °C. A standard vitamin mixture was prepared by mixing these vitamin stock solutions and diluting the mixture with water, the final concentrations of the vitamins in the mixtures varied and ranged from 10 µM to 110 µM. Cell culture medium samples were analysed untreated. Spiked cell culture medium samples were prepared by adding 4 µL of a 4.5 mM standard vitamin mixture to 356 µL cell culture medium sample. Descriptive statistics were calculated according to the normal procedures.

5.3 RESULTS AND DISCUSSION

5.3.1 METHOD DEVELOPMENT

The vitamins pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), thiamine (B1), and nicotinamide (B3) with pKa values of 5.6, 4.1, 9.6, 5.5, and 3.6, respectively [25], are all positively charged at acidic pH. Therefore, a BGE with a low pH should be used. Phosphate has a pKa value at 2.2 and thus a high buffering capacity at this pH, which is significantly lower than the pKa values of the vitamins. In addition, it is inorganic, thus showing very little UV absorbance. Hence, phosphoric acid BGEs were investigated. At a low pH, the separation window for cationic substances is wide, which increases the separation of cell culture medium matrix component from the vitamins. One of the attributes a robust method needs to adhere to, is good migration time repeatability. Selecting a BGE with a high buffering capacity improves the migration time repeatability [26]. For a robust method, the BGE composition should be consistent. For that reason, the pH of the BGE should be set by using calculated concentrations of buffer components, rather than setting the pH afterwards by the addition of acids or bases. This approach controls the ionic strength and reproducibility. In order to ensure sharp peaks, electromigration dispersion should be suppressed by selecting a co-ion with a mobility close to that of the analytes [27]. The mobilities of pyridoxal, pyridoxine, and nicotinamide were determined to be 26.5, 27.1, and 33.9 x10-9 m2/Vs, respectively, by Terekhova et. al. [28] and the mobilities of pyridoxamine and thiamine are expected to be in the same range. Two

co-ions with mobilities close to this range were selected; Tris and glycine. The pKa of phosphoric acid is 2.2, a higher buffering capacity is thus obtained with a BGE pH close to this value. When mixing phosphoric acid and Tris in a ratio of 2:1, and phosphoric acid and glycine in a ratio of 1:1, a pH of 2.3 is obtained. Three BGEs were prepared; A) 50 mM phosphoric acid, 25 mM Tris, pH 2.3, B) 50 mM phosphoric acid, 50 mM glycine, pH 2.3, and C) 100 mM phosphoric acid, 100 mM glycine, pH 2.3, see Table S1 in the Supporting Information for buffer properties. A standard vitamin mixture containing vitamin B1, B3, and the B6 complex pyridoxine, pyridoxal, and pyridoxamine was analysed with these three BGEs, see Figure 1 A-C for the obtained electropherograms and Table S2 in the Supporting Information for measured mobilities. The migration time repeatabilities of the vitamins achieved with BGE A, B, or C were all within 7.0% RSD, 1.6% RSD, or 0.7% RSD, respectively. As expected, the migration time repeatability increased with increasing buffering capacity. Using glycine in the BGE unfortunately caused the vitamin peaks to split into double peaks. The eigenmobility of the BGEs containing glycine is close to the mobilities of the vitamins, which could cause an interference [29], however, interference caused by the eigenmobility of the buffer is expected to influence only one of the vitamin peaks, not all peaks. Although the mechanism is not fully understood, it is clear that a different co-ion should be selected. When using Tris in the BGE, not all vitamins were baseline separated. An alternative co-ion for Tris is T EthA. T-EthA interacts with the capillary wall, forming a dynamic coating, controlling the EOF and slightly reversing it [30], which could improve resolution. In addition, the dynamic coating improves robustness as small changes in pH do not affect the EOF as strongly as on an uncoated capillary, it prevents adsorption of cell culture medium components, and it increases migration time reproducibility. For T-EthA, a pH of 2.3 is obtained when mixing phosphoric acid and T-EthA in a ratio of 1.8:1. A standard vitamin mixture was analysed with a BGE consisting of 100 mM phosphoric acid and 55 mM T-EthA at pH 2.3 (BGE D). This BGE had a buffering capacity of 69.1 mM, which is about equal to the 50 mM phosphoric acid / 50 mM glycine BGE (Table S1). By changing the co ion to T-EthA, an improvement in resolution and peak shape was achieved. Due to the high current produced when using this BGE (82 µA at 20 kV), the applied voltage was reduced to prevent excessive Joule heating. Changing the voltage from 20 to 13 kV did not affect the resolution of the vitamin peaks, it only increased the migration times. The migration time repeatabilities for the vitamins using T-EthA in the BGE were all within 0.4% RSD, which is significantly better than when using Tris or glycine as co-ion.

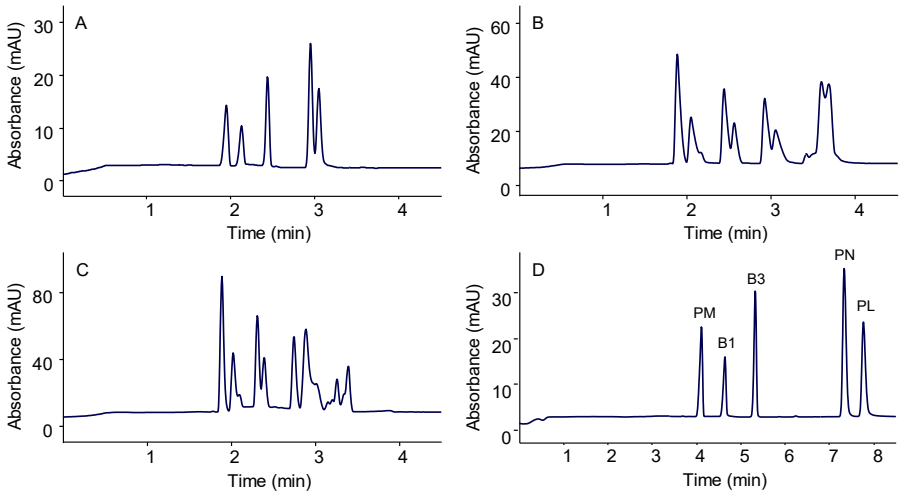

Figure 1. Electropherograms of a standard vitamin mixture (conc. 2.5 – 4.5 mM) analysed with A) BGE A: 50 mM phosphoric acid, 25 mM Tris (current 52 µA), B) BGE B: 50 mM phosphoric acid, 50 mM glycine (current 52 µA), or C) BGE C: 100 mM phosphoric acid, 100 mM glycine (current 84 µA), at 20 kV, or with D) BGE D: 100 mM phosphoric acid, 55 mM T EthA (current 46 µA) at 13 kV. PM – pyridoxamine, B1 – thiamine, B3 – nicotinamide, PN – pyridoxine, PL – pyridoxal.

Another factor affecting the robustness of the method is the capillary preconditioning. To investigate the influence of the preconditioning solvent on the robustness, the capillary was flushed with either 0.1 M phosphoric acid or with 1 M NaOH prior to analysing a standard vitamin mixture six times. The migration time repeatabilities of the vitamins analysed after the phosphoric acid flush were all within 0.8% RSD, while the migration time repeatabilities of the vitamins analysed after the NaOH flush were all within 0.4% RSD. The peak area repeatabilities after the phosphoric acid or NaOH flush were all within 8.2% RSD or 2.6% RSD, respectively. Using an NaOH flush significantly increases both the migration time and the peak area repeatabilities compared to a phosphoric acid flush. It was also observed that the migration times slightly increased after the NaOH flush. The T-EthA forms a dynamic positive coating on the capillary wall, slightly reversing the EOF. After flushing with NaOH, more silanol groups on the capillary wall are deprotonated then after the phosphoric acid flush, which results in more T-EthA adsorbing to the capillary wall, explaining the slightly longer migration times. A trend in decreasing migration times was observed after both preconditioning protocols, however, it was less prominent after the NaOH flush, which indicates that after the NaOH flush a more stable T-EthA coating was formed.

Preliminary method validation was performed for the final method with optimised capillary preconditioning and BGE. Method precision was tested using six consecutive runs of a standard vitamin mixture. The migration time repeatabilities for the vitamins in the mixture were within 0.4% RSD and the repeatabilities of the peak area were all within 2.6% (Table 1). Linearity was investigated over the range 10 – 110 µM by triplicate analysis of six standard vitamin mixtures at different concentrations (10 µM, 30 µM, 50 µM, 70 µM, 90 µM, and 110 µM). Calibration plots were linear over this range with R2 larger than 0.99 for all vitamins (Table 1, and Figure S1, Supporting Information). The lowest level, 10 µM, is the lower range limit QL [31] and was prepared in duplicate and injected in total six times, in order to have good precision and accuracy on the lower range limit of quantification QL. Accuracy was determined as recovery of the calculated concentrations from the actual concentrations. Overall, the recovery was 95 – 105%, although the recovery at the lowest concentration level of pyridoxamine was 94% (Table 2). Generally, for the vitamin determination in complex cell culture media a recovery of 90 – 110% and a precision of 10% RSD are required. This method performs well within the acceptable range.

Table 1. Preliminary method validation data: precision on migration time and peak area of six consecutive runs, and linearity data over the range 10 – 110 µM.

	Precision (n=6)		Linearity (n=21)		
	Migration time	Area	Slope	Intercept	Correlation coefficient (R^2)
PM	0.4%	1.9%	0.11	0.14	0.996
B1	0.4%	2.6%	0.08	0.23	0.996
B3	0.3%	2.3%	0.10	0.13	0.998
PN	0.3%	1.5%	0.25	0.34	0.998
PL	0.3%	1.5%	0.20	0.24	0.998

Table 2. Method accuracy expressed as percentage recovery

Conc.	n	PM	B1	B3	PN	PL
10 µM	6	94%	95%	95%	95%	96%
30 µM	3	102%	101%	100%	100%	98%
50 µM	3	102%	103%	103%	103%	104%
70 µM	3	103%	102%	102%	101%	101%
90 µM	3	96%	97%	98%	98%	100%
110 µM	3	101%	101%	100%	100%	99%

5.3.2 APPLICATION TO CELL CULTURE MEDIUM

In order to show the applicability of the developed method for vitamin monitoring in biopharmaceutical processes, three different cell culture media were tested. Two of these media were the commercially available ActiPro medium and ActiSM medium. These cell culture media are commercially available with a proprietary composition but are described as chemically defined and animal-derived component-free media developed to provide high yields of recombinant proteins in bioprocesses using Chinese hamster ovary (CHO) cell lines [32]. The ActiSM medium is generally used for developing the bioprocess, while ActiPro is used for the production process. The third medium was modified FMX-8 medium (FMX-8 MOD). The FMX-8 culture medium was developed for the production of recombinant proteins under chemically defined culture conditions and its formulation was published [33]. To prepare FMX 8 MOD, several components commonly present in cell culture medium were added to the well-defined FMX 8 culture medium. The exact composition of FMX 8 MOD was described in [24], FMX-8 MOD contains the cationic vitamins nicotinamide (16.2 µM), thiamine (4.3 µM), and pyridoxine (12.9 µM). The three media were analysed untreated (Fig 2A), as well as spiked with a standard vitamin mixture (Fig 2B). The

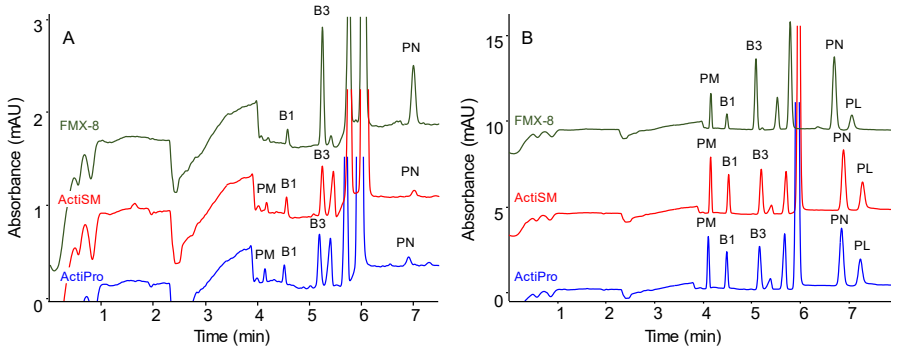

Figure 2. Electropherograms of A) FMX 8 MOD, ActiSM, and ActiPro medium and B) FMX 8 MOD, ActiSM, and ActiPro medium spiked with standard vitamins (50 µM) analysed with BGE: 100 mM phosphoric acid, 55 mM T-EthA. Applied voltage 13 kV, resulting current 40 – 46 µA. PM – pyridoxamine, B1 – thiamine, B3 – nicotinamide, PN – pyridoxine, PL – pyridoxal.

vitamins are separated from the components in the complex cell culture medium matrices and detected in the cell culture medium samples. This shows the potential of the method as a platform method to monitor the concentrations of the vitamins in biopharmaceutical processes. A complete validation always needs to be performed with samples from the actual bioprocess under monitoring. The data given here demonstrate that this additional/complementary validation will be straightforward.

5.4 CONCLUSION

Summarising the results, the cationic vitamins B1, B3, and the B6 complex pyridoxine, pyridoxal, and pyridoxamine can be separated using a 100 mM phosphoric acid and 55 mM T-EthA BGE at pH 2.3 with good resolution. The dynamic T-EthA coating is most stable after a capillary preconditioning with 1 M NaOH. A good precision was obtained with migration time repeatabilities within 0.4% RSD and peak area repeatabilities within 2.6% RSD. The method was linear over the range 10 – 110 µM, with good accuracy of 94 – 104%. These performance results were well within the required limits of 90 – 110% accuracy and ≤ 10% RSD. The method shows to be applicable for the monitoring of vitamins in biopharmaceutical processes, the vitamins are separated from the complex cell culture medium matrix. This shows the great potential of using this method for at-line analysis for the monitoring of the biopharmaceutical process. A full validation should be performed with process samples from the actual bioprocess under monitoring. Looking at the preliminary validation data, no issues are expected for the final validation.

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SUPPLEMENTARY INFORMATION

Table S1. BGE composition and properties as calculated by Peakmaster[1]. PA: phosphoric acid, Gly: glycine. The pH is 2.3 for each BGE.

BGE	Ionic strength (mM)	Conduc-tivity (S/m)	Buffering capacity (mM)	System eigen-mobility	Co-ion mobility ($x10^{-9}$ $m^2/V/s$)
A: 50 mM PA, 25 mM Tris	30.9	0.34	40.5	49.5	24.4
B: 50 mM PA, 50 mM Gly	31.9	0.35	68.2	18.3	18.0
C: 100 mM PA, 100 mM Gly	62.5	0.54	125.4	14.6	18.2
D: 100 mM PA, 55 mM T-EthA	61.4	0.49	69.1	41.7	23.8

Table S2. Apparent mobilities ($x10^{-9}$ $m^2/V/s$) of the vitamins obtained with the different BGEs. At pH 2.3, the EOF could be assumed virtually zero, making these values also the electrophoretic mobilities. For BGE D, a negative EOF is likely present, decreasing the apparent mobilities of the vitamins

BGE	B1	B3	PL	PM	PN
A: 50 mM PA, 25 mM Tris	32.3	28.9	24.3	35.0	25.0
B: 50 mM PA, 50 mM Gly	28.1	23.5	18.6	36.3	19.0
C: 100 mM PA, 100 mM Gly	29.4	24.8	20.0	36.0	23.6
D: 100 mM PA, 55 mM T-EthA	22.2	19.4	13.3	25.1	14.1

[1] https://web.natur.cuni.cz/gas/peakmaster.html

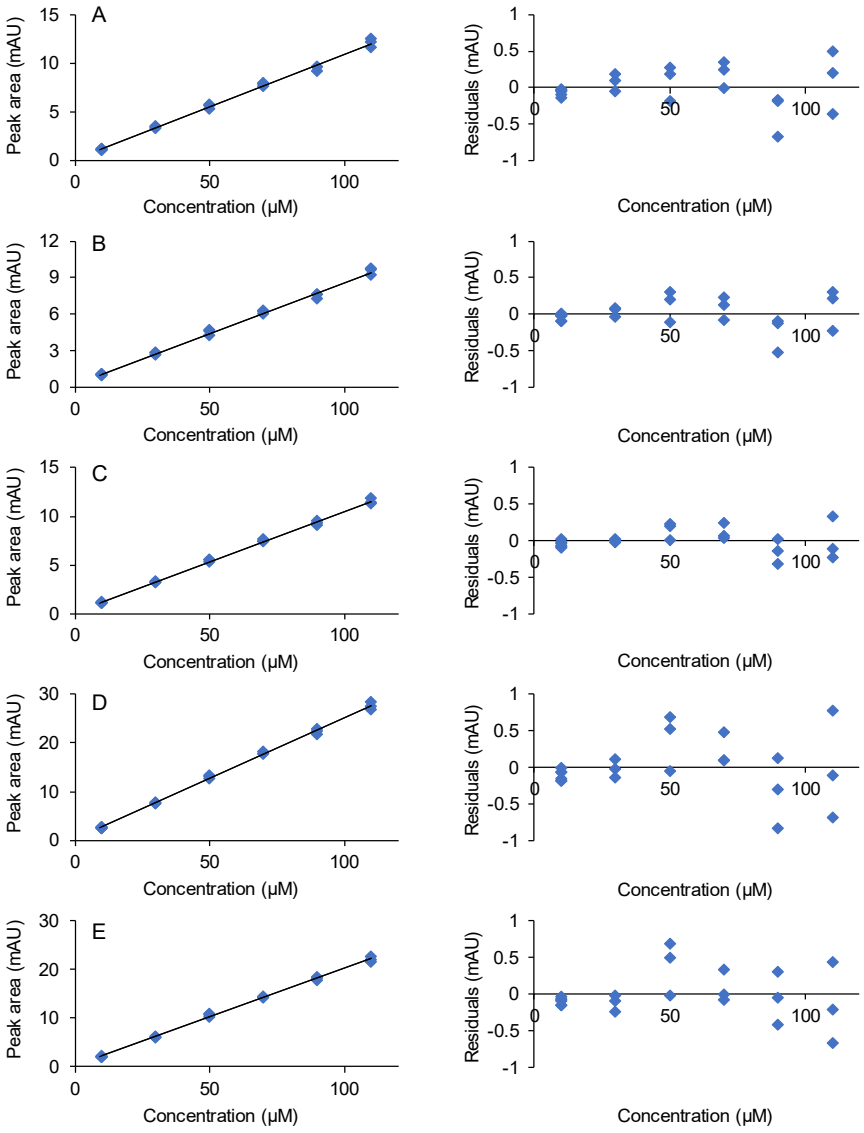

Figure S1. Calibration curves (left) and residual plots (right) of A) pyridoxamine, B) thiamine, C) nicotinamide, D) pyridoxine, and E) pyridoxal.

Chapter

Design of experiments for micellar electrokinetic chromatography method development for the monitoring of water-soluble vitamins in cell culture medium

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ABSTRACT

Biopharmaceutical production takes place in complex processes which should be thoroughly understood. Therefore, the iConsensus project focuses on developing a monitoring platform integrating several process analytical technology tools for integrated, automated monitoring of the biopharmaceutical process. Water-soluble vitamin monitoring using (microchip) capillary electrophoresis (CE) is part of this platform. This work comprises the development of conventional CE methods as the first part towards integrated vitamin monitoring. The vitamins were divided based on their physical–chemical properties to develop two robust methods. Previously, a method for the analysis of cationic vitamins (pyridoxine, pyridoxal, pyridoxamine, thiamine and nicotinamide) in cell culture medium was developed. This work focused on the development of a micellar electrokinetic chromatography method for anionic and neutral vitamins (riboflavin, d-calcium pantothenate, biotin, folic acid, cyanocobalamin and ascorbic acid). By employing multivariate design of experiments, the background electrolyte (BGE) could be optimised within one experiment testing only 11 BGEs. The optimised BGE conditions were 200 mM borate with 77 mM sodium dodecyl sulphate at a pH of 8.6. Using this BGE, all above-mentioned cationic, anionic and neutral vitamins could be separated in clean samples. In cell culture medium, most anionic and neutral vitamins could be separated. Combining the two methods allows for analysis of cationic, anionic and neutral vitamins in cell culture medium samples. The next step towards integrated vitamin monitoring includes transfer to microchip CE. Due to the lack of fast and reliable methods for vitamin monitoring, the developed capillary methods could be valuable as stand-alone at-line process analytical technology solutions as well.

6.1 INTRODUCTION

Biopharmaceuticals are produced in complex processes where many factors influence the yield and quality of the product. For good process understanding and to optimise conditions to ensure high product quality and yield, process monitoring is essential. Process analytical technology (PAT) is a tool to increase knowledge of the effect of process parameters on the state of the culture and on the critical quality attributes of the product [1]. For the purpose of increased process understanding, manufacturing efficacy and robustness, the European project iConsensus focuses on developing an integrated control and sensing platform for biopharmaceutical cultivation processes [2]. The vision of this monitoring platform is an automated platform, including sampling, sample preparation and analysis, employing several on-/at-line PAT tools for the (near) real-time detection of physical factors, reagents and mAb quality attributes in the bioreactor. The platform will include a monitoring information database collecting all the data and modelling tools for immediate data processing, allowing for efficient feedback control of the cultivation process. A schematic overview of the platform can be seen in Figure S1.

Some factors in the bioreactor can be analysed by sensors or affinity-based techniques, others require separation techniques. A miniaturised separation technique would simplify the integration into the automated, integrated monitoring platform. Cell culture medium is a complex matrix consisting of the medium components, including carbohydrates, amino acids, vitamins, lipids, salts, trace elements, growth factor, polyamines, buffers, surfactants, antifoams [3], as well as components produced or leaked by the cells, such as metabolites, proteins, nucleic acids, lipids and membrane debris. This complex sample matrix requires a technique with high separation power. Additionally, sample preparation should be limited for integration in the monitoring platform. Capillary electrophoresis (CE) has proven to be a valuable technique for biopharmaceutical analysis [4] and has a number of benefits for the analysis of upstream process samples. CE can handle the highly complex cell culture matrix, has high resolving power, requires little sample preparation, has low solvent and sample consumption and provides rapid analysis. Additionally, the simple setup provides the possibility for miniaturisation and automation on microchip CE. CE was therefore selected as separation technique for the monitoring platform.

Cell cultivation processes producing biopharmaceuticals require numerous nutrients. These nutrients are present in the cell culture medium. Because they are consumed by the cells and could degrade over time, usually a feed medium, containing these nutrients, is administered during the process [3]. A group of essential nutrients in cell

culture medium that should be monitored by this platform are water-soluble vitamins that play important roles in cellular processes, cell growth, cell death or productivity [5, 6]. The addition of vitamins to the cell culture medium showed to increase the yield of mAbs [3] and the presence of B vitamins affects the colour of the drug substance, which is a product quality attribute [7, 8]. As vitamins affect product quality, they are considered critical process parameters. Their monitoring is essential to understand their impact on the cell culture process and the final product.

For this purpose, CE methods should be developed for use in the integrated, automated monitoring platform. The vitamins should be monitored in complex upstream process samples, the methods should allow for unattended analysis for the duration of the upstream cell process campaign (generally 7–14 days [9]), and the platform will be operated by process operators rather than analytical chemists. This sets more stringent requirements on the methods, especially on robustness, repeatability and reproducibility. Additionally, the methods must be developed as integrated sample preparation, separation and detection. This sets more stringent requirements on sample preparation and compatibility of all solvents and chemicals with the materials of the integrated platform.

Vitamin monitoring in cultivation processes comprises some challenges, such as their trace amount concentration, the complex matrix and their diversity in terms of chemical structure and properties [10] (Table S1) complicating a one-size-fits-all derivatisation or sample clean-up, and additionally, many water-soluble vitamins are unstable and can degrade under various conditions, such as light exposure, temperature or acidic or alkaline conditions [11]. Sampling, storage and transferring to analytical labs, for example, could all cause degradation of the vitamins, and thus negatively influence the reliability of the results. This emphasizes the need for rapid analysis by this automated, integrated monitoring platform.

There are numerous CE and liquid chromatography applications for vitamin analysis in food and pharmaceutical formulations [12]. However, the concentrations of vitamins in food and pharmaceutical formulations (mg–g/tablet) are generally higher than in cell culture medium (≤1 mmol/L), and the sample matrices are less complex. Additionally, extensive sample preparation steps are frequently used, which are unfeasible in the automated, integrated monitoring platform. These methods can therefore not be readily used. Only very limited applications for vitamin analysis in biopharmaceutical samples are published. Some applications employ liquid chromatography coupled to mass spectrometry [13, 14]. As these techniques are challenging to miniaturise, they are out of scope for the monitoring platform. To the best of our knowledge, only one CE application for vitamin analysis in bacterial growth medium was published [15]. This method shows the potential of CE for

vitamin monitoring in complex cell culture medium samples; however, only five vitamins (thiamine [B1], pyridoxine [PN], riboflavin [B2], nicotinamide [B3] and folic acid [B9]) were analysed using this method. This shows the need for additional methods for vitamin monitoring in biopharmaceutical cultivation processes.

The project involved the simultaneous development of the analytical methods, the microchip instrumentation and the instrumentation for the integration of all different modules. As the microchip instrumentation was developed in parallel to the CE methods, part 1 towards automated, integrated vitamin monitoring comprised the development of conventional CE methods. Part 2 will comprise of the transfer of the methods to microchip CE and their integration into the monitoring platform. Previous work illustrated the transferability of CE methods developed on conventional CE to the prototype microchip CE instrument [16]. As the iConsensus project is currently still in the exploratory phase, and considering the lack of analytical methods for vitamin monitoring in biopharmaceutical processes, the capillary methods could be valuable as stand-alone, at-line PAT solutions as well.

Developing a robust method for the simultaneous analysis of all water-soluble vitamins is challenging due to their differences in physical–chemical properties. To improve robustness, it may be beneficial to divide the vitamins into groups based on their physical–chemical properties and develop multiple methods. For CE analysis it would be useful to divide the vitamins based on their charge. At acidic conditions, the vitamins PN, pyridoxal (PL), pyridoxamine (PM), B1 and B3 are positively charged. A CE method for the analysis of these vitamins in cell culture medium samples was previously developed [17]. A CE method for the remaining anionic and neutral vitamins needed to be developed. For the separation of neutral vitamins, micellar electrokinetic chromatography (MEKC) proved suitable for separation [18, 19]. However, the published methods were developed for food and food supplements, which have an entirely different sample matrix than upstream biopharmaceutical process samples, impacting the separation optimization. In addition, sample cleaning procedures such as solid phase extraction employed for food samples are not feasible in the intended automated set-up for cell culture medium monitoring.

In this work, an MEKC method for the monitoring of the anionic and neutral vitamins (B2, D-calcium pantothenate [B5], biotin [B7], B9, cyanocobalamin [B12] and ascorbic acid [C]) in biopharmaceutical processes was developed. The method must be developed for future integration into the automated monitoring platform, thus the method must be simple, robust, with no or limited sample preparation, transferrable to microchip CE and with a precision of ≤10% RSD.

6.2 MATERIALS AND METHODS

6.2.1 CHEMICALS

The vitamins C, B1 HCl, B2, B3, B5, PN HCl, PL HCl, PM 2HCl, B7, B9 and B12 were purchased from Sigma Aldrich. Phosphoric acid 85%–95%, boric acid, sodium tetraborate decahydrate (borax) and sodium dodecyl sulphate (SDS) were from Sigma Aldrich. Acetonitrile was obtained from VWR. Dimethyl sulfoxide (DMSO), monosodium phosphate (NaH2PO4) and disodium phosphate (Na2HPO4) were purchased from Merck. HyClone ActiPro medium was purchased from Cytiva. Cell culture medium based on FMX-8 medium [20] with certain modifications (FMX-8 MOD) [21] and vitamin-free cell culture medium were provided by the Department of Industrial Biotechnology, KTH Royal Institute of Technology. Water was of Milli-Q grade quality (18.2 MΩ cm).

6.2.2 INSTRUMENTAL CONDITIONS

Experiments were conducted on an Agilent 7100 CE system with diode array detector. OpenLAB CDS ChemStation (Rev. C.01.09) software was used for instrument control, data acquisition and data analysis. Bare fused silica capillaries with 50 µm i.d. were purchased from Agilent Technologies. Capillaries were cut to a total length of 33 cm with an effective length of 24.5 cm. The separation voltage ranged from 10 to 20 kV depending on the conductivity of the used background electrolyte (BGE) and was applied in a ramp of 0.5 min. Positive polarity mode was used (detector placed at the cathodic side of the capillary). Samples were introduced hydrodynamically at 30 mbar for 5 s, followed by the injection of a BGE plug. Separations were carried out at 20°C. The detector signal was recorded at 197, 210 and 270 nm. Before first use, the capillary was flushed successively with 1 M NaOH, water and BGE at 1 bar for 20 min each. At the beginning of each working day, the capillary was flushed successively with 0.1 M NaOH, water and BGE at 1 bar for 10 min each. Prior to each injection, the capillary was preconditioned with BGE at 1 bar for 2 min.

6.2.3 BGE PREPARATION

BGEs containing borate and SDS at alkaline conditions were tested. The buffers were prepared by mixing appropriate amounts of stock solutions of SDS, boric acid or borax. The tested SDS concentrations ranged from 10 to 100 mM and the tested borate concentrations ranged from 80 to 285 mM, the pHs of the BGEs were adjusted by varying the ratio of boric acid and borax. The BGE was optimised using design of

experiments (DoE). The design was created, and the data was processed using the DoE software MODDE (version 12.1, Sartorius). The parameters were optimised in a central composite orthogonal design; the centre point was prepared in triplicate. The final BGE consisted of 200 mM borate (104 mM boric acid and 24 mM borax) and 77 mM SDS, which had a pH of 8.6. For high accuracy, the BGE should be prepared by weighing 3.21 g boric acid, 4.57 g borax and 11.09 g SDS on an analytical balance, and dissolving that in water to a total volume of 500 mL.

6.2.4 SAMPLE PREPARATION

Stock solutions of vitamins were prepared in water, except for vitamins C, B2, B7 and B9. Vitamins B7, B9 and B2 were dissolved in DMSO, 1 M NaOH and 0.1 M NaOH, respectively. It should be noted that B2 is unstable in alkaline solutions, however, protected from light the solution should be stable for the duration of the experiment. Vitamin C was dissolved in a solution of 0.1% phosphoric acid with 5% acetonitrile, in which it is most stable [22]. All stock solutions were stored in at −20°C, except B7, which, due to the DMSO, had to be stored at RT and was therefore prepared freshly before each experiment. Working solutions were prepared daily by mixing these vitamin stock solutions and diluting the mixture with a 50 mM phosphate buffer (35 mM Na2HPO4, 15 mM NaH2PO4 and pH 7.2). The NaOH present in the mixture causes alkaline conditions, which could negatively affect the stability of the vitamins. The phosphate buffer was used to ensure the sample has a pH close to that of upstream process samples. The final concentrations of the vitamins in the mixtures varied and ranged from 10 to 95 µM. Cell culture medium samples were analysed without sample pre-treatment.

6.3 RESULTS AND DISCUSSION

6.3.1 METHOD DEVELOPMENT

A method for cationic vitamins was previously developed [17], and this work focuses on the method development for the anionic and neutral vitamins. In order to ensure a charge on the anionic vitamins, an alkaline BGE was selected. For the separation of neutral molecules, MEKC was employed. MEKC is a CE mode that combines the principles of electrophoresis and chromatography by the addition of a charged surfactant, often SDS, to form micelles in the BGE. The micelles act as a pseudo-stationary phase and the separation is based on the mobility (electrophoretic) as well as the retention by the micelles (chromatographic). The mobility is determined by the charge-to-size ratio, whereas the retention is determined by the partitioning of

analytes between the micelles and the BGE and electrostatic attraction or repulsion between charged analytes and the negatively charged micelles. The surfactant SDS was added to a 200 mM borate buffer, pH 9 at various concentrations ranging from 0 to 100 mM to evaluate its effect, see Figure 1. The neutral vitamin B12 is well separated from the EOF at a concentration of 20 mM SDS. Higher SDS concentrations increase the separation window by increased the interaction of analytes with the micelles. This could be required for the separation of the vitamins from the matrix components. At 100 mM SDS, a good separation was achieved for most vitamins. The migration time of B1 was most affected by the SDS concentration; its migration time at 100 mM SDS was around 20 min and not recorded in the electropherogram in Figure 1D.

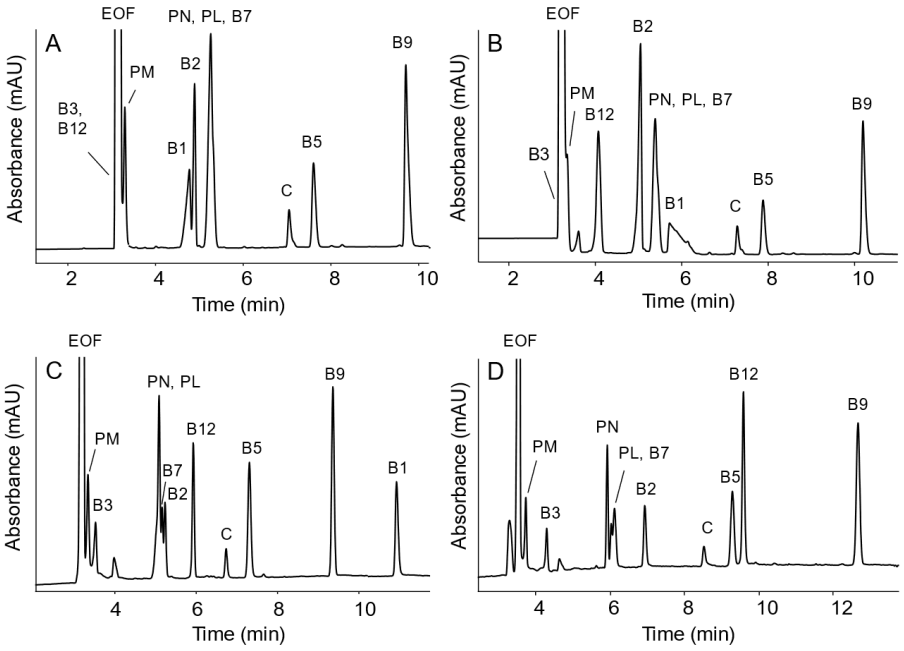

Figure 1. Effect of the sodium dodecyl sulphate (SDS) concentration on the separation of 11 water-soluble vitamins. All background electrolytes (BGEs) consisted of 200 mM borate, pH 9 containing (A) 0 mM SDS, (B) 15 mM SDS, (C) 50 mM SDS or (D) 100 mM SDS. B1, thiamine; B2, riboflavin; B3, nicotinamide; B5, D-calcium pantothenate; B7, biotin; B9, folic acid; B12, cyanocobalamin. Note: these experiments were performed with not yet optimised capillary conditioning and on different capillaries, sometimes resulting in fluctuating migration times. PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine.

To optimise the separation, multivariate DoE were employed. This approach optimises all significant factors simultaneously rather than optimising one factor at a

time. DoE reduces the number of experiments required, provides information over a design space rather than over single experiments, including information on the influence of or the interaction between factors, and will result in a more accurate optimum [23]. To create a DoE, first the objective, the factors and the responses should be defined. The objective is the optimisation of the BGE to maximise the separation, the factors are the parameters affecting the separation, and the responses are the resolutions between the vitamins. To define the factors, previous knowledge was used to determine which parameters affect the separation and what the appropriate ranges were. The ranges were determined in feasibility experiments. Parameters in the BGE affecting the separation of the vitamins are the borate concentration, the SDS concentration and the pH. Previous experiments showed that the borate concentration did not significantly affect the separation (data not shown); therefore, the borate concentration was kept constant at 200 mM. The SDS concentration should be high enough to ensure the separation of the neutral vitamin B12 from the EOF and to provide a sufficiently large separation window but is limited by the increase in analysis time. The SDS concentration was therefore a factor in the design optimised in the range 50–100 mM. The pH has a strong effect on the separation and was therefore defined as a factor. The vitamins B7, PN and PL co-migrated at all tested SDS concentration. The pKa values of vitamin B7, PN and PL are 4.4, 9.4 and 8.0, respectively (Table S1). At alkaline conditions, B7 is fully charged, whereas the charge of PN and PL is dependent of the pH. The separation of B7 from PN and PL could be enhanced by reducing the charge on PN and PL by lowering the pH. This is however limited for robustness purposes. The EOF should not be significantly impacted by small pH variations. At a pH < 2.5 or in the pH range 8–10, the EOF is least affected by variations [24]. Additionally, boric acid has a pKa value of 9.2, for sufficient buffering capacity, the pH should be in the range 9.2 ± 1. Therefore, the range of the factor pH was 8–9.

An appropriate design should be selected. For the purpose of optimisation, a design that incorporates quadratic models should be chosen as they possess flexibility and can imitate a wide range of response functions. Ideally, each factor should be explored at five levels to establish a trustworthy quadratic model [23]. A central composite orthogonal design is such a model. It comprises a full factorial design (all corner points of the design), symmetrically arranged axial experiments and three replicates of the centre point to determine the experimental error. See Figure S2 for an illustration of the design. The axial points are located slightly outside of the design space, ensuring all factors are investigated at five levels, facilitating the accurate estimation of quadratic terms. With this design, the BGE optimisation (borate:

200 mM, SDS 50–100 mM, pH 8–9) could be performed with only 11 experiments (Table S2).

After performing the experiments, the responses were entered in the software MODDE to create models providing information on the relationship between the factors (i.e. pH and SDS concentration) and the response (i.e. resolution of critical vitamin pairs). Vitamin pairs that could possibly co-migrate were classified as critical vitamin pairs. Models for the relationships between the resolution and the pH and SDS concentration were only created for these critical vitamin pairs. The 12 critical vitamin pairs were: PM–B3, PM–PL, PM–PN, PL–PN, PL–B7, PN–B7, B2–B7, B2–C, B2–B5, B2–B9, B5–C and B9–B12. Each vitamin has a different degree of interaction with the SDS micelles, and due to the different pKa values, the pH will not affect the mobility of the vitamins equally. As the factors influence the resolution of each peak pair differently, separate models were created for each vitamin pair. The resolutions at the tested corner, axial and centre points were used to create the models in MODDE. The software created the models based on the effects of the separate factors (the effect of a single factor independent of other factors), the interaction between the factors (if the effect of one factor depends on the level of the other factor) and the quadratic factors (representing the curvature or non-linearity of the relationship between a single factor and the response) on the resolution. The magnitude of a model term indicates the size of the effect that term had on the resolution. The centre point provided information on the experimental error and thus on the confidence intervals of the terms. This data was used to adjust the models when required. When the confidence interval of a model term intersected zero, the effect was considered non-significant and deleted from the model. The hierarchy among the model terms dictates that a separate factor term remains in the model, even if it is non-significant, when it is significant in an interaction or quadratic term. Figure S3 shows the model terms present in each model, their magnitude and their confidence intervals. In order to create a good model, it is advantageous if the response is normally distributed. This was checked for each model, and if the response was not normally distributed, the response was transformed logarithmically. If the response data was skewed to the right (a few resolutions were much larger than the others), a positive logarithmic transformation was used, and if the response data was skewed to the left (a few resolutions were much smaller than the others), a negative logarithmic transformation was used (see Figure S3). The models predicted the values for resolutions at all possible BGE combinations in the design space.

In order to get an overview of the resolutions of the different critical vitamin pairs over the entire design space, a Sweet Spot Plot was created. A Sweet Spot Plot

indicates the number of specifications met at all areas in the design space. The software can create this plot after given defined criteria for the specifications. In this case, the specifications were the number of baseline-resolved critical vitamin pairs. Baseline separation is generally achieved with a resolution (R) of ≥1.5. With as many requirements as 12 critical peak pairs, it is likely that not all specifications are met simultaneously and compromises should be made. These compromises should be made on the vitamins that are analysed with the cationic vitamin method previously developed. The vitamin pair PN and PL is analysed with the previously developed method [17]. As a resolution of 1.5 was not achieved for any of the combinations in the design space, the specification for the resolution of these vitamins was reduced to R ≥ 1.0. The Sweet Spot Plot is depicted in Figure 2. All specifications were met in the sweet spot depicted by the light green area of the plot, with the cross indicating the selected BGE conditions: 200 mM borate with 77 mM SDS at a pH of 8.6. At these conditions, all vitamin peaks were separated (Figure 3). The sweet spot was located in the centre of the design space, indicating the design covered the appropriate range and the design did not have to be extended. The Sweet Spot Plot in Figure 2 illustrates that DoE could be used to determine a range of conditions where specifications were met. By selecting conditions in the centre of the sweet spot, the method is most robust against small changes in BGE conditions. The SDS concentration is easily kept within the sweet spot range. The pH has a narrower range of ±0.05, which can be maintained by preparing the BGE by weighing the appropriate amounts of boric acid and borax. The buffering capacity of borate at pH 8.6 and the high buffer concentration also minimise pH variability. When a narrower pH range is required for separation, it is advisable to exchange the BGE frequently. In the final monitoring platform, the BGE will be exchanged every run.

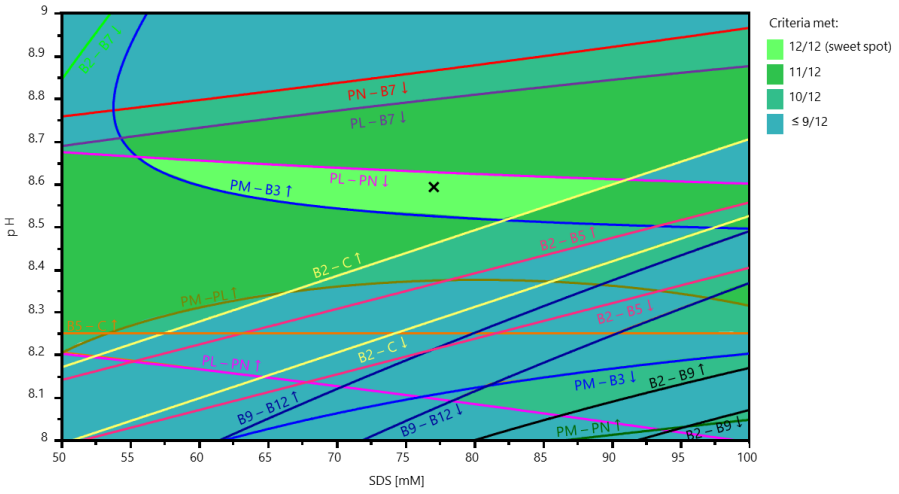

Figure 2. Sweet Spot Plot of resolutions of critical vitamin pairs, criterium is R ≥ 1.0 for the resolution between pyridoxine (PN) and pyridoxal (PL) and R ≥ 1.5 for all other critical vitamin pairs. The arrows indicate at which side of the border the criterium was met. The cross indicates the selected conditions. B1, thiamine; B2, riboflavin; B3, nicotinamide; B5, D-calcium pantothenate; B7, biotin; B9, folic acid; B12, cyanocobalamin. PM, pyridoxamine.

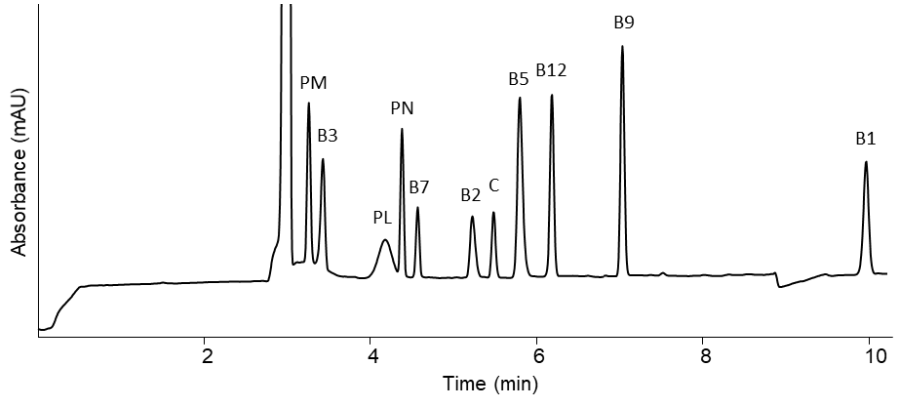

Figure 3. Standard vitamin mixture analysed at background electrolyte (BGE) optimum: BGE: 200 mM borate, 77 mM sodium dodecyl sulphate (SDS) and pH 8.6. Separation length 24.5 cm, applied voltage 11 kV, injection at 30 mbar for 5 s, UV signal at 197 nm. B1, thiamine; B2, riboflavin; B3, nicotinamide; B5, D-calcium pantothenate; B7, biotin; B9, folic acid; B12, cyanocobalamin. PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine.

Many vitamins are unstable and can degrade under various conditions, such as light exposure, temperature or acidic or alkaline conditions [11]. Aspects such as sample handling and time between sampling and analysis could therefore influence the

results. This emphasises the need for PAT and rapid, automated analysis. The unstable character of the vitamins complicates method validation, as the time in the autosampler will differ for every sample. Additionally, method validation has to be performed on real samples in the final automated, integrated monitoring platform. In the monitoring platform, each sample will be sampled, prepared and transferred in the same way, reducing variations due to degradation. As this platform is being developed in parallel, full method validation cannot yet be performed. To give an indication on the method performance, some experiments were performed on linearity and repeatability. Measures were taken to limit variations due to degradation, such as sample preparation from stock solutions just before analysis. Stock solutions could however not be freshly prepared before each analysis, as this would induce a larger variation due to the use of different stock solutions, as well as increased hands-on time and used materials.

A standard vitamin mixture was analysed in six consecutive runs. The migration time and peak area repeatabilities of the anionic and neutral vitamins were within 0.13% and 7.3% RSD, respectively. This was within the 10% required for process monitoring. Linearity was determined using a five-point calibration curve, evenly distributed over the concentration range stated in Table S3. Levels 1 and 5 were prepared in duplicate and all levels were analysed in triplicate. The calibration curves were linear with R2 greater than 0.93, except for vitamin C. As strong antioxidant, vitamin C is very instable, which could cause the lower R2. Accuracy was inferred from specificity, precision and linearity (ICH draft guideline Q2R2). The LOQs were estimated from 10× the signal of the noise using a six-point calibration curve in the concentration range close to the LOQs (Table S3). These experiments show the potential of the method for integration into the monitoring platform.

6.3.2 CELL CULTURE MEDIUM

To show the applicability of this method to cell culture medium samples, various concentrations of vitamin were spiked into vitamin-free cell culture medium, see Figure 4. The vitamins PN, PL, B2, B5, B12, B9 and B1 were separated from medium components. Vitamin B7 co-migrated with a medium component, but the increase in peak area correlated with the increase in concentration. In the 197 nm signal, vitamin B2 migrated between two matrix components and resolution was poor, this vitamin was separated from matrix components in the 270 nm signal. Quantification of vitamin B7 might be possible using different detection wavelengths or background correction, this should be further explored. Vitamin C was not observed in the electropherograms in the 10–50 µM spikes. In cell culture medium, vitamin C is generally present in a range of 0.1–1 mM. When the lower concentration of this

range was spiked in vitamin-free medium (95 µM, see inset in Figure 4B), vitamin C could be detected.

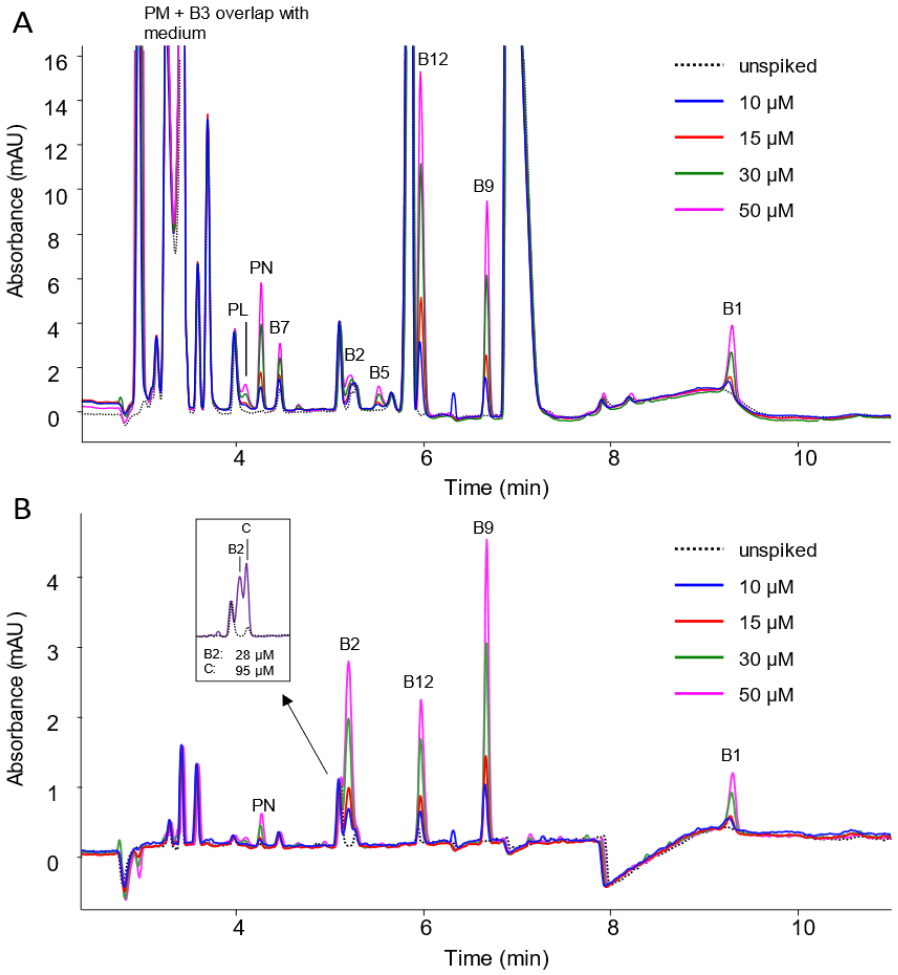

Figure 4. Electropherograms of vitamin-free cell culture medium unspiked or spiked with 10, 15, 30 or 50 µM vitamins: (A) UV detection at 197 nm; (B) UV detection at 270 nm (inset of electropherogram of 28 µM B2 and 95 µM C spiked in vitamin-free cell culture medium. Analysed with background electrolyte (BGE): 200 mM borate, 77 mM sodium dodecyl sulphate (SDS) and pH 8.6. Separation length 24.5 cm, applied voltage 11 kV, injection at 30 mbar for 5 s. Separate electropherograms are shown in Figure S4.

A standard vitamin mixture was also spiked into two other cell culture media: ActiPro and modified FMX-8 (FMX-8 MOD). ActiPro medium is commercially available with a proprietary composition. ActiPro is described as a chemically defined and animal-

derived component-free medium developed to provide high yields of recombinant proteins in bioprocesses using Chinese hamster ovary cell lines [25]. The FMX-8 culture medium was developed for the production of recombinant proteins under chemically defined culture conditions and its formulation was published [20]. To prepare FMX-8 MOD, several components commonly present in cell culture medium were added to the well-defined FMX-8 culture medium. The exact composition of FMX-8 MOD was described in Ref. [21]. Figure 5 shows the electropherograms of the cell culture media (blue) compared to the cell culture media with standard vitamins added to the media (red).

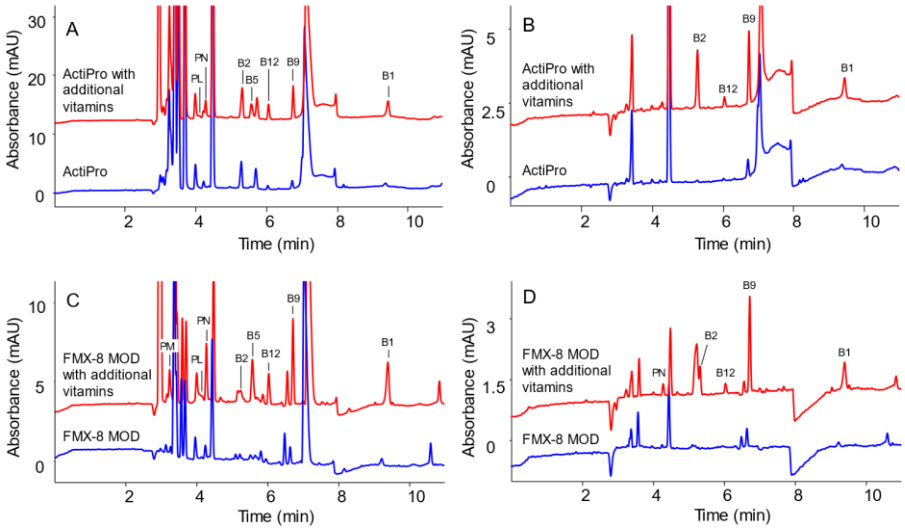

Figure 5. Electropherograms of cell culture medium samples or cell culture medium samples with a standard vitamin mixture added to the medium. (A + B) ActiPro medium, (C + D) FMX-8 MOD medium detected at (A + C) 197 nm and (B + D) 270 nm. Analysed with background electrolyte (BGE): 200 mM borate, 77 mM sodium dodecyl sulphate (SDS) and pH 8.6. Separation length 24.5 cm, applied voltage 11 kV, injection at 30 mbar for 5 s.

The FMX-8 MOD medium was shipped and stored for longer time, affecting vitamin degradation, and the ActiPro medium has a proprietary composition. Therefore, the vitamin concentrations in the media were uncertain. An increase in vitamin peak areas could be observed after the addition of the vitamin standards. This method can be used for the determination of the aniconic and neutral vitamins. The cationic vitamins were analysed with better resolution and/or precision with the vitamin method previously developed [17]. The combination of both methods allows for the analysis of a broad range of vitamins.

6.4 FUTURE PERSPECTIVE AND CONCLUDING REMARKS

As the first part towards integrated vitamin monitoring, CE methods were developed for implementation into an automated platform. Due to the diversity of vitamins, two methods were developed based on the physical–chemical properties of the vitamins. Previously, a method for PN, PL, PM, B1 and B3 was developed, where the vitamins were baseline separated in cell culture medium samples with good precision [17]. In this work, an MEKC method was developed for the monitoring of the remaining anionic and neutral vitamins. Using multivariate DoE for BGE optimisation, 10 B-vitamins and vitamin C were baseline-resolved in clean samples using a very limited number of experiments. Although full method validation should be performed in the final automated, integrated monitoring platform with real samples, preliminary method performance data showed good linearity and precision. For the anionic and neutral vitamins, the migration time repeatabilities were ≤0.1% RSD, and the peak area repeatabilities were ≤7.3% RSD, well within the 10% limit. Vitamin C is a strong oxidizing agent and therefore highly unstable. This resulted in poorer validation results, with R2 for linearity of 0.81. The integration of the method in the final monitoring platform, with immediate analysis after sampling in a closed system, will avoid large variations due to degradation. Information about the method robustness could also be derived from the Sweet Spot Plot (Figure 2). The pH needs to be kept within ±0.05. Therefore, the final buffer should be prepared by weighing the required amounts of boric acid and borax, in order to keep the pH variability as small as possible. In addition, the running buffer should be exchanged frequently. In the final monitoring platform, the BGE will be exchanged before every run. Most anionic and neutral vitamins were separated from medium components. Combining the two developed methods allows for the monitoring of the nine water-soluble vitamins. Although vitamin B7 was not resolved, quantification might be possible using different detection wavelengths or background correction. This should be further investigated. Future work includes additional experiments to improve the selectivity for vitamin B7 under alternative detection conditions. Selectivity might also be improved by the addition of an organic modifier, which alters the interaction of vitamins with the SDS micelles [19].

For integrated monitoring, the developed methods should be implemented into the automated, integrated monitoring platform. This includes the transfer of the methods to microchip CE. Previous work showed that the transfer from conventional CE using fused silica capillaries, to microchip CE using silica chips is straightforward [16]. Attention should be paid to detection. The low concentrations require sensitive detection, and UV detection would probably be insufficiently sensitive, especially

with the small sample volumes and short pathlengths in microchip CE. Alternatives are electrochemical detection, fluorescence detection or mass spectrometry. Mass spectrometry is associated with high costs and large instrument setups and therefore out of scope for the monitoring platform. Fluorescence detection requires derivatisation, which will be challenging for the diverse vitamins. Therefore, electrochemical detection or indirect fluorescence detection has the best potential. Automated monitoring also includes automated reprocessing of the data, which will require appropriate calibration methods to be developed. The monitoring platform should contain automated bioreactor sampling, sample preparation and sample transfer to the microchip. The limited sample preparation required simplifies this.

Although the methods were developed for the integration in the automated monitoring platform, they could be used as stand-alone technique as well. Considering the lack of rapid (near) real-time analytical tools for vitamin monitoring in upstream cell culture processes, the developed capillary methods could be valuable PAT solutions.

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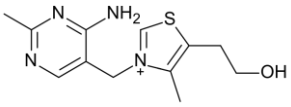
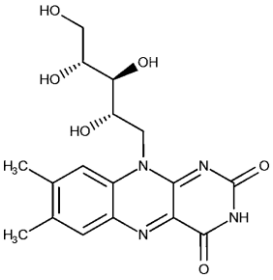
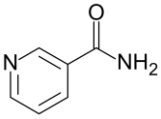
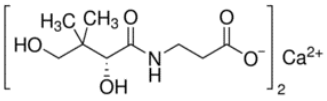
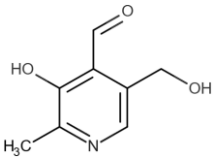
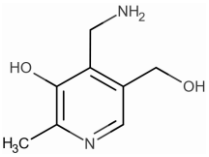
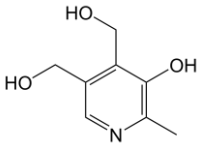
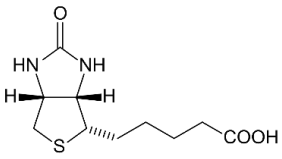
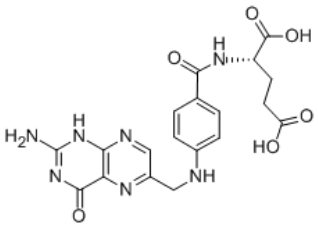
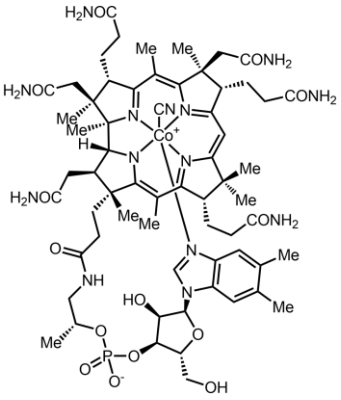
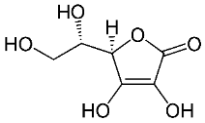
SUPPLEMENTARY INFORMATION

Table S1. Vitamins with their molar mass, strongest acidic pKa (pKa), strongest basic pKa (pKb), and their molecular structure.

Vitamin	Mw (g/mol)	pKa [a]	pKb [a]	Structure
B1; Thiamine	337.3	9.2	4.8	
B2; Riboflavin	376.4	6	-0.2	
B3; Nicotinamide	122.13	13.4	3.6	
B5; D-calcium pantothenate	238.27	4.4	-2.8	
PL; Pyridoxal	167.16	8	4.1	
PM; Pyridoxamine	168.19	7.8	9.8	
PN; Pyridoxine	169.18	9.4	5.6	

B7; Biotin	244.31	4.4	-1.9	
B9; Folic acid	441.14	3.5, 4.3 [b]	2.1	
B12; Cyanocobalamin	1355.39	1.8	8.7	
C; Ascorbic acid	176.12	4.2	-3	

[a] https://go.drugbank.com

[b] https://pubchem.ncbi.nlm.nih.gov

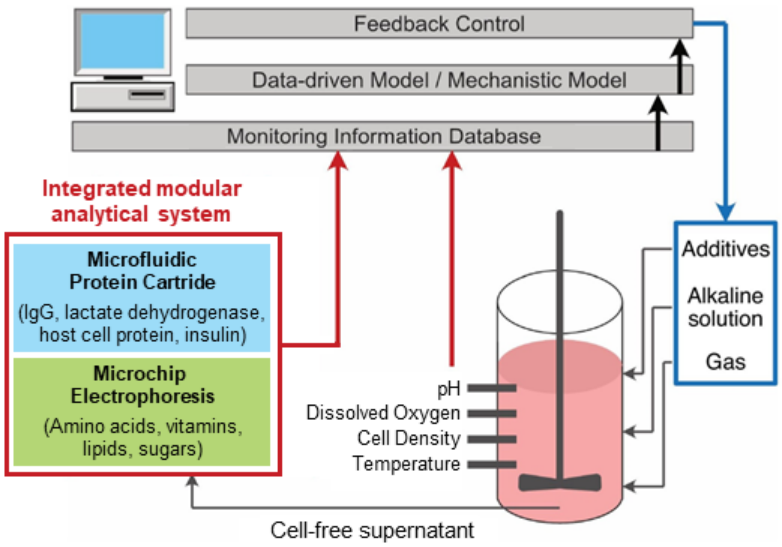


Figure S1. Integrated modular analytical system, the modules contain one or several multiplexed miniaturised quantification techniques. Adapted from ref. [1] under the Creative Commons CC BY licence (Elsevier).

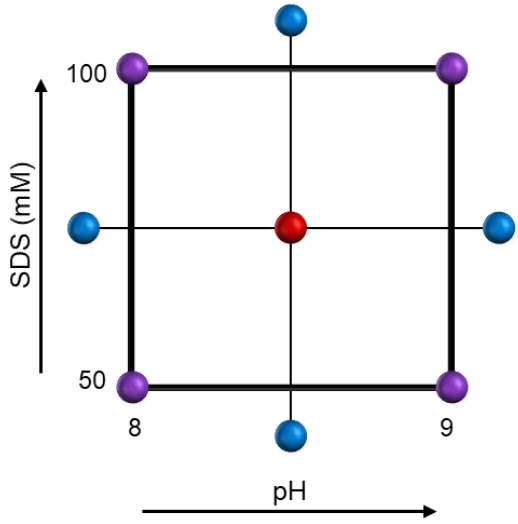


Figure S2. Central Composite Orthogonal design with all corner points (purple), axial points (blue), and the centre point (red). The black square represents the design space.

[1] Pinto, I. *et al.* Knowing more from less: miniaturization of ligand-binding assays and electrophoresis as new paradigms for at-line monitoring and control of mammalian cell bioprocesses. Current Opinion in Biotechnology, 2021, 71, pages 55–64. https://doi.org/10.1016/j.copbio.2021.06.018

Table S2. Central Composite Orthogonal design for BGE optimisation in the range 50 – 100 mM SDS and pH 8 – 9.

Experiment	SDS (mM)	pH	Point type
1	50	8.0	Corner
2	100	8.0	Corner
3	50	9.0	Corner
4	100	9.0	Corner
5	46	8.5	Axial
6	104	8.5	Axial
7	75	7.9	Axial
8	75	9.1	Axial
9	75	8.5	Centre
10	75	8.5	Centre
11	75	8.5	Centre

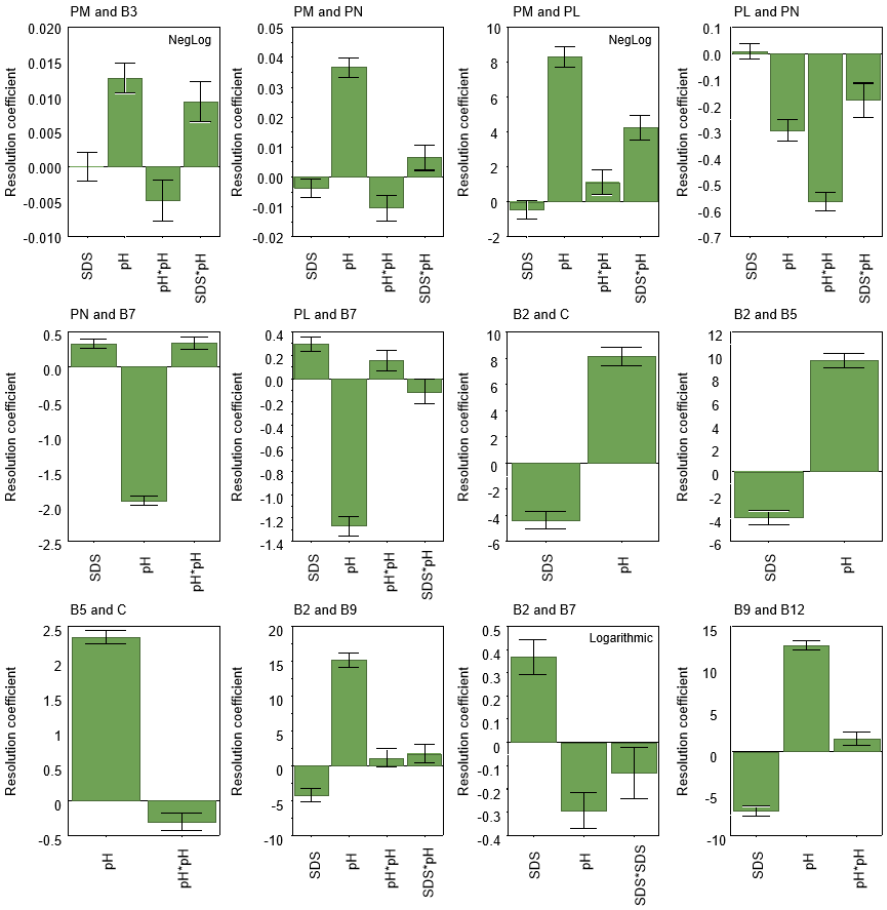


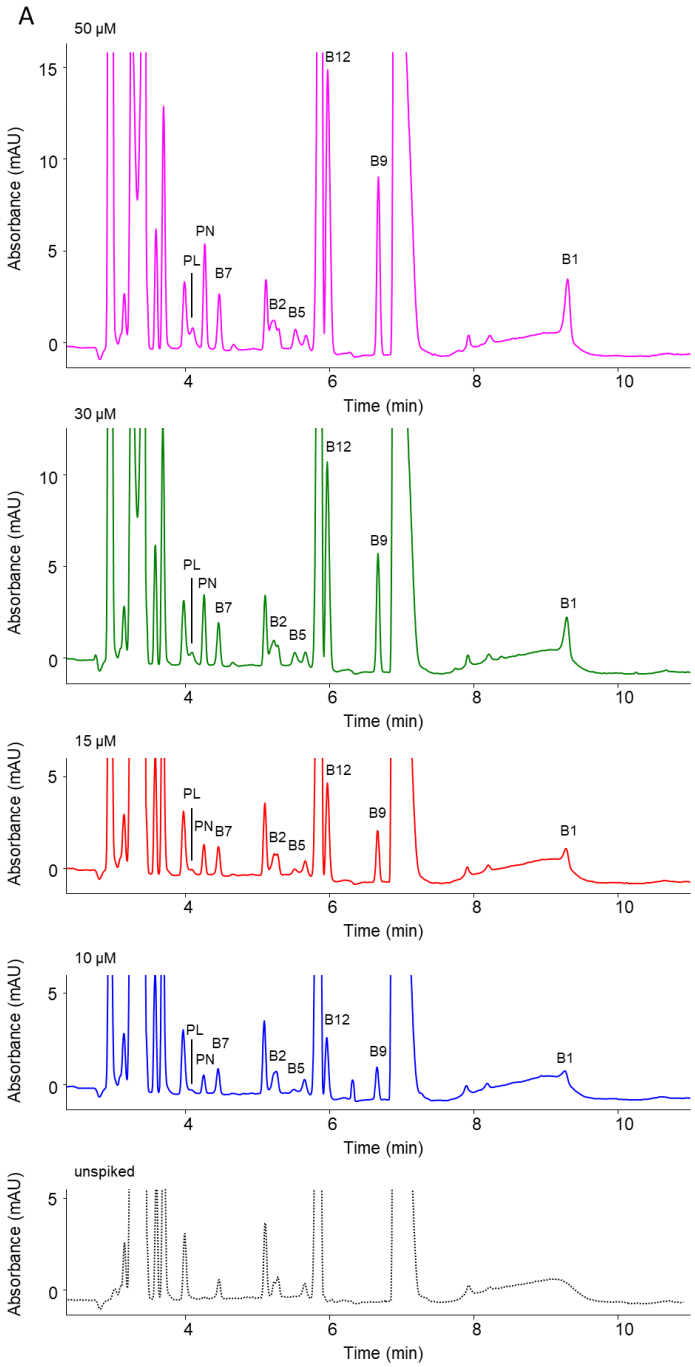
Figure S3. Model terms and transformation for the vitamin pairs. Terms: SDS and pH as separate factors, SDS*pH as interaction effect between SDS and pH, and pH*pH and SDS*SDS as quadratic terms. If the response was transformed, it is indicated in the figure; Logarithmic [logarithmic transformation: 10Log(Y)] or NegLog [negative logarithmic transformation: -10Log(100-Y)].

Table S3. Preliminary results on precision, linearity, and LOQ. Migration time and peak area repeatability (n=6), linearity data was derived from a five-point calibration curve with levels 1 and 5 prepared in duplicate and all levels analysed in triplicate. Data for vitamins B2 and C are based on detection at 270 nm.

	Migration time RSD%	Peak area RSD%	Conc. range (µM)	Slope	Intercept	R^2	LOQ (µM)
B2	0.13%	7.6%	24 – 107	0.28	-1.61	0.926	5.4
B5	0.09%	2.6%	63 – 277	0.10	0.37	0.996	16.1
B7	0.09%	2.1%	25 – 109	0.07	-0.05	0.987	14.9
B9	0.10%	3.7%	24 – 108	0.66	0.21	0.995	1.4
B12	0.07%	4.3%	7 – 32	0.92	-0.62	0.994	1.2
C	0.06%	5.6%	47 – 209	0.18	-2.09	0.805	16.5
B1 [a]	0.13%	35.1%	18 – 81	0.73	1.82	0.853	2.5
B3 [a]	0.05%	2.9%	24 – 107	0.11	1.39	0.972	
PL [a]	0.24%	4.4%	24 – 108	0.17	0.21	0.981	19.9
PM [a]	0.07%	7.7%	25 – 108	0.14	0.18	0.985	17.4
PN [a]	0.07%	4.4%	24 – 107	0.31	-0.18	0.978	3.6

[a] These vitamins are to be determined with the cationic vitamin method [2].

[2] Van der Burg, D. *et al.* Analysis of cationic vitamins in cell culture medium samples by capillary zone electrophoresis. Journal of Analytical Methods in Chemistry, 2022. https://doi.org/10.1155/2022/2819855


A
50 µM
30 µM
15 µM
10 µM
unspiked
Absorbance (mAU)
Time (min)
PL
PN
B7
B2
B5
B12
B9
B1

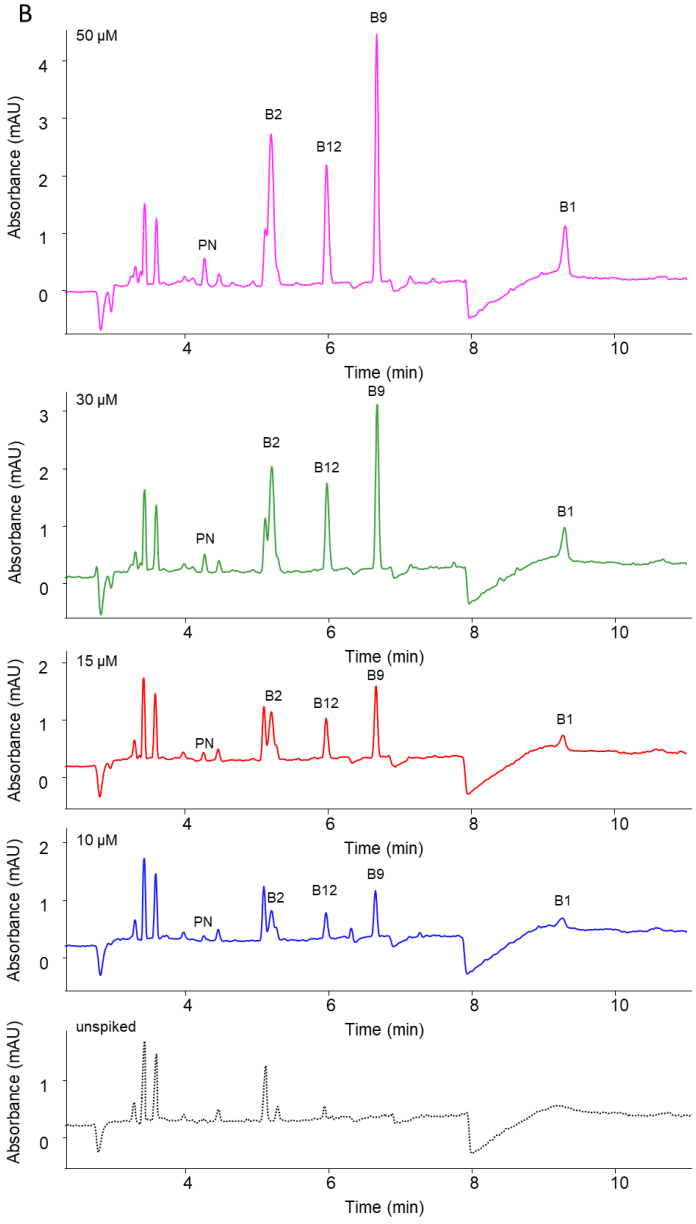


Figure S4. Separate electropherograms from Figure 4. Electropherograms of vitamin-free cell culture medium unspiked or spiked with 10, 15, 30, or 50 µM vitamins. A) UV detection at 197 nm, B) UV detection at 270 nm. Analysed with BGE: 200 mM borate, 77 mM SDS, pH 8.6. Separation length 24.5 cm, applied voltage 11 kV, injection at 30 mbar for 5 s.

Chapter 7

Concluding remarks and Future perspectives

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The use of CE in the biopharmaceutical industry has been steadily increasing over the past years. The flexibility to change the separation chemistry by altering the BGE enables the analysis of a wide variety of compounds, including proteins, peptides, small molecules, metabolites, and nutrients. Due to its ability to handle complex matrices, limited sample pretreatment requirements, small sample volumes, rapid analysis, and simple instrumental setup, CE is a valuable tool for biopharmaceutical process monitoring and implantation into the PAT toolbox. It has proven powerful for the determination of protein concentration (CZE, CE-SDS), molecular size (CE-SDS), glycosylation (CGE, CZE), and nutrients. Especially CZE has proven to be very tolerable against samples with complex matrices such as the cell culture medium samples in USP.

The work in this thesis further underlines the applicability of CE for biopharmaceutical process monitoring. Four methods were successfully developed for the monitoring of nutrients (mono- and disaccharides and vitamins) and for the determination of the mAb concentration during the USP process using AQbD principles. The developed methods show good potential as stand-alone PAT tools as well as for integration in the automated monitoring platform. All methods allow for fast analysis and require limited sample preparation. The vitamins and mAb methods require no sample pretreatment or only a simple dilution. The saccharide method requires fluorescent derivatisation, which was developed to allow for automation in a closed system. By applying AQbD during method development, the BGE compositions and the fluorescent derivatisation reaction could be optimised with a limited number of experiments. Optimisation with help of DoE optimised all significant factors simultaneously, reducing the number of experiments required. In addition, it provided information on the relationship between the tested factors and the response over the design space, including information on the influence of or the interaction between factors. Optimisation with DoE generally helps to find the real optimum [1].

A challenge for analysing USP samples is the prevention of adsorption of matrix components to the capillary wall, as adsorption could cause an irreproducible EOF and therefore a non-repeatable method. Section 1.5 describes various ways of preventing adsorption of analytes or matrix components to the capillary wall, such as the use of capillary coatings, high buffer concentration, and the use of BGE additives. A combination of these techniques was used for the methods developed in this thesis.

Prevention of adsorption is extra important for the mAb method, as the analyte itself could adsorb to the wall, resulting in non-symmetrical peak shapes and potentially in inaccurate results. Various capillary coatings were investigated to ensure robust, repeatable use without peak tailing. The final method used a dynamic T EthA coating in combination with poloxamer as BGE additive to prevent adsorption. The BGE had a low pH, such that most mAbs will have a strong positive charge and the method can be applied as platform method. As a low pH minimises the negative charge on the silica capillary wall, and aids in prevention of adsorption due to electrostatic repulsion. For the saccharide method, adsorption of matrix components was prevented by using high buffer concentrations, and by the addition of surfactants (i.e., poloxamer, SDS). The method for cationic vitamin analysis employs a combination of a low pH BGE, a high buffer concentration, and T EthA as BGE additive to prevent adsorption of matrix components. For the analysis of anionic and neutral vitamins, a low pH BGE could not be used, instead a high concentration of SDS minimised adsorption.

PAT requires (near) real-time, and ideally, automated analysis. Miniaturisation to MCE is one step towards automated analysis. MCE is an upcoming technique and being more frequently used for biopharmaceutical process analysis. It offers great potential for integration in automated systems, with integrated sample preparation, analysis, detection, and data processing. Common chip designs are outlined in 1.3.2 *Microchip capillary electrophoresis*. The design of chips is however very flexible, and could incorporate the coupling of various channels for sample concentration, purification, mixing, dilution, reaction, etc. Various studies show the possibilities of on-chip sample preparation, some examples include the addition of channels for on-chip mixing and reacting of sample with derivatisation reagents [2,3], on-chip reaction by using a heated reactor coil [4], incorporating a solid-phase extraction reversed-phased monolith channel for on-chip preconcentration, fluorescent derivatisation, and purification [5], and integration of nano-porous membranes for protein purification and concentration [6]. Alternatively, for simpler chip designs, sample preparation could be integrated using robotic liquid handling systems. Various materials can be used for microchip fabrication, such as quartz, glass, and polymers. Advantages of glass substrates are their good optical properties, well-understood surface chemistry, and well-developed microfabrication methods. Polymer substrates have lower manufacturing costs and could therefore allow disposable use [7]. The substrate material in the channels plays an important role in both separation efficiency and fluid movement control, for example for sample injection, as the surface chemistry in the channels affects the EOF. Fluid movement in microchips, for example for injection, is generally controlled using EOF, and the

EOF strongly influences electrophoretic separations. The EOF could be controlled by employing channel coatings [8].

Due to the small dimensions and sample volumes on chip, UV detection is often insufficiently sensitive. Coupling of MCE to LIF offers great sensitivity and additional selectivity. Derivatisation is however often required. Alternatives are electrochemical detection or MS detection. Electrochemical detection has the advantage of low cost and small size. Coupling MCE to MS offers great opportunities for structural characterisation and good sensitivity. An issue of MS is the large size of the instrumentation, making it less convenient to place close to the bioreactor for automated monitoring. High pressure MS (HPMS) does not require large vacuum pumps, significantly reducing the instrument size. MCE-HPMS is used in the commercialised cell culture medium analyser the "REBEL" from 908devices, analysing over 30 nutrients and metabolites in near-real-time [9].

MCE was one of the selected analytical techniques for implementation in the iConsensus monitoring platform. As the microchip instrumentation was being developed in parallel to the CE methods, method development was started on conventional CE. The developed methods must be transferred to MCE for implantation into the automated monitoring platform. To simplify this transfer, fused silica chips were selected as their surface chemistry is similar to fused silica capillaries. Switching to polymeric chips might reduce costs, however, additional method development would be necessary. During capillary method development, the sample can be injected at the detector end of the capillary, also called the short end. This results in a separation length of 8.5 cm, which is similar to the 7.9 cm separation length on the chips. Chapter 4 showed the good transferability of the saccharide method from capillary to silica microchips. Saccharides were separated and detected on MCE in less than six minutes.

The mAb method is ready for transfer to MCE. This method injects sample at the short end of the capillary, resulting in a separation length similar to that on chip. The capillary method uses UV detection. On the small chip dimensions, this will likely be insufficiently sensitive. Hence, the transfer of the mAb method to MCE should include transfer to a more sensitive detection technique. The three aromatic amino acids, tryptophan, tyrosine, and phenylalanine, are native fluorescent [10] and present in the Fc regions of immunoglobulins [11]. Native fluorescence detection is therefore possible for the vast majority of mAbs. Studies comparing HPLC-UV and HPLC-LIF for mAbs show a significant increase in sensitivity [12,13]. The CE-UV method is therefore expected to be easily transferable to MCE-LIF.

In the vitamin methods, a separation length of 24.5 cm was used. The cationic vitamin method is a CZE method, and fundamentally, the resolution in a CZE separation is

independent of the capillary length. The separation length does play a role in the MEKC method for the anionic and neutral vitamins due to the additional chromatographic separation. Resolution on the current microchips available within the iConsensus project, with separation lengths of 7.9 cm, might be insufficient. Alternative MCE chip geometries, such as serpentine and cyclic geometries, have been employed to achieve longer separation lengths on microchips required for applications utilising chromatographic principles like MEKC and chiral separations [14–16]. The low concentrations of the vitamins require sensitive detection. UV detection would probably be insufficiently sensitive, especially with the small sample volumes and short pathlengths in microchip CE. Alternative detection techniques must be explored. Fluorescence detection requires derivatisation, which will be challenging for the chemically diverse vitamins. Electrochemical detection or indirect fluorescence detection are good alternatives to be explored. Although out of scope for the iConsensus monitoring platform due to high costs and large instrument setup, MS detection offers sensitive detection. Especially MCE coupled to miniaturised HPMS could have great potential as stand-alone, easy-to-use monitoring device. Considering the lack of rapid (near) real-time analytical tools for vitamin monitoring in upstream cell culture processes, and the instability of vitamins, which negatively affects the reliability for offline analysis with long times between sampling and analysis, these vitamin methods could be valuable stand-alone PAT tools as well.

This thesis describes the development of (M)CE applications for upstream bioprocess monitoring. The saccharide (Chapter 4) and amino acids [17] methods illustrate a good proof-of-concept for implementation of the electrophoretic methods into the automated, integrated monitoring platform developed within the iConsensus project. Overall, CE has taken an increasingly important role in biopharmaceutical process analysis and PAT solutions and especially MCE has great potential for online, automated process monitoring.

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