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Analytical Quality by Design Method Development for Vaccine Characterization

LARS GEURINK



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Abstract

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Vaccines that are safe, efficacious, and can be rapidly developed are needed to prevent and to react to emerging global infectious disease threats such as influenza, Polio, and Coronavirus diseases. Fast and reliable analytical methods are required without delay to support vaccine process and product development, characterization, and quality control testing. The traditional analytical methods for vaccines are laborious and often lack analytical power, causing slow, expensive, or sometimes failing vaccine development. Capillary electrophoresis (CE) is a technique that has great potential for biopharmaceutical analysis, although there has been limited application in vaccine development.

Several novel CE methods were explored, developed, and applied for viral vaccine analysis, making use of the analytical quality by design (AQbD) process and tools. AQbD is a framework of science- and risk-based decision making to achieve in-depth method understanding and to set up fit-for-purpose and in-control analytical methods.

Commercial kits for capillary gel electrophoresis (CGE) and imaging capillary isoelectric focusing (icIEF) for antibodies analysis were applied and improved for vaccine analysis. Analytical mechanisms were studied, such as the effect of gel buffer composition on separation, and an AQbD CGE method development strategy was established. The strategy was successfully applied to develop CGE methods for the analysis of seasonal and universal influenza, and sabin inactivated polio vaccine proteins. An icIEF method was also developed, validated, and applied for the universal influenza vaccine protein.

A capillary zone electrophoresis (CZE) method development for intact adenovirus concentration determination started with background electrolyte (BGE) and capillary design and screening. An BGE with tris and tricine and a neutral capillary resulted in optimal and robust separation and limited adsorption. The CZE method was validated for seed release, in-process control, product release, and stability testing. The precise, accurate, fast, and robust CZE method was applied for all process intermediates and used at different locations. Process impurities and product degradation could also be characterized.

Additionally, CZE methods for chloride and bromide analysis in complex matrices, and a CGE method for host cell DNA characterization were developed for characterization as well as to support process development.

Development of CE methods using AQbD reduced lead times and costs. The developed CE methods were easier to use, were more accurate and precise, and were more selective for product and process impurities compared to the previously used analytical methods for vaccines. The use of CE and AQbD helped improve on vaccine safety, efficacy, and quality.

Keywords: viral vaccines, adenovirus, influenza, polio, COVID, corona, capillary electrophoresis, analytical quality by design, analytical method development, CGE, icIEF, CZE, crude cell suspension analysis, in-process sample analysis, quality control testing, biopharmaceutical characterization

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All we have to decide is what to do with the time that is given to us.

Gandalf – J.R.R. Tolkien, *The Fellowship of the Ring*

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Van Tricht, E., **Geurink, L.**, Pajic, B., Nijenhuis, J., Backus, H., Germano, M., Somsen, G.W., Sanger-van de Griend, C.E. (2015) New capillary gel electrophoresis method for fast and accurate identification and quantification of multiple viral proteins in influenza vaccines. *Talanta*, 144(2015):1030–1035
- II. **Geurink, L.**, van Tricht, E., Dudink, J., Pajic, B., Sanger-van de Griend, C.E. (2021) Four-step approach to efficiently develop capillary gel electrophoresis methods for viral vaccine protein analysis, *Electrophoresis*, 42(2021):10–18
- III. Van Tricht, E.¹, **Geurink, L.**¹, J., Backus, H., Germano, M., Somsen, G.W., Sanger-van de Griend, C.E. (2017) One single, fast and robust capillary electrophoresis method for the direct quantification of intact adenovirus particles in upstream and downstream processing samples. *Talanta*, 166(2017):8–14
- IV. Van Tricht, E.¹, **Geurink, L.**¹, Galindo Garre, F., Schenning, M., Backus, H., Germano, M., Somsen, G.W., Sanger-van de Griend, C.E. (2019) Implementation of at-line capillary zone electrophoresis for fast and reliable determination of adenovirus concentrations in vaccine manufacturing. *Electrophoresis*, 40(2019):2277–2284
- V. **Geurink, L.**, van Tricht, E., Dudink, van der Burg, D., Schepink, G., J., Pajic, B., Dudink, J., Sanger-van de Griend, C.E. (2022) Sixteen capillary electrophoresis applications for viral vaccine analysis. *Electrophoresis*, 43(2022):1068–1090

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Abbreviations

Ab	Antibody
Ad[n]	Adenovirus [subtype]
AEX	Anion-exchange
AF4	Asymmetrical flow field flow fractionation
API	Active pharmaceutical ingredient
AQbD	Analytical quality by design
ATP	Analytical target profile
BGE	Background electrolyte
bnAb	Broadly neutralizing antibody
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
CMP	Critical method parameter
COVID	Corona virus disease
CQA	Critical quality attribute
CS	Control strategy
CZE	Capillary zone electrophoresis
DNA	Deoxyribonucleic acid
DoE	Design of experiments
DP	Drug product
DS	Drug substance
ELISA	Enzyme-linked immunosorbent assay
FMEA	Failure-mode effect analysis
HA	Hemagglutinin
HIV	Human immunodeficiency virus
ICH	International conference on harmonization
icIEF	Imaging capillary isoelectric focusing
IPC	In-process control
LC	Liquid chromatography
LCL	Lower control limit
LNP	Lipid nanoparticle
LOD	Limit of detection
LOQ	Limit of quantitation
mAb	Monoclonal antibody
MS	Mass spectrometry
NA	Neuraminidase

nCOV	Novel coronavirus
OD260	Optical density at 260 nm
OFAT	One factor at a time
PCR	Polymerase chain reaction
PNGase F	Peptide N-glycosidase F
PS-20	Polysorbate-20
PVA	Polyvinyl alcohol
QbD	Quality by design
QC	Quality control
qPCR	Quantitative polymerase chain reaction
QTPP	Quality target product profile
mRNA	Messenger ribonucleic acid
RP	Reversed phase
RPN	Risk priority number
RSD	Relative standard deviation
RSV	Respiratory syncytial virus
SDS-PAGE	Sodium dodecyl-polyacrylamide gel electrophoresis
sIPV	Sabin inactivated polio vaccine
SRID	Single radial immunodiffusion
SSC	System suitability control
SST	System suitability test
tITP	Transient isotachopheresis
UCL	Upper control limit
UF/DF	Ultrafiltration and diafiltration
UV	Ultraviolet
VLP	Virus like particle
VP	Virus particle
WB	Western blot
WHO	World health organization

1. Introduction

Vaccination

Infectious diseases were estimated to cause 10.2 million death in 2019 [1]. Recent outbreaks of severe acute respiratory syndrome (SARS) [2002 – 2003], flu H1N1 [2009 – 2010], middle east respiratory syndrome (MERS) [2012], Polio [2014], Ebola [2014 – 2016 & 2018 – 2020], Zika [2015 – 2016], coronavirus diseases (COVID-19) [2019 – to date], Monkeypox [2022 – to date] in addition to persisting cases of for example acquired immunodeficiency syndrome by the human immunodeficiency virus (HIV), respiratory syncytial virus (RSV) disease, pneumonia, diarrhoea and cervical cancer indicate the urgent need of treatments against infectious diseases [2, 3]. The world health organization (WHO) reckons that vaccination is one of the most effective ways to prevent infectious diseases [4]. Vaccination is the administration of a biological preparation to acquire immunity to a particular infectious disease. There are many different types of vaccines with different characteristics, see Table 1. In this these studies a seasonal influenza viro-some subunit vaccine, a universal hemagglutinin (mini-HA) subunit vaccine, a Sabin inactivated poliovirus vaccine (sIPV), and adenovirus vector vaccines (AdVac®) were used.

Table 1. Overview of vaccine types, characteristics, and examples.

Type	Characteristics	Examples
Attenuated	A viable pathogen with reduced virulence	Yellow fever vaccine, measles vaccine, mumps vaccine, rubella vaccine, and bacterial disease typhoid vaccine
Inactivated	A non-viable pathogen without virulence after treatment with chemicals, heat, or radiation	Inactivated polio vaccine (IPV), hepatitis A vaccine, rabies vaccine and most influenza vaccines
Toxoid	An inactivated toxin of which toxicity is suppressed while maintaining the immunogenic properties	Tetanus vaccine and diphtheria vaccine
Subunit	A purified antigenic part of the pathogen	Hepatitis B vaccine, human papillomavirus vaccine, edible algae vaccine, hemagglutinin and neuraminidase subunits of the influenza virus vaccine, plague immunization vaccine.
Conjugate	A combination of a weak antigen with a strong antigen carrier so that the immune system has a stronger response to the weak antigen.	Haemophilus influenzae type B vaccine
Outer membrane vesicle	A lipid vesicle released from the outer membranes of Gram-negative bacteria.	Serotype B meningococcal vaccine
Heterotypic	A pathogen from another organism that causes no or mild disease	Jenner pox via cowpox vaccine, tuberculosis via Mycobacterium bovis vaccine

Type	Characteristics	Examples
Genetic: Viral vector	A safe virus with a gene coding for an antigen	ChAdOx1 vaccine, Sputnik V vaccine, Jcovden (Ad26.COV2-S), Convidecia COVID vaccine, Zabdeno/Mvabea Ebola vaccine, Recombinant vesicular stomatitis virus (rVSV)-ZEBOV vaccine
Genetic: Bacterial	A safe bacterium with a gene coding for an antigen	
Genetic: RNA	A messenger-RNA coding for an antigen that can be packaged within, for example, a lipid nanoparticle	COVID-19 RNA lipid nanoparticles vaccines
Genetic: DNA	A DNA coding for an antigen, inserted into human or animal cells by, for example, electroporation	ZyCoV-D vaccine
T-cell receptor peptide	A peptide with the sequence of a T-cell receptor, to down regulate the presence of this specific T-cell	Valley Fever vaccine, stomatitis vaccine, and atopic dermatitis vaccine

Influenza

Annual epidemics of the flu are caused by the influenza virus. Influenza often results in fever, runny nose, sore throat, etc., but can also be deadly for risk groups such as the elderly. There are four different influenza strains, of which A and B are causing the seasonal epidemics in humans. The Influenza virus has a pleomorphic envelope and a spherical form of about 100 nm in diameter. Eight or seven negative-sense single-stranded RNA segments are encapsidated and encode for 9 or 10 major proteins depending on the strain. Hemagglutinin (HA) and neuraminidase (NA) are the most targeted influenza proteins due to their immunogenic properties [5, 6]. Due to the antigenic drift of HA and NA via mutations in the RNA sequence, three new influenza strains (2 A and 1 B strain) are targeted for vaccination each year [7]. These strains are selected by the WHO [7-10] based on their HA and NA sequence class. The subunit vaccine in this thesis consists of the HA and NA protein produced on a human cell line, Per.C6®, purified, and reformulated into a lipid virosome [11]. Easy, adjustable, and broadly applicable analytical test methods are needed to support the fast annual redevelopment of seasonal influenza vaccines.

A universal flu vaccine that protects against a broad range of influenza strains and subtypes would potentially eliminate the need for an annual redevelopment of the influenza vaccine [12, 13]. For this purpose, a universal mini-HA subunit vaccine is being developed to induce broadly neutralizing antibodies (bnAbs). It consists of a homo-trimeric protein and has multiple deglycosylation sites with a high degree of variability in the glycosylation composition. The quality and integrity of the mini-HA protein is critical for bnAbs induction.

Polio

Poliomyelitis, in short polio, is caused by the poliovirus that infects the central nervous system and causes flaccid paralysis. The virus contains positive-sense RNA and four major proteins (VP1 – VP4) of which three form the capsid. Polio is almost eradicated due to a global vaccination approach. However, the conventional inactivated (wild type strains) polio vaccine (IPV) has the biosafety risk of infecting a person, thereby hindering to completely eradicate polio. The attenuated Sabin polio strain does not entail this risk and is recommended by the WHO [14]. To develop the Sabin inactivated polio vaccine (sIPV), the Sabin polio virions are grown on human cells, purified, and inactivated with formaldehyde.

Adenovirus vectors

Adenoviruses are a type of viruses that can be used as viral vector vaccines, and are used to combat COVID-19 [15] and Ebola [16] currently. In the adenovirus vector vaccines, the adenoviruses are used to deliver the transgene to the host cell in order to trigger an immune response against the transgene protein. Adenoviruses are non-enveloped viruses with icosahedral symmetry (252 capsomers) and diameters of 70 – 90 nm [17, 18]. Double stranded DNA is encapsidated by proteins and about 13 different types of proteins are present in the virion [19] with molecular weights ranging from 5 to 120 kDa. The total molecular weight of an adenovirus is about 150 MDa. A common type of adenovirus is type 5 (Ad5). The use of Ad5 vaccine might imply the risk of broadly existing immunity. Alternative adenovirus types for vaccination are type 26 (Ad26) and 35 (Ad35) [20, 21]. The different adenovirus types differ in protein and DNA composition and, therefore, have different physicochemical properties [19]. The AdVac® [20, 21] platform was established to be able to rapidly introduce new antigens of for example HIV, RSV, Ebola virus, or nCOV-2 into the Ad26 genome and develop a vaccine.

The adenovirus vector vaccines are produced in bioreactors with cultured human cells by adenovirus vector seed inoculation (e.g., HEK293, Per.C6®) [22-32]. Purification of the adenovirus can be performed via cell lysis, clarification of the adenovirus particles from the cell debris and host cell DNA (HC DNA), anion exchange (AEX)-filtration, and ultrafiltration and diafiltration (UF/DF) [22, 23, 26, 33-38]. After purification, the adenovirus vaccines are formulated and packaged before storage, distribution, and clinical use [39-45].

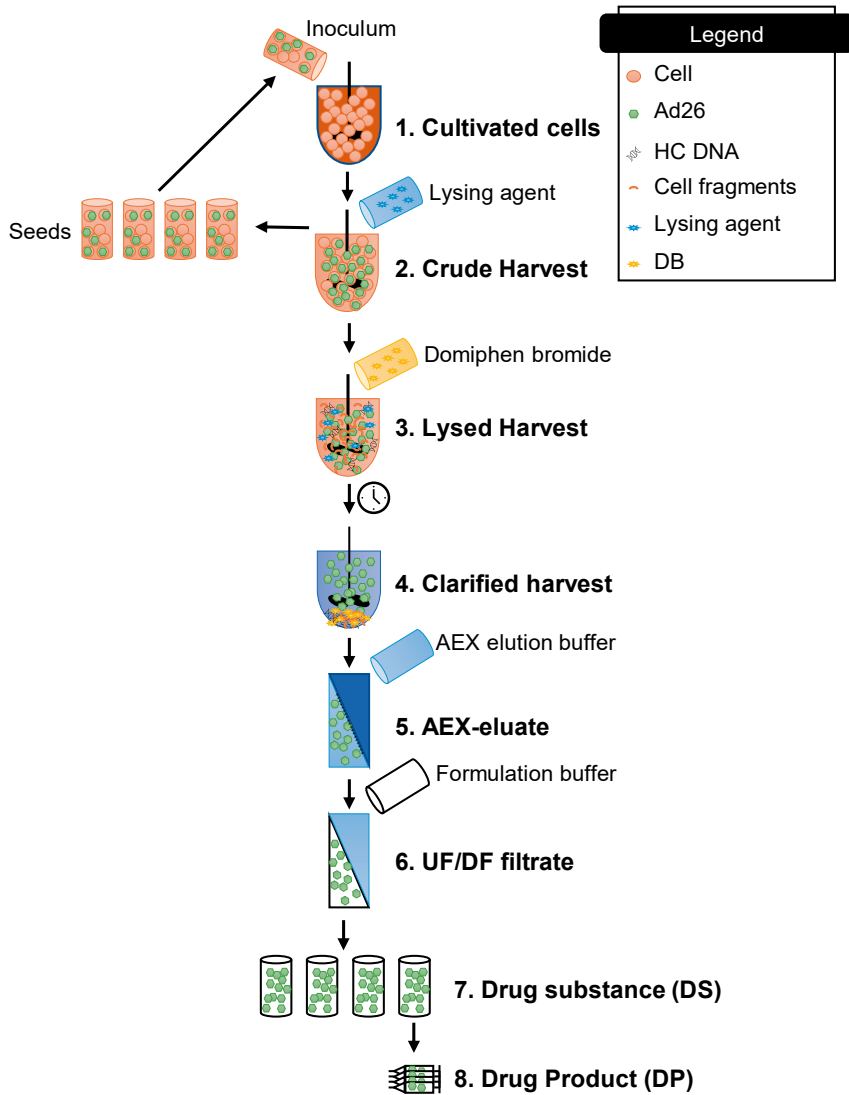


Figure 1. A schematic presentation of the adenovirus vaccine production process.

Quality by design product development

Safe, efficacious, and consistent high-quality vaccines are required for effective vaccination and infectious disease prevention [46, 47]. Such vaccines can only be obtained if the production process is scientifically sound and quality controlled [48]. In general, all these vaccines are produced in a similar way, i.e.: active pharmaceutical ingredient (API) production in a bioreactor (upstream processing), purification of the API into drug substance (DS) (downstream processing), and formulation, fill, and finish into drug product (DP). Nonetheless, the details of each step are very different and mostly depend on the API characteristics and production cell chosen.

The production development may follow a quality by design approach in which a quality target product profile (QTPP), a description of the intended product and its characteristics, is established. Critical quality attributes (CQAs) of the DP are identified theoretically and by characterization testing as early as possible (e.g.: during vaccine design). Viral vaccines have many CQAs in common for safety, efficacy and quality, and examples are given in Figure 2.

After vaccine design, production must be scaled up and the process and the product are developed. During process and product development critical process parameters (CPPs) and critical material attributes (CMAs) with impact on CQAs are identified through characterization of in-process samples. From the knowledge gained regarding the relationship between CQAs and CPPs and CMAs, a control strategy (CS) is designed to produce a safe, efficacious, and quality vaccine product [47, 49-51]. The vaccine production process, based on the CS, is validated and proven acceptable ranges (PARs) for the CPPs are determined.

During vaccine design a broad range of analytical tests is applied to characterize the physicochemical properties of the API. Often little sample volume and time is available. Focus is mostly on the potential efficacy of the new molecular entity. Therefore, broadly applicable analytical methods that use low sample volumes are preferred.

For process and product characterization often many process parameters are studied resulting in a high number of very diverse samples to be tested. High throughput and matrix-compatible analytical test methods are therefore preferred. Toxicological studies in cells, animals and the first human clinical trials provide insights in safety to the characterization results. Additionally, throughout process and product development, new insights are obtained regarding API behaviour and product specific CQAs. So, an increasing number of innovative technologies and developed analytical test methods with increasing quality and resolution are needed to support process and product development and characterization towards phase 3 clinical material production.

Analytical test methods are needed for the production and release QC testing and are part of the CS. For QC testing methods with a high reliability are required and is achieved by selecting simple and robust technologies and intensive method development.

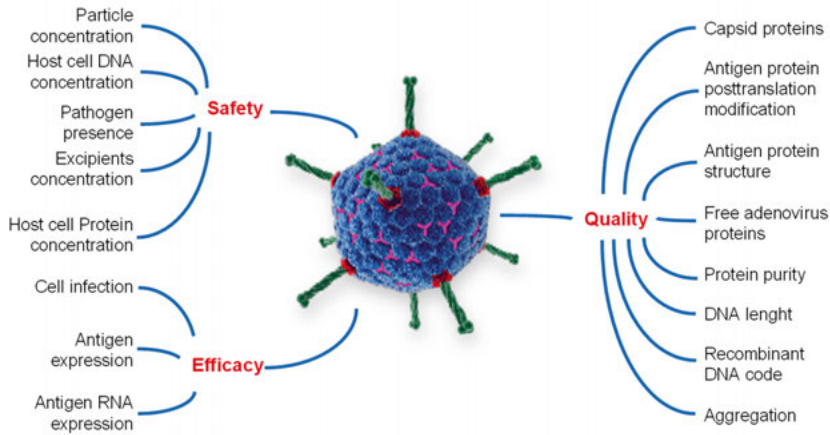


Figure 2. Schematic representation of potential viral vaccine CQAs.

Analytical method development

Each of the areas process and product development, characterization, and QC testing have their own requisites, e.g.: is the produced API content within specification, how does the bioreactor temperature influence the total amount of an impurity, etc. Analytical methods are needed to answer these questions.

An analytical test method is a process in which a knowledge gap is filled or answered, by sampling, performing an analytical test, and converting the result into data that are evaluated and that contribute to existing information and knowledge.

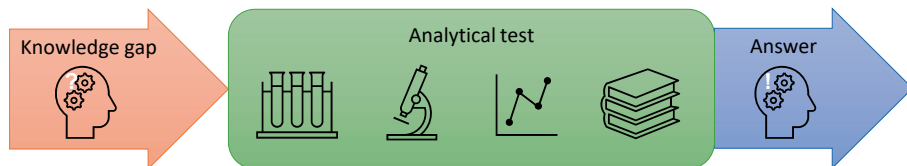


Figure 3. Schematic presentation of the definition of an analytical test.

Reliability of the test result is of utmost importance. Determination of the method performance characteristics (ICH Q2R1) [52] yields a partial under-

standing of the method reliability. The identification of sources of variation and errors results in a much better understanding of the method reliability and is achieved during method development. Conscientiously and purposefully reducing error propagation through an understanding of the technique mechanisms and its sources of variation results in highly reliable methods.

Unreliable methods and poor method development can cause delays in clinical trial material manufacturing, process development, or production. These delays can be very costly, as many people are involved in root-cause analysis of the analytical failure and re-analysis, and project or release of product can be delayed. An in-company survey showed that a method development process from “identified need” to “start testing” for QC took about 55 weeks on average. A minor adjustment to a method took more than a month, even for a relatively simple adjustment as a specificity check for a new adenovirus vector for an existing adenovirus platform method. For some assays 15 – 30% of the assay runs were invalid (failed assay acceptance criteria such as positive and negative controls), only about 25% were meeting method expectations for the intended purpose, and more than 50% of the methods to be validated required major redevelopment and amended validation studies.

The causes for the long development time and high amount of assay defects were investigated. The different and personal method development processes were analysed and compared. Over 70 different method development failure-modes were identified with each causing either long development lead times, rework, or both. As a solution, a new practical standardized and aligned analytical method development process and supportive tools were designed based on the AQbD principles [53-55].

Analytical quality by design (AQbD)

AQbD is a framework (paradigm) of science- and risk-based decision making to achieve reliable and adequate in-depth method understanding and to set up fit-for-purpose and in-control analytical test methods.

The analytical methods developed in this thesis were developed according to the AQbD approach and served as test cases for the novel AQbD process. The AQbD process started with determining the purpose of the analytical test result (e.g.: the CQA and error tolerance for the intended study) and was translated into a list of analytical method performance characteristics (e.g.: ICH Q2(R1)), together called the analytical target profile (ATP). Focusing on the purposes and list of requirements, analytical technologies were selected based on the potential of each technology for each method requirement. When a technology was selected, the critical method parameters (CMPs) were identified. Criticality and risk assessments were performed to identify knowledge gaps. Knowledge gaps were resolved by experiments. Design of

experiments (DoE), including multifactorial designs, were performed to identify CMPs, to obtain a deeper understanding of the method mechanisms (e.g.: separation, detection, interactions, etc.), and to set optimal and robust parameter settings. A CS was defined based on the understanding of the method strengths and sources of uncertainty. The developed methods were validated based on the ICH Q2(R1) method performance characteristics to formally prove the quality and applicability of the method for the intended purpose. Trending was performed as part of the CS in order to maintain control of the method quality during use.

Vaccine analysis

Traditionally, technologies such as plaque assays [56-61], median tissue culture infective dose assays [56, 57, 62, 63], Polymerase chain reaction (PCR) assays [56, 64, 65], sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [66-69], gel electrophoresis [70] and Western blotting (WB) [71, 72], have been used to analyse vaccines. However, these technologies can be cumbersome, or laborious, often show poor precision, and generally do not fulfil the increasing regulatory requirements. The result is that the development of a vaccine can be slow, expensive, and often fail, due to insufficient characterization and, therefore, lack of product understanding.

For small-molecule pharmaceuticals an extensive analytical toolbox is available for rapid process development and product characterization, for example Spectrophotometry, Fourier Transformed Infrared Spectroscopy, Nuclear Magnetic Resonance Spectroscopy, Capillary Electrophoresis (CE), Liquid Chromatography (LC), and Mass Spectrometry (MS). These tools are increasingly being used for the characterization of biopharmaceuticals such as antibodies (Abs) and protein therapeutics. Examples of often used separation technologies are CE-MS and LC-MS for glycan analysis [73-78], capillary gel electrophoresis (CGE) [79-87] and capillary isoelectric focusing (cIEF) [88, 89] for mAb purity and heterogeneity analysis, and capillary Western blotting [90]. More and more these techniques replace or complement techniques such as SDS-PAGE, WB, Enzyme-linked immunosorbent assay (ELISA), etc. Challenges for bio-pharmaceutical analysis are:

- Analysis under native conditions
- Prevention of adsorption
- Limited sample availability
- Variable sample matrix
- Biomolecule heterogeneity and stability
- Lack of appropriate standards

Efforts to improve the vaccine analytical toolbox are limited and therefore it can be said that the vaccine analytical toolbox is in an exploratory phase [91]. A reversed phase liquid chromatography (RP-LC) method [8, 9] was developed to replace single radial immunodiffusion (SRID) and SDS-PAGE for the concentration determination of Hemagglutinin in influenza vaccine. An RP-LC-MS method for in-depth virus protein and proteolysis analysis was developed in the group of Heck [19, 92] for virus particle composition and degradation markers. An RP-LC method for protein profiling and virus-like particle analysis was developed by Vellekamp and others to determine virus particle composition and purity [93-96].

CE has been applied for the analysis of viruses, for example: virus concentration and integrity [22, 97-119], viral protein concentration [120], composition, integrity, and identity [121-132], etc. Looking at the use of CE, there are a limited number of recent developments on viral vaccine analysis. Oita investigated Polio particle concentration determination with micellar electrokinetic capillary chromatography [110-114]. Bettonville, *et al.*, developed capillary electrophoresis methods for the analysis of human papillomavirus virus like particles [133, 134]. Rustandi, *et al.*, made automated capillary western blotting, capillary zone electrophoresis (CZE) and cIEF methods for vaccine analysis [120, 123-128, 135-137]. In addition to the use of CE for API analysis, CE was also used for characterization of viral vaccine impurities, such as chloride [138], and bromide [139, 140], etc.

Capillary electrophoresis

CE is a technique in which charged components are separated through a background electrolyte (BGE) solution in an electric field applied over a narrow bore capillary (10 – 100 μm diameter) [141-145]. High voltages can be applied, resulting in high efficiencies and fast separations. Working with a narrow-bore capillary provides the opportunity to work with low volumes and many different detectors (e.g.: conductivity, ultra-violet or visible light, total capillary imaging, laser induced fluorescence, and/or with hyphenation such as mass spectrometry (MS)). CE can be performed on chips [146-149] and can be coupled directly to bioreactors as a process analytical technology (PAT) [150, 151].

To develop a fit-for-purpose CE method, a science-based understanding of the fundamentals is obtained and applied to make decisions working towards the goal of the method. Selection of the appropriate BGE and capillary dimensions and coating are key for a successful separation. The BGE should be composed such that:

- analytes are stable, soluble, and/or in native form.
- selectivity for size, charge, and/or affinity is provided.

- there is sufficient buffering capacity despite electrolysis during electrophoresis in order to maintain electrophoretic and electroosmotic mobilities.
- in-capillary concentration can be possible, e.g.: field-amplified sample stacking or transient isotachopheresis (tITP).
- the background noise for detection is reduced.
- compatibility with hyphenated technologies is obtained.

The BGE can contain detergents to improve solubility and/or reduce adsorption, polymers to increase viscosity and/or achieve a sieving mechanism, components that improve selectivity via affinity (e.g.: ligands, cyclodextrins, antibodies / antigens), ampholytes to achieve a pH gradient, etc. The ionic strength of the buffer should be limited to avoid excessive Joule heating and consequently loss of efficiency and capillary burn-through. The temperature of the capillary and for the samples can be optimized to minimize excessive Joule heating and improve the separation and sample stability. Additionally, the capillary conditioning and the injection procedure need to be optimized to avoid adsorption, contamination, and carry-over and to enhance sensitivity. See Figure 4 for a schematic representation of a CE set up including the most important CMPs.

Many of the challenges for biopharmaceutical analysis are covered by CE. A very broad range of separation matrices and BGEs can be selected to be able to work natively in CE. Adsorption may easily be found and counteracted, since a minimal number of materials come in contact with the sample. Low sample amounts, in the order of nL and attomoles, of biomolecules are sufficient for CE analysis. The efficiency in CE is mostly impacted by longitudinal diffusion and is limited for large biomolecules. The highly efficient separation of CE has the potential to separate large biomolecules with only one charge difference (e.g.: an additional sialic acid, N-terminal lysine, or protein oxidation).

Albeit many possibilities, once the suitable settings for the BGE and capillary are found, a capillary electrophoresis method is easily standardized and therefore made robust, and reproducible, as BGE and standards can be prepared in bulk, sample preparation can be automatized, and pre-programmed injection sequences, electrophoresis methods, processing methods and reports can be used.

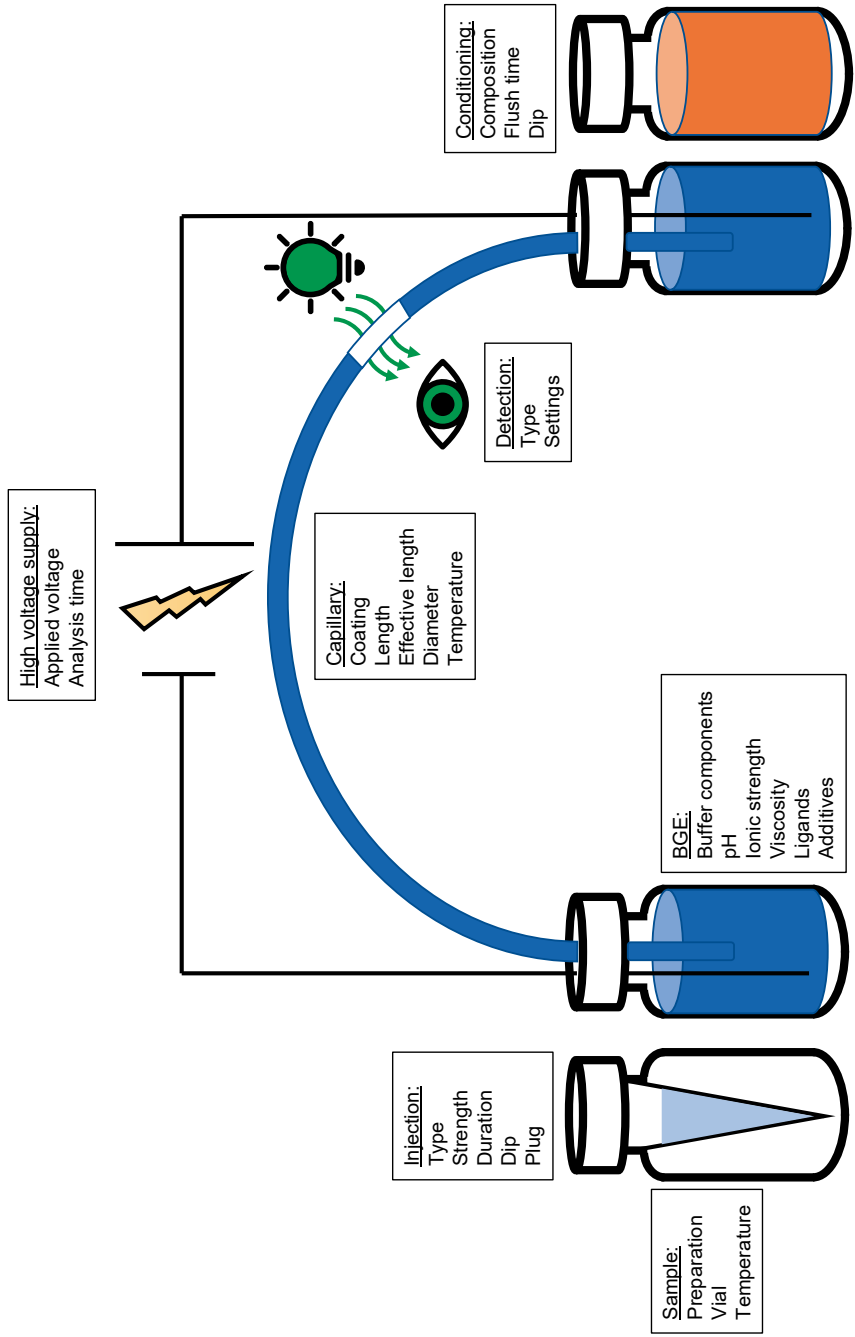


Figure 4. Schematic overview of a CE set up and the CMPs.

2. Aim

The aim of the research presented in this thesis was to investigate the applicability of CE and develop and apply CE methods for rapid and precise vaccine characterization. To achieve this, an AQbD approach was to be developed and applied.

The specific aims for the studies described in the included papers were:

- I. To develop an alternative method for fast, accurate and precise determination of the HA concentration in virosome samples and in inactivated virus. Preferably, the method would allow for the identification and quantitation of HA1, HA2, matrix protein, and nucleoprotein in in-process samples.
- II. To identify the most important CMPs and to propose and test an AQbD approach for the development and validation of CE-SDS methods for vaccine protein analysis (test objects: influenza G1 mini-haemagglutinin protein vaccine (mini-HA) purity and inactivated polio vaccine (IPV) identity).
- III. To develop a method that allows precise and accurate adenovirus concentration determination in in-process samples containing variable amounts of cell debris, cell lysate, host cell proteins, host cell DNA, salts, detergents, and/or additives.
- IV. To design a CS for rapid in-process adenovirus concentration determination with CZE, to compare the CZE method with qPCR, and to evaluate the CZE method performance upon use of the CZE method.
- V. To demonstrate the applicability of CE methods for process and product development, characterization, and QC testing of seasonal influenza virosomal vaccines, universal subunit influenza vaccines, Sabin inactivated polio vaccines, and adenovirus vector vaccines.

3. CGE

Feasibility

“Before you say you can’t do something, try it.”

Kiichiro Toyoda

The applicability of CGE was explored to support vaccine development.

First, The commercial Sciex CE-SDS MW and IgG purity assay kit [152, 153] was applied for determination of the quality of Abs to be used in immunochemical methods used for in-situ antigen expression determination. CE-SDS is a specific application of capillary gel electrophoresis (CGE) developed for monoclonal Abs (mAb) and a capillary analogue for the slab gel application SDS-PAGE. The negatively charged ionic detergent SDS is added to denature the proteins. The denatured protein sample is injected into a capillary pre-filled with a BGE composed of a linear polymer dextran gel, comprising a tris-borate buffer, SDS, glycerol, and EDTA [154]. The SDS added to the sample and in the BGE causes the protein to have a uniform size/charge ratio [87, 155, 156]. The dextran forms an entangled network with dynamic pores (i.e. sieve). The separation of proteins is mainly based, but not limited to [157], the sieving mechanism. Proteins are, therefore, mostly separated based by size rather than charge.

This specific CE-SDS commercial application is designed for analysis on the SCIEX 800 plus instrument. In absence of an SCIEX 800 plus instrument, the CE-SDS application was translated to an Agilent 7100 system and performed similarly. The different Abs resulted in the expected distinct peak patterns and heterogeneous Abs were identified (paper V). Antibody heterogeneity was correlated with WB and ELISA signal interference. This result indicated the power of the CE-SDS application to support immunochemical method critical reagent control.

Second, an analytical method for the analysis of influenza HA protein was needed that was selective, precise, accurate, applicable for the many different HA variants, and compatible with the many different matrices from the process and has a high throughput. None of the used methods could adhere to all the requirements. CE had the potential to adhere to all these characteristics as an alternative method for SDS-PAGE, SRID, and RP-LC [8, 9] (paper I).

Feasibility was tested for the CE-SDS application in paper I. The CE-SDS application resulted in peak profiles comparable to SDS-PAGE for reduced and reduced and deglycosylated samples. However, not all proteins were separated, peaks were close to the limit of quantitation, and the analysis took at least 2 days. Method development was needed to improve separation, method sensitivity, and throughput.

Method development

A CGE method for the influenza protein analysis was developed from the CE-SDS application (paper I). Sample pre-treatments were explored and optimized using multifactorial DoE. Influenza protein separation and sensitivity were further improved by the optimization of the CGE conditions. Dilution of the gel buffer with milli-Q water yielded improved peak efficiencies and resolution of viral protein. The gel buffer dilution and the type of injection were optimized using multifactorial DoE. The developed CGE method was validated for HA quantitation (paper I and V). Repeatability of 8% – 10% relative standard deviation (RSD), and accuracies of 93% – 109% relative to SRID, in the range of 7 – 76 µg/mL HA were found. In-process samples could be analysed as well and circa 100 samples could be analysed within 4 days. None of the tested strains (> 8) required method adjustments indicating the method's broad applicability as an influenza platform method. Annual development could be saved. Additionally, the influenza nucleoprotein and matrix protein were separated and could be quantified as well. An effective length of 8.5 cm was introduced without impacting the method performance, reducing the analysis time about threefold, see Figure 5 (paper V).

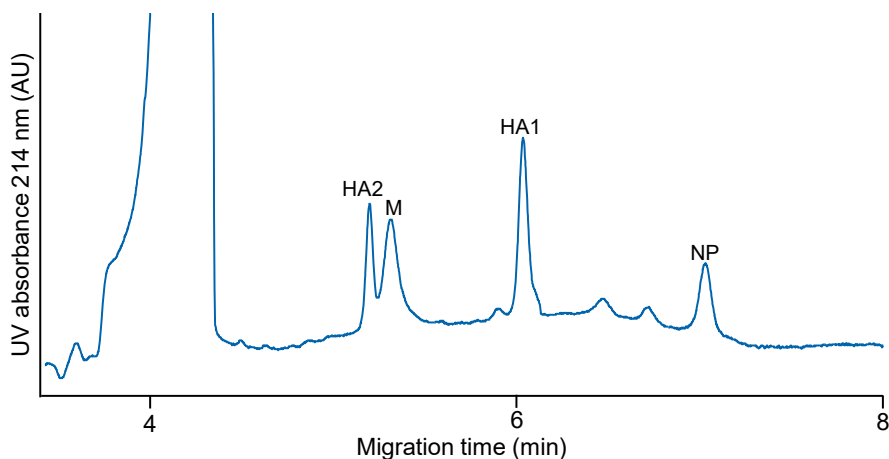


Figure 5. Electropherogram of influenza reference standard NIBSC B/Brisbane/60/2008 analysed with CGE with an effective length of 8.5 cm, and peaks hemagglutinin subunit 1 (HA1), hemagglutinin subunit 2 (HA2), matrix protein (M), and nucleoprotein (NP) (Paper V).

Gel buffer dilution mechanism

Influenza protein separation and sensitivity improved upon gel buffer dilution. The gel buffer dilution effects were different between electrokinetic and hydrodynamic injection. The mechanism of the effects of gel buffer dilution and capillary temperature on method performance were studied to understand these differences and potentially apply the insights for effective method optimization (paper II).

The gel buffer is composed of an entangled network with dynamic pores formed by dextran and acts as a molecular sieve. The sieving mechanism was expected to be impacted by decreasing the dextran concentration through gel buffer dilution [154, 156, 158]. Additionally, dilution of the gel buffer decreased the ionic strength, the viscosity, and the conductivity. Increasing the temperature decreased the viscosity and increased the current and diffusion. In a CE system with multiple solutions, such as a capillary filled with gel buffer and a sample plug, the electric field strength in each solution is inversely proportional to the conductivity of each solution. The ion velocity and the electrical current are proportional to the electric field strength. For gel buffers with a lower conductivity, the applied voltage for separation could be increased without introducing excessive Joule heating and consequently efficiency loss. The higher applied voltages decreased the migration times, hence shorter analysis times were achieved.

During electrokinetic injection a higher local electric field strength over the gel buffer caused a lower local electric field strength in the sample vial.

Consequently, proteins in the sample migrated slower into the gel buffer in the capillary, hence a lower number of proteins were injected.

A lower viscosity resulted in larger hydrodynamically injected sample volumes. Consequently, the injected protein amount and the sample plug length increased, and the separation length decreased. During separation a low conductivity in the buffer resulted in a high local electric field strength in the gel buffer and a relatively low local electric field strength in the sample plug. The difference in electric field strength between the sample and the gel buffer decreased, decreasing sample stacking. In total, gel buffer dilution and capillary temperature changes impacted the efficiency through the injected band width, band broadening, and decreased stacking. The gel buffer dilution and temperature may be optimized to find an optimal balance between the sensitivity and efficiency. An overview of the different effects of gel buffer dilution is provided in Figure 6. In this figure the effects of the capillary temperature on the viscosity, conductivity and diffusion are not presented.

The study in paper **II** focused on explaining the different effects of gel buffer dilution between electrokinetic and hydrodynamic injection on efficiency by looking at a macro level to viscosity, ionic strength, conductivity, etc. Potential effects of dilution on a molecular level was not studied, e.g.: dilution of the dextran polymer network on the sieving mechanism, and dilution of borate adducts formation on the selectivity of the method [157]. These factors could have a profound effect and should be considered for future CGE method development. In summary, a desired separation and analysis time could be obtained by optimizing the gel buffer composition and the capillary temperature, and the optimal solution is very much dependent on the analyte, sample, and the analytical objective.

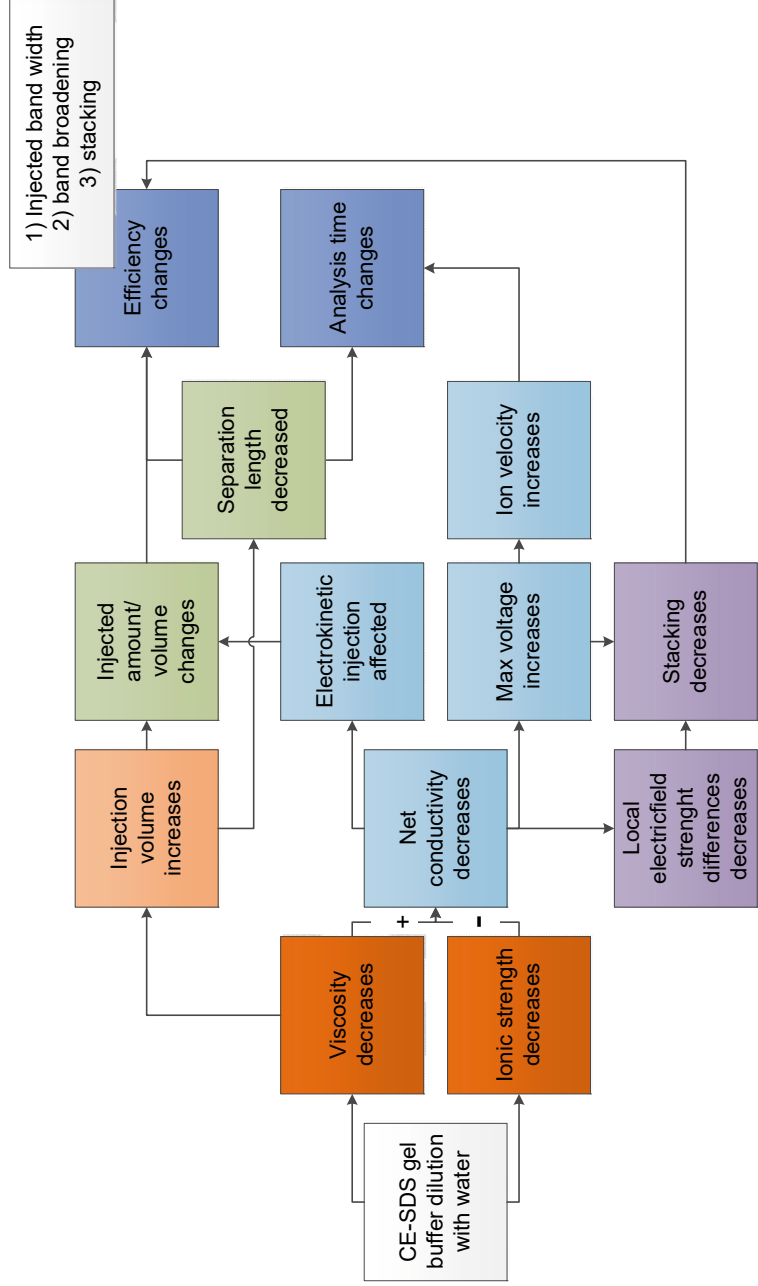


Figure 6. Schematic overview of gel-buffer dilution effects on the physical properties of the BGE, the injection, CGE separation, and analysis efficiency (Paper II). CE-SDS gel buffer dilution decreased the viscosity and ionic strength, that each have opposite effects on the conductivity. A net conductivity decrease was observed. The analysis time changed due to decreased separation lengths and increased applied voltages. The efficiency changed due to changed injected amounts and volumes, and decreased stacking.

AQbD CGE development strategy

An AQbD method development strategy with the purpose to improve CGE method development lead times and quality for viral protein analysis was defined from the experience and understanding gained (paper **II**).

CMPs and their respective effects were identified and listed from literature, previous CE-SDS Ab application, and CGE influenza method development (papers **I**, **II**, and **V**). For sample pre-treatment, a combination of dilution, de-salting, denaturation, reduction, alkylation, deglycosylation, and derivatization can potentially be applied to improve the selectivity and sensitivity (paper **II**). The most optimal combination depends on the nature of the protein and the purpose of the method. The need for a pre-treatment was verified by feasibility testing. For each pre-treatment optimization was performed by including the CMPs: incubation time, the incubation temperature, the pH, and the reagent(s) concentration(s) (paper **II**).

The CMPs for separation were the capillary internal diameter, conditioning, injection volume, applied voltage, mode of injection, gel buffer dilution, capillary temperature, and effective length, etc. The separation CMPs with most impact on resolution, sensitivity, and run time and that required optimization were the gel buffer dilution, the capillary temperature, and the effective length. Most other separation CMPs as well as the detection CMPs did not require optimization and could be based on best practices, method purpose, and experience (paper **II** figure 3 (A)).

Based on the CMP assessment a four-step approach development strategy was proposed (paper **II**), see Figure 7. The first step was feasibility testing. During feasibility, the sample stability was determined, and the need for sample preparation and separation was assessed. After the feasibility, sample preparation and separation were optimized with multifactorial DoEs. When optimal conditions were found the method was validated for its intended purpose.

In total, the knowledge of and focus on optimizing CMPs as defined in the four-step strategy, had the potential to greatly improve the method development time and quality.

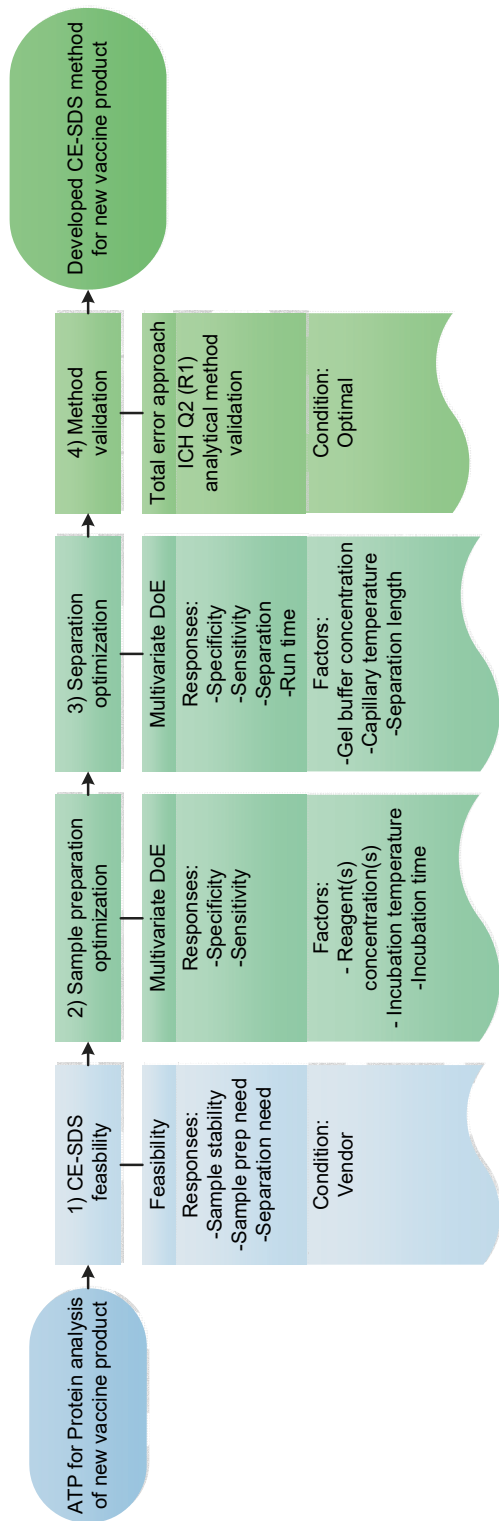


Figure 7. Schematic overview of the four-step approach for AQbD method development for viral vaccine protein analysis with CGE (paper II).

AQbD CGE method development strategy application

The use of the defined strategy was proven by the development of a CGE method for purity determination of mini-HA (paper **II**) and identity determination of sIPV (paper **II**). A CGE method for group 1 mini-HA glycoprotein trimer protein primary structure purity for process development was developed and validated according to the four-step approach. Feasibility results confirmed mini-HA stability and the need for deglycosylation and reduction pre-treatment. Different combinations of PNGase F (N-deglycosylation), O-glycosidase, and sialidase with and without reduction resulted in very distinct peak patterns uncovering N- and O-glycosylation as well as sialylation patterns on the trimer and monomer (paper **V**), see Figure 8. The combination of reduction and PNGase F, O-glycosidase, and sialidase (Figure condition I) was selected. Optimal and robust sample pre-treatment and separation conditions were found with multifactorial DoE and validated (paper **II** and **V**). For the analysis of mini-HA the gel buffer dilution had limited effect on the total analysis time, including conditioning, and the separation. Therefore, the undiluted gel buffer was selected to minimize gel solution handling and minimize the risk for errors.

The CGE method was applied to characterize stressed mini-HA samples (paper **V**). Under all tested stress conditions (thermal, oxidative, and basic stress), a change in the peak profile and thus mini-HA purity could be determined, see Figure 9. The CGE method could, therefore, be used in degradation and stability studies.

In summary, a robust, optimal, and controlled method was developed by applying the four-step approach that could help identify and test product CQAs during the early phases of vaccine design and process development. The same was achieved for protein analysis of a trivalent sIPV vaccine with CGE within 4 days (paper **II** and **V**). Both examples illustrate the potential of the four-step AQbD method development approach for viral vaccine protein analysis with CGE.

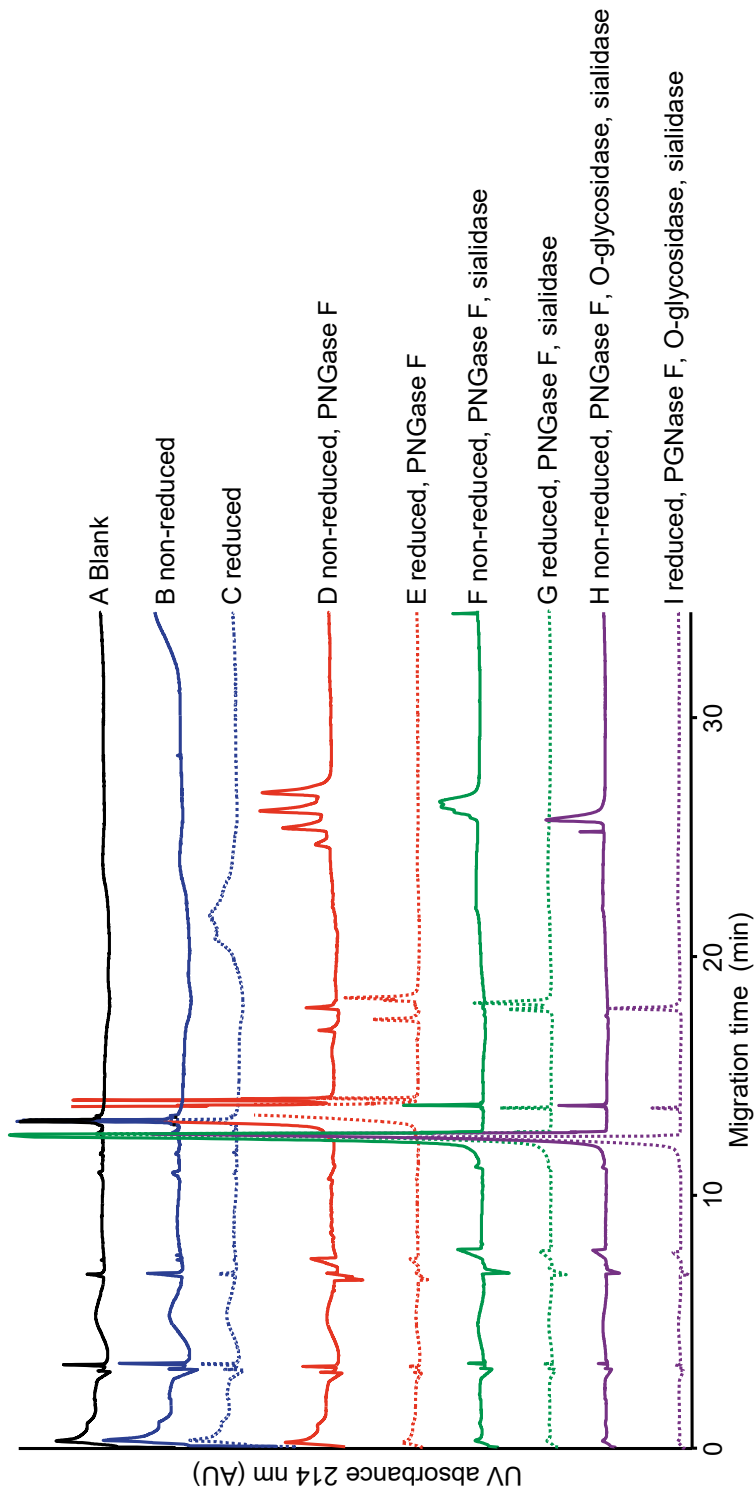


Figure 8. Electropherograms of mini-HA subjected to different sample pre-treatments and analysed with CGE (paper V). Different combinations of PNGase F (N-deglycosylation), O-glycosidase, and sialidase with or without reduction resulted in very distinct peak patterns uncovering N- and O-glycosylation as well as sialylation patterns on the trimer (non-reduced) and monomer (reduced).

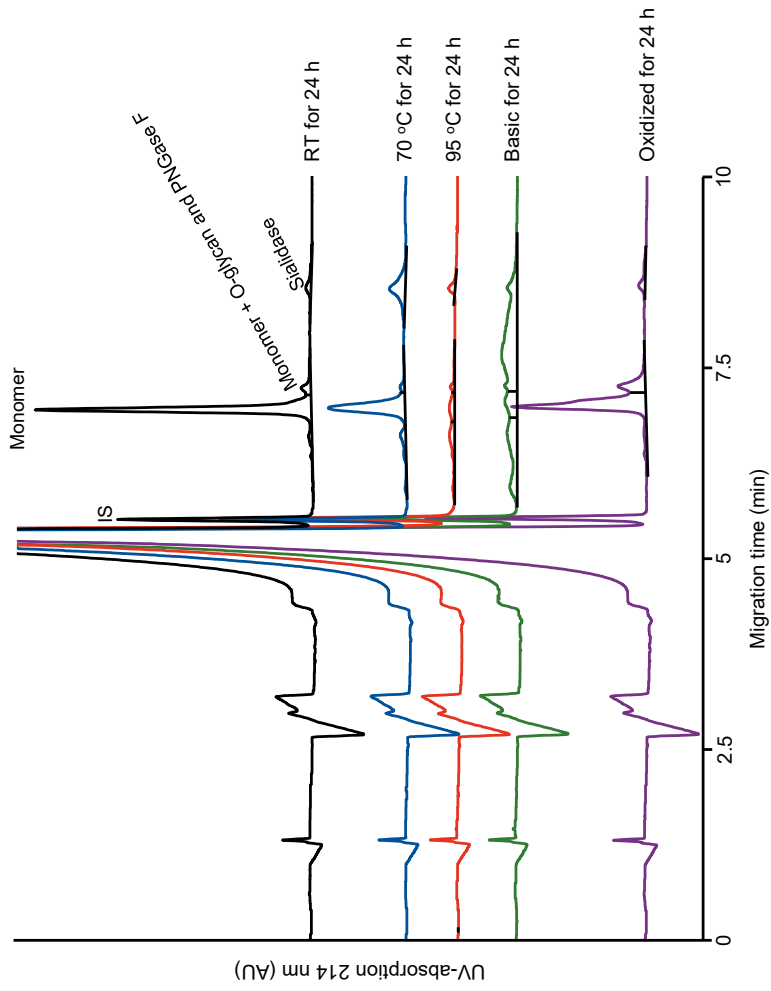


Figure 9. Electropherograms of mini-HA subjected to different types of degradation conditions for 24 h analysed with CGE (paper V). All stress conditions result in a decrease of the monomer peak and appearance of new peaks compared to the control conditions, i.e.: incubated at room temperature for 24 h.

4. CZE adenovirus concentration determination

Analytical target profile

“You can’t hit a target if you don’t know what it is.”

Tony Robbins

Adenovirus vaccines are dosed on adenovirus concentration and, therefore, accurate and precise adenovirus quantitation throughout the process in different process matrices is important. Experts with backgrounds in adenovirus production, purification, formulation, and analytical development and pharmaceutical release testing were gathered to determine the purposes and the quality requirements for adenovirus concentration determination. Process and product requirements were translated into analytical test requirements, such as method performance characteristics. In addition, analytical operational and business requirements, such as ease of operation, throughput, and costs, were added. An aligned ATP for the adenovirus concentration determination method was created to start making decisions towards a fit-for-purpose analytical test method, see Table 2.

Table 2. ATP for an adenovirus concentration determination method. The numbers refer to numbered descriptions, (1) process and product development and characterization, and (2) in-process control (IPC) testing.

Method performance characteristic	Targets
Description	1) For adenovirus production process development and product characterization a precise, accurate, robust, degradation sensitive, and a high-throughput method is needed for the quantitation of adenovirus particles in all process intermediates, DS, and DP. 2) A proper adenovirus amount should be loaded upon the AEX- filters, to avoid filter blockage, excessive viral dilution, and impurity break through and to avoid product degradation whilst testing, a precise, accurate, robust, and fast method for adenovirus quantitation is required for in-process control testing (IPC).
Specificity	Detection of Ad26 and Ad35 without significant interference of sample matrix components in the samples: Crude harvest (1) Lysed harvest (1) Clarified harvest (1 and 2) AEX-eluate (1 and 2) UF/DF filtrate (1) DS and DP (1)
Precision	Intermediate precision $\leq 10\%$ RSD
Accuracy	90% – 110% spiked recovery, $\leq 10\%$ bias
Range	10^{10} – 10^{12} virus particles (VP)/mL
Time from sampling to reported result	< 2 days for process development (1), < 4 h for IPC testing (2)
Throughput	1 sample per run for IPC testing, > 7 samples per process development run. > 300 samples a month for process development.
Future requirement	QC testing

Technique selection

“May your choices reflect your hopes, not your fears.”

Nelson Mandela

With a clear purpose and target for the determination of the adenovirus concentration, potential analytical technologies were evaluated. First, the method characteristics of the ATP were prioritized in order to make balanced decisions. A list of potential technologies was created based on pharmacopoeias, contract research organisation catalogues, scientific literature, vendor applications, and in-house experiences. The potential of each technology for each method characteristic was filled and scored against the method characteristic target, i.e.: meets requirement (green), is at risk to meet the requirement (orange), does not meet the requirement (red).

Up till then, OD260 [159, 160], AEX-LC [37] and qPCR [161] methods were used for adenovirus concentration determinations. However, OD260 is nonspecific, suffers from matrix interference, and cannot be used for in-process testing. The AEX-LC method suffered from adsorption of the adenovirus particles. Adsorption was worse for in-process samples, so AEX-LC could not be used for in-process testing. The qPCR method was very sensitive but had poor precision (10% –30% RSD) and accuracy (70 – 130% spike recovery). An increased testing format (3 runs with each 3 replicate samples) improved the precision but not the accuracy, and caused the method to be laborious, costly, and time consuming. In summary, the ATP and technology selection table provided insight in the reasons why previous methods did not adhere to the expectations.

The analysis of viruses is challenging. Samples are highly complex and variable ranging from $10^8 - 10^{12}$ Ad26 virus particles (VP)/mL, $10^0 - 10^6$ (lysed) cells/L, 50 – 500 mM NaCl, $10^{-9} - 10^0$ g/L nucleotides and proteins, $10^{-6} - 10^{-1}$ % (w/v) sugars, organics, or detergents. The virus particles are not stable in all sample matrices, nor when subjected to shear stress or acidic, or organic environments [96]. Both the separation technique and the sample pre-treatment should account for the characteristics of the analyte, the sample and the analytical test goal. In addition, sample preparation was preferably reduced to a minimum to decrease error propagation and the hands-on time.

Several analytical techniques were considered, see Table 3. CZE has the potential for fast, precise, and accurate analysis of biopharmaceuticals. Additionally, native analysis is possible. Risk factors for CZE analysis could be large particles blocking the capillary, such as cell debris and DNA aggregates, or high ionic strengths in the samples, such as used for AEX-filtration. When large particles would be present sample pre-treatment matching the type of particles could be considered. The high ionic strength in samples

could also be used for in-capillary sample concentration as with transient isotachopheresis (tITP). Although there was limited experience, it was no surprise that CZE had the potential to adhere fully to the ATP requirements. As no other technique had the potential to adhere to the requirements CZE was selected for development. qPCR was the best back-up option as it had no “does not meet” on a priority 1 requirement. Both techniques were further developed to make sure clinical production deadlines were met.

Table 3. Technique selection decision matrix for an adenovirus quantitation method.

Requirements			Techniques							
Characteristic	Target [range]	Priority	OD260	AEX-LC-UV	RP-UPLC-UV	SEC-LC-UV	qPCR	CE-UV	Virocyt (Virprep-plus)	AF4-UV
Specificity	Ad particles	1	Aromatic peptides and nucleotides	Ad particles	Ad protein	Ad particles & protein aggregates	Ad Encapsulated DNA	Ad particles	Ad and Ad protein epitope	Ad particles
Matrix	Crude Lysed Clarified AEX-eluate UF/FD filtrate DS and DP	1	DS/DP	DS/DP	Crude Lysed Clarified AEX-eluate UF/FD filtrate DS/DP	DS/DP	Crude Lysed Clarified AEX-eluate UF/FD filtrate DS/DP	Crude Lysed Clarified AEX-eluate UF/FD filtrate DS/DP	Crude Lysed Clarified AEX-eluate UF/FD filtrate DS/DP (uncertain)	DS/DP
Range	$10^{10} - 10^{12}$ VP/mL	1	$> 10^{10}$ VP/mL	$> 10^{10}$ VP/mL	$> 10^{10}$ VP/mL	$> 10^{10}$ VP/mL	$> 10^9$ VP/mL	$> 10^{10}$ VP/mL	$> 10^7$ VP/mL (uncertain)	$> 10^{10}$ VP/mL
Accuracy (spike recovery)	90% – 110%	2	80% – 120%	80% – 120%	80% – 120%	80% – 120%	70% – 130%	90% – 110%	unknown	80% – 120%

Requirements			Techniques							
Characteristic	Target [range]	Priority	OD260	AEX-LC-UV	RP-UPLC-UV	SEC-LC-UV	qPCR	CE-UV	Virocyt (Virprep-plus)	AF4-UV
Precision (intermediate precision)	10% RSD	2	20%	10% – 20%	10% – 20%	10% – 20%	10% – 35%	5%	9% (uncertain)	20%
Time to result (IPC testing)	< 4 h	3	< 4 h	4 h	4 h	4 h	4 h	1 h	uncertain 1 h	4 h
Expected sample load per month	> 300	2	> 300	300	300	300	< 300	> 300	> 300	300
Future requirement	QC testing	2	yes	yes	yes	yes	yes	yes	maybe	no
Development investment	Medium	3	low	low	low	low	low	low	high	low
Equipment available	yes	1	yes	yes	yes	yes	yes	yes	no	yes
Risk of not meeting requirements			Nonspecific (matrix interference) and sensitivity	Adsorption	Protein unrepresentative of VP concentration	Adsorption and protein aggregates interference	Precision and accuracy upstream	Matrix separation	Uncertain of all characteristics, DNA probe might not enter capsid	Adsorption

Critical method parameters

“Critical thinking and curiosity are the key to creativity”

Amala Akkineni

The most important CMPs for a CZE method are the BGE composition and the capillary surface type [141-145]. Before designing an effective BGE and selecting an adequate capillary, a literature search was conducted to identify additional potential CMPs and to create an overview of commonly used BGEs and buffers. Only 22 applicable publications were found ranging from the analysis of viruses to microorganisms at the of starting method development. Mann, *et al*, described a CZE method for Ad 5 analysis with a phosphate buffered BGE in a PVA capillary [108]. About half of the applications used a capillary coating, both dynamic [108, 113, 117, 118, 162-169] and static [170, 171], e.g.: neutrally coated capillaries, were used. Many applications used a BGE based on borate in combination with SDS [99, 103, 104, 109, 112-114]. SDS in the BGE is not suitable for the determination of intact adenovirus particles, as the particles degrade in the presence of SDS. However, potentially a detergent might be beneficial for the solubility of the adenovirus particles. For the analysis of other biological compounds triethylene-tetramine [167], or successive multiple ionic-polymer layers [162, 172-176] were used previously. Coatings and detergents can be used to prevent adsorption and can also influence the separation [173, 175, 177]. So, the BGE composition, and the capillary type were confirmed to be the most important CMP, potentially detergents and a capillary coating are required, and no additional CMPs were identified to be considered during application feasibility screening.

Screening

“Better well invented than badly stolen”

Anonymous

A screening experiment to test the feasibility of different capillaries and BGE combinations was performed (paper **III**). The screening experiment was designed to find capillary and BGE combinations with the potential for repeatable adenovirus quantitation.

A tris-tricine BGE was designed from CE fundamentals and experience and knowledge about the analyte. A tris and tricine BGE was proposed because:

- Tris has been used in stable adenovirus formulations

- Tricine was used in AEX-LC without any signs of adenovirus particle instability previously
- The pK_{as} of both tris and tricine are at least 1 pH unit above the expected adenovirus pI. At pI the analyte carries no net charge and solubility is usually reduced. At pHs below the pI adenovirus particles were considered unstable
- the pK_{as} of tris and tricine are close and in combination, tricine acts as a buffering co-ion and tris as a buffering counter-ion, resulting in a high buffering capacity of the BGE which improves robustness. A high buffering capacity maintains the pH longer when electrolysis occurs
- tris and tricine are both low-conductive components, so high concentrations of the buffer components can be used without causing excessive Joule heating as was verified by making an Ohm's plot
- The risk for electromigration dispersion is reduced by the high buffer concentrations and potentially by better matching co-ion
- The BGE can be accurately and robustly prepared by using precise concentrations by weighing in. A pH measurement is used to control the preparation.

In addition to the designed tris-tricine BGE, other BGEs from literature were also screened. A wide range of BGEs was selected ranging in pH, ionic strength, co- and counterions, and surfactants. Since proteins and virus particles are known to adsorb to silica [178], PS-20 was taken along as an alternative detergent for SDS and probably a capillary coating needed, most likely a neutral coating. The different BGEs were tested on a wide range of different capillaries, i.e.: uncoated, dynamically coated, and statically coated, charged and neutral.

To determine experiment reliability and evaluate the potential of combinations of BGEs and capillaries to analyse adenovirus particles several representative samples were taken into account:

- A cell lysate, worst case "dirty sample" with cell debris
- An AEX-eluate, worst case sample with a high salt concentration
- A DS, a purified adenovirus best case sample with little to no interference expected from matrix components
- An IgG, a model protein of which often results were already published, taken along as a system control
- A blank, as negative control

Each sample was spiked with the internal standard o-phthalic acid to confirm experiment performance and to identify potential instrumental issues. A UV-spectrum was recorded to identify the peaks observed. Performance of the

buffer and capillary combinations was scored on the repeatability of observing the adenovirus, the internal standard, and the antibody. The highest scores were obtained when an adenovirus peak was observed. Lower scores were obtained for conditions where the internal standard and/or IgG was observed but no adenovirus particles. The reason for not observing the adenovirus particles may be overcome when adjusting the settings.

The neutrally coated capillaries performed best in combination with either a tris-tricine or a phosphate buffered BGE. Due to the potential benefits listed above, the tris and tricine BGE was selected to continue development with. Performance on neutrally coated polyvinyl alcohol (PVA) and polyacrylamide (PAA) capillaries was later confirmed by testing on a fluorocarbon coated (μ SIL-FC) capillary. The PVA coated capillary from Agilent could be bought at the right length (no capillary cutting), was easy to install, had a stable pH-range, and was insensitive to exposure to air. Additionally, PVA coated capillary with extended light path or bubble cell were an option. Therefore, the PVA coated capillary with extended light path from Agilent was selected to continue development with.

Method development

“I never lose, I either win or learn”

Nelson Mandela

A failure-mode effect analysis (FMEA) was used to facilitate further CZE adenovirus concentration method development, see Table 4. A diverse team of operators and scientists worked together to identify as many CMPs as possible. The current knowledge regarding the effects of the CMPs on method performance characteristics were captured in the FMEA in the form of failure-modes, e.g.: inadequate BGE composition (CMP) could cause the adenovirus to adsorb to the capillary wall and cause inaccurate and non-repeatable (method characteristics) results. The risk of each failure-mode was scored based on the impact (I), the probability (P) and detectability (D) of the failure-mode. The multiplication of these three factors formed the risk priority number (RPN). The RPN was used to sort the CMPs and to focus on the most important CMPs during method development. For the example of the BGE composition, accurate and repeatable test results were an absolute necessity, hence the failure-mode impact I was scored 10 out of 10. In literature limited viral and adenoviral applications were found. However, these applications did not show the level of quality needed, e.g.: accuracy, repeatability, robustness, non-purified sample applicability. The probability P of inaccurate and imprecise results caused by the BGE composition was high and was scored 8 out of 10. The chance that we would detect inaccuracy and

imprecision caused by the BGE was also very low when only testing a sample and D was scored 6 out of 10. For the BGE composition the RPN was 480, which was at that time the highest RPN. To improve method reliability the risks were mitigated to reduce the probability of occurrence and improve detection of unreliable results. Screening experiments were performed previously (section 2.4) and the Agilent PVA capillary and a tris-tricine BGE were chosen (paper III), Table 4 experiment 1. Consequently, the probability and risk of inaccurate and imprecise results decreased. So, setting the CMP to a robust and optimal setting reduced the probability of the failure-mode occurring. The setting was found by performing experiments but may also be based upon previous experiences and best practices, e.g.: detection settings for the CGE AQbD development strategy (Section 1.3).

During the screening studies migration times and peak widths increased, and corrected peak areas decreased with the number injections on the capillary. This could be caused by precipitation, aggregation, and/or adsorption. Since the effect increased with the number of injections and the BGE was fresh for each injection, in-capillary precipitation and aggregation was not expected. In-sample aggregation and precipitation were excluded due to constant concentration and aggregation level determination with other methods such as OD260, qPCR, and protein analysis with RP-LC, DLS and AF4-MALS. Therefore, it was concluded that these effects were caused by increasing adsorption of sample components to the capillary wall.

The characteristics of the adsorption were further studied by repeated injections of samples on a capillary. This was done for each process intermediate and DS at different adenovirus concentrations, each on a fresh capillary. Migration time and peak width increases were observed for repetitive injections for all samples. Only for DNA-containing process intermediates the corrected peak area decreased and after 15 injections no adenovirus peak was observed anymore. In addition, spikes in the electropherogram, current drops and column blockages were observed. It was concluded that adsorption was enhanced by in-process sample matrix components and was caused by degraded PVA coating. Since ions such as chloride were not impacted it was derived that small changes in the capillary coating occurred such as damage of small patches of PVA coating. Subsequently, adenovirus particles together with matrix components, such as DNA, could adsorb to the small, exposed silica parts of the capillary wall.

A screening experiment was set up to identify CMPs that affected the degree of adsorption, see also Table 4 experiment 2. The CMPs that were screened were:

- BGE additives type (non-ionic detergents, dynamic coating)
- BGE component(s) concentration(s) (incl. a pH range)
- Capillary conditioning solution composition (formulation buffer, acidic, basic, reductive, detergent)

- Capillary conditioning component concentration
- Capillary conditioning duration
- Capillary conditioning flow direction of the flow
- Sample preparation (dilution, filtration, centrifugation)
- Capillary coating
- Capillary temperature

The addition of PS-20 to the BGE, reversed flushing with phosphoric acid between injections, and a lower capillary temperature separately decreased, but did not fully prevent, adsorption. A combination of all three was required and optimization of these factors reduced adsorption to a minimum, see Table 4 experiment 3. Mitigation strategies to prevent adsorption were designed based on the experimental results and incorporated, see Table 4. Mitigation by setting CMPs to experimental optimal conditions only decrease the probability of adsorption happening, hence minimizing the occurrence. The impact cannot be changed, therefore, detection if adsorption occurs was required to make sure inaccuracy and imprecision via adsorption were flagged at the start of an analysis. Bracket controls were implemented to identify inaccuracy and imprecision within runs. Trending prevented use of bad capillaries in consecutive assay runs, see Table 4 mitigation 4.

The above situation showed that multiple CMPs may impact the same failure-mode. An additional line with capillary temperature was added to Table 4 section 1 in order to illustrate that a single CMP may also have different types of failure-modes. The failure-mode is different, so the RPN is different and different types of mitigations may be needed.

Interestingly, in this example the CMP capillary temperature was set based on the experiment for adsorption and not based on the experiment for adenovirus instability. Both failure-modes were affected by the same CMP and are therefore interacting. It is important to evaluate both the failure-modes before setting the CMP. In the end, the most optimal and robust settings for both the failure-modes should be selected. Multifactorial DoE is helpful to be able to find such robust optimum between interacting CMPs and failure-modes.

Method development was continued with separation condition optimization, see also Table 4 experiment 3 for examples for some of the CMPs. A full factorial DoE was performed to find optimal conditions for the tris concentration, tricine concentration, applied voltage, and the capillary effective length, in order to improve separation and separation time (paper III). The optimal conditions (125 mM tris, 338 mM tricine, -20 kV applied voltage, 8.5 cm effective length) were deemed robust in a range of 113 – 138 mM tris, 338 – 413 mM tricine and -15 to -25 kV applied voltage.

Under these optimal conditions, interference of the matrix components was observed in DNA-containing samples, that was not solved by optimizing

the separation conditions. Investigations using enzymatic removal (DNase treatment), physical removal (centrifugation), UV-spectrum analysis, and fluorescent labelling, indicated that these peaks were DNA associated, but did not contain adenovirus. Since isolated DNA migrated at an earlier migration time than these interfering peaks, it was suggested that the peaks were DNA associated aggregates.

Benzonase is a DNase and cleaves DNA into small fragments. Sample treatment with Benzonase resulted in a shift in migration time of the interfering peaks. Therefore, a Benzonase pre-treatment for DNA-containing samples was optimized in a full factorial design (paper III). The adenovirus peak and the interfering peaks were separated in DNA-containing samples pre-treated with 8 units/mL Benzonase, 1.4 mM MgCl, and incubation for 30 min at 37 °C. Since Benzonase pre-treatment is an enzymatic pre-treatment, many factors could influence the efficiency. Therefore, the robustness was tested for these optimal conditions with DNA-containing samples from another process. The effects of 7 – 9 U/ml Benzonase, 1.0 – 1.8 mM MgCl, and incubation time of 25 – 35 min at 37 °C on separation between the adenovirus peak and the interfering peaks were tested. A migration time difference of 0.7 – 1.0 min between the adenovirus and the interfering peaks was found in this design space. The DNA-containing pre-treatment was deemed robust.

In summary, method development was facilitated using an FMEA. The FMEA was used continuously to find knowledge gaps, prioritize experimenting, capture the gained knowledge and understanding such as the relation between CMPs and the method performance characteristics, evaluate method reliability, and develop an effective CS, during the different phases of the method development.

Table 4. A small part of the FMEA for the adenovirus concentration determination method, with I = impact, P = probability, D = detectability, RPN = risk priority number (I×P×D), with phase 1) BGE and capillaries feasibility screening, phase 2) adsorption effect screening, phase 3) optimization, phase 4) detectability mitigations.

CMP	Failure mode	Characteristic	I	P	D	RPN	Experiment	Mitigations	I	P	D	RPN
Phase 1)												
BGE composition	Inadequate BGE composition could cause the matrix components or adenovirus to adsorb, precipitate, or aggregate and cause an inaccurate and nonrepeatable results	Accuracy / precision	10	8	6	480	1) Screening of BGE and capillary	1) Set BGE to 200 mM Tris 200 mM tricine	10	6	6	30
							2) Screening to reduce adsorption	2) Add PS-20 to BGE	10	4	6	
							3) Optimize conditions	3) Set BGE to 125 mM tris and 338 mM tricine and 0.2% w/v PS-20	10	3	2	
								4) Implement bracket controls and peak width and migration time trending	10	3	1	
Capillary quality	Improper choice of capillary or inadequate capillary conditioning could cause the adenovirus to adsorb to the capillary wall and cause an inaccurate and nonrepeatable results	Accuracy / precision	10	8	6	480	1) Screening of BGE and capillary	1) Select PVA capillary	10	6	6	30
							2) Screening to reduce adsorption	2) Between injections conditioning	10	4	6	
							3) Optimize conditions	3) Optimized between injection conditions	10	3	2	
								4) Implement bracket controls and peak width and migration time trending	10	3	1	

CMP	Failure mode	Characteristic	I	P	D	RPN	Experiment	Mitigations	I	P	D	RPN
Capillary temperature	Inadequate capillary temperature could cause adenovirus instability and degradation and cause an inaccurate results	Accuracy	10	4	4	160	3) Optimize conditions	Initial: Controlled capillary temperature at 25 °C	10	4	4	10
								3) Set capillary temperature to 15 °C	10	1	4	
								4) Implement negative and positive controls and use pre-programmed instrument methods and assay sequences	10	1	1	
Phase 2)												
Between injections capillary conditioning	Inadequate capillary conditioning could cause the adenovirus to adsorb to the capillary wall and cause an inaccurate and nonrepeatable results	Accuracy / precision	10	8	6	480	2) Screening effects for adsorption	2) Use acidic flush	10	6	6	30
								3) Set between injections capillary conditioning to 10 mM phosphoric acid reversed flush at 1 bar for 1 min	10	3	6	
								4) Implement bracket controls and peak width and migration time trending	10	3	1	
Capillary temperature	Inadequate capillary temperature could cause the adenovirus to adsorb to the capillary wall and cause an inaccurate and nonrepeatable results	Accuracy / precision	10	6	6	360	2) Screening effects for adsorption	Initial: Controlled capillary temperature at 25 °C	10	6	6	30
								3) Set capillary temperature to 15 °C	10	3	6	
								4) Implement bracket controls and peak width and migration time trending	10	3	1	

Validation

The optimal and robust CZE method was validated for IPC testing (paper **III**). Validation experiments were carried out by at least one future operator, to reflect actual method performance. Future operators were made aware of best practices for the method at hand before running into validation. Special attention to best practices was paid during method development and should be captured explicitly in the FMEA, CS, and, eventually, trained to the future operators.

Specificity was determined by confirming the peak identity and verifying the presence of intact adenovirus particles. The Ad26 and Ad35 were both separated from all the matrix components in all the process intermediates. Ad26 vectors with different genomic inserts could not be separated from each other as the insert does not change the charge/size ratio for the adenovirus particle. All different adenovirus types (strains) tested so far differed in migration times due to small protein modification that influenced the charge/size ratio and thus migration (paper **III**, paper **V**).

The method repeatability ($n = 18$) was 2.1% – 4.8% RSD for the corrected peak area and 0.55 – 0.82% RSD for the migration time. The intermediate precision ($n = 18$) was found to be 7.8% RSD for the corrected peak area and 2.5% RSD for the migration time. The accuracy was 95% – 110% spiked recovery (three replicates at three levels). In conclusion, all predefined acceptance criteria were met, and the method was considered suitable for the determination of the adenovirus concentration in vaccine products.

Thereafter, the CZE method was also validated for seed release and DS/DP release and stability testing (paper **V**). Seeds are DNA-containing samples and required the additional Benzonase pre-treatment. The method performance for seed release, IPC, and DS/DP release and stability testing were very similar. The precision of the seed release method was slightly higher due to the additional pre-treatment. The pre-treatment had no effect on the accuracy.

A total error approach was used to establish a more predictive measure of method performance for future test results [179-182]. Both random variation (precision) and systematic variation (bias) are based on measurement uncertainty. The total error approach focuses on the probability of assay results to be around the (conventional) truth. The difference is that the uncertainty is associated with the results of a measurement and is retrospective and the total-error accounts for the whole distribution of test results with the analytical procedure and is prospective. The total-error approach is, therefore, more suitable as a method performance characteristic for future testing. The total error was determined by calculating the β -expectation tolerance intervals of spiked sample results at each concentration level, with 3 replicates per run and 4 assay runs. The total error increased with lower adenovirus particle concentrations due to closeness to the limit of quantification (LOQ), see

Table 5. At higher adenovirus concentrations the total error for DS/DP and IPC testing is between 4% – 18% from the spiked sample expected true value. For seed samples the total error was higher due to the sample pre-treatment. Overall, the total error of the method is rather low and fit-for-purpose.

Table 5. Beta-expectation tolerance intervals for adenovirus quantitation in seed release, IPC, and DS/DP release samples.

Adenovirus concentration level (10¹¹ VP/mL)	Ad26 seed Relative β-expectation tolerance interval (%)	Ad26 IPC Relative β-expectation tolerance interval (%)	Ad26 DS/DP release Relative β-expectation tolerance interval (%)
0.25	-25 – 17	-31 – 26	-9 – 15
0.50	-20 – 3.0	-18 – 16	-7 – 16
1.50	-22 – 7.5	-7 – 11	-1 – 4
1.94	-20 – 25	-4 – 4	-4 – 4

Implementation

The qPCR method was used for the development of adenovirus products before the CZE method was available. So, a comparability study was needed in order to implement the CZE method. Comparability was performed by means of an equivalence study for all process intermediates (Paper IV). In total 131 samples were tested with both qPCR and CZE, see Figure 10 for a bivariate plot for qPCR versus CZE for different process intermediates. The 95% prediction confidence interval of the difference between the CZE and qPCR measurement were in general $-0.18\log_{10}$ to $0.16\log_{10}$. For previous qPCR comparability studies acceptance limits of $-0.2\log_{10}$ to $0.2\log_{10}$ were used. The CZE results fell well within these acceptance limits, so CZE and qPCR results were deemed interchangeable.

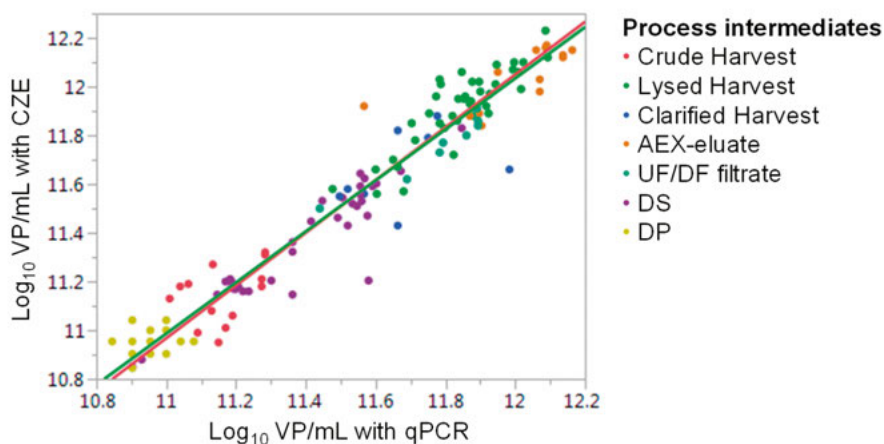


Figure 10. Deming regression plot of CZE and qPCR results for different process intermediate samples. Equivalency is illustrated by closeness of the results and the fit (green line) to the $y = x$ line (red) (paper IV).

Control strategy

“Control is an illusion.”

“Only two things are under your control: your actions and your attitude.”

Mo Gawdat – Solve for happy

The purpose of a CS is to reduce the risk for analytical errors and to increase the chance of detecting errors before finalizing the analysis. A CS is a planned set of controls based on method understanding and the purpose of the analysis.

A CS for the CZE method was designed during method development. The scientific input from all experiments was used to build an adequate CS and improve the method reliability. Optimization experiments resulted in robust and optimal conditions resulting in a low probability and prevention of failing to provide reliable results. Detection methods, like bracket controls and trending, were put in place to flag improper test results and prevent non-reliable result reporting. Other detection mitigations were:

- Control samples: Positive/ negative controls, reference standards/material, bracket controls etc.
- Internal standards
- Standardized (assay) recording sheets
- Instrument monitoring, error signaling, and error recording
- Trending
- Review: lab notebook, done by /checked by, co-signing, etc.

The FMEA was used to identify the biggest risk factors that might result in unreliable, inaccurate, and imprecise results. For example, the quality of the capillary coating was shown to be a high-risk factor (section method development, Table 4). The consequent and calculated risk of increased capillary coating degradation was acceptably controlled and accepted in favour of fast IPC analysis with no other sample pre-treatment than dilution (paper IV). A system suitability test (SST) was designed to verify the method performance before sample testing. Bracket injections were implemented to verify method performance during a run. Before sample analysis, the system current (BGE in capillary), blanks (no contamination or carry-over), repeatability (6 system suitability control sample (SSC) injections), and quantitation (SSC vs. calibrant) were verified within 1 h. When all the acceptance criteria pass, the samples can be analysed. When the acceptance criteria fail, troubleshooting needs to be done, and the root-cause resolved prior to analysis restart.

IPC test results are preferably reported < 4 h after sampling to quickly proceed with the downstream processing and avoid product degradation and process yield loss due to prolonged hold-times. To decrease the risk of delaying the process, the SST was planned prior to when the sample was expected. The acceptance criteria on the SST were purposefully set rather strict in order to reduce the risk of analytical failure during sample analysis, when time was critical. With this strategy, the remaining time needed to analyse the sample, to interpret the data, and to report the results was not more than 2 h (paper IV). This stringent CS resulted in 3 out of 525 invalid analytical runs, i.e., sample results could not be reported in time. An invalid rate of 0.6% is uncommonly low for these types of analysis.

In addition to prior- and within analytical run method performance verification with the SST, trending was applied to verify method performance between runs and post analysis. The adenovirus concentration of the SSC was monitored over these 525 runs. The adenovirus concentration was within the predicted confidence intervals, < 15% (paper IV). A bias of 4% was observed (paper IV). The repeatability of the SSC within run was on average 1% RSD, and a 100% of the runs was < 5% RSD (predefined acceptance criterion). This is in line with the validation results, indicating full control of the method and meeting the method requirements.

Method life cycle

“Progress cannot be generated when we are satisfied with the existing situation.”

Taiichi Ohno

For process development and product characterization analysis, another 1656 assays were run with a similar SST set-up, see Figure 11 for the SSC concentration control chart. In phase 0 the adenovirus concentration response was not yet trended, and the method was in development and validation. During phase 0 the SSC target concentration was set with respect to the requirements of IPC testing to 1.00×10^{11} VP/mL with specification limits at 0.85×10^{11} VP/mL and 1.15×10^{11} VP/mL. Interpretation of trending results were assisted by target limits, calculated control limits, and Shewhart tests (not shown in Figure 11). In phase 1 the variation was as expected, and the same bias as for IPC testing of 4% was observed. A risked prioritized list of possible CMPs having impact on the accuracy was created with the FMEA. Evaluation of the list suggested a high change of improper pipetting causing the bias. Operator assay execution was carefully observed to identify possible root-causes for the observed bias. It was noted that each operator pipetted differently, and they all introduced a pipette tip pre-rinsing step. This was trained as a best practice during a vendor pipetting training. During the training operators were trained for best practices pipetting milli-Q water. However, the adenovirus samples and the diluent were more viscous than water and clung to the pipette tip wall. The optimal pipetting procedure for these types of samples was found to cease pipette tip pre-rinsing and to include a sample pipette tip wash out. This procedure was implemented for all CZE applications and operators were retrained and the bias and the variation decreased significantly during phase 2.

In phase 3 the throughput of the assay increased. New operators had limited CE and vaccine analysis experience, which was thought to be a potential cause of the increased variation and bias. In addition, the SSC were prepared in bulk with a mono-channel pipette 1000 μ L instead of 200 μ L pipette. A mono-channel pipette of 1000 μ L has a higher variation than a 200 μ L pipette. By reintroducing the mono-channel pipette 200 μ L for SSC dilution the variation decreased proportionally in phase 4.

In phase 5 a re-evaluation of method execution took place, and it was found that the correct way of pipetting needed retraining. In phase 6 the method was executed for a long period by the same operators. In phase 7 many new operators were trained quickly in response to the COVID emergency for process development. The number of operators increased from 7 in start of phase 7 to 12 end of phase 7. All taken together, pipetting had a large

impact on the method precision. Appropriate pipettes should be selected, and operators should be made aware and receive adequate (recurrent) training.

Important to note is that during process and product development, sample composition and the purpose of the analysis changed. A certain amount of flexibility and ruggedness should be anticipated during analytical method development. However, not all changes can be anticipated but may cause the method to be out of control and provide unreliable results. The implementation of siliconized vials for DP was an example of such a change. Continuous involvement of the analytical experts in process and product development study plans resulted in anticipation. A theoretical assessments and feasibility test provided the evidence to continue testing reliably and in-control.

When changes are not so obvious and shared in advance, careful evaluation of SST, sample, and trending results, are of essence to identify potential erroneous results due to process and product changes. Continuously looking out for “out of the ordinary” observations, performing investigations and improving the method precision and ruggedness, yielded a higher control of the analytical test method. Consequently, less time was spent on troubleshooting impacting project and process timelines.

Each issue that caused a delay in normal assay execution was logged. Costly and reoccurring issues could be targeted and solved by systematic, lean, problem solving. For example, the PVA capillaries used for adenovirus quantitation were prone to degradation and adsorption (paper **III**). Often the SSC was started and indicated malfunctioning of the capillary. This caused operators about 4 h a week extra. The vendor was informed regarding the issues. Together with the vendor the reason for higher degrees of malfunctioning in batches was researched. Root-causes were found and respectively tackled. In addition, control measures were put in place at the vendor to evaluate batch quality for adenovirus content determination. This provided an earlier feedback loop to the vendor and prevented the use of poor capillaries during analysis.

Systematic removal of roadblocks in assay execution resulted in less resources spend on an assay runs and troubleshooting.

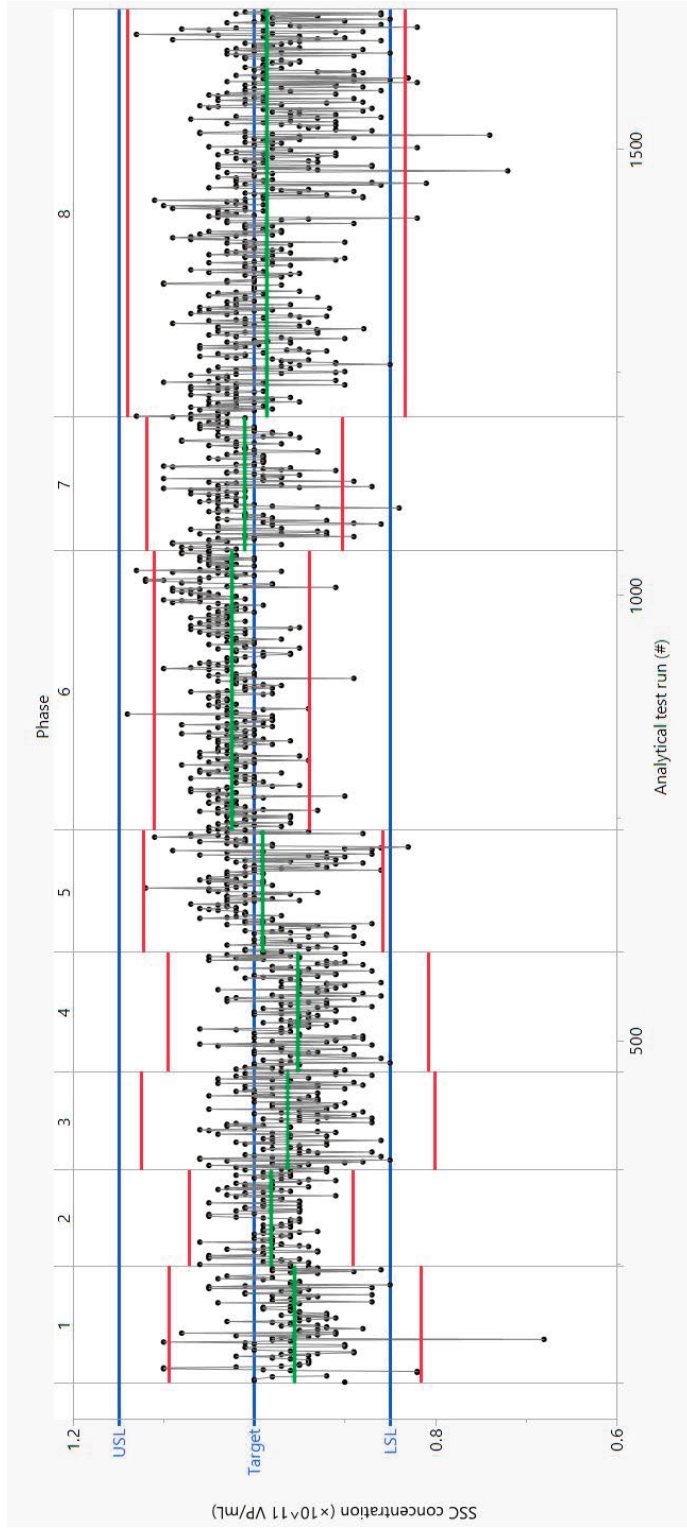


Figure 11. Control chart of the SSC concentration response determined in 1656 assay runs. The SSC concentration results from each consecutive test run are depicted as black dots and connected chronologically with a grey line. The targeted value, lower specification limit (LSL) and upper specification limit (USL) are depicted as blue lines. The different phases are separated with grey lines and annotated at the top. The phases' averages are depicted as green lines, and the phases' lower control limits (LCL) and upper control limits (UCL), i.e.: 3 x standard deviation, are depicted as red lines.

Applications

“Recycle, recycle. It is fun to recycle.”

Peppa pig

The CZE method was applied for many different purposes (paper V), a few are highlighted here. For each of these applications previously obtained knowledge regarding the method during method development and captured in the FMEA could be re-used and amended.

Throughout the vaccine production process, it was important to know how many adenovirus particles were present, and whether the adenovirus particles were stable, or degraded. In CZE, the adenovirus particles are separated based on their size and charge. Adenovirus particle modifications, disintegration, and aggregation change the size and/or the net charge of the virus particle. Indeed, the CZE method could be used to determine adenovirus particle loss during formulation studies, and in stability studies, and to characterize degradation pathways and identify new CQAs.

At the start of downstream processing, the adenovirus producing cells are lysed and domiphen bromide is added for host cell DNA precipitation and LH clarification. However, domiphen is a cationic detergent and interacts with the negatively charged adenovirus as well, changing the charge, size, and solubility of the adenovirus particles. This interaction was studied with CZE to understand the interaction and optimize the clarification process. Increased domiphen concentrations resulted in increased adenovirus particle peak widths and migration times in CZE. At higher domiphen concentrations several peaks were visible, and the adenovirus concentration decreased with time, indicating precipitation of the adenovirus particles and loss of yield. Based on CZE results, robust and optimal harvest clarification domiphen concentrations could be selected to maximize DNA removal and minimize adenovirus particle loss. At the selected domiphen concentration, no adjustment to the CZE method was needed.

For novel low dose vaccines, the method required adjustment of the range and with that of the sensitivity. The injection volume could easily be increased 35-fold due to the combination of the tris-tricine BGE and the presence of chloride in the sample. This use of tricine in the BGE created a tITP stacking mechanism, where low net charge, slow-migrating tricine acted as a terminating electrolyte. The chloride in the sample acted as a leading electrolyte. Approximately 50% of the capillary could be filled with sample, resulting in LOD of 5×10^8 VP/mL (0.8 pmol/L) and an LOQ of 1.5×10^9 VP/ml (2.5 pmol/L) for DS and DP samples. However, the capillary robustness decreased with the exposure to high amounts of sample. Therefore, a robust method with a 10x higher injection volume was validated for quantitation of adenovirus particles in low dose vaccines, (Paper V, Table 1 application 16).

In addition to Ad26 and Ad35 other adenovirus types were explored. This CZE method could separate all tested adenovirus serotypes, see Figure 12. Therefore, the method could potentially be used for identity testing, and possibly also content testing. For the quantitation of other types of adenovirus separation optimization for better quantitation is advised. The knowledge obtained during development of the current CZE method could readily be applied to optimize the method for other adenovirus types.

The Ad26 CZE method detects many other anions than the adenovirus. One of these anions is chloride. Chloride has no significant UV-absorbance but could be detected indirectly. With a separation time of 0.5 min a precise and accurate method for the quantitation of chloride could be established quickly for Ebola emergency process validation.

In addition to chloride also DNA and protein could be detected. In one of the examples a new peak was observed in LH and CH samples. Concomitantly AEX-filter blockage was observed during purification. In the UV-spectrum of the new peak an absorption maximum at 280 nm was observed and was, therefore, suggested to be a protein. Further investigation of the AEX-filter blockage revealed that the cause of the AEX-filter blocked was an HCP. Future AEX-filter blockages were prevented by observing the extra HCP peak in the CZE electropherogram.

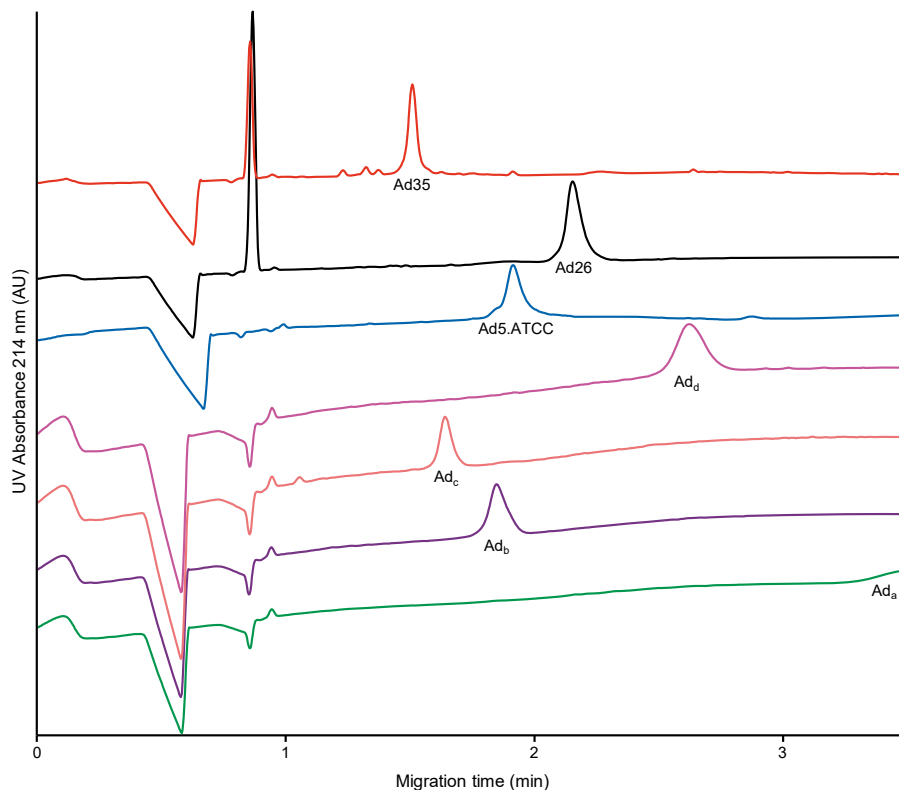


Figure 12. Electropherograms of different adenovirus serotypes analysed with CZE. The migration time of each adenovirus serotype was different (paper V).

Transfer

After method development the methods were made ready for execution. During the COVID pandemic, the CZE adenovirus content methods were executed by new operators, on other instruments, in other labs, in other countries, etc. Training and method transfer between the development and the execution labs were required.

Training and transfer were prepared by updating the FMEA considering the new operators and locations. This meant that more extensive risk mitigation was potentially needed if expertise and standardized mitigations (procedures) were lacking.

One of the first tasks during the method installation was a gap assessment to identify everything that is needed to be set in place before analysis. The FMEA and test procedure were used for completeness and mutual risk understanding. The gap assessment was translated into a plan to close the gaps. The activities that took the most time were started with, i.e.:

1. Lab quality level (e.g.: biosafety level)
2. Instrument (and software) quality level
3. Materials (consumables, solutions, references, etc.) in
4. Operator trained
5. Proven analytical test performance.

Common practice in industry is that the receiving operators are being trained by a standardized approach like a 1 “see”, 2 “do under supervision”, 3 “do without direct supervision” training. This limited training approach mainly focused on test procedure execution and assumes knowledge and experience with the technology used. When implementing a new technology, a holistic view on training, including teaching the fundamentals and best practices of a technique, the specific instruments and the software’s is required (Paper V). The test procedure itself is then the last step in the training. This approach, in combination with enough trial runs for the Ad26 CZE method, resulted in operators with know-how and experience regarding the technology and method. The number of and time spend on troubleshooting was significant lower and the holistic training approach set the basis for the operators to perform troubleshooting with minor support and, eventually, independently. After transfers, intense method maintenance and expert coaching during the first period of method operation were made part of analytical method transfer.

5. icIEF mini-HA purity

A method for mini-HA charge purity was needed. Several technologies were considered and imaging capillary isoelectric focusing (icIEF) was selected for development (Paper V), see Table 6.

Feasibility of icIEF with standard conditions was tested for the analysis of mini-HA. The mini-HA is known to have 3 N-glycan sites and 1 O-glycan site, with potential sialic acids on each of them. Because of the sialic acids distribution heterogeneity in a sample a broad range of unresolved peaks was observed during feasibility. A neuraminidase treatment was implemented to remove N-glycans.

Process development samples may contain high amounts of salts, up to 200 mM, which may disrupt the electric field required for icIEF. To avoid the effects of high salt concentrations, a desalting sample pre-treatment was implemented based on on-filter buffer exchange.

An FMEA was used to prioritize CMP optimization and different icIEF master mix compositions were tested to optimize peak separation. First the concentration of urea was optimized. The apparent pIs shifted about 1 pH when 6 M urea was added to the master mix. A 1.5 M urea was selected to have maximum icIEF peak efficiency and minimal protein unfolding effects determined with circular dichroism. Second, several ampholyte mixtures were tested to optimize separation. A mixture of 25% ampholyte pH 3 - 10 and 75% Servalyte pH 4 - 9 resulted in the highest number of peaks present and gave a repeatable peak pattern. A 13 mM iminodiacetic acid anodic spacer was added to the master mix to obtain a robust separation in the detection window for 10 minutes focusing. The rest of the standard protocol as suggest by the vendor were used and did not require optimization. An electropherogram with the final conditions is given in Figure 13.

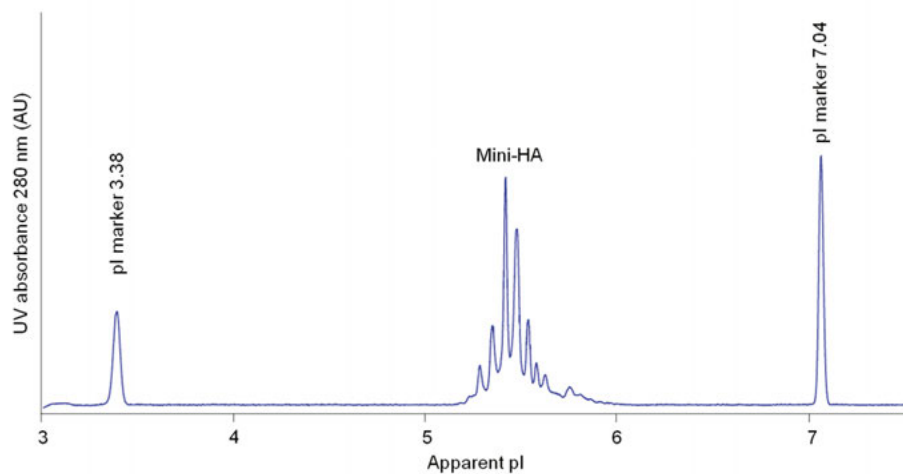


Figure 13. Example electropherogram of a mini-HA analysed with icIEF (paper V).

The icIEF method was validated for process and product development and characterization (paper V). A pI repeatability of 0.0% – 0.7% RSD, % peak area repeatability of 0.5% – 0.7% RSD, and an LOQ of < 0.1 mg mini-HA/mL was obtained. The method was proven to be stability indicating and was used in stability studies for degradation pathway analysis and CQA identification.

Table 6 Technique selection for mini-HA charge purity determination.

Requirements		Techniques				
Characteristic	Target [range]	Priority	cIEF	icIEF	CZE	IEEX
Specificity	mini-HA charge variants	1	Mini-HA charge variants	Mini-HA charge variants	Mini-HA charge and size variants	Mini-HA charge variants
Matrix	10 mM – 150 mM NaCl + 5% sucrose	1	< 50 mM NaCl	< 50 mM NaCl	< 500 mM NaCl	10 mM – 150 mM NaCl + 5% sucrose
LOQ	> 0.1 mg/mL mini-HA	2	1 mg/mL	0.1 mg/mL	0.1 mg/mL	0.1 mg/mL
Time to result	1 day	3	1 day	1 day	1 day	1 day
Expected sample load per month	10	3	> 10	> 10	> 10	> 10
Development time	3 months	2	3 months	3 months	6 months	6 months
Development costs	medium	2	medium	low	high	high
Equipment available	yes	2	yes	yes	yes	yes
Risk of not meeting requirements			Sensitivity	Access to instrument	Little experience and no platform methods available	Little experience and no platform methods available

6. AQbD gain

“You can work hard, but if you don’t work smart, you’ll work for the rest of your life.”

Iron man

In total 16 CE applications for viral vaccines analysis were developed (Paper V). The first application for influenza HA quantitation was developed without making use of the AQbD process (paper I). While further researching the CE-SDS separation mechanism and establishing a standardized method development strategy a first version of AQbD was retrospectively used (paper II). A full cycle of AQbD (understand & design, performance, and use & improve) was applied for the development of the adenovirus quantitation method with CZE (paper III and IV). AQbD was also used for extending the scope of the Adenovirus quantitation CZE method, and analysis of HC DNA with CGE, mini-HA with icIEF, bromide with CZE and chloride with CZE (paper V). In addition, the established AQbD strategy to develop efficiently CGE methods for viral vaccine protein analysis was applied for the development of CGE method for mini-HA purity and sIPV identity determination (paper II). The AQbD process was continuously evaluated, and lessons learned were applied during the development of the 16 CE applications for viral vaccine analysis. The current AQbD process flow is depicted in Figure 14.

Applying the AQbD principles overall resulted in a decreased analytical method development lead time. The development lead time from scratch to a validated method decreased from 55 weeks to 20 weeks on average, while the number of methods developed per year increased a 4-fold and the workforce about 2-fold. Minor method adjustments lead times decreased from > 1 month to between < 1 week and < 1 month, depending on the type of adjustment and test at hand.

The overall assay quality improved. The percentage invalid assay runs per analytical test method were 0% – 35% in QC and decreased to 0% – 11% on average for all assay runs performed for process and product development and characterization. Before AQbD was used, about 25% of the methods did not adhere to the expectations of the analytical scientist in need of the test results and > 50% of the validation studies resulted in redevelopment and

validation study amendments. After AQbD implementation almost all developed methods adhered to the expectations. About 10% of the analytical test methods required redevelopment at a later stage, for example during amended validation studies, or because of adjusted ATPs or change of materials. Overall, a major gain regarding method development throughput and quality was achieved in 6 years of implementing and continuous improving AQbD method development. Much more can be still gained by full adaptation of this scientific, functional and pragmatic approach of AQbD method development.

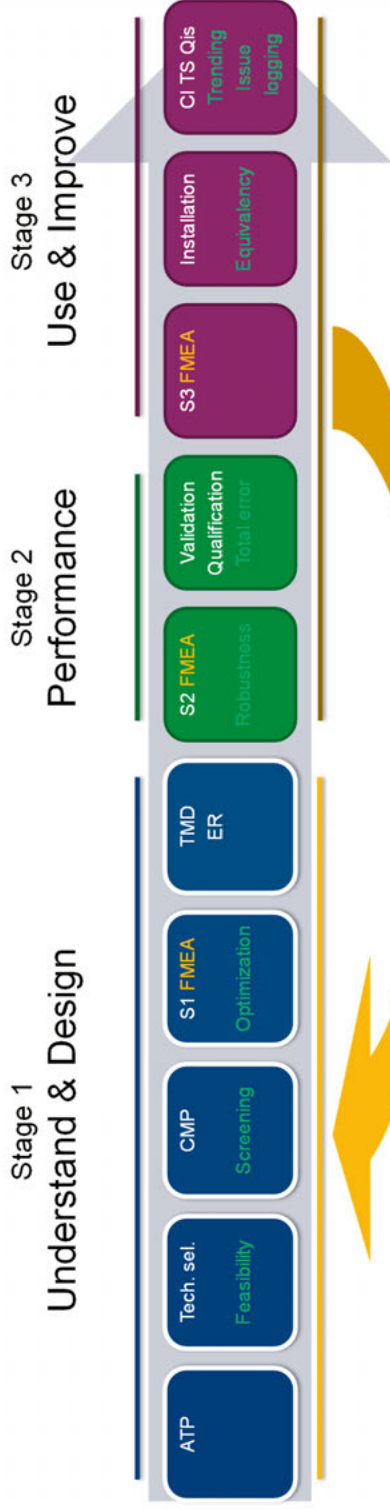


Figure 14. Schematic overview of the improved A QbD approach. In stage 1 the purpose of the method is established and a technique is selected, followed by method understanding by performing DoE, and method design by CMP identification and FMEA risk assessment. In stage 2, the performance of the method is assessed via FMEA updates, robustness testing, and method validation. In stage 3, the method is installed, the performance is monitored, and subsequent method improvements and adjustments are being made. Findings in both stage 2 and 3 could be a cause to feedback into a stage 1 process step.

7. Further discussion

Throughout the development of these CE applications, similar challenges were observed. Large biomolecules are expected to result in efficient, narrow peaks due to low diffusion coefficients. However, sometimes broad peaks were observed. The reason for this is not inefficient separation but rather the consequence of large biomolecule heterogeneity and the large number of possible modifications. CE is capable to determine the type and extent of heterogeneity and has the potential to link this to biological function and safety. Additionally, spikes in the electropherogram due to analyte aggregation and adsorption of the analyte to the capillary were more often observed. Both could be counteracted by improving solubility through proper BGE design and selecting an adequate capillary.

For the CZE adenovirus method development, the design of an effective BGE was the key to success. The stability of the virus particles, the $pK_{a,s}$ of the buffering components with respect to the adenovirus pI, the buffering capacity, the conductivities, electromigration dispersion, and robust preparation were considered for BGE buffer selection. For the adenoviruses, the tris and tricine combination was optimal. For other viruses and large biomolecules tris and tricine may be very effective as well. The concentrations may require optimization. Other low conducting buffer components, such as MOPS, bis-tris, MES, HEPES etc. may be more effective, depending on the analyte, sample, or purpose of the method.

In CE, the only material sample components come into contact with is the capillary wall. Proteins, virus particles, and other biological components have strong adsorption to the silica of the capillary wall, hence coated capillaries were used. Several capillaries were screened, and the three types of neutral capillaries had good potential for further development. PVA was selected from these three to be the most practical in use. Other neutral capillaries may be used as well, though it is important to note that the CS to limit adsorption was optimized for the PVA neutral capillary and would require redevelopment if another neutral capillary would be implemented.

Poor PVA coatings were often the reason, but not the root-cause, for test issues and resulted in adsorption and nonrepeatable results. A close collabo-

ration with the capillary and coating manufacturers was needed to bring together the knowledge regarding the processes of making the capillary and coating and the product and analytical test details and find the root-cause. In general, it is important to understand the effect critical reagents and materials may have on the analytical test results. A close collaboration with vendors is beneficial but may not be sufficient and additional in-depth research may be needed for specific analytes and methods.

Pure standards, high-grade materials and reagents, and best practice preparations are needed for good methods. Ultra-pure or well-characterized standards for peak identification are hardly available for viruses and vaccines. Because of the analyte heterogeneity in combination with the nL sample volumes and the μL BGE volumes used in CE, peak identification by fraction collection in CE is challenging. BGEs, such as those used for the mentioned applications, and complete viruses are not compatible with other techniques such as MS. Nonetheless, good efforts are being made to couple MS to CE for protein analysis [171, 174, 183-186].

The use of high-grade chemicals and solution preparation best practices are important to improve assay ruggedness and robustness. Low-grade chemicals could potentially cause unwanted effects on the sample and BGE. Solution preparation best practices for capillary electrophoresis start with weighing in chemicals, instead of determining volumes or adjusting the pH or ionic strength manually. In addition, it is good practice to filter and/or sonicate solutions when chemicals are dissolved, or particles are to be expected. Reversed pipetting techniques could be beneficial in avoiding air bubble introduction during liquid transfer. The effects of solution temperature, solution viscosity, or pipette tip adsorption on pipetting accuracy should not be overlooked.

In assay kits the standards, materials, reagents, and sometime best practices are provided and are out of developer or operator control. When the kit quality is adequate, fast development times may be possible and an assay may be easily and robustly executed, e.g.: CE-SDS application for the analysis of mAbs. However, no adequate assay kits were available for the analysis of viral vaccines and from scratch method development or existing method adaptation was needed. In our experience, designing a method from scratch, using the AQbD principles, often resulted in a better method understanding and control and, consequently, led to a more robust and fit-for-purpose assay compared to off-the-shelf applications. A fundamental understanding of the technology and the critical mechanism creates the opportunity to re-use the knowledge for new method developments, method adjustments for other scopes, method improvements, and troubleshooting. Such an investment would be beneficial for assay kits as well and could result in effective and efficient method development strategies such as the viral vaccine protein analysis with CGE AQbD method development strategy.

The risk of having standardized AQbD approaches, like a CGE method development strategy, is that decisions could be made empirically or even without proper scientific understanding. Running DoE and multivariate DoE without a predefined goal or mechanistic understanding of CMP and their effects will result in a false sense of method control. Such a checkbox exercise will not result in optimal and robust methods. Another risk can be that applying the AQbD process as an “obligation” from the ICH Q14 or USP <1220> guidelines becomes a purpose in itself, instead of a tool to support a scientific process.

Eventually, the analytical test is often not performed by the developer. The test result’s reliability is strongly dependent on how well the method was executed. Therefore, adequate training of operators is important. Before training the method procedure, knowledge, expertise, and experience, needs to be gained by fundamental training on the product, technology, instrument(s) and software, followed by best practices. When this is established, on the job training of the method procedure can effectively be performed. Sufficient trial runs need to be performed before method validation and analysis of real test samples. The more in-depth training of operators yielded less assay issues, and less laborious troubleshooting. The above is generally valid but becomes more visible when implementing new technologies.

Good science, method development, and test result interpretation cannot be replaced by automated robots (maybe by artificial intelligence one day). There will always be a need for evaluation of the test results with respect to the patient.

In total, the time we took to invest in understanding CE technology and develop applications resulted in a wide range of CE applications and strategies supporting viral vaccine analysis. CE took viral vaccine testing beyond what was previously possible and improved process and product understanding and, overall, the control of viral vaccine production with respect to safety, efficacy, and quality. In an emergency situation such as the COVID pandemic, fast, easy to develop and powerful analytical technologies was a game changer. During the COVID pandemic, the adenovirus CZE method played an important role. The CZE method could immediately be applied for rapid production process upscaling and development. Adenovirus yields were pushed to a maximum and could be achieved in a short time frame due to fast and precise CZE analysis. Through method understanding, the method could be quickly adapted to these higher concentration samples and the consequently changed process sample matrices. Minor adaptations regarding tiered sample dilution were applied to limit assay variability and operator errors for QC testing. Production was scaled up, and production and testing were transferred overseas in a very short time frame. All COVID re-

strictions, such as no traveling and highly reduced onsite access, made adequate transfer even more challenging. A well-designed CS, based upon the scientific insights and daily operator behaviours, combined with tailored holistic approach of trainings resulted in quick and robust implementation of CZE abroad. This significantly shortened the test cycles during trial and transfer production. In summary, this example illustrates the power of both the scientific AQbD attitude and the potential of CE.

8. Conclusions

Novel CE methods were successfully explored, developed according to AQBd, and applied for rapid and precise characterization of influenza, polio, and adenovirus vector vaccines.

In Paper **I**, the applicability of a commercial CE-SDS Ab analysis kit was shown for fast, accurate, and precise determination of the HA concentration and the characterization of other influenza proteins in in-process samples. The sample pre-treatment, injection, and separation conditions were optimized to improve the separation, sensitivity, and time from sample to reported results. Gel buffer dilution and temperature optimization improved separation. The novel CGE method was validated and applied to support annual influenza vaccine development and was superior to the previously used SDS-PAGE, SRID, and RP-HPLC methods.

In paper **II**, an effective AQBd CGE method development strategy was established based on studies to identify and to understand the effects of the most important CMPs. Separation CMPs were explored and the effects of gel buffer dilution and temperature on the viscosity, ionic strength, and conductivity and, thereby, the impact on separation, peak efficiency, and analysis time was determined for electrokinetic and hydrodynamic injection. A four-step approach was established to develop a viral vaccine protein CGE method within week(s). The strategy was effectively applied for the development of mini-HA purity and sIPV identity CGE methods. The influenza proteins, the mini-HA glycoprotein, and the sIPV proteins were distinctly different analytes, illustrating the broad applicability of this AQBd method development strategy.

In paper **III**, a fast, precise, accurate, and robust CZE method for the adenovirus concentration determination in all process intermediates was developed applying AQBd principles. A tris-tricine BGE was designed considering the physicochemical and electrophoretic properties of the analyte and sample and was optimized with multifactorial DoEs. A PVA-coated capillary was selected for both electrophoretic as well as practical reasons.

An effective CS to limit adsorption was designed and comprised a combination of addition of a detergent to the BGE, of reversed flushing with phos-

phoric acid, and of decreasing the temperature to 15 °C. A stringent SST, bracket controls, and trending of the SSC were incorporated to flag onset of capillary degradation and significant adsorption. For crude, DNA-containing samples a Benzonase sample pre-treatment was optimized with multifactorial DoE.

The adenovirus concentration determination with CZE was validated. The time from sampling to reported result was < 2 h (5 min run time per sample), precision was $\leq 5\%$ RSD, with accuracies of 90 – 110%. The short time from sampling to reported result combined with high precision of the method significantly improved the production process throughput, resulting in more and higher quality products. Additionally, the CZE method could distinguish between different adenovirus strains and has the potential to detect impurities and viral degradation.

In Paper IV, the effective CS for the rapid analysis of in-process control samples of adenovirus vaccines proved to be successful for in-process testing. CZE and qPCR method comparability was demonstrated, and qPCR was replaced by CZE. An SST was designed with stringent fit-for-purpose acceptance criteria to prepare for fast, precise, accurate, and robust sample analysis. Only 3 out of 525 test runs were not reported in time due to test issues during sample analysis, other issues were flagged and solved prior to testing.

The performance of the 525 runs were within the expected predicted confidence intervals from method validation. All taken together, an effective and fit-for-purpose CS was designed to produce high amounts of high-quality adenoviruses.

In Paper V, sixteen CE applications for vaccine process and product development, characterization, and QC testing were developed.

CGE methods were developed and validated to control the quality of critical mAbs for bioassay use, influenza HA quantitation and protein characterization, mini-HA purity testing, sIPV protein identity testing, and HC-DNA characterization. A commercial CE-SDS application was optimized for HA, mini-HA, and sIVP protein analysis (paper I and II). The analysis time of the determination of the HA concentration was further decreased with switching from long-end to short-end injection, without loss of method performance. Mini-HA glycosylation could be characterized by using combinations of different deglycosylation enzymes.

An icIEF method was developed for mini-HA charge purity determination. Impurities and degradants of mini-HA were observed during forced degradation studies with the CGE and icIEF method.

The CZE method for the determination of the adenovirus concentration for in-process control testing (Paper III and IV), was additionally validated for product release and stability testing and bioreactor seed release test-

ing. With the CZE method, new insights were obtained regarding adenovirus degradation, domiphen adenovirus interaction, surface charge, aggregation and precipitation, and host cell protein process filter blockage.

A chloride concentration determination method could be rapidly derived from the CZE adenovirus method that was more sensitive and selective for complex process samples than existing methods.

Another CZE method was developed for the determination of the bromide concentration in presence of high amounts of chloride. The method was used to determine bromide clearance throughout adenovirus purification.

An analytical quality by design approach was used to develop these CE applications. AQBd was an important tool that supported and documented scientifically sound and risk-based decision making with respect to a clearly defined method purpose.

9. Future perspectives

The analytical toolbox for vaccines still holds a lot of expansion potential for CE.

Currently, the icIEF method was only applied for viral proteins. IcIEF was tested for intact adenovirus particles as well but was thus far unsuccessful. The challenge in icIEF analysis of virus particles is the stability and solubility of the virus particles and the characterization and the identification of the observed peaks.

The CZE method was used for in process hold-time and degradation studies. So far, only the peak area was used to determine loss of adenovirus. Additionally, migration time changes and the appearance of new peaks were observed. The new peaks were not yet identified and could potentially result in the characterization and quantitation of degradation pathways. This has to be explored further and may result in a very sensitive method to detect early modifications of the adenovirus particles.

Interestingly, isolated cellular DNA peaks were observed in the adenovirus CZE method. This suggests that CZE could very well be used for determination of free DNA of in-process samples.

Both the chloride and bromide analysis with CZE illustrate the power of CE to determine inorganic ions with CE in complex matrices. In general, small inorganic and organic ions, such phosphate, citric acid, etc., could be easily determined with CZE in for example bioreactors [150, 151, 187-189].

For other vaccine types, CE is becoming an indispensable asset as well. For mRNA lipid nanoparticle (LNP) vaccines often limited sample volumes are available. CE would be ideal to study the size, charge and abundance of the LNPs, mRNA, and impurities of both the production process and the product itself [190-193]. Potentially the LNP morphology could be studied if emphasizing on size separation, or the loading degree by determining the ratio of the RNA concentration (RNA stain) in the LNPs and the LNP concentration. Similarly, virus like particles (VLP) such as adenovirus associated viruses [194-196] and human papillomavirus VLP [133, 134] could be analysed and characterized with CE. Different strains may be produced and identified with CZE, similar to adenovirus vectors, or CGE, similar to sIVP vaccines. Historically, vaccine content was determined by total protein determination, OD280, BCA, Lowry, etc. However, these techniques are non-specific and often suffer from matrix interference, especially for in-process

samples. A procedure to uniformly coat the proteins such that the size/charge ratio is equal, such as with SDS, could be used to separate the protein from the matrix with CZE. This would result in a specific and fast total protein concentration determination.

Beside content, purity, and identity, the potency of vaccine products may be studied. The potency was used to be determined by cell infection and consequent cell death. Currently, often antigen expression is requested by health authorities to be more specific for the mode of action of the vaccine rather than the vector. Rustandi, *et al.*, have used capillary Western blotting (cWB) for automated antigen expression quantitation after cell culture infection with the vaccine product [127, 135]. cWB uses specific antibodies and is as specific as other bioassays. However, cWB is much faster and quantitative than for example Western Blot. cWB may be applied to all types of vaccines that induces antigen expression. Alternatively, affinity CE [197-202] or other in capillary technologies such as Flow Induced Dispersion Analysis [203] can be used for antibody antigen interactions.

In addition to vaccine development, CE could be used for diagnostic purposes. Antigen and PCR tests were for example used during the COVID pandemic. Both are highly specific, but the PCR test is laborious and expensive, and the antigen test is not very sensitive. CE has the potential to be specific, sensitive, cheap, and fast. CE could replace or complement the PCR test in the laboratory. A home test should be possible since the CE technology is rather simple and can already be performed on printable chips and efforts are being made to couple them to batteries or mobile phones [204]. A good understanding of separation of viruses from body fluids would be necessary to set up a development strategy for such CE tests. Sample pre-treatment such as for the antigen test may be used as well.

Overall, AQbD method development and CE have taken an increasingly important role for the characterization of viral vaccines and has the potential of becoming a game changer for infectious diseases prevention and control.

10. Popular scientific summary

Vaccines teach one's body to fight viruses and bacteria. Harmless parts of the viruses and bacteria are in the vaccine. Making safe vaccines that work is difficult. Tests are used to prove that the vaccines are safe and working. With these tests we determine which parts, how many parts, and whether the right parts, are in the vaccine. Developing these tests and developing vaccines cost a lot of money and time.

In this study, faster and cheaper tests were explored and developed. Capillary electrophoresis (CE) is a technique that we used to separate the vaccine parts by their charge and size in a narrow open glass tube, the capillary. In the capillary, the parts move through a solution with electrolytes, the so-called background electrolyte (BGE). At the end of the capillary ultraviolet light is shone through the capillary and on the vaccine parts. The parts block a part of the light. Based on the leftover light it can be measured which parts and how much of the parts were in the vaccine. CE is a fast and cheap technique.

A limited number of CE tests have been developed for vaccines until now. So, new CE tests were needed. An analytical quality by design (AQbD) approach was followed to develop new tests based on knowledge and experience. Newly required knowledge was created throughout experimentation. The knowledge about CE and the vaccine was used to create tests that answered the right questions and that prevented mistakes during testing. The new knowledge was reused to rapidly create new CE tests for similar questions.

First, an existing CE test with a gel BGE was used to find out whether critical test materials were good for use. The gel BGE was used to separate the vaccine parts by size, like a sieve. Second, the gel BGE was modified to determine how many flu parts were in an influenza vaccine. The effects of gel BGE modification on separating the parts was studied. A plan to quickly make good new gel BGE CE tests was made. Following the plan, CE tests were developed to determine impurities in a novel flu vaccine, and to confirm whether the right polio parts were in a novel polio vaccine.

Another type of CE test was developed to determine how many adenoviruses were in a vaccine. Adenoviruses are small packages that bring the virus or bacterium genetic parts into your body to teach the body to fight the disease. A novel BGE was explored to separate the adenoviruses package from

other vaccine parts. The result was a fast, cheap, and accurate CE test. We could use the same CE test to determine other parts of the vaccine and to find out when the adenovirus breaks down.

In total, new CE tests that were faster and cheaper were rapidly developed and resulted in safer and better vaccines that could be made faster and cheaper.

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