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A Practical Approach to Implementing ICH Q14: Tools for Analytical Quality by Design in Capillary Electrophoresis Method Development

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ABSTRACT

The ICH Q14 guideline introduces a structured framework for analytical method development based on Analytical Quality by Design (AQbD) principles, aiming to ensure robust, reliable, and fit-for-purpose methods throughout the product lifecycle. However, implementing ICH Q14 remains challenging due to the lack of complete examples and training resources, making it difficult for organizations to translate theory into practice. Although previous studies have applied AQbD to capillary electrophoresis method development, many have focused only on specific aspects such as the design of experiments (DoEs) or analytical target profile (ATP), leaving a gap in providing comprehensive, practical tools for the entire analytical lifecycle. This manuscript presents a novel, user-friendly approach to implementing ICH Q14 and AQbD, offering ready-to-implement tools and methodologies that simplify the process of method design, optimization, validation, and implementation. Through a stepwise process, the approach provides practical solutions for integrating AQbD principles into everyday workflows, bridging the gap between theoretical concepts and real-world applications. The approach has been thoroughly tested in diverse industrial settings, demonstrating its reliability and effectiveness. This work aims to facilitate the adoption of AQbD in analytical method development by providing structured tools, lessons learned, and best practices that align with ICH Q14 guidelines.

Abbreviations: Ad, adenovirus; Ad26, adenovirus serotype 26; Ad35, adenovirus serotype 35; AEX, anion exchange; AEX-HPLC, anion exchange HPLC; AF4, asymmetric flow field-flow fractionation; APCS, analytical procedure control strategy; AQbD, analytical quality by design; ATP, analytical target profile; BGE, background electrolyte; CCD, central composite design; CH, clarified harvest; CMP, critical method parameter; CQA, critical quality attribute; CR, crude harvest; CRS, chemical reference substances; DF, diafiltration/ultrafiltration product; DM-β-CD, dimethyl-β-cyclodextrin; DNA, deoxyribonucleic acid; DoE, design of experiments; DS, drug substance; DSP, downstream processing; FCCD, face centered central composite design; FDA, Food and Drug Administration; FMEA, failure-mode effect analysis; HA, hemagglutinin; HDMS-β-CD, hexadecyl-modified β-cyclodextrin; ICH, The International Council for Harmonization; IPC, in-process control; LH, lysed harvest; LSL, lower specification limit; MODR, method operable design region; nCMP, non-critical method parameter; OD260, absorbance spectrophotometry at 260 nm; OFAT, one factor at a time; PAR, proven acceptable ranges; pCMP, potential critical method parameter; QC, quality control; qPCR, quantitative polymerase chain reaction; QTPP, quality target product profile; rCE-SDS, capillary gel electrophoresis with sodium dodecyl sulfate under reducing conditions; RP-LC, reversed phase liquid chromatography; RSM, response surface model; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; SRID, single radial immunodiffusion; SSC, system suitability control; SST, system suitability test; S-γ-CD, sulfated γ-cyclodextrin; TETA, triethylenetetramine; USL, upper specification limit; USP, upstream processing; UV, ultraviolet.

1 | Introduction

The International Council for Harmonization (ICH) Q14 guideline introduced a structured framework for the development of analytical procedures by Analytical Quality by Design (AQbD) principles [1]. ICH Q14 aims to ensure that analytical methods are robust, reliable, and fit-for-purpose throughout the entire product lifecycle. Despite its clear benefits, the practical implementation of ICH Q14 is challenging due to the lack of complete examples and official training material, adding to the difficulty of translating theory into practice for many organizations.

In recent years, several studies have successfully applied AQbD principles to capillary electrophoresis method development, even before the publication of the ICH Q14 guideline [2–29]. Many existing studies, though not specifically focused on CE, focus primarily on demonstrating theoretical benefits [9, 30–36] or only parts of the AQbD process, such as an analytical target profile (ATP) or a design of experiments (DoEs) [5, 12, 13, 17, 22–24, 27, 37–49]. Although these studies have made valuable contributions to the field, there remains a gap in providing a complete overview of the whole analytical life-cycle and practical tools to facilitate the implementation of ICH Q14 and AQbD principles. In this manuscript, we present a novel, straightforward, practical approach to implementing ICH Q14, providing tools and methodologies specifically designed to simplify the adoption of AQbD principles in analytical method development. Our approach offers ready-to-implement solutions that align with the ICH Q14 guideline, facilitating the design, optimization, validation, and implementation of new analytical methods in a more accessible and reproducible manner. Our focus is on practical applicability and ease of integration into everyday workflows by breaking down the workflow in smaller, ready to use steps, and on bridging the gap between theoretical AQbD concepts and real-world implementation.

Here, a stepwise approach is presented that has been thoroughly tested over many years across various companies, demonstrating its reliability and effectiveness in diverse industrial settings. Structured and user-friendly tools are discussed, continuously comparing our approach with the ICH Q14 guidelines. Throughout, we share lessons learned and best practices for each of the steps to smoothen the roll-out of AQbD within an organization, illustrated with examples from the current literature.

2 | ICH Q14 and AQbD

The ICH Q14 guideline describes a systematic approach that integrates AQbD principles to ensure the robustness and reliability of analytical methods. This process begins with defining the ATP, which outlines the purpose of the method and sets performance criteria, ensuring that the method is aligned with the product's critical quality attributes (CQAs). Next, knowledge and risk management tools are used to identify the method parameters that could impact the performance and to prioritize the parameters that need to be experimentally investigated. During the method development phase, systematic experimentation, including DoE, is recommended to evaluate the influence of method parameters on method performance. This approach allows to explore a range of conditions and optimize the method efficiently. From this, a design space can be established, defining the boundaries within which the method performs reliably. By operating within this design space, flexibility is ensured without compromising the method's robustness. A comprehensive control strategy is then implemented, comprising of suitable controls and system suitability tests (SSTs), to ensure the method consistently meets its predefined criteria during routine use. The method can subsequently be validated, confirming that it adhered to the ATP by evaluating parameters such as accuracy, precision, linearity, and robustness. Finally, the method is subjected to lifecycle management, with continuous monitoring and adjustment to maintain its performance over time. This lifecycle approach ensures that the method remains effective as part of ongoing quality control (QC).

Although the annexes of ICH Q14 describe mock examples of each of the steps described above, we encountered difficulty in implementing the ICH Q14 approach. Figure 1 shows a simplified and structured proposed stepwise approach for using AQbD, in compliance with ICH Q14. The flow sketched in Figure 1 represents the full method development cycle. Although it is well understood that in reality, method development is not a linear process but may involve several reiterations at different levels, we presented the flow in a linear manner for transparency purpose. Similarly, the equal length of the steps does not mean that these steps take equal time or effort. On many occasions, these steps are not performed within the same time frame or by the same individuals. Where there is a significant amount of prior knowledge, method development typically starts midway. The "Use" and "Improve" steps are typically at QC or initiated by QC; however, often support from the method development team is requested. In the following paragraphs, we will demonstrate this work flow with examples from literature. The most complete example in literature comes from a capillary zone electrophoresis method development for adenovirus (Ad) quantification, using AQbD and ICH Q14, based on a series of published studies and theses [6, 8, 28, 29, 50].

3 | The AQbD-Flow

3.1 | Method Request and ATP

The whole process of analysis starts with formulating a question for which the answer implies the need for analysis. Although this is obvious, it is often a bypassed step in the whole process. An appropriate ATP can only be described if the full purpose of the analysis, and thus the original question asked, is understood, as well as the ultimate role of the analytical results that will be created. In the ATP, the intended purpose of the analytical procedure and the characteristics of the product are captured and translated into the performance criteria (e.g., accuracy). The ATP example in ICH Q14 [1] and most ATPs described in literature [2, 3, 32] only describe the method requirements as listed in ICH Q2 R2 [51], such as accuracy, precision, specificity, and reportable range. However, a more comprehensive approach should also consider additional factors, such as business requirements, alongside method performance. It is furthermore useful to prioritize the ATP requirements to support the technologies selection. The ATP should not only capture the requirements from the requester but also incorporate the needs of the method developer and the

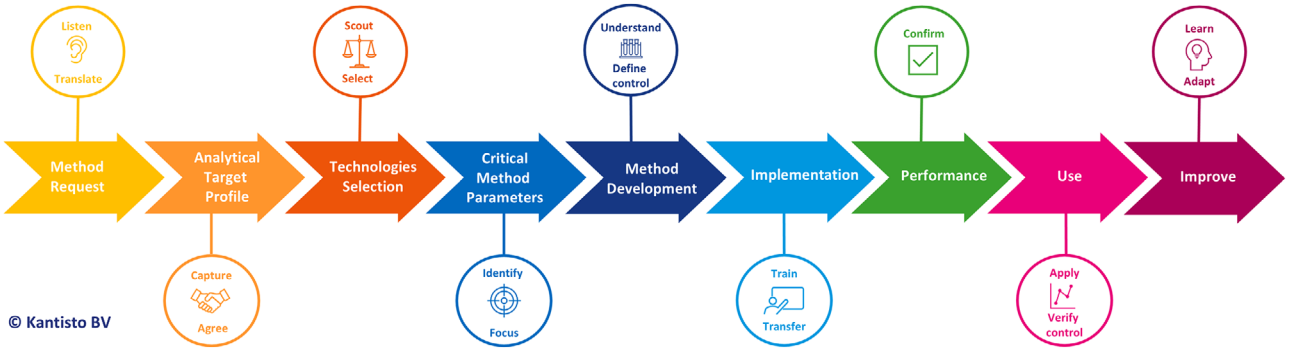

FIGURE 1 | Analytical quality by design workflow, translating the ICH Q14 components into user-friendly, smaller, and ready-to-use steps. Note that equal length of the steps does not imply equal effort, as some steps may require more time or resources than others. *Source:* Reprinted with permission, Kantisto B.V (https://www.kantisto.nl).

end-user, with analytical development in the driver's seat to guide the process to obtain a complete and accurate ATP (see Table 1). The ATP should be defined on the basis of a quality attribute or the characteristics of the process or product, remaining independent of specific techniques or analytical capabilities. The ATP serves as a critical handshake between the method developer and the project team, ensuring mutual agreement on the purpose and intended use of the method. By establishing this shared understanding, both analytical development and the project team can identify the need for further method development should the ATP requirements evolve. Additionally, the ATP provides clear guidance for method development, validation, and transfer, steering the selection of suitable analytical techniques. This approach also enhances understanding of variability sources and clarifies how input parameters influence the reportable results. A well-defined ATP minimizes additional work and frustrations, brings clarity and focus on achieving the target rather than pursuing a "perfect" analysis, and reduces the need for assumptions throughout the method development process.

The ATP used for the method development for determining Ad particles is summarized in Table 1. A method was requested to determine the Ad particle concentration throughout the whole upstream and downstream processing. The time-to-result, that is, the whole process of sampling, sample registration, analysis, result review, and reporting to the requester, was an important criterion because the production process is on-hold during an in-process control (IPC) analysis. Long lead times could lead to product degradation in the cell culture or process media. The accuracy of the measured Ad concentration was another important requirement, because the determined concentration was used to further steer and plan the production process. To develop an accurate ATP, it is crucial to gather information not only from the requester but also from the method developer, the current end-user, and potentially future end-users. End-users, who are often from a different group or location than the method developers, will ultimately use the developed method to generate results.

The following references show other examples of published ATPs with business requirements [10, 12, 20, 52] or ATPs without business requirements [2, 3, 14, 16, 53] for CE development. Simeoni et al. [52] used an ATP for capillary gel electrophoresis with sodium dodecyl sulfate under reducing conditions (rCE-SDS), aiming to replace SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The ATP not only specifies method requirements like accuracy and precision but also includes operating conditions, environmental factors, and business requirements. Pasquini et al. [20] outlined an ATP for a micellar electrokinetic chromatography method aimed at determining sitagliptin. In addition to specifying requirements for accuracy, precision, and sensitivity, they emphasized the importance of a short analysis time. Borman et al. [37] use an ATP to select a suitable method to determine three CQAs for the analysis of Ad vectors (infectivity, identity, and transgene expression), and they include business drivers in their ATP like analysis time, number of samples, complexity of the technology, and business risks. Jackson et al. [38] show how an ATP can be derived from a quality target product profile (QTPP). They also give three ATP examples, focusing on how to set meaningful requirements for specificity, accuracy, and precision.

3.2 | Technologies Selection

After the ATP is defined (combinations of), suitable technologies are selected that are estimated to be able to fulfill the quality requirements described in the ATP. The technologies selection process should prevent that commonly used and established technologies are automatically chosen, without considering better or newer, state-of-the-art technologies. We suggest including business requirements like required sample throughput, available budget, end-user, and time-to-result, as these requirements could play a critical role in selecting the right technologies or method development choices. The technologies should encompass the whole analysis flow, that is, sample preparation, separation, detection, calibration, and quantification (if required). Each technology is scored insomuch, and it can fulfill the separate ATP requirements. Prioritization of the ATP requirements helps rank the selected technologies and selecting the most appropriate ones.

During technologies selection, common pitfalls often arise from preconceived ideas or rigid preferences, such as "I need an SEC method for aggregates" or "I need an RP method for purity", which can overly constrain choices. Other challenges include adherence to established practices ("We always do it like this"), assumptions about regulatory expectations ("The FDA says this is the gold standard"), personal biases ("My professor says that this technique is not robust"), or limited prior experience ("I

TABLE 1 | The analytical target profile for the adenovirus particle concentration determination.

	Requirements		Priority	Adenovirus particle concentration determination (in VP/mL)
Requester	Aim of study/method			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. An accurate adenovirus amount should be loaded upon the anion exchange (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)
	Product		1	Adenovirus type 26 (Ad26) and 35 (Ad35)
	Product quality attribute			Virus particle concentration determination (in VP/mL)
	Process intermediate(s)		1	Crude harvest, lysed harvest, clarified harvest (IPC), anion exchange product, diafiltration product, drug substance, and drug product
	Analyte(s)			Adenovirus type 26 and type 35
	Matrix		1	The matrix could contain cell debris, residual DNA, salt, protein, and/or formulation buffer components dependent on the process intermediate
	Requester requirements	Time-to-result	2	< 2 days for process development, < 4 h for IPC testing
Method developer	ICH Q2R2 characteristics	Type of test		Content/potency
		Specificity	2	Detection of Ad26 and Ad35 without significant interference of sample matrix components or other Ad types used in the facilities
		Accuracy	3	≤ 10% bias
		Precision	2	Intermediate precision ≤10% RSD
		Concentration range	3	10^{10}–10^{12} VP/mL
End-user	End-user			Quality control
	End-user requirements	Throughput	3	One sample per run for IPC testing, > 7 samples per process development run, > 300 samples per month for process development

Abbreviations: ICH, International Council for Harmonization; RSD, relative standard deviation.
Source: Adapted from [28, 29] with permission from the authors.

tried this for xyz and it did not work"). Additionally, a lack of accessible resources, like application notes, studies, or kits, can further impede the unbiased assessment of alternative methods.

A key lesson is the importance of being a true partner in development and avoiding the automatic use of traditional methods or favored technologies. It is essential to challenge the reliance on perceived authority or past practices to ensure that the most appropriate technology is chosen. Technologies selection should involve experts, not just project or line management, to ensure that decisions are based on expertise and not on routine. Authorities may request "state-of-the-art" methods, so it is important to encourage the use of newer technologies where they offer clear benefits. Demonstrating the successful use of new technologies can also justify further budget allocation and continued investment.

The technologies selection of the method development for Ad particles is given in Table 2. Seven possible technologies were selected that might be suitable to meet the requirements from the ATP in Table 1 based on in-house methods, pharmacopoeia, contract research organizations, prior knowledge, and scientific literature: quantitative-polymerase chain reaction (Q-PCR) [54], anion-exchange chromatography (AEX-LC) [55], asymmetric flow field-flow fractionation (AF4), CZE, reversed phase liquid chromatography (RP-LC) [56], size exclusion chromatography (SEC), and absorbance spectrophotometry at 260 nm (OD260) [57, 58]. The techniques were evaluated and scored against the ATP requirements, according to priority, based on a theoretical assessment and/or feasibility experiments. Colors were used to indicate the estimated risk (high, medium, and low) to not meet the requirement from the ATP. Three out of the seven techniques met the priority 1 ATP requirements "Process intermediate(s) and matrix," meaning that only qPCR, RPLC, and CZE were possibly able to quantify the Ad concentration in all process intermediates and in the final product, independent of the sample matrix. All techniques were able to directly or indirectly quantify the Ad concentration albeit that OD260 is not specific for virus particles and AF4 cannot distinguish among different Ad types. However, direct quantification (intact virus particles) was preferred over indirect quantification (measuring a specific protein or encapsulated DNA content) as indirect quantification required assumptions about protein or DNA copies per virus particle, potentially introducing a bias. The qPCR method was available in-house, but the time-to-result, the precision, and accuracy did not meet the ATP requirements. The CZE method was assessed to meet all the requirements of the ATP and was the technique of choice to continue method development. However, the CZE method development had to be started from scratch in a field where little experience was available at the time. This comprised a risk, and therefore the qPCR method was optimized in parallel to lower the risk of not delivering a fit-for-purpose method in time.

No further technology examples were found in the literature for CE method development, as mostly technologies selection was done before focusing on the presented CE method. Borman et al. [37] published a study on analytical technologies selection for Ad vector infectivity, identity, and transgene expression analysis. They first provide an overview of potential technologies alongside the quality attributes each can assess, detailing the advantages and limitations of each approach. Each technology was then evaluated against the ATP requirements using a 10-point scoring system to determine whether the technologies meet, partially meet, exceed, or fail to meet these requirements. This example furthermore illustrates that with different requirements on the method, even if the analyte is the same, different technologies are appropriate.

3.3 | Critical Method Parameters (CMPs)

A CMP is a method parameter (e.g., the capillary type) that has a significant effect on one or more ATP requirements (e.g., specificity or accuracy) and the reportable results of a method [48]. It should be noted that the word "critical" in this connotation does not imply adverse or disapproving judgment. The word "critical" in English also has a neutral meaning in expressing both merits and faults, and in science, the adjective expresses importance and/or seriousness. In other words, a CMP is important to the progress or success of the method. It is therefore important to control and optimize all CMPs to fully understand the mechanisms of action and to assure a robust analytical procedure. Identifying CMPs cannot be done without scientific knowledge and experience on the technologies and the analytical request.

ICH Q14 suggests identifying analytical parameters (by Ishikawa diagrams [60]) and to use risk assessment tools as described in ICH Q9 Annex 1 to assess the potential impact and to identify and prioritize experiments [1]. It is laborious to perform a risk assessment on all method parameters because many analytical procedures consist of more than 50 parameters that could potentially be optimized. In our AQbD-flow, non-critical method parameters (nCMPs) are filtered out first by using a criticality assessment tool prior to performing a risk assessment. This approach aligns with ICH Q8 on pharmaceutical development [59], where CQAs are defined prior to conducting a risk assessment. The process is described in detail in the following paragraphs.

3.3.1 | Mapping Method Parameters

Method parameters can be mapped by using an Ishikawa diagram (e.g., 1, 49), a mind map (e.g., 39), or just a simple list. Although usually focusing on the instrumental parts first, the 6Ms (method, material, machine, mankind, mother nature, and measurement) can aid in categorizing the method parameters. Alternatively, one may use general sections from an analytical procedure to categorize the method parameters like materials/chemicals, sample treatment, and so forth. For the Ad CZE method example, a mind map was used to visually map all method parameters, such as the materials/chemicals, equipment, sample treatment conditions, the separation conditions, and data processing parameters, but also other parameters like the temperature in the lab and the sample matrix. A total of approximately 60 parameters were mapped for the CZE method for Ad determination (Figure 2).

Other examples of Ishikawa diagrams for CE method development are presented in [22, 61]. Orlandini et al. [61] presented

TABLE 2 | Technologies selection based on the ATP from Table 1.

	Requirements		Specification for virus particle concentration determination (in VP/mL)	Priority	OD260	AEX-LC	RPLC	SEC	qPCR	CZE	AF4
Requester	Process intermediate(s) and matrix		Crude harvest, lysed harvest, clarified harvest (IPC), anion exchange product, diafiltration product, drug substance, drug product. The matrix could contain cell debris, residual DNA, salt, protein, and or formulation buffer components dependent on the process intermediate	1	Only DS/DP	Only DS/DP	All	Only DS/DP	All	All	Only DS/DP
Method developer	Requester requirements	Time-to-result	< 4 h for IPC testing	2	< 4 h	4 h	4 h	4 h	4 h	1 h	4 h
		Specificity	Detection of Ad26 or Ad35 without significant interference of sample matrix components or other Ad types used in the facilities	1	Aromatic peptides and nucleotides	Adenovirus particles	Adenovirus protein	Adenovirus particles and protein aggregation	Adenovirus encapsulated DNA	Adenovirus particles	Adenovirus particles
		Accuracy	Accuracy (spiked recovery) ≤ 10% bias	2	80% –120%	80%–120%	75%–125%	80%–120%	70%–130%	90%–110%	80%–120%
		Precision	Intermediate precision ≤ 10% RSD	2	20%	10%–20%	10%–20%	10%–20%	10%–35%	5%	20%
		Concentration range	10^{10}–10^{12} VP/mL	3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
End-user	Quality Control		Reportable value for product release	1	> 10^{10} VP/ml	>10^{10} VP/mL	> 10^{10} VP/mL	> 10^{10} VP/mL	>10^{10} VP/mL	> 10^{10} VP/mL	?
	End-user requirements	Throughput	1 sample per run for IPC testing, > 7 samples per process development run, > 300 samples a month for process development	2	> 300	300	300	300	< 300	> 300	300
	Equipment availability		GMP qualified equipment	1	Available	Available	Available	Available	Available	Available	Not in QC

Note: Techniques: Quantitative-polymerase chain reaction (q-PCR), anion-exchange chromatography (AEX-LC), capillary zone electrophoresis (CZE), Asymmetric flow field-flow fractionation (AF4), size exclusion chromatography (SEC), reversed phase liquid chromatography (RPLC), and absorbance spectrophotometry at 260 nm (OD260). The colors represent the risk to not meet the ATP requirements: red, high risk; orange, medium risk; green, low risk.
Source: Adapted from [28, 29] with permission from the authors.

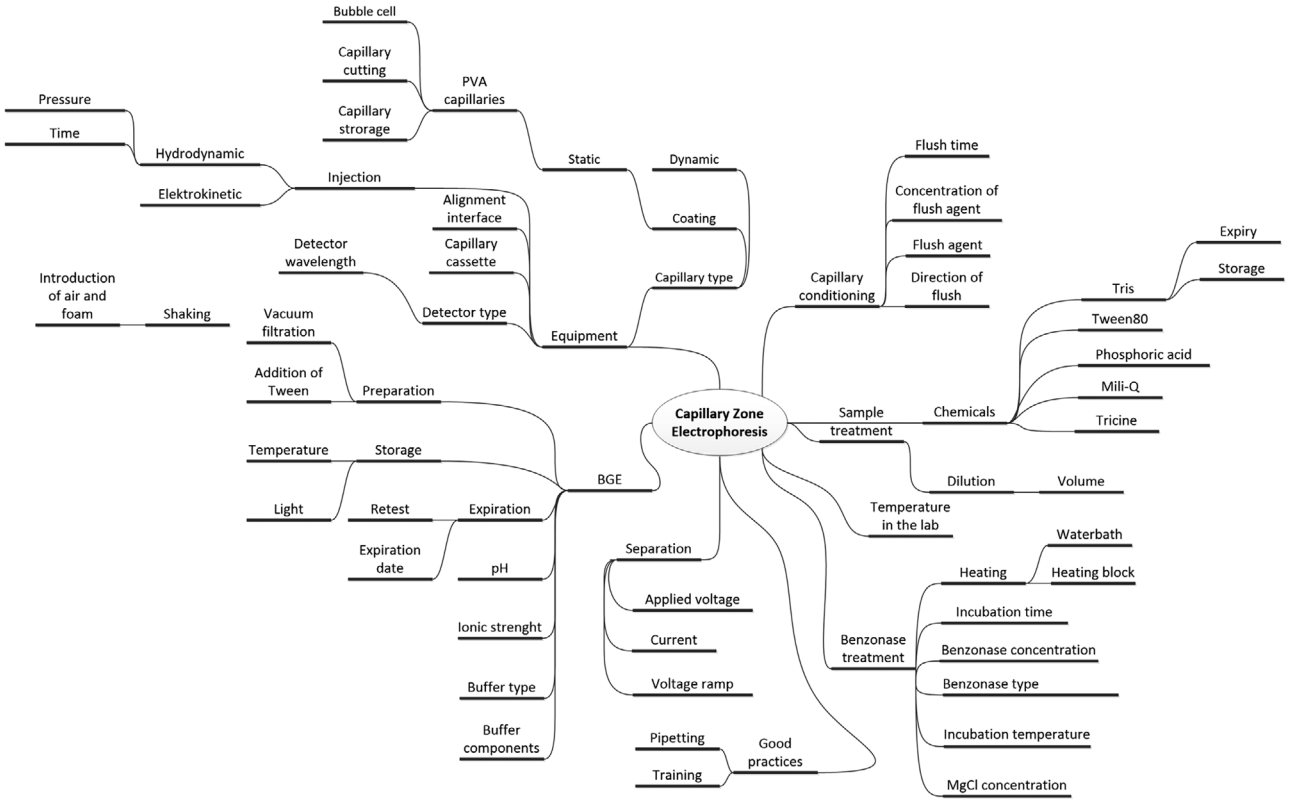

FIGURE 2 | Part of a mind map for the CZE method. *Source:* Adapted from [29] with permission from author.

TABLE 3 | Scoring table for criticality assessment.

		Certainty		
		Low	**Medium**	**High**
Effect	Low	pCMP	nCMP	nCMP
	Medium	pCMP	pCMP	CMP
	Significant	CMP	CMP	CMP

Note: Blue: CMP, orange: PCMP, and green: NCMP.
Abbreviations: cmp, critical method parameter; nCMP, non-critical method parameter; pCMP, potential critical method parameter.
Source: Adapted from [28, 39] with permission from authors.

an Ishikawa diagram for an MEEKC method, distinguishing between parameters requiring optimization through DoE and those fixed based on preliminary experiments or prior knowledge. Pasquini et al. [22] published an Ishikawa diagram for a chiral CE method. They used the diagram to classify each method parameter as a controlled parameter (*C*), a noise parameter (*N*), or a parameter to be investigated experimentally (*X*).

3.3.2 | Criticality Assessment

After listing all method parameters, the first filtering step is the criticality assessment. The goal of this assessment is to categorize each method parameter as CMP, nCMP, or potential critical method parameter (pCMP). A scoring table like in Table 3 can be used to score the overall effect (from low to high) of a method parameter on any of the ATP requirements and the certainty on the overall effect (from low to high). For example, it was highly certain that the buffer pH has a significant effect on the separation of the Ad from its matrix components (ATP requirement: specificity) and was therefore a CMP. Another example: The capillary length does not affect the resolution in CZE, so this method parameter was scored as noncritical.

nCMPs can be set from prior knowledge or expertise and ignored during the risk assessment and method development because they have a low effect on the ATP requirements. For pCMPs, it is recommended to perform feasibility experiments to get more knowledge (i.e., certainty) to be able to categorize the pCMP as either CMP or nCMP for the method at hand. Rescoring the parameters is essential when new knowledge is obtained during the AQbD process, which might change an nCMP into a pCMP or CMP in a later stage. At the final stage of the AQbD process, sufficient knowledge is available about the method and all method parameters should either be nCMP or CMP. Table 4 provides part of the criticality assessment performed for the Ad particle determination as illustration. For the Ad CZE method example, all pCMP (2) and CMPs (14) were included in the risk assessment because all these parameters have a medium or significant effect on the ATP requirements. Filtering through the criticality assessment meant that the following risk assessment was performed on 16 method parameters only instead of all 60 method parameters, excluding 44 nCMPs in an early stage in the process.

No other published examples of criticality assessments for CE method development were found.

TABLE 4 | An overview of part of the CZE method parameters evaluated in the criticality assessment.

Method parameters	Effect (low, medium, significant)	Certainty (low, medium, high)	Proceed to risk assessment?	Score
Separation buffer pH	Significant	High	Yes	CMP
Separation buffer type	Significant	High	Yes	CMP
Capillary coating	Significant	High	Yes	CMP
Capillary conditioning	Significant	High	Yes	CMP
Sample type and matrix	Significant	High	Yes	CMP
UV detection wavelength	Significant	High	Yes	CMP
Injection mode	Significant	High	Yes	CMP
Capillary storage	Significant	High	Yes	CMP
Capillary cassette temperature	Significant	High	Yes	CMP
BGE degassing	Significant	Medium	Yes	CMP
BGE filtration	Significant	Medium	Yes	CMP
Injection volume	Medium	High	Yes	CMP
Applied voltage	Medium	High	Yes	CMP
Sample tray temperature	Medium	High	Yes	CMP
Sample treatment (removal of DNA)	Medium	Low	Consider	pCMP
Benzonase storage	Medium	Medium	Consider	pCMP
CE vials	Low	Medium	No	nCMP
CE UV lamp equilibration time	Low	High	No	nCMP
Pipette step for sample transfer	Low	High	No	nCMP
Capillary length	Low	High	No	nCMP
Capillary diameter	Low	High	No	nCMP
Capillary detection window diameter	Low	High	No	nCMP
Sample buffer concentration	Low	High	No	nCMP
Benzonase type	Low	High	No	nCMP

Note: Blue: CMP, orange: PCMP, and green: NCMP.

Abbreviations: BGE, background electrolyte; CMP, critical method parameter; nCMP, non-critical method parameter; pCMP, potential critical method parameter; UV, ultraviolet.

Source: Adapted from [28, 29] with permission from the authors.

3.3.3 | Risk Assessment

The criticality assessment identifies each method parameter as nCMP, pCMP, or CMP based on its effect and the certainty of the scoring. Subsequently, a risk assessment is performed to determine and reduce the risks of a (p)CMP setting being wrong and resulting in decreased performance or incorrect results. To define a risk, we need to understand how a CMP affects the ATP requirements by understanding the cause and effect. For example, if the CE capillary is not properly coated (cause), then matrix and/or virus adsorption to the capillary wall could occur (cause), impacting the bias and precision (effect on ATP requirements). If we understand the cause and effect, we can begin scoring the probability that this cause and effect will actually happen. The final risk score is calculated by multiplying probability and the effect and can range from very low risks to very high risks. There are different scoring systems for low and high probabilities and low and high effects [1, 62]. The scoring system itself is of limited interest as the tool's main purpose is to rank risks, allowing method development to begin with the most impactful parameters. The classical failure-mode effect analysis (FMEA) also includes scoring the detectability [62, 63]. We have learned that mitigating the probability is usually sufficient to reduce the overall risk score, and therefore we recommend not scoring the detectability to simplify the risk assessment process.

Table 5 shows part of the risk assessment for some of the CMPs of the Ad CZE method development example with the highest risk scores. In this stage, prior to method development, feasibility and screening experiments were defined for each of the method parameters with a high risk score. For example, the separation buffer pH had a potential effect on multiple ATP requirements (e.g., precision, bias, and selectivity) and was therefore assessed separately in the risk assessment (rows 2, 3, and 5 in Table 5). The separation buffer type, separation buffer pH, sample type and matrix, and capillary coating were identified as high-risk CMPs (with scores of 60–80 out of 100) and were prioritized to study first during method development, after which the risk assessment was updated. As an example, the capillary coating was identified as a CMP with a high risk score of 80, as Ad could adsorb to the capillary's inner wall, affecting precision and bias (Table 5, row 1). Consequently, multiple capillaries and coatings were tested with various background electrolytes (BGEs) during method development (see chapter 3.4.1). A PVA-coated capillary from Agilent was selected as a mitigation strategy, as it demonstrated good virus recovery and separation. This PVA capillary was preferred over other neutral-coated options due to the availability of a high-sensitivity bubble cell (improved S/N) and ease of operation (precut capillaries that do not require storage in water to prevent air contact). Following this mitigation, the risk score was reduced from 80 to 20 by selecting the PVA-coated capillary (Table 5 row 1).

After the experiments were completed, the risks (effect multiplied by probability) were reassessed, significantly reducing the risk score ranges from 60–80 to 6–30. The ultimate goal of the risk assessment and following method development is to reduce the risks of all CMPs and pCMPs by implementing appropriate mitigations. A risk can be mitigated in several ways: (1) *Define*, for example, by setting the incubation temperature between 35°C and 39°C; (2) *Describe*, such as gently pipetting up and down with a P100 pipette for 10 cycles; and (3) *Control*, for instance, by using an Ad control sample with a known concentration of 10^{11} VP/mL to confirm accuracy within 90%–110% (see also chapter 3.4.4). An analytical procedure is considered in control when all CMPs are known, the cause-and-effect relationships are well understood, and all risks have been mitigated.

The risk assessment is a recurring process, and during each cycle, it has a different purpose (Figure 3). The first time the risk assessment is executed during the AQbD process, it is focused on prioritizing the initial experiments based on the parameters with the highest risk. During the actual method development step (see Section 3.4), the risk assessment is used to find mitigations for all CMPs, and it is used to define a control strategy for each of the CMPs. Other reasons to reevaluate the risk assessment include changes to the ATP, increased knowledge and experience (e.g., from troubleshooting), the identification of new CMPs, or transitioning to a new AQbD stage. Ultimately, the risk assessment document is a living document and a compact version of the method development report. Although some CE publications discuss the use of risk assessments within an AQbD approach [19, 20], no other examples providing an example of a risk assessment table were found.

3.4 | Method Development

ICH Q14 suggests, in their advanced approach, to explore method parameter ranges and their interactions using multivariate experiments (e.g., DoEs) [1]. The outcome of multivariate method development could be a fixed set point for a method parameter (e.g., tris concentration of 200 mM) or a range (e.g., PAR of MODR). An MODR (method operable design region) consists of combined ranges for two or more variables where the analytical procedure is demonstrated to be suitable for its intended use. Proven acceptable ranges (PAR) for the analytical method can be established through the univariate assessment of individual parameters.

In the presented AQbD-flow, three types of DoEs are used: screening designs, optimization designs, and robustness designs. Screening designs can be used when the criticality assessment and risk assessment resulted in a large amount of CMPs and pCMPs. A screening DoE is a fast and effective tool to identify the pCMPs and CMPs with the most effect on a response (e.g., the separation between the main analyte and its impurities). The optimization DoE is used to find an optimal set point or a range for all the CMPs (e.g., incubation temperature between 36°C and 39°C, or 0.04% w/v Tween-80). The robustness design can be used to test the effect of slightly changed settings of the CMPs, demonstrating that small changes do not impact the method responses or result. Both the optimization and robustness designs should not only study the main effects of method parameters but also interaction effects and frequently the quadratic effects. In the context of analytical method development, the focus during DoE should shift toward the reportable value (e.g., % purity, concentration), rather than merely optimizing separation descriptors (e.g., resolution, tailing factor, and S/N). This shift aligns with the goal of ensuring that the analytical procedure meets its intended purpose with robust performance, taking into account factors such as measurement uncertainty and the consistency of reportable results. By emphasizing the final reportable value, method development becomes more aligned with real-world applications, where ensuring accuracy, precision, and reliability of the result is crucial for decision-making throughout the product lifecycle. Despite the well-established benefits of DoE in enhancing method development and improving analytical procedures, its adoption remains limited. One reason for this could be the perceived complexity and resource demands of DoE, which can deter its widespread use in environments where quick method development is prioritized. Additionally, many analysts still rely on traditional approaches, such as the one factor at a time (OFAT) method, due to familiarity and (perceived) shorter timelines, even though DoE can offer more reliable and efficient results in optimizing methods.

The risk assessment such as in Table 5 is used to facilitate the method development. For each of the CMPs, experiments are defined to generate more knowledge or to mitigate possible cause and effect (the risk). After each experiment, the risk is rescored. If the risk score is acceptably low, then the CMP can be considered "in control." If the risk score is still too high after rescoring, then more experiments or other control measures (e.g., introducing a control sample) are needed to reduce the risk.

TABLE 5 | A selection of risks from the risk assessment of the Adenovirus CZE method development.

				Risk score *before* mitigation					Risk score *after* mitigation		
Row no.	**CMP**	**ATP requirement affected**	**Possible cause and effect**	**Effect (1–10)**	**Probability (1–10)**	**Score (before mitigation**	**Proposed experiments**	**Mitigations**	**Effect (1–10)**	**Probability (1–10)**	**Score (after mitigation**
1	Capillary coating	Bias and precision	Virus adsorption to the capillary wall could impact bias and precision	10	8	80	Test multiple capillaries and coatings and select capillary + coating with good virus recovery	A PVA coated capillary was selected to prevent adsorption of the adenovirus. Optimized capillary conditioning in between injections	10	3	30
2	Separation buffer pH	Bias	A pH that is too low or too high could cause virus degradation	10	8	80	Find the optimal pH range for adenovirus stability	The optimal pH range was determined as pH 6.0–8.5	10	1	10
3	Separation buffer pH	Specificity/selectivity and bias	The pH of the BGE could impact the selectivity and therefore the separation of the adenovirus from its matrix components or other Ad types	7	10	70	Screen several buffer types (with different pHs) to evaluate the impact on the selectivity. Select a BGE and pH and perform a robustness experiment	A BGE containing 125 mM tris and 338 mM tricine at pH 7.7 was selected and optimized. This BGE and pH allowed for baseline separation of all peaks and could be reproducibility prepared (robust)	10	1	10
4	BGE composition	Bias and precision	Inadequate BGE composition could cause the matrix components or adenovirus to adsorb, precipitate, or aggregate and cause an inaccurate and imprecise results	10	8	80	Screening of BGE and capillaries screening to reduce adsorption optimize conditions	Use tris/tricine buffer Add PS-20 to BGE. Set BGE to 125 mM tris and 338 mM tricine and 0.2% w/v PS-20. Introduce an adenovirus control sample to monitor accuracy and precision	10	1	10

(Continues)

TABLE 5 | (Continued)

		Risk score *before* mitigation					Risk score *after* mitigation				
Row no.	**CMP**	**ATP requirement affected**	**Possible cause and effect**	**Effect (1–10)**	**Probability (1–10)**	**Score (before mitigation**	**Proposed experiments**	**Mitigations**	**Effect (1–10)**	**Probability (1–10)**	**Score (after mitigation**
5	Separation buffer pH	Precision	Variations in the BGE pH might impact the migration time precision of the method	6	10	60	Prepare the BGE in such a way that the pH is reproducible	Tris and tricine at fixed concentrations (instead of titrated) to reduce the pH and ionic strength variation	6	1	6
6	Sample type and matrix	Bias and precision	Sample matrix components like DNA, protein, or salts could impact the precision and bias	10	8	80	Evaluate whether a sample treatment is required for a set of representative samples from DSP and USP	A sample treatment was implemented for USP samples to remove residual DNA. The precision and bias were determined and acceptable for three representative samples: CH, AEX, and DS	10	3	30
7	Detection wavelength	LOD and LOQ	If the wrong detection wavelength is selected, this could result in low or no S/N values	10	8	80	Evaluate and select a wavelength that allows for adenovirus detection and quantification	A wavelength of 214 nm was selected to analyze adenovirus	10	1	10

Note: The risk is scored before and after the mitigations. The effect is scored from 1 (low effect) to 10 (high effect). The probability is scored from 1 (low probability) to 10 (high probability). Red: high risk score, orange: medium risk score, and green: low risk score.
Abbreviations: AEX, anion exchange; ATP, analytical target profile; BGE, background electrolyte; CMP, critical method parameter; USP, upstream processing.
Source: Adapted from [28, 29] with permission from authors.

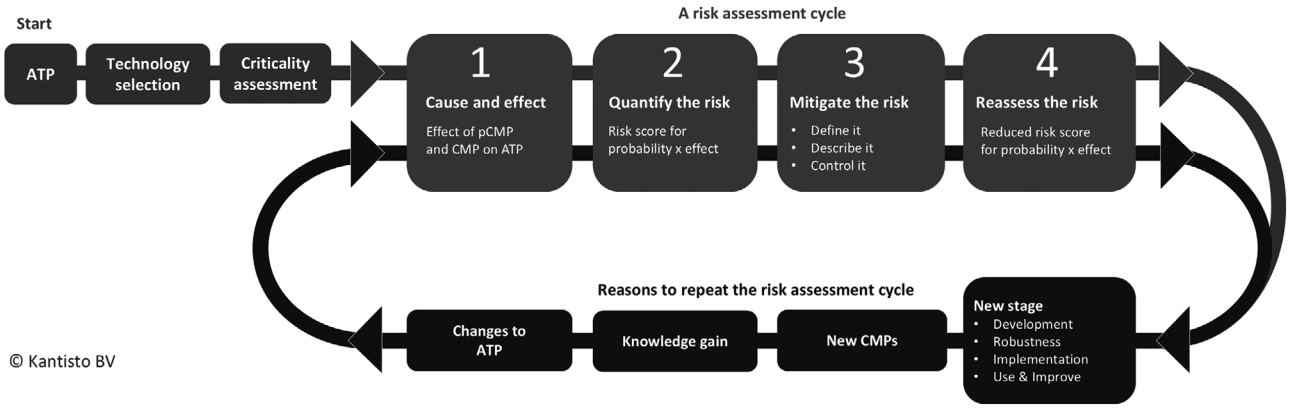

FIGURE 3 | Recurrent risk assessment cycle. The cycle starts with the ATP followed by a technologies selection and a criticality assessment to define the nCMPs, pCMPs, and CMPs. A risk assessment is performed on the pCMPs and CMPs by (1) describing the cause of effect, (2) quantifying the probability and effect, (3) defining mitigations for the risks (4) reassessing the risk by rescoring the probability and effect. Reasons to reevaluate the risk assessment include changes to the ATP, increased knowledge and experience (e.g., from troubleshooting), the identification of new CMPs, or transitioning to a new AQbD stage. The recurrent risk assessment cycle starts from the critical method parameters step onwards in Figure 1 and is frequently adapted and reassessed during all following steps in the AQbD process. ATP, analytical target profile; CMP, critical method parameter; pCMP, potential critical method parameter. *Source:* Reprinted with permission, Kantisto B.V.

3.4.1 | Screening Experiments

The compositions of the BGE and the type of capillary were CMPs for the Ad CZE method. Ad particles and/or matrix components (e.g., proteins) can adsorb to the inner walls of bare fused silica capillaries [64, 65]. To prevent undesired adsorption, various uncoated, dynamically and statically coated, charged, and neutral capillary coatings were screened in combination with a wide range of BGEs, ranging in ionic strength, pH, co- and counterions, and surfactants [6, 28, 29, 66]. A set of representative samples and controls were defined to evaluate the potential of the BGEs and capillaries to analyze Ad particles. A blank was used as negative control, a crude harvest sample (with cell debris) was used as worst-case sample, an anion exchange (AEX) sample was selected for its high salt concentration, a purified Ad sample was taken as best-case sample, and a known IgG was taken as system suitability control (SSC) sample. Each sample was spiked with *o*-phthalic acid as internal standard. The tested capillaries included neutral-coated capillaries, fluorocarbon-coated capillaries, polyvinyl alcohol (PVA)-coated capillaries, and bare-fused silica capillaries, paired with the following additives: triethylenetetramine (TETA), polysorbate-20, or SDS. The capillaries were evaluated on the basis of their ability to detect and potentially separate the Ad from its matrix components. The tested BGEs were selected on the basis of literature research as well as a BGE designed from CE fundamentals and best practices [15, 66]. The designed BGE contained a mixture of tris and tricine. Tris was previously used in Ad formulations, whereas tricine was used in AEX chromatography. Both tris and tricine buffer in the desired pH range, thus resulting in a BGE with strong buffering capacity. The combination of tris and tricine results in low-conducting buffers, so the concentrations can be increased to improve buffering capacity and sensitivity and reduce potential electromigration dispersion. The screening showed that, as expected, a capillary coating was essential to prevent virus adsorption. Additionally, a pH range of 6.0–8.5 was necessary to avoid virus degradation. The best initial results were obtained with the BGE made of 200 mM tris–200 mM tricine (pH 8.1) and PVA-coated capillary with an effective length of 24.5 cm, based on scoring the detection and repeatability of the Ad peak area and migration time, as well as the performance of the IgG and the internal standard (see Figure 4). Using tris–tricine at fixed concentrations (rather than titrating the buffer) reduced the risk scores for the CMP buffer pH from 60–80 to 6–10 (see rows 2 and 5 in Table 5), because the probability that the bias and precision were affected was reduced significantly.

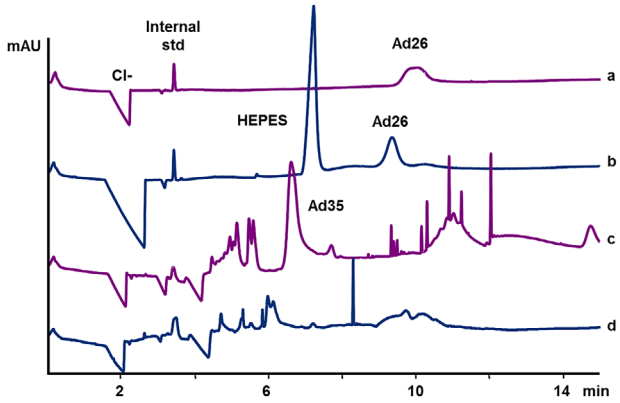

FIGURE 4 | Typical electropherograms obtained during method optimization for (a) Ad26 reference material, (b) Ad26 AEX sample, (c) Ad35 CR sample, and (d) Ad35 CR sample after repeated injection. Conditions: BGE 200 mM tris–200 mM tricine (pH 8.1), PVA-coated capillary with effective length of 24.5 cm; applied voltage, −15 kV. Other conditions see reference [6]. *Source:* Reprinted from *Talanta* [6] with permission from Elsevier.

Despite using a PVA coated capillary, repeated injections of the crude harvest sample impacted the Ad peak area and migration time (see figure 2 in reference [6]). This effect was mainly seen for crude harvest samples containing cell debris and cellular DNA, but all types of Ad samples analyzed thereafter were impacted. The decrease in peak area and shifts in migration time for the Ad may be due to adsorption caused by the degradation of the

PVA coating or by the adsorption of matrix components onto the capillary wall. A second screening design was set up to reduce the impact of adsorption by studying the Ad peak area and migration time by varying the cassette temperature, BGE additives (non-ionic detergents and dynamic coating), capillary conditioning (duration, rinsing direction, type of reagent, and concentration), and BGE composition. Adsorption could only be prevented by using a combination of the following settings: a PVA-coated capillary with a BGE containing 0.2% (v/v) polysorbate-20, a capillary cassette temperature of 15°C, and rinsing between runs with 10 mM phosphoric acid in the opposite direction of the separation. These conditions allowed for the analysis of over 400 samples on a single capillary without any loss in Ad peak area, resulting in repeatability of Ad corrected peak area between 2.5% and 5% relative standard deviation (RSD). These results were well within the ATP requirement for precision: < 10% RSD. The knowledge obtained from the second screening design reduced the risk scores for the CMP coating to an acceptable risk score of 30 (see row 1 in Table 5).

The following publications provide additional examples of screening and scouting approaches for CE method development: [12, 20, 67]. Milan et al. [67] described an example of an OFAT screening strategy for the development of chiral CE method, followed by a face centered central composite design (FCCD) for the optimization process. The OFAT approach was successfully applied to screen the influence of five analytical parameters (BGE concentration, CD concentration, applied voltage, system temperature, and injection pressure) on three analytical responses (chiral resolution and migration times of the enantiomers). Niedermeier et al. [12] used a fractional factorial resolution V+ screening design to study the capillary temperature, applied voltage, ammonium acetate concentration, acetic acid concentration, and HDMS-βCD concentration for a nonaqueous capillary electrophoresis method. Coefficient plots were used to identify the CMPs.

3.4.2 | Method Optimization

For the Ad CZE method development, a full factorial optimization design was used to study the tris concentration, tricine concentration, separation voltage, and capillary effective length, aiming to optimize Ad separation efficiency and minimize total run time. This was particularly important to meet the ATP requirement of achieving results in less than 4 h. The tris concentration was varied from 50 to 200 mM, whereas the tricine concentration ranged from 250 to 400 mM. The applied voltage was tested simultaneously at −15, −20, −25, and −30 kV. The study was conducted using effective capillary lengths of either 24.5 cm (long-end) or 8.5 cm (short-end). The best results were achieved at 125 mM tris, 338 mM tricine (pH 7.7), analyzed with an effective capillary length of 8.5 cm. Figure 5 shows the contour plot of the selected optimum. The separation between the Ad peak and the impurities remains robust across variations in separation voltage (−15 to −25 kV), tris concentration (100–200 mM), and tricine concentration (250–400 mM). The pH was maintained between 7.5 and 7.7 under all tested conditions. Total run time was reduced from 15 to 3.5 min (see figure 3b in reference [6]), which was crucial for delivering a time-to-result of < 4 h. The results of this optimization design, and specifically of the mitigations implemented, led to risk scores as low as 10 out of 100 after reevaluating the risks associated with buffer pH and BGE composition (refer to rows 3 and 4 in Table 5).

The following publications describe other CE methods that were optimized by DoE: [5, 12, 19, 20, 27, 42, 46–49, 53, 67, 68]. Yao et al. [42] optimized a CZE method for the quantification of *Escherichia coli* L-asparaginase and its acidic variants by using a three-level central composite design (CCD). Regressions coefficient plots were used to find factors (i.e., method parameters) with significant effect on three different responses (peak to valley ratio, RSD of peak area, and migration time). Subsequently, contour plots were used to find the optimal BGE concentration and pH for the three responses described above. Niedermeier et al. [12] employed a central composite face-centered design to optimize the chiral separation of four phenothiazines by non-aqueous capillary electrophoresis. Probability maps of the design space were evaluated to optimize the voltage, hexadecyl-modified β-cyclodextrin (HDMS-β-CD) concentration, and ammonium acetate concentration. Pasquini et al. [20] used a DoE with a response surface model (RSM) to optimize the composition of the BGE, namely, the concentration of borate buffer and of the added organic solvents. Contour plots and risk of failure maps were used to define the MODR for the solvent-modified micellar electrokinetic chromatography method.

3.4.3 | Method Robustness

The ICH Q14 describes that if evaluation of robustness was already conducted during development, it does not need to be repeated during validation as discussed in ICH Q2 [1]. ICH Q2 further underlines that robustness testing is part of method development rather than part of method validation [51].

In analytical methods, robustness can be viewed as experimental robustness or holistic (or system) robustness. Experimental robustness, often assessed via DoE, tests the impact of deliberate changes in CMPs, identifying tolerance ranges where results remain reliable. In contrast, holistic robustness emphasizes the method's reliability and steadiness in routine use, focusing on control strategies and mitigations rather than parameter testing. Although experimental robustness isolates parameter effects, holistic robustness ensures consistent performance through comprehensive control of the entire process. Together, these approaches provide a balanced view of robustness in method development.

The robustness testing of the CZE method, performed using the same DoE model developed during method optimization, assessed the allowable variability in critical parameters such as tris concentration, tricine concentration, and separation voltage. Variations within the established tolerance ranges did not negatively affect the method's performance, demonstrating that the method remains robust despite small deviations in these parameters. This testing was crucial for ensuring that the method could tolerate typical variations encountered during routine use while still meeting the ATP requirements. The robustness of the selected crude sample pretreatment conditions for the Ad CZE method was tested with a full factorial design. The benzonase concentration was varied from 7 to 9 U/mL, the $MgCl_2$

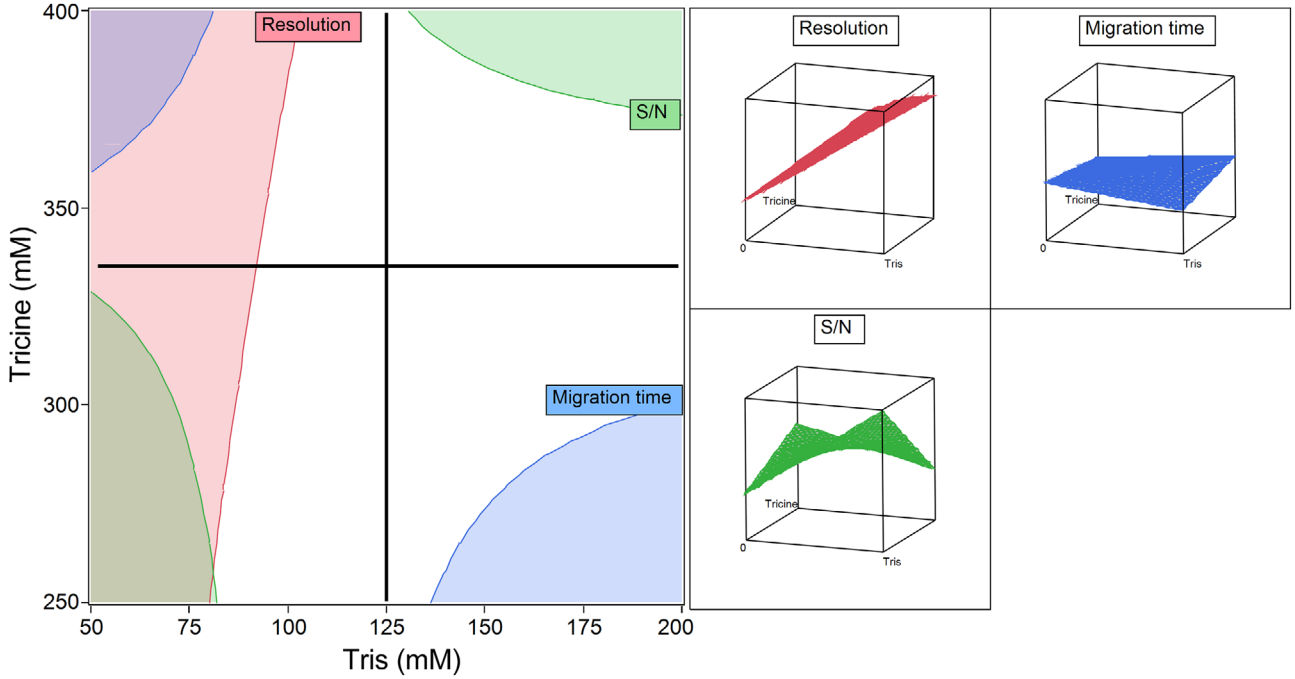

FIGURE 5 | Contour plot of the selected optimum (cross marker). A least squares fit of the tris and tricine concentration at an applied voltage of −25 V. The output variables were migration time, S/N, and resolution. The red contour is resolution with a value of 2.5, the green contour is the S/N with a value of 150, and the blue contour is the migration time (run time) with a value of 6 min. *Source:* Reprinted from *Talanta* [6] with permission from Elsevier.

concentration from 1 to 1.8 mM, and the incubation times at 37°C from 25 to 35 min. The robustness DoE showed that the sample treatment conditions were robust at the selected conditions for small variations due to operator errors or day-to-day variance.

Continuously, during screening, optimization, and robustness, testing the risk assessment (from Table 5) was used and updated to define knowledge gaps, to prioritize experiments, and to capture increasing knowledge and understanding for all CMPs. Ultimately, the relationships between the method parameters and the reportable values were understood. All the risk scores were lowered from 60–80 to 6–30, reducing the risk of a CMP to affecting the ATP requirements to acceptable levels. In general at the end of the method development step, good understanding of the CMPs and their influence on the reportable results should be established. The final method should be verified against the ATP requirements and all ATP requirements should be met.

Additional examples of robustness studies with CE can be found in references: [5, 12, 15, 17, 22–24, 27, 42–45]. Cârcu-Dobrin et al. [43] demonstrated the robustness of a chiral capillary electrophoresis method for Verapamil enantioseparation using a Plackett–Burnham design. Five parameters were evaluated: BGE concentration, pH, cyclodextrin concentration, voltage, and temperature. Their findings indicated no significant effect of any parameter on chiral resolution or enantiomer migration time, confirming the method's robustness. Niedermeier et al. [12] used a Plackett–Burman design to test robustness across capillary temperature, voltage, ammonium acetate, acetic acid, and HDMS-β-CD concentrations for the analysis of desipramine, revealing significant impacts of ammonium acetate on migration time and resolutions, and CD concentration on enantiomer resolution; therefore, careful BGE preparation is recommended. Harnisch et al. [53] assessed the robustness of a capillary electrophoresis method developed for determining impurities in dapoxetine hydrochloride. To ensure robustness beyond the design space, they applied a Plackett–Burman design to examine additional parameters across specified ranges. They evaluated sodium phosphate buffer concentration, pH, sulfated γ-cyclodextrin (S-γ-CD) concentration, dimethyl-β-cyclodextrin (DM-β-CD) concentration and manufacturer, voltage, and capillary temperature. Scaled and centered coefficient plots showed, as expected, that S-γ-CD concentration and voltage significantly impacted the current. However, the results for DM-β-CD were unexpected, as the manufacturer significantly affected analyte migration times and resolution values for both the EOF and impurities. To ensure consistency in QC, the authors recommend using DM-β-CD from the same manufacturer.

3.4.4 | Control Strategy

The analytical procedure control strategy (APCS) of the analytical method ensures that the analytical procedure continues to meet the ATP requirements during routine use throughout the entire life-cycle. The APCS is the result of a combination of all the knowledge obtained during method development (screening, optimization, and robustness) and all the mitigations for all the CMPs in the risk assessment. ICH Q14 mentions that the APCS should be defined before validation (ICH Q2) and should be confirmed after validation has been finalized [1]. Every analytical method needs at least an SST with SSC samples to monitor critical aspects of the analytical method. Usually negative controls (blank) and positive controls (sample or reference) are included

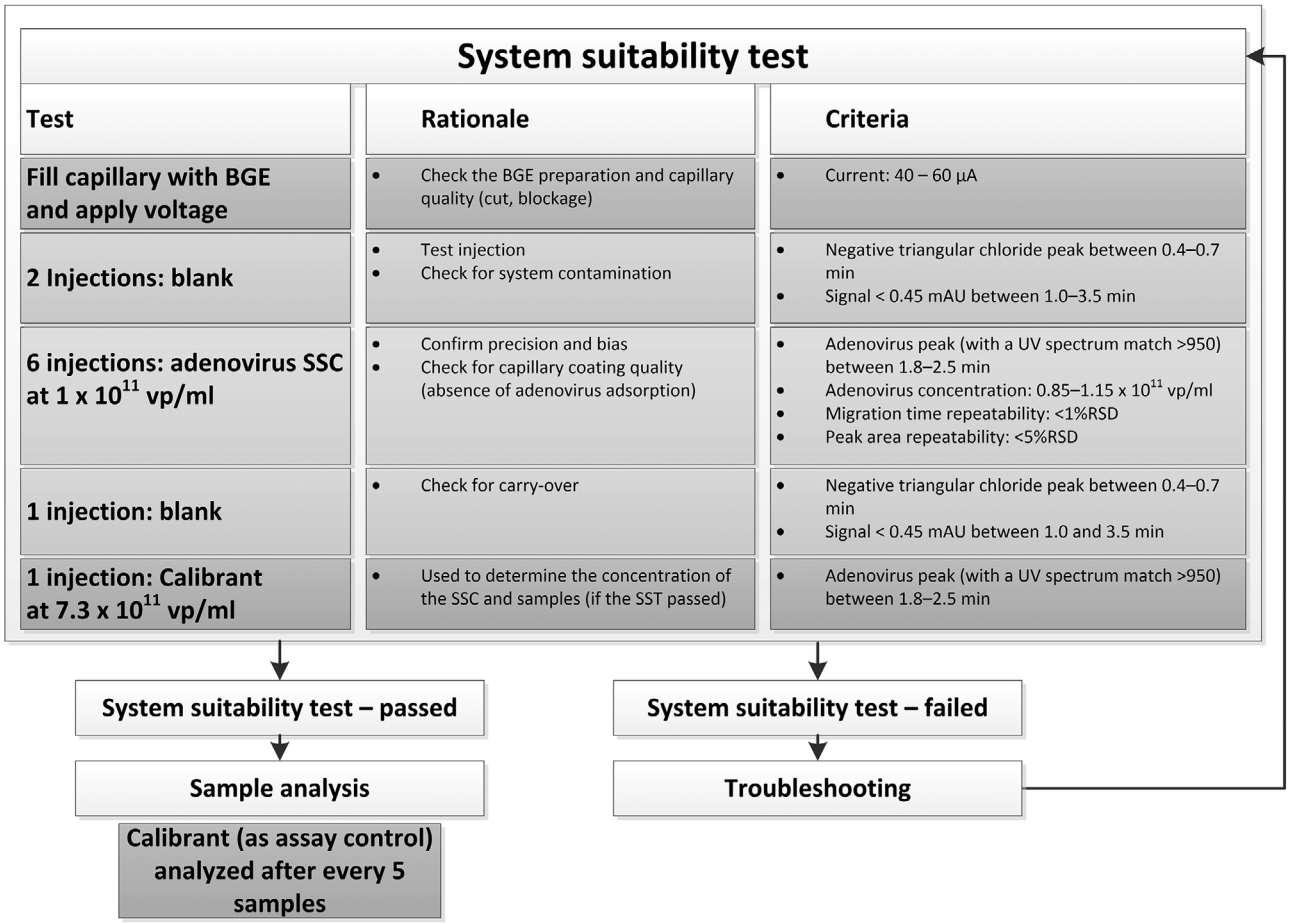

FIGURE 6 | System suitability test to assure proper performance of the CZE method during an analytical run. *Source:* Reprinted from *Electrophoresis* [8] with permission from Wiley.

in the control strategy to monitor the equipment, the calibration, the sample and control preparation, the separation, the data processing, and/or the result. The SST with all its acceptance criteria should be based on the CMPs that require control as identified in the risk assessment and the ATP. This automatically means that there is no such thing as a generic SST, and all methods should have SSTs that are meaningful and verify fitness for intended purpose on the day of analysis.

Figure 6 shows the SST for the developed CZE method for the Ad concentration determination. For this method, during the time from pulling a sample for IPC testing until return of the result to production, the process is on hold. Therefore, the time to result should be as short as possible. During method development, this resulted in the choice to offer capillary lifetime rather than of introducing a complex sample preparation procedure (other than sample dilution) for downstream process and product samples. As the clock starts ticking when the IPC sample arrives in the lab, the system suitability will be performed earlier on the day the IPC sample is expected. To reduce the risk that the actual IPC sample fails, the requirements on the SST were set rather stringently. The SST starts with filling the capillary with BGE after which a voltage is applied. Method development and robustness showed that currents between 40 and 60 µA are acceptable for this application, demonstrating that the capillary is not blocked and cut at the right length. Next, two blank samples are analyzed to check for contamination. An SSC (Ad sample) with a known concentration is injected six times to verify method performance before each analytical run, ensuring a precision of ≤ 5% RSD for the Ad peak area and a total error (bias and intermediate precision combined) of ≤ 15% for virus particle concentration, in line with the ATP requirements (see Figure 6 and Table 1). Thereby, the peak width is an indicator for the quality and status of the capillary wall. After the SSC, another blank is measured to check for carry-over. Then, a one-point calibration is performed by analyzing the calibrant. Additionally, the calibrant is measured after every five test samples and at the end of each sequence. A reduction in Ad concentration in the control sample during the sequence signals degradation of the capillary coating and/or Ad adsorption. A concentration change of ± 10% is acceptable for this application. Samples are only analyzed after a valid SST. When the SST criteria fail, investigation and troubleshooting are executed after which the SST is restarted. Routine application of the CZE method (n = 525 analytical runs) revealed an initial SST failure rate of approximately 20% (n = 105). This indicates that troubleshooting and retesting of the SST were necessary in one out of every five runs before sample analysis can proceed. Despite the 20% failure rate for SST runs, 99.4% of the sample data was generated on the same day. The stringent SST requirements effectively prevented the analysis of samples on a CE system that was not functioning properly, reducing the risk of a failed IPC sample analysis and ensuring compliance with ATP requirements

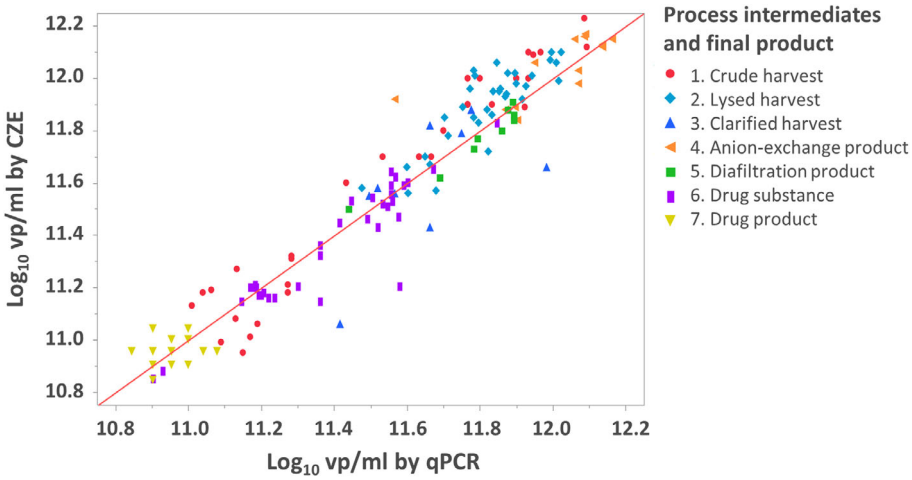

FIGURE 7 | Comparison of the qPCR results (log10 VP/mL) versus CZE results (log10 VP/mL) of six process intermediates and the final product. The red line represents the unit line. *Source:* Reprinted from *Electrophoresis* [8] with permission from Wiley. qPCR, quantitative polymerase chain reaction.

and short hold-times for the production process. Trending and monitoring of ATP requirements (e.g., accuracy), CMPs (e.g., lot number of capillary), and/or responses (e.g., resolution between Ad peak and impurities) are crucial to control the analytical method over time [69].

3.5 | Method Implementation

The optimized CZE method was installed and implemented in different laboratories at analytical development, process development, analytical support, and QC (development and manufacturing). A common approach for training new operators in the industry is a standardized 1–2–3 approach: (1) observe, (2) perform under supervision, and (3) perform without direct supervision. This limited approach is often very well suited for established technologies like HPLC, because the operators are educated on or familiar with other HPLC applications. When implementing a new technology, such as CE in this example, it is necessary to take a holistic approach to training, covering the fundamentals and best practices of the technique, along with instruction on the specific instruments and software, and eventually training the specific application. The CMPs, along with their control strategy and mitigations, form the basis for training new users and represent the most essential information in an analytical procedure. It is recommended to perform a co-validation between the receiving and sending laboratory to prevent that the validation is performed by the most experienced method developers and therefore show flattered results rather than setting realistic expectations for future use, see chapter 3.6.

The optimized CZE method was implemented to replace qPCR for the determination of the Ad concentration in process samples. Comparability of CZE and qPCR was assessed through an equivalence study for all process intermediates. A total of 131 different upstream and downstream processing samples were tested using both qPCR and CZE, as illustrated in Figure 7, which shows a bivariate plot of qPCR versus CZE for various process intermediates. The 95% prediction confidence interval for the difference between CZE and qPCR measurements generally ranged from −0.18 log10 to 0.16 log10 VP/mL. Previous qPCR comparability studies established acceptance limits of −0.2 log10 to 0.2 log10 VP/mL. The CZE results fell comfortably within these acceptance limits, indicating that CZE and qPCR results were interchangeable.

Other examples of method application and implementation are published here: [12, 22, 42, 52, 53, 70]. Simeoni et al. [52] executed a technology bridging between rCE-SDS and SDS–PAGE. A method bridging study demonstrated that the new release method (rCE-SDS) is exchangeable with the current SDS–PAGE method for detecting therapeutic monoclonal antibody variants under reducing conditions, with rCE-SDS offering higher sensitivity, enabling consistent impurity quantification, and allowing specification limits alignment with SDS–PAGE, while also providing additional information on NG-HC content. Van Tricht et al. [70] developed a capillary gel electrophoresis method to quantify viral proteins in influenza virus and virosome samples as an alternative to SDS–PAGE, HPLC, and single radial immunodiffusion (SRID). The CGE method was successfully applied to B/Brisbane inactivated virus and virosome samples. The hemagglutinin (HA) concentration in the virosome and inactivated virus samples, determined by CGE, closely matched the SRID-determined titers, confirming the method's accuracy and reliability. Niedermeier et al. [12] applied a developed nonaqueous CE (NACE) method to commercially available levomepromazine chemical reference substances (CRS) and Neurocil tablets. The method showed advantages over previous techniques, particularly in its ability to detect dextromepromazine down to the 0.01% level and reduce oxidation of the sulfoxide. The NACE demonstrated good applicability for determining the stereochemical purity of levomepromazine.

3.6 | Method Performance

ICH Q14 recommends that analytical procedures are validated in accordance with ICH Q2(R2). The ATP serves as a foundation for deriving the appropriate analytical procedure attributes and acceptance criteria for analytical procedure validation. For practical reasons and in line with a risk-based approach, it may not

be necessary or feasible to validate the entire MODR. However, the sections of a PAR or MODR intended for routine use in the analytical procedure must be supported by validation data. Validation approaches for MODRs can be found in annex B of ICH Q14. Validation of the analytical procedure is required only for performance characteristics not addressed by data from the development phase [1].

In method validation, several common errors can compromise the reliability and applicability of analytical procedures. One frequent issue is the use of incorrect significant figures, which can distort statistical calculations, data accuracy, and interpretation. Another problem arises when validation efforts focus too narrowly on certain parts of the method, overlooking critical steps such as sample preparation. Sample preparation is a frequent source of errors and uncertainties, yet it is often excluded from validation procedures. Additionally, some validations are designed to showcase the maximum capability of a method rather than providing realistic expectations for routine performance. This approach can result in overestimating the method's reliability under typical operating conditions. Moreover, validations are often conducted by highly experienced operators instead of the intended end-users, which may mask challenges that less skilled personnel might face. Finally, a "checklist" mentality can dominate the process, where the focus is on meeting formal requirements rather than evaluating whether the method is truly fit for its intended purpose. Addressing these issues is essential to ensure that validation results genuinely reflect the method's performance in real-world applications.

The Ad CZE method was validated according to ICH Q2. Specificity was established by confirming peak identity and verifying the presence of intact Ad particles. Both adenovirus serotype 26 (Ad26) and adenovirus serotype 35 (Ad35) were effectively separated from all matrix components in each of the process intermediates, as well as from other Ad types (Figure 12 in [50]. The method's repeatability ($n = 18$) yielded a RSD of 2.1% to 4.8% for the corrected peak area and 0.55% to 0.82% for the migration time. The intermediate precision ($n = 18$) showed an RSD of 7.8% for the corrected peak area and 2.5% for the migration time. The accuracy, assessed through spiked recovery (three replicates at three levels), ranged from 95% to 110%. In conclusion, all predefined acceptance criteria from the ATP were met, confirming that the method is suitable for determining Ad concentration in vaccine products.

After method validation, it can be concluded that the method's performance, specifically regarding precision and bias, was acceptable during the validation experiments. However, these experiments do not guarantee that future results obtained using the method will maintain the same quality. Hubert et al. [71] and Rozet et al. [72–74] proposed an alternative statistical methodology to predict the quality of future results by evaluating the total error of an analytical method. The total error was assessed for the Ad CZE method by using an accuracy profile. The total error, which is the sum of the random error (precision) and the systematic error (bias), of an analytical method predicts the method's ability to produce accurate future results. Figure 8 shows the accuracy profile of the CZE method. The β-expectation tolerance intervals, derived from the intermediate precision and bias of the analytical method, are statistical intervals in which

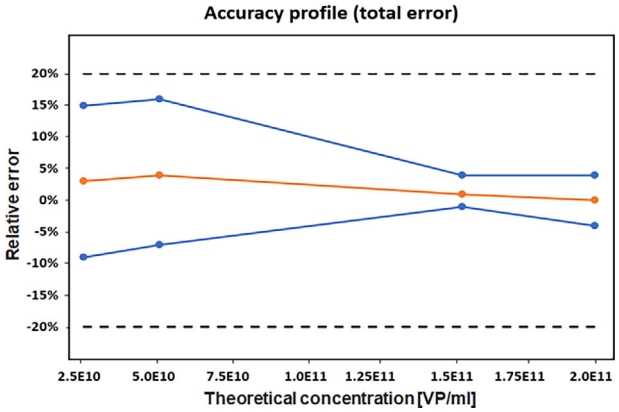

FIGURE 8 | Accuracy profile of the adenovirus concentration determination. The orange line represents the relative bias. The blue lines represent the 90% β-expectation tolerance intervals (prediction for future results) and the dotted black lines represent the acceptance limits of 20%. *Source:* Reprinted from [29] with permission from the author.

it is expected that each future result will fall with β probability (typically 90%). The LOQ and range of the method can be determined by selecting the lowest and highest concentration levels (with tolerance intervals) that are within the acceptance limits.

The following publications describe more examples of CE method performance experiments [5, 12, 67].

3.7 | Use and Improve

ICH Q14 emphasizes a risk-based approach to developing and continuously monitoring methods, focusing on controlling CMPs and using change control and revalidation as necessary. Continuous improvement and systematic updates are encouraged to maintain method suitability as new data, equipment, or regulatory expectations evolve. By managing knowledge gained during development and routine use, ICH Q14 aims to sustain method performance and ensure that analytical procedures remain fit for purpose throughout the product lifecycle [1].

In practice, this means recognizing that an analytical method is never truly "finished." The original developer of the method should remain responsible for its application whenever possible, even after transferring it to another lab, as it is only through this transfer that the true robustness and effectiveness of the method can be fully assessed. In the "Use and Improve" phase, two critical elements are essential: (1) continuously monitor the performance of a method, and (2) learn from issues and troubleshooting, and actively seek opportunities for method improvement. Although analytical methods are initially developed with specific purposes and ATP requirements, users frequently apply these methods for different or expanded applications beyond their original intent. This adaptive use necessitates periodic assessment to confirm that the procedure continues to meet ATP specifications and maintains accuracy, precision, and robustness under new conditions. Deviations from ATP criteria can indicate the need for method development or reassessment. Certain scenarios, such as the introduction of a new product, a different sample matrix, a varied

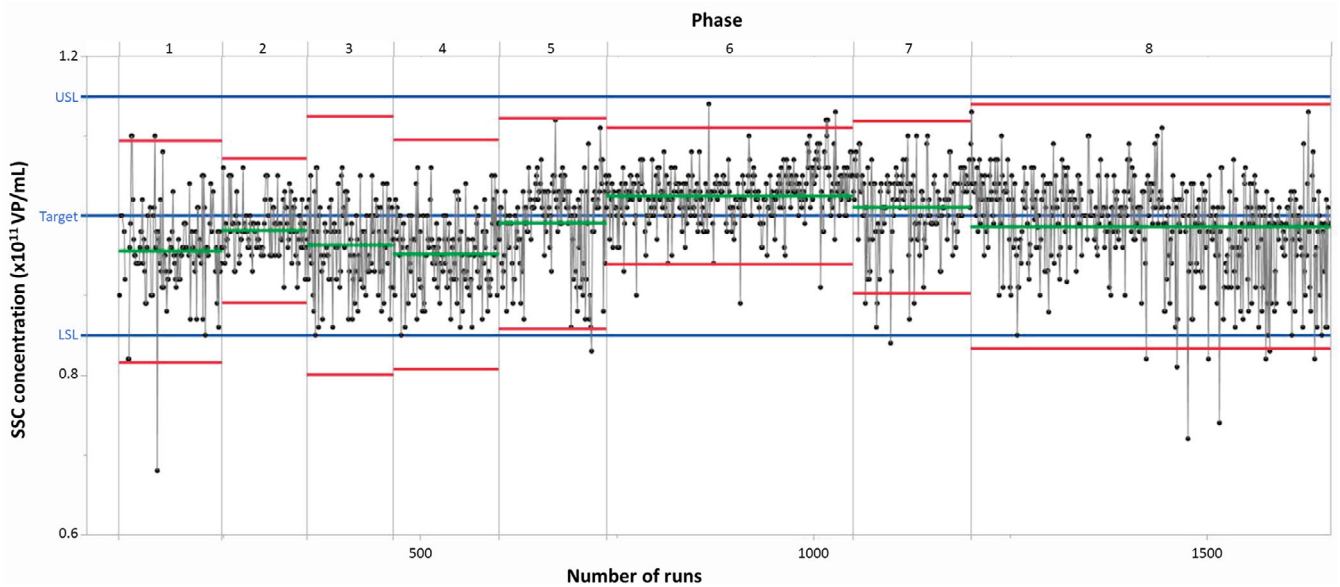

FIGURE 9 | Control chart of the SSC concentration response determined in 1656 assay runs. The SSC concentration results from each consecutive test run are depicted as black dots and connected chronologically with a gray line. The targeted value, lower specification limit (LSL) and upper specification limit (USL) are depicted as blue lines. The different phases are separated with gray 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), that is, 3× standard deviation, are depicted as red lines. *Source:* Reprinted from [28] with permission from the author.

concentration range, or new product combinations with potential interference from impurities, are especially relevant as they can directly impact the validity of the analytical result. In such cases, method revalidation, modification, or even the development of a new analytical procedure may be warranted to ensure that the method remains suitable and compliant with regulatory and scientific standards. This proactive approach to life-cycle management enables a more resilient analytical procedure that adapts to evolving requirements in pharmaceutical development.

Figure 9 shows a control chart of 1656 results of the Ad SSC of the CZE method. The trending results were continuously evaluated by interpretation of target limits, control limits, and Shewhart tests. The lower specification limit (LSL) of $0.85 \cdot 10^{11}$ VP/mL and the upper specification limit (USL) of $1.15 \cdot 10^{11}$ VP/mL were defined in phase 0 based on the total error of 15% from the method performance (Figure 8). In phase 1, a negative bias was observed of 4%. Although within limits, an investigational study was performed to identify the root cause and take corrective measures before the bias would move outside the acceptable range. The risk assessment was reevaluated to identify CMPs that could impact accuracy. After intensive investigation, pipetting was recognized as a potential root cause of the observed bias, as typical Ad samples are viscous and can have a variable surface tension. To investigate further, multiple operators were observed during the routine use of the CZE method. It was noted that each operator pipetted differently, and all used a prerinsing step. The prerinsing was introduced after a retraining from a pipette vendor, based on the good practices of pipetting Milli-Q water. However, the viscosity and surface tension of the Ad samples and diluent differ significantly from water, resulting in a bias. A new pipetting routine for these Ad samples was introduced, and Phase 2 shows that the results were well within the acceptance limits after retraining the operators. In all of the other phases, specific reasons were found that explained increased variability (i.e., precision) or bias. Every time, the risk assessment was reviewed to understand the effect of the CMPs on the ATP requirements and to find the root cause. Maintaining control over an analytical method requires various approaches, such as retraining operators, clarifying or rewriting the analytical procedure, and implementing new control measures. The risk assessment should continuously be updated with the latest insights and knowledge and is the basis for a method-specific control strategy.

It is recommended that the causes for invalid SSTs, results, or runs are logged. The causes of SST failure for the CZE method were monitored, and initially in 50% ($n = 53$) of the cases failure was attributed to instrument malfunction. Other causes were as follows: 27% ($n = 28$) material defects (e.g., capillary coating), 15% ($n = 16$) software issues, 4% ($n = 4$) unclarities in the test procedure, and 4% ($n = 4$) operator errors. Mapping the down-time of the equipment and the time spent to solve the issue can help prioritize the causes that need to be fixed first by continuous improvement. In this case, most of the failures attributed to instrument malfunction were significantly reduced with operator retraining on CE best practices and Ad sample handling.

At another point in time, a lower Ad concentration sensitivity was required, and the ATP was adapted accordingly. Thanks to the AQbD method development process, thorough understanding of the CE principles and well-documented method development and risk assessments resulted in a fast adaption of the method. Sensitivity was enhanced by large-volume injection and transient-isotachophoresis on-column sample concentration where tricine in the BGE acts as a terminating electrolyte and chloride, always present in any sample matrix, acts as a leading electrolyte. Without further effort, an LOD of $5 \cdot 10^8$ VP/mL (0.8 pmol/L) and

an LOQ of 1.5•10^9 VP/mL (2.5 pmol/L) were readily achieved for drug substance and drug product [50].

4 | Conclusions/Concluding Remarks

This article highlights the advantages of applying AQbD in capillary electrophoresis method development, underlining its role in creating robust and reproducible analytical procedures. Although demonstrated here for CE, this approach is equally applicable to any analytical technology, offering a structured framework for optimizing performance, minimizing risk, and ensuring consistent quality across various methods. The presented AQbD-flow with its tools such as criticality assessment, risk assessment, and DoE is complementary to sound knowledge and experience of the analytical technologies used, as well as of the application field for which the method is needed. Use of the tools does not replace science but helps better document and transfer all knowledge gained. To the experienced technology expert, the AQbD-flow might seem superfluous, as an expert makes swift, often subconscious, decisions on CMPs and risks. However, the AQbD tools will support the expert in capturing those thoughts and transferring the knowledge to others, as the method development expert is usually not the person performing the (QC) analysis or might not be around when troubleshooting or method life cycle management is required. Knowledge transfer is of utmost importance as the safety of the patient through well-characterized and quality-controlled medicines cannot be dependent on the availability of a single expert.

A common misconception is confusing DoEs with AQbD. Although DoE is a statistical tool used for process optimization and identifying key variables, AQbD is a broader, more comprehensive framework. It incorporates DoE as part of a systematic, risk-based approach but also integrates knowledge management, risk assessment, and lifecycle management, providing a holistic view of method development. The key benefits of the AQbD approach presented here include ensuring that all method developers follow a consistent strategy and use a common language. The AQbD approach also helps in focusing on the end user, which in pharmaceutical analysis typically is a skilled operator in a manufacturing QC environment and not a technology expert. Best practices and robustness should be built into the final method and not be dependent on the operator. An AQbD focus furthermore prevents methods from being developed for the wrong purpose, ensures traceability of data, and facilitates the seamless integration of completed tools into the method development report, enhancing both clarity and documentation.

Although the implementation of AQbD and ICH Q14 guidelines offers significant advantages, they should not become mere checkbox exercises. These frameworks are tools designed to systematically enhance method robustness through deep scientific understanding and proactive risk management. However, they must be complemented by field-specific expertise in the underlying technologies and analytes. Following procedural steps without truly understanding the science behind them risks undermining AQbD's goal of enhancing method performance and adaptability.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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