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DEVELOPMENT OF AN ACCURATE, PRECISE AND ROBUST METHOD FOR DETERMINATION OF THE PROTEIN CONTENT OF BIOPHARMACEUTICAL THERAPEUTICS

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Development of an accurate, precise and robust method for determination of the protein content of biopharmaceutical therapeutics

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Abstract

The development of a biopharmaceutical consists of R&D, CMC manufacturing and clinical trial phases. The quality of the biopharmaceutical product and its assessment via biophysical and biochemical analysis are essential components of this process. The corresponding analytical methods are involved in stages of the biopharmaceutical development, serving as a foundation for process and production development, while also playing a role in assessing clinical outcome (i.e. PK) and approving the final product, from first-in-human (FIH) trials to routine market supply. The different analytical methods include a broad spectrum of techniques from standard methods as described by European and United States pharmacopeia to very specific methods for detailed characterization (e.g., *in*-vivo CQA determination by mass spectrometry).

Here, we focus on determining the protein concentration of the active pharmaceutical ingredient (API). This protein content determination method is included in the standard set of analytical methods and is applied in all phases throughout the development process. At a first glance, the analytical procedure for protein content determination "would appear simple". It's importance, however, is reflected in the fact that patient dosing is directly related to the protein concentration of the drug substance (DS) and drug product (DP) of the medicinal product. Hence, the quantity of the API is one of the key elements of specifications for each medicinal product recommended by all regulatory bodies and described in ICH guideline 6B (1).

The requirements for the protein content determination method are thus very strict, and there is an eminent need for high precision and accuracy. Meeting both criteria is challenging and requires a sophisticated development strategy as pointed out in this article. This is particularly true if dose correction is needed, due to an inaccurate method, as this may, in turn, increase the need for reevaluating submitted dossiers, including specifications and CTAs. In the worst case scenario, an inaccurate protein concentration could impact clinical trial design and execution, ultimately, triggering additional clinical studies. Overall, the potential consequences would unduly increase the workload, costs and risk of the development program, relative to those of a thorough analytical development strategy.

Here, we present a concept for developing, qualifying and validating a precise, accurate and robust method for protein content determination, one that can be adapted to the specific requirements of the product and applied universally in different phases of biopharmaceutical development, including execution at multiple laboratories and sites.

Introduction

The determination of protein concentration during the development of a therapeutic biopharmaceutical is an essential and critical step. Relative to sophisticated technologies like HDX or site-specific mass spectrometric analysis, however, protein content methods are thought of as "simple" due to the inherent simplicity of the underlying physical principles, analytical instrumentation, and speed of measurement.

Protein content determination is the essential step in fill-and-finish step for DP, as the protein content has a direct impact on the amount of therapeutic agent administered to patients. During the process of manufacturing biopharmaceutical, the protein concentration of in-process-control (IPC), DS, and DP samples needs to be determined for different concentration ranges and corresponding buffers/formulations, which imposes requirements to a universal protein content method.

The protein concentration, which is determined throughout the entire process and all phases of biopharmaceutical production, will be used by many different laboratories for a variety of intended purposes. As such, the method must be highly robust, and method transfer must be straightforward.

Accurate determination of protein content is especially important during the development phase, (in PK studies, etc.) with regards to subcutaneous (sc) formulations where the total administered amount is typically an order of magnitude higher than is the case for intravenous (iv) formulations. The dose is derived from the extractable volume in combination with the protein concentration. Biopharmaceuticals often show a direct correlation between dose and corresponding activity, and variability of the dose could therefore lead to a pharmacokinetic profile outside of the intended activity range.

Additionally, the determined protein content directly impacts all other assays, such as potency and bioassays. A difference caused by inadequate precision or accuracy of the protein content method will impact the result of bioassays and potency assays during characterization, comparability, or release testing and could trigger out of specification events.

Overview of analytical methods for protein content determination

Several methods can be used for determining protein concentration during biopharmaceutical development (Table 1).

Table 1: Overview of protein content determination methods

ANALYTICAL CATEGORY	ASSAY
COLORIMETRIC ASSAY	LOWRY (2) COOMASSIE (3) / BCA (4) AMIDOBLACK (5)
AMINO ACID QUANTIFICATION	HYDROLYSIS AND CHROMATOGRAPHIC QUANTIFICATION BY UPLC (6)
IMMUNOLOGICAL METHODS	ELISA (7)
EMISSION / ABSORPTION	FLUORESCENCE DYES (FLUORESCAMINE, NANOORANGE) (5) UV ABSORPTION AT 205 AND 280 NM (8)
MASS SPECTROMETRY	STABLE ISOTOPE LABELING (9)
CHROMATOGRAPHY	PROTEIN A - SEC (10), RP-HPLC (11, 12)

Different categories of methods are available. The protein content can be determined, for example, indirectly via a colorimetric reaction. Here, the established assays (e.g., Coomassie, Lowry / BCA, Amidoblack) are based on a similar principle. A colorimetric dye binds to specific parts of the protein, a process that triggers a shift of the absorption within the colorimetric dye. The dye binds to the protein in a specific ratio, and the shift in wavelength is thus directly correlated to the protein content and can be linearly extrapolated to determine the protein concentration. Colorimetric assays are very sensitive to low protein amounts (typically ranging between 1 and 1750 µg/mL) and can be applied in almost any laboratory, given its widespread commercial availability (2; 3; 4; 5). The same principle applies to fluorescent dyes. Here, the absorption maximum of the dyes is not limited to the visible range of the spectrum but rather extends into the UV range for detecting fluorescence emission in fluorescent dyes. Due to its higher sensitivity, this method can be applied to even lower concentrations on the order of e.g.,10 ng/mL (5, 8).

Methods based on immunological binding, such as ELISA assays, can be applied where a high specificity for the protein of interest is needed. Such assays are used for determining the proportion of active ingredient in the presence of other UV/VIS active substances, including other proteins, formulation components, etc. (7).

Another way of specifically detecting the amount of the protein of interest in complex matrices or in serum is mass spectrometry. This approach involves using stable isotope labels for the purpose of generating specific peptides corresponding to regions of the therapeutic protein. These peptides serve as a calibration curve enabling precise quantification by mass spectrometry, even in complex mixtures (9). Chromatographic quantification by HPLC represents an option for quantifying protein content by affinity chromatography (e.g., Protein A for mAbs) (10), size exclusion (10) or reverse phase / hydrophobic interaction chromatography (HIC) (11; 12). HPLC is applied for the determination of specific proteins of interest in protein mixtures. Here, the limit of quantification is very low and specific for the protein of interest when the protein can be compared to control samples. However, the gold standard for determining the protein content of biotherapeutics in solution is amino acid analysis after hydrolysis. The protein is incubated at temperatures (e.g., 110 °C) in highly concentrated hydrochloric acid (6 M). Afterwards, the amino acids are labeled with a fluorescent dye and subsequently separated and quantified via liquid chromatography (6). The outstanding advantage of the amino acid analysis is the independency of the matrix and formulation of the samples of interest. Despite the accuracy, this approach is laborious and is not often chosen for use throughout the development process. In this article, we will focus on the most commonly used method of protein content determination in the development of biologic drugs, which combines a simple physical principle with inexpensive analytical equipment and straightforward execution: protein content determination via UV absorption at 280 nm.

The UV-based method is the industry standard and a must-have method in every analytical laboratory supporting R&D, and process and manufacturing development, as well as QC release and stability testing for biopharmaceuticals (13). Protein content determination by UV absorption has multiple advantages for application during the development of a biopharmaceutical. On the one hand, the method is not influenced by most of the excipients commonly utilized in the biopharmaceutical formulations that might interfere with colorimetric and fluorescent dyes. Furthermore, the concentration range can be adapted to almost any concentration by selecting a suitable dilution scheme (described in more detail later). On the other hand, the execution of an established UV method can be executed quickly and in an analytical laboratory with little investment in instruments, relative to, say, amino acid analysis. Importantly, the procedure for protein content analysis can be qualified and validated by UV in a GMP-compliant fashion and in a regulated GMP environment, which is more time consuming, but more cost-efficient than most other methods (14).

Protein content determination by UV absorption

Light absorption principle

Proteins consists of a specific arrangement of amino acids, which determine the primary, secondary, tertiary, and/or quaternary structure. Furthermore, the amino acids are responsible for a characteristic UV absorbance between around 205 to 290 nm (3). Two specific fundamental molecular structures efficiently absorb light in proteins. The peptide bond, which connects the amino acids to each other, absorbs light at a wavelength between 190 and 220 nm and is typically measured at a single wavelength of 205 or 210 nm. Three amino acids contain aromatic side chains: tryptophan, tyrosine and phenylalanine. These aromatic rings have side chain specific absorption maxima (phenylalanine λ_{max} 257 nm; tyrosine λ_{max} 274.6 nm and tryptophan λ_{max} 279.8 nm). For convenience 280 nm typically is used for measurement protein concentration because the molar absorptivity of Trp is substantially greater than Phe/Tyr, making it the most reliable wavelength to measure the absorbance related to the aromatic side chains (3). The absorbance of light can be used to determine protein concentration because the amount of absorbed light is proportional to the amount of protein. The physical principle underlying this correlation is the Lambert-Beer-Law:

$$E_\lambda = \log10 (I_0 / I_1) = e_\lambda * c * d$$

E_λ = Extinction (absorbance of light at wavelength λ)
I_0 = Intensity incoming light
I_1 = Intensity transmitting light
c = protein concentration
e_λ = decadic extinction coefficient at wavelength (λ) (specific for the tested substance)
d = thickness of layer (here cuvette path length)

To measure the absorbance, the substance of interest is placed in a cuvette and analyzed in a UV spectrophotometer at a certain wavelength. The spectrophotometer measures the intensity of the light after its passage through the cuvette, relative to a reference sample or blank measured in parallel or after the analysis of a blank substance in the same cuvette.

Extinction coefficient (e_λ)

The proportionality of the absorbance and the protein content is specific for every protein and related to the primary sequence of the amino acids. The precise determination of the protein-specific extinction coefficient is an essential step. This extinction coefficient can be evaluated theoretically based on the amino acids sequence of the protein (15). The extinction coefficient is not only based on the primary sequence of the amino acids but can be influence by the higher-order structure, including secondary and tertiary structure as well as oligomerization and aggregation. For this reason, measurement may be performed under denaturing conditions. Additionally, e_λ can be influenced by protein modifications like glycosylation or pegylation (16). Hence, it is recommended to verify the product specific extinction coefficient experimentally, for example by amino acid analysis (17).

The extinction coefficient is a constant for a given molecule in a given matrix and therefore the extinction coefficient should be determined and verified in the same matrix or buffer used for protein content determination due to the fact that the matrix or buffer has a direct impact on the secondary, tertiary and quaternary structure of proteins which influences the extinction coefficient (18).

The extinction coefficient applied by the innovator company is unknown to the biosimilars development team, and this will increase the complexity of a comparability exercise during a biosimilarity assessment. During a biosimilarity demonstration, all methods that rely on the extinction coefficients of both the biosimilar and originator products could be impacted unless the biosimilars development team determines the extinction coefficient.

At the same time the protein content is one of the most important factors during a biosimilarity exercise, because the filling amount, which consists of filling volume and protein concentration, needs to be as similar as possible between originator and biosimilar candidate. This way, both drugs behave similarly in activity assays and PK studies, which subsequently is the basis for demonstrating that the biosimilar can be applied as a substitute product. It needs to be demonstrated experimentally that the determined extinction coefficient and related protein concentration of the originator and biosimilar candidate are comparable, although the formulation between both products might differ, which might result in a slightly different absorption behavior.

Impact of formulation and background absorption (I_0 / I_1)

Biopharmaceutical products not only consist of the API but also are comprised of selected excipients to stabilize the protein. The formulation of a DP and buffer components of in-process controls contain several excipients - typically salts, surfactants/detergents, sugars, and amino acids. All these components might contribute to the absorbance measurement of the protein solution and impact the evaluation of the protein concentration measurement. Most of these substances have an absorbance maximum below 250 nm or higher than 300 nm, and therefore, measurement of the absorbance at 280 nm has further been established as the most common wavelength to specifically determine the concentration of the protein of interest.

Best practice is not to use the absorbance at 280 nm directly, but to determine the absorbance at 320 nm in addition and to subtract the absorbance at 320 nm from 280 nm to correct for background absorbance (3).

Furthermore, to prevent artificial absorbance, the diluent without protein must be checked for the absorbance maximum as a control. Therefore, a wavelength scan from 180-700 nm is recommended, and if absorbance is detected, background subtraction by measuring the blank performed to correct for the offset.

In any case, one critical step during the protein concentration determination is the usage of a suitable blank. Background absorption influences directly the resulting intensity and might cause under- or overestimation of the protein concentration, and thereby negatively affect the accuracy and precision of the method.

Limitation based on the path length (d) and linearity of the photometer

The third parameter of the Lambert-Beer-Law (Equation [1]), which impacts the absorbance measurement and the protein concentration calculation, is the path length (d).

The path length describes the length of the sample through which the light travels, determined by the specific properties and filling volume of the cuvette.

There are a number of different cuvettes available with path lengths varying from 1 mm up to 20 mm. Other features of the cuvette may also vary, such as width, depth and shape, leading to typical filling volumes ranging from <0.01 mL to as much as 3.5 mL.

An additional limitation stems from the photometer. The intrinsic linearity of the wavelength detectors typically ranges between 0.2 and 2 absorption units (AU).
The selection of a suitable cuvette and path length, in combination with the linearity of the wavelength detector of the photometer, therefore determines the minimum and maximum range for the protein concentration, that can be analyzed via a specific UV absorption setup. For protein concentrations outside the relevant range the protein solution may need to be diluted, depending on the protein concentration itself and on the combination of instrument and cuvette (i.e. path length) (13; 19).

Evaluation of different approaches

If the product-specific extinction coefficient is not available, the protein concentration of a solution of interest can be determined relative to a calibration curve.

Calibration curve

A calibration curve can be established by using a reference standard of the protein of interest with a known concentration. This standard will be used to establish a calibration curve and the protein concentration of the solution of interest will then be determined relative to the calibration curve. During the biopharmaceutical development, this reference standard can be obtained from the most representative manufacturing lot or from a working reference standard. Within a biosimilarity process, an originated sample can also serve as this reference standard. One disadvantage here lies in the availability of suitable reference material for the protein of interest. Also, differences in formulation buffer composition (between the reference material and the protein of interest) may not easily be accounted for and may bias determination of the concentration.
In general, the use of a calibration curve minimizes the intersession variability, however, as potential deviation is negated by normalizing the protein content values to the calibration curve.

Critical parameters to consider for method development

Very high protein concentrations up to 300 g/L

Several molecule-specific factors need to be considered when developing a protein content method based on UV absorbance. Very high DS concentrations (up to 300 g/L (13; 20)) may be present, especially in biopharmaceuticals used in subcutaneous formulations. A highly concentrated protein solution in combination with excipients, that stabilize the protein in solution, often results in a highly viscous solution (21). Such highly concentrated protein solutions must be adequately diluted for protein absorbance to fall within the linear range of the UV absorbance method (i.e. the combination of photometer and cuvette). In general, dilution of the protein solution adds an additional step or steps to the procedure, thereby introducing a potential source of error or high degree of variability, and should be prevented or minimized as much as possible (13). One way to prevent dilution steps for highly concentrated protein solutions or to limit dilution to a single step is to decrease the path length of the cuvette (19). If the path length is shorter, the volume of protein solution (i.e. the number of molecules) for the light beam to pass through is lower, which yields a less intense signal and UV absorbance. With a shorter path length, the test concentration of the protein can be increased and no dilution or only one dilution step is needed. Path lengths from 0.125 mm to 2 cm are commonly available, and decreasing the path length by a factor of ten allows users to increase the protein concentration by the same factor. For example, if using a cuvette with a path length of 1 mm, a protein concentration of 200 g/L can be diluted in one step to 10 g/L by applying a dilution factor of 20. If an extinction coefficient of 1 is assumed in this example, the absorbance will be 1 AU, which is within the linear range of a typical UV spectrometer. If, for the same protein solution and setup, a cuvette with a 1 cm path length is used, the resulting absorbance will be 10 AU, which is outside the linear range of a typical UV spectrophotometer, and a two-step dilution workflow might be necessary. However, high viscosity in combination with small volume and narrow path length could require very precise pipetting techniques as well as an in-depth cleaning procedure for the cuvettes.
Smart selection of path length and dilution scheme can therefore decrease method variability and increase the precision and accuracy of the UV absorbance method.

Very low protein concentrations down to 0.1 g/L

Unlike high-concentration protein solutions (antibodies, etc.) and high-potency therapeutics (enzymes, etc.), peptides and small proteins (parathormone, filgrastim, etc.) are formulated at low concentrations in the range of 0.1 g/L or even lower (e.g., botulinum toxin). Here, the opposite routine might be helpful, as low-concentration samples such as these could potentially be measured by extending the path length. If so, precision and accuracy of the protein concentration determination could be high. If the protein concentration is even lower (µg/mL - ng/mL), protein concentration can be measured reliably by using multiple other techniques, as outlined in the first paragraph, e.g., by labeling the protein with UV active or fluorescence dyes to increase sensitivity.

Dilution factor and volumetric vs. gravimetric dilution approach

During the development of a biopharmaceutical the API will be present at several different concentrations and under various solution conditions. For example, concentrations are typically lower for in-process control samples, which have only a few excipients in their buffers. The DS is typically presented at a high protein concentration and the DP will constitute the most complex formulation. The protein concentration needs to be determined for all of these different types of samples, ideally with the same analytical workflow applicable to all sample types (13).
A harmonized workflow for samples with different concentrations and formulations can be achieved by diluting all samples with a suitable diluent to a final testing concentration range. Whenever possible, a method that is capable of measuring a broad range of protein concentrations without dilution should be used (13).
Two main procedures are available for diluting a protein solution: A volumetric dilution or a gravimetric dilution.

A **volumetric dilution** is indicated when the viscosity of the protein solution and the diluents is low. This involves mixing a specific volume of the protein solution with a specific volume of the diluent. Important factors when setting up a precise volumetric dilution are the starting concentration of the protein solution of interest and the corresponding dilution factors. In general, a dilution factor higher than 50 should be avoided, and a two-step dilution workflow might be considered to provide more reliable results. To further increase the precision and reproducibility of the method, the initial uptake volume of the solution of interest can be increased, whereas pipetting volumes less than 10 µL should be avoided.
For highly viscous protein solutions and diluents, the volumetric dilution (i.e. pipetting of the liquids) introduces a potential risk and variability to the dilution process. An alternative way to dilute highly viscous samples is to perform a **gravimetric dilution**.

A defined volume of diluent is placed in the vial and the weight is determined with an analytical balance. Afterwards, a defined volume of the solution of interest is added to the diluent and the final weight is determined. In addition, the weights can also be corrected for the corresponding density of the diluent and the solution of interest to further increase accuracy of the dilution procedure (22). The difference of the weights in relation to the overall weight determines the gravimetric dilution factor.

The same parameters (i.e. uptake volume, dilution factors and one vs. multiple dilution steps) should be considered for the gravimetric dilution, as outlined above for the volumetric dilution.

In addition to the establishment of a robust dilution procedure (one enabling the application of the protein content analysis procedure to all types of samples), a second generation of techniques evolved for measuring protein concentrations between 0.15 and 242 g/L without any dilution step (13). Two different approaches are feasible for determining the concentration of high-concentration protein solutions **without dilution.**

The first approach is based on the classic spectrophotometer setup in combination with very small path lengths, with or without a cuvette, and using an instrument such as a Nanodrop (Thermo Fischer Scientific, Waltham, MA). The use of cuvettes with very short path lengths (minimum: 0.125 mm) makes it possible to load the protein solution of interest directly, while keeping absorbance within the linear range of the spectrophotometer. Path lengths of 0.05 and 1 mm are available for the NanoDrop device, which reduces the path length even further to allow for direct measurement of highly concentrated samples without dilution (19).
The second approach is called “slope spectroscopy” and is based on the linear correlation of absorbance and path length. The SoloVPE (C Technologies Inc., Bridgewater, NJ) varies the path length during the measurement and records the absorption at 280 nm multiple times. The linear correlation is then used for determining the protein concentration based on the slope of these measurements.

Especially for DS samples of sc formulations, which have high protein concentrations, a dilution step might introduce potential risk for higher variability and inaccurate precision and accuracy (13).
Several handling approaches and considerations were studied for mitigating problems that arise when diluting highly concentrated samples, and these are discussed below.

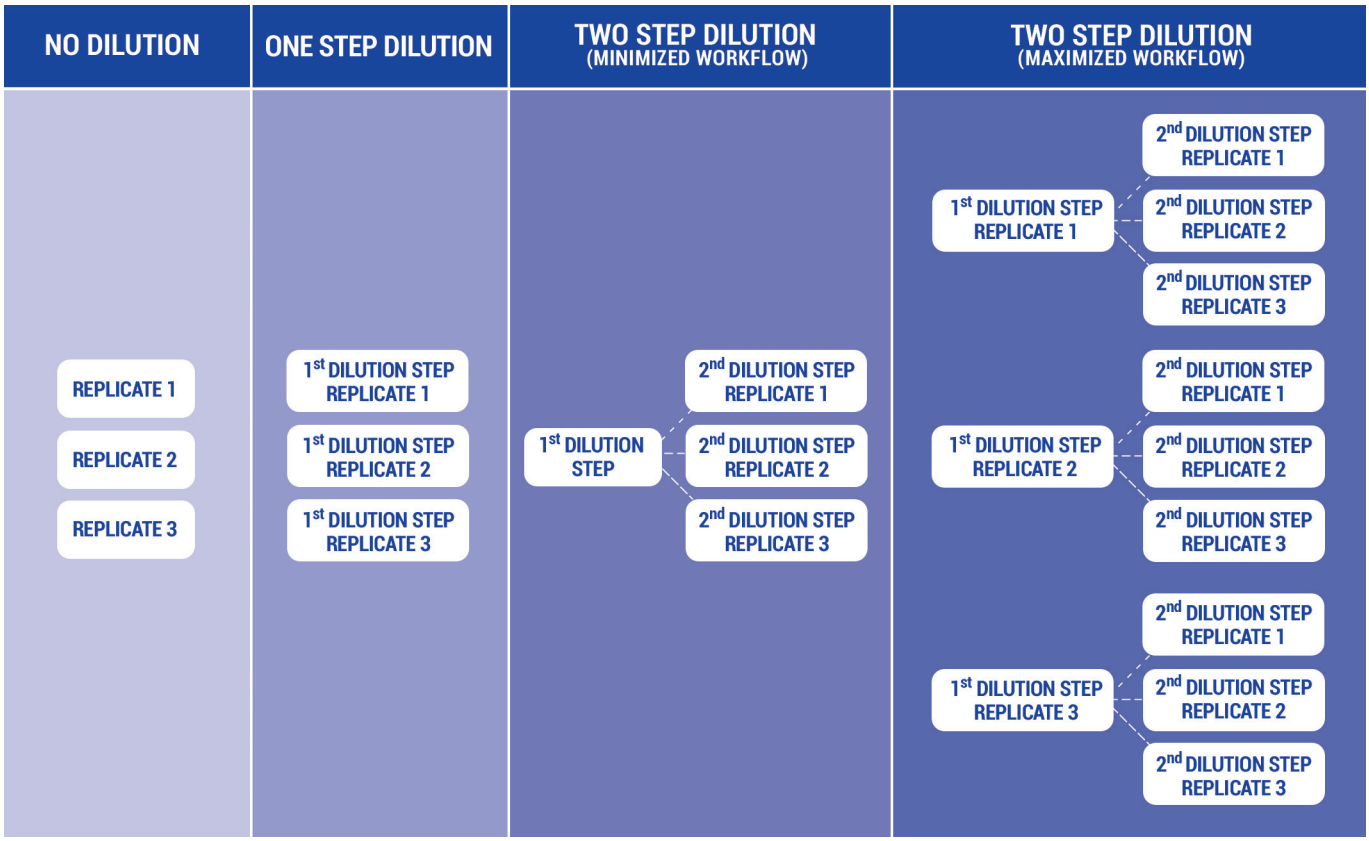

Figure 1: Replicate design and selection

Number of replicates

The number of replicates per dilution step needs to be defined based on the number of dilution steps (see Figure 1).

If one dilution step is applied, three independent dilutions and three independent protein concentration determinations are suggested. The mean of all nine analyses is then determined.

If two dilution steps are applied, the number of replicates can vary between the first and second dilution step. The less time-consuming approach comprises one initial dilution step followed by three secondary dilution steps. Again, the protein concentration is determined as the mean of these three independent triplicate analyses (minimized workflow, Figure 1). Alternatively, the first dilution step can be performed in triplicate, with each of the three replicates undergoing the second dilution step (one to three replicates), which, in total, would result in nine values (maximized workflow, Figure 1) being included in the mean protein concentration determined for the solution of interest.

For solutions of interest with very low protein concentrations, no dilution is required and each sample is typically analyzed in independent triplicates, whereby the mean of the three values is determined. This makes the next section very important, as the source of inaccurate and imprecise protein concentration determinations for low concentration protein solutions is most substantially related to correct selection of the container dilution vessel.

Selection of container used for dilution

In solution, all proteins present a unique molecular surface defined by their primary, secondary and tertiary structure and by conformational dynamics. Among other characteristics, this surface is composed of charged/uncharged and polar/apolar regions, which may interact with material surfaces, depending on an individual protein's properties and compatibility with the materials to which it is exposed. Some surfaces promote protein adsorption, which results in a loss of protein molecules in solution. This effect leads researchers to underestimate the protein concentration of the solution of interest, which can be problematic because low-concentration biologics are often high potency.

To identify incompatibilities in material choices, different containers, transfer materials, and/or cuvettes should be tested during the development of a protein-specific method. The first step is to test vials made of glass or polypropylene, as these are considered to have less potential for binding many proteins. Some manufacturers offer coated vials to specifically increase recovery and prevent protein adsorption effects.

Diluents

Use of an appropriate diluent is also dependent on the container material and can support recovery during the dilution steps. A diluent containing components that saturate the surface of the container before the protein solution is added could increase recovery. However, the addition of surfactants, detergents and other excipients that prevent absorption along the container surface need to be considered carefully, because UV absorbance at 280 nm must be low in order to minimize background absorption and interference with the analytical procedure.

Table 2 shows an example of the impact that the combination of container material (glass vs. polypropylene), and diluent selection (PBS with and without detergent) has for a specific protein (IgG) with a target protein concentration of 100 g/L.

Table 2: Selection of container and diluent

OPERATOR	ANALYTICAL SESSION	PROTEIN CONCENTRATION [g/L]			
		GLASS VIAL		POLYPROPYLENE VIAL (COATED)	
		PBS	PBS PLUS DETERGENT	PBS	PBS PLUS DETERGENT
1	1	98.04	97.30	103.36	100.63
	2	96.36	98.29	99.63	102.51
	3	97.34	97.97	97.76	100.06
2	1	94.55	96.90	97.86	98.87
	2	95.94	97.31	96.04	99.70
	3	103.88	99.06	95.52	99.90
MEAN [g/L]		97.68	97.80	98.36	100.28
CV [%]		3.3	0.8	2.9	1.2

In this case study (Table 2) a gravimetric two step dilution is performed and the diluent containing detergent decreased the variability of the method. Using the glass vial resulted in lower protein concentrations than using the coated polypropylene vial. Therefore, the coated polypropylene vial in combination with detergent-containing diluent gave the most accurate and precise results.

Pipetting styles

Attention should be paid to pipetting techniques when pipetting highly viscous solutions or when an interaction between the protein and the container surface is observed during method development. The manufacturers of pipettes provide detailed instructions and recommendations for how to pipette solutions, and several guidelines are available that detail good pipetting practices (23, 24, 25) (Table 3).

Table 3: Pipetting recommendations by pipette vendors.

ASPECTS TO BE CONSIDERED	PIPETTING GUIDELINES		
	THERMO FISCHER (23)	GILSON (22)	EPPENDORF (21)
RELEASE OF LIQUID (WALL VS DIRECT)	WALL	N.A.	VOLUME < 10 µL = WALL OR DIRECT VOLUME >10 µL = WALL
PREWETTING OF THE PIPET TIP	YES, 3x	YES	YES, 3x
REFERENCE	-	ISO 8655	-

The general recommendation is to prewet the pipette tips and release the liquid along the vessel wall in order to utilize capillary action.
A more detailed description of the pipetting process is needed, however, for method training and/or optimization. In the following case study (Figure 2), three different pipetting styles were applied with a detergent-containing diluent (because the protein was known to have a strong interaction with the container surface).

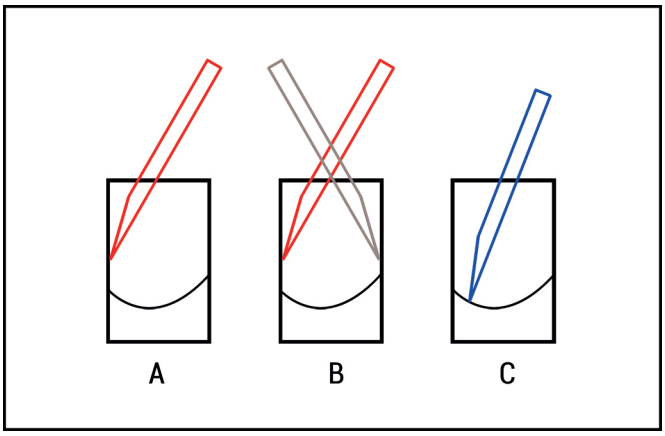

Figure 2: Comparison of different pipetting styles. Prewet and release of diluent and sample at the same wall position (A), Prewet and release of diluent and sample at different wall positions (B), Release of sample directly above the prefilled diluent (C).

Table 4 shows the results of the case study depicted in Figure 2, comparing different pipetting styles to reach a known target protein concentration.

Table 4: Protein concentration depending on the pipetting style.

OPERATOR	STYLE	MEAN [g/L]	CV [%]
1	A	97.98	0.29
2	A	97.07	0.35
1	B	94.61	0.66
2	B	95.34	0.39
1	C	99.49	0.25
2	C	99.33	0.60
KNOWN PROTEIN CONCENTRATION			TARGET 100.00 g/L

The lowest protein concentration was obtained when two different operators (1+2) applied pipetting style B. Although pipetting style B follows the recommendations of the manufacturers (i.e. release the protein along the surface of the vial), the protein was observed to adsorb to the vessel surface.

When pipetting style A was applied, which involves saturating the wall of the container by releasing the detergent-containing diluent and then, in a second step, releasing the protein solution at the same spot on the container wall, the protein concentration was found to increase, although it was still lower than the expected target level.

Finally, in pipetting style C, the diluent was placed in the vial and the protein solution was released directly above the diluent without any interaction with the container surface. This technique increased recovery compared to pipetting styles A and B, and yielded results very close to the expected target concentration.

From method setup to release testing

Method setup, development, and optimization

Taking the suggestions and considerations of the last sections into account, the development of a protein content method might follow the sequence described below (see Figure 3).

After passing the required decision points shown in Figure 3, the next step is to test the final setup through confirmation runs. These confirmation runs should include the maximum number of variables expected during later application. The best case scenario will include several different operators, instruments, diluent batches, cuvette batches, and analytical sessions. When final recovery and variability are acceptable, the method can be considered fit for its intended purpose. The next step is to qualify the method to verify applicability.

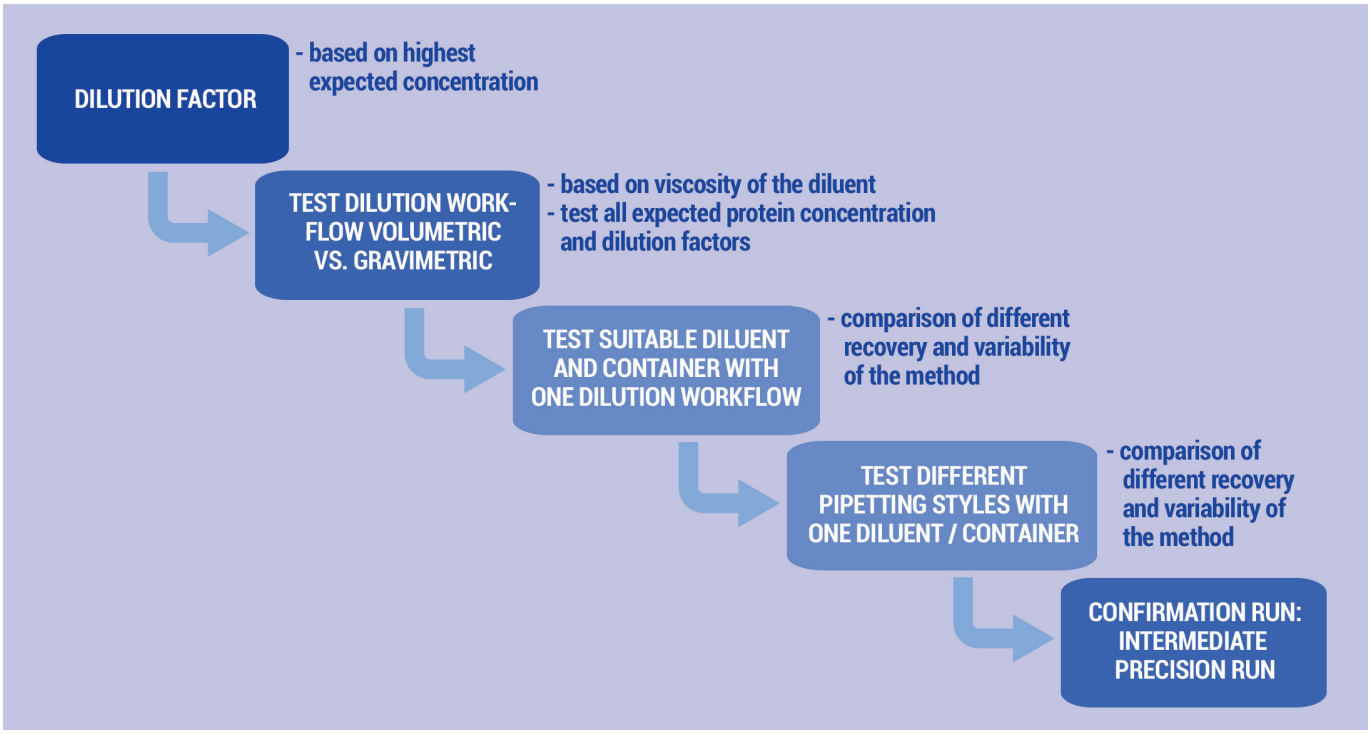

Figure 3: Schematic route to develop a protein content method

Qualification and validation of the method

Qualification and validation

Method qualification covers the critical parameters of precision, specificity and linearity based in the recommendation of the ICH guideline Q2(R1) (26) for validating methods (see Table 5). The method should exhibit sufficient precision as determined by repeatability and intermediate precision testing, as well as by demonstrating method specificity in the form of negative results from absorbance testing on the diluents and the formulation.

The linearity of the method should range from 50% up to 150% in equidistant intervals. The final test concentration after all dilution steps is used as the 100% level. If the method passes all three parameters, it is sufficiently developed to be validated for the purpose of release testing, etc.

The method validation parameters shown in Table 5 are derived from the ICH Q2(R1) (26) guideline released by the EMA in June 1995.

Table 5: Overview of method qualification and validation parameters

	METHOD QUALIFICATION		METHOD VALIDATION	
PARAMETER	RELATIVE IMPORTANCE OF QUALIFICATION PARAMETERS	NUMBER OF RUNS / ANALYTICAL DESIGN	VALIDATION PARAMETER BASED ON ICH Q2 (R1)	NUMBER OF RUNS / ANALYTICAL DESIGN
ACCURACY	-	NOT COVERED DURING QUALIFICATION	+	TAKEN FROM LINEARITY (RECOVERY OF 50% AND 150% TO 100% VALUE)
PRECISION - REPEATABILITY	+	THREE INDEPENDENT ANALYSIS	+	THREE INDEPENDENT ANALYSIS
PRECISION - INTERMEDIATE PRECISION	+	THREE INDEPENDENT ANALYSIS CARRIED OUT FROM AT LEAST TWO OPERATORS AND AT TWO DIFFERENT DAYS	+	THREE INDEPENDENT ANALYSIS CARRIED OUT FROM AT LEAST TWO OPERATORS AND AT TWO DIFFERENT DAYS
SPECIFICITY	+	AGAINST DILUENT AND PRESENT FORMULATIONS	+	AGAINST DILUENT AND PRESENT FORMULATIONS AGAINST OTHER PRODUCT MANUFACTURED AT SAME SITE
DETECTION LIMIT	NOT REQUIRED			
QUANTITATION LIMIT	NOT REQUIRED			
LINEARITY	+	50% - 150% OF NOMINAL CONCENTRATION AFTER DILUTION	+	50% - 150% OF NOMINAL CONCENTRATION AFTER DILUTION
RANGE	-	NOT COVERED DURING QUALIFICATION	+	SUFFICIENT PRECISION SHALL BE SHOWN AT 50% AND 150% NOMINAL LOADING CONCENTRATION

Precision
Determination of precision is similar to tests performed during method development and qualification. The highest possible number of aspects should be varied for intermediate precision testing (e.g., operator, equipment, reagents, sessions, sites).

Linearity and range
The linearity must also be tested at dilutions ranging from 50% to 150% in equidistant steps, based on the final diluted concentration. Linearity analysis is performed in triplicate at each concentration level, because this allows researchers to determine recovery at each concentration level relative to 100% level, and consequently, to derive the accuracy parameter from the same data set. The three replicates at each level can also be used for determining the CV. The overall range can be assessed from the linearity data together with the recovery of the accuracy parameter and in combination with the precision at each concentration level.

Specificity
In terms of specificity, the diluent and formulations should not exhibit any absorbance during testing that would otherwise result in under- or over-estimation of the protein concentration.
In addition, one may be required to show that the method is able to distinguish between products manufactured at the same site may be necessary as well. If a similar product is manufactured at the same site, and the final concentrations differ from each other, this product could be used to establish specificity relative to similar products. Applying the same dilution factors would cause the protein concentration of the alternative product to fall outside of the ranges determined during precision testing. Consequently, it can be concluded that this is not the protein of interest.

Robustness
The robustness of the method should focus on operator-specific issues such as the dilution factor, as well as on instrument- and method-related factors such as wavelength variability and reagents in the diluent that are critical during dilution (sugars that raise the viscosity, detergents that minimize absorption effects, etc.) (Table 6).

Table 6: Parameter examined for robustness testing

PARA METER	DESCRIPTION	NOMINAL VALUE	LOWER VALUE (L)	UPPER VALUE (H)
1	WAVELENGTH [nm]	280	278	282
2	SUGAR CONTENT IN DILUENT [%]	5.0	4.8	5.2
3	DETERGENT CONTENT IN DILUENT [%]	0.10	0.05	0.15

Robustness testing can be set up using a Plackett-Burman (Ferreira SLC, 2017) design, as shown in Table 7 below. In this example, three critical parameters have been selected to be increased (H = upper/high value) or decreased (L = lower value) from the regular applied setting. The matrix in Table 7 can be used to verify the influence of different combinations of changed settings or parameters that could impact the results and variability of the analytical method.

Table 7: Plackett-Burman design for robustness testing. H = upper/high value, L = lower value.

EXPERIMENT	PARAMETER 1	PARAMETER 2	PARAMETER 3
#1	H	H	L
#2	L	H	H
#3	H	L	H
#4	L	L	L

Tech transfer and co-validation

Handing over a protein content method is always a very critical step. Several aspects and parameters influence the outcome of a protein content method.

Table 8: Reproducibility design at each transfer site

ANALYTICAL SESSION	INDEPENDENT REPLICATE	OPERATOR	CUVETTE / DILUENT	DAY	INSTRUMENT	LABORATORY
1	1	1	A	1	1	1 & 2
1	2	1	A	1	1	1 & 2
1	3	1	A	1	1	1 & 2
2	1	2	B	2	2	1 & 2
2	2	2	B	2	2	1 & 2
2	3	2	B	2	2	1 & 2
3	1	1/2/3*	A/B/C*	1/2/3*	A/B/C*	1 & 2
3	2	1/2/3*	A/B/C*	1/2/3*	A/B/C*	1 & 2
3	3	1/2/3*	A/B/C*	1/2/3*	A/B/C*	1 & 2
* IF POSSIBLE, A THIRD VARIANT SHALL BE USED, IF NOT AVAILABLE THE FIRST OR SECOND VARIANT CAN BE REPEATED.						TOTAL: 18

Extensive operator training is required during technology transfer and/or co-validation. As shown in the section "Pipetting style," small differences in the pipetting style can cause significant differences in protein concentration results. As a result, onsite training provided by the donating laboratory at the receiving site is highly recommended. The first step should be to perform a gap analysis focusing on the aspects outlined in the section on critical parameters to be considered. The vials, reagents used in the diluent, the balance (in gravimetric dilutions), pipettes, pipette tips, cuvettes and the photometer are all critical where alignment between the sites is needed.

After establishing the method and providing training at the receiving site, handover is to be verified, either during method validation (co-validation approach) or after validation is completed (formal GMP method transfer). A comparative measurement is mandatory in both cases. Essentially, each site performs an intermediate precision study as shown in the example below (Table 8).

Testing at each site is followed by determination of the total protein concentration and CV of all 18 replicates. As a general guideline, the difference in protein content between laboratories should be within 5% and the CV of the 18 replicates should be ≤5%.

Release testing

Finally, when the protein content method has been successfully validated and most likely transferred to multiple sites, it will be used in a number of different applications, including (release) testing for IPC, DS and DP samples during manufacturing and stability testing, and for providing the protein concentration for additional tests (e.g., physicochemical, potency, bioassay).

To ensure precise method execution, the critical steps should be monitored and controlled via system suitability testing (SST), and the criteria implemented in the corresponding SOP. The following are some suggested points to be described in the SOP.

- The description of the pipetting style should be highly detailed, perhaps with the support of illustrations.
- If performing a gravimetric dilution, limits for the two weighing steps can be included (not less/more than a total weight of x mg).
- If performing a volumetric dilution, the total filling of the vials can be controlled via visual inspection or by weight.
- The instrument and the blank/diluents should not exhibit (background) absorbance above a certain level and to be verified in advance, prior to any testing.
- The excipients in the diluents need to be carefully monitored, as any impurities they contain may cause interference for example.
- After cleaning the cuvettes, a blank should be run to ensure they are clean.

Conclusion

The requirements for protein content determination via UV absorption at 280 nm are established on the basis of both the specific characteristics of the protein of interest and the demands of the health authorities (1).

In order to meet all of these requirements, development must be designed to take all influencing factors into account. In this article we present guidelines describing the most critical aspects to be considered during the development and execution of a protein content determination method. One of the primary parameters determining the final execution setup derives from the protein of interest itself and the corresponding manufacturing process. High protein concentrations (as may be observed during the production of monoclonal antibodies administered subcutaneously to the patient) require the development of a reliable dilution strategy suitable for DS, DP and IPC samples.

In addition, specific molecular properties determine the type of dilution and the equipment to be used (vials and diluent). Table 9 shows a matrix that supports the selection and development of a precise, robust protein concentration method.

For high concentrations of monoclonal antibodies, a one- or two-step gravimetric dilution in combination with a medium or narrow pathlength represents a promising starting point for method development.

Extensive, successful method development, qualification and validation determine the precision and robustness of a protein content method based on UV absorbance at 280 nm. Equally importantly, however, precision and robustness contribute to practical execution and affect the operational steps needed to achieve reliable results.

For this kind of method, which is affected more than other methods by operator handling, general handling and operating procedures often are not detailed enough to allow for precise training and execution across different laboratory sites.

To ensure consistency and robustness in method application, work instructions for any UV-based protein content method must provide highly detailed handling procedures, ideally supported by illustrations or practical videos.

Table 9: Recommended UV methods suitable for different expected concentrations

CONCENTRATION	VISCOSITY	DILUTION STEPS	VOLUMETRIC / GRAVIMETRIC	PATHLENGTH	DILUENT	VIALS
0,01 g/L - 1 g/L	low	none	n.a.	wide	TO BE TESTED	
0,1g/L - 10 g/L	low	one-step	volumetric	medium		
10-50 g/L	low	one-step	volumetric	medium		
10-50 g/L	high	two-step	gravimetric	medium		
50-300 g/L	low to high	two-step	gravimetric	narrow		

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