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White paper

Use of SDS-PAGE for Residual Protein Determination

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Use of SDS-PAGE for Residual Protein Determination

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Abstract
In recent years, Almac has increasingly integrated biocatalysis into the manufacturing of active pharmaceutical ingredients (APIs). These biologically driven processes, while efficient and sustainable, inherently introduce host cell derived impurities and recombinant proteins into the production stream. As a result, the detection and quantification of residual proteins-host cell proteins (HCPs) and recombinant enzymes-are critical to ensuring the quality, safety, and efficacy of the final API.

Absorption based methods for protein analysis, such as ELISA, Bradford, and BCA assays, often face limitations in specificity, sensitivity, or compatibility with complex sample matrices. To overcome these challenges, Almac has developed a robust and versatile sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) method tailored to accommodate a wide range of APIs and intermediates. This technique enables both qualitative and semi-quantitative assessment of residual proteins, offering a reliable alternative for routine quality control in biocatalytic API production.

1. Introduction

Biocatalysis has revolutionised pharmaceutical manufacturing, offering environmentally friendly and selective synthesis routes. However, the use of recombinant enzymes introduces host cell proteins (HCPs) as well as recombinant proteins into the process stream, necessitating rigorous residual protein testing.

SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) is a discontinuous electrophoretic system developed by Ulrich K. Laemmli which is commonly used as a method to separate proteins with molecular masses between 5 and 250 kDa. The combined use of sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) and polyacrylamide gel eliminates the influence of structure and charge, and proteins are separated by differences in their size. The mechanism is shown in Figure 1:

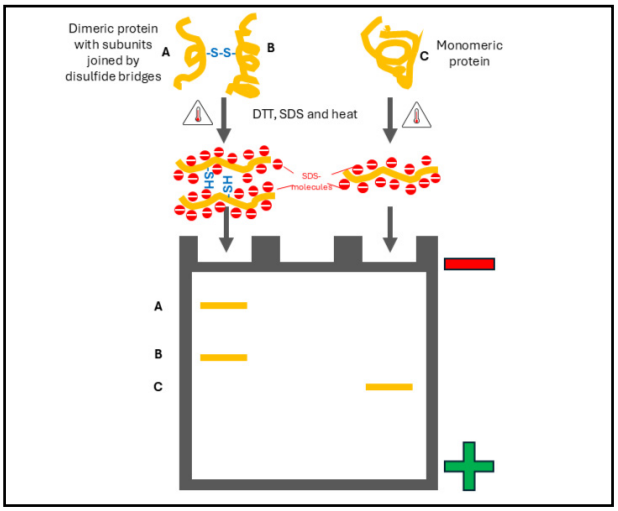

Figure 1: SDS PAGE Principle

SDS-PAGE together with Elisa are two validated methods featured in the US pharmacopeia recommendations for residual host cell protein measurement in biopharmaceuticals[1].

SDS-Page has the advantage that soluble and insoluble proteins are measured, and it has high robustness. The range of detection lies between 5 – 1,500 µg/mL CFE (cell free extract) based on different staining methods.

SDS-PAGE, as a classic protein separation technique, offers numerous advantages, including high resolution, sensitivity, and a broad applicability. However, its inability to retain protein native conformation, limited separation capabilities for properties other than molecular weight, stringent sample preparation requirements, and limitations in quantitative analysis underscore the need for careful consideration of its limitations when selecting this separation technique.

SDS-PAGE is typically employed for qualitative analysis. Although the relative abundance of proteins can be estimated by the density of the bands, the technique has limitations in quantitative analysis. Staining intensity can be influenced by various factors, such as protein characteristics and staining efficiency, making precise quantification challenging but not impossible[2].

The methodology presented in this article has the distinct benefit of determining the specific levels of the protein/enzyme content as well as identifying the presence of individual protein species. Herein, SDS-PAGE with imaging analysis was investigated as a suitable methodology for determining residual protein within an API.

2. Materials and methods

2.1. Reagents and equipment-SDS-PAGE Electrophoresis

XCell Surelock Mini Cell with Invitrogen PowerEase Touch 120 W power supply were used in this study. Main reagents used in the study are presented in Table 1.

Table 1: Chemicals and reageants

Material	Specification
Precast gels for SDS-PAGE analysis	NuPAGE™ 4-12% Bis-Tris Gel (12 or 10 well)
Enzyme markers	Cell Free Extract (CF) used for biotransformation
Protein ladder	SEEBLUE PLUS2 PRE-STAINED PROTEIN STANDARD
PBS buffer	1 x phosphate buffered saline, pH 7.4
Sample Diluent	DMSO
SDS-PAGE sample buffer	NuPAGE™LDS Sample Buffer (4 x)
SDS-PAGE-running buffer	NuPAGE™MES SDS running buffer (20 x)
SDS-PAGE-reducing agent	NuPAGE Sample Reduing Agent (10 x)
Silverstaining kit	SilverQuest™

2.2. Experimental development

Preparation of enzyme standard solution: a total of 10 mg catalyst (the CFE of the used biocatalyst and its cofactor recycling system in the same ratio as used in the reaction) was weighed out and dissolved in PBS buffer to make up a solution of 10 mg/mL.

From that the necessary dilutions to make solutions at 50 µg/mL (100ppm), 25 µg/mL (50ppm) and 12.5 µg/mL (25ppm) were prepared.

The preparation of the test solutions is listed in Table 2:

Table 2: Preparation of test solutions

	A (sample)	B (spiked sample	C (Enzyme limit 100ppm)	D (blank)
Sample	5 µL	5 µL	0 µL	0 µL
SDS-PAGE sample buffer	12.5 µL	12.5 µL	12.5 µL	12.5 µL
Sample reducing agent	5 µL	5 µL	5 µL	5 µL
Enzyme standard spike (50 µ/mL)	0 µL	5 µL	5 µL	0 µL
DMSO	0 µL	0 µL	5 µL	5 µL
PBS Buffer	27.5 µL	22.5 µL	22.5 µL	27.5 µL
∑	50 µL	50 µL	50 µL	50 µL

[1]USP <1132> Residual Host Cell Protein Measurement in Biopharmaceuticals.

[2]Quantitation and speciation of residual protein within active pharmaceutical ingredients using image analysis with SDS-PAGE, Journal of Pharmaceutical and Biomedical Analysis 207 (2021) 114393.

In all these experiments, excess sample was prepared at a concentration of 500 mg/mL in DMSO to ensure detection of trace protein impurities. The drawback is that excess product can obscure impurities that have low molecular weight and migrate close to the product and cause interference.

2.3. Run conditions

After vortexing, the samples were heated at 98 °C for 10 min and applied to an SDS-PAGE gel alongside marker ladder.

15 µL of each sample (A to D, + standard curve of enzyme marker at 100 to 25 ppm) was pipetted into each well of a 12 well minigel.

The proteins were separated by 1 D gel electrophoresis (under standard settings - 180 V, 45 min) and the gel stained by silverstaining using the Thermo Fisher silverstaining kit according to manufacturer's instructions.

3. SDS-PAGE Analysis

3.1. Development Run 1

Two samples were analysed: as representative for chemical production of the desired product (Sample 01 with no residual protein from production = negative control) and representative for biocatalytic production of the desired product (Sample 02 with residual protein to be tested). The results of the development run (run 1) are shown in Figure 2:

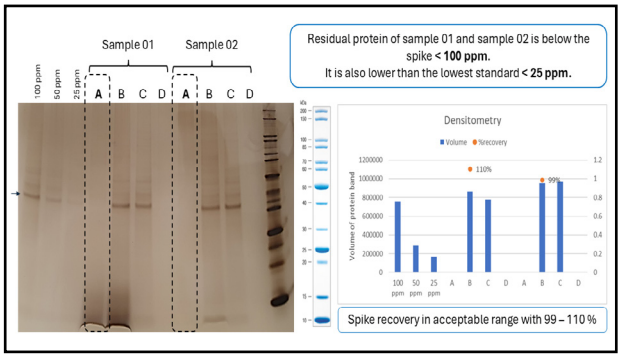

Figure 2: Results of development run 1

The residual protein of sample 01 and sample 02 was below the spike < 100 ppm. The samples were also lower than the lowest standard < 25 ppm.

3.2. QC verification Run 2

Run 2 was performed with the same experimental condition as run 1 using additional sample produced to further verify suitability of the method for GMP use.

For run 2, 15 µL of each solution (A - C) was pipetted into each well of a 10 well minigel as shown in Figure 3:

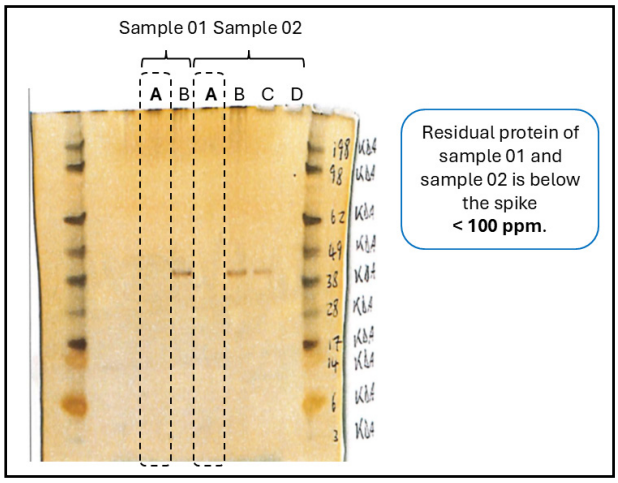

Figure 3: Results of QC run (run 02)

The residual protein of sample 01 and sample 02 was below the spike < 100 ppm. Since this was validated as limit test, no standard curve was necessary or densitometry measurements.

4. Results and Discussion

No protein bands were detected in either the samples or the blanks. In Run 1, the spike and recovery results—based on the band intensity of Sample B (spiked) and Sample C (reference)—were within an acceptable range (99% and 110%, respectively). This suggests that residual protein levels are below the lowest spike concentration (100 ppm), and that the API does not interfere with the intensity of the spiked protein.

Additionally, since a standard was included down to 25 ppm and is still faintly visible, while no protein bands are visible in the sample, it can be inferred that residual protein levels are below the lowest standard applied (25 ppm). However, because the 25 ppm band is very faint, it is likely near the detection limit of the sample preparation and

analytical method used.

For GMP analysis, method suitability was confirmed using a 100 ppm limit, as demonstrated in Run 2, which was deemed acceptable for that specific process.

5. Solvent compatibility and Robustness of SDS-PAGE

At Almac, SDS-PAGE has been successfully applied across numerous APIs, however for each development careful consideration for sample preparation is required.

Due to the nature of API isolation, which often involves acid/base precipitation or extraction with organic solvents, it is likely that most proteins in the crude biocatalyst mixture are denatured. This denaturation alters their solubility, shifting some from water-soluble to water-insoluble forms. Consequently, any analytical method for residual protein detection must be robust against a wide range of chemical environments and capable of detecting proteins at low concentrations.

SDS-PAGE is particularly well-suited for this purpose; it tolerates a variety of impurities - including residual APIs, solvents, salts, and pH variations.

For solvents incompatible with SDS-PAGE—such as certain hydrophobic organics—the addition of glycerol (e.g., 25 %) has been shown to improve sample retention and compatibility.

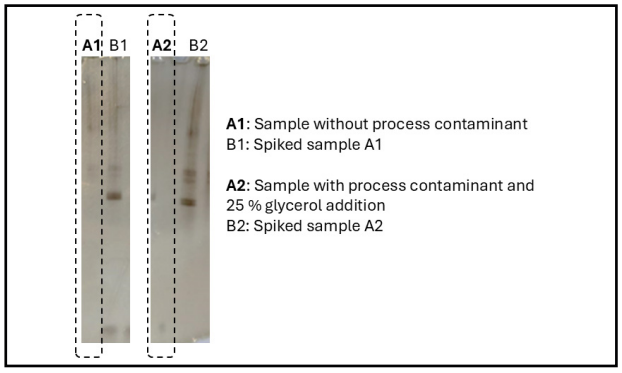

Figure 4: Glycerol - Development run

To further evaluate the robustness of the SDS-PAGE method, trials were conducted using DMSO at concentrations of 10% and 20%. These trials aimed to simulate conditions where the API or residual proteins are only soluble in partially aqueous environments. The results demonstrated that SDS-PAGE maintained its resolution and sensitivity even in the presence of high DMSO concentrations. Protein bands remained distinct, and no significant interference was observed, confirming the method's suitability for complex sample matrices.

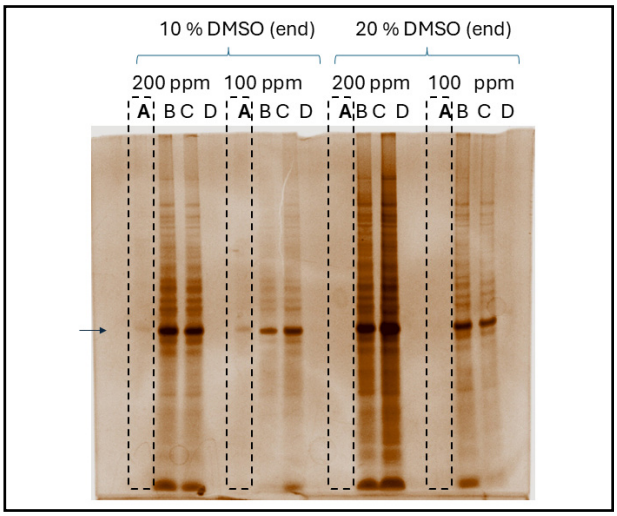

Figure 5: DMSO - Development run

Residual protein of the sample was below the lowest standard < 100 ppm. At 20 % DMSO (end concentration in the SDS-PAGE sample). No protein was visible on the gel in the sample with the spiked sample showing the expected protein levels, therefore this was used in all further experiments for this project.

6. Troubleshooting

One other key advantage of SDS-PAGE is its superior ability to uncover unknown impurities within complex sample matrices. The method's robustness allows it to tolerate a variety of contaminants, such as residual APIs, solvents, and pH variations, which can obscure the presence of impurities in other analytical techniques. This capability is crucial for the accurate determination of residual proteins, providing a comprehensive assessment of sample purity and significantly

enhancing quality control measures. An example of a low molecular weight impurity that has been missed by ELISA studies is shown in Figure 6:

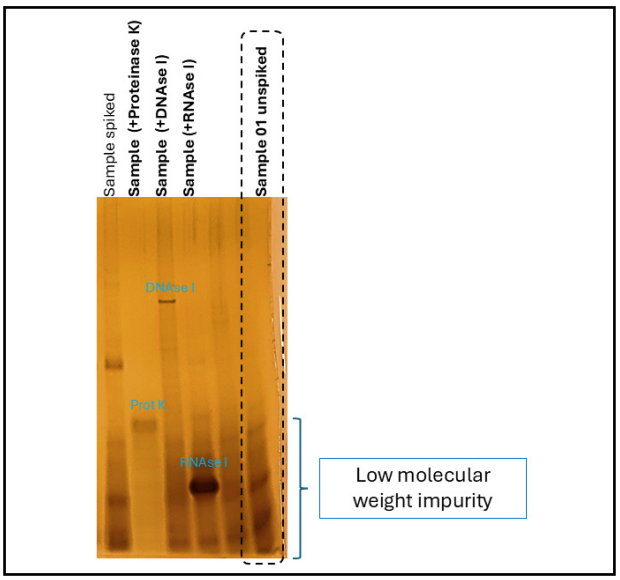

Figure 6: Low moluecular weight impurity

No overexpressed proteins were detected in the samples. Total protein analysis indicated that the overall protein concentration was approximately equivalent to the 100 ppm reference standard (data not shown). However, interpretation was challenging due to the presence of low molecular weight contamination, which appeared as a smear across the gel.
To investigate the nature of this impurity, additional experiments were conducted using DNase, RNase, and Proteinase K. The impurity was eliminated only by protease treatment, suggesting that it was protein-based. As a result, the workup process was refined to prevent the formation of this protein impurity.

7. Conclusion

The residual protein limit test method was successfully developed and implemented for routine quality control. This study presents a simple and effective approach for evaluating API purity by measuring residual proteins remaining after production.

SDS-PAGE, with image analysis, is a validated, cost-effective, and informative method for residual protein quantification in APIs. It complements other protein quantitation assays and provides critical insights into protein speciation, aiding in process optimisation and quality control.

Key advantages of this method include high-resolution protein separation, approximate molecular weight estimation, and ease of implementation. In conclusion, the developed method is suitable for use as a limit test or for in-process enzyme monitoring across various compounds, offering a robust tool for ensuring manufacturing consistency.

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