AN INTERNSHIP REPORT

ON

"Analytical Techniques for Qualitative and Quantitative Analysis of Monoclonal Antibodies (mAbs)"

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By

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CERTIFICATE

This is to certify that the report titled "*Analytical Techniques for Qualitative and Quantitative Analysis of Monoclonal Antibodies (mAbs)"* submitted by Avadh Jani to the Atmiya University, Rajkot for the degree of "Master of Biotechnology" is reviewed by me.

Name of mentor: Mr. Ronit Bose

Date: 27, March 2023

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1. AIM

Qualitative and quantitative analysis of monoclonal antibodies (mAbs) using various analytical techniques.

2. OBJECTIVE and SCOPE

The purpose of this report is to summarize and draw conclusions from qualitative and quantitative analysis of monoclonal antibodies (mAbs).

The scope of this document covers the qualitative and quantitative analysis of monoclonal antibodies (mAbs) performed at Enzene Biosciences Ltd. The analytical technique includes:

a) Protein concentration by Protein A HPLC
b) Purity analysis by Size exclusion chromatography (SE-HPLC) and Reverse phase chromatography (RP-HPLC)
c) Host cell protein (HCP) and Residual Protein A (rPrt A) analysis by ELISA

3. INTRODUCTION

3.1 Monoclonal Antibodies (mAbs):

Monoclonal antibodies (mAbs) are monospecific antibodies. These antibodies are produced from clone of single lymphocyte directed against a single antigenic determinant or epitope [2]. They were first generated in mice in 1975 using a hybridoma technique. The first licensed monoclonal antibody was Orthoclone OKT3 (muromonab-CD3) which was approved in 1986 for use in preventing kidney transplant rejection [2].

Since then, mAb based therapeutics are playing an important role in the treatment or prevention of many important diseases such as cancers, autoimmune disorders and infectious diseases. They are engineered to serve as substitute antibodies that can restore, enhance or mimic the immune system's attack on cancer cells. They are designed to bind to antigens that are generally more numerous on the surface of cancer cells than healthy cells. Cancer cells that are coated in monoclonal antibodies are more easily detected and targeted for destruction [2].

The drug (mAbs) helps the immune system in following ways: Flagging cancer cells, triggering cell membrane destruction, blocking cell growth, preventing blood vessel growth, blocking immune system inhibitors, directly attacking cancer cells, delivering radiation treatment, delivering chemotherapy, binding cancer and immune cells.

Development of mAbs as therapeutic drugs is becoming rapidly emerging area in biopharmaceutical industries. The global mAbs market was 95.1 Billion USD in 2017 and is estimated to reach 131.33 Billion USD by 2023. Even though, the development of mAb products has good prospects, the structure of mAbs are far more complex than those of small molecule drugs including the primary structure, higher order structure, glycosylation and charge variants [2]. Other than these intrinsic heterogeneities, the mAbs introduced to bioprocess manufacturing are susceptible to further chemical modifications and degradation [2]. Thus, to guarantee the quality and consistency of mAbs each step of manufacturing from protein expression to storage phase should be controlled and characterized. Based on the platform knowledge of the Critical Quality Attributes (CQAs) of mAbs an analytical platform is established to support the development of therapeutic mAbs.

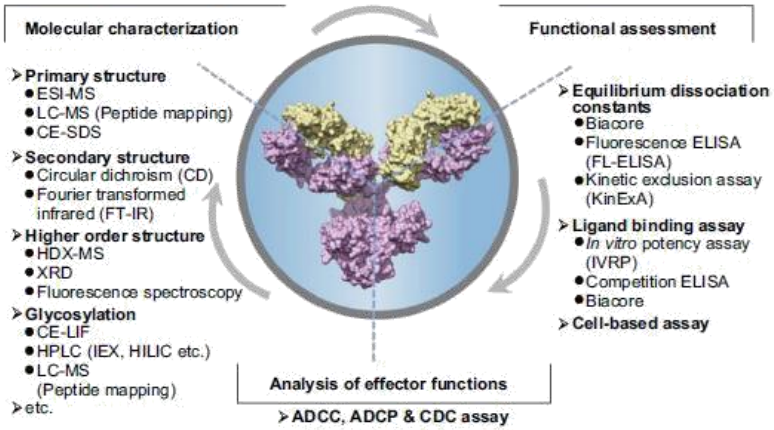

Figure 1: Overview of analytical methods (The example mAb is a representative IgG1) [2]

3.2 Production Processes for monoclonal antibodies:

Nowadays, mAbs are produced mostly from engineered microorganisms at commercial scale. Various systems of production are used, including animal cells, microorganisms, plants and mammary glands [3].

In general, process of commercial production of mAbs begins with generation of mAb by immunizing an animal or by molecular biology methods involving construction and identification of a stable high producing clone. After the clone development, the cell line selection is done which is a critical step to maintain the cells to secrete the desired protein with the correct conformation at high levels. Generally, CHO cell lines are used.

3.2.1 Upstream Process: The mAb cell culture process uses a proprietary, chemically defined basal culture medium. In the seeding steps, (Refer Figure *2*) Working Cell Bank (WCB) is expanded to a volume of culture that contains enough cells to meet the target initial cell density of the production bioreactor (Refer Figure *2*). [4]

The production bioreactor (Refer Figure *2*) is inoculated to achieve a range of initial Viable Cell Concentration and cultivated at controlled conditions for temperature, pH, and dissolved oxygen (DO). A nutrient feed is added at a defined time post-inoculation and multiple discrete glucose feeds are used to maintain the glucose concentration. Antifoam solution is added as required for foam control. [4]

Cultures are clarified by various centrifugation steps to remove the bulk of suspended cells and cell debris. [4]

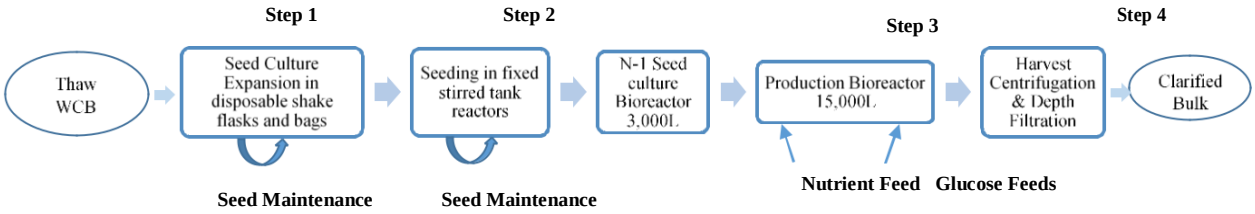

Figure 2: Generalized Upstream Process flow diagram [4]

3.2.2 Downstream Process: The downstream process captures mAb from the clarified harvest and purifies the antibody by a combination of chromatography unit operations. It also includes orthogonal steps devoted to virus inactivation and removal. The antibody is formulated through a diafiltration and ultrafiltration step to a composition and concentration suitable for drug product manufacturing. The formulated product is 0.2 µM filtered, filled into the appropriated containers and stored [4].

The general downstream process of mAb includes some important steps which are presented in

Figure 3

1. Protein A Affinity Chromatography: This step is important for the capturing of the antibodies.
2. Low pH viral inactivation: It involves the inactivation of enveloped viruses which are present in therapeutic protein products derived from mammalian cell culture.

3. Cation Exchange Chromatography: Results in the reduction of HCP, HCD to acceptable levels for subsequent processing by AEX chromatography.
4. Anion Exchange Chromatography: Remove HCP, DNA, Leached Protein A and endotoxins.
5. Small Virus Retention Filtration: Removal of small viruses such as minute virus of mice (MVM) and larger virus, generally present in product derived from mammalian cell culture.
6. Ultrafiltration/Diafiltration: Formulation and concentration of mAb to drug substance specifications.
7. Final Filtration, Filling and Freezing: Sterilize filtration and dispensing for drug substance storage.

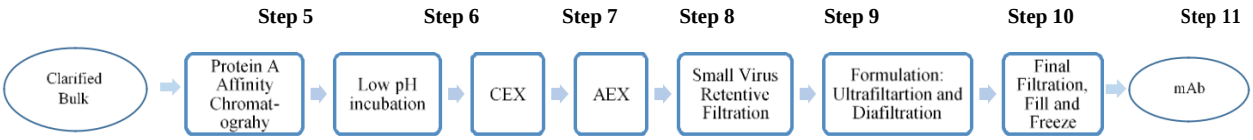

Figure 3: Generalized Downstream Process Flow Diagram [4]

The quality of the product needs to be assessed throughout the production process. Several analytical techniques (HPLC, ELISA) are routinely used for quantification and purity control of the product.

HPLC :-

High performance liquid chromatography (HPLC) is a technique used to separate different constituents of a compound using high pressure to push solvents through the column. It is the most widely used technique to identify, quantify and separate components of a mixture.

Before the existence of HPLC, LC analysis was carried by gravitational flow of the eluent that takes several hours for the analysis to be completed. These analysis systems use high-pressure pump that generates rapid flow of eluent, and thus results in dramatic improvement in the analysis time.

Components of HPLC System:

1. Solvent Reservoir: A reservoir holds the solvent, the mobile phases.
2. High-pressure pump: It has high-pressured pump (solvent delivery system or solvent manager) is used to generate and provide a specified flow rate of mobile phase, typically ml/min.
3. Injector: An injector (sample manager or autosampler) can introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample in the HPLC column.
4. Column Compartment: The compartment has the HPLC column. Column contains the chromatographic packing material needed for the separation. The packing material is called the stationary phase because it is fixed in the column hardware.
5. Detector: The detector is used to give the output in the form of the chromatogram. It is needed to see the separated compound as they elute form the HPLC column. The detector is wired to the computer data station, which records the electrical signal needed to generate the chromatogram on its display and to identify and quantitate the concentration of the sample components.

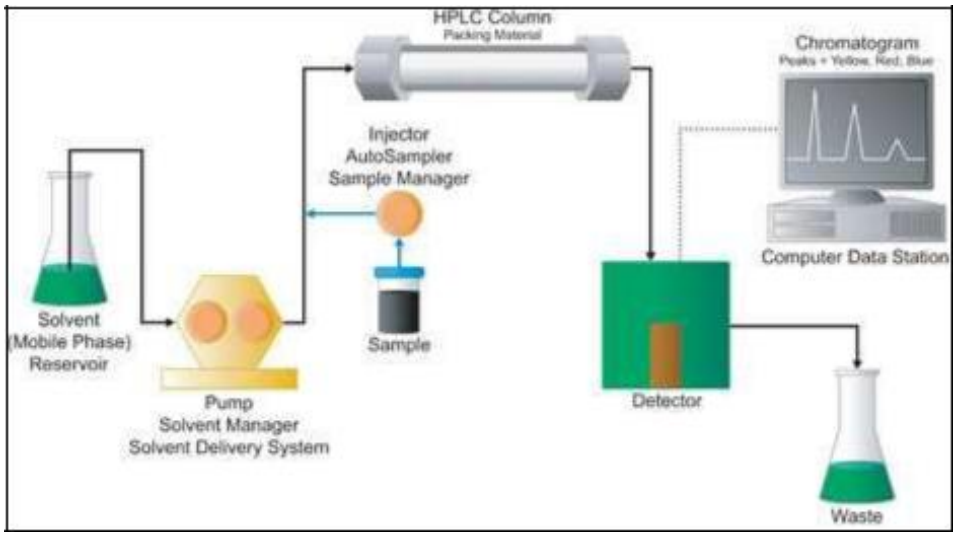

Figure 4: High Performance Liquid Chromatography (HPLC) system

Since sample compound characteristics can be very different, several kinds of detector have been developed.

Table 1: Types of HPLC detectors

Detectors	Features
UV, Visible and Photo Diode Array (PDA) Detectors	Sample absorbs part of the UV light. Standard UV detector range: 195-370nm, Visible: 400-700nm, both UV and Vis: 195-700nm
Refractive Index (RI) Detector	It measures change in refractive index. It is suitable for sugars, alcohols or organic ions.
Evaporative Light Scattering Detector (ELSD)	For good sensitivity, for non-volatile analytes. For lipids, sugars and high molecular weight analytes.
Multi-Angle Light Scattering Detector (MALSD)	Used to determine the molecular weight directly without the need of the calibration curve, used of SEC analysis.
Conductivity Detector	Measures Electronic resistance and value is directly proportional to the concentration of ions, used for ion chromatography
Fluorescence Detector	Used for fluorescence compounds. It used specific wavelength, analyte atoms are excited and then emit light signal.
Chemiluminescence Detector	Instead of light source to excite analyte atoms, the excitation is initiated by chemical reaction
Optical Rotation Detectors	Separates R and L type optical isomers

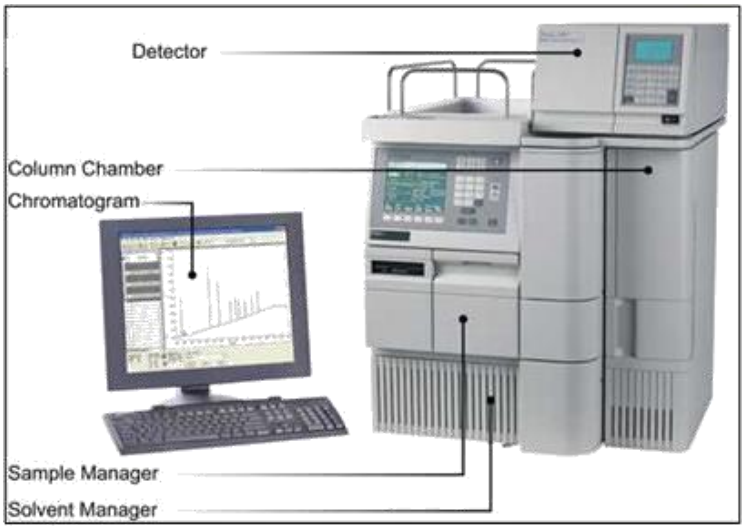

Figure 5: A Typical Analytical HPLC System

SEC HPLC

5.2 Size exclusion chromatography (SE-HPLC)

5.2.1 ***Objective:*** To determine high molecular weight (HMW) and low molecular weight (LMW) impurities of recombinant proteins (monoclonal antibodies) by Size-Exclusion High Performance Liquid Chromatography (SE-HPLC).

5.2.2 ***Principle:*** Molecular weight related impurities constitute the key product-related impurities as they typically exhibit a high degree of immunogenicity. Typically, 1–3 % mAb aggregates are observed. This can be monitored by SE-HPLC technique.

The size exclusion separates on the basis of the molecular size. The molecules smaller than the fractionation range enter the pores of the resin while molecules larger than the fractionation range are excluded from entering the pores, separating the HMW and LMW impurities.

SEC is classified into Gel Permeation Chromatography (GPC) and Gel Filtration Chromatography (GFC). GFC is used widely to measure the molecular weight distribution of molecules (proteins and polysaccharides).

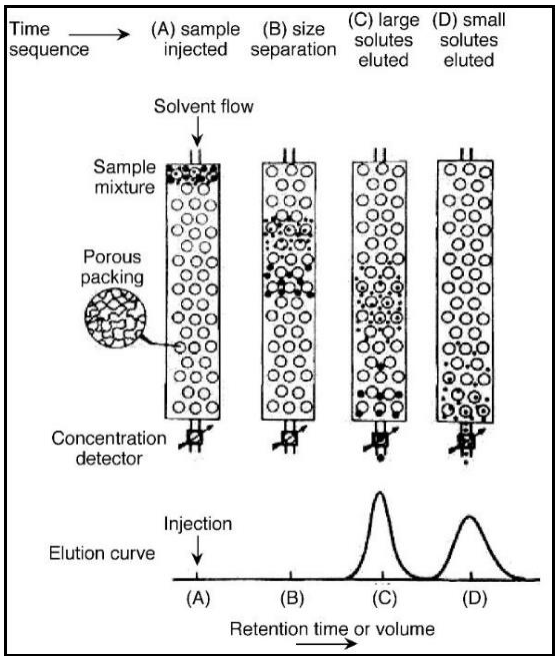

Figure 8: Schematic representation of separation of LMW and HMW impurities

SEC is an important technique for the impurities removal and consist of following factors:

- Allows contaminating material to be removed in relatively small volume.
- It is different from dialysis, as it has the advantage of speed, which is necessary for certain experimental situations.
- Separates on the basis of molecular size.
- Stationary phase is composed of uncharged porous particles.
- Molecules smaller than fractionation range can enter the pores of the resin, while molecules larger than the fractionation range are excluded from entering the pores.
- Small molecules (buffer salts, small molecules) enters the pores and macro molecules (proteins, nucleic acids, DNA) pass the resin bed so we can collect desalting fraction at the end.
- For effective separation, resin bed should be sufficiently tall and voluminous to fully separate the emergence from the end of the column.

5.2.3 Materials

1. Instrument- Alliance WATERS e2695 series HPLC system
2. Equipment and materials- Pipettes, vortex, TSK Gel SEC Column (5µM pore size, 7.8mm ID*30cm length), pH Meter

   Glass-wares: Measuring cylinder, Glass bottles, Beakers, PVDF membrane filter (0.2µM), Solvent filtration apparatus
3. Chemicals- Reference Standard, test sample, Milli Q Water, Analytical grade Sodium phosphate monobasic monohydrate, Sodium phosphate dibasic dihydrate, Sodium Chloride

5.2.4 Method

SE-HPLC was performed using TSK Gel SW Type steel column. The method follows isocratic elution. Chromatogram was acquired and processed (integrated) for purity determination.

A. Sample and Standard Preparation

- *Preparation of reference standard:* The reference standard (5mg/ml) was diluted at a concentration of 1 mg/ml in Milli Q water and injected. Reference standard is considered as the criteria for system suitability.

- *Sample preparation:* The test sample was diluted at a concentration of 1mg/ml in MilliQ based on the concentration obtained from OD_{280nm}. After dilution, the samples were filled in HPLC vials for analysis.
- *Sample Injection and analysis:* Injected 10 µL of sample and used isocratic flow from 0 to 100 % over 30 min to elute the sample.

B. Chromatographic conditions:

Table 5: SE-HPLC chromatographic conditions

S. No	Parameters	Specifications
1	Mobile Phase	100 mM phosphate buffer+200 mM NaCl, pH 6.7
2.	Elution Condition	Isocratic elution
3.	Column specifications	TSK Gel SW Type (5µM pore size, 7.8mm ID*30cm length)
4.	Flow rate	0.5 mL/min
5.	Detection wavelength	217 nm
6.	Column temperature	25 °C
7.	Sample temperature	5°C±3
8.	Injection Volume	10-100 µL
9.	Run Time	30 min

C. Setting Up of HPLC System:
STEPS:-

- Our samples would come in batches hence we would have to name according to it and put it on different groups
- Then we would first find the concentration of the samples
- According to the concentration we would dilute the samples to 1 mg/mL and put them in the micro centrifuge tubes
- After mixing we would transfer them in vials with inserts to be of 300µL or 150µL, according to your concentrations
- Then we would open EMPOWER3, a software which would connect our computer with HPLC system
- We would write down the sequence and check the injection volume, vials position, blanks and standard vials in the carousel
- After the cycle is complete we would check the chromatograms and integrate it as per MOA.
- SE HPLC used also called gel- filtration or gel-permeation chromatography (GPC), uses porous particles to separate molecules of different sizes.

5.2.5 Observation and Results:

Chromatogram was obtained and analyzed by manual integration method. LMW and HMW peaks were identified and purity determines in terms of area %.

Retention time of main peak in test sample and reference sample was comparable.

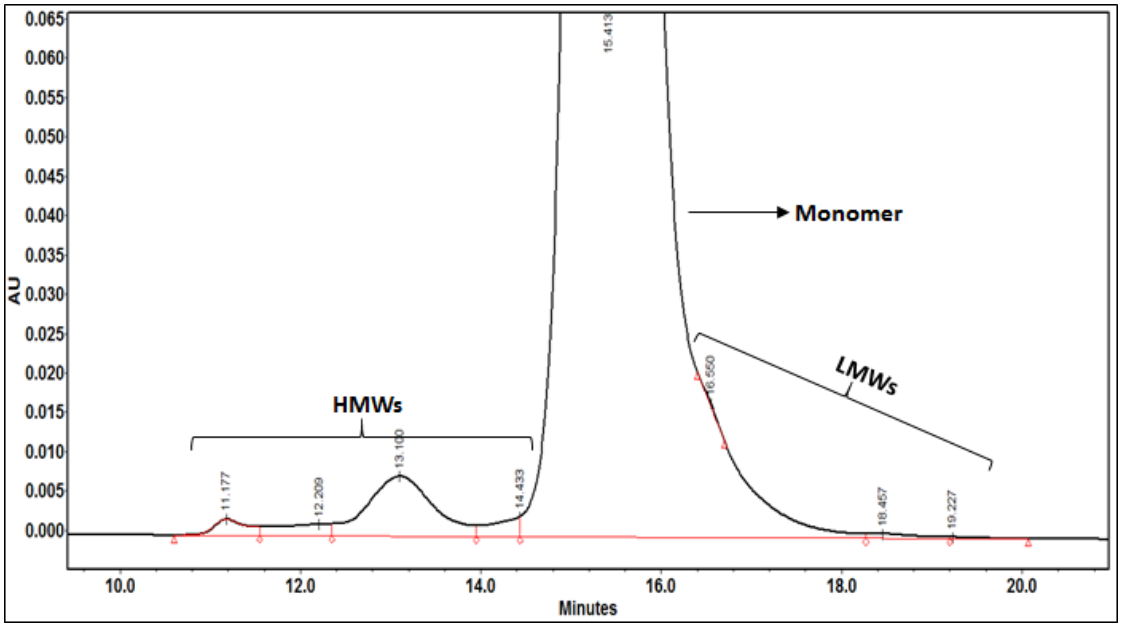

Figure 1: Representative profile of integrated reference standard, using SE-HPLC (Zoomed view)

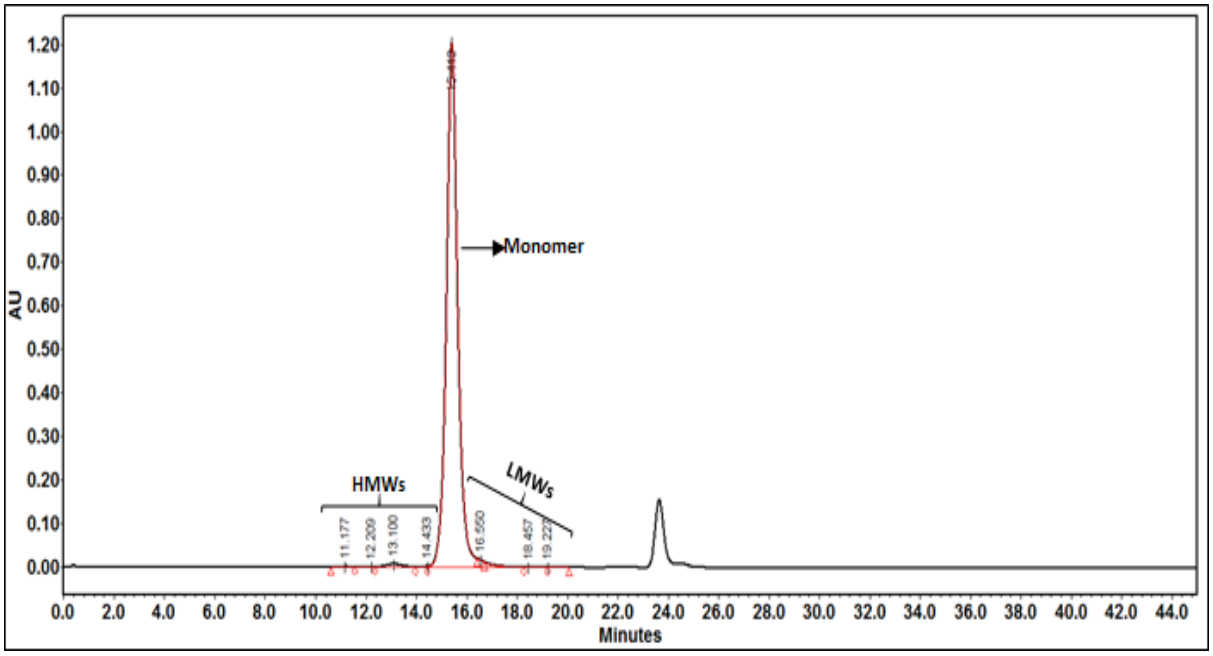

Figure 2: Representative profile of integrated reference standard, using SE-HPLC (Un-zoomed view)

Table 1: Representative data of Reference Standard using SE-HPLC

HMW (%)	Monomer (%)	LMW (%)

1.4	98.5	BQL

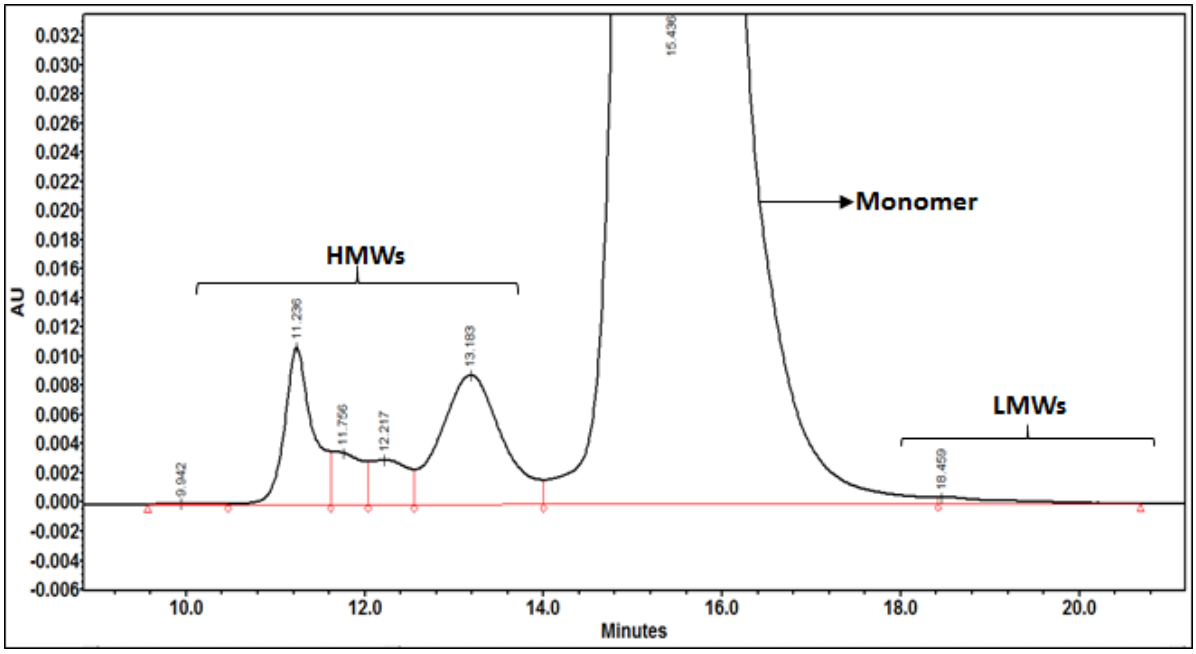

Figure 3: Representative profile of integrated test sample, using SE-HPLC (Zoomed view)

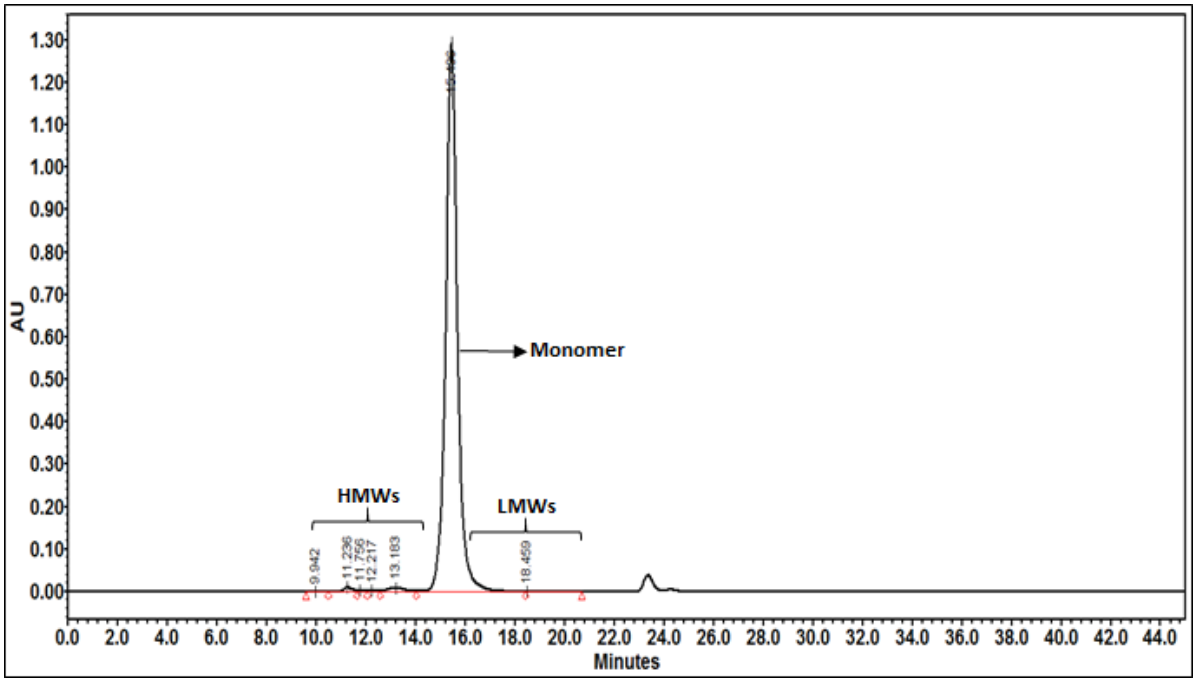

Figure 4: Representative profile of integrated test sample, using SE-HPLC (Zoomed view)

Table 2: Representative data of test sample using SE-HPLC

HMW (%)	Monomer (%)	LMW (%)
1.9	98.0	0.1

NR CE-SDS :-

- CE-SDS separates proteins based on differences in the hydrodynamic size under non-reducing and reducing conditions. The method involves heat denaturing of a specified concentration of protein in the presence of SDS. Once denatured, the sample is separated by size in a capillary containing a replaceable SDS polymer matrix. This method resolves impurities by size, and subsequently quantifies the heterogeneity and size variants which may exist in a given sample.
- We quantitated the molecular weight variants using CE-SDS (Capillary Electrophoresis Sodium Dodecyl Sulfate)
- We performed CE-SDS under non-reducing (NR) conditions to determine protein purity.
    - The charged solutes, the ions, migrate through the tube, with the highly charged ions migrating the fastest and the lesser charged ions, the slowest.
    - Neutral molecules are not affected by the electric field and move through the tube under the influence of just the EOF and are not separated from each other.
    - The larger the diameter of the tube, the more joule heat is generated, which causes spreading of the zones giving poor separations.
    - Molecules in the centre of the tube migrate faster than those near the wall because the viscosity of the electrolyte is lower in the centre.
    - These separations are facilitated by the use of high voltages, which may generate electroosmotic and electrophoretic flow of buffer solutions and ionic species, respectively, within the capillary.
    - One of the fundamental processes that drive CE is electro-osmosis.
    - This phenomenon is a consequence of the surface charge on the wall of the capillary.
    - The fused silica capillaries that are typically used for separations have ionizable silanol groups in contact with the buffer contained within the capillary.
    - The pl of fused silica is about 1.5.
    - The degree of ionization is controlled mainly by the pH of the buffer.
    - In untreated fused silica capillaries most solutes migrate towards the negative electrode regardless of charge when the buffer pH is above 7.0.
    - At acidic buffer pH, most zwitterions and cations will also migrate towards the negative electrode.

- To ensure that a system is properly controlled, it is often necessary to measure the EOF.
- This is accomplished by injecting a neutral solute and measuring the time it takes to reach the detector.
- The basic apparatus for CGE is identical to that of capillary zone electrophoresis (CZE) and consists of a capillary column, an on-column detector, and a high voltage power supply.
- The major difference between the two techniques is the separation media: a sieving matrix is employed in CGE while a background electrolyte solution is utilized in CZE.
- Polyacrylamide (PA) has been widely used in slab gel electrophoresis of proteins, and consequently it is frequently utilized in CGE. Initially, PA gels were synthesized in-situ inside capillaries. Typically, a capillary column was prepared by mixing acrylamide (monomer), N,N'-methylenebis(acrylamide) (Bis, cross-linker), ammonium peroxy-disulfate or ammonium persulfate (radical initiator), N,N,N',N'- tetramethylethylenediamine (TEMED, catalyst) and other background electrolytes, introducing the mixture into the capillary, and allowing the solution to polymerize inside the capillary.
- While this worked in general, problems occasionally arose when PA shrank during polymerization, breaking PA gel into segments and/or forming bubbles inside the column.
- Additionally, a good column could work well for only the first a few runs, as large molecules and particulate materials accumulated at the injection end of the column, which deteriorated and eventually shut down the separation.
- When PA sieving matrices are used to run CGE, capillary walls often need to be coated for achieving high quality separations. Poly(N,N-dimethylacrylamide)-grafted PA , a derivative of PA, was prepared by Zhang et al. [36] in 2006, and when this polymer was used to sieve proteins, capillary wall coating could be avoided. This is because poly(N,NN dimethylacrylamide)-grafted PA is capable of coating capillary walls dynamically.
- Microfabricated (or microchip) devices are developed with a goal to perform and integrate multiple analytical processes (e.g. sample pretreatment, solution distribution/mixing, separation, detection, etc.) on a chip platform [76, 77]. Due to the short column length and high separation efficiency, microchip CGE is generally fast, typically from a few seconds to a few minutes.

Results:-

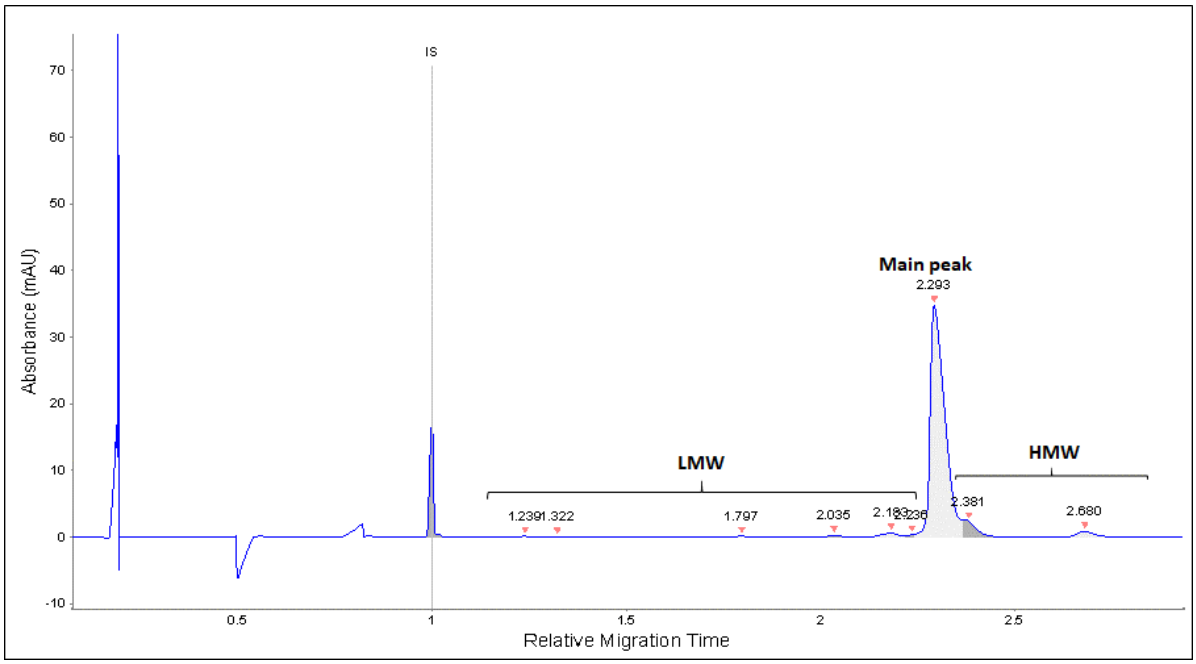

Figure 5: Representative profile of reference standard, using NR CE-SDS

Table 3: Representative data of Reference Standard using NR CE-SDS

LMW (%)	Main peak (%)	HMW (%)

3.7	88.6	7.8

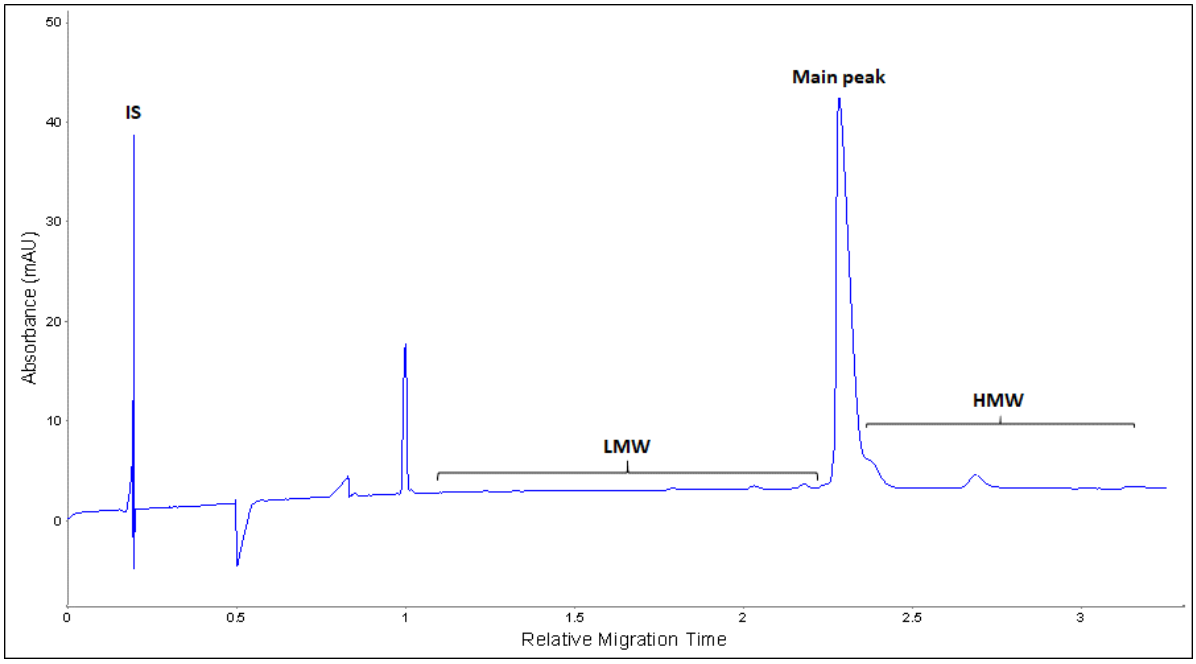

Figure 6: Representative profile of test sample, using NR CE-SDS

Table 4: Representative data of Reference Standard using NR CE-SDS

LMW (%)	Main peak (%)	HMW (%)
2.9	87.9	9.2

REFERENCES: -

1. Dr. Vikash Kumar Dubey, Proteomics & Genomics
2. www.wikipedia.
3. Yuri Kazakevich, Rosario Lobrutto , HPLC for Pharmaceutical Scientists, page no. 12
4. Chi-san Wu, Handbook of Size Exclusion Chromatography and Related Techniques, Second Edition, page no. 8-10.
5. Szablocs Fekete, Alain Beck, Jean-Luc Veuthey, Davy Guillarme, Theory and practice of size exclusion chromatography for the analysis of protein aggregates, Journal of Pharmaceutical and Biomedical Analysis, (2014) page no. 161-173
6. https://www.pharmatutor.org
7. www.harvardapparatus.co
8. Shapiro AL, Vinuela E, Maizel JV. Biochem. Biophys. Res. Commun. 1967; 28:815–820. [PubMed: 4861258]
2. Weber K, Osborn M. J. Biol. Chem. 1969; 244:4406–4412. [PubMed: 5806584]
3. Guttman A, Nolan J. Anal. Biochem. 1994; 221:285–289. [PubMed: 7810868]
4. Shieh PCH, Hoang D, Guttman A, Cooke N, J Chromatogr A. 1994; 676:219–226. 5. Guttman A. TrAC Trends Anal. Chem. 1996; 15:194–198. 6. Jo Schmerr M, Jenny A, Cutlip RC, B JChromatogr. 1997; 697:223–229. 7. Manabe T. Electrophoresis. 199
9; 20:3116–3121. [PubMed: 10596819]
8. Hu S, Jiang J, Cook LM, Richards DP, Horlick L, Wong B, Dovichi NJ. Electrophoresis. 2002; 23:3136–3142. [PubMed: 12298085]
9. Hjerten S. J. Chromatogr. 1983; 270:1–6. 10. Cohen AS, Karger BL. J. Chromatogr. 1987; 397:409–417. [PubMed: 3654832]
11. Ganzler K, Greve KS, Cohen AS, Karger BL, Guttman A, Cooke NC. Anal. Chem. 1992; 64:2665–2671. [PubMed: 1284102] 12. Widhalm A, Schwer C, Blaas D, Kenndler E. J. Chromatogr. 1991; 549:446–451
. 13. Okada H, Kaji N, Tokeshi M, Baba Y. Anal. Sci. 2008; 24:321–325. [PubMed: 18332537] 14. Verhelst V, Mollie JP, Campeol F, A JChromatogr. 1997; 770:337–344. 15. Lausch R, Scheper T, Reif OW, Schlosser J, Fleischer J, Freitag R, A JChromatogr. 1993; 654:190–195. 16. Kaneta T, Yamamoto D, Imasaka T. Electrophoresis. 2009;
30:3780–3785. [PubMed: 19862753] 17. Luo S, Feng J, Pang H-m, A JChromatogr. 2004; 1051:131–134. 18. Michels DA, Hu S, Dambrowitz KA, Eggertson MJ, Lauterbach K, Dovichi NJ. Electrophoresis. 2004; 25:3098–3105.
[PubMed: 15472976] 19. Griebel A, Rund S, Schonfeld F, Dorner W, Konrad R, Hardt S, Lab Chip. 2004; 4:18–23.
20. Lo CT, Throckmorton DJ, Singh AK, Herr AE, Lab Chip. 2008; 8:1273–1279. 21. Hatch AV, Herr AE, Throckmorton DJ, Brennan JS, Singh AK. Anal. Chem. 2006; 78:4976–4984. [PubMed: 16841920]