Analytical Characterization of Protein-based Biopharmaceuticals

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Abstract

Biopharmaceuticals are produced through biotechnological processes using molecular biology methods, encompassing several classes, such as nucleic acids and proteins. The main goal of this project was to develop, understand and implement fit for purpose analytical methodologies to characterize biological large molecules, such as proteins. This project includes the characterization of model proteins (Bovine Serum Albumin, Ovalbumin and Lysozyme) and a monoclonal antibody, ARD_2019, through the development of a Karl Fischer Titration (KFT) to determine the process water content biological compounds and the development of Size Exclusion Chromatography (SEC) together with Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) methodologies to determine protein purity, size variants and stability of proteins. An Ion Exchange Chromatography (IEX) was also developed and implemented to assess the presence of different charged variants. By KFT, it was possible to determine the process water content of BSA [6.87 %(w/w)] and ARD_2019 [0.55 - 3.02%(w/w)]. The SEC method was first optimized for the model proteins with known molecular weights, and later applied to ARD_2019, which allowed the estimation of its molecular weight, and the High and Low Molecular Weight species. An SDS-PAGE methodology was also developed. The results obtained for the isolated antibody are in accordance with SEC results. By SDS-PAGE was possible to observe both antibody heavy (50 kDa) and light (25 kDa) chains. The determination of charge heterogeneity analysed by Cation Exchange Chromatography was performed with both pH and salts gradients. For the ARD_2019 mAb it was possible to determine the presence of 8 different charged variants.

Keywords: Biopharmaceuticals; Biocharacterization; Bovine Serum Albumin; Lysozyme; ARD_2019 protein; Analytical Methodologies.

Introduction

In the 20th century, the pharmaceutical industry has deeply evolved due to a developing appreciation of the principles of organic chemistry together with an increased knowledge in molecular biology, in disease-associated pathways and disease mechanisms [1]. The United States and/or European Union witnessed over the period from January 2014 until July 2018, the approval of 155 biopharmaceutical products, being 81 (52%) of the these genuinely new to the market, with the remaining products representing biosimilars, me-too products (a product introduced by a company after it has seen that other companies are successful with the same type of product) and products previously approved elsewhere [2]. Biopharmaceutical proteins are emerging as an important class of drugs for the treatment of various diseases including cancer, inflammatory diseases and autoimmune disorders, among others. Several examples include insulin, interferons, interleukins and other cytokines, which regulate aspects of immunity, inflammation and other processes of central importance to maintaining a healthy state [3]. There are a wide variety of biopharmaceutical proteins formats, enhanced by new nucleic acid modalities and cellular therapies that are being propelling on the market. However, monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), recombinant proteins, produced by engineered chinese hamster ovary (CHO) cells and, therefore, extracted from mammalian cells, have been emerged as the largest group of biopharmaceuticals, dominating the biopharmaceutical market, attributed to its obvious benefits in terms of safety and efficacy [2]. The most important attributes in biomolecules characteristics arise from their macromolecular nature, which provides the structural complexity that is often required for specificity. This structural complexity makes the characterization biomolecules critical quality attribute more challenging, since the structural integrity of the protein needs to be assured. Many biologics such as monoclonal antibodies and other recombinant therapeutic proteins are larger and complex proteins. Therefore, the analytical methodologies for the characterization of these molecules must be able to evaluate their primary structure and the high ordered secondary, tertiary and quaternary structures, that define the protein three-dimensional structure shape and function. The characterization of post translational modifications (PTMs), glycosylation, oxidation, phosphorylation, sulphation, lipidation, disulphide bond formation, and deamidation are crucial aspects of the analysis, since these phenomena can impact in the three-dimensional structure of the protein and influence the immunogenicity of biologics [4].

Materials and Methods

Development of a Karl Fischer (KF) Titration Method

In the present work, a KF oven (874 Oven Sample Processor and 801 Stirrer) method was developed to analyze process residual water, using the model proteins (BSA, Ovalbumin and Lysozyme). To perform this procedure, 20 mg of each protein were weighed in a glove box, with a relative humidity between 7 and 8%, since these proteins are hygroscopic. The samples were weighed out directly into vials that are then hermetically sealed, with PTFE-coated septa, and placed in the oven apparatus, preventing the water content from being falsified by atmospheric moisture. The analysis was performed with a temperature ramp between 50 ºC and 220 ºC, to realize what would be the optimum temperature of the protein to avoid its denaturation while determining the water content. To verify if the equipment is properly working, three (3) samples of a Lactose Standard at 5% (w/w) H_2O (STD) were weighed and analyzed at 150 ºC. After confirming the equipment is properly working, control samples (empty vials) were also analyzed, prior to each sample measurement, at the same temperature defined after temperature ramp assignment (120 ºC). In order to demonstrate the repeatability of the method, six samples of BSA were then weighed (10 mg), in a glove box at a relative humidity between 7 and 8%, and the vials were then placed in the oven apparatus. To verify that the equipment is not saturated with water after the analysis of all samples, another lactose standard was weighed and analyzed at 150 ºC. For all the Lactose Standards analyzed it was evaluated if the water content was within, 5.10% (w/w) ± 0.20% (w/w). After establishing the conditions that should be used to determine the process residual water content of proteins, as shown in Table 1, the same conditions were used for the determination of the water content of ARD_2019. It was weighed 10 mg of the ARD_2019 sample, in a glove box at ≤ 5% Relative Humidity (RH). For both model and ARD_2019 proteins, the water content results were analyzed by the software indicated, in order to quantify process residual water.

Size Exclusion Chromatography (SEC) Methodologies

A SEC method was developed using a gel filtration standard containing the proteins Thyroglobulin (670 kDa), IgG (150 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), and Vitamin B12 (14 kDa). This was reconstituted with 500 µL of water, diluted to 5 mL with water and then 100 µL were transferred to HPLC vials with an insert. It was also weighed 10 mg of 0.1 mg/mL 4-aminobenzoic acid (PABA) (0.14 kDa), and transferred to a 15 mL conical centrifuge tube, adding 10 mL of water and vortex to mix. After a 1:10 dilution with water Milli-Q, the PABA solution was also introduced into an HPLC vial, making up to 3 quarters of the volume. Bovine Serum Albumin (BSA) was also used at a concentration of 2 mg/mL in water, in order to condition the column.

An Alliance HPLC was used with a TSKgel G3000SWxl column, 30 cm × 7.8 mm, 5 µm, 250 Å. The mobile phase was 92.5% of 25 mM sodium phosphate monobasic (NaH_2PO_4), 50 mM sodium chloride (NaCl) and 7.5% of 2-propanol (v/v), at a pH of 6.8 (adjusted with sodium hydroxide, NaOH).

After preparation, the mobile phase was filtered (Nylon membrane filter 0.2 µm, 47 mm). The flow rate was set to 0.8 mL/min. The gel filtration standard, BSA and PABA, already introduced into HPLC vials, with the autosampler temperature set to 2-8 °C, were injected (10 µL) into the column (maintained at ambient temperature) and separated by running mobile phase for 40 minutes. The elution of the proteins and the organic compound from the column was monitored at a wavelength of 220 nm. Regarding to ARD_2019 characterization by SEC, all the conditions of the method were maintained. The ARD_2019 sample preparation (18 mg) was performed at a relative humidity ≤ 5%, as it is a hygroscopic product, and in which theoretically 10 mg of the sample contained 120 µL of water, having been added 222 µL of the mobile phase (92.5% of 25 mM sodium phosphate monobasic (NaH_2PO_4) and 350 mM sodium chloride (NaCl) and 7.5% of 2-propanol (v:v)). From this diluted sample, an additional 1:3 dilution in water (ARD_2019 final concentration = 0.12 mg/mL) was performed and the sample solution was then transferred to an HPLC vial with an insert, counting on an injection volume of 10 µL. Elution of the protein was also monitored at a wavelength of 220 nm.

Size Exclusion Chromatography Degradation Studies

Two different stress conditions, pH and temperature, were tested. For this purpose, the protein selected for the study was BSA (1 mg/mL). The study was performed at 2 different conditions: 0.3 mg/mL of BSA subjected to 80 °C for 30 minutes, and (2) 0.3 mg/mL of BSA with the addition of 0.5% of formic acid (CH2O2). A control sample was also prepared with 0.3 mg/mL of BSA subjected to any stress condition (in its native form).After degradation, fresh BSA protein samples at the same concentration (0.3 mg/mL) were spiked with degraded protein already centrifuged for 5 minutes at 13.4 rpm, at the levels of 0.5% (w/w), 1% (w/w) and 2% (w/w). Additionally, three control solutions were prepared at 2% (w/w) of BSA for each stress degradation condition (final volume was 300 µL). Samples were then centrifuged prior to transfer to HPLC vials with inserts of 150 µL, and analysed by size exclusion chromatography at the same conditions as previously described.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To confirm the results obtained by SEC, the degradation samples were also analysed by SDS-PAGE. The BSA protein was again chosen for further comparative analysis of these two techniques and was prepared as well as for Size Exclusion Chromatography Degradation Studies, and subjected to the same stress conditions, pH and temperature. Additionally, the purity and the characterization of the various fragments of the monoclonal antibody ARD_2019 was also performed by SDS-PAGE, in reduced conditions. For both studies, electrophoresis was performed on a 12% (v/v) polyacrylamide gel in 2.5 M Tris-HCl, pH 8.8, containing 10% SDS (m/v). The stacking gel had a polyacrylamide concentration of 5% (m/v) in 0.5 M Tris-HCl, pH 6.8.

The 3 dried powder samples of ARD_2019 were prepared in a glove box, with a relative humidity under 5%. After weighing and then diluting with water Milli-Q, the samples were at a concentration of 83 mg/mL. To match the concentration of the dried powder samples with the fresh monoclonal antibody, which was at a concentration of 15 mg/mL, a 1:3 dilution with H2O was made, obtaining a final concentration of 27.7 mg/mL (15 mg/mL of antibody and the remainder being the excipients that support the formulation). Given the high concentration of the antibody, a dilution was performed to a final concentration of 2 mg/mL. To all ARD_2019 samples previously prepared it was necessary to add the denaturation solution, sample buffer, prepared with a total volume of 20 mL (5 mL of stacking gel, 8 mL 10% SDS, 1 mL β-mercaptoethanol, 2 mL Gycerol, and 4 mg of Bromophenol blue, and was added in the same ratio of each sample, total volume of 10 µL, in a proportion of 1:1 to protein mixture. The standard protein mixture with a range of known molecular weights between 10 kDa and 250 kDa, used in the SDS-PAGE analysis gel performed for the degradation studies, and a standard protein mixture with known molecular weights of 17 kDa, 44 kDa, 158 kDa and 670 kDa, used to ARD_2019 gel electrophoresis, were prepared in the same way. After the addition of the sample buffer, and in order to speed up the denaturation process, the samples were introduced in a dry bath for 10 minutes at 95 °C and then were subjected to a short spin in the centrifuge. Samples and the standard proteins mixture were loaded (10 µL each) with a pipette using gel loading tips, with special attention to avoid cross contamination between different samples in the different gel wells. This way, the electrophoresis started at constant 120 V, 400 mA, for 70 minutes. After electrophoresis was completed, the running buffer was discarded and was removed the gel cassette and washed with water Milli-Q for the subsequent gel colouring, with the staining solution Coomassie Blue R-250, for 30 minutes in the stirrer plate. Finally, the staining solution was removed and added the detaining solution (7.5% (v/v) glacial acetic acid and 45% (v/v) methanol) in the gel for 30 minutes in the stirrer plate. The gel was kept in this solution until the analysis.

Ion Exchange Chromatography

The evaluation of charge heterogeneity of the ARD_2019 was supported by Cation Exchange Chromatography using either pH and salt gradients. For the development of these technique, a Protein Pak Hi Res CM, 7µm, 4.6 × 100mm column was used. The first step was the preparation of the equipment and the mobile phases, since the samples were diluted in mobile phase A. Relatively to the pH gradient, the optimized method encompassed a mobile phase A (10 mM Tris buffer, pH 7.5) and mobile phase B (10 mM Tris buffer, pH 10.5) and regarding to the salt gradient it was used the mobile phase A, and adding to mobile phase B, 300 mM NaCl, where for both the pH values (7.5 and 10.5), it was adjusted with HCl and NaOH. The mobile phases were filtered using a Nylon membrane filter 0.2 µm, 47 mm, before being placed in the equipment. Additionally, the storage solution was prepared: 0.05% (w/w), sodium azide in high purity water, which inhibit undesired microbial growth. The preparation of the sample was performed in a glove box with relative humidity under 5%, where it was weighted 20 mg of the sample. The ARD_2019 sample presents 120 µL of water in 10 mg and was diluted with 238 µL of mobile phase (10 mM Tris buffer, pH 7.5). Finally, from this diluted sample, a new 1:3 dilution in mobile phase was performed: 150 µL of this solution were then transferred to an HPLC vial with an insert, counting on an injection volume of 10 µL, and the elution of the protein was monitored at a wavelength of 220 nm. For each elution, the column was pre equilibrated with, at least, three column volumes of mobile phase A; prior to sample injection, water washes were carried out through the column as salts may precipitate and the column clogging. After the injection of the monoclonal antibody sample onto the column, a linear increasing in the percentage of mobile phase B was delivered. The linear gradient at a flow rate of 0.8 was started with 100% of mobile phase A, increasingly linearly to 100% of mobile phase B, in 25 minutes, and kept for 5 minutes; the percentage of mobile phase A was then increased to 100% in the preparation for subsequent analyses, summing up a 40 min of run.

Results and Discussion

Model Proteins Characterization – Karl Fisher Oven

For the purpose of quantifying process residual water present in the proteins under study, a Karl Fischer Oven method was developed. Thus, the first step was the implementation of a temperature ramp, between 50 ºC and 220 ºC as shown in Figure 1.

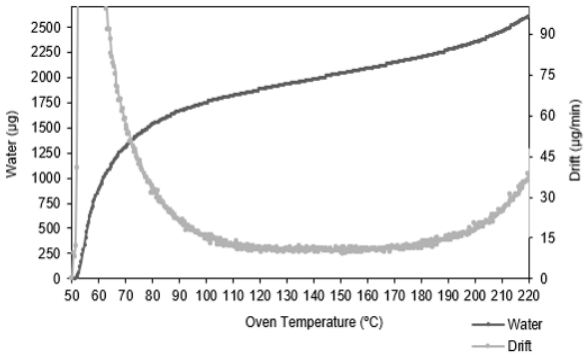

Figure 1: Karl Fischer Oven (874 Oven Sample Processor and 801 Stirrer) method development. Temperature ramp of Bovine Serum Albumin. The standard used for temperature ramp analysis (50 ºC and 220 ºC) was Lactose Standard at 5% (w/w) H_2O, analysed at 150 ºC.

The temperature ramps performed for the model proteins (Bovine Serum Albumin, Ovalbumin and Lysozyme) were intended to infer the optimal temperature for determining process residual water. Similarity of profiles for each of the temperature ramps, verified for all model proteins, led to a temperature set of 120 ºC with a lactose system at 150 ºC, based on the drift that each protein presented when subjected to a temperature range. Optimum temperature selection was made due to the absence of oscillations at this point of temperature, since it is indicative, by the results obtained, that the denaturation of the proteins occurs from temperature higher than 180 ºC, at which the drift increases. In addition, 120 ºC is close to the water release temperature (boiling point at 100 ºC), preponderant to the release of process residual water. The repeatability assessment was performed by analysing six different BSA sample preparations, as shown in Table 1.

Table 1: Repeatability method by Karl Fischer Oven of six Bovine Serum Albumin samples. The standard used for water content analysis (120 ºC) was Lactose Standard at 5% (w/w) H_2O, analysed at 150 ºC

Sample	Water Content (µg)	Sample Size (mg)	Water Content [(%(w/w))]
BSA 1	839.83	11.12	7.01
BSA 2	840.94	11.05	7.07
BSA 3	896.38	11.89	7.03
BSA 4	865.65	11.71	6.88
BSA 5	802.43	11.11	6.68
BSA 6	809.92	11.43	6.56

From the individual values obtained for each of the BSA samples preparation, their average was calculated, obtained a process water content of 6.87% (w/w). The results and average obtained also allowed the calculation of the %RSD (Relative Standard Deviation), with a value of 3. According to pharmacopeia guidelines, the acceptance criteria for the %RSD is ≤ 10%, which proves the method is repeatable.

A study for the determination of the total water content in inulin using the volumetric KF titration, corroborates the advantage of the Oven technique, especially as regards to the amount of sample [5]. In the development of volumetric KF method, for the determination of the total water content in inulin, a 250 mg of inulin was used, substantially higher when compared to the amount of BSA sample used in the present development, which was about 12 mg.

Size Exclusion Chromatography

In order to analyse the chromatographic profile of the proteins present in the standard gel filtration, a TSKG3000SWxl column was used, being the chromatographic profile obtained, as shown in Figure 2.

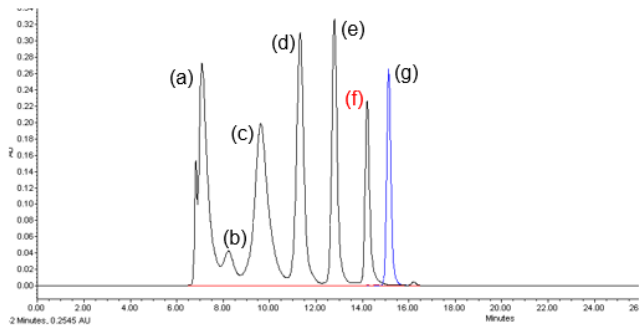

Figure 2: Size Exclusion Chromatographic profile of standard gel filtration with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220 nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC. (a) Thyroglobulin (670 kDa), (b) IgG aggregate, (c) IgG IgG (150 kDa), (d) Ovalbumin (44 kDa), (e) Myoglobin (17 kDa), (f) Vitamin B12 (14 kDa), (g) PABA (4-aminobenzoic acid) (0.14 kDa).

Aiming to mitigate secondary interactions (adsorption between the analyte and packing material) and electrostatic interactions, the mobile phase of 25 mM sodium phosphate monobasic and 350 mM sodium chloride was used at a pH 6.8, considering that the pH stability of the column is between 2.5 and 7.5 [6]. Based on the theoretical molecular weights of the model proteins (Thyroglobulin, IgG, Ovalbumin and Myoglobin), a calibration curve of the logarithm of the molecular weight was made as a function of the practical retention times, not being included in this calibration curve Vitamin B12, since Vitamin B12 does not have the same structure as a globular protein and therefore was considered as an outlier, and PABA, since it only served to evaluate the performance of the column. It was possible to establish a calibration curve fitted by the equation $LogMW = -0.2815RT + 4.841$, to later determine the molecular weights of other proteins, particularly as regards to the molecular weight of the protein under study, ARD_2019.

Degradation Studies

In order to evaluate the sensitivity of the Size Exclusion Chromatographic method to detect small variations in the level of impurities, aggregates or proteolysis, a degradation study was implemented. In this degradation study, the BSA protein was the chosen model protein.

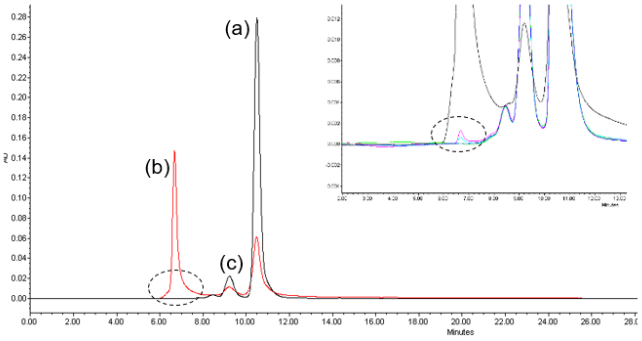

Figure 3: Size exclusion chromatographic profile of spikes of 0,5% (w/w), 1% (w/w) and 2% (w/w) at 80 ° C with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220 nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC. (a) BSA main peak, (b) High molecular weight (HMW) species, (c) HMW species.

Table 2: Bovine Serum Albumin size exclusion chromatographic results of the different spikes, 0.5% (w/w), 1% (w/w) and 2% (w/w), of fresh BSA at temperature stress condition (80 ºC) with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220 nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC. (a) BSA main peak, (b) High molecular weight (HMW) species, (c) HMW species

Stress Condition	Main Peak Retention Time (min)	Main Peak % Area	HMW species Retention Time	HMW species %Area
80 ºC spike 0.5% of fresh BSA	10.493	88.17	6.697	0.01
80 ºC spike 1% of fresh BSA	10.488	87.83	6.674	0.14
80 ºC spike 2% of fresh BSA	10.495	86.75	6.677	0.38

In the chromatographic profile obtained for the native protein, the main peak has a retention time of 10.491 minutes and a percentage area of 88.13% (area). When then subjected to 80 ºC, it is apparent that retention times and percentage area do not vary relative to the main peak by the slight increase in the percentage of the spikes, as shown in Table 2. In the chromatographic profile of the protein, when subjected to a temperature of 80 °C, a peak corresponding to High Molecular Weight (HMW) species was observed, as shown in Figure 3. The purpose of these experiments was to evaluate the method's sensitivity to detect small variations that could occur according to the different spiking percentages of the sample subjected to stress conditions, since there are variations in HMW species peak, as shown in Figure 3, and the results demonstrated in Table 2.

In contrast to temperature stress condition, the addition of formic acid to protein, with impact on pH, reveals the appearance of Low Molecular Weight (LMW) species in chromatographic profiles in comparison to the chromatographic profile of the native protein as shown in Figure 4, where the results are demonstrated in Table 3.

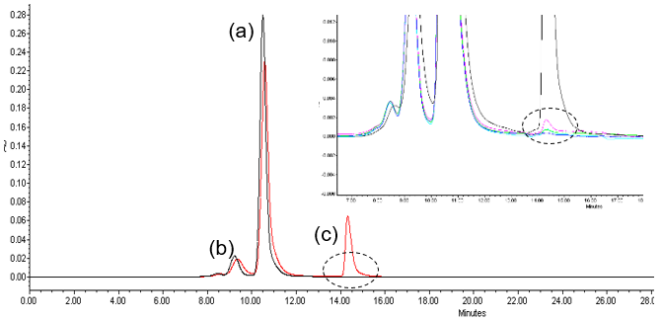

Figure 4: Size Exclusion Chromatographic profile of spikes of 0.5% (w/w), 1% (w/w) and 2% (w/w) with formic acid with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC. (a) BSA main peak, (b) HMW species, (c) LMW species.

Table 3: Bovine Serum Albumin size exclusion chromatographic results of the different spikes, 0.5% (w/w), 1% (w/w) and 2% (w/w) of BSA with 0.5% of formic acid at pH stress condition (0.5% formic acid) with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220 nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC.

Stress Condition	Main Peak Retention Time (min)	Main Peak % Area	LMW species Retention Time	LMW species % Area
BSA spiked with 0.5% Formic acid	10.491	87.24	14.31	0.24
BSA spiked with 1% Formic acid	10.497	86.87	14.309	0.84
BSA spiked with 2% Formic acid	10.497	86.45	14.317	0.88

In the case of the addition of formic acid, compared to the temperature stress condition, the variation of the values of the different spikes is higher, as shown in Table 3. These small variations are presented in the peak of low molecular weight species, being indicative of proteolysis or impurities. Thus, it is possible to infer that the size exclusion chromatography method for the two stress conditions, temperature and pH, can detect small variations, proving the sensitivity of the method.

In order to confirm the results obtained by size exclusion chromatography for the proteins subjected to stress conditions, a SDS-PAGE analysis was performed.

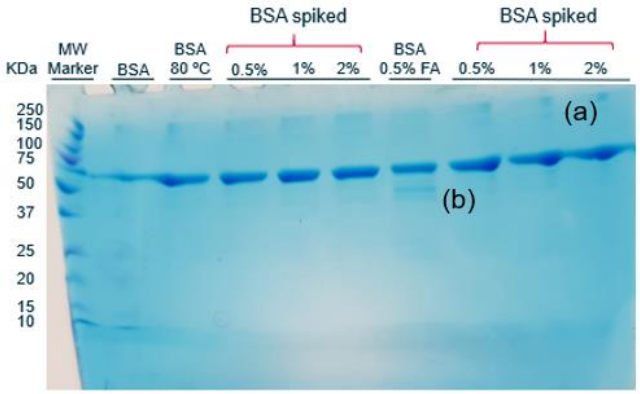

Figure 5: SDS-Polyacrylamide Gel electrophoresis: 12% (v/v) polyacrylamide gel in 2.5 M Tris-HCl, pH 8.8, containing 10% SDS (m/v). The stacking gel had a polyacrylamide concentration of 5% (m/v) in 0.5 M Tris-HCl, pH 6.8. Electrophoresis conditions: 120 V, 400 mA for 70 minutes. The samples were prepared under non reduced conditions, with the addition of sample buffer (5 mL of stacking gel, 8 mL 10% SDS, 1 mL β-mercaptoethanol, 2 mL Gycerol, and 4 mg of Bromophenol blue). The proteins were visualized by Coomassie Blue R-250. The samples used were obtained from different spikes, 0.5% (w/w), 1% (w/w) and 2% (w/w), of BSA with 0.5% of formic acid at pH stress condition (0.5% formic acid) and of fresh BSA at temperature stress condition (80 ºC). (a) High molecular weight species, (b) Low molecular weight species.

The samples were the same with respect to the two stress conditions under study. With the information obtained from the SDS-PAGE, which besides evaluating the purity of the components, provides their molecular weights, it was possible to confirm the results obtained by SEC, since it was observed bands corresponding to HMW (a) and LMW (b) species (Figure 5). In the electrophoresis gel it is possible to observe more intense bands common to all samples corresponding to the molecular weight of the protein, 67 kDa. Bands with higher and lower molecular weights, relative to those of reference, although attenuated, are visible, could be indicative of aggregation and proteolysis phenomena compared to chromatograms shown in Figure 3 (b) and Figure 4 (c), respectively.

ARD_2019 Characterization – Karl Fischer Oven

Regarding the method for the determination of water content by Karl Fischer Oven, 3 different ARD_2019 dried powder, were analysed. The results obtained for the water content determination were concordant between the different trials.

The results obtained for the water content was 0.55 - 3.02% (w/w), which are acceptable values for this material.

Size Exclusion Chromatography

The ARD_2019 protein was analysed by Size Exclusion Chromatography and the chromatographic profile obtained, as shown in Figure 6, was performed at the same wavelength used to analyse the model proteins, 220 nm.

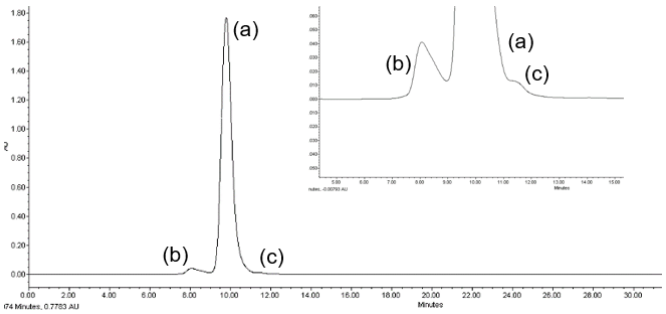

Figure 6: Size exclusion chromatographic profile of ARD_2019 with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC. (a) ARD_2019 main peak, (b) HMW species, (c) LMW species

The ARD_2019 chromatographic profile shows the elution of the native protein (main peak), High Molecular Weight and Low Molecular Weight species, in which retention times and percentage of areas are shown in the Table 4.

Table 4: ARD_2019 Size Exclusion Chromatographic results with method conditions optimized: 25 mM sodium phosphate monobasic and 350 mM sodium chloride, pH 6.8. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220 nm. The column used was TSKgel G3000SWxl at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC.

Sample	Main Peak Retention Time (min)	Main Peak % Area	HMW species Retention Time (min)	LMW species Retention Time (min)	HMW species % Area	LMW species % Area
ARD_2019	9.79	96.6	8.06	11.3	3.11	0.46

The equation from the calibration curve obtained from the model proteins, by SEC, $LogMW = -0.2815RT + 4.841$, was used to calculate the molecular weight of the ARD_2019 native protein, using the retention time obtained (9.79 minutes). Thus, the value of 122 kDa was obtained for the total molecular weight of the protein, approximate value of the theoretical value of the total molecular weight of an antibody, 150 kDa.

Analysis of the high molecular weight species was performed as these could be other high molecular weight proteins or aggregates. Using the retention time of the HMW species (8.06 minutes), it was obtained a value of 372 kDa, which makes approximately three times the molecular weight of the native protein, suggesting that aggregation of the protein itself may have occurred. The presence of Low Molecular Weight (LMW) species is also visible, with a lower percentage of area [(0.46%

(area))], wherein the calculated molecular weight was 45.71 kDa taking into account its retention time (11.3 minutes), indicative of proteolysis phenomenon, influenced by protein instability processes caused by the low pH or temperature stress conditions.

In the case of the presence of HMW species (b), this phenomenon is justified from self-association of the native monomer (a) (unaltered conformationally and active) that occurs when conformationally stable monoclonal antibody molecules form complexes through non-covalent interactions. This phenomenon of self-association may lead to reversible or irreversible aggregation, depending on the nature of the native protein.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The purpose of the characterization of ARD_2019 by SDS-PAGE that separates different size proteins according to their electrophoretic mobility, was (1) to compare the results obtained with the ones obtained by SEC, and (2) to evaluate the purity of the antibody. In the electrophoresis gel performed, as shown in Figure 7, four samples were run, fresh antibody and the three trials, under reducing conditions, showing the separation of the chains. Considering the molecular marker as a reference for calculating the molecular weight of the antibody, the bands corresponding to the antibody light chains, 25 kDa, and the antibody heavy chains, 50 kDa are visible. The electrophoresis gel shows consistent results regarding the size exclusion chromatographic technique, since it evidences the purity of the trials, given the absence of bands of molecular weight superior to the heavy chain, confirming the hypothesis of possible aggregation.

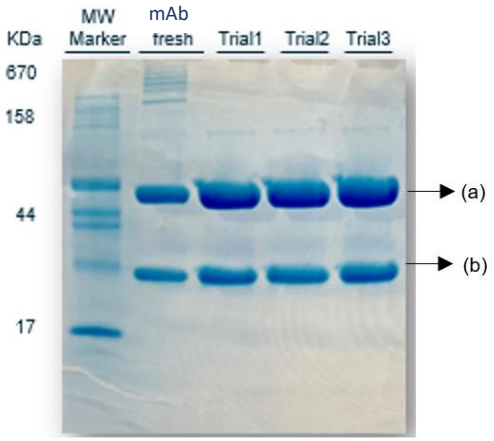

Figure 7: SDS-Polyacrylamide Gel electrophoresis: 12% (v/v) polyacrylamide gel in 2.5 M Tris-HCl, pH 8.8, containing 10% SDS (m/v). The stacking gel had a polyacrylamide concentration of 5% (m/v) in 0.5 M Tris-HCl, pH 6.8. Electrophoresis conditions: 120 V, 400 mA for 70 minutes. The monoclonal antibody (hBU12 fresh) and the three trials at a concentration of 2 mg/mL, were prepared under non reduced conditions, with the addition of sample buffer (5 mL of stacking gel, 8 mL 10% SDS, 1 mL β-mercaptoethanol, 2 mL Gycerol, and 4 mg of Bromophenol blue). The proteins were visualized by Coomassie Blue R-250. (a) Antibody Heavy Chain (HC), (b) Antibody Light Chain (LC).

In the characterization of monoclonal antibodies, where fragments are revealed by SDS-PAGE under reducing conditions, and in the case of pure ARD_2019 samples, the heavy (50 kDa) and light (25 kDa) chains of the antibody are expected to be observed, as shown in Figure 7. Referring to an analysis performed by Z.C. Zhu *et al*., under non-reducing conditions, in the specific case of the lgG 4 antibody, its heavy and light chain fragments are also preserved and revealed in the electrophoresis gel [7].

Ion Exchange Chromatography

In order to evaluate the charge heterogeneity of the monoclonal antibody, a Cation Exchange Chromatography was performed. Parameters such as the buffer and pH range were optimized, obtaining an optimization profile. Initially, the first condition tested was pH gradient, using the Tris buffer (10 mM), with a pH range between 7.5 and 10.5, considering the isoelectric point (pI) of the antibody, 8.2 (Figure 8).

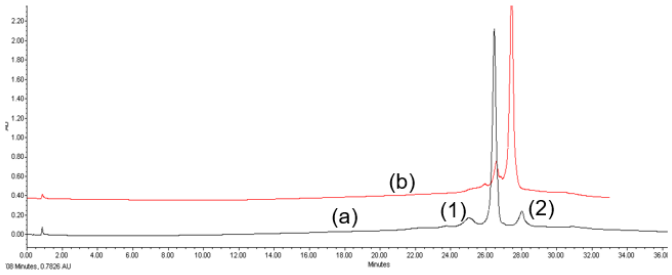

Figure 8: Cation exchange chromatographic profile of ARD_2019 by pH gradient with method conditions optimized: mobile phase A :10 mM Tris buffer, pH 7.5 and mobile phase B: 10 mM Tris buffer, pH 10.5. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220 nm. The column used was Protein Pak Hi Res CM at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC. (a) pH 7.5 - 10.5, optimized profile (b) pH 7 - 9.5, (1) acidic species, (2) basic species.

In Figure 8 are presented the comparison of two chromatographic profiles obtained with different pH variations. Both assumed a linear gradient between the mobile phase A (10 mM Tris buffer, pH 7.5) and the mobile phase B (10 mM Tris buffer, pH 10.5). Initially the percentage of mobile phase A was higher than the mobile phase B, allowing conditioning of the column. The positively charged mobile phase will bind to the negatively charged carboxymethyl group of the stationary phase, and the antibody will have to compete to bind, and therefore protein desorption occurs, and it is eluted. Thus, it is possible to observe in the chromatographic profile the acidic (1) and basic (2) antibody species, relative to the main peak. In the case of a pH range between 7 and 9.5 the basic species are not visible, and it was necessary to extend the pH range of the buffer (7.5 and 10.5).

A salt gradient with impact on the electrostatic surface of the protein was also tested, giving the information on the different charged variants of the antibody. In this case, the mobile phase A was 10 mM Tris Buffer, pH 7.5, and the mobile phase B was 10 mM Tris Buffer + 300mM NaCl, pH 7.5, having obtained the optimized chromatographic profile as shown in Figure 9.

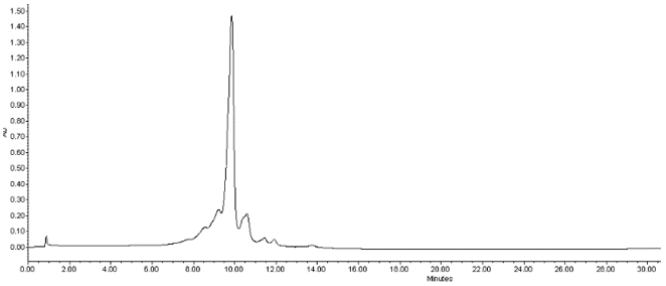

Figure 9: Cation ion exchange chromatographic profile obtained for ARD_2019 by salt gradient with method conditions optimized: mobile phase A: 10 mM Tris buffer, pH 7.5 and mobile phase B: 10 mM Tris Buffer + 300 mM NaCl, pH 7.5. The injection volume was 10 µL. The flow rate was 0.8 mL/min and monitored at a wavelength of 220nm. The column used was Protein Pak Hi Res CM at a temperature of 25 ºC; Autosampler Tray Temperature: 2-8 ºC.

In this situation, the additional factor is the salt, and the higher the net charge of the protein, the higher the ionic strength that is needed for elution, in which protein must compete to be able to bind to the negatively charged carboxymethyl group. Contrary to what occurs in the pH gradient, in the salt gradient, initially with 100% of mobile phase A, up to 30 min of run there is a gradual incremental change of mobile phase B, which contains 300 mM NaCl, with 65% mobile phase A and 35% mobile phase B.

The cation exchange chromatographic methodologies during this thesis, allowed the evaluation of the charged variant profile of the antibody under study. With these methods it was possible to identify 8 different charge variants for ARD_2019 mAb.

Conclusions

The analytical characterization of the model proteins (BSA, Ovalbumin and Lyzozyme) was made through different analytical techniques. The process residual water was carried out by a Karl Fischer Oven method. Initially, a temperature ramp between 50 ºC and 220 ºC was performed, to infer the optimal temperature that should be considered. The optimal temperature determined was 120 ºC. All the model proteins presented a similar temperature ramp profile. Since this method was repeatable for BSA protein, was also used to determine the water content of the ARD_2019 monoclonal antibody, using the same optimal working temperature, 120 ºC. Values between [0.55 and 3.02% (w/w)] for the water content were obtained.

The evaluation and characterization of the physicochemical parameters, size variants and process related impurities was performed by the development of a Size Exclusion Chromatographic method. A standard gel filtration was used, and the best results were obtained when applying a flow rate of 0.8 mL/min, 25 mM sodium phosphate monobasic and 350 mM sodium chloride, 7.5% IPA buffer, an injection volume of 10 µL and a pH value of 6.8, monitored at a wavelength of 220 nm. This chromatographic profile revealed better peak shape, peak sensitivity and resolution. Based on theoretical molecular weights of the known proteins present in the standard gel filtration and their retention times, a calibration curve was made. The optimized SEC method was also applied to the ARD_2019 antibody, to determine its molecular weight and to characterize the antibody size variants. In this profile it was observed, besides the main peak (122 kDa), High Molecular Weight species (HMW) (372 kDa) and Low Molecular Weight species (LMW) (45.71 kDa), with the molecular weights obtained from the calibration curve equation. The molecular weight values obtained allowed to infer that they could be indicative of (1) aggregation, in the case of HMW species, and (2) proteolysis, in the case of LMW species, which was also corroborated by the analysis of the electrophoresis gel obtained for ARD_2019 antibody.

After optimizing the method using the model proteins and applying it to the ARD_2019 antibody, a degradation study was carried out to evaluate the sensitivity of the SEC method to detect small variations in the level of impurities, aggregates or proteolysis. For this study, the BSA protein was subjected to stress conditions, temperature (80 ºC) and pH (0.5% formic acid). Regarding the chromatographic profile obtained for the temperature stress condition, it showed that when increasing the spiking levels, it was observed an increasing in the area corresponding to HMW species, which may be associated with the aggregation phenomenon. The same was verified with the addition of formic acid to the BSA protein, being observed the increase in the percentage area of the LMW species with the increase in the percentage of spikes, which can be associated with proteolysis phenomena. Thus, it can be concluded that the stress conditions analysed, pH and temperature, can be detected by SEC, which confirms the sensitivity of the method. The same samples, subjected to the same stress conditions, were also analysed by SDS-PAGE, in order to confirm the results obtained by Size Exclusion Chromatography, by evaluating the purity of the electrophoresis gel of the BSA protein. The results obtained corroborate the results achieved by the SEC method, since bands with higher and lower molecular weights, relative to those of reference, were visible, corresponding to aggregation and proteolysis phenomena.

The electrophoretic pattern of ARD_2019 protein was also analysed by SDS-PAGE, under reducing conditions. The gel showed consistent results with the Size Exclusion Chromatography method, regarding the purity of the antibody, in which only intense bands, corresponding to the HC (50 kDa) and LC (25 kDa) were visualized.

Cation Exchange Chromatography was also performed in order to evaluate the isoform pattern. The charge heterogeneity of the ARD_2019 antibody was evaluated by either pH gradient and salt gradient. In the pH gradient, the pH and buffer were optimized, where 10 mM Tris buffer with a pH range of 7.5 and 10.5 was used. These conditions may be considered optimal for the characterization of ARD_2019 antibody, since the chromatographic profile demonstrated well resolved acidic and basic antibody species. With an optimized profile, the conditions of the method were extrapolated for salt gradient analysis, where ionic strength was added by the addition of 300 mM sodium chloride at pH 7.5. It is possible to conclude that the Cation Exchange Chromatographic method, was reliable and consistent for charge heterogeneity analysis, since it was possible to analyse acidic and basic species, and charge variants of ARD_2019 protein.

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