Universidade de Aveiro Departamento de Química
2015

Diana Patrícia Gil da Silva

Produção de amostras de ovos hiperimunes seguido de fracionamento por sistemas aquosos bifásicos

Production of hyperimmune egg samples followed by aqueous biphasic systems fractionation

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo de Métodos Biomoleculares, realizada sob a orientação científica da Doutora Mara Guadalupe Freire Martins, Investigadora Coordenadora no Departamento de Química, CICECO, da Universidade de Aveiro, e do Doutor Ricardo S. Vieira Pires, Investigador Auxiliar do Centro de Neurociências e Biologia Celular da Universidade de Coimbra.

Aos meus pais e à minha irmã…

O júri

Presidente

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Palavras-chave Anticorpos hiperimunes, Anticorpos de aves, IgY, Líquidos iónicos, Sistemas aquosos bifásicos, *Tags* de fusão.

Resumo

Os anticorpos pertencem à família das Imunoglobulinas (Ig), e que se encontram no plasma e no fluído extracelular dos vertebrados, sendo produzidos pelas células B de modo a identificar e neutralizar as moléculas estranhas e patogénicas (antigénios) ao nosso organismo. A produção de anticorpos monoclonais e policlonais e a sua capacidade para se ligarem a antigénios específicos possibilitou a sua aplicação em análises quantitativas e/ou qualitativas, em métodos de purificação de antigénios e na modulação de efeitos fisiológicos em investigação, de diagnóstico ou terapêutica. Os anticorpos das aves (IgY) têm sido muito estudados neste tipo de aplicações por serem uma fonte mais económica e existirem em maior quantidade. A produção de IgY para aplicação em imunização passiva tem vindo a ser estudada nos últimos anos, e isto deve-se ao fato de existirem muitos microrganismos resistentes a antibióticos, doenças que não reagem aos fármacos aplicados e de indivíduos que são incapazes de responder ao método de vacinação tradicional. Para a produção de anticorpos específicos, expõem-se a ave a um determinado antigénio, através de injeção, levando posteriormente a uma resposta imunitária humoral e assim à produção de ovos hiperimunes. O grande objetivo deste trabalho consistiu na produção de ovos hiperimunes, na qual se acompanhou o perfil dos mesmos ao longo do tempo, seguido da avaliação da estabilidade de IgY em soluções aquosas de sais e polímeros utilizados em sistemas aquosos bifásicos que poderão ser estratégias promissoras para a sua purificação.

Keywords Hyperimmune antibodies, Hen antibodies, IgY, Ionic liquids, Aqueous biphasic systems, Fusion tags

Abstract

Antibodies are glycoproteins that belong to the family of immunoglobulins (Ig). They are found in plasma and extracellular fluids of vertebrates and are produced by B cells to identify and to neutralize pathogens and foreign molecules (antigens) in our body. The production of monoclonal and polyclonal antibodies and their ability to bind to a specific antigen, allows their use in quantitative and/or qualitative analyses of specific antigens, in purification methods and in modulating physiological effects in research, diagnosis or therapy. Hen antibodies (IgY) have been studied for such type of applications because they can be produced at a lower cost and in larger amounts. The production of IgY for use in passive immunization has been suggested because there are many antibiotic-resistant microorganisms, diseases that do not respond to the applied drugs and individuals who are unable to respond to vaccination. The major objective of this work consists on the injection of hens with specific antigens (produced with the aid of fusion tags) which leads to a humoral immune response and to the production of hyperimmune eggs, which were followed a long time. Finally, the stability of IgY was evaluated in aqueous solutions of salts and polymers that could be used in the creation of aqueous biphasic systems for purification purposes.

Contents

Abbreviations

ABS: Aqueous biphasic system

C: Constant domain

$[C_nC_nmim]^+$: Imidazolium

$[C_nC_npip]^+$: Piperidinium

$[C_nC_nPy]^+$: Pyridinium

$[C_nC_nPyr]^+$: Pyrrolidinium

CD: Circular Dichroism

DSC: Differential Scanning Calorimetry

E. coli: Escherichia coli

FA: Freund's adjuvants

FCA: Freund's complete adjuvant

FIA: Freund's incomplete adjuvant

GFP: Green fluorescent protein

GST: Glutathione-S-transferase

H: Heavy chain

HDL: High density lipoproteins

HiTraqQ: Ionic exchange chromatography

H. pylori: Helicobacter pylori

HR: Hinge region

Ig: Immunoglobulins

IMAC: Immobilized-Metal affinity chromatography

KSI: Ketosteroid isomerase

L: Light chain

LDL: Low density lipoproteins

ILs: Ionic liquids

MBP: Maltose-binding protein

MW: Molecular weight

$[N_{nnnn}]^+$: Quaternary ammoniun

NusA: N-utilization substance A

OD: Optical density

ON: Overnight

PEG: Polyethylene glycol

pI: Isoelectric point

RT: Room temperature

RT-PCR: Real-time polymerase chain reaction

SO: Sypro Orange

SUMO: Small ubiquitin related modifier

TrxA: Thioredoxin A

V: Variable domain

WSF: Water-soluble fraction

YFP: Yellow fluorescent protein

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1. Introduction

1.1 Scopes and objectives

The administration of preformed antibodies in humans or animals has become very attractive as a protective strategy against pathogenic viruses and bacteria, being also applicable in the control of drug-resistant microorganisms, diseases that does not respond to drug therapy and when the immune system is unable to respond to conventional vaccines (1,2).

Immunoglobulins (Ig), also known as antibodies, are produced by B cells and provide a versatile and specific response against antigens (3). In the past few years, mammals are being use for the production of monoclonal and polyclonal antibodies because they have the ability to specifically bind to an antigen and are thus used in numerous applications such as: in qualitative and/or quantitative analysis in solution, cells and tissues; in antigen purification methods; and in modulation of physiological effects in research, diagnostic or therapeutics (4).

In this work, we attempted the production of specific antibodies in Japanese Quails (*Coturnix japonica*), a model bird recently introduced in our research lines. Quails are small and less expensive than chickens, have a short generation interval and show genetic variation for growth traits in most populations (5). The implementation of an avian facility was also required and, for that, the European guidelines on the protection of animals used for scientific purposes were followed (Directive/2010/63/EU) (6). Standard immunization protocols in quails were implemented in order to produce hyperimmune eggs while presenting specific immune responses against model protein antigens, which were followed by ELISA assays.

This work is divide in two principal objectives: the establishing of immunization procedures in *Coturnix japonica* and the study of the IgY stability in aqueous solutions of phase-forming components used in the creation of aqueous biphasic systems (ABS), which have been studied for the purification of IgY by liquid-liquid separations. To attain the first objective, we prepared model immunogens and monitored their specific reactivity; whereas in the second objective we characterized the stability of IgY by thermostability assays in aqueous solutions of ABS components, such as polymers and salts, in order to obtain *a priori* information on which type of ABS can be used for the purification of IgY.

1.2. Antibodies

Antibodies are glycoproteins constituted by four polypeptides that belong to the family of immunoglobulins (Ig). They exist in the plasma and extracellular fluids of vertebrates and are produced by B cells in order to identify and neutralize pathogens and foreign molecules (antigens) to our body (3,4,7–9). Therefore, antibodies provide a versatile and specific response against antigens (3). The antibody/antigen binding triggers a biological activity that, depending on the antigen, can be a cell lysis, augmented phagocytosis or even allergic reactions (3).

1.2.1. Structure and function of antibodies

Structurally, immunoglobulins have a common global organization and consist of four separate polypeptide chains that interact with each other through S-S bonds, arranging themselves in a functional molecule in a Y-shaped form (4,9,10). Each Ig molecule contains two light chains and two heavy chains with approximate molecular weights of 25 kDa and 55 kDa, respectively (**Figure 1**) (4,9,10). The heavy chain is composed of one variable domain (V_H), followed by three constant domains (C_{H1}, C_{H2} and C_{H3}) and a flexible hinge region positioned between C_{H1} and C_{H2} (**Figure 1**) (1,2,9). In mammals there are five main heavy chain, namely gamma (γ), alpha (α), mu (μ), delta (δ) and epsilon (ε), resulting in different heavy chain classes that determine various Ig functions: IgG the main Ig of the immune system responding pathogenic invasions; IgA functioning at the level of the urinary, respiratory and intestinal tracts; IgM and IgD promoting an initial immune response to pathogens; IgD in the initial response to the immune system; and IgE in response to allergens (7–10).

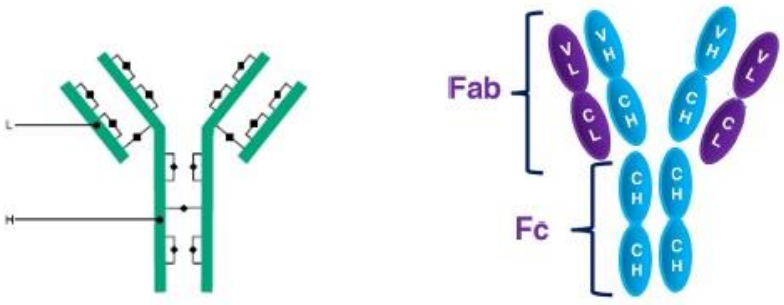

Figure 1: Molecular structure of immunoglobulins: L, light chain; H, heavy chain; Fab, antigen-binding domain; Fc, crystallisable fragment [adapted from (10,11)].

The light chain is composed of a variable domain (V_L) followed by a single constant domain (C_L). Two classes are present in mammals, namely kappa (κ) and lambda (λ) (9,10).

In terms of relevant structural domains of immunoglobulins, one can distinguish between Fab and Fc domains or fragments. Fab (antigen-binding domain) is a monovalent fragment of the antibody that contains the antigen binding site. The Fc (crystallisable fragment) determines the antibody biological activity by mediating the binding to cell-receptors and downstream responses (4). The two domains are linked by a hinge region consisting of proline, serine and threonine, which allows a rotational movement in Fab for an improve antibody/antigen bind (4). This region is easily cleaved by two proteolytic enzymes: papain and pepsin (4,9,12). Papain cleaves the antibody in the links located above the S-S bonds generating two fragments: Fab (antigen-binding domain monovalent) and a Fc fragment, while pepsin cleaves the links below the S-S bonds forming a single $F(ab')_2$ (bivalent binding domain antigen) and a Fc fragment partially digested (**Figure 2**) (4,9,12).

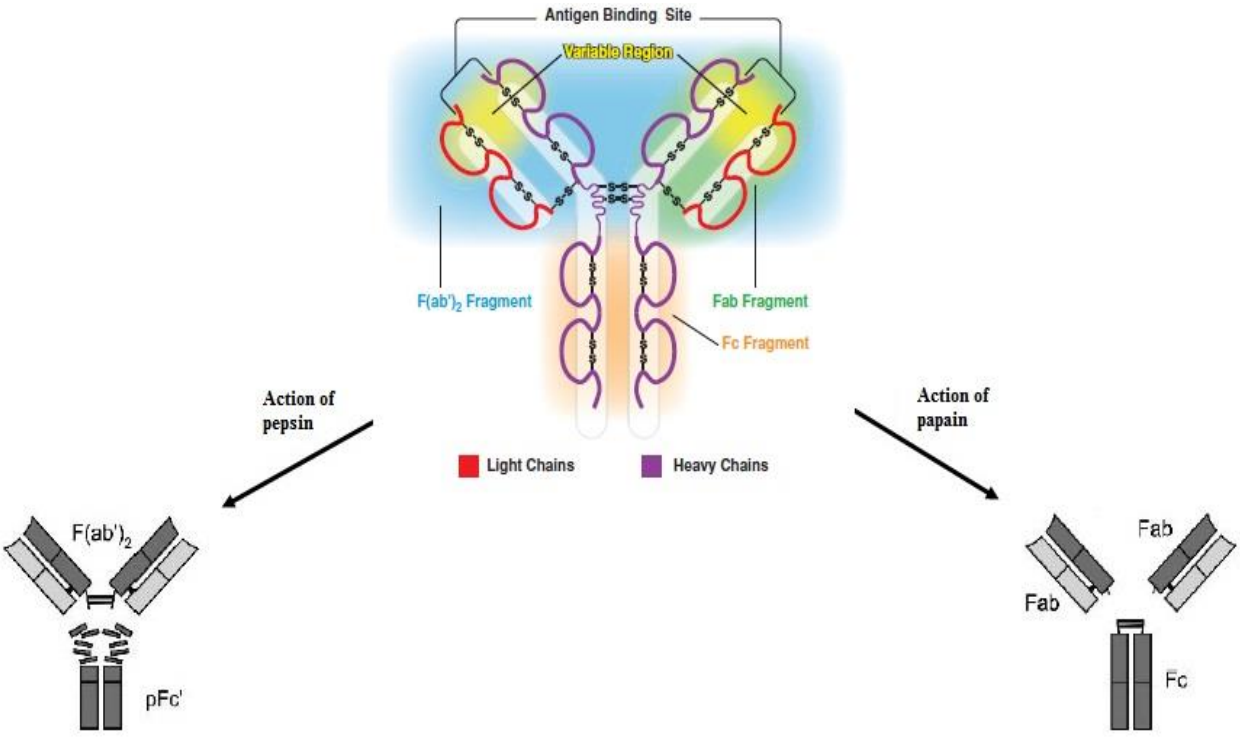

Figure 2: Structural domains after proteolytic processing of Immunoglobulin G [adapted from (4,9)].

1.2.2. Monoclonal and polyclonal antibodies

By immunizing animals such as rabbits, goats, sheep or apes, polyclonal and monoclonal antibodies are generated. These can be used in therapy, diagnosis and research (4,8,9).

Polyclonal antibodies are a heterogeneous mixture that recognize a multiplicity of epitopes of the same antigen being produced by different B cell clones, so they have different specificities and affinities (**Figure 3**) (9,10). Therefore, multiple clones of polyclonal antibodies show high levels of labelling to only one antigen, since many antibodies are produced for different epitopes of the same protein (8,9). However, with different epitopes, the antibody may ultimately connect to multiple protein and not to the protein-antigen of interest (8).

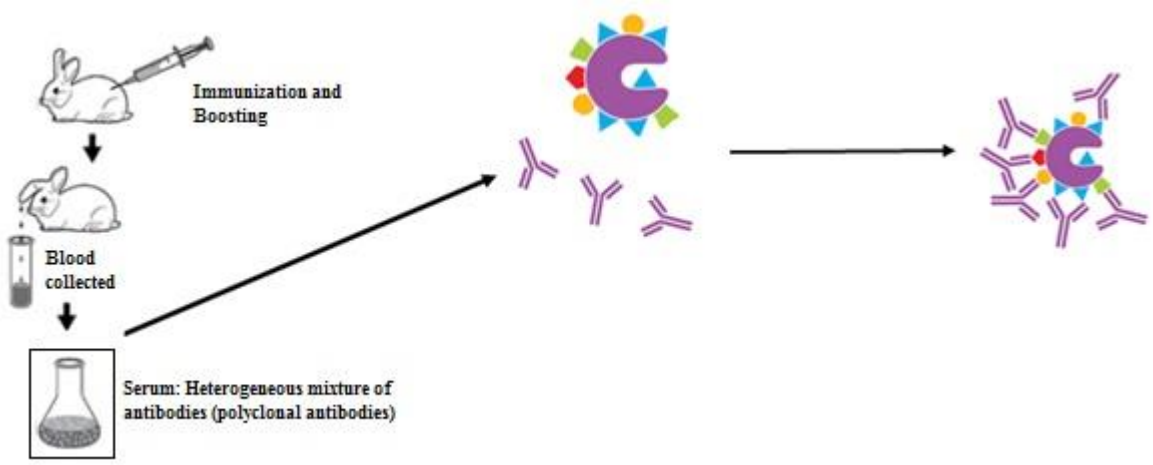

Figure 3: Production scheme of polyclonal antibodies by immunization of rabbits followed by antigen-binding to multiple epitopes [adapted from (10)].

Monoclonal antibodies are a homogeneous population of Ig, resulting from the expansion of a single B cell (4,8–10). These antibodies were initially found during the analysis of serum from patients with multiple myeloma, since clonal expansion of plasma cells leads to an increase of the same antibody (8). With this discovery, Köhler and Milstein in the mid-1970s [cited in (4,8)], have developed a technique for the production of monoclonal antibodies with the fusion of B lymphocytes with immortal myeloma cells, generating hybridomas (4,8).

With the specificity of the monoclonal antibodies it is possible to evaluate the changes in molecular conformation, interactions in protein/protein, phosphorylation state and identification of a protein family; yet, small changes in the epitope affects the function of

the monoclonal antibody (4,8). For the production of these antibodies it is necessary to take into account the process of selection, cloning and the low levels of labelling (due to the fact that connection has weak affinity) (4,8).

1.3. Hen IgY

Nowadays, the production of antibodies is dependent on model animals. In this work, we used the Japanese quail (*Coturnix japonica*). Currently, the most used antibodies are from mammalian sources. However, in 1893, Klemperer (2) demonstrated that with the immunization of chickens, specific antibodies are transferred to eggs and could be used to treat tetanus infections (2).

Immunoglobulins are found in chicken serum and transferred to the egg to confer protection to the offspring (1,13). Three classes of immunoglobulins are found in the hen eggs: IgA, IgM and IgY (1,2). During the egg formation, IgY is transferred to the yolk through a specific receptor present on the surface of the yolk sac membrane, while IgA and IgM will be deposited in egg white (1,2).Avian IgA and IgM are very similar to their mammalian counterparts in terms of molecular weight, structure and electrophoretic mobility, while IgY is functionally equivalent to mammalian IgG and thus represents the main Ig class in birds (1,2). The antibodies present in the yolk (IgY) have been extensively studied in passive immunization, diagnostic and therapy (1,14).

1.3.1. Molecular structure of IgY

IgY is functionally homologous to IgG, however, in terms of molecular weight, IgY is heavier (about 180 kDa) than IgG (150 kDa) (1,2,15). In IgY, two identical heavy chains (H) and two identical light chains (L) connected by S-S bonds exist, and where in the L chain a variable domain (V_L) and a constant domain (C_L) are found (1,2). Unlike IgG, between these two areas there is not an intra-chain S-S connection making these molecule less stable and, additionally, the structure also has less extension on β sheet which also compromises its overall stability (1,2). The H chain is constituted by a variable domain (V_H) and four constant domains (C_{H1}, C_{H2}, C_{H3} e C_{H4}). Between C_{H1} and C_{H2} domains does not exist in the hinge region (HR) that confers flexibility to the Fab fragment; instead proline and glycine residues that give some flexibility to the molecule are found (1,2). Therefore, it is possible to say that,

in terms of constant domains, C_{H2} and C_{H3} of IgG are equivalent to C_{H3} and C_{H4} domains of IgY (**Figure 4)** (1,2).

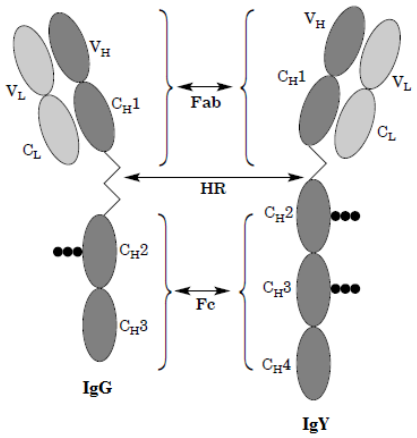

Figure 4: Contrast between the molecular structure of IgG and IgY (16).

1.3.2. Physicochemical properties of IgY

For the two immunoglobulins, the isoelectric point (pI) of IgY is lower (5.7 to 7.6) than that of IgG (6.1 to 8.5) (2,16). IgY also presents a larger Fc fragment (C_{H2}, C_{H3} and C_{H4}) than IgG (C_{H2} and C_{H3}) and therefore IgY is more hydrophobic (2,16).

Different studies demonstrated that pH affects the stability of IgY. At pH 3.5 there was a loss of activity of the IgY and, at pH 3, no activity was observed due to changes in the protein conformation (1,2,16,17). On the other hand, at pH 11, IgY is relatively stable, but with increasing pH to 12 a loss of activity was verified. Thus, IgY is stable between pH 4 and pH 11 (1,2,16,17). For the improvement of the stability of the protein stabilizers may be added, such as simple and complex carbohydrates, as well as polyols (1).

In terms of the enzymatic activity, IgY is resistant to digestion of trypsin and chymotrypsin, but it is very sensitive to pepsin digestion (2,16). The sensitivity of IgY to pepsin depends on the pH and enzyme/substrate ratio used; with a pH value ≥ 5 IgY is resistant, been able to maintain their activity on the antigen-binding site and cell agglutination (1,2). However, at a pH ≤ 4.5, there is total loss of activity occurring complete hydrolysis of the protein (1,2). With trypsin, there is the loss of polypeptides, maintaining

antigen binding activity and cell agglutination, but with chymotrypsin digestion their high activity is preserved (2).

The heat resistance of IgY is influenced by the presence of carbohydrates and several studies revealed that with the increase of temperature and heating time, the binding activity of the antibody/antigen decreased. So, IgY is stable at temperatures between 60°C and 70°C, while at temperatures above 70°C a decrease of the protein activity is observed and at temperatures above 75°C proteins suffer full denaturation (1,2). High pressure, on the other hand, seems not to influence the stability and activity of IgY, at least up to 4,000 kg per cm^2 (2).

In l998, Chansarkar (2) observed loss of antigen binding activity of IgY as well a decrease of solubility in processes of freezing and pulverizing (2). However, a study conducted in 2006 by Fu (2), reported no changes in the activity of IgY (2). The processes of freezing and spray-drying are considered less destructive processes since they use low temperatures (2).

1.3.3. Immunization of laying hens for production of specific IgY

A hyperimmune egg contains a large amount of specific antibodies (IgY) produced in the bird's blood after the exposure to an antigen (1,18). For the production of specific IgY it is necessary to inject a combination of antigen-adjuvant at regular intervals, to take into account factors such as the antigen (the dose and molecular weight), the type of adjuvant used, the route of administration and frequency of immunization for an efficient immunization (2,16,18). **Figure 5** shows how this process occurs.

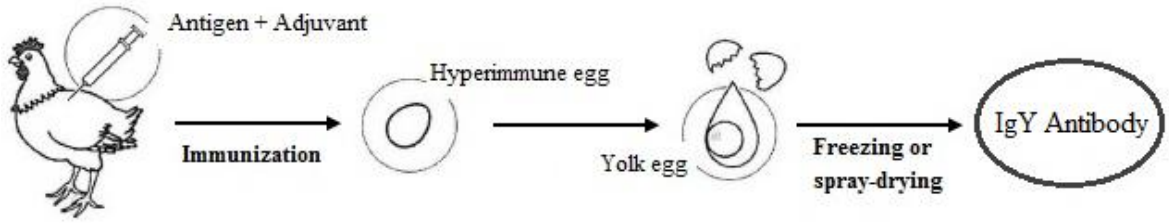

Figure 5: Immunization process of a bird [adapted from (18)].

The immune response begins when the organism is in the presence of a foreign body designated antigen (2). Hence, the amount of antigen used will influence the immune response, specifically, high or low amounts can lead to suppression, sensitization and

tolerance (2,16,18). According to the studies conducted by Schwarzkopf in 2001 (2,18), and by Cook in 2010 (18), it was shown that the recommended dose of injected antigen for the immunization of chickens is between 0.01 mg to 1 mg (2,18).

The selected antigen may be proteins, peptides and polysaccharides or may be a multi-antigen, like bacteria, fungi or viruses (2,18). In the case of small antigens, like peptides (<10 kDa), it is required the conjugation of a carrier protein such as γ-globulin bovine (2,18). Due to its low immunogenicity, nucleic acids and lipids need to be conjugated to a carrier protein (2,18). It is necessary to take into account the purity of the sample injected to decrease the possibility of cross-reactivity or negative responses by the animal (18).

For an effective immune response it is necessary to add an adjuvant to form an emulsion in order to allow a controlled interaction of the antigen for producing an adequate humoral response (18). The adjuvant, typically, consists of oil, detergent and bacterial extract (mycobacteria). The most commonly used is Freund's adjuvant (FA) that showed good results in the production of antibodies (1,2,18). Two types of FA are known: the complete Freund adjuvant (CFA) and the incomplete Freund adjuvant (IFA); the differences between them is that CFA is a bacterial extract while IFA is not (2,18). Several studies demonstrated that CFA leads to better results in the production of antibodies (2,18). However, in studies with rabbits it was found an inflammation at the injection site being necessary TO substitute CFA by IFA although this last one did not reveal to be efficient. Thus it is necessary to use a combination of these two types of adjuvants in hen immunization where in the first and second injections the authors used CFA; and in the third and fourth injections the authors used IFA (2,18).

1.3.3.1. Immunization frequency

The most usual route of application for the production of IgY is intramuscular and subcutaneous injection. The last one is less used because it is most painful for animal and the results are less effective (1,2,16,18,19). During the injections, for example in chickens, it is necessary to not exceed the maximum volume of 1 mL and it should be administered in four different locations to not cause distress to the animal (18).

In order to have a good adaptation of the immune system, the frequency of the injections must be from 1 to 8 weeks and the most usual is between 3 and 4 weeks, although it is essential to known the immunogenicity of antigen and the type of adjuvant used (2,18). Then,

it is advisable to make a new injection when the IgY production reaches a plateau or decreases (18).

1.4. Purification of IgY from egg yolk

For the extraction/purification of IgY from egg yolk there are several methods and commercial kits (20,21); however, it is difficult to isolate and purify IgY from the complex egg yolk matrix.

Egg yolk is composed mainly of lipids (triglycerides, phospholipids and cholesterol) and proteins present in about 15-17% (2). The proteins are composed of high density lipoproteins (HDL: α- and β-lipovitellins), phosvitin (glicophospoprotein), livetins (globular glycoproteins free of lipids and water soluble) and low density lipoproteins (LDL) (2). Livetins are divided into three classes: α, β and γ-livetins, where the IgY is the most predominant form of γ-livetin (2).

Several methods have been implemented for the extraction and purification of egg yolk antibodies and the most used involve protein precipitation with ammonium sulphate, dextran sulphate or polyethylene glycol (PEG) and separation by ion exchange chromatography (21). So, for the separation of IgY it is necessary to remove lipoproteins and to recover the water-soluble fraction (WSF) (2). Many methods have been used for the purification of IgY as the dilution of egg yolk in water, where the aggregation of the egg yolk lipoproteins with low ionic strength occurs, followed by centrifugation (1). However, to obtain a more effective purification of these antibodies it is necessary to take into account the pH and the extension of dilution, and in studies made in 1994 by Nakai (1), the best results were obtained with dilutions of about six times with water at pH 5.0 (1). Organic solvents and coagulants of lipoproteins (PEG or dextran sulphate) have also been widely used for IgY purification (1). Additionally, natural polysaccharides, such as sodium alginate, xanthan gum, λ-carrageenan and pectin, are also potential alternatives for IgY extraction, as they can efficiently precipitate about 90% of the lipoproteins of yolks (1).

The choice of the IgY extraction method is influenced by the quality of extraction (preservation of antibody activity, purity and recovery yield), if it is for an industrial or laboratory scale, and the cost-effectiveness of the technology (2).

In the recovery of the WSF an additional step to separate the γ-livetin (IgY) from α and β-livetins (water-soluble proteins) and from the remaining LDL based on precipitation,

chromatography and filtration could be used (2). After this first step the use of chromatography (gel-filtration, ion-exchange, thiophilic, affinity chromatography) or a repeated precipitation of IgY is used (22). With these combined steps, the IgY purified can be used for passive immunization and other human related applications. Nevertheless, the development of an economic and easily scaled up method for IgY purification is the major goal (22).

1.5. Advantages of the use of IgY relative to IgG

The production of antibodies in birds are of a more economic nature, they produce a large amount of antibodies, do not compromise the animal welfare and is in accordance with the principles of the 3Rs (reduction, refinement and replacement) defined by Russell (18) in 1959 (1,18). So, the production of IgY is less invasive because it requires only the eggs collection, the number of injections is reduced and the yield of antibodies in egg yolk is higher compared to the amount in rabbits serum, for instance (1,18).

The phylogenetic distance between birds and mammals allows the IgY to be recognized by more epitopes when the immunogen used is a highly conserved protein from mammals, being thus possible to obtain a better immune response with less amount of antigen (1,17). In immunodiagnostic applications, the use of IgY is also very advantageous since the mammalian IgG interferes with immunoassays. For instance, the rheumatoid factor reacts with IgG from different mammals (17).

Table 1 shows the main differences between the antibodies from rabbits (IgG) and antibodies from chickens (IgY). Finally, and in addition to the advantages described above, the hen immunization and the subsequent egg collection, is a process that can occurs at the industrial level making the IgY large-scale production possible (1).

Table 1: Comparison between IgG and IgY (16,17,21).

	Rabbits (IgG)	Chickens (IgY)	Quails (IgY)
Antibody source	Blood Serum	Yolk egg	Yolk egg
Isolation	Expensive	Simple, cost-effective	Simple, cost-effective
Type of antibody	Polyclonal	Polyclonal	Polyclonal
Antibody sampling	Invasive	Non-invasive	Non-invasive
Antibody amount	200 mg/40 mL blood	100-150 mg/egg	12-30 mg/egg
Collection frequency	Every 15 days: 40 ml / bleed	Every day: 300 eggs / year	Every day: 360 eggs / year
Ab amount/year	5200 mg	27000 mg	6480 mg
Specific Ab	≈5%	2-10%	2-10%
Avidity	Moderate	High	High
Cross reactivity	High	Low	Low

1.6. *Coturnix japonica* (Quail)

Over the years, *Coturnix japonica* has been used as a model laboratory animal in scientific research in genetics, nutrition, toxicology and pathology fields (23,24).

In this work, we used *Coturnix japonica*, because they have several advantages comparing with chickens. Their small size permits an ease housing and reduced feed costs. They have a short life cycle since the females began to lay eggs at an average of six weeks of age and generally lay 5 to 7 eggs *per* week, while being able to produce eggs for at least for one year. They are more disease resistant, respond positively to laboratory manipulation and respond quickly to changes in the environment (24,25). On the other hand, they aged more quickly (laying birds should be replaced each year), shell damage occurs frequently in the eggs, the chicks are very small and tender and they are sensitive to noise (24,25). So, a facility was set up with an appropriate environment, with all the conditions to proceed with the immunization experiment. **Figure 6** depicts some pictures from the facility.

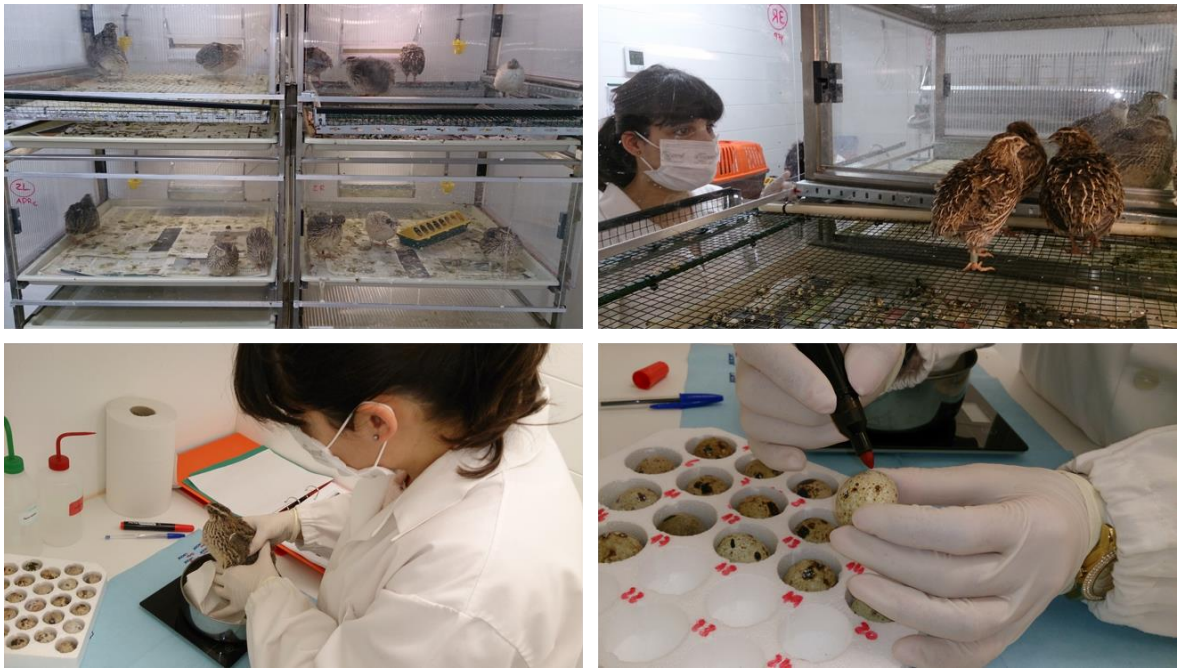

Figure 6: Pictures from the facility used for the immunization and control of *Coturnix japonica* as an experimental laboratory animal.

1.7. IgY in biomedicine and biotechnology

Nowadays, the specific IgY for a specified antigen can be produced in large scale by producing hyperimmune eggs (2). The use of IgY for passive immunization showed to be efficient in the treatment of infectious diseases caused by various pathogens, such as intestinal diseases (2).

Passive immunization comprises the transfer of preformed antibodies from an individual to another protecting against infection (**Figure 7**). In the active immunization the individual generates its own antibodies when exposed to a particular antigen (1,18). With passive immunization we obtain immediate protection but for a limited period and can last from a few weeks to three or four months (1,2).

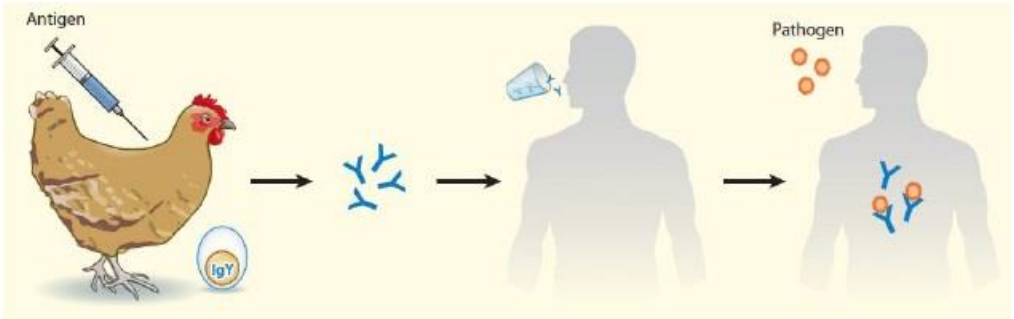

Figure 7: Passive immunization process (1).

The administration of preformed antibodies in humans or animals has become very attractive as a protective strategy against pathogenic viruses and bacteria being also applicable in the control of resistant organisms (2). Therefore, the immunotherapy has been applied as a cancer diagnostic agent and therapy, in the inactivation of toxic substances such as drugs, and in neoplastic and infectious diseases (17).

In medicine, IgY can be administered systematically or by intravenous route, being the oral administration of IgY a useful treatment against microorganisms in humans and animals (17). IgY shown to be effective in the treatment of diseases caused by pathogenic organisms *in vitro* and in clinical trials, and show successful results in preventing colonies of *pseudomonas aeruginosa* in patients with cystic fibrosis (17). In patients infected by *Helicobacter pylori* (H. pylori), responsible for causing gastritis and gastric ulcers, and with the increase of bacteria resistance to antibiotics, studies with IgY have been very important, verifying, in animal models, a reduction in bacterial adhesion (17).

IgY may also be used in veterinary medicine because with the increasing use of antibiotics during the animal growth also increases the resistance of antibiotic-resistant bacteria (17). Consequently, IgY have been used as a food additive in order to combat specific pathogens and improve growth and feed efficiency (1). Additionally, these feed additives have the advantage of being more easily accessible because they did not present high costs and have large amounts of polyclonal antibodies (17). The administration of these inhibit the enzyme uricase, reduce emissions of nitrogen in poultry, reduce *Escherichia coli* (E. coli) that causes diarrhoea in pigs, *Salmonella* in mice and calves and *Campylobacter*, *Clostridium*, and *Salmonella* in poultry (17).

IgY can be used in immunoaffinity chromatography that involves the isolation and purification of target molecules using immobilized antibodies. So, this chromatography can be used to purify specific molecules from complex starting materials. However, this technique has a high cost to be used in large scale due to the production of antibodies and immobilization efficiency (17).

1.8. Production of recombinant proteins

Living cells are mostly constituted by proteins and they have an important role in cell processes like cell signalling, immune responses, cell adhesion and cell cycle (26). To produce specific antibodies it is necessary to have pure model immunogens (antigen) and in

reasonable amounts. In the last 30 years, the use of recombinant DNA technology has allowed the study of structure and function of numerous proteins of interest and contributed to significant advances in the areas of biomedicine and biotechnology (27–29). The principle of this technology consists in the introduction of genes from one organism (e.g. human) into the genome of another organism, said host (e.g. *Escherichia coli*), allowing thr subsequent expression of the encoded protein by the host cell machinery (27–29). The most widely used system in the production of recombinant proteins include bacteria (e.g. *Escherichia coli*), yeast (e.g. *Saccharomyces cerevisiae, Pichia pastoris*), insect cell lines (e.g. *Spodoptera frugiperda*), mammalian cell lines (*Chinese hamster ovary*, *CHO*, *Human Embryonic Kidney 293*, *HEK*) and even plants (*Solanaceae* family) (27–29). Due to the versatility of *E. coli*, this system is the most explored in this technology because it allows the production of proteins with different origins and in significant amounts (26,28).

Fusion tags are molecular tools used for protein study to overcome some inherent limitations associated with the production of recombinant proteins (28,29). Tags are typically proteins, protein domains or peptide sequences that are genetically inserted in amine (N) or carboxylic acid (C) terminals of the target protein (28,29). The different types of tags determine different properties and behaviour of the target-tag fusion protein (28,29). In general, the use of fusion tags improve the expression level, enhance solubility and stability in solution (soluble fraction increases), while further allowing the monitoring, purification and detection by cell expression assays (e.g. Western blot) (26,28,30,31).

For the application of these tags, the choice of the tag should be considered since this may compromise the structure and topology of the target protein (26,30,31). Therefore, the size should be chosen critically, because larger tags tend to improve solubility, as well as the sequence and amino acid composition, because longer sequences have more flexibility which is not desirable in all situations (26,30,31).As a final point, these tags are very versatile because they enable the use of several fusion tags in tandem for purification in subsequent steps (30).

In the sections below the parameters of solubility, model immunogens produced during this work, and affinity purification are presented and discussed.

1.8.1. Fusion tags that promote solubility

As mentioned earlier, there are various fusion tags that can be used to promote the solubility of target proteins. These include: glutathione S-transferase (GST), maltose-binding protein (MBP), thioredoxin A (TrxA), small ubiquitin related modifier (SUMO), ketosteroid isomerase (KSI), N-utilization substance A (NusA) and TrpΔLE (26,27). In this work we used **GST**, **NusA**, **TrxA** and a fluorescent tag, the **VenusYFP** as a model immunogen for antibody production and their subsequent characterization.

The tag GST has 26 kDa from *Schistosoma japonicum* that is being used to promote the solubility and the purification of fusion proteins produced in various prokaryotic and eukaryotic systems (26,27).

NusA is a hydrophilic protein with 55 kDa that acts in the regulation of cellular transcription termination essentially having a role in anti-termination (26,27). This protein makes the translation slower, allows more time for the folding process and promotes the solubility of all hydrophobic proteins (27). Moreover, NusA presents different structural, physico-chemical and biological properties, making of this protein an ideal tag for the production of soluble proteins (26,27).

Thioredoxins are oxido-reductases that reduce disulfide bonds through the exchange of thio-disulfide (27). TrxA is a highly soluble cytoplasmic protein of 12 kDa, found in *E. coli* (26,27). It is used as a N- or C-terminal fusion tag increasing the solubility of the recombinant protein (26,27). These fusion protein does not have intrinsic affinity properties and alone it does not facilitate the purification, being necessary the use of small affinity tags to facilitate the protein crystallization (26,27).

Through fusion, a protein with fluorescent proteins is possible to visualize by biological processes, such as protein expression, localization, degradation and interaction with in vivo imaging and in vitro fluorescence labelling (32). Green fluorescent protein (GFP) came from the jellyfish *Aequorea victoria* and demonstrated a high stability of mature over various environmental conditions, spontaneous autocatalytical generation of the fluorophore and the possibility of spectral manipulation by mutagenesis (33). GFP as three types of variants, and the variant used in this work was the yellow fluorescent protein (YFP) with 26.4 kDa, that is relatively acid-sensitive and exceptionally reduced by Cl^- (30,34). In this context, mutations are necessary to decrease the sensitivity to pH and Cl^- (34). One of the mutations

is named by VenusYFP that greatly accelerates oxidation of the chromophore, the rate-limiting step of maturation at 37°C (34).

Affinity tags are efficient tools for purification of recombinant proteins (26,27). They can be divided into peptides or proteins that bind to small ligands immobilized on a solid support (e.g. His6 tag which allows selective binding to resins with immobilized Ni or Co) and tags that bind to immobilized molecules (e.g. MBP tag for binding to an amylose resin) (26). Elution of the fusion tag-protein of different solid supports is typically accomplished through a competitive ligand (26). Then, it is necessary to consider the cost implications as well as the interference from the elution agent with the eluted protein sample (26). The size of the affinity tag may also compromises the manipulation and subsequent characterization of the protein and, so, this is another factor to consider (26).

In conclusion, the selection of these protein tags as model immunogens was based on the increased protein solubility, which further helps on protein purification, and on their stability, easy-to-produce and increase on the protein´s immunogenicity (26).

1.8.2. Tag removal

The presence of fusion tags may compromise the structure and function of the recombinant protein of interest and, often, its removal is desirable (26,27). For this end, specific amino acid sequences are inserted between the tag and the native protein that enable an enzymatic or chemical cleavage (26,27).

Enzymatic cleavage is more efficient than chemical cleavage because the last one requires conditions that compromise the stability of the protein of interest (26). In the enzymatic cleavage two groups of proteases can be used: endoproteases and exoproteases (26). The must use endoproteases are the serine proteases (factor X activated from blood coagulation (factor Xa), enterokinase and thrombin-α) and viral proteases (tobacco etch virus - TEV - and the human rhinovirus 3C protease) and the exoproteases more used are the metallocarboxypeptidases and aminopeptidases (26).

1.9. Aqueous biphasic systems (ABS)

1.9.1. Extraction of biomolecules using ABS

In 1958, Albertsson (35,36) demonstrated the existence of Aqueous Biphasic Systems (ABS) as liquid-liquid extraction techniques (35,36). The author showed that a concentrated solution of an organic polymer, like polyethylene glycol (PEG), and an inorganic salt such as sodium or potassium phosphate could form two immiscible liquid phases, with the organic polymer in the upper phase and the inorganic salt in the bottom one (37). These systems formed when two incompatible hydrophilic solutes are dissolved in water above certain concentrations (37). In general, ABS can consist of two immiscible aqueous phases like polymer-polymer, polymer-salt and salt-salt combinations (35,36,38). They are mostly constituted by water having an environment more appropriate and biocompatible for the extraction and purification of biologically active molecules, such as proteins, antibodies and enzymes (36,38). Additionally, ABS are a simple technique, easily operated, could lead to high extraction efficiencies and a high degree of purity, and with low cost components (36–38).

ABS consisting of two-polymers display two phases very similar in terms of polarity (depends on the amount of water in each phase) (36,39). On the other hand, polymer-salt ABS have a hydrophobic phase enriched in polymer and a more hydrophilic phase, mainly composed of a high charge density salt (36,39). The hydrophilic polymer most commonly used in these systems is polyethylene glycol (PEG) since it is highly biodegradable, has low toxicity, low volatility, low melting point, is soluble in water and has low cost (40,41). Although PEG presents these characteristics, its nature is very hydrophilic, that limits its applicability in the extraction/purification of various biomolecules (41). Therefore, various studies have been made in order to tune the physical and chemical properties of PEG, such as variation of polymer chain length and its functionalization (41,42).

1.9.2 ABS phase diagrams

The ABSs are ternary systems consisting of water and combinations of two solutes. The representation of these ternary systems, under a particular set of conditions (e.g. pH, temperature) can be made on an orthogonal phase diagram (**Figure 8**) (36). In such diagram, the binodal curve (that passes by D-C-B-A) separates the monophasic from the biphasic regime (36,37). For a given mixture (M) at the biphasic region, the points D and B denote

the composition of each phase. These diagrams allow to identify ideal phase compositions in order to prepare ABS for extraction/separation processes (36).

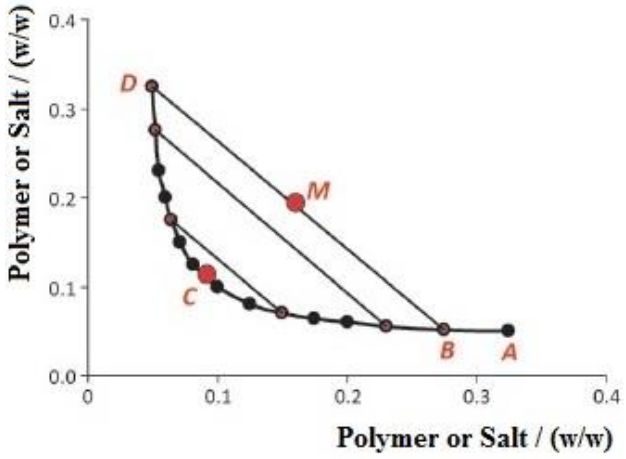

Figure 8: Representation of an ABS ternary phase diagram (36). M is the total mixture composition that falls into the biphasic region and the binodal curve is the curve that passes by D-C-B-A. The compositions of each phase are represented by the points D and B, the end-points of the tie-lines represented by the parallel lines (36).

In 2003, Rogers and his research group (36,38) proposed the use of ionic liquids (ILs) to form ABS as an alternative to the polymers commonly used. This strategy enabled the generation of ABS with a broader range of polarities and reduced viscosity (35,36,38). In the following years, ILs have been largely investigated as substitutes for hydrophilic polymers in ABS (43). With the replacement of an inorganic salt with high charge density and/or by substitution of the polymer by an IL, it has been possible to cover a wide range of phase polarities allowing therefore the tuning of the extraction performance and selectivity (36,39).

1.9.3 Ionic liquids

Ionic liquids (ILs), discovered in 1914, are organic melted salts at temperatures below 100°C constituted by large organic cations and anions that may be organic or inorganic (44–48). These salts are designated as "green solvents" and they present unique properties, such as a low vapour pressure (non-volatile at atmospheric conditions) high electrical conductivity, high thermal stability and high solvation capacity for organic and inorganic compounds (43,48,49). In this perspective, ILs are considered as good substitutes for volatile

organic solvents currently used in large-scale applications (50). ILs have been studied in several areas of research, such as in catalysis, electrochemistry, in analytical chemistry and in separation processes (50).

IL properties can be modified according to their chemical structure, or in other words, they can be synthesized with various combinations of anions and cations for a specific task or application – "designer solvents" (43,45). The ILs more used are represented in **Figure 9**.

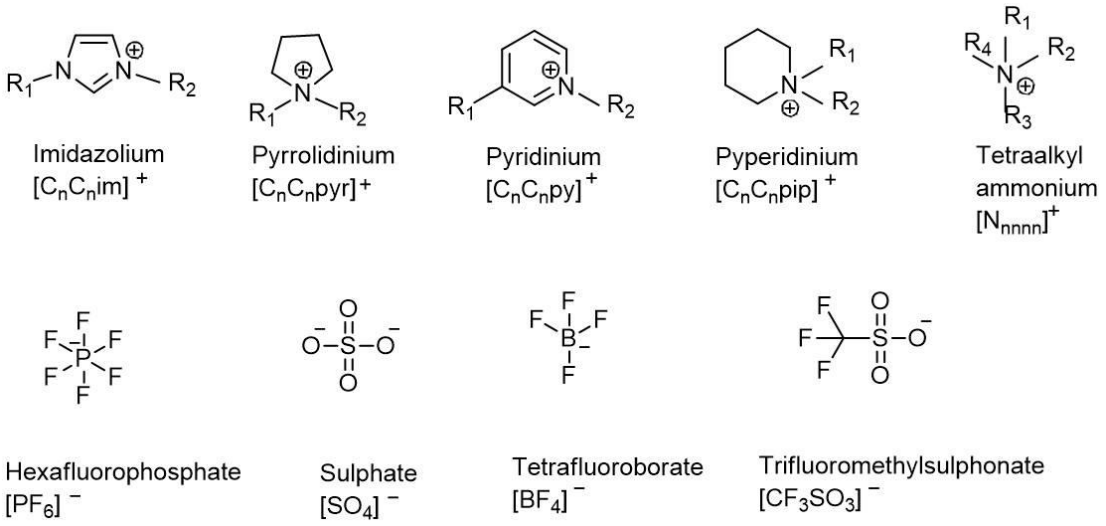

Figure 9: Chemical structures of anions and cations of ILs (45).

2. Experimental section

2.1. Immunogen expression and purification

2.1.1. Preparation of BL21 (DE3) star and DH5α competent cells

To obtain competent cells, each strain was inoculated from a glycerol stock in 5 mL of LB medium and incubated overnight (ON) at 37°C with agitation. In the next day, 2.5 mL of this culture were inoculated on 250 mL R-LB medium (1% (w/v) tryptone; 0.5% (w/v) NaCl; 0.04% (w/v) NaOH and 0.24% (w/v) $MgSO_4$) and allowed to grow under the same conditions until an Optical Density (OD) of 0.4-0.6 at 600 nm. The cells were collected by centrifugation at 3000 rpm for 5 minutes and the supernatant discarded. The pellet was resuspended in 100 mL of TFB I (30 mM $C_2H_3KO_2$; 100 mM RbCl; 10 mM $CaCl_2$; 50 mM $MnCl_2$; 15% (v/v) glycerol; pH 5.8 adjusted with acetic acid) and incubated on ice for 5 minutes. The cell suspension was centrifuged again at 3000 rpm for 5 minutes and the cell pellet resuspended in 10 mL TFB II (10 mM MOPS; 75 mM $CaCl_2$; 10 mM RbCl; 15% (v/v) glycerol; pH 6.5 with KOH or NaOH) and further incubated for 30 minutes on ice. Final cell suspension was divided in 130 µL aliquots that were flash-frozen in liquid nitrogen and stored at -80°C.

2.1.2. Transformation of BL21 (DE3) star and DH5α competent cells

Transformation of competent cells was performed using the heating shock method. One microliter of plasmid encoding the gene of interest was added to 130 µL of competent cells (pGEx4T2-GST; pCoofy18-VenusYFP; pCoofy15-NusA; pCoofy23-Trx); the mixture was incubated on ice for 30 min. Next, a heat-shock was induced by transferring the cells rapidly to 42°C for 45 seconds and then again to ice. To allow recovery of the cells and consequent expression of the genes of interest, 1 ml of LB medium was added and tubes were placed at 37°C with orbital shaking at 170 rpm for one hour. Finally, the cells were spread on LB-Agar supplemented with the appropriate antibiotic (50 µg/mL kanamycin) and incubated ON at 37°C.

2.1.3. Expression and purification of model immunogens

Transformed BL21 (DE3) star cells were resuspended from agar plates with 5 mL of LB medium. The OD600 was measured for reference and a sample of the cell suspension used to inoculate 1 L of LB medium. The culture was grown at 37°C with orbital shaking at 170 rpm for two/three hours (depending on the protein) until an OD600 of 0.4 – 0.6. Protein

expression was induced with 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and in some cases 2% ethanol was additionally used; cultures were incubated at 30°C with orbital shaking at 170 rpm for three hours. Cells were then pelleted at 3000 rpm for 15 minutes at 4°C (Avanti™ J-26 XPI centrifuge, Beckman Coulter™) and stored at -20°C until use. Cell pellets were processed after resuspension in binding buffer (respectively **VenusYFP:** 50 mM TrisHCl pH 7.5, 150 mM KCl, 5 mM β- ME (β- mercaptoethanol); **NusA:** 50 mM TrisHCl pH 7.5, 150 mM NaCl; **TrxA:** 50 mM TrisHCl pH 8.0, 150 mM NaCl) supplemented with protease inhibitors (1 µg/mL leupeptin, 1 µg/mL pepstatin A, 0.1 mM PMSF, phenylmethylsulfonyl fluoride). Cells were disrupted using an Emulsiflex-C3 homogenizer. The lysate was centrifuged at 17500 rpm for 30 minutes at 4°C (Avanti™ J-26 XPI centrifuge, Beckman Coulter™) and cleared lysate supplemented with 10 mM Imidazol. The lysate was then loaded onto a HisTrap™ HP 5 mL column (GE Healthcare Life Sciences) with flow rate of 0.5 mL/min using a peristaltic pump (Bio-Rad). The column was previously pre-loaded with 0.5 M $NiSO_4$ at 2.5 mL/min and pre-equilibrated with binding buffer supplemented with 10 mM Imidazol. Protein elution was performed by a multi-step gradient of imidazole (**VenusYFP:** 10 mM, 20 mM and 150 mM; **NusA:** 10 mM, 20 mM, 150 mM and 500 mM; **TrxA:** 25 mM, 100 mM, 150 mM and 500 mM) and fractions containing the protein of interest were pooled and quantified on NanoDrop® ND-1000 Spectrophotometer. The protein was further purified by size exclusion chromatography using a Superdex™ 200 10/300 GL column (GE Healthcare Life Sciences) equilibrated in running buffer (**VenusYFP:** 50 mM TrisHCl pH 7.5, 150 mM KCl; **NusA:** 50 mM TrisHCl pH 7.5, 150 mM NaCl; **TrxA:** 50 mM TrisHCl pH 8.0, 150 mM NaCl); eluted fractions were pooled and quantified using a NanoDrop® ND-1000 Spectrophotometer.

2.1.4. Denaturing Gel Electrophoresis

The analysis of protein expression was made by electrophoresis on polyacrylamide gel under denaturing conditions using sodium dodecyl sulphate (SDS). A 12% running gel was prepared (1.65 mL distilled deionized water; 1.25 mL1,5M Tris pH 8.8; 2.0 mL 30% acrylamide/bisacrylamide solution – Grisp; 50 µL 10% (w/v) SDS; 50 µL 10% (w/v) ammonium persulfate and 2.5 µL TEMED (N, N, N', N' – Tetramethylethylenediamine – Sigma-Aldrich)). The gel was added into the compartment between the glass and the plate, avoiding bubble formation. Isopropanol was added on top of the running gel to allow

polymerisation and the levelling of the gel surface. After polymerization, the isopropanol was removed. During polymerization, the 5% stacking gel was prepared (1.05 mL distilled deionized water; 190 µL 1,5M Tris pH 6.8; 250 µL 30% acrylamide/bisacrylamide solution – Grisp; 15 µL 10% (w/v) SDS; 15 µL 10% (w/v) ammonium persulfate and 2.5 µL TEMED (N, N, N', N' – Tetramethylethylenediamine – Sigma-Aldrich)) and added over the running gel and the 10-well comb put in place. Finally, the comb was removed and the wells washed with water. The protein samples were diluted in Loading Buffer 6x (350 mM Tris-HCL pH 6.8; 30% (v/v) glycerol; 10% (w/v) SDS; 0.6 mM DTT; 0.012% (w/v) bromophenol blue) and boiled for 10 minutes. Protein samples were ran at 120-180 V in electrophoresis buffer (0.2 M Tris Base; 0.2 M Bicine and 0.1% (w/v) SDS) for 60 minutes. The proteins were stained using PageBlue Protein Staining Solution – Thermo Scientific. Precision Plus Proteins™ Unstained – Bio-Rad were used as protein standards.

2.2. Producing polyspecific antibodies in *Coturnix japonica*

2.2.1. Bird immunization procedure

Immunization procedures for antibody production were performed in Japanese quails (*Coturnix japonica*). Two adult female quails (housed in pairs) were used *per* experiment and immunized in four moments with two-week intervals (Day-0, -15, -30 and -45).

Injectable samples were prepared by standard emulsification procedures and 100 µg of purified immunogen were used *per* bird. Briefly, immunogen samples were mixed in equal volumes of the adjuvant to obtain injectable emulsions; the first immunization (Day-0) and first boost (Day-15) used complete Freund's adjuvant (CFA) and for the second (Day-30) and fourth boost (Day-45) incomplete Freund's adjuvant (IFA) was used.

Intramuscular injections were performed in two sites of bird's pectoral muscles, with no more than 150 µL *per* injection site. Bird body weight and behaviour was monitored along the full immunization protocol (typically 80-90 days).

2.2.2. Processing hyperimmune eggs

During the immunization protocol, quail eggs were collected daily and yolk samples corresponding a two days of posture were prepared (1 sample = yolk pool of two days). Yolk samples were subsequently processed by precipitation with 3.5% polyethylene glycol, MW 6000 (PEG 6000) (VWR Chemicals) in PBS to remove major lipid content. Briefly, samples

were incubated with 3.5% PEG 6000 for 10 minutes with agitation and centrifuged at 13000 g at 4°C for 20 minutes; the supernatants were collected and an additional spin was performed to remove pellet contaminants. Finally, the total protein concentration of each yolk sample supernatant was measured using NanoDrop® ND-1000 Spectrophotometer and normalized with PBS for downstream ELISA assay.

2.2.3. ELISA assays

Yolk samples collected along the immunization protocol (80-90 days) and processed as described above were used to monitor specific antibody response against each model immunogen. ELISA plates (F96 MaxiSorp Nunc - Immuno Plate) were coated ON at 4ºC with 0.2 µg of purified immunogen *per* well. Unbound proteins were washed with Tris-buffered saline, 0.1% Tween® 20 (TBS-t) and plate wells were blocked at room temperature (RT), for 2h, with TBS-t supplemented with 1% bovine serum albumin (BSA) (Capricorn Scientific GmbH). After blocking, the plate was washed 3 times with TBS-t and 100 µL of yolk processed samples (IgY, primary antibody) were added to each well and incubated at RT, for 2h with agitation. Blocking buffer was then washed off with TBS-t and 100 µL of secondary antibody diluted 1:50000 (α-chicken IgY horseradish peroxidase (HRP) conjugated, Rabbit – Sigma-Aldrich) were added to each well and incubated at RT for 1h with agitation. Finally, ELISA development was performed with ABTS substrate (ABTS Tablets from Roche Diagnostics GmbH) prepared in ABTS Buffer. The secondary antibody was washed off with TBS-t and 100 µL of ABTS substrate were added in each well and incubated at 37°C, for 30 minutes with agitation for colour development. The colorimetric signal was analysed by measuring the absorbance at 405 nm using a microplate reader and respective software (BIO-TEK® PowerWave XS with software KC Junior™).

2.3. IgY Thermostability Assays

2.3.1. Isolation of IgY from chicken and quail eggs by means of PEG precipitation followed by Hitrap™ Q HP

15 mL of yolk were added to 15 mL of PBS plus sodium azide at 0.04%. In the next day, the sample was thawed and 15 mL of PBS and PEG 6000 was added to a final concentration of 3.5%, mixed on a vortex and incubated at RT for 20 minutes with agitation. Then, the samples were centrifuged at 14000 g at RT for 20 minutes, the supernatant collected and

filtered through a filter unit assembled with filter paper (Membrane Filters (Cellulose acetate) 1.2 µm Whatman™ GE Healthcare Life Sciences) and a 0.2 µm filter (Membrane Filters (Cellulose acetate) 0.2 µm Whatman™ GE Healthcare Life Sciences). After the filtration, PEG 6000 35% was added to a final concentration of 12%, vortex and incubated at RT for 20 minutes with agitation. The filtrated solution was centrifuged again in the same conditions as before and the pellet was resuspended in 10 mL of 50 mM TrisHCl pH 8.0. At the solution was added PEG 6000 to a final concentration of 12%, vortex and incubated at RT for 20 minutes with agitation. The sample was centrifuged again and the pellet was resuspended in 5 mL of 50 mM TrisHCl pH 8.0 and incubated at 4°C ON with agitation. Next day, the solution was centrifuged at 14000 g at 4°C for 20 minutes and loaded onto HiTrap™ Q HP 5 mL (GE Healthcare Life Sciences) at 1 mL/min pre-equilibrated with 50 mM TrisHCl pH 8.0 and, then, applied a linear gradient of salt (0-1 M NaCl) at 1 mL/min in 50 mM TrisHCl pH 8.0. The fractions containing the protein of interest were pooled and quantified by a NanoDrop® ND-1000 Spectrophotometer. The protein was further purified by size exclusion chromatography using a Superdex™ 200 10/300 GL column (GE Healthcare Life Sciences) equilibrated in buffer (50 mM TrisHCl pH 8.0; 150 mM NaCl); eluted fractions were pooled and quantified by a NanoDrop® ND-1000 Spectrophotometer.

2.3.2. IgY Thermostatility assays

In a Multiplate™ PCR Plates 96-weel (Bio-rad Laboratories, Inc), for a final volume of 40 µL were added 30 µL of aqeuous solutions of phase-forming components of ABS, reagent **X** (**X** = PEG 400 at 0%, 5%, 10%, 20%, 40%, 50% and 60%; PEG 600 at 0%, 5%, 10%, 20%, 50%, 69%; PEG 1000, 1500 and 8000 at 0%, 5%, 10%, 20%, 30%; PEG 6000 at 0%, 5%, 20%, 40%, 50% and 60%; $C_6H_5K_3O_7$ at 0%, 5%, 10%, 15%, 20% and 25%; K_2HPO_4 + acid citric at 0%, 5%, 10%, 15%, 20%, 22,5% and 25%; Na_2SO_4 at 0%, 5%, 10%, 15%, 20%, 25%), 8 µL of protein (IgY from chicken and/or quail) and 2 µL of Sypro® Orange Protein (Sigma Life Science). The plate was sealed with iCycler iQ® optical tape (Bio-Rad Laboratories, Inc) and analysed on CFX384 real-time PCR detection system with CFX Manager software (Bio-Rad Laboratories, Inc). The conditions used on CFX384 real-time PCR detection system were temperatures between 35°C - 95°C at a rate of 1°C/min.

3. Results and discussion

3. Results and discussion

The use of hen antibodies has a huge potential on biomedical and biotechnology applications because they have become very attractive as a protective strategy against pathogenic viruses and bacteria (1). Extensive literature reports on the purification of antibodies can be found (20,51). However, for these applications, high-purity level antibodies are required. In this context, the use of Aqueous Biphasic Systems (ABS) has been studied as an alternative purification method (36). ABS consist of two immiscible aqueous phases, mostly constituted by water, providing thus a more appropriate and biocompatible environment for the extraction and purification of biologically active molecules such as proteins, antibodies and enzymes (36,38).

In this work we foreseen the use of ABS for the purification of antibodies from egg yolk. For this, we initially designed an experimental set up to produce hyperimmune eggs and characterized the stability of IgY in aqueous solutions typically used for ABS formation.

Japanese quails (*Coturnix japonica*) were chosen instead of chicken because they are of a smaller size meaning reduced feed costs, they have a short life cycle, they produce eggs for at least for one year, they are more disease resistant and respond positively to laboratory manipulation (24,25). In order to produce hyperimmune eggs, we have chosen different well characterized and well behaved model proteins to be used as immunogens.

3.1. Immunogen Expression and Purification

For quails immunization we purified well known protein affinity or reporter tags, namely NusA, GST, VenusYFP and TrxA.His-tag immunogen versions previously obtained in the lab were used for expression and were purified by a first step of Immobilized-Metal Affinity Chromatography (IMAC). The His-tag was not cut from the proteins of interest since the respective molecular weight was assumed to not interfere significantly with the antibody production. After that, the specific antibodies were finally purified by Size Exclusion Chromatography (SEC) (52).

3.1.1. N-utilization substance A (NusA)

In **Figure 10** it is shown the SDS-PAGE results from NusA on IMAC.

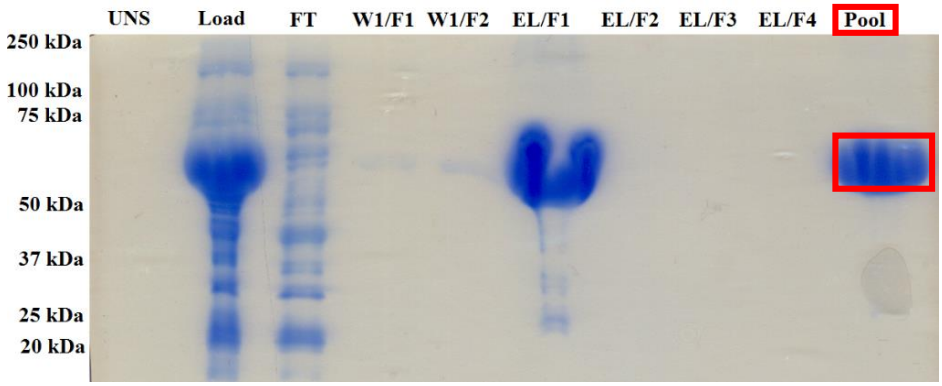

Figure 10: SDS-PAGE of Affinity Chromatography (HisTrap Ni-NTA) fractions; W, washing steps; EL, elution steps using imidazol-containing buffer; Pool, final sample ready for SEC step. The elution steps were obtained with a multi-step gradient of imidazole: W1/F1 with 10 mM; W1/F2 with 20 mM; EL/F1 and EL/F2 with 150 mM and EL/F3 and EL/F4 with 500 mM.

From **Figure 10**, it can be see that when the sample was loaded onto the column it was highly impure; after the column washing step the sample was eluted and in the end a pool was made, with the protein of interest (NusA), for the SEC step. In general, the NusA is identified as the protein with the molecular weight (MW) of 55 kDa (27).

Figure 11 depicts the obtaining results from SEC chromatography for NusA and **Figure 12** shows the respective SDS-PAGE results.

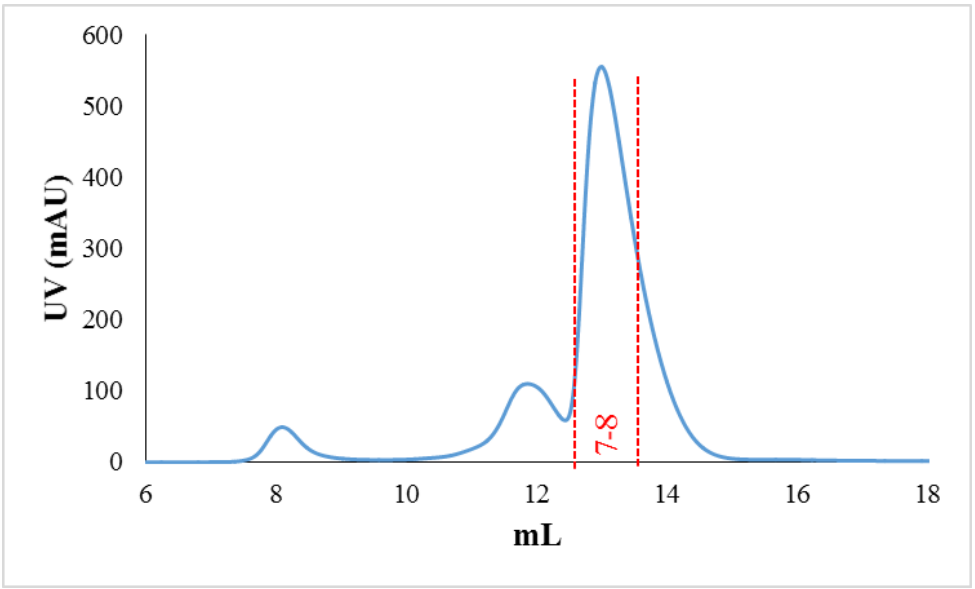

Figure 11: Size Exclusion Chromatography (SEC) profile of NusA model protein. Fractions 7-8 correspond to samples with highest purity.

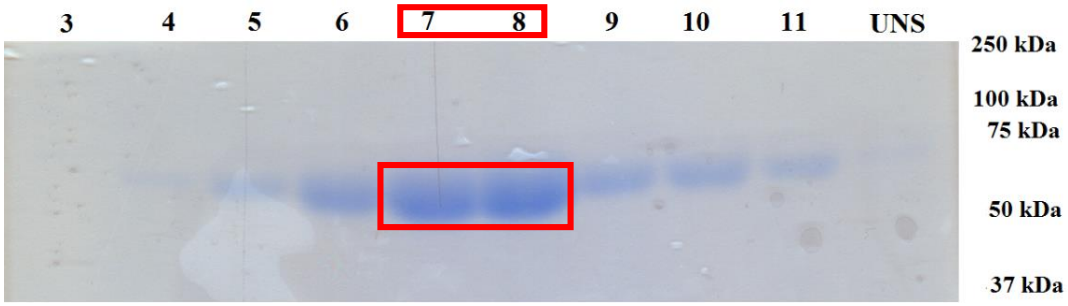

Figure 12: SDS-PAGE of the elution fractions from SEC.

After comparing the results from SDS-PAGE and the profile from SEC, a pool with the samples 7 and 8 was made since they present the highest purity level as shown by SDS-PAGE. The pool was then quantified obtaining 2.01 mg/mL of protein concentration. This pool was stored at -20°C for subsequent use.

3.1.2 Thioredoxin A (TrxA)

In **Figure 13** it is shown the SDS-PAGE results from TrxA on IMAC.

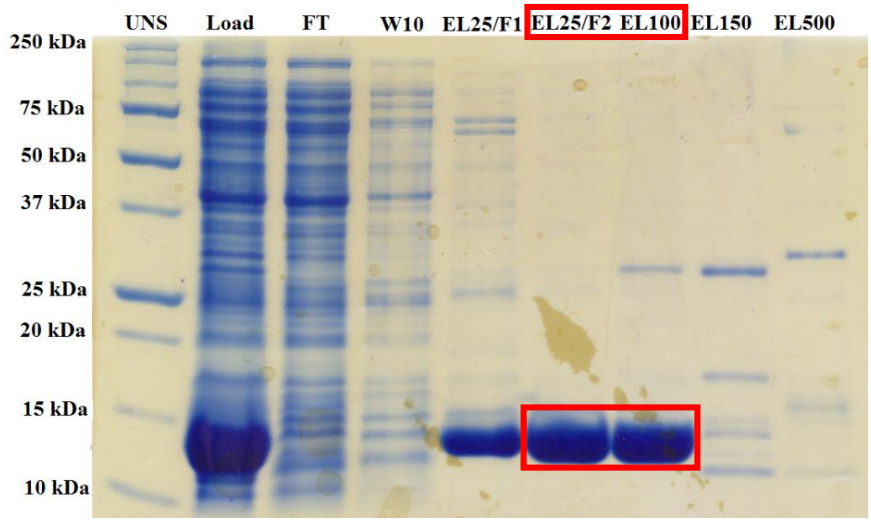

Figure 13: SDS-PAGE of Affinity Chromatography (HisTrap Ni-NTA) fractions; W, washing steps; EL, elution steps using imidazol-containing buffer. We made a pool with the sample EL25/F2 and EL100 for SEC step. The elution steps were obtained with a multi-step gradient of imidazole: W10 with 10 mM; EL25/F1 and EL25/F2 with 25 mM; EL100 with 150 mM and EL500 with 500 mM.

When the sample was loaded onto the column it was highly impure; after a column washing step, the sample was eluted and in the end a pool was made with the samples EL25/F2 and EL100 because they contain the protein of interest (TrxA). This pool was then purified by SEC where TrxA can be identified as the protein with 12 kDa of MW (27).

In the SEC step 3 runs were performed although only the first is shown, since all the runs led to the same profile. **Figure 14** shows the obtained results from SEC chromatography for TrxA and **Figure 15** depicts the respective SDS-PAGE results.

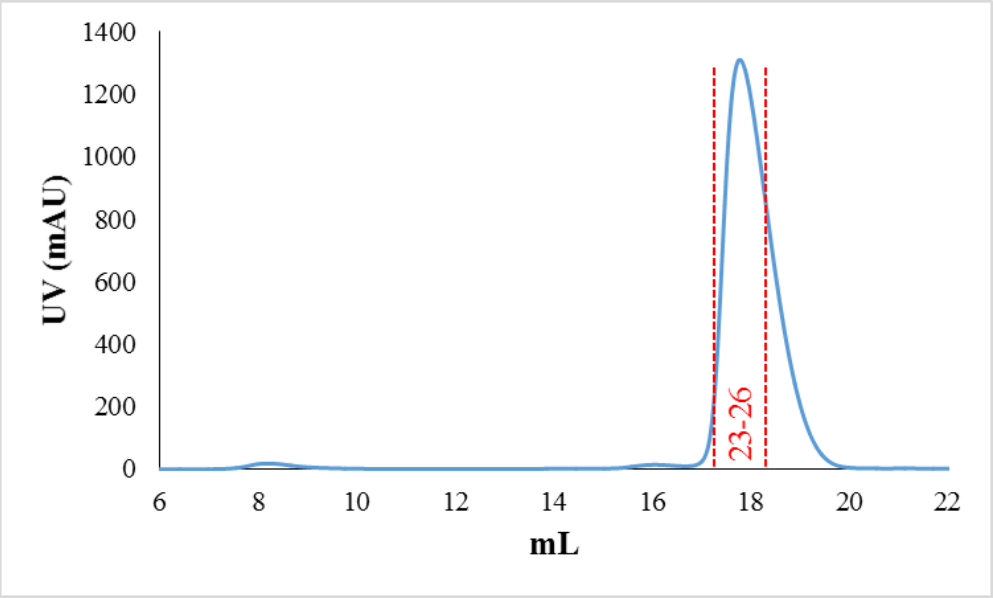

Figure 14: Size Exclusion Chromatography (SEC) profile of Trx model protein. Fractions 23-26 correspond to samples with highest purity.

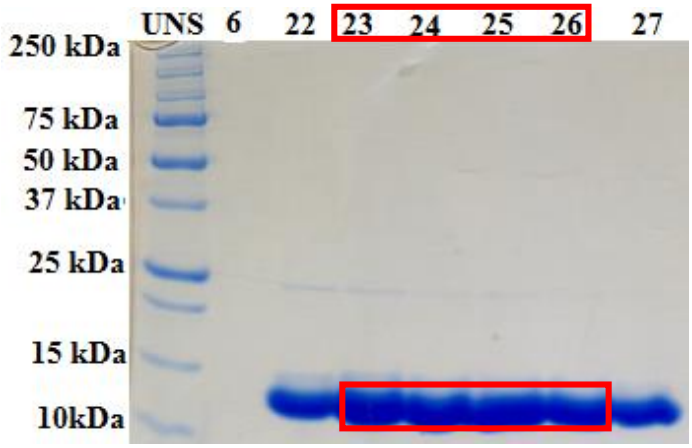

Figure 15: SDS-PAGE of the elution fractions from SEC.

After comparing the results from SDS-PAGE and the profile from SEC, pools with the samples 23-26 for Run 1, 6 and 7 for Run 2, and 6 and 7 for Run 3 was chosen because they present the highest purity as shown in the SDS-PAGE results. The protein concentration found was 2.00 mg/mL. The pool was stored at -20°C for subsequent use.

3.1.3. Glutathione-S-transferase (GST)

For the expression and purification of GST, the obtained results after SEC are shown in **Figure 16**.

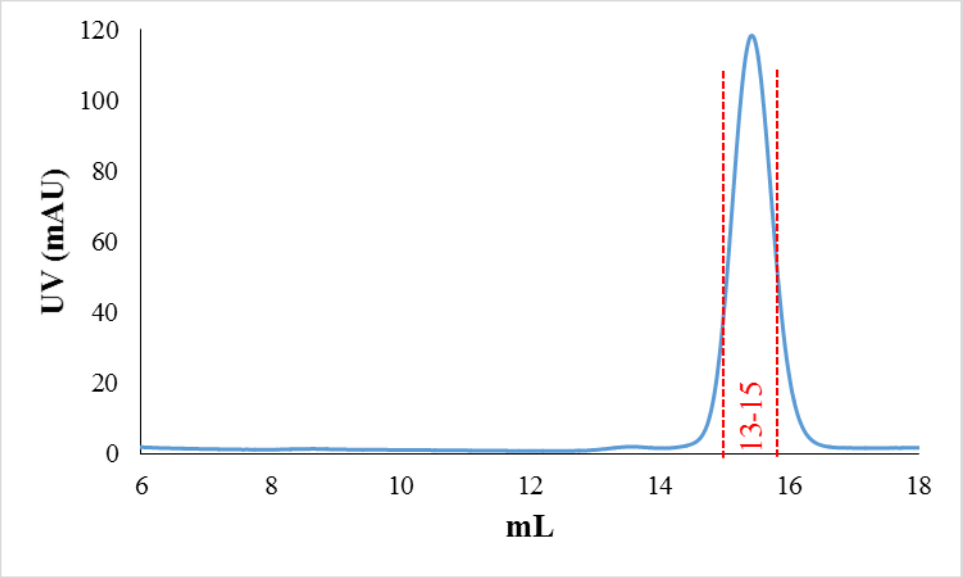

Figure 16: Size Exclusion Chromatography (SEC) profile of GST model protein. Fractions 13-15 correspond to samples with highest purity.

After the SEC chromatography a pool with samples 13-15 was made. GST has 26 kDa of MW (27). The pool has 0.54 mg/mL of protein. Finally, the sample was stored at -20°C for subsequent use.

3.1.4 Yellow Fluorescent Protein (YFP)

Figure 17 shows the SDS-PAGE results for VenusYFP from IMAC.

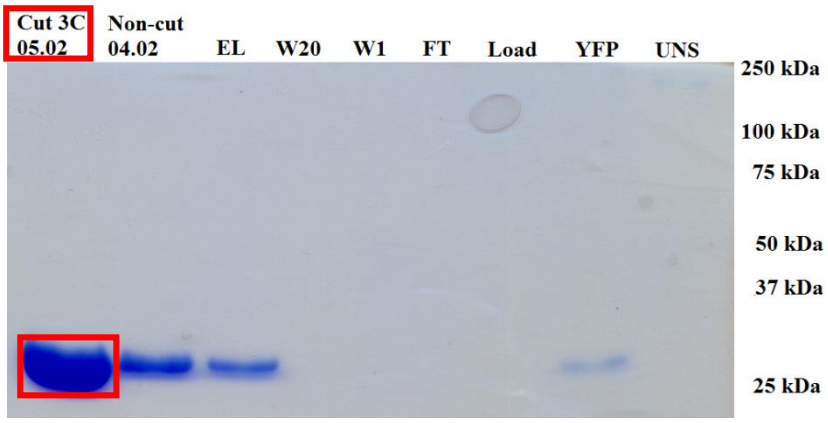

Figure 17: SDS-PAGE of Affinity Chromatography (HisTrap Ni-NTA) fractions; W, washing steps; EL, elution steps using imidazol-containing buffer. We used the sample Cut 3C 05.02 for SEC step. The elution steps were obtained with a multi-step gradient of imidazole: W1 with 10 mM; W20 with 25 mM; EL with 150 mM.

After washing the column the sample was eluted and in the end the protein was cut with 3C (1:100) to remove the His-tag. The cutted VenusYFP was then loaded onto the SEC column, where it is seen shown in **Figure 18** and identified in **Figure 19** with a MW of 26.4 kDa (30).

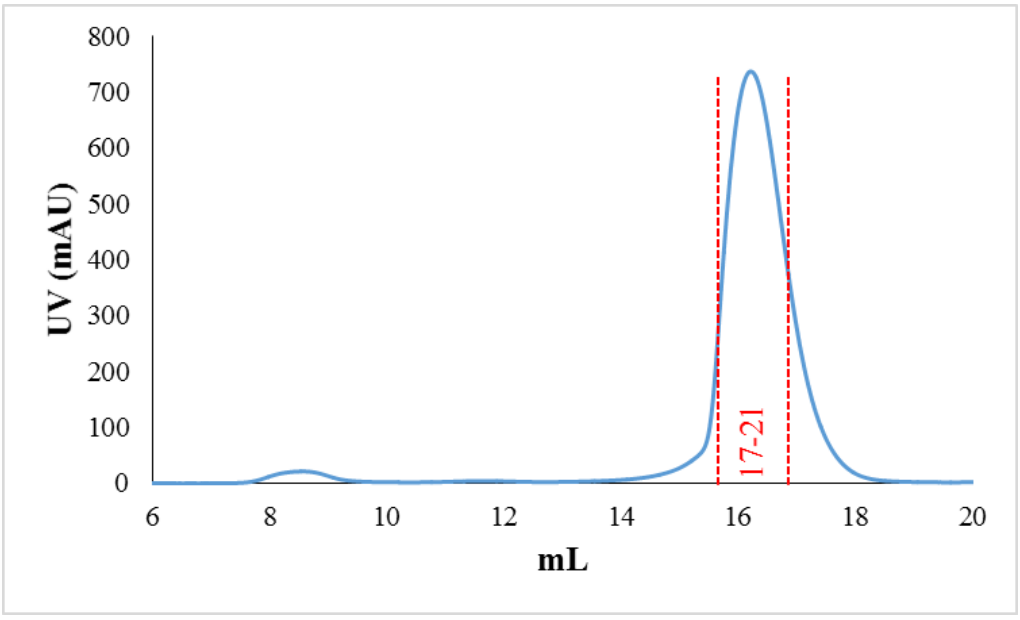

Figure 18: Size Exclusion Chromatography (SEC) profile of YFP model protein. Fractions 17-21 correspond to samples with highest purity.

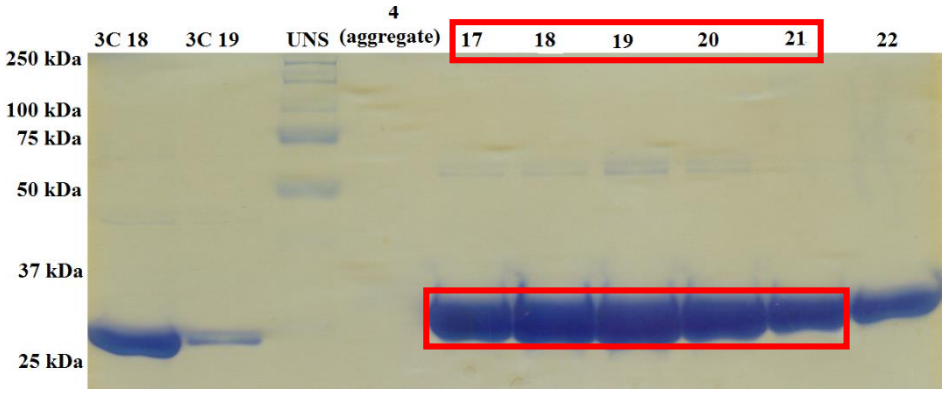

Figure 19: SDS-PAGE of the elution fractions from SEC.

After comparing the results from SDS-PAGE and the profile from SEC chromatography a pool with the samples that present the highest purity level was performed: for run 1, samples 1.17-1.21; for run 2, samples 2.3-2.7; for run 3, samples 3.1-3.4; for run 4, samples 4.5-4.9 and for run 5, samples 5.5-5.8. For this pool 2.82 mg/mL of protein were quantified. The pool was stored at -20°C for subsequent use.

For immunogen expression and purification results, all immunogens were obtained with high purity level (purity above 90%) as estimated by Size Exclusion Chromatography (SEC) and SDS-PAGE. The high level of purity from the model immunogens will guarantee that immunized birds will produce antibodies against the protein of interest (model immunogens) and reduce reactivity against other residual protein contaminants. Finally, this high purity level and knowledge on the protein content allows to test the anti-protein reactivity monitored by ELISA assays.

3.2. Monitoring Japanese Quail Immunizations

3.2.1 Birds immunization and reactivity

In **Figure 20** it is shown a time-line that includes the main stages of Japanese quail development.

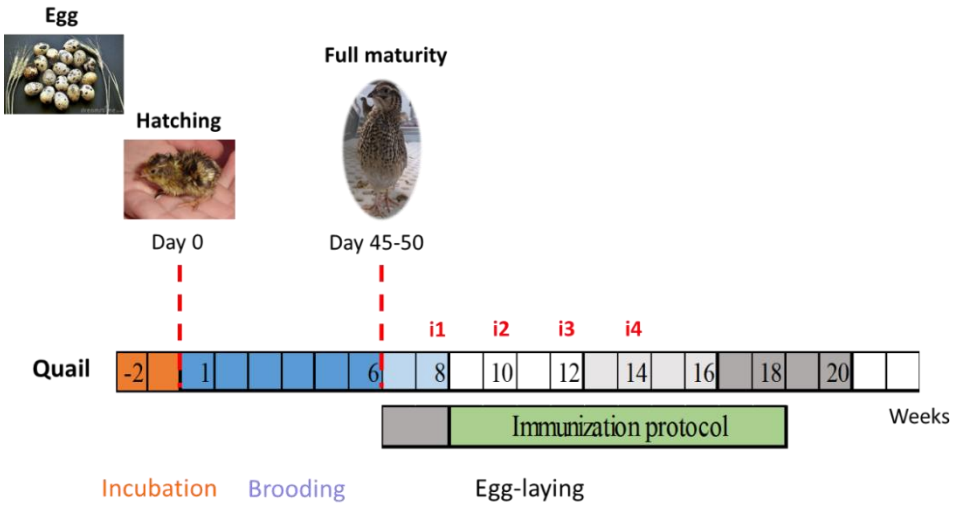

Figure 20: Time-line for Japanese quail development. Three main stages are depicted: egg incubation, bird brooding and egg-laying. The duration of the immunization protocol used in the work is represented by the green bar. Each square in the time-line bar (orange, blue, white and grey) represents a period of one week.

After 6 weeks of development, quails reach full maturity and the immunization process can be initiated. During the following 10 weeks, the immunization protocol continues. In this process we used two birds *per* experiment (2-bird system), meaning that each different immunogen was used to immunize two animals. The general welfare of the animals during the immunization procedure was regularly monitored, namely through the analysis of body weight evolution and egg-laying capacity.

As shown in **Figure 21**, during the immunization process using the three immunogens, the egg-laying capacity and the body weight of each experimental animal was relatively stable. Interestingly, towards the end of these three procedures, a decrease in the egg-laying capacity was observed. The reason is that, in GST and NusA, one of the experimental birds died (Q0314-01 for GST and Q0314-10 for NusA) thus reducing the total number of eggs. Indeed, the weight loss observed in the compromised bird reflects the importance of such monitoring in the control of the animal welfare. For Trx (**Figure 21**, **B3**), on the other hand, the drop in egg laying capacity cannot be explained by the body weight evaluation, since both birds immunized with Trx seem to keep a typical body weight evolution (an S-shaped curve profile).

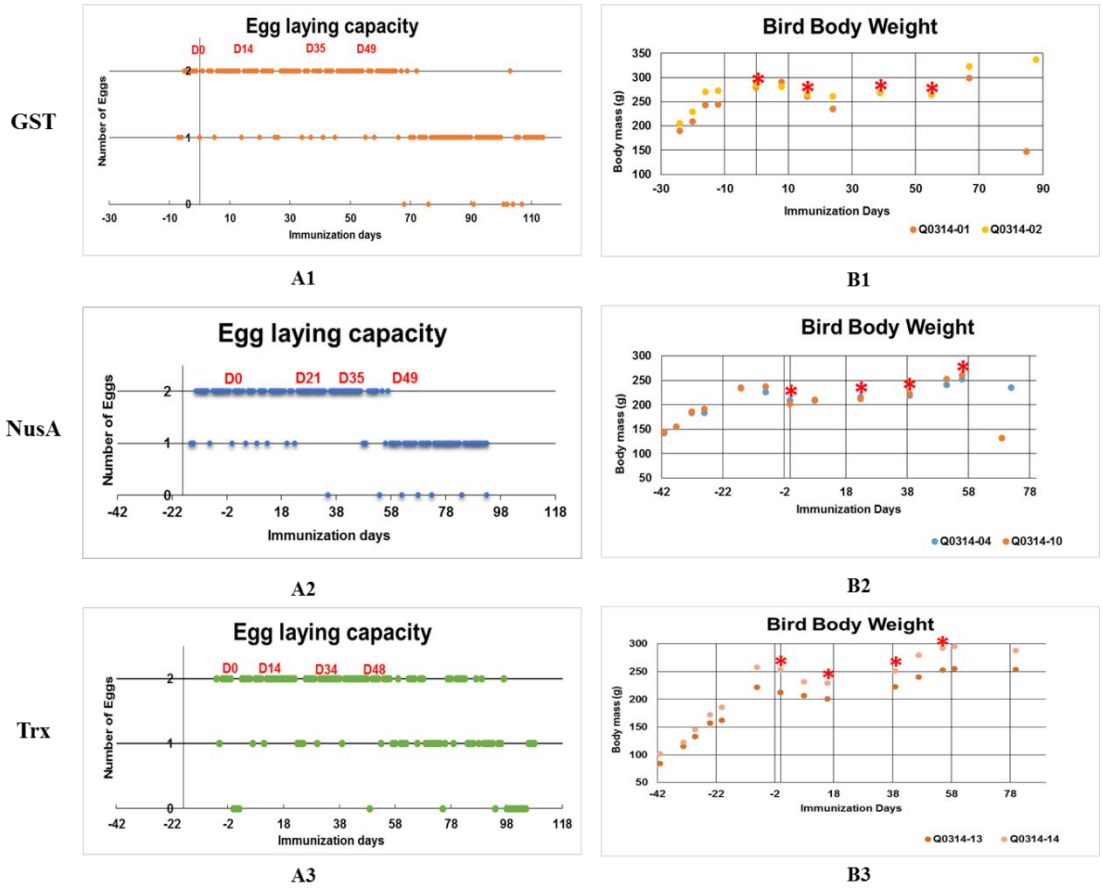

Figure 21: Plots **A1**, **A2** and **A3** show the number of eggs layed *per* day by the 2-bird system immunized respectively with the immunogens GST, NusA and Trx. Each injection event is labelled Dn, with n being days post immunization start. Plots **B1**, **B2** and **B3** show individual bird body weight evolution (animal IDs are shown); the red star indicates the injection events.

As described above, a 2-bird system was used for immunization procedures with immunogens GST, NusA and Trx. However, the loss of one of the experimental animals can also dictate the loss of reactivity against the immunogen. This is showed in **Figure 22**, where the anti-GST specific signal is abruptly reduced upon the loss of a single animal.

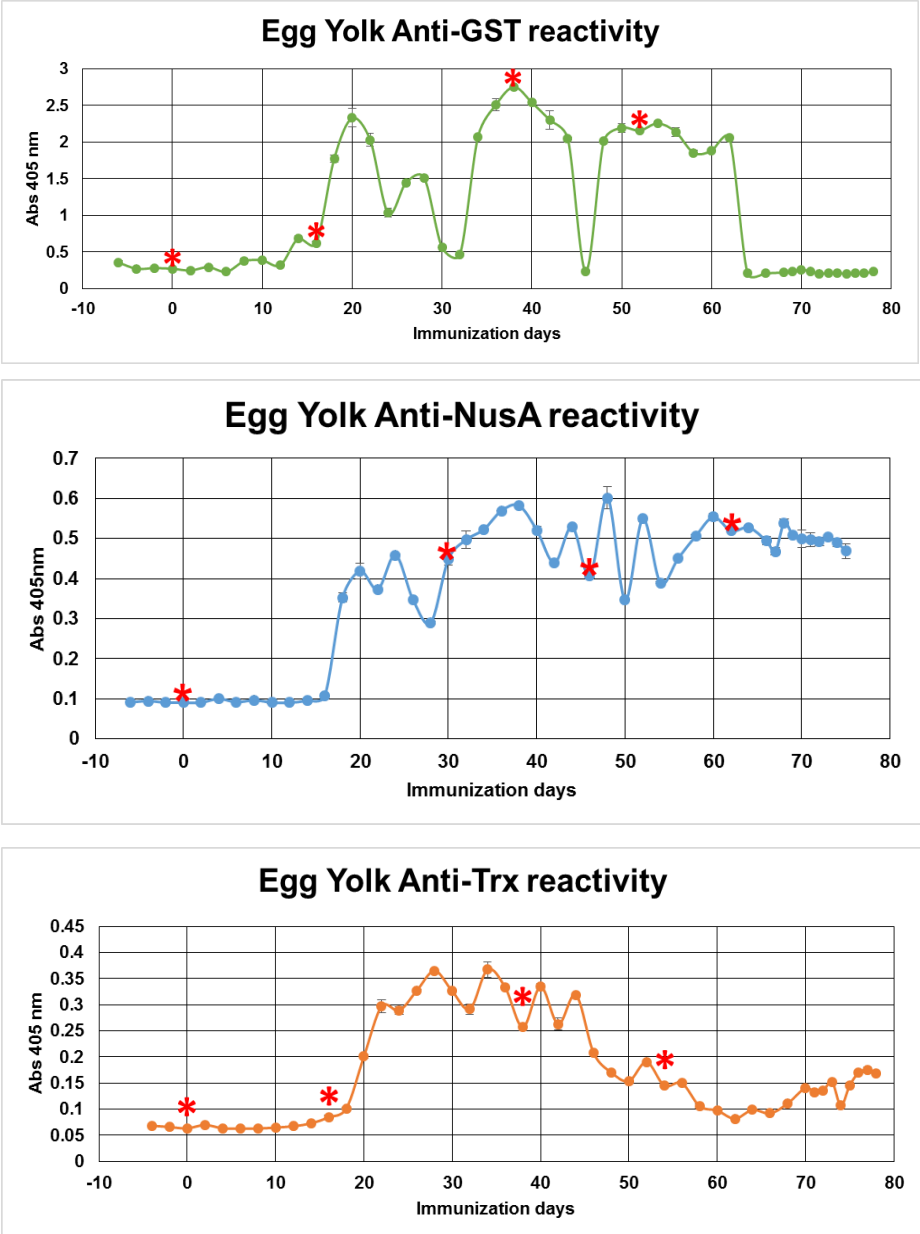

Figure 22: Anti-protein reactivity monitor by ELISA assay performed with egg yolk samples collected along the immunization procedure for each model protein used in this project. Each egg yolk sample analysed corresponded to a pool of four eggs (average of two days).

Comparing the reactivity plot (**Figure 22**) with the body weight plot and the egg-laying capacity plot (**Figure 21 A1** and **A2**) for GST, a decrease on the egg-laying capacity and body weight for the bird Q0314-01 was observed. Indeed, the absence of anti-GST reactivity after D46 post-immunization can either be explained by a technical error or because in the

2-bird system the remaining bird did not developed reactivity against GST. This is likely the case for GST procedure, since the lack of signal continuous until the accomplishment of the procedure.

Due to the problems described before, for VenusYFP, a 3-bird system was used. **Figure 23** depicts the obtained results.

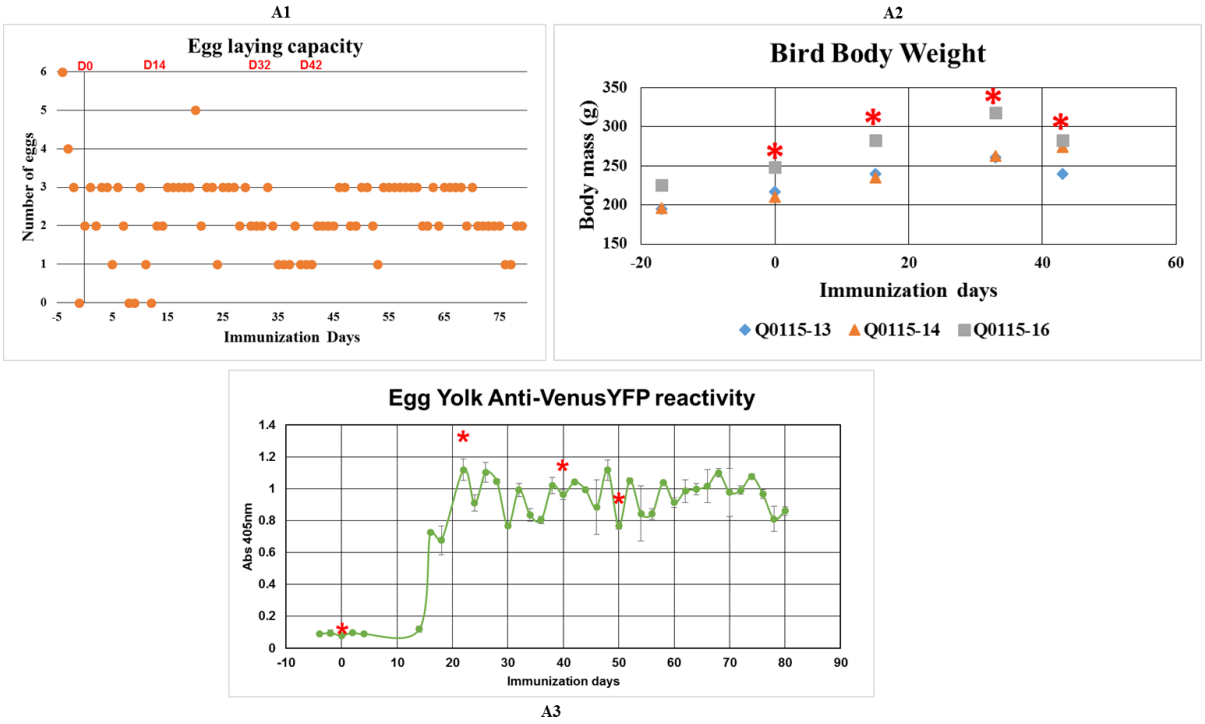

Figure 23: Plot **A1** show the number of eggs layed *per* day by the 3-bird system immunized. Each injection event is labelled Dn, with n being days post immunization start. Plot **A2** show individual bird body weight evolution (animal IDs are shown); the red star indicates the injection events. Plot **A3** show anti-protein reactivity monitor by ELISA assay performed with egg yolk samples collected along the immunization procedure for each model protein used in this project. Each egg yolk sample analysed corresponded to a pool of four eggs (average of two days).

Using the 3-bird system we verified that, when one bird ceased laying eggs, the other ones also tend to cease egg laying. Because of that it was necessary to separate the birds in order to re-establish the egg laying behaviour. This is actually a natural tendency of grouped experimental animals and still requires additional optimizations for our particular systems with quails.

The evaluation of immunogen specific reactivity was performed by ELISA assay; this is a standardized quantitative method, common in antibody-antigen characterization. The antigen is typically coated on the bottom of multiwell plate and antibody-containing samples are screened for reactivity (53). The ELISA plots showed two reactivity peaks: one after the second immunization event and another one after the third immunization event, namely in NusA and GST procedures. Indeed, this is the expected reactivity profile of an immune response; nevertheless, in NusA and Trx procedures, the two reactivity peaks are less evident. The reactivity profiles demonstrate a clear immunogen specific response; each model protein used in this work is a different antigen, thus dictating unique reactivity behaviour. Even though the birds were immunized with the same quantity of proteins we observed different behaviours in reactivity. Therefore, the samples of the different proteins were diluted in order to avoid ELISA signal saturation. For this, some samples were chosen to test different dilutions in order to obtain a less saturated signal. The saturated signal is related with the fluorogenic substrates because they have a higher sensitivity and allow the measurements of the levels of antigen concentrations in the sample with more accuracy and precision (53). In order to determine the optimal egg yolk sample dilution for the ELISA assay and avoid signal saturation, different sample dilutions were tested (see **Annex 1**).

Figure 24 shows the normalized anti-protein reactivity monitoring by ELISA.

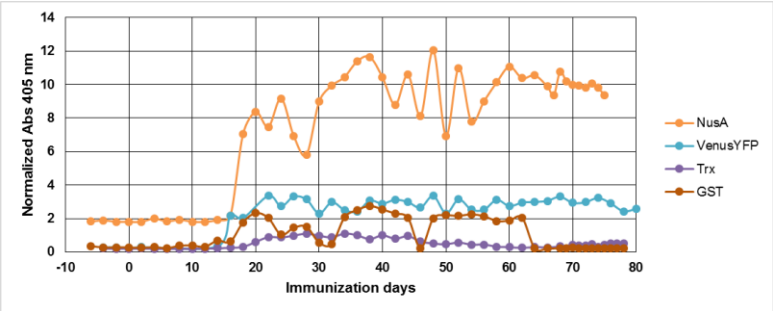

Figure 24: Normalized anti-protein reactivity monitor by ELISA assay performed with egg yolk samples collected along the immunization procedure for each model protein used in this project. Each egg yolk sample analysed corresponded to a pool of four eggs (average of two days).

Corresponding to results where no dilutions were applied so that a more direct comparison between reactivity signals of egg yolk samples can be attained. However, the anti-NusA

signal is already higher in the pre-immune samples indicating a previous contact of the experimental birds with this immunogen. This is not surprising since NusA is found in *E.coli* (54), and thus it is likely that our experimental birds have been previously exposed to the NusA antigen.

To conclude, for the particular immunization procedure using 2-birds systems and taking four immunization examples, between days 20 and 40, higher titers of specific antibodies are obtained. This time is highly important for future procedures involving the collection of hyperimmunized eggs.

3.3. Isolating of IgY from chicken and quail eggs by means of PEG precipitation followed by HiTrapQ affinity purification

Although the production of IgY display several advantages, in what concerns IgY purification one problem arises: the separation of IgY from the other components of egg yolk is not easy to achieve (20). Over the years, a number of methods have been described for the isolation and purification of IgY, but, in this project, for first step the method described for Polson et al (55) was used, where polyethylene glycol (PEG) is used for the precipitation of lipoproteins. PEG is a mild precipitation agent that concentrates the proteins until they exceed their solubility limit (20,51). With this method, all the contaminants are not precipitated and an additional separation technique is still required to obtain IgY of high purity (20). Therefore, for IgY purification to the following protocol with three purification steps was used: 1) PEG precipitation; 2) Anionic exchange chromatography; and 3) Size exclusion chromatography (20,56).

Figure 25 shows the SDS-PAGE analysis of different samples along the purification steps of ionic exchange chromatography and size exclusion chromatography. The sample Pellet PEG 12_2, corresponds to the IgY-enriched pellet that was ressuspended in 50 mM Tris-HCL pH 8.0 and subsequently loaded on the column for ionic exchange chromatography; even though the sample was highly impure, both the IgY heavy chain (65-68 kDa) and IgY light chain (25 kDa) are clearly enriched.

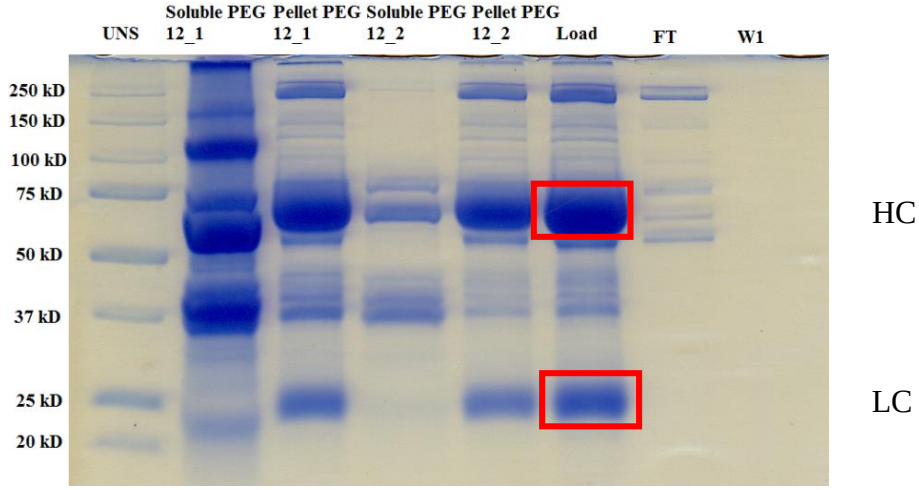

Figure 25: SDS-PAGE from PEG precipitation samples and elution fractions from HiTraQ for chicken IgY. Soluble PEG 12_1 and Soluble PEG 12_2 consists of yolk solids and fatty substances; Pellet 12_1 and Pellet 12_2 correspond to the IgY-enriched pellet.

Elution from HitrapQ column was performed by a salt gradient and monitored by OD280. **Figure 26** shows the chromatographic profile for this purification step.

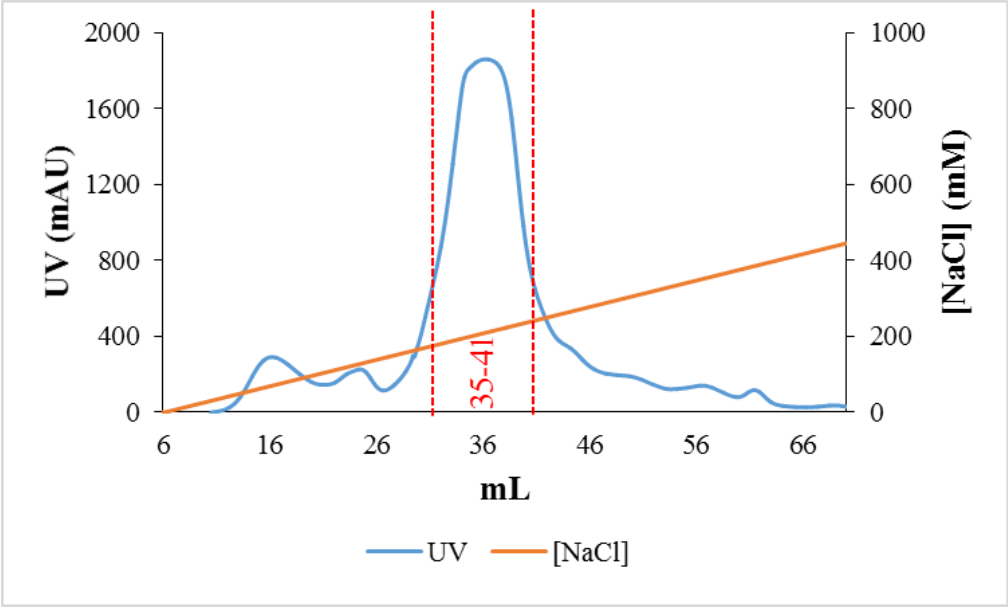

Figure 26: Ionic Exchange Chromatography profile (HiTraQ) from chicken IgY. Fractions 35-41 correspond to samples with highest purity.

With the obtained profile we collect the samples that belong to the peak and analysed them by SDS-PAGE (**Figure 27**) in order to select the ones with a lower level of contaminants.

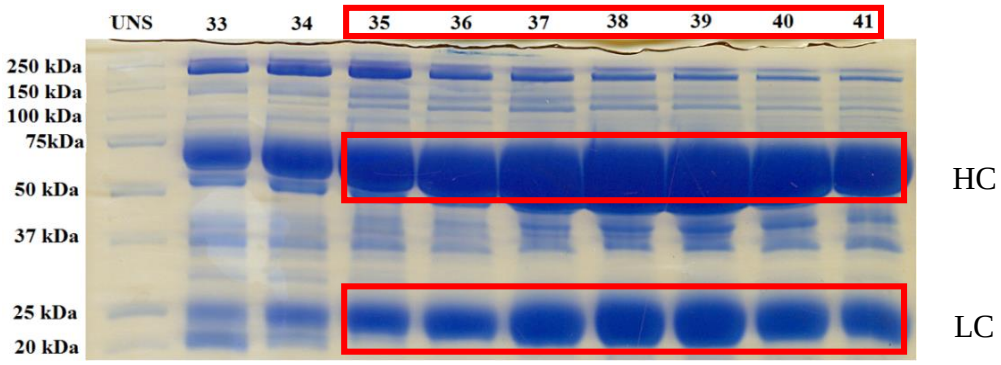

Figure 27: SDS-PAGE from Ionic Exchange Chromatography from chicken IgY. Looking for this gel and to the curve from chromatography, a pool with the samples with higher purity levels (35 to 41) to proceed with Size Exclusion Chromatography (SEC) was chosen.

The samples marked by a red rectangle in **Figure 27** display a large content of IgY and a reduced level of other protein contaminants. Therefore, a pool was made with these samples (9.99 mg/mL of final concentration) and was submitted to the third purification step to further purify the IgY. A size exclusion column was used for this last step; due to the high amount of protein at this stage and given the loading limitation of the size exclusion column, seven individual runs were performed, each using 500 µL of sample.

Figure 28 and in **Figure 29** show, respectively, the obtained chromatographic profiles from SEC and the SDS-PAGE analysis of the eluted fractions.

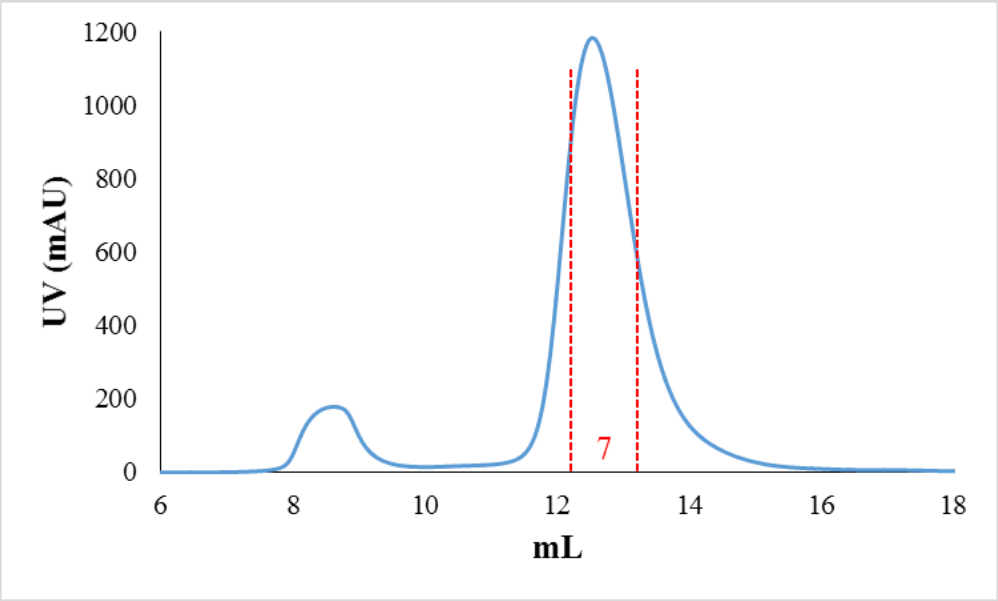

Figure 28: SEC profile from chicken IgY Run1. Fraction 7 correspond to samples with highest purity.

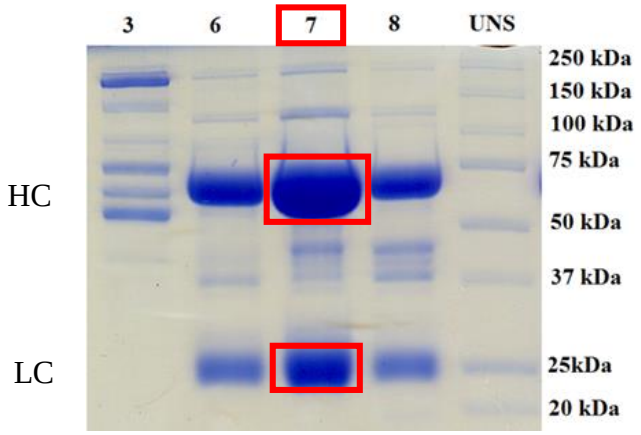

Figure 29: SDS-PAGE from SEC from chicken IgY Run1. Looking for this gel and to the curve from chromatography a pool with the sample of higher purity was made (sample 7).

McCannel and Nakai in 1990 (56), after the PEG precipitation step, observed that the sample contained a large number of contaminants; this is indeed the case demonstrated in **Figure 25**. The authors concluded that with the used of another method of purification the contaminants were removed (20). By further purifying IgY by size exclusion chromatography, the level of protein purity was remarkably improved and ended up with a IgY sample at 4.12mg/mL (**Figure 29**). Even though minor contaminants are still observed by SDS-PAGE, one can estimate the protein purity to be above 95%.

For the extraction/purification of IgY from the quail eggs, a similar procedure was applied. **Figure 30** shows the different fractions obtained during the PEG precipitation. The sample Pellet PEG 12_2, corresponds to the IgY-enriched pellet that was ressuspended in 50 mM Tris-HCL pH 8.0 and subsequently loaded on the column for ionic exchange chromatography; even though the sample was highly impure, both the IgY heavy chain (65-68 kDa) and IgY light chain (25 kDa) are clearly identified.

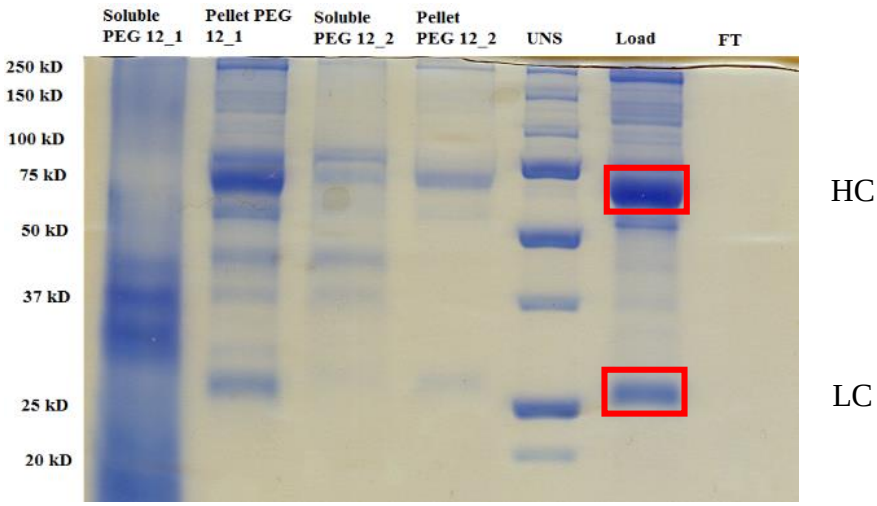

Figure 30: SDS-PAGE from PEG precipitation samples and elution fractions from HiTraQ for quail IgY. Soluble PEG 12_1 and Soluble PEG 12_2 consists of yolk solids and fatty substances; Pellet 12_1 and Pellet 12_2 correspond to the IgY-enriched pellet.

Elution from HitrapQ column was performed by a salt gradient and monitored by OD280. **Figure 31** shows the chromatographic profile for this purification step.

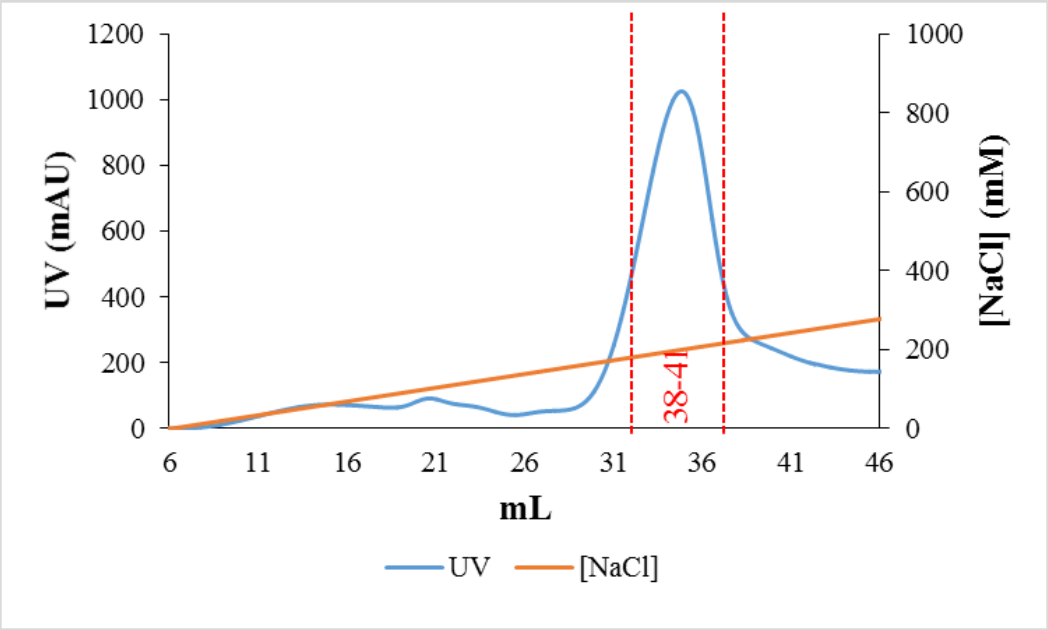

Figure 31: Ionic Exchange Chromatography profile (HiTraqQ) from quail IgY. Fractions 38-41 correspond to samples with highest purity.

From the obtained profile, samples corresponding to the protein peak were collected and then analysed by SDS-PAGE (**Figure 32**).

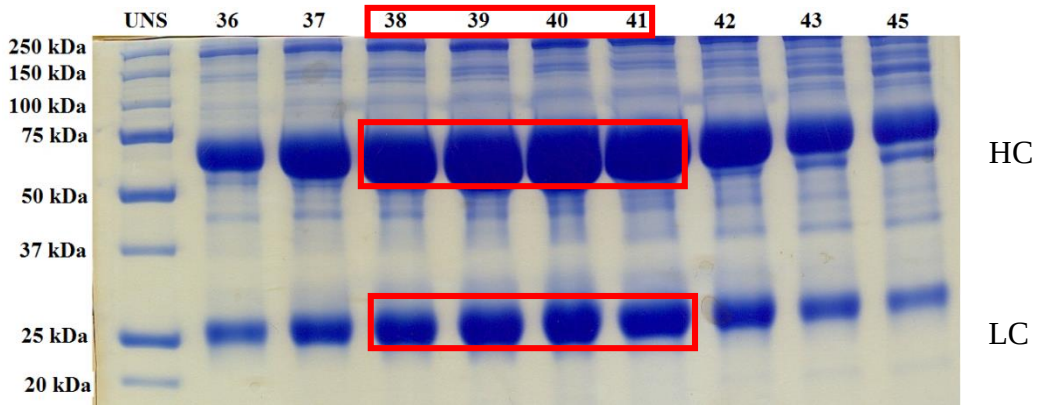

Figure 32: SDS-PAGE from Ionic Exchange Chromatography from quail IgY. Looking for this gel and to the curve from chromatography, a pool with the samples with higher purity levels (38 to 41) to proceed with Size Exclusion Chromatography (SEC) was chosen.

The samples marked by a red rectangle in **Figure 32** are rich in IgY and with a reduced level of other contaminant proteins. Therefore, a pool was made with these samples (4.07

mg/mL of final concentration) and was submitted to a third purification step to purify IgY. Three individual runs were performed, each using 500 µL of sample.

Figure 33 and in **Figure 34** depict, respectively, the obtained chromatographic profile from SEC and the SDS-PAGE results of the eluted fractions.

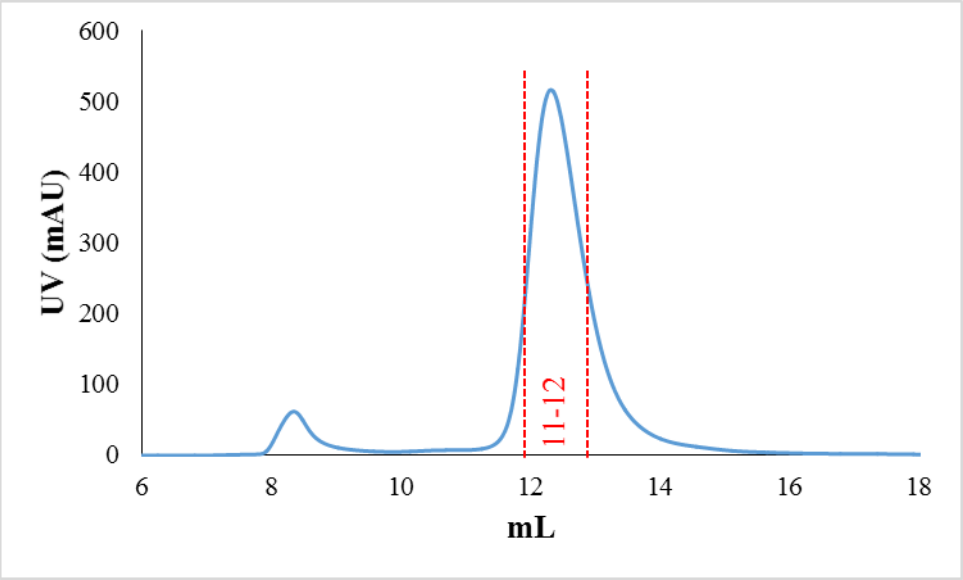

Figure 33: SEC profile from Run1 from quail IgY. Fractions 11-12 correspond to samples with highest purity.

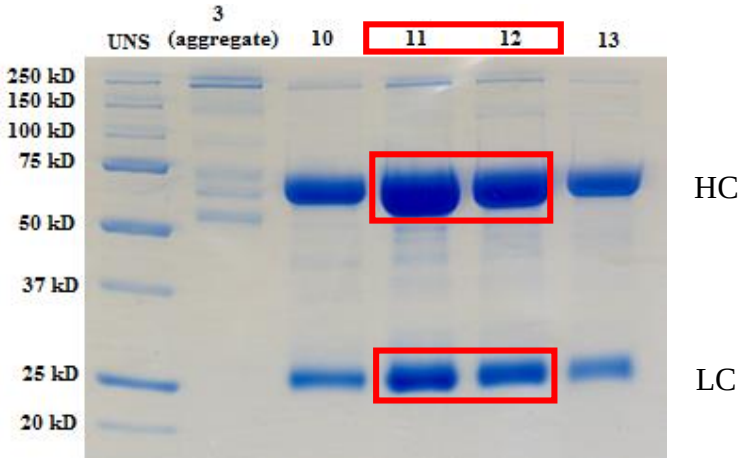

Figure 34: SDS-PAGE from SEC from quail IgY Run1. Looking for this gel and to the curve from chromatography a pool with the sample of higher purity was made (sample 11 and 12).

The samples from quail eggs, after the chromatographic step, contain some contaminants (**Figure 34**); yet, 1.58 mg/mL of IgY was obtained when making a pool with the samples presenting the higher level of purity, and as verified with IgY from chickens. When comparing the results from chicken and quail IgY purification (**Figure 29** and **Figure 34**, respectively), it can be concluded that the protocol implemented seems to be more efficient for quail IgY purification. Indeed, the final quail IgY sample presents less residual contaminants as observed by SDS-PAGE (**Figure 34**).

Aqueous Biphasic Systems (ABS) have been largely used to purify biomolecules, and can be applied to purify IgY (36–38). In order to better characterize the stability of chicken and quail IgY in ABS, the IgY purified samples obtained were used to perform thermostability assays as described in the next section.

3.4. IgY Thermostability Assays

The thermostability assay is an efficient screening tool to identify suitable buffer conditions and to analyse protein-ligand interactions (57). The protein thermal stability can be evaluated by using a fluorescent protein-binding dye: the protein thermal unfolding can be followed by the increase of fluorescent signal, as the dye interacts with the exposed hydrophobic core regions of the protein (58). In this work, the dye used was SYPRO Orange (SO) because this dye has excitation and emission properties compatible with real-time polymerase chain reaction (RT-PCR) instrumentation (58).

In this section, we studied the stability of chicken and quail IgY (samples obtained in section above) by thermalshift assays. The rationale behind these experiments was to identify and individually evaluate the IgY stability in well-known ABS components, such as polymers and salts, in order to extrapolate further conclusions for ABS that could be used in the purification of IgY from egg yolk.

The following components and concentrations were chosen: PEG 400, 600, 1000, 1500, 6000 and 8000 at concentrations of 5, 10, 20, 50 and 70 wt%; Na_2SO_4 at concentrations of 0, 5, 10, 15, 20 and 25 wt%; aqueous solution buffered at pH 4.5 of K_2HPO_4 + citric acid at concentrations of 5, 10, 15, 20 and 25 wt%; and $C_6H_5K_3O_7$ at concentrations of 5, 10, 15, 20 and 25 wt%.

Figure 35 shows the stability results for IgY in PEG 600 aqueous solutions. For both chicken and quail IgY, the protein maintains its stability up to 20% of PEG 600. For

concentrations above that, in this work 50%, only the signal from PEG 600 was read because polymer binds to the probe. Even so, in general, IgY from chicken and quail is stable up to 20% of PEG 600. However, we also figured out that the quail IgY has a higher stability temperature (70ºC) than chicken IgY (66ºC).

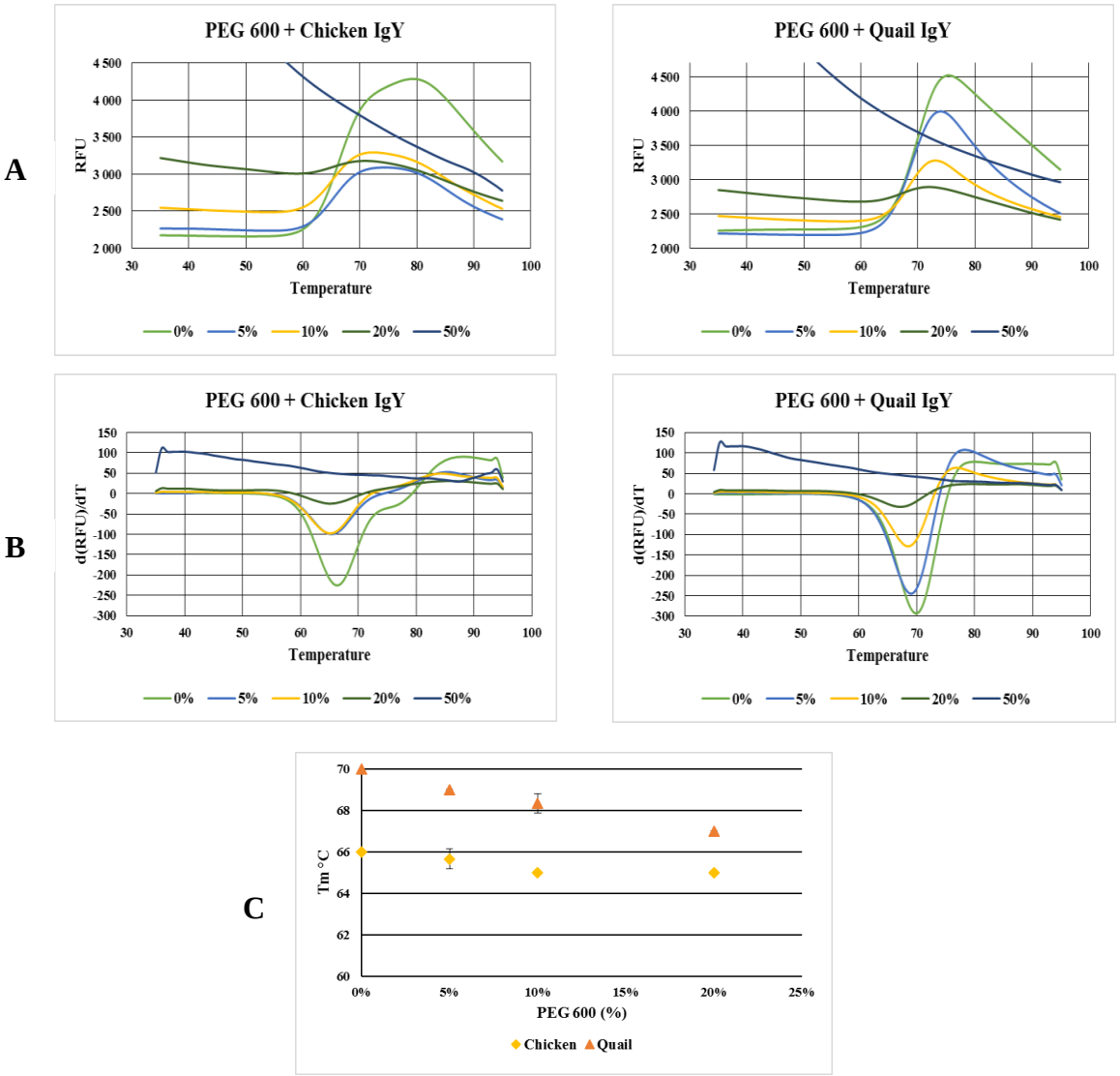

Figure 35: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability of chicken and quail IgY in different PEG 600 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; and **C)** IgY melting temperature (Tm) variations in the tested conditions.

Figure 36 depicts the melting temperature of IgY in presence of aqueous solutions of PEG 1500.At 30% of PEG 1500, the binding of the polymer to the probe was found. However, it is safe to state that both types of IgY are stable up to 20% of PEG 1500. It is thus possible to conclude that this ABS phase-forming component does not interfere significantly with the stability of the protein and can be used in further purification steps.

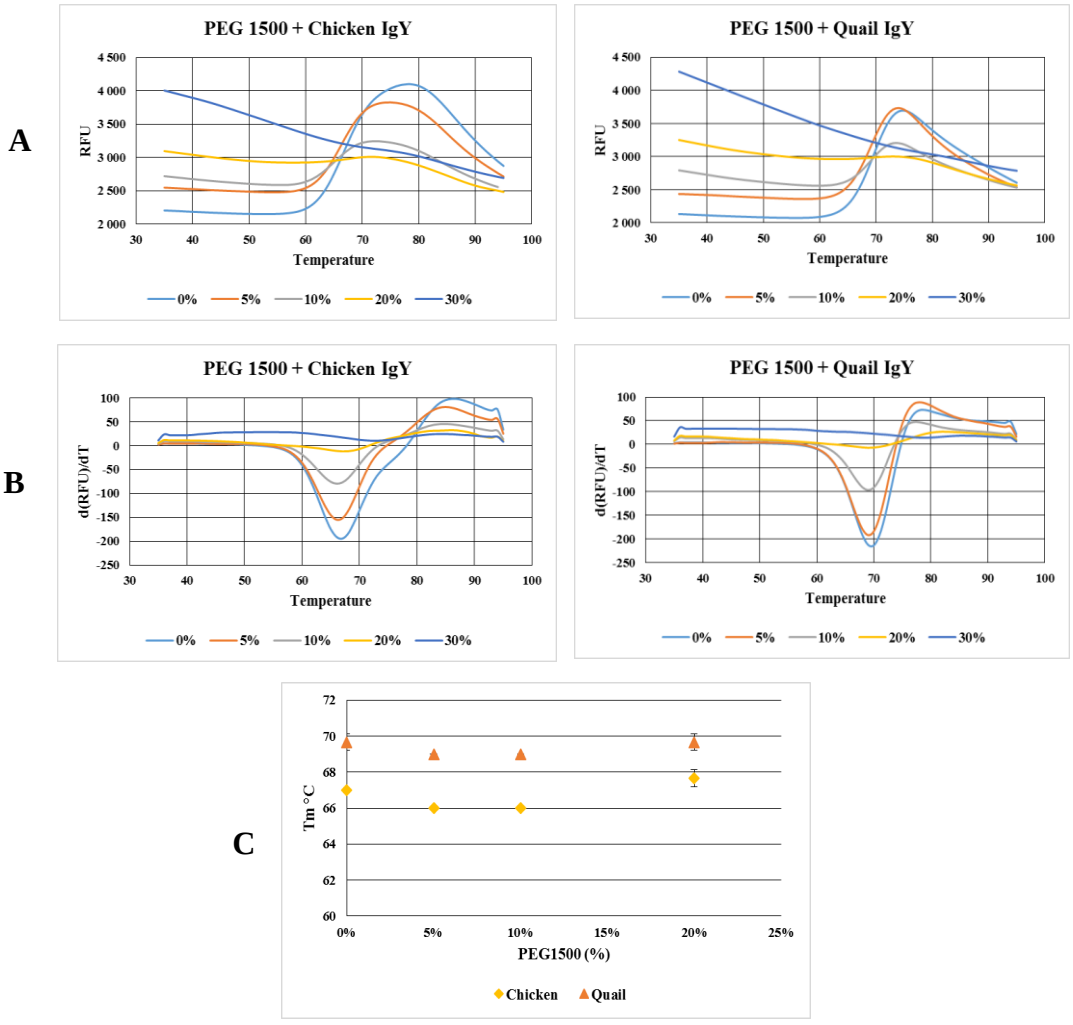

Figure 36: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different PEG 1500 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.

Other PEGs were tested but the results were not viable because they bonded to the probe masking the signal derived from IgY. These results can be seen in **Annex 3**, **Annex 4** and **Annex 5**.

We also tested some salts and the obtained results for Na_2SO_4 are shown in **Figure 37**. Interestingly, IgY from both birds are more stable as the concentration of the salt increases, and more evident for quail IgY.

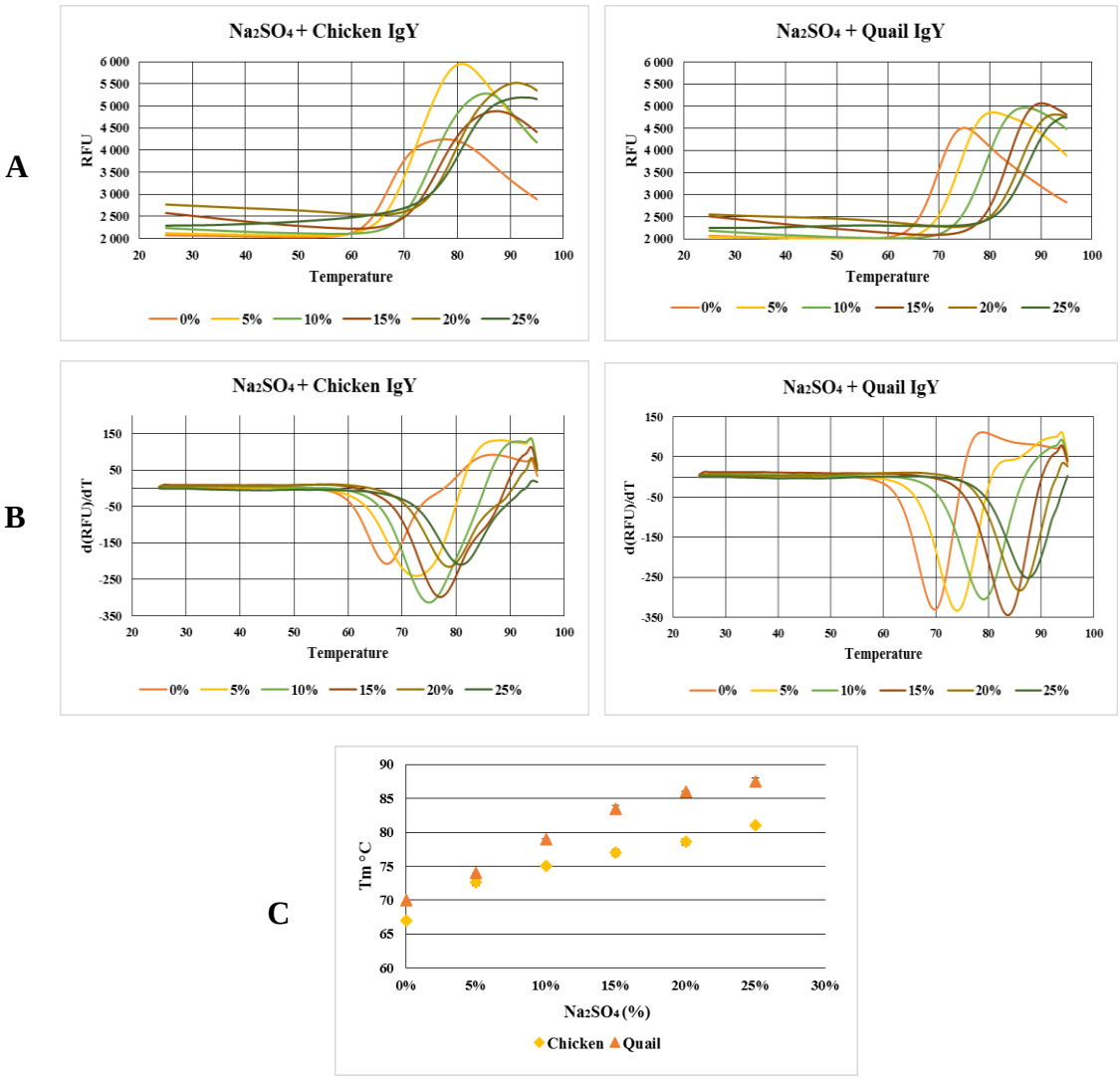

Figure 37: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different Na_2SO_4 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX

probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.

Similar results were obtained with $C_6H_5K_3O_7$, results shown in **Figure 38**. Therefore, this salt is also suitable for IgY stabilization.

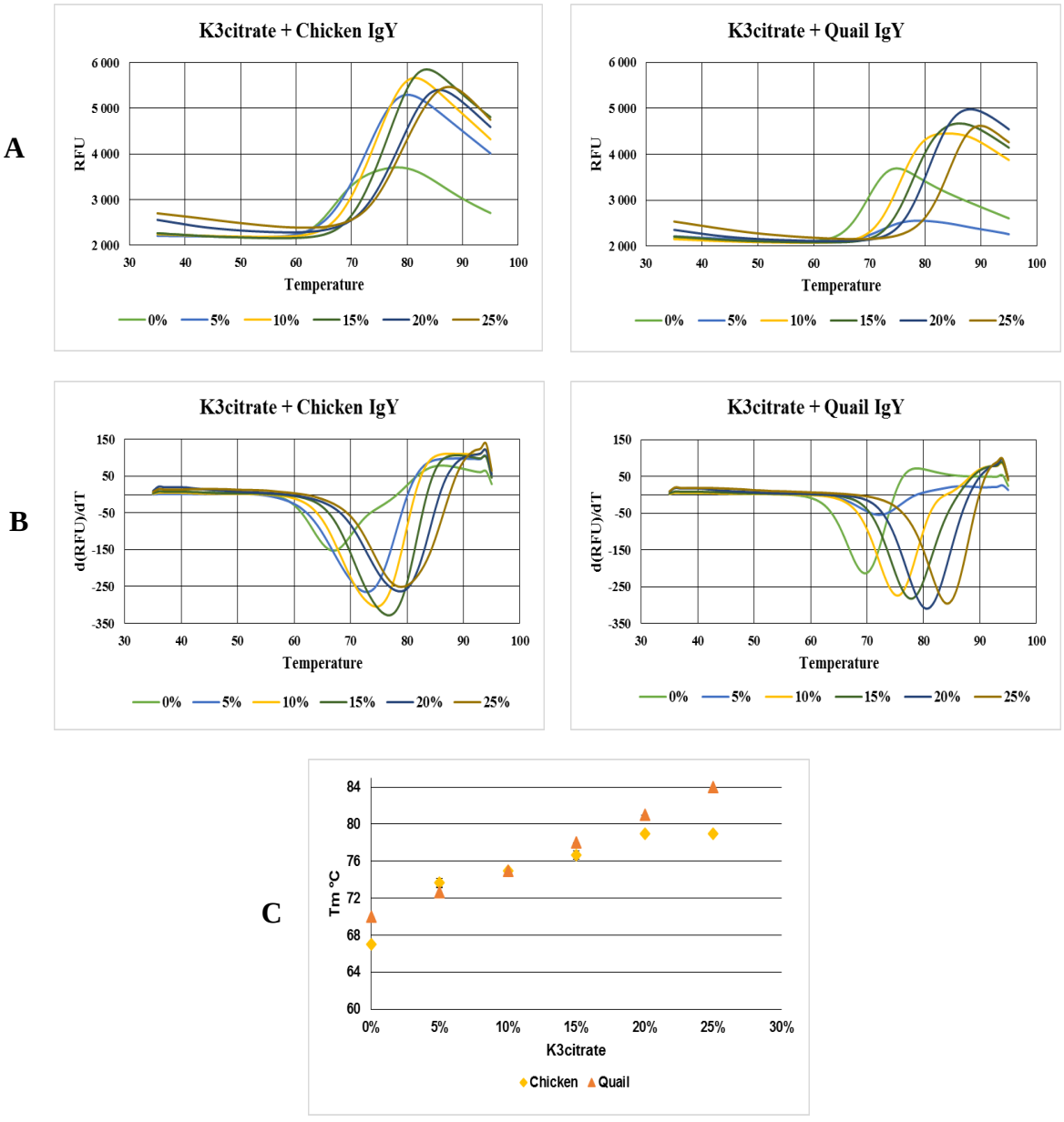

Figure 38: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different $C_6H_5K_3O_7$ concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO

dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.

Table 2 shows the obtained results for the different phase-forming components of ABS. **Annex 6** shows the obtained results for the buffered K_2HPO_4 + citric acid mixture

Table 2: Feasibility on using phase-forming components of ABS for the purification of IgY evaluated by ThermalShift Assays. ✓ correspond to the stability from IgY from both birds; × correspond to not the stability from IgY from both birds; ≈ the stability from IgY from both birds maintain the same and; - don't obtained results for IgY from both birds.

Phase-forming components	Chicken IgY	Quail IgY	
$C_6H_5K_3O_7$	✓	✓	
K_2HPO_4 + acid citric	×	×	
Na_2SO_4	✓	✓	
PEG 400	×	×	Up to 20%
PEG 600	≈	≈	Up to 20%
PEG 1000	-	-	
PEG 1500	≈	≈	Up to 20%
PEG 6000	-	≈	Up to 20%
PEG 8000	≈	≈	Up to 10% - 20%

The thermalstability assay allowed to identify the components $C_6H_5K_3O_7$ and Na_2SO_4 as the most effective ones out of the group of compounds evaluated in this work. We also identify some technical limitations for the study of PEGs, concluding that other equipment, such as Circular Dichroism (CD) (59,60) and Differential Scanning Calorimetry (DSC) (61), need to be used for the characterization of such compounds through the stability of IgY.

4. Final Conclusions

4. Final Conclusions

This work is divided into two principal objectives: establishment of immunization procedures in *Coturnix japonica* and study of the IgY stability in phase-forming components of ABS envisaging the antibodies fractionation.

In order to achieve to the first objective, the model immunogens were prepared and the specific reactivity was monitored. An in-house facility was implemented in order to exploit the production of antibodies in Japanese quails (*Coturnix japonica*). In order to produce the antibodies it was necessary to implement a standard immunization protocol and for that model proteins were used, namely NusA, Trx, GST and VenusYFP. A 2-bird system was used for immunization procedures with immunogens GST, NusA and Trx, and for Venus-YFP, a 3-bird system was used. For GST and NusA a decrease in the egg-laying capacity of the system was observed because one of the experimental birds died (reducing the total number of eggs and the weight loss). For Trx, the drop in egg laying capacity cannot be explained by the body weight evaluation, since both birds immunized with Trx seem to keep a typical body weight evolution. For Venus-YFP, it was used a 3-bird system and it was verified that when one bird ceased laying eggs, the other ones also tend to cease egg laying. Therefore, in future experiments, it is necessary to separate the birds in order to re-establish the egg laying behaviour.

In order to test the reactivity against anti-immunogens, ELISA assays were used and, for all prepared immunogens, the reactivity profiles demonstrate a clear immunogen specific response. Since each model protein used in this work is a different antigen, the results dictate unique reactivity behaviour.

In summary, and for the particular immunization procedure using two birds systems and taking four immunization examples, between day 20 and day 40 high titers of specific IgY are obtained. This time should thus be considered as the optimum window time for eggs collection.

To achieve the second objective, it was necessary to purify IgY using well-known protocols. Then, the stability of IgY in aqueous solutions of polymers and sals used as phase-forming components of ABS was addressed using Thermostability assays. This assay allowed to identify $C_6H_5K_3O_7$ and Na_2SO_4 as the most effective ones out of the group of compounds evaluated since they increase the stability of both chicken and quail IgY. Some experimental limitations were found with high concentrations of polymers, being thus

required the use of other assays to evaluate the protein stability, like Circular Dichroism (CD) and Differential Scanning Calorimetry (DSC)(61).

In conclusion, all the proposed goals were achieved and, for future work, other model proteins using the implemented standard immunization protocol can be investigated while foreseeing their biomedical and biotechnological applications.

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5. Bibliography

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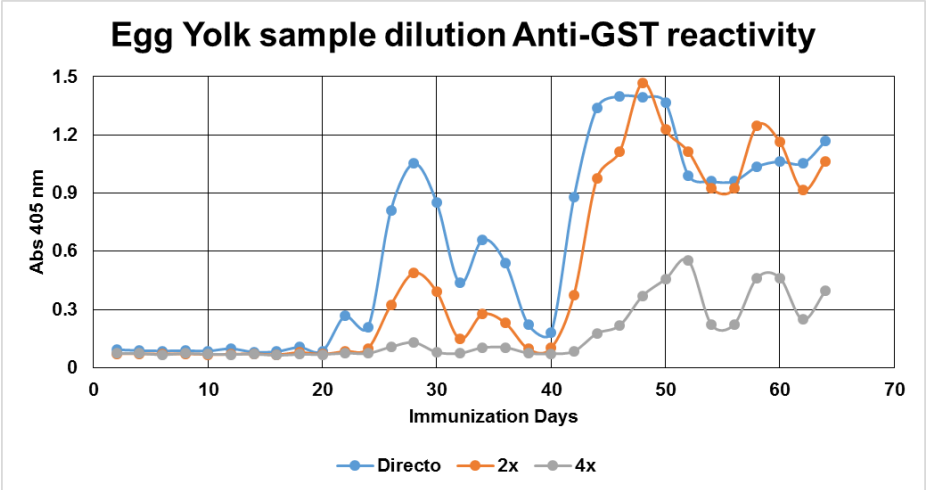
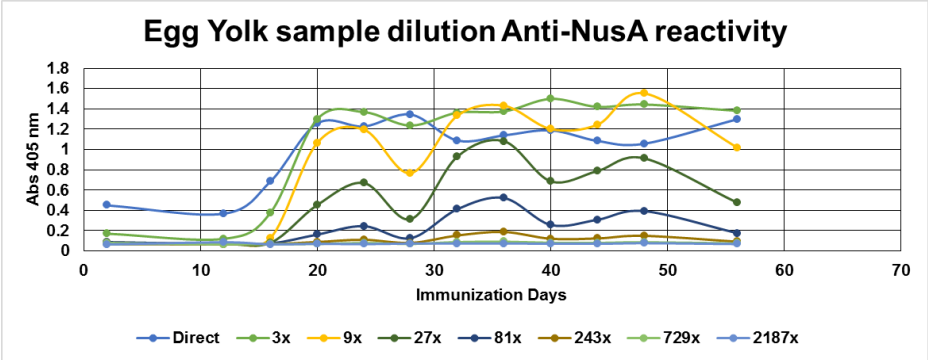
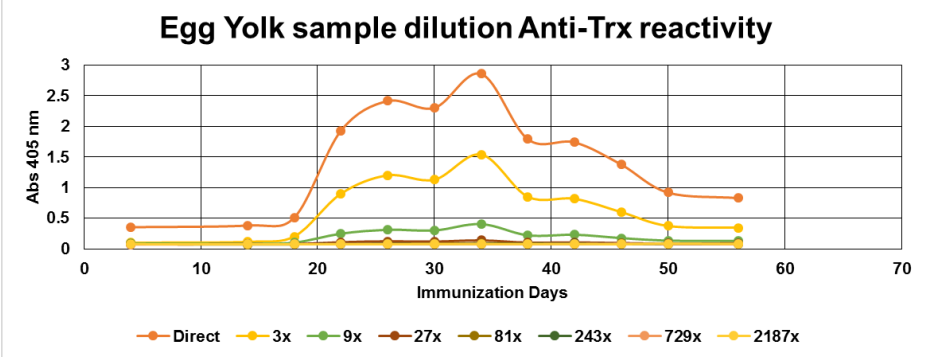
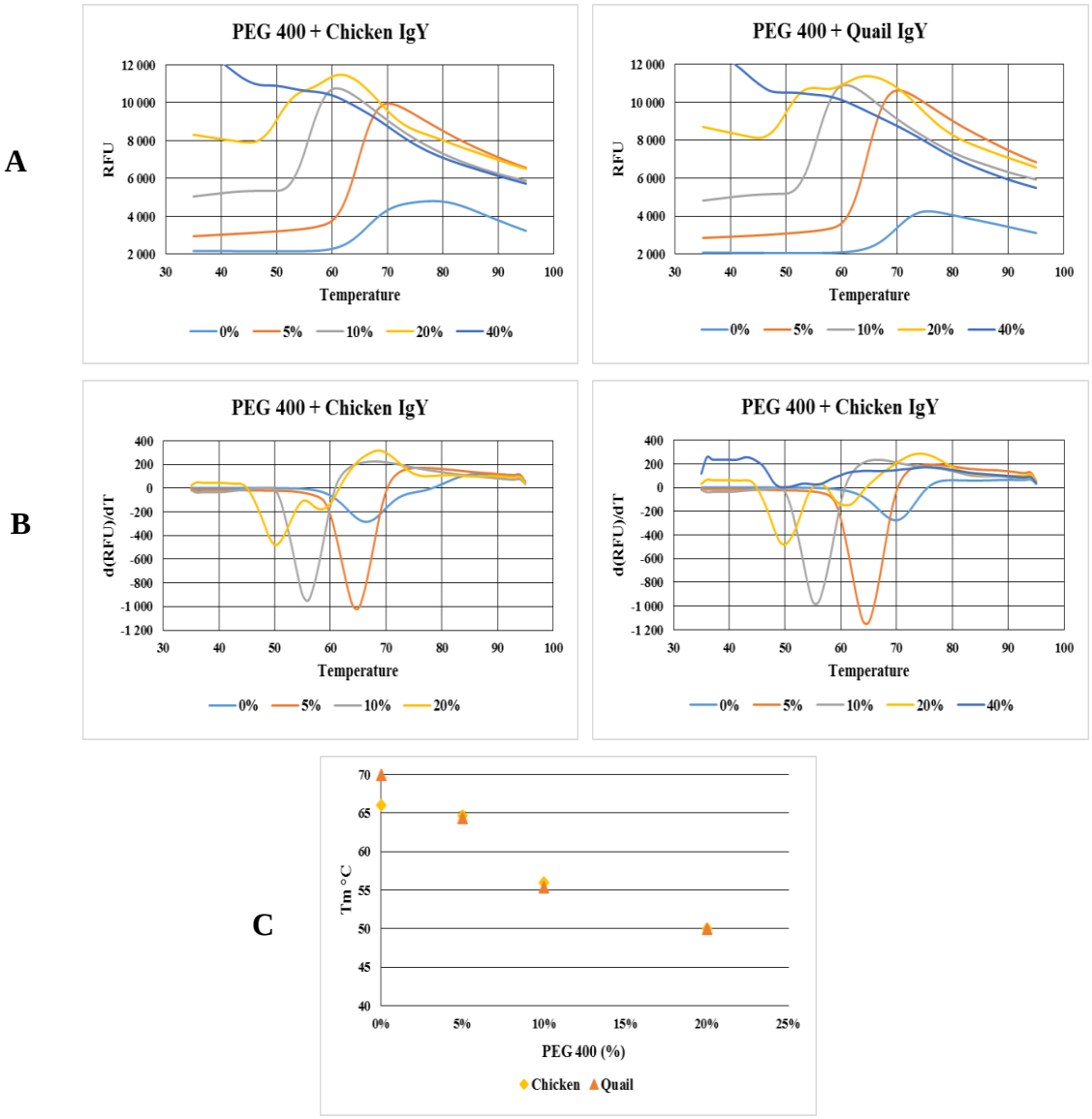
6. Appendix



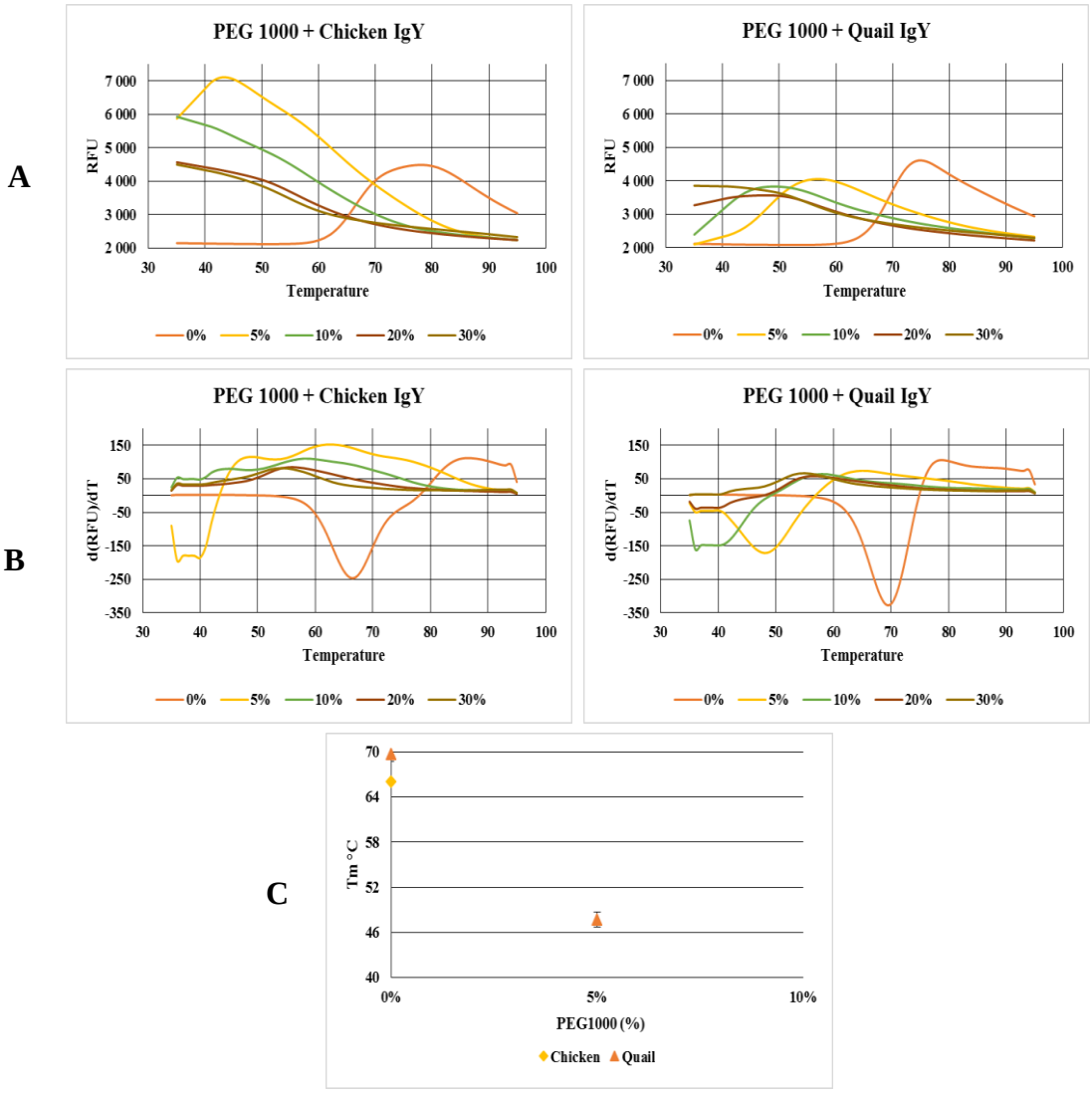




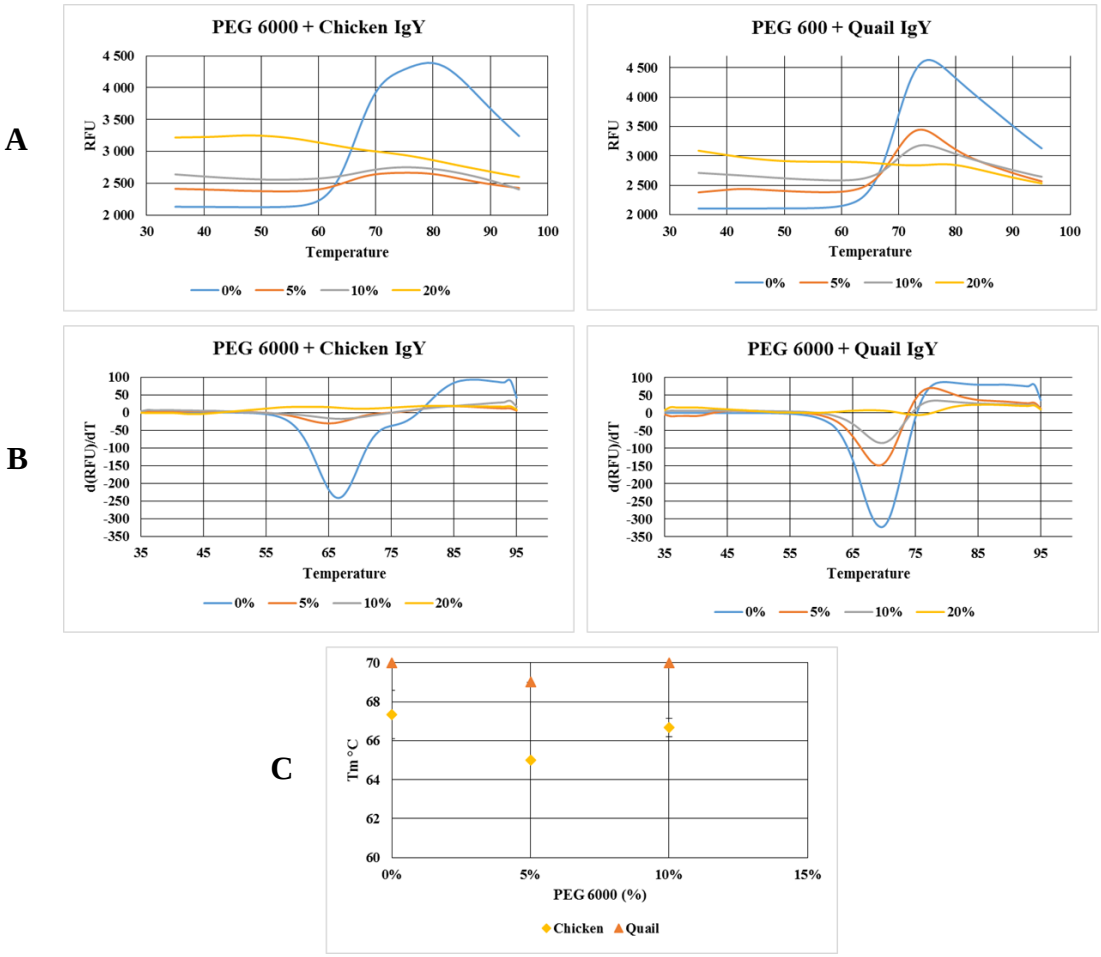
Annex 1: Diluted anti-protein reactivity monitored by ELISA from egg yolk samples collected along the immunization procedure.



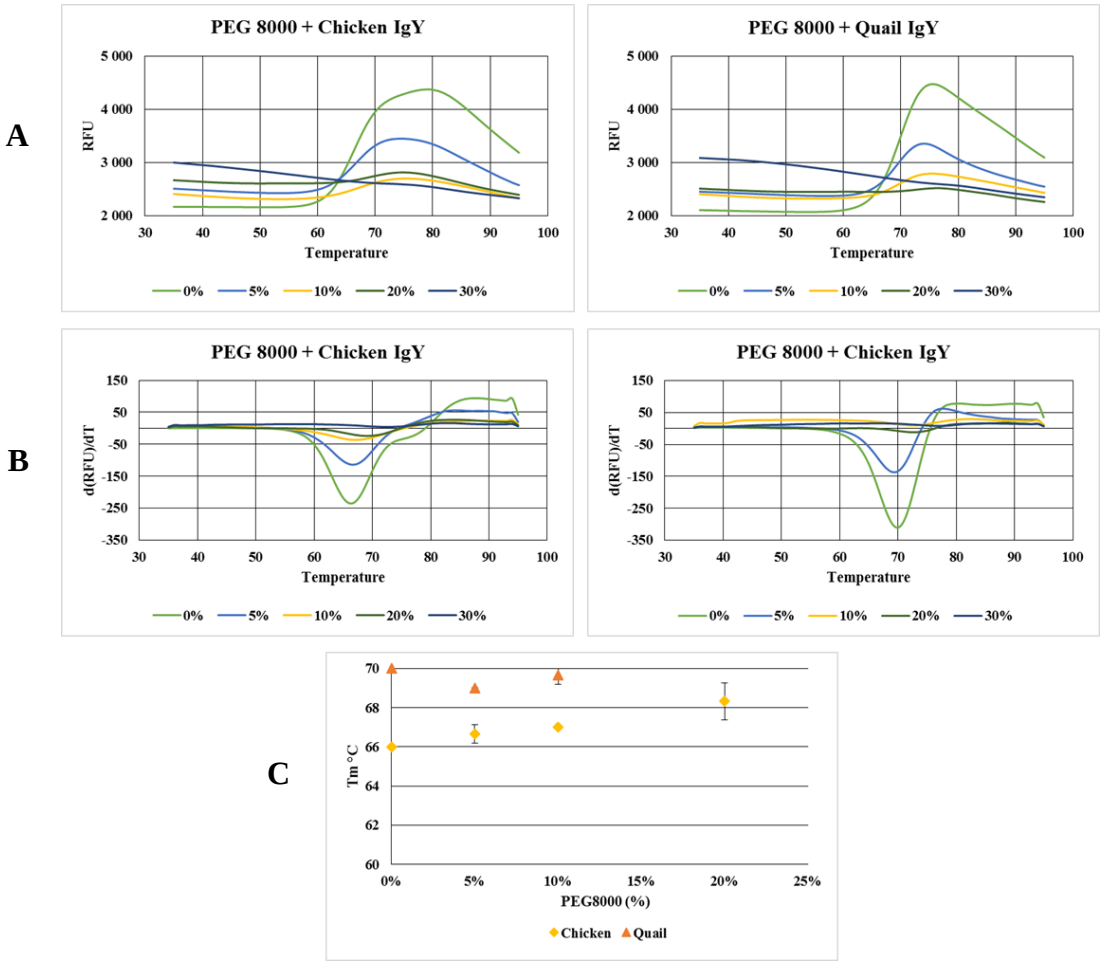
Annex 2: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different PEG 400 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.



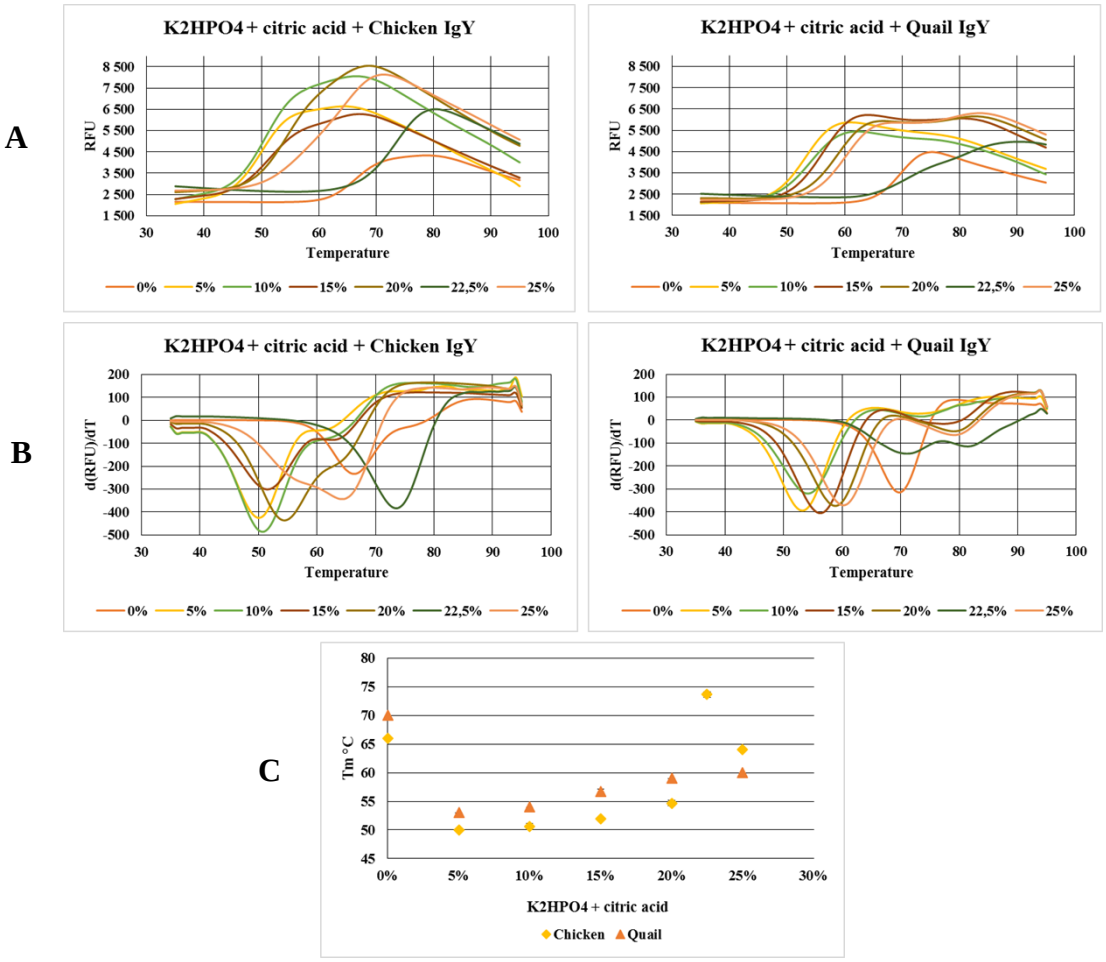
Annex 3: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different PEG 1000 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.



Annex 4: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different PEG 6000 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.



Annex 5: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different PEG 8000 concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.



Annex 6: Results obtained from ThermoFluor Assays (with Sypro Orange probe) to study the stability from chicken and quail IgY in different K_2HPO_4 + citric acid concentrations. **A)** Experimental thermal melting curves plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR; **B)** Inversed first derivative plot for chicken and quail IgY using SO dye and HEX probe from RT-PCR and **C)** IgY melting temperature (Tm) variations in the tested conditions.