# Purification strategies for equine Chorionic Gonadotropin

By

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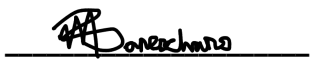
# Declaration

I, Martie Boneschans, hereby declare that the dissertation I herewith submit for the Master of Science Degree, majoring in Biochemistry, at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.



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# Abstract

Equine Chorionic Gonadotropin (eCG) is a glycoprotein hormone secreted by the endometrial cups of pregnant mares during days 37 to 120 of gestation. In equids it exhibits luteinizing hormone (LH) activity, while in non-equine species eCG exhibits both LH - and follicle stimulating hormone (FSH) like activity. Due to the high sialic acid content of eCG the 60 kD protein possess a long half-life in mammalian plasma. This, together with its dual hormonal activity (LH and FSH) makes eCG an extremely effective hormone for use in animal reproduction. Recombinant production of the hormone is still in its initial phases, therefore eCG, for commercial use, is still primarily purified from the serum of pregnant mares. Equine serum albumin remains the biggest challenge in the purification of eCG due to its relative abundance in the serum and similar molecular weight (70 kDa). This study investigated some of the strategies involved in the isolation of eCG in order to identify possible areas for improvement.

We compared three different acid precipitation steps as an initial phase in the isolation of eCG from eCG-spiked equine serum. Precipitation with metaphosphoric acid (MPA) showed the highest purification factor compared to precipitation with 15% (v/v) –and 25% (v/v) trichloroacetic acid (TCA) but resulted in high eCG losses. We also investigated the use of albumin affinity chromatography in the purification process but found that both albumin and eCG bound to the albumin affinity resin, raising our suspicions about possible interaction between the proteins. Ultrafiltration, using filtration units with a MWCO of 30 kDa, proved an ineffective method in the isolation of eCG as we found that eCG exhibits an affinity for the porous membrane of the filtration unit. During cation exchange chromatography we found that when eCG was mixed with BSA, the proteins exhibited changes in their column binding properties. This was confirmed with anion exchange where neither a continuous salt – nor pH gradient could separate eCG and albumin. This further supports our notion of a possible interaction between the two proteins as albumin is widely known as a carrier protein for thyroid hormones and other molecules. We found that Lectin affinity (LAC) - and hydrophobic interaction chromatography (HIC) did succeed in separating eCG and albumin but we could not elute most of the eCG from the LAC column, therefore future work should include further optimization of the method. HIC was performed on small scale and future work should look in to using higher concentrations of protein.



**Keywords:** eCG, equine chorionic gonadotropin, glycoprotein hormone, purification, protein precipitation, affinity chromatography

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# Chapter 1: Literature Review

## 1.1 Introduction to eCG

Equine chorionic gonadotropin (eCG), originally known as Pregnant Mare Serum Gonadotropin (PMSG), is a glycoprotein hormone belonging to the same family as LH (Luteinising Hormone), FSH (Follicle Stimulating Hormone) and TSH (Thyroid Stimulating Hormone). In mammals LH, FSH and TSH are regulated by the anterior pituitary gland, where it is secreted by the somatotrophic and gonadotrophic cells. These hormones play principal roles in controlling mammalian reproduction, growth and metabolism (Bhardwaj et al. 2017).

eCG was discovered and described by Cole and Hart in the 1930's when they found that the serum of pregnant mares stimulated maturation of the immature genital system in rats. They injected rats, approximately 28 days old, with up to 10 ml crude pregnant mare serum. After repeating this six times a day for a period of two days the rats were sacrificed and their reproductive tracts weighed and examined. Their results showed an increased maturation effect with pregnant mare serum obtained between days 40 to 120 of gestation (Cole and Hart 1930). In 1966, Denis Gospodarowicz and Harold Papkoff described the first method of isolating eCG from the crude serum of pregnant mares (Gospodarowicz and Papkoff 1967).

A few years after its discovery eCG was applied to humans (1941) in order to aid in stimulating follicular growth and inducing ovulation (Kane 1942). In 1945 the first successful treatment with eCG was described. The protein was however withdrawn from human treatment in 1972 due to the development of antibodies induced by its use and increased availability of less immunogenic pituitary gonadotropin extracts (Lunenfeld 2004; Kara et al. 2019).

eCG is the equine equivalent to human Chorionic Gonadotropin (hCG) and functions to maintain progesterone production in pregnant mares, which helps to ensure foetal development by maintaining the ideal uterine environment. eCG shows LH-, and FSH-like activity in non-equid species, stimulating follicle development, ovulation and the formation of the corpus luteum, whilst only having LH activity in mares. Due to the high sialic acid content of eCG the 60 kD protein possess a long half-life in mammalian plasma, for instance in sheep the half-life of eCG was recorded at 21 hours, in horses at 26 hours and in cows, 45.6 hours (Martinuk et al. 1991b; Gonzalez et al. 1994) This, together with its dual hormonal activity (LH and FSH) makes eCG an extremely effective hormone for use in animal production (Alvarez et al. 2017). An international standard for PMSG/eCG was defined in

1938, with one International Unit (IU) described as the activity contained in 0.25 mg of the standard preparation (Burn et al. 1950; Lunenfeld 2004).

During the nonbreeding season of cattle, sheep, pigs and goats a gonadotropin is required to bring the animals into oestrous. eCG is the most common gonadotropin used and is usually administered at the end of a progestogen treatment. One of the main veterinary uses of eCG today is to control the reproductive activities of cows. eCG, 1) improves reproductive performance during early post-partum stages; 2) induces ovulation and therefor increases pregnancy rates in non-cyclic cows; 3) improves the conception rate in cows with delayed ovulation; and, 4) is used in protocols that synchronise ovulation for fixed-time artificial insemination because of its beneficial effects on embryo development and survival (De Rensis and López-Gatius 2014). The generally accepted dose for inducing a single ovulation in cattle is ranged between 300 and 600 IU of eCG, while doses required to induce multiple ovulations are approximately 2500 IU (Sá Filho et al. 2010; Okouyi and Hanzen 2016; Chandran et al. 2017). Similarly, in ewes and does, a dose between 400 and 600 IU of eCG should induce a single ovulation (Kaşikçi et al. 2011; Martinez-Ros et al. 2019). In pigs a larger dose of approximately 1000 IU administered by intramuscular (IM) injection (cattle, sheep and goats also receive eCG IM) bring about ovulation (Stančić et al. 2015; Zhao et al. 2021).

The animal welfare group AWF (Animal Welfare Foundation) reports that as eCG is only obtainable from mares early in the pregnancy, the appalling practise of inducing abortions have been adopted by some “blood farms”, in Southern America, as the foal is considered an unnecessary by-product and abortion allows mares to be impregnated twice a year. They have applied to the EU Commission to ban the sale of all eCG products obtained through a cruel production method and urged the consideration to implement the use of alternatives to the hormone (Animal Welfare Foundation 2022).

Bioactive eCG requires a very specific glycosylation profile that makes recombinant production of the protein challenging. In our group, Lekena *et al*., was able to establish a stable Chinese hamster ovary (CHO-K1) cell line to extracellularly express a tethered recombinant eCG (reCG) containing six Histidines (His) fused to the C-terminal end of the protein. The histidines would allow for the use of affinity chromatography with nickel ($Ni^{2+}$) and cobalt ($Co^{2+}$) resins in order to isolate the reCG, but the purification was unfortunately unsuccessful (Lekena 2019). An Argentinian group reported success, in 2021, in recombinantly producing eCG with significant *in vivo* bioactivity. They used lentiviral vectors as a delivery method to CHO-K1 cells and were able to produce reCG in sufficient quantities for commercial use (Villarraza et al. 2021). The work of both groups will hopefully in the

future negate or at the very least greatly ease dependence on pregnant mare serum to obtain the protein and thereby alleviate many if not all of the animal ethics issues involved in the production of eCG. However, at present, eCG is still primarily purified from the serum of pregnant mares and this study aims to investigate some of the strategies involved in isolating the protein.

## 1.2 Equine reproduction

### 1.2.1 The equine oestrous cycle

In the southern hemisphere mares usually enter their oestrous cycle in spring through summer (October – March). Equine oestrous cycles span a period of 21 days ± 3 days. The oestrous phase can be 4-10 days with the dioestrous phase usually lasting 12-18 days. During the oestrous phase an oocyte is released from the follicle in the process known as ovulation. Ovulation can happen at any time during this phase but it usually occurs 24-48 hours before the end of oestrous. For this reason breeding or inseminations are usually done, every other day, from day 2 or 3 of oestrous (Satoh and Hoshi 1932; Cummings 1942; Vilhanová et al. 2021). Oestrous can be indicated by certain behavioural changes in the mare such as an increased interest and vocalisation towards the stallion, increased urination and lifting of the tail. In the same way dioestrous is signalled by normal urination and posture indicating reduced interest towards the stallion (McDonnell 2017; Crabtree 2022).

### 1.2.2 Physiological changes during the equine oestrous cycle

Increased daylight hours during spring causes the hypothalamus of the mare to release gonadotropin releasing hormone (GnRH) which in turn stimulates the anterior pituitary gland to produce FSH, the hormone responsible for inducing follicle development and the maturation thereof in the ovaries. Follicular growth coincides with increases in oestrogen levels which causes proliferation and vascularization of the endometrial wall and induce the clinical signs of oestrous. This elevation in oestrogen levels causes stimulation of LH secretion and inhibits further FSH secretion (**Fig 1.1**). The LH surge causes the follicle to rupture and release an oocyte. Once the oocyte is released the ‘empty’ follicle forms the corpus luteum (CL) which mainly produces progesterone, a hormone responsible for further proliferation and vascularization of the endometrial wall as well as the cessation of uterine contractions (**Fig 1.2**). Progesterone therefore inhibits oestrous behaviour and secures an intra-uterine environment suited for foetal development. In time (after approximately 14 days) prostaglandin will cause the CL to degenerate affecting a decrease in progesterone levels

and signalling shedding of the endometrial wall if no fertilization occurs, which indicates the start of a new cycle.

In the case of fertilization the production of eCG by trophoblastic cells in the endometrial cups, of the mare, functions to maintain CL integrity, thereby maintaining progesterone levels. The endometrial cups are circular structures contained in the endometrium of pregnant mares around the point of attachment of the foetus, as shown in **Fig. 1.3**. eCG is found in the highly viscous gel secreted by these endometrial cups and it is also present in the blood of the mares during day 37 to 120 of gestation, with the highest concentration reached between days 50 and 70. Progesterone support begins to be taken over by the placenta as early as day 50 of gestation but the CL does not start to degenerate until approximately 100 days later. The mare typically foals at approximately day 350 of gestation (Satih and Hoshi 1932; Cummings 1942; Andersson 2008; Nath 2014).

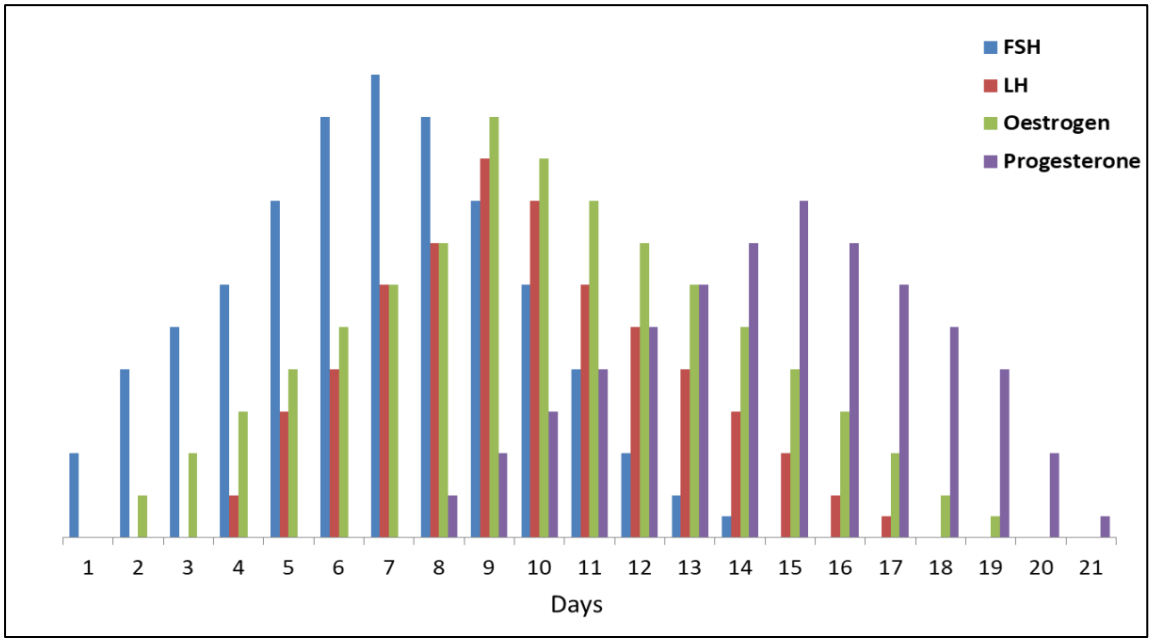


**Figure 1.1:** Graph indicating relative levels of FSH, LH, Oestrogen and Progesterone during the unfertilized equine oestrous cycle (adapted from information in Satih and Hoshi 1932; Cummings 1942; Andersson 2008; Nath 2014).

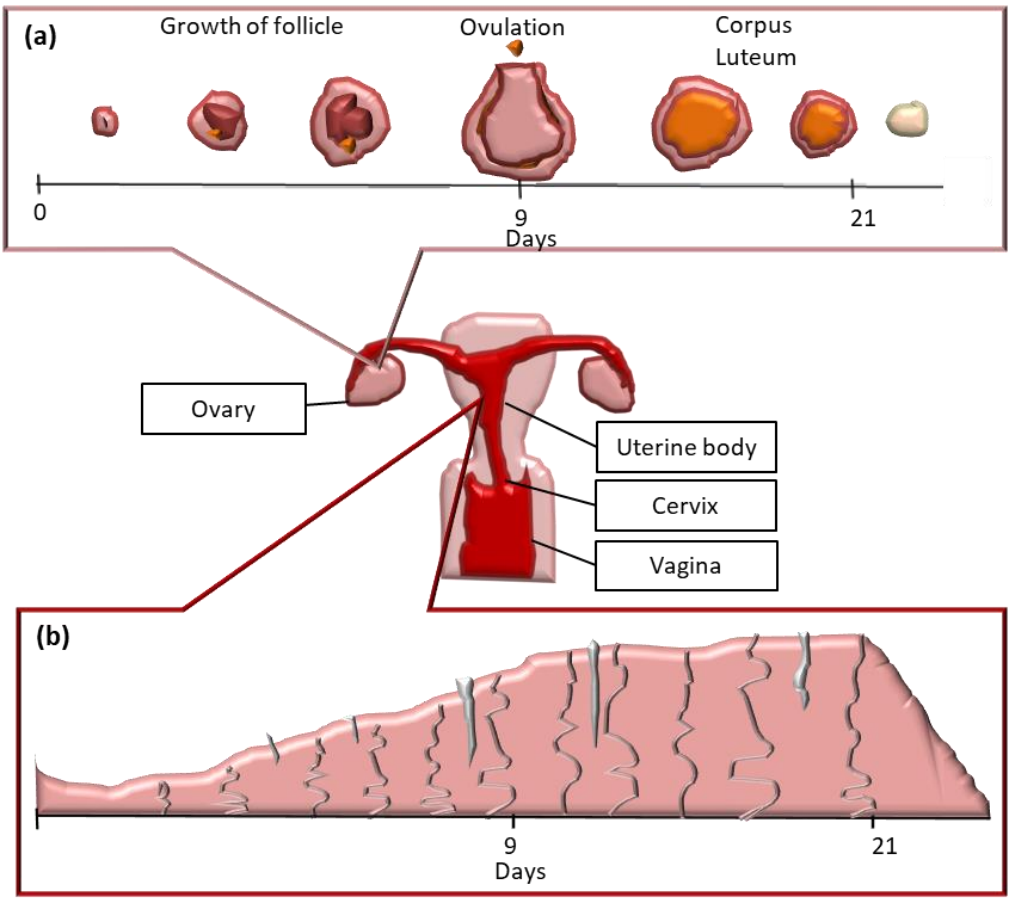


**Figure 1.2:** Physiological changes in the follicle **(a)** and endometrial wall **(b)** during the unfertilized equine oestrous cycle (adapted from Anderson 2008).

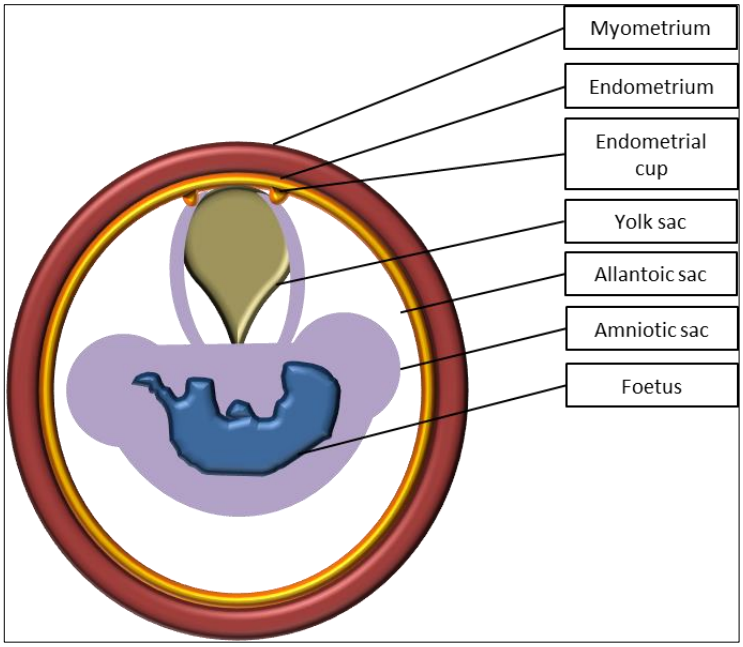


**Figure 1.3:** Diagram of the foetus and conceptus at approximately day 34 of gestation indicating the possition of the endometrial cups responsible for the secretion of eCG (adapted from Ginther 1998)

## 1.3 eCG characteristics

### 1.3.1 The α subunit of eCG

eCG is a heterodimeric protein, consisting of two subunits, α and β, that are dissimilarly glycosylated and non-covalently associated. All glycoprotein hormones, including LH, FSH and TSH, share the same α-subunit structure within a species, with the β-subunit being unique and specific for receptor binding (Bousfield et al. 1987). This is partly due to the fact that a single gene encodes the α-subunit, within the same species, while different genes encode the β-subunits (Combarnous 1992).

The α subunit of eCG is unconditionally required for the protein to manifest both LH and FSH activity (Chopineau et al. 1993). The subunits have no known biological activity when apart, with only the complete heterodimer providing hormonal activity because of its extensive glycosylation, sialylation and attached carbohydrate moieties (Ulloa-Aguirre et al. 1988). The α-subunit is composed of 96 amino acids, as shown in Figure 1.4.

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | 10 | | | | | | | | | | 20 |
| Phe | Pro | Asp | Gln | Glu | Phe | Thr | Thr | Gln | Asp | Cys | Pro | Glu | Cys | Lys | Leu | Arg | Glu | Asn | Lys |
| | | | | | | | | | 30 | | | | | | | | | | 40 |
| Tyr | Phe | Phe | Lys | Leu | Gln | Val | Pro | Ile | Tyr | Gln | Cys | Lys | Gly | Cys | Cys | Phe | Ser | Arg | Ala |
| | | | | | | | | | 50 | | | | | | | | | | 60 |
| Tyr | Pro | Thr | Pro | Ala | Arg | Ser | Arg | Lys | Thr | Met | Lys | Val | Pro | Lys | Asn | Ile | Thr | Ser | Glu |
| | | | | | | | | | 70 | | | | | | | | | | 80 |
| Ser | Thr | Cys | Cys | Val | Ala | Lys | Ala | Phe | Ile | Arg | Val | Thr | Val | Met | Gln | Asn | Ile | Lys | Leu |
| | | | | | | | | | 90 | | | | | | | | | | |
| Glu | Asn | His | Tht | Gln | Cys | Tyr | Cys | Ser | Thr | Cys | Tyr | His | His | Lys | Ile | | | | |

**Figure 1.4:** Amino acid sequence of eCGα (adapted from Combarnous 1992)

Bhardwaj and colleagues performed homology modeling and molecular docking analysis on eCGα and its interaction with a GnRH antagonist ganirelix. Their study gained great structural and functional insights into the subunit (Bhardwaj et al. 2017). Figure 1.5 displays their 3D homology modeling results.

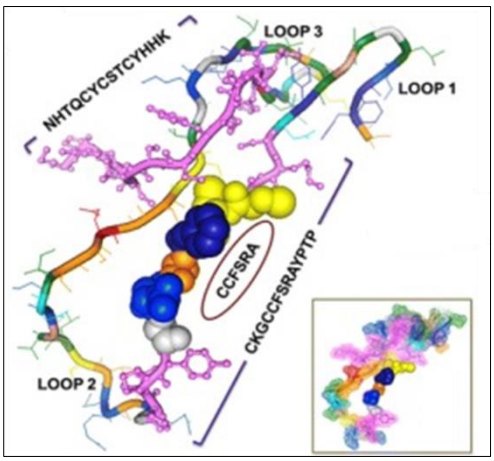


**Figure 1.5:** 3D Homology Model of the α-subunit of eCG, indicating three alpha subunit loops in the structure as well as the region of "CCFSRA" as probable receptor binding site and the location of two conserved motifs with amino acid sequences of "CKGCCFSRAYPTP" and "NHTQCYCSTCYHHK" (copied from Bhardwaj et al. 2017)

### 1.3.2 The β subunit of eCG

The β-subunit of eCG is composed of 149 amino acids with a carboxy-terminal peptide (CTP) formed from residues 122 to 149 as shown in Figure 1.6 (Legardinier et al. 2005). In 1985, Bousfield *et al.* reported that eLH and eCG share the same CTP (Bousfield et al. 1985). Later in 1987 Sugino *et al.* found that the two hormones in fact share identical primary structures of their beta subunits, suggesting that a single gene encodes both beta subunits (Bousfield et al. 1987; Sherman et al. 1992; Bousfield et al. 2001). There is however a difference in molecular weight between eLH and eCG in their heterodimeric forms. This is thought to be due to variations in the carbohydrate content of the beta subunits. Approximately 45% of eCG's molecular weight can be attributed to carbohydrates while in eLH it is only about 30% (Martinuk et al. 1991).

| 1 | | | | | | | | | 10 | | | | | | | | | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ser | Arg | Gly | Pro | Leu | Arg | Pro | Leu | Cys | Arg | Pro | Ile | Asn | Ala | Thr | Leu | Ala | Ala | Glu | Lys |
| | | | | | | | | | 30 | | | | | | | | | 40 |
| Glu | Ala | Cys | Pro | Ile | Cys | Ile | Thr | Phe | Thr | Thr | Ser | Ile | Cys | Ala | Gly | Tyr | Cys | Pro | Ser |
| | | | | | | | | | 50 | | | | | | | | | 60 |
| Met | Val | Arg | Val | Met | Pro | Ala | Ala | Leu | Pro | Ala | Ile | Pro | Gln | Pro | Val | Cys | Thr | Tyr | Arg |
| | | | | | | | | | 70 | | | | | | | | | 80 |
| Glu | Leu | Arg | Phe | Ala | Ser | Ile | Arg | Leu | Pro | Gly | Cys | Pro | Pro | Gly | Val | Asp | Pro | Met | Val |
| | | | | | | | | | 90 | | | | | | | | | 100 |
| Ser | Phe | Pro | Val | Ala | Leu | Ser | Cys | His | Cys | Gly | Pro | Cys | Gln | Ile | Lys | Thr | Thr | Asp | Cys |
| | | | | | | | | | 110 | | | | | | | | | 120 |
| Gly | Val | Phe | Arg | Asp | Gln | Pro | Leu | Ala | Cys | Ala | Pro | Gln | Ala | Ser | Ser | Ser | Ser | Lys | Asp |
| | | | | | | | | | 130 | | | | | | | | | 140 |
| Pro | Pro | Ser | Gln | Pro | Leu | Thr | Ser | Thr | Ser | Thr | Pro | Thr | Pro | Gly | Ala | Ser | Arg | Arg | Ser |
| Ser | His | Pro | Leu | Pro | Ile | Lys | Thr | Ser | | | | | | | | | | | |

**Figure 1.6:** Amino acid sequence of eCGβ with the CTP indicated from Pro 122 to Ser 149 (adapted from Bousfield 1987 and Sherman 1992).

A disruption in the FSH-like activity of a tethered reCG, where amino acids 94 to 96 (Gln,Ile,Lys) of the beta subunit was changed to three Ala residues, was reported by Park *et*

*al*. and similarly Combarnous revealed that amino acids 102 to 104 also play a critical part in signal transduction to FSH receptors(Combarnous 1992; Park et al. 2010).

### 1.3.3 Subunit glycosylation

The α-subunit of eCG has an average molecular weight of 16.96 kDa and contains two complex-type *N*-glycans located at asparagines (Asn) 56 and 82 (Christakos and Bahl 1979). Min *et al*. replaced Asn 56 with Gln in a reCG (recombinant eCG) transfected in CHO-K1 cells. They found that the mutant eCG had significantly less LH-like activity but interestingly had increased FSH-like activity compared to the wild type hormone. This finding was in contrast to earlier beliefs that the *N*-linked oligossacharide on Asn 56 was essential for both LH- and FSH activity (Min et al. 1996).

The 43.72 kDa β-subunit holds one *N*-glycan at Asn 13 with the CTP *O*-glycosylated at twelve serine or threonine residues (**Fig 1.7**) (Bousfield et al. 2001). Min *et al.* also deleted the CTP of the β subunit of a single chain reCG that they created in CHO-1K cells to investigate its role in the LH- and FSH activity of ecG. The deletion showed only slightly reduced LH activity with FSH activity remaining at about half of the wild type hormone. This indicated that both LH- and FSH activity were not greatly impacted in the *O*-de-glycosylated mutant (Min et al. 2004).

The *N*-glycosidically linked carbohydrate chains, of the beta subunit of eCG is comprised of mono-, di-, tri- and tri'-antennary *N*-acetyllactosamine type glycans that are partly α1-6 fucosylated at the Asn-bound GlcNAc residue. These glycans also possess α2-6 and α2-3 linked *N*-acetyl and *N*-acetyl-4-0-acetylneuraminic acid residues as its sialic acid elements. The *O*-linked chains are formed by tri-, tetra-, penta- and hexa-saccharides (Damm et al. 1990).

Although the beta subunits of both eLH and eCG are glycosylated at identical positions, Bousfield *et al.* reports that there are differences in the length, structure and composition of the carbohydrate chains (Bousfield et al. 2001). eLH as well as eCG induced marked increases in ovarian weight and oestrogen production in 27-day old rats, indicating that both hormones possessed the dual LH/FSH activity first only credited to eCG. However, eLH appears to have decidedly less of an effect than eCG (Moudgal and Papkoff 1982). Investigation of the terminal residues, of the *N*- and *O*-linked oligosaccharides, on the beta subunits indicate that this difference in hormonal effectiveness can be attributed to the extent of its sialylation. The *N*-linked oligosaccharides on eCG usually terminate with a sialic acid in the sequence Siaα2,3 or 6Galβ1,4GlcNAc where *N*-linked oligosaccharides on eLH usually have one or two branches terminating in a sulphate with the sequence $SO_4$-4GalNAcβ1,4GlcNAc (Smith et al. 1993). This results in 10% of the carbohydrate content of

eCG consisting of sialic acid which infers a very low isoelectric point (pI) of 1.8 to the hormone. This is a property exclusive to eCG which can be used in purification (Müller et al. 2011b). Sialic acid residues on the *N*- and *O*-glycans of the beta subunit of eCG also play an important role in its extended life span within the serum compared to eLH (Matsui et al. 1994).

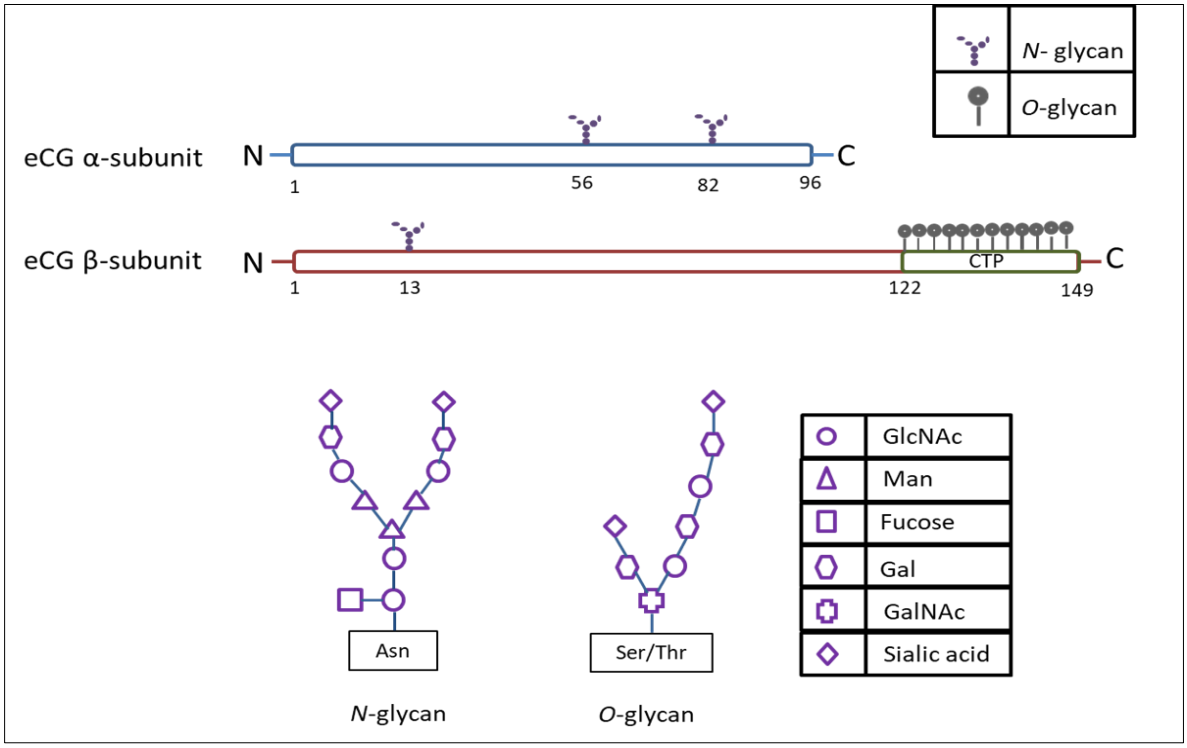


**Figure 1.7:** Schematic representation of *N*- and *O*- glycosylation sites on the α- and β subunits of eCG (adapted from Legardenier 2005).

### 1.3.4 eCG concentration in equine serum

The concentration of eCG in the serum of pregnant mares are affected by a number of different factors such as the number of foetuses present and the breed, size and parity (the amount of prior successful pregnancies) of the mare. In a 1938 study, Cole reported that the maximum concentration of eCG (then called PSMG) was inversely proportional to the size of the mare and that a fourfold difference was found between ponies and larger breeds. He also noted that mares of smaller breeds reached the highest eCG concentrations at earlier stages of gestation compared to larger breeds (Cole 1938). These findings were confirmed by Day and Rowlands, almost ten years later, and they showed that there were significant variations in eCG concentrations between individual mares at comparable stages of gestation. No positive correlation was found between the age of the mare and eCG production but they did note that successive pregnancies in Shetland ponies caused a marked reduction in eCG levels (Day and Rowlands 1947). In a subsequent study increased

levels of eCG was found in the serum of mares carrying twin foetuses, with one foetus in each horn of the uterus (Amoroso et al. 1948).

Allen compared the eCG concentration between a group of six Welsh Mountain pony mares and ten thoroughbred mares between days 20 and 150 of gestation for the Welsh ponies and days 20 and 120 for the thoroughbreds. The data he obtained is shown in Figure 1.8.

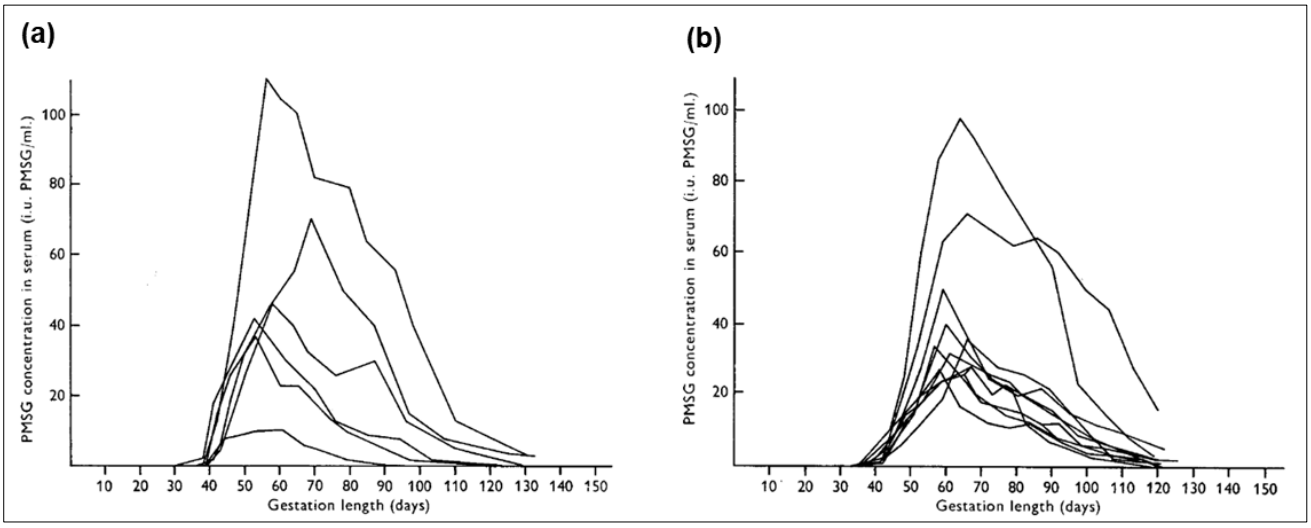


**Figure 1.8:** eCG (or PMSG) concentration in the serum of **(a)** six Welsh Mountain pony mares versus **(b)** ten thoroughbred mares (copied from Allen 1969).

He used a quantitative haemagglutination inhibition assay and found a great disparity in eCG concentration not only between breeds (therefore the sizes of the mares) but also between the individuals of a specific breed, confirming Day and Rowlands earlier report in 1947. eCG was first detected around days 35 to 41 of gestation for both breeds which sharply increased to a maximum at approximately days 55 to 60 for the ponies and days 60 to 65 for the thoroughbreds. eCG in both breeds slowly decreased till days 120 to 130 when very low levels were detected in the serum. Strikingly a difference of 102 IU eCG(PMSG)/ml was found between a multiparous pony and a maiden pony with reduced variances in the thoroughbreds. In fact it was noted that in the ponies the highest concentrations were produced by three maiden mares in their first pregnancy, whereas two thoroughbred multiparous mares showed the highest overall eCG production. Therefore the ponies showed an effect of parity on eCG concentration levels but none was found within the thoroughbreds (Day and Rowlands 1947; Allen 1969a).

Later Allen investigated the effect of foetal genotype on eCG production in mares and donkeys (**Fig 1.9**). Mares carrying horse foetuses showed maximum eCG levels of 101-143 IU/ml at day 60 of gestation, with donkeys carrying donkey foetuses at much lower maximum concentrations of 53 IU/ml and 32 IU/ml at days 47 and 55 respectively. The mares carrying donkey foetuses showed the lowest maximum eCG levels of 17 to 22 UI/ml on approximately day 48 of gestation. Remarkably the donkey carrying a hinny foetus showed the highest maximum eCG concentration of 203 IU/ml at day 60 of gestation suggesting that this interspecific genetic combination might be preferable to a horse carrying a horse foetus as a commercial source of eCG (Allen 1969b).

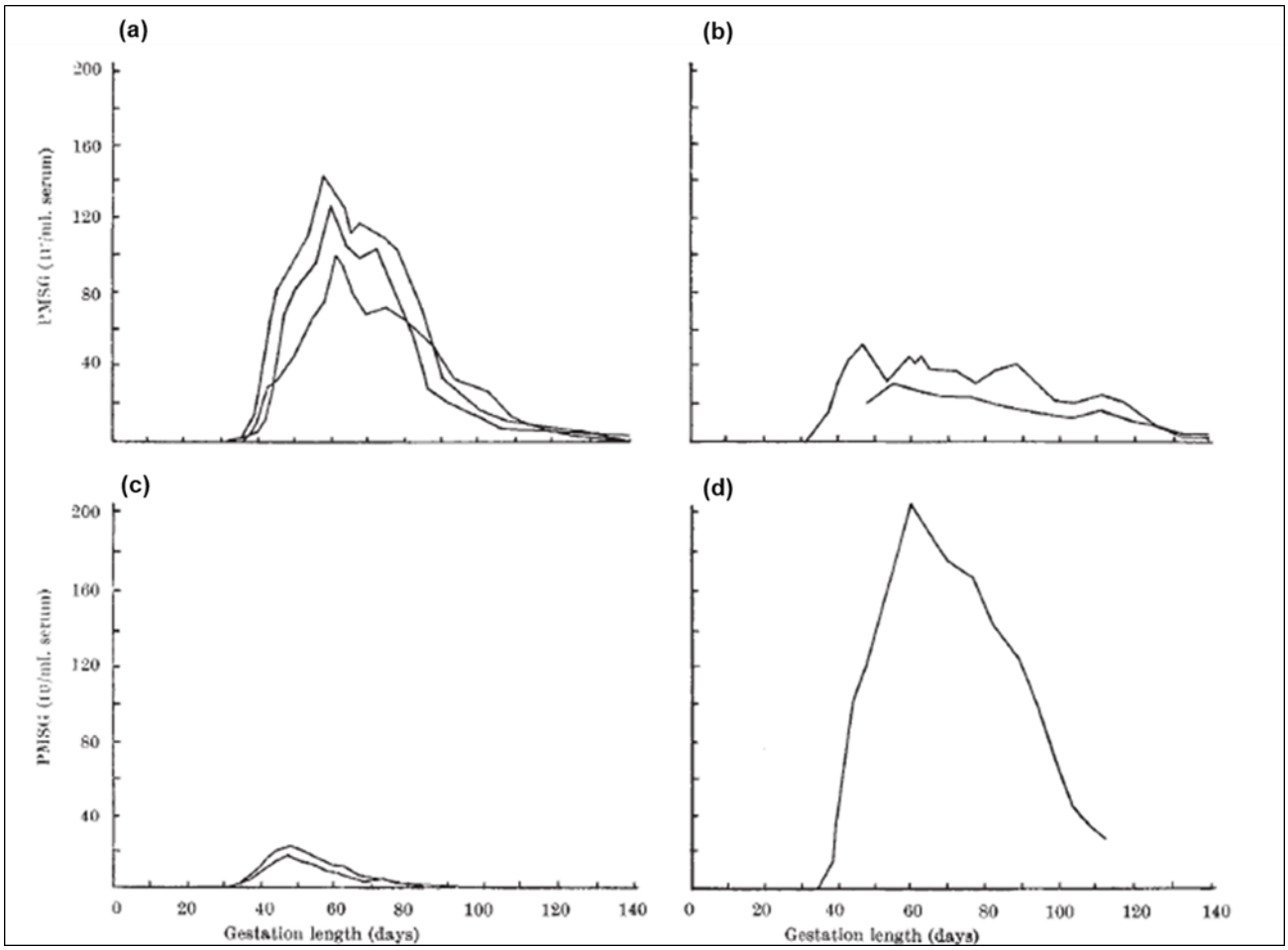


**Figure 1.9:** eCG (PMSG) concentration in the serum of mares and donkeys carrying a **(a)** horse foetus (horse female x horse male), **(b)** donkey foetus (donkey female x donkey male), **(c)** mule foetus (horse female x donkey male) and **(d)** hinny foetus (donkey female x horse male) (copied from Allen and Short 1997).

## 1.4 Equine serum albumin

Equine serum albumin (ESA) is the most common protein found in equine serum as is the case with most mammals. The protein has a molecular weight of 67.5 kDa which is very

close to eCG's weight of 60 kDa (Svedberg and Sjögren 1930). The 583–residue polypeptide is a heart-shaped protein comprising of three helical domains each of which is divided into two subdomains (**Fig 1.10**) (Majorek et al. 2012)

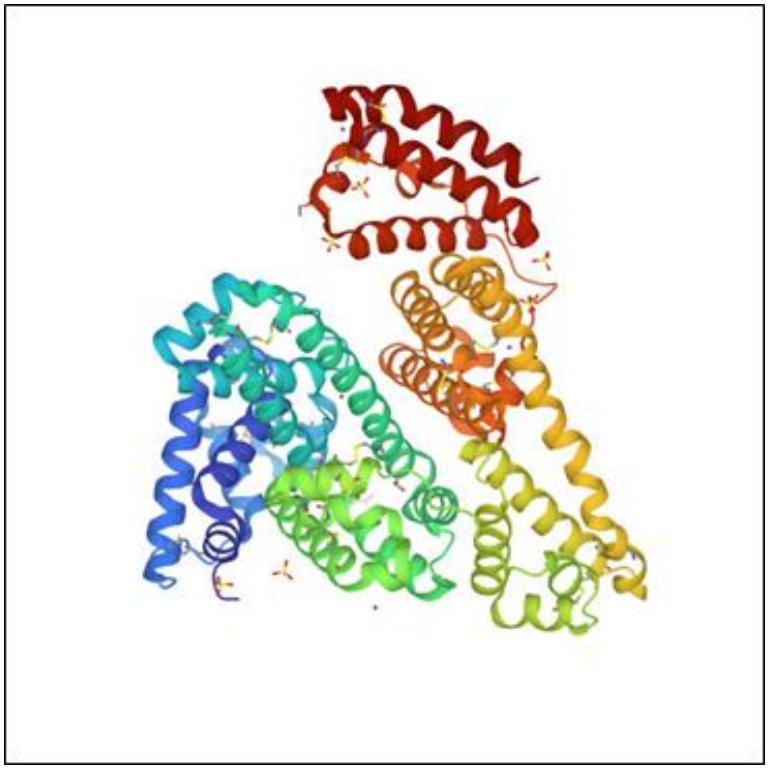

**Figure 1.10:** Protein structure of Equine Serum Albumin (Majorek, PDB 3V08).

Serum albumin functions as an enzyme, antioxidant, transport protein and ligand to many substances and proteins in the serum (Kajal and Pathania 2021). In mammals, albumin is produced in the liver and is water soluble with a pI of approximately 5 (Peters 1985). One of the most important steps in the purification of eCG from pregnant mare serum is the depletion of ESA, however since ESA is much more abundant in the serum (albumin makes up to 60% of proteins in horse serum) and of such similar molecular weight, to eCG, this can also be the most challenging step.

## 1.5 The purification of eCG from pregnant mare serum: A historic overview

In 1937 Cartland and Nelson described the first method for producing highly purified eCG from the serum of pregnant mares. The process firstly included a serum preparation step where the pH was adjusted to 6 in order to allow a flocculent precipitate of inactive proteins to separate. Subsequent precipitation was performed with various concentrations of alcohol and acetone ranging from 30-90% v/v. The hormone remained in solution in acetone up to

50%v/v and alcohol up to 60%, but completely precipitated in solutions of 70-80% acetone and alcohol. Therefore, most of the plasma proteins could be removed by precipitating the crude plasma with 60%v/v alcohol or 50% acetone, and then eCG would be obtained by precipitating the supernatant with 70% alcohol or acetone.

The precipitate containing active eCG was washed with alcohol and ether and vacuum dried to produce a white, water-soluble powder yielding 70-90% of the eCG present in the crude plasma. Their purified protein contained a high concentration of sodium citrate which they corrected by re-dissolving the active precipitate in water and then repeating the above-mentioned process but making the final step a precipitation at 65% acetone (**Fig 1.11**). This method showed a 130-fold purification of eCG. (Cartland and Nelson 1937).

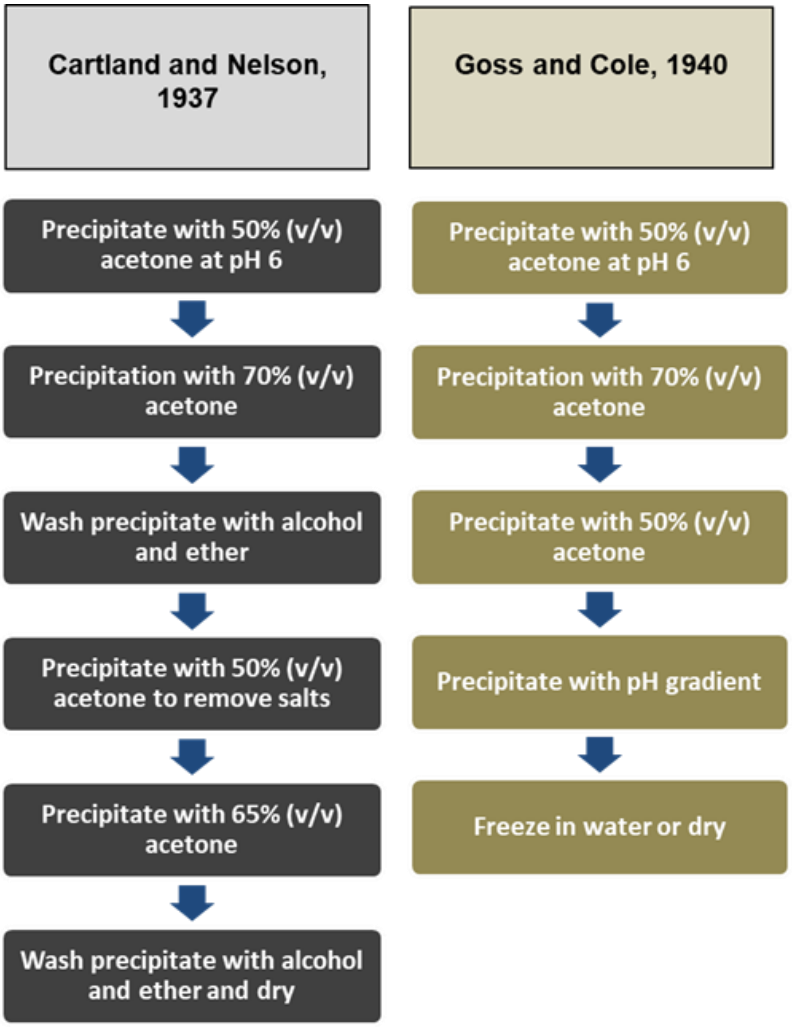


**Figure 1.11:** Schematic representation of the steps involved in the purification processes described by Cartland and Nelson as well as Goss and Cole (adapted from Cartland and Nelson 1937; Goss and Cole 1940).

Goss and Cole expanded on the research of Cartland and Nelson by suggesting that the first active precipitate, obtained after precipitation with 70% acetone, should be redissolved in 50% acetone at a pH of 6 to obtain a further 2-fold purification (**Fig 1.11**). This 50% acetone solution is then pH fractionated in steps until eCG precipitates in a higher purity between pH 5.5 and 4.5 (Goss and Cole 1940).

A few years later (1967) Gospodarowicz and Papkoff described the two-step process that would later be known as the classical method to purify eCG from the serum of pregnant mares (**Fig 1.13**). In their method the pH of the serum is first adjusted to 3 with metaphosphoric acid ($HPO_3$) to precipitate most of the major plasma proteins after which the pH of the supernatant is increased to 4.5 by adding sodium hydroxide (NaOH). Two cold (-20 °C) ethanol precipitations follows, the first with 50% (v/v) ethanol after which 75% (v/v) ethanol is added to the supernatant in the second precipitation. Following this the precipitate is then dissolved in water and dialyzed against distilled water to be lyophilized. In the second purification step a final eCG extract is produced by performing gelfiltration, using a Sephadex G-100 column which increases the specific activity fivefold (from 600 IU/mg, obtained after the 75% ethanol precipitation, to 3000 IU/mg) and then lastly performing cation-exchange chromatography with a sulfoethyl Sephadex C-50 column to increase specific activity another six times (2500 IU/mg to 15 800 IU/mg) (**Fig 1.12 (1)**) (Gospodarowicz and Papkoff 1967).

Subsequent eCG purification methods have attempted to improve either on the number of steps required for this classical method or the amount of chemical reagents needed whilst improving the yield of protein.

Schams and Papkoff employed the processes of the second purification step, in the classical method, to further purify commercially available eCG. In their study commercially available eCG, with an activity of 2500 IU/mg, was first applied to a cation exchange sulfoethyl Sephadex C-50 column and then to a Sephadex G-100 column for gel filtration. This yielded a 35% increase in activity (10 500 IU/mg) (Schams and Papkoff 1972).

In 1978, Christakos and Bahl used three similar chromatography steps to purify a crude preparation containing 1600 IU/mg eCG. Firstly gelfiltration with a Sephadex G-100 which gave a twofold purification (3200 IU/mg eCG), followed by anion exchange chromatography on a DEAE-Sephadex A-50 column which yielded 10 300 IU/mg protein and lastly multi-modal chromatography (this type of chromatography combines hydrophobic interaction chromatography and ion-exchange chromatography) on hydroxyapatite to yield an eCG end product of 13 700 IU/mg (**Fig 1.12 (2)**) (Christakos and Bahl 1979).

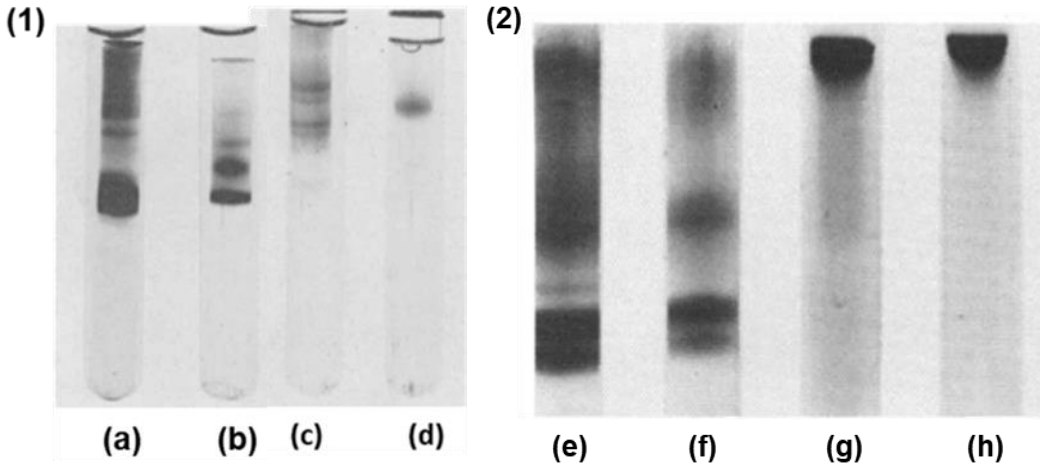


**Figure 1.12: (1)** Polyacrylamide gel electrophoresis of eCG fractions. **(a)** Unfractionated, **(b)** 600 IU/mg obtained after precipitation with 75% ethanol, **(c)** 3000 IU/mg obtained after gel filtration, and **(d)** 15 800 IU/mg obtained after cation exchange chromatography; in the classical purification method described by Gospodarowicz and Papkoff. Electrophoresis performed at pH 8.3 in Tris-glycine buffer for 30 minutes at 200 V, stained with amido black (copied from Gospodarowicz and Papkoff 1967). **(2)** Polyacrylamide electrophoresis of eCG fractions obtained by Christakos and Bahl from **(e)** crude preparation containing 1600 IU/mg eCG, **(f)** after gel filtration on Sephadex G-100 (3200 IU/mg), **(g)** after DEAE-Sephadex A-50 column chromatography (10 300 IU/mg) and **(h)** multi-modal chromatography on hydroxyapatite (13 700 IU/mg). Electrophoresis was done on a 7% polyacrylamide gel, at pH 8.3 for 1 hour at 5mA. Visualization was done via Coomassie blue staining (copied from Christakos and Bahl 1979).

The group also used the same three columns on urea-dissociated PMSG and obtained purified homogeneous preparations of the α- and β-subunits. However recombination proved problematic and full binding activity of the proteins could not be regained, suggesting that the subunits are not as stable as human chorionic gonadotropin (hCG) or simply that optimal recombination conditions were not met (Christakos and Bahl 1979).

After testing chromatography on a Quaternary Aminoethyl (QAE)-Sephadex A50 (anion exchange) column and a Sephadex G-200 (gelfiltration) column, Moore and Ward found an optimal one-column purification method using hydroxyapatite to further purify partially purified eCG preparations. A crude preparation of 2500 IU/mg eCG was purified to an activity of 12 000 IU/mg in the non-adsorbent fractions (Moore and Ward 1980).

In their effort to produce eCG *in vitro* from equine trophoblast cells Aggarwal *et al*. found that a small adjustment to Gospodarowicz and Papkoff's classical method allowed for better purification from the culture medium reclaimed from monolayers of horse chorionic tissue. They suggested that a pH of 7.5 be used at the 50% ethanol precipitation step instead of 4.5 to prevent partial eCG precipitation at this point. Interestingly the eCG produced *in vitro* had

a smaller molecular weight compared to that of the hormone purified from serum which was believed to be due a lower carbohydrate content of the *in vitro* produced protein. This also coincided with reduced bioactivity, in FSH and LH assays, for eCG purified from culture medium (Aggarwal et al. 1980).

In 1998 Gonzáles and co-workers developed an alternative method where the initial enrichment step was substituted by a direct adsorption to chromatographic media in a batch-wise fashion. This would eliminate the exposure of eCG to solvents and could be performed at room temperature on large scale. 10 L of plasma (adjusted to pH 4) was first concentrated to a volume of 5 L by ultrafiltration and then buffer exchanged (to 100 mM Sodium acetate buffer, pH 4) by diafiltration. 300 g of DEAE-Sephacel (a weak anion exchanger bead) was added to 10 L of the diafiltrate and incubated with gentle mixing for 1.5 hours. After the gel settled the supernatant was discarded and some washing steps followed. eCG was eluted using 100 mM sodium acetate buffer at pH 4.5 with 0.3M NaCl. They reported an excellent recovery of more than 90% of the protein which was comparable with the classical methaphosphoric-ethanol precipitation process but showed a 1.5 times higher yield. Finally cation exchange chromatography was performed on the eluate using S-Sepharose giving a total eCG recovery of more than 70% with a final potency of approximately 4000 IU/mg (González et al. 1998).

Müller *et al.* described a purification method that improved on the classical method by decreasing the amount of ethanol needed by 66%. The classical method was followed at first but after the precipitation with 50% ethanol the crude eCG was desalted and concentrated by diafiltration. Consequently magnetic anion exchanger beads with *N,N*-diethyl-ammonium functional groups (DEAP) were synthesized for the final chromatography step in the purification process. This process reached a protein yield of 79% with a product having 1200 IU/mg activity which would be suitable for commercial use (**Fig 1.13**) (Müller et al. 2011b). The group was however still unsatisfied with the fact that precipitation steps were still needed in order to produce an intermediate product suitable for chromatography and set out to investigate the direct application, of eCG affinity-liganded magnetic adsorbents, to the serum of pregnant mares (**Fig 1.13**).

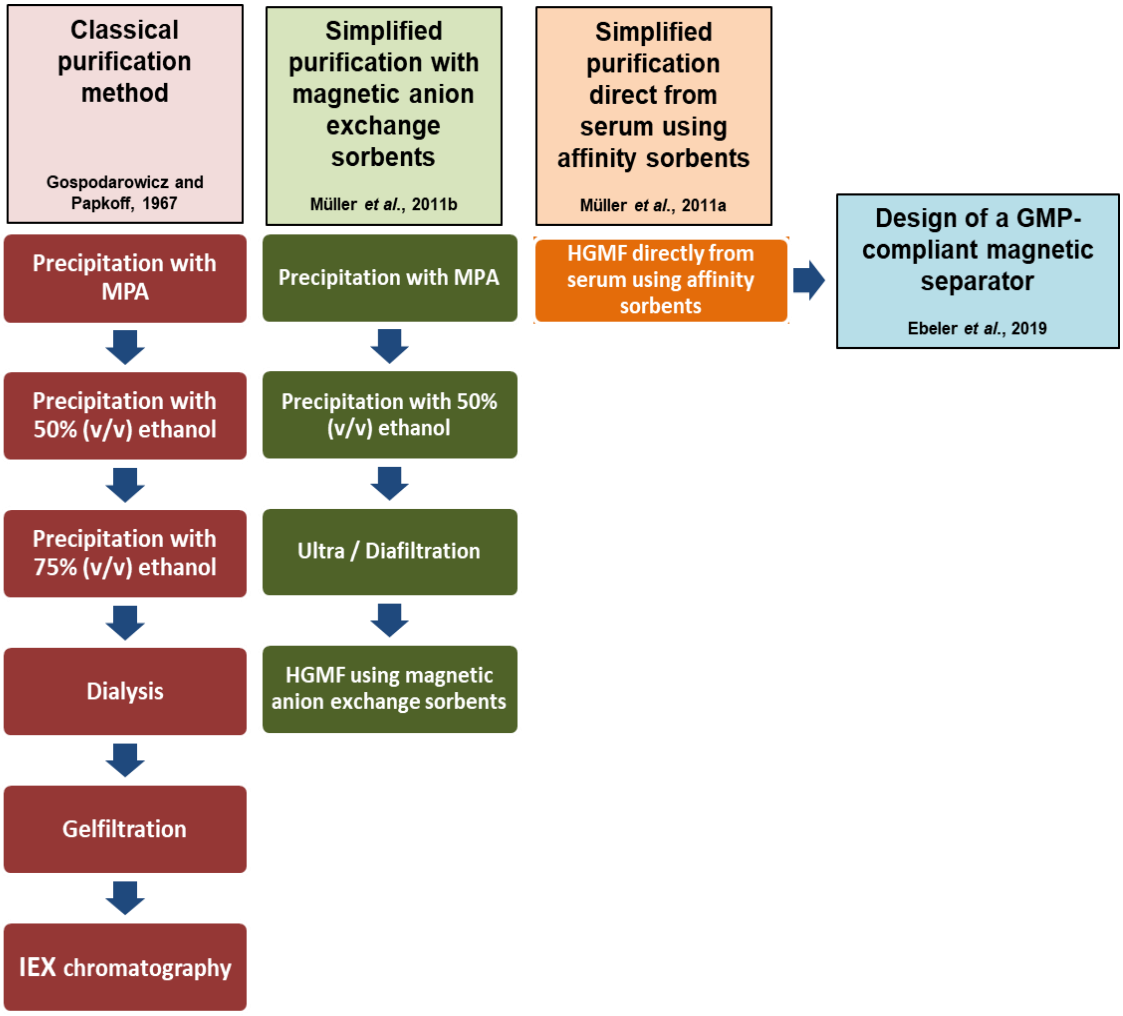


**Figure 1.13:** Schematic representation of the steps involved in the classical eCG purification and modifications to it by Müller *et al*. and Ebeler *et al.* (adapted from Müller *et al*. 2011a)

The beads were synthesized by immobilizing eCG-specific mAB onto the carboxylated M-PVA through *N*-hydroxysuccinimide (NHS) ester activation. This process was performed on small scale using an automated high gradient magnetic fishing (HGMF) 'rotor-stator' device. The process involved firstly mixing the magnetic affinity sorbents (2g) with 1 L of serum and then capturing the eCG-enriched sorbents with a magnetic filter that depleted the serum. After up to seven washing steps (with 0.1 M, sodium phosphate buffer at pH 7) elution was performed using 0.1 M ammonium citrate, pH 3. This process improved on the purification factor of the previously presented process but presented a lower eCG yield of 50% (compared to 79%) and the adsorbents lost up to 30% binding capacity over 30 process cycles, lowering their reusability (Müller et al. 2011a). Later in 2019, Ebeler and co-workers designed a HGMF-device, that was Good Manufacturing Practice (GMP) compliant with an automated clean-in-place (CIP) routine, to purify eCG based on the strategy mentioned above (**Fig 1.13**). The device comprised of a separation compartment surrounded by an

electromagnet. Densely perforated metal discs, which serve as matrix elements, were stacked in the separation compartment. They used a double counter-current binding process, where fresh unloaded magnetic particles ($M_0$) were first incubated in serum ($S_1$) that had previously been incubated with loaded magnetic particles ($M_1$) before being incubated in fresh serum ($S_0$), (**Fig 1.14**) to increase protein yields to over 95% (Ebeler et al. 2019).

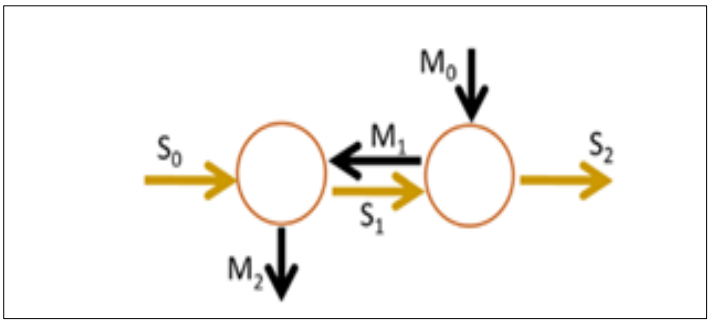


**Figure 1.14:** Schematic representation of the double counter-current binding process used by Ebeler and co-workers. The fresh serum ($S_0$) is firstly incubated with preloaded magnetic particles ($M_1$) and then with unloaded magnetic particles ($M_0$) after which eCG is eluted from the particles. (copied from Ebeler *et al.* 2019)

A one step purification process was proposed by Sharifi and co-workers who immunized rabbits by injecting 25 IU of eCG in order to prepare polyclonal antibodies. eCG was subsequently extracted directly from plasma via the solid-phase extraction (SPE) method and the end product had a concentration of 5250 mg/ml (Bradford method with serum albumin and gamma globulin for standards). The group suggested that the reduced amount of solvents needed for the purification process would be a more cost effective method for the production of eCG (Sharifi et al. 2014).

Baieli *et al*. synthesized an affinity matrix to purify eCG directly from plasma. Firstly chitosan beads were fixed with wheat germ agglutinin (WGA), which they previously found successful in purifying glycoconjugates (Baieli et al. 2017). The matrix was then incubated with equine plasma, for 4 h at 25°C after which eCG was eluted using 1.0 M D-GlcNAc, pH 7 & 1% (v/v) Tween 20. After three consecutive cycles an average yield of 60% eCG with a specific activity (1290 UI/mg) suitable for commercial use, was obtained. The matrix also maintained its chromatographic performance when the process was scaled up 500 times in volume (Baieli et al. 2020).

Today research is on-going in the search for an optimal method to purify eCG from the serum of pregnant mares in quantities suitable for commercial use. This is mainly due to the fact that recombinant production of the protein is still in its commencement phase and pregnant mare serum therefore remains the primary source of eCG.

## 1.6 Strategies used today in the purification of eCG from serum

### 1.6.1 Acid and/or alcohol precipitation

Mammalian blood plasma is a complex biological material containing hundreds of proteins that perform a myriad of physiological functions. Total plasma protein concentration in adult non-thoroughbred horses range between 53-73 g/L (Rossdales Laboratories 2022). The most abundant proteins in equine plasma, as is the case in most mammals, are albumin (60%), globulins (30%) and fibrinogen (4%) Only 1% of whole plasma proteins is attributed to low abundance proteins such as hormones, enzymes and pro-enzymes (Anderson and Anderson 2002; Farrugia 2010).

This huge dynamic range of individual protein concentrations (up to 10 orders of magnitude) makes the purification, identification, and quantification of the low abundance proteins much more challenging. As we have seen, some processes have been developed to tackle this problem. One of the most extensively used of these processes is known as fractionation or precipitation by the use of organic solvents such as ethanol, acetone and isopropanol. This process mostly exploits the solubility of the proteins and by adjusting the sample pH the protein pI also influences the resultant precipitate and supernatant (Yang et al. 2020).

As previously noted the classical purification method developed by Gospodarowicz and Cole start with a metaphosphoric acid (MPA) precipitation step (Gospodarowicz and Papkoff 1967).

MPA is used to precipitate especially water soluble proteins, a property which distinguishes it from other phosphoric acids. The acid has a general formula of $(HPO_3)n$, where “n” denotes the number of phosphoric acids present in the cyclic structure of MPA (**Fig 1.15**). MPA is a transparent, hygroscopic solution that dissolves very slowly in cold water, eventually changing to phosphoric acid ($H_3PO_4$). Its $pK_a$ of 2.1 makes MPA perfect for purifying eCG (Briggs 1940; Chemistry 2017; Tyner and Francis 2017).

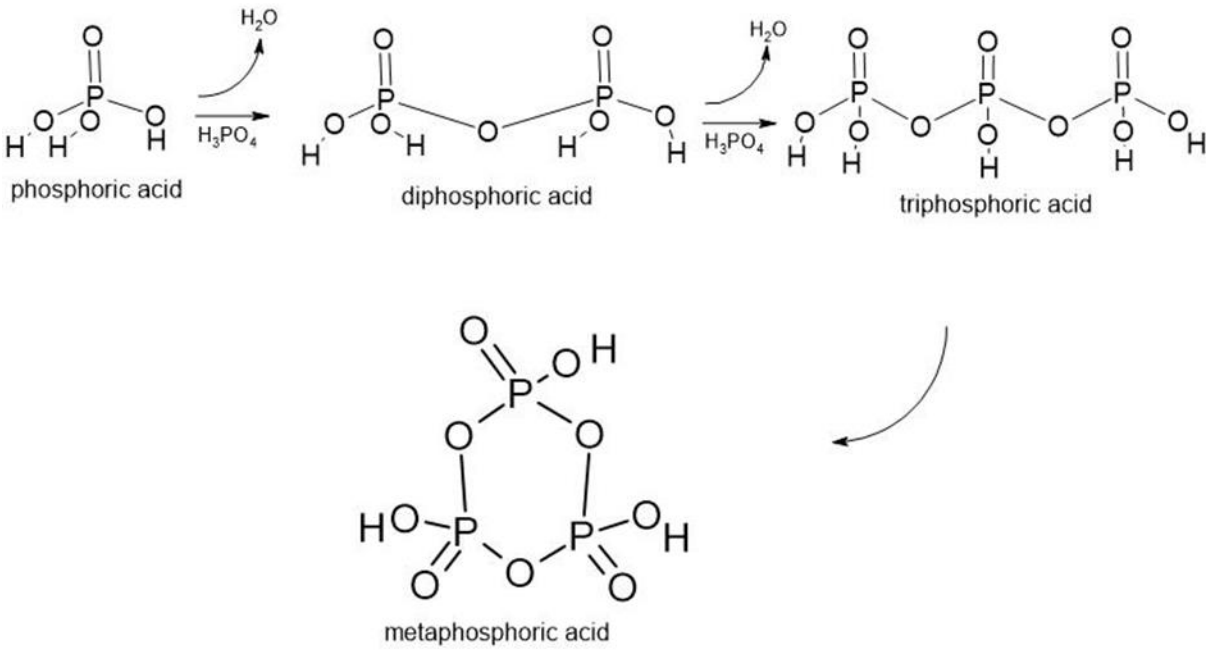


**Figure 1.15:** Diagram showing the production of MPA by heating phosphoric acid (prepared using Chemsketch Freeware).

Another acid used for protein precipitation is Trichloroacetic acid (TCA) which is much less expensive than MPA. The molecular formula is $C_2HCl_3O_2$ which is the result of adding chlorine to acetic acid (**Fig 1.16**). TCA is usually a white to colourless crystalline substance with a sharp odour. Concentrations of 10% (w/v) and 20% (w/v) usually yield the best precipitation results as it is thought that this halogenated derivative of acetic acid induces an acid-state (A-state or molten globule) in the protein that causes the protein to precipitate.

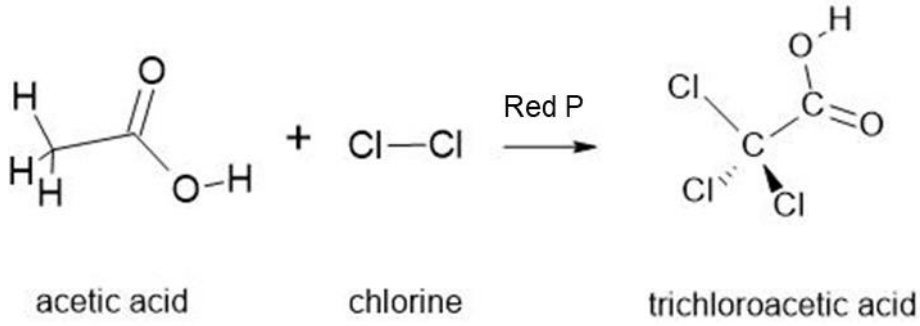


**Figure 1.16:** Diagram of the production of TCA from acetic acid and chlorine (prepared using Chemsketch Freeware).

This A-state also appears to be completely reversible. In fact, the precipitation extent versus concentration can be plotted as a U-shaped curve with 15% TCA as the optimal concentration (Sivaraman et al. 1997; Rajalingam et al. 2009; Koontz 2014).

We also found one group who combined Isopropanol with 1% TCA to deplete human serum albumin and since the removal of albumin is a logical target in the purification of eCG we wondered whether this method could be adopted for use with horse serum (Liu et al. 2014).

### 1.6.2 Albumin affinity chromatography

Affinity chromatography allows for the selective purification of analytes. This is achieved through the means of liquid chromatography where an affinity ligand is used as a stationary phase in a column.

Cuatecasas *et al.* were the first to introduce affinity chromatography for the purpose of purification in 1968 (Cuatrecasas et al. 1968). There are numerous different types of affinity ligands such as enzymes, proteins, antigens, hormones, biomimetic dyes, enzyme substrates or inhibitors etc. The type of ligand immobilized to the resin of the column is determined by the purpose or the compound and matrix from which it is to be purified.

Albumin affinity chromatography is widely performed using metal ion-, immuno-, and dye-affinity ligands, in human serum.

Odaba *et al*. developed a bead with 2-methacrylamidohistidine as a bio-ligand and discovered that the chelation of Cu(II) onto the beads effected an increase in the maximum adsorption of human serum albumin (HSA) with only trivial adsorption of other proteins such as fibrinogen and γ-globulin. It was also possible to reuse the Cu(II) chelated beads without losses in adsorption capacities (Odaba et al. 2001).

Raoufinia and co-workers used immuno affinity chromatography (IAC) to isolate HSA. They immunized rabbits to produce a suitable polyclonal HSA-antibody which was functionalized to cyanogen bromide (CNBr) -activated Sepharose beads. SDS-PAGE and Western blot analyses showed a 98% purity of HSA isolated, indicating that IAC was a robust method of isolating HSA (Raoufinia et al. 2016).

IAC ligands are extremely specific but unfortunately their use is also expensive due to high production costs, the extensive purification steps involved and storage precautions. To circumvent these drawbacks dye ligands is considered as one of the important synthetic alternatives to anti-bodies. The former is able to bind various proteins and can easily be immobilized, especially on matrices bearing hydroxyl ($OH^-$) -groups. Dye ligands are also commercially available and inexpensive. These ligands are synthesized to interact with the active sites of proteins by mimicking the structure of cofactors and substrates for those

proteins. Protein separation is usually performed on dye affinity systems in the form of spherical sorbents or as affinity membranes.

The observation of an unexpected interaction between Blue Dextran (Cibacron Blue and dextran conjugate) and certain kinases during size exclusion chromatography, initiated dye affinity chromatography (Dean et al. 1985). Earlier studies showed that it was possible to purify several proteins e.g., phosphofructokinase, glutathione reductase, erythrocyte pyruvate kinase and several coagulation factors when performing size exclusion chromatography with Blue Dextran (Kopperschläger et al. 1968; Kopperschläger et al. 1971; Bohme et al. 1972). It was found that the dye, Cibacron Blue F3G-A, bound the proteins in each case. In 1971, Roschlau and Hess were the first to immobilize Cibacron Blue on Sephadex G-200 directly for use in the purification of yeast pyruvate kinase (Röschlau and Hess 1972). Today dyes used in dye-ligand affinity include Reactive Red 120, Congo Red, Reactive Blue 4, Mimetic Blue 1 A6XL, Green HE4BD and Cibacron Blue F3GA (Denizli and Pişkin 2001).

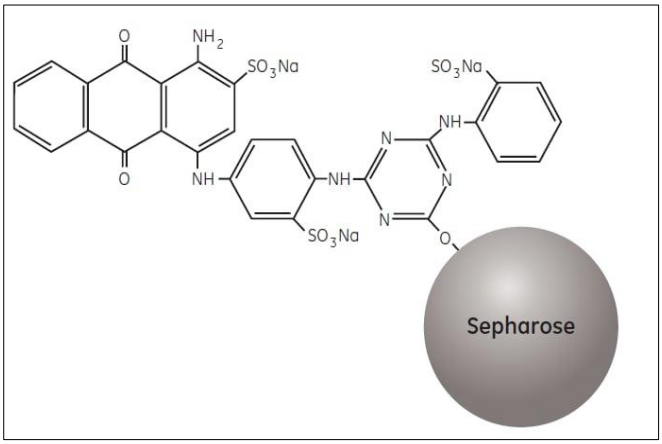


Figure 1.17 shows one application of such a dye fixed to a resin, displaying an affinity for albumin. The Cibacron Blue F3GA is covalently attached to the sepharose.

**Figure 1.17:** Partial structure of Cibacron Blue F3G-A linked to Sepharose to create Blue Sepharose (copied from Merck 2022).

### 1.6.3 Ultrafiltration

Ultrafiltration (UF) is a membrane-based, size exclusion process, where a fluid is placed under pressure on one side of the perforated membrane of a particular pore size. Molecules between 1 kDa – 1000 kDa can be separated by UF if they differ in size by at least one order of magnitude. UF is much less expensive than other purification or separation techniques, it is also easy to use and has a high throughput of product with easy scalability. Relatively mild operating conditions such as low temperatures, no chemical additives or phase changes and low pressures, avoids denaturation and deactivation of the biological products (Christy and Vermant 2002).

UF membranes are usually made of certain polymers, metallic or ceramic materials. Ceramic membranes are mostly employed in the biotechnological and pharmaceutical industry due to their sturdiness and ability to endure chemical or thermal sterilisation. Metallic membranes are mostly only used in small scale applications. Polymeric membranes are the workhorses of UF applications. There are many different polymers that can be used in UF membranes but most commercially available membranes are either polyethersulfone or polysulfone (Cui 2005).

Sieving, in UF, is only one of the mechanisms that affect protein transmission. System hydrodynamics, trans membrane pressure coupled with membrane resistance, hydrophilicity as well as the porosity and morphology of the membrane all affect the flux of the protein solution. Electrostatic charge also plays an important role, hence protein to protein interactions, solution pH and ionic strength become critical (Wan et al. 2005).

### 1.6.4 Ion exchange chromatography (IEX)

IEX is one of the most popular separation methods in liquid chromatography and its history stretches back to Biblical times. Exodus 15: 22-25, from the Holy Bible, reads as follows:

*22 Then Moses led Israel from the Red Sea and they went into the Desert of Shur. For three days they travelled in the desert without finding water. 23 When they came to Marah, they could not drink its water because it was bitter. (That is why the place is called Marah) 24 So the people grumbled against Moses, saying, "What are we to drink?" 25 Then Moses cried out to the LORD, and the LORD showed him a piece of wood. He threw it into the water, and the water became fit to drink.*

This shows the first recorded use of ion-exchange chromatography in that Moses, unwittingly, rendered the water drinkable by removing salt-bearing minerals containing calcium, magnesium and sodium. Other ancient records also indicate early knowledge of desalination techniques such as Aristotle's *Problematica* where the use of sand filters, to obtain drinking water from the sea and other impure origins, is discussed. Sir Francis Bacon wrote a method of obtaining fresh water at the coast of Barbary in *Sylva Sylvarum: A natural history in ten centuries*:

*Digge a hole of the sea-shore somewhat above high-water mark and as deep as low-water mark, which when the tide cometh will be filled with fresh water and potable.*

Generally the recognition of the phenomenon of ion exchange is accredited to two English agricultural scientists, Thompson and Way, who were the first to observe the adsorption of

ammonium ions to certain soils. They found that an ion exchange function was performed by complex silicates in the soil and they prepared materials of this type in the laboratory, from sodium silicate and sodium aluminate (Thompson 1850; Way 1850). Materials of this type was used by Gans, in 1906, to soften water and treat sugar solutions (Gans 1905). Folin and Bell performed the first analytical application of ion-exchange in 1917. They separated ammonium from urine using a synthetic zeolite (Folin and Bell 1917). Whitehorn was the first to use ion exchange in column chromatography, in 1923, when he showed that other amines besides ammonia were also readily adsorbed by a synthetic aluminosilicate (Whitehorn 1923). The first use of an anion exchange column was for the determination of sulfate in natural -and industrial wasters (Bahrdt and Anal 1927).

In 1935, Adams and Holmes synthesized the first organic ion exchangers. They discovered that crushed phonograph records displayed ion exchange properties (Adams and Holmes 1935). The middle 1940's saw the development of polystyrene-divinylbenzene-based resins, through the copolymerization of styrene cross-linked with divinylbenzene (**Table 1**). Polystyrene-divinylbenzene resins offered more stability, strength and exchange capacity than their predecessors and it was through this innovation that the complete demineralization of water was made possible. In the late 1940's a quaternary ammonia was attached to the polystyrene-divinylbenzene support but modern day chromatography was only introduced in 1975 with the industrialization of the technique by Small *et al*. who developed a column that allowed conductivity detection (Small et al. 1975).

**Table 1.1:** Historical development of ion-exchange chromatography (adapted from Lucy 2003; Kumar and Jain 2013).

| Scientist/Source | Breakthrough | Year |
|---|---|---|
| Holy Bible | Moses decalcifies water | ~ 1400 BC |
| Aristotle | Aristotle discovers that sea water loses part of its salt contents when percolated through certain sands | ~ 330 BC |
| H.S. Thompson | Experiments with passing a solution of manure through a filter made of ordinary garden soil and finds that the ammonia is removed from solution | 1845 |
| H.S. Thompson & J.T. Way | Recognition of the phenomenon of ion exchange and description of its basic characteristics | 1850 |
| R. Gans | Discovers that zeolites can be used to soften hard water. Invents processes of synthesizing zeolites and designs the equipment – This zeolite water softener is later used to recover gold from sea water | 1906 |
| O. Folin & R. Bell | First analytical application of ion exchange | 1917 |
| J. Whitehorn | The first use of ion exchange in column chromatography | 1923 |
| A. Bahrdt & Z. Anal | The first use of an anion exchange column | 1927 |
| B.A. Adams & E.L. Holmes | Synthesizes the first organic ion exchanger | 1935 |
| G.F. D'alelio | Invents sulphonated polystyrene polymerization cation exchangers | 1942 |
| G. E. Boyd, J. Schubert, & A. W. Adamson | Demonstrates the applicability of ion exchange for adsorption of fission products in traced amounts | 1942 |
| C. H. McBurney | Invents aminated polystyrene polymerization anion exchangers | 1947 |
| A. Skogseid | Prepares potassium-specific polystyrene cation exchanger chelating resin | 1947 |
| J. A. Marinsky, L. E. Glendenin, & C. D. Coryell | Discovers promethium, an element found in nature, attributed to ion exchange | 1947 |
| D. K. Hale, D. Reiechenberg, N. E. Topp, & C. G. Thomas | Development of carboxylic addition polymers as weak acid cation exchanger | 1949-1956 |
| R. M. Barrer & D. W. Breck | New zeolites as molecular sieves with ion exchange properties | 1951-1956 |
| H. P. Gregor, K. W. Pepper, & L. R. Morris | Invents and develops chelating polymers | 1952-1971 |
| M. A. Peterson & H. A. Sober | Develops cellulose ion exchangers | 1956 |
| F. Helfferich | Lays foundation for the new theoretical treatment of ion exchange | 1959 |
| T. R. E. Kressmann & J. R. Millar | Invents and develops of isoporous ion exchange resins | 1960 |
| J. Weiss | Thermally re-generable ion-exchange and water desalination based on it | 1964 |
| H. Small, T.S. Stevens & W.C Bauman | Industrialization of ion exchange chromatography | 1975 |

Ion exchange chromatography separates biomolecules based on their net surface charge. Charge properties like the charge density, surface charge distribution and the differences in the overall charge of the molecule affect the degree of interaction that takes place.

Therefore, ion exchange chromatography involves the binding of a charged sample molecule to an oppositely charged functionalized group attached to a resin/matrix. This binding is electrostatic and reversible which grants a valuable separation method.

The amphoteric nature of proteins makes them excellent molecules for separation by ion exchange chromatography as their net charge change gradually as the pH of their environment changes. As we know, the isoelectric point (pI) of a protein is the pH at which the molecule carries no net charge. At a pH below its pI, a protein will have a positive net charge and easily bind to a cation exchanger whereas at a pH above its pI the molecule will exhibit a negative net charge and bind an anion exchanger. The choice between using an anion –or cation exchanger, for protein separation, typically only depends on the stability of the protein at a pH above or below its pI. Elution of the biomolecules usually involves changing the ionic strength of the buffer, therefore allowing the salt ions (usually $Na^+$ and $Cl^+$) to compete with the bound proteins on the resin and in some instances also adjusting the pH. This technique is powerful and provides separation even for biomolecules differing by a single charged amino acid (Grodzki and Berenstein 2010; Acikara 2013).

### 1.6.5 Lectin affinity chromatography (LAC)

In the last decade, the use of lectin affinity enrichment has been a very popular approach for studying glycosylation profiles. Lectins are proteins that interact with specific carbohydrate moieties of oligosaccharides linked to other biomolecules. They are found in bacteria and animals but have most comprehensively been studied in plants. In plants they act as a defence mechanism by identifying foreign pathogens through attaching to their surface oligosaccharides. These proteins usually bind glycans weakly but gain high avidity through the binding of multiple subunits, which strengthens the interaction. The proteins do however exhibit extremely high specificity as they can recognize the structure of a glycan down to a single sugar residue (Weis and Drickamer 1996; Hirabayashi et al. 2015). Different analytical techniques have been developed, in the past, based on the affinity of lectins towards glycans. Today we have lectin blotting or lectin microarrays, lectin histochemistry and enzyme-linked lectin assays which all incorporate the use of lectin proteins to provide quantitative and qualitative analysis for glycosylation profiles (Dan et al. 2016). Apart from these techniques, lectin-based affinity sorbents can be used in glycoprotein enrichment.

Lectins are categorized into classes based on their specificities toward different glycans and their biological origins. Special thought should thus be given to choosing a suitable lectin for analytical needs. Lectins can be immobilized to insoluble resins in many formats like on magnetic beads, monoliths or on particles to be used in cartridge, capillary or column setups for use in solid phase extraction (SPE) or dispersive solid phase extraction (dSPE)(see **Fig**

**1.18**). Other methods include filter-based enrichment, where a membrane with a specific MWCO is used in combination with free lectins or lectin-sorbents to separate glycosylated and non-glycosylated biomolecules (Hashim et al. 2017; Hendrickson and Zherdev 2018).

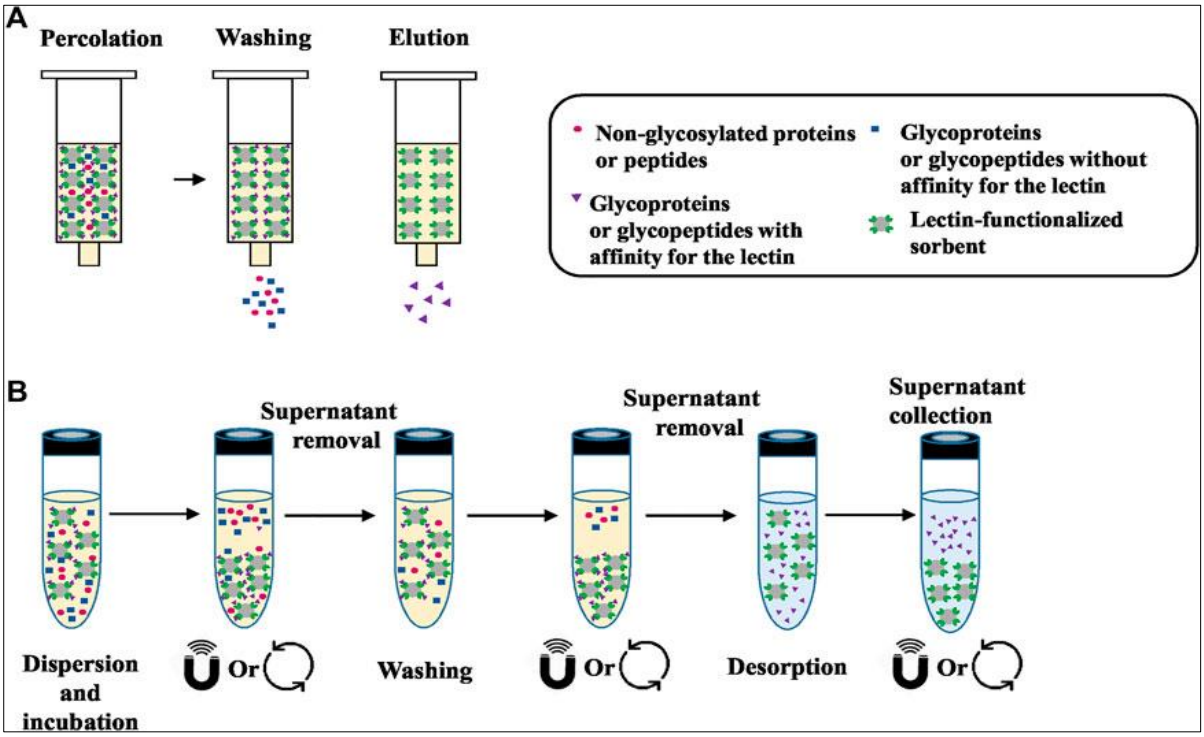


**Figure 1.18:** Diagram depicting the general workflow of **(A)** lectin-based SPE and **(B)** lectin-based dSPE. The sample is initially mixed in solution with lectin-functionalized sorbents in order for glyco-molecules to bind the sorbents. Centrifugation or the application of a magnetic field can separate compounds with no affinity for the lectins to the supernatant. After some additional washing steps, desorption of the target glycoproteins is performed (copied from Goumenou et al. 2021).

Research shows that lectin affinity enrichment has, in the past, been applied more for the isolation of *N*-glycosylated proteins but the isolation of *O*-glycosylated proteins are also possible (Durham and Regnier 2006; Chalkley et al. 2009; Trinidad et al. 2012; Nagel et al. 2013; Vakhrushev et al. 2013; Darula et al. 2016; 2017).

### 1.6.6 Hydrophobic interaction chromatography (HIC)

The well-known fact that hydrophobic proteins tend to interact, or self-associate, in aqueous solutions form the basis for many biological interactions, such as protein-substrate interactions, protein folding and protein transport across cell membranes (Janson 1998). HIC exploits this fact and is used both in preparatory and analytical scale protein purifications. In this technique the hydrophobic regions in macromolecules bind hydrophobic ligands on chromatography resins. Researchers Van Oss *et al.* (1986) suggested that, despite the complex mechanism involved in hydrophobic interactions, van der Waals forces were the

major contributing factor. This makes the use of HIC advantageous since minimal denaturing of the macromolecule takes place leaving its biological activity intact (van Oss et al. 1986).

These interactions usually occur in an environment with a high salt concentration because this favours hydrophobic interactions. In water, nonpolar molecules self-associate or aggregate and in doing so effect a net increase in the entropy of the environment as the highly ordered structures of water molecules around each protein become less structured when aggregation takes place.

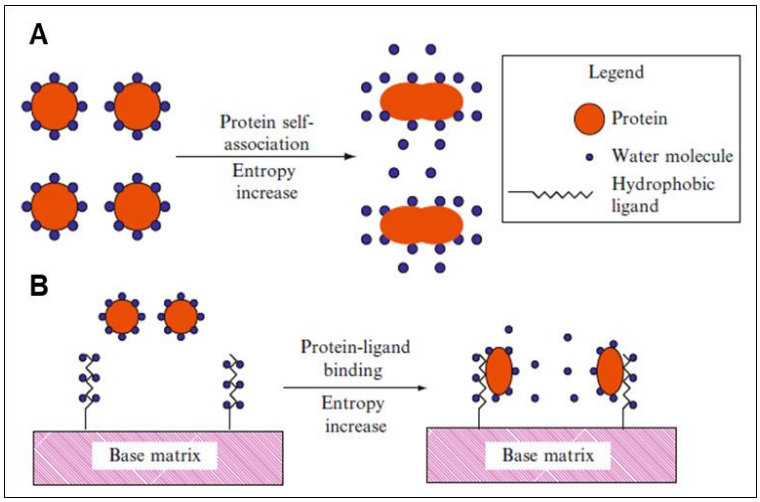


**Figure 1.19:** Diagram showing the principle of hydrophobic interactions between **(A)** proteins in aqueous solution and **(B)** proteins and a hydrophobic ligand functionalized to a HIC adsorbent (copied from McCue 2009).

The highly ordered molecules around individual macromolecules are disrupted as the surface area of the hydrophobic sites, of the protein that is exposed to the polar solvent, is decreased (**Fig 1.19 A**). Increased entropy is the favoured thermodynamic state and therefore these hydrophobic interactions occur spontaneously. The same principle applies to the interaction between hydrophobic proteins and hydrophobic ligands attached to adsorbents in HIC (**Fig 1.19 B**) (McCue 2009).

The addition of organic solvents or salts, to the solvent, can weaken or strengthen hydrophobic interactions between the adsorbents and the proteins. The well-known Hofmeister series indicates the effects of different ions on hydrophobic interactions (Hofmeister 1988). Anions in the Hofmeister series are listed below in decreasing lyotropic (ions which promote hydrophobic interactions) order:

$PO_4^{-3} > SO_4^{-3} > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^-$

Cations in the Hofmeister series are listed below in increasing chaotropic (ions which weaken/disrupt hydrophobic interactions) order:

$NH_4^+ > Rb^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$

To clarify, in the above series ammonium ions most strongly promote hydrophobic interactions while barium ions weaken them (Påhlman et al. 1977). The two most commonly used lyotrophic salts, in aqueous solution, are sodium chloride and ammonium sulfate. The strength of the hydrophobic interactions in aqueous solution can also be altered by the use of organic solvents. Organic solvents commonly used to weaken hydrophobic interactions include acetonitrile, alcohols and glycols. These are usually added during the elution process in order to elute strongly bound proteins of interest (Melander and Horváth 1977; Fausnaugh and Regnier 1986). The pH of the mobile phase also affects protein retention in HIC. An increase of the pH usually results in decreased hydrophobic interactions between proteins and hydrophobic ligands. This is likely due to the change in the net charge of the protein with increased pH levels. Similarly the opposite is true for a decrease in the pH of the mobile phase (Porath et al. 1973; Štrop et al. 1983; Montel et al. 1995).

The mobile phase properties are not the only conditions that affect HIC performance, stationary phase characteristics such as the chemical nature of the backbone, substitution level of the resin and the type of hydrophobic ligand also play a part as well as the temperature of the system (Porath et al. 1973; Melander et al. 1984; Arakawa 1986; Lin et al. 2000).

The most commonly used ligands for HIC are linear chain alkanes (such as butyl and octyl) and some simple aromatic groups (such as phenyl) (see **Fig 1.20**). The strength of the hydrophobic interaction between the proteins can be increased by increasing the alkyl chain length, however very long chain lengths can result in the irreversible adsorption of proteins which then necessitates harsher elution conditions and cause denaturation of the proteins. Therefore ligands with intermediate binding strength would be more useful as they provide elution by simply decreasing the salt concentration (Yon 1978; Hofstee 1979).

In this study we investigated the current purification strategies for the purification of eCG using eCG-spiked equine serum. Several of the purification steps we investigated include methods first mentioned in the classical method of Gozpodarowicz and Papkoff.

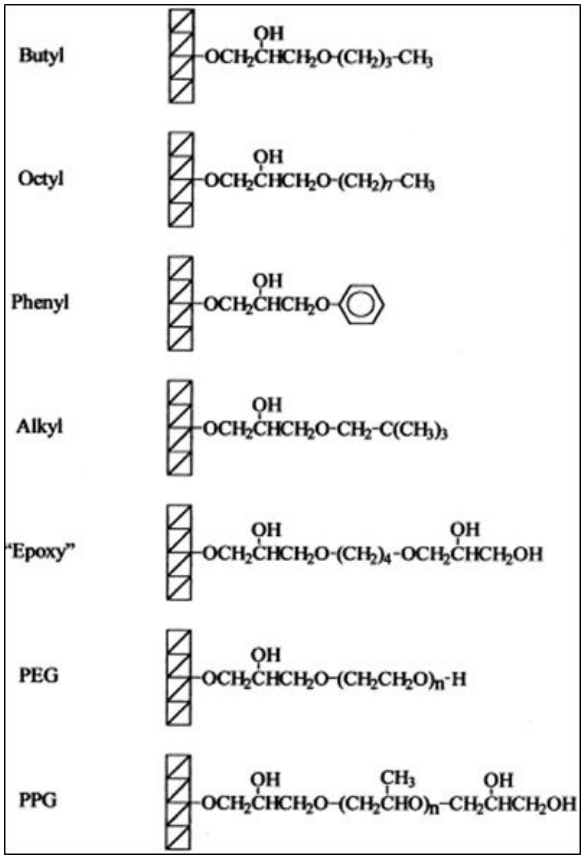


**Figure 1.20:** Schematic of the structures of some HIC ligands (copied from Queiroz et al. 2001)

## 1.7 Research aim and objectives

This study aims to investigate purification strategies for equine chorionic gonadotropin (eCG).

The objectives of this study (**Fig 1.21**) are to:

1. Investigate the efficiency of three serum precipitation methods.
2. Investigate the efficiency of albumin affinity chromatography in the isolation/depletion of albumin from equine serum.
3. Investigate the efficiency of ultrafiltration in the purification process.
4. Investigate the efficiency of four different chromatographic techniques in the isolation of eCG from equine serum.

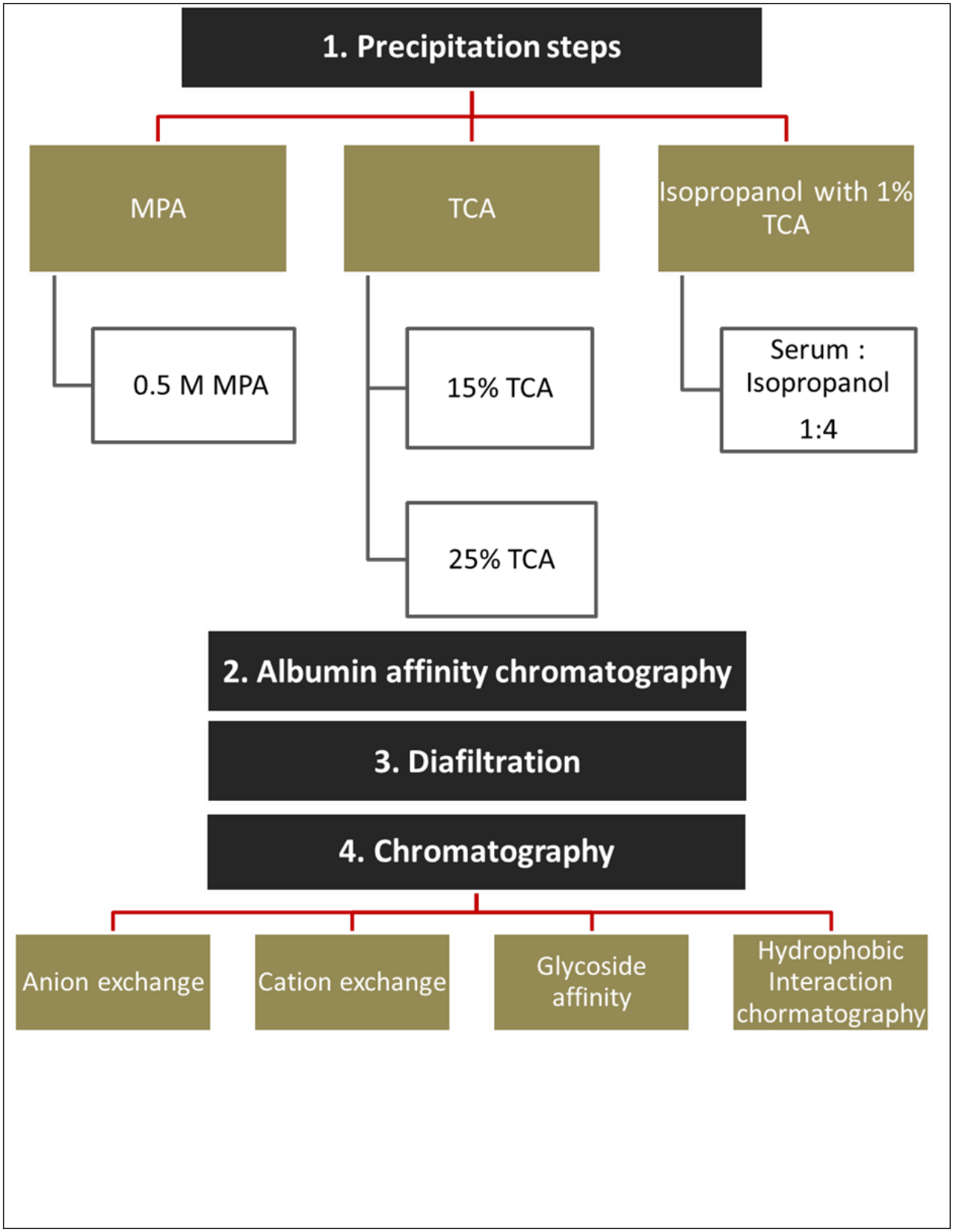


**Figure 1.21:** Diagram showing the objectives of the current study.

# Chapter 2: Experimental materials and methods

## 2.1 Laboratory analysis

### 2.1.1 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein visualisation via SDS-PAGE was performed using the Mini-PROTEAN™ Tetra system (Bio-Rad) operated according to the manufacturer's instructions.

Sample preparation was done by mixing 15 µl of sample with 15 µl of a denaturing solution (50 µl of beta-mercaptoethanol added to 950 µl of Laemmli sample buffer) and then boiling at 100 °C for 10 minutes in a heat block. When loading the samples onto the gel, 6 µl of the Page Ruler™ prestained protein ladder was usually loaded in the first lane followed by 12 µl of sample per lane. The 12% polyacrylamide gel were electrophoresed in 10% TGS buffer (25 mM Tris, 129 mM gycine, 0.1% w/v SDS; pH 8.3), obtained as a premix from BioRad, at 100 V for 2 hours. Gels are stained overnight in Coomassie brilliant blue R-250 solution and then destained in a "destaining solution" made up of 100 ml glacial acetic acid (Sigma Aldrich), 300 ml methanol and 500 ml $d$$H_2O$. Gels were visualized and photographed with the Chemidoc™ MP Imaging system from BioRad.

### 2.1.2 Quantitative analysis

Total protein was determined using a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific) according to the manufacturer's specifications. The eCG activity of samples were assessed by the use of a Pregnant Mare Serum Gonadotropin Enzyme-Linked Immunosorbent Assay (PMSG ELISA) kit, from Demeditec Diagnostics, according to the manufacturer's instructions.

This PMSG ELISA kit employs the sandwich principle to determine eCG activity. Standards ranging from 0 to 800 mIU/ml are provided (**Table 2.1)**. A standard curve is created each time the test and it used to determine the quantities within the unknown samples.

**Table 2.1:** Standard concentrations as provided in the PMSG ELISA kit (Demeditec Diagnostics).

| Standard sample | Concentration eCG/PMSG [mIU/ml] |
|---|---|
| 0 | 0 |
| 1 | 25 |
| 2 | 100 |
| 3 | 200 |
| 4 | 400 |
| 5 | 800 |

## 2.2 Sample preparation and storage

### 2.2.1 Blood collection and storage

Ethics approval, for the project, was granted by the Interfaculty Animal Ethics Committee with Project Number: UFS-AED2016/0081. Anipharm (Pty) Ltd kindly provided the blood samples used in the current study. Approximately 150 ml of blood was taken from two different non-pregnant mares. After a 30 minute period of incubation, at room temperature, most of the fibrinogen and clotting factors formed a precipitate. This together with other plasma constituents was removed by centrifugation at 2500 xg for 10 minutes. The serum was stored in 50 ml conical tubes at -20 °C.

### 2.2.2 eCG-spiked equine serum

Serum samples (49 ml) were thawed and spiked with 1200 IU/ml (1 ml) of commercially available eCG (Chronogest®) after thawing. Edta-free protease inhibitors, from Roche, were also added before the sample was considered suitable for use or further storage at -20 °C.

## 2.3 Precipitation steps

### 2.3.1 Precipitation with MPA

An equal volume of 0.5 M MPA (Merck) was added to equine serum spiked with commercial eCG. After mixing by inversion, samples where centrifuged, in an Eppendorf 5804 R centrifuge, for 15 minutes at 9000 xg, at a temperature of 4°C. Thereafter, the pH of the

obtained supernatants was raised to 7 by the addition of NaOH and it was fractionated with 50% & 75% ethanol (Gospodarowicz and Papkoff 1967). This involved adding an equal volume of 50% ethanol and incubating at 4°C for 2 hours. The samples were then centrifuged again at 9000 xg for 15 minutes, at a temperature of 4°C. The subsequent supernatant was retained and an equal volume of 75% ethanol was added. After centrifugation, at 9000 xg for 15 minutes at a temperature of 4°C, the supernatant was discarded and the precipitate was re-suspended in 20 mM Sodium phosphate, pH 7 to be stored until analysis.

Later in the study we negated the fractionation with 50% ethanol to reduce th loss of eCG at this step. Al supernatants and precipitants were stored at 4°C before analyses by SDS-PAGE, PSMG ELISA and in some instances BCA assay were performed.

### 2.3.2 Precipitation with TCA – comparing 15% concentration with 25%

Concentrations of 15% (v/v) and 25%(v/v) TCA were prepared from 100% (v/v) TCA stock (Merck). To test and compare the effectiveness of both concentrations 5 ml of the respective TCA concentration was added to 5 ml of sample and after mixing it was centrifuged at 9000 g's for 15 minutes at a temperature of 4°C. The precipitate was discarded. An equal volume (10 ml) of 75% ethanol was added to the supernatant and samples were incubated at 4°C for 2 hours after which it was centrifuged again at 9000 g's, for 15 minutes, at 4°C. The resulting precipitate was re-suspended in 20 mM Sodium Phosphate buffer, pH 7 and stored at 4°C. Analyses by SDS-PAGE, ELISA and BCA were performed shortly after.

### 2.3.3 Precipitation with Isopropanol containing 1% TCA

1% TCA was prepared from 100% TCA stock. 10 ml of 1% TCA (Merck) was added to 10 ml of isopropanol (Merck) to create an organic solvent solution. One volume of sample was added to four volumes of the organic solvent solution and mixed vigorously by vortex. Samples were then centrifuged at 1500 g's, for 5 minutes, at 5°C.

The supernatant was buffer exchanged via diafiltration using filtration units from Amicon® with a molecular weight cut off (MWCO) of 30 kDa. To perform the diafiltration the unit containing the supernatant was centrifuged at 4000 xg, for 10 minutes, at 4°C where after 20 mM sodium phosphate buffer, pH 7 was added. The sample was then stored at 4°C until analyses were done.

The pellet that formed after precipitation with isopropanol containing 1 % TCA was re-dissolved in 10% methanol after which it was centrifuged at 2000g, for 2 minutes at 5°C. The

precipitate was discarded and the supernatant was buffer exchanged as described above before storage.

## 2.4 Albumin affinity chromatography

Equine serum was spiked with commercial eCG (Chronogest®) as described in Section 2.2.2. The pH of the samples were lowered to 2.3 with TCA (Merck) after which the samples were centrifuged at 9000 g's, for 15 minutes, at 4 °C. The precipitate was discarded and the pH of the supernatant restored to 7 with the addition of NaOH.

The HiSreen Blue FF column (Cytiva) was manually equilibrated with 5 column volumes (CV) of 20 mM sodium phosphate, pH 7. 2 ml of sample was manually loaded onto the column and six fractions of 1.5 ml were collected of the flow through, wash, eluate and post-elute wash. The buffer used to elute the albumin was 20 mM sodium phosphate, pH 7 with 2 M NaCl. All fractions were stored at 4°C for no longer than 3 days before analyses was performed. After use, the column was washed with 4 CV of 70% ethanol and stored in 20% ethanol at 4°C. All SDS PAGE- and quantitative analyses were performed as described in Sections 2.1.1 and 2.1.2.

The column was regenerated between use by washing with 5 CV of 0.1 M NaOH followed by washing with 4 CV of 70% ethanol. A last wash cycle of 5 CV was performed with the equilibration buffer of 20 mM sodium phosphate, pH 7 where after the column was deemed ready for use.

## 2.5 Ultrafiltration

The samples obtained after albumin affinity chromatography (performed as in section 2.3) were analysed by SDS-PAGE to determine the presence of protein in each phase (loading-, wash-, eluate- and post-elute wash fractions) of the chromatography. Phases containing proteins were pooled and ultra-filtrated.

15 ml of sample was filtrated using ultrafiltration units from Amicon with a MWCO of 30 kDa. The filtration units were centrifuged at 4000 xg, for 30 minutes, at a temperature of 4°C. After centrifugation the resultant samples were concentrated to approximately 700 µl each, giving a concentration factor of 21.4. The retentate was removed from the

membrane by re-suspension in 20 mM sodium phosphate, pH 7. The filtration unit was discarded after use. The retentate and filtrate were stored at 4°C for further analyses by SDS-PAGE, BCA and PSMG ELISA.

## 2.6 Selected chromatography methods

### 2.6.1 Sample preparation

Bovine serum albumin or BSA (Roche) was prepared by mixing 0.2 µg of the commercially available compound with 1 ml of the appropriate binding buffer for the specific column. eCG was prepared by dissolving the commercially available, lyophilized powder (Chronogest®) in 5 ml of the appropriate binding buffer specific to the experimental column. When performing ion exchange with only eCG as sample, 1 ml of the above mentioned preparate was used.

When combining eCG and BSA for ion exchange chromatography, 0.2 µg of BSA was dissolved in 1 ml of eCG preparate obtained by the addition of 5 ml of the appropriate binding buffer to the commercially available, lyophilized powder. Spiked serum was obtained as described in Section 2.2.2. The serum was precipitated with TCA, as mentioned in Section 2.3.2, to deplete some of the highly abundant proteins and gain a clarified homogenised protein solution.

### 2.6.2 Cation exchange chromatography

A one millilitre sulfopropyl fast flow column (Cytiva) was used to perform manual cation exchange chromatography. The column was firstly washed with 10 ml of the elution buffer (20 mM Citrate buffer, pH 6.2 with 1 M NaCl) and then equilibrated with 10 ml of the binding buffer (20 mM Citrate buffer, pH 3.2). 1 ml of sample (BSA/eCG/eCG and BSA mixture/eCG-spiked equine serum) was loaded. The flow through fraction was collected. After this the column was washed with 5 ml of binding buffer. A stepwise pH gradient was used to elute the proteins starting with 5 ml of 20 mM Citrate buffer, pH 4.2. The pH gradient was applied as follows: pH 4.2 > pH 5.2 > pH 6.2 with the addition of 1 M NaCl for final elution. All fractions, 5 ml each, were collected and stored at 4°C before analysis by SDS-PAGE, PMSG ELISA and BCA.

### 2.6.3 Anion exchange chromatography

A one millilitre quaternary ammonium fast flow column (Cytiva) was used to perform anion exchange chromatography manually and by use of the Äkta Prime Plus. We tested three different methods of elution (**Table 2.2**) during the anion exchange chromatography.

**Table 2.2:** Buffers and elution methods used during anion exchange chromatography

| Method of elution | Binding buffer | Elution buffer |
|---|---|---|
| Stepwise pH gradient | 20 mM Citrate buffer, pH 6.2 | 20 mM Citrate buffer, pH 3.2 with 1 M NaCl |
| Continuous NaCl gradient | 20 mM Sodium phosphate buffer, pH 7 | 20 mM Sodium phosphate buffer, pH 7 with 1 M NaCl |
| Continuous pH gradient | 20 mM Sodium phosphate buffer, pH 7 | 20 mM Sodium phosphate buffer, pH 4 with 1 M NaCl |

The column was firstly washed with 10 ml of the applicable elution buffer (**Table 2.2**) and then equilibrated with 10 ml of the applicable binding buffer (**Table 2.2**). 1 ml of the sample (BSA/eCG/eCG and BSA mixture/eCG-spiked equine serum) was loaded. The flow through fraction was collected. After this the column was washed with 5 ml of the applicable binding buffer. The proteins were eluted using a stepwise pH gradient, continuous pH gradient or continuous NaCl gradient as shown in Table 3. Fractions of 5 ml were collected at all phases of the chromatography (wash, elution, post elution wash) and stored at 4°C before further analysis was performed.

### 2.6.4 Lectin affinity chromatography

For this chromatography experiment we used a one millilitre HiTrap® Con A 4B column from Cytiva. As the name suggests, this column comes prepacked with Con A which is a tetrameric metalloprotein isolated from the jack bean (*Canavalia ensiformis*). This specific lectin binds molecules containing α-D-glucopyranosyl, α-D-mannopyranosyl, and sterically related residues. Con A requires the presence of both $Ca^{2+}$ and $Mn^{2+}$ to maintain its binding characteristics and therefore these ions are included in the commercial columns in large excess.

We manually performed LAC on eCG-spiked equine serum. The column was cleaned with 10 ml of the elution buffer (20 mM Tris, 500 mM NaCl, 300 mM methyl-D-glucoside, pH 7.4) and then equilibrated with 10 ml of the binding buffer (20 mM Tris, 500 mM NaCl, 1 mM

$MnCl_2$, 1 mM $CaCl_2$, pH 7.4). 1 ml of the eCG-spiked equine serum (obtained as described in Section 2.2.2) was loaded onto the column and the flow through fraction collected. Following this the column was washed with 5 ml of the binding buffer before the proteins were eluted with 5 ml of the elution buffer. Fractions of all phases of the chromatography was collected and stored at 4°C until analysis via SDS-PAGE, BCA and PSMG ELISA.

### 2.5.5 Hydrophobic interaction chromatography

A one millilitre Octyl fast flow column (Cytiva) was used to manually perform HIC on eCG-spiked equine serum. After cleaning the column with 10 CV of elution buffer (50 mM sodium phosphate, pH 7.0) it was equilibrated with 10 CV of the binding buffer (50 mM sodium phosphate with 1.5 M ammonium sulphate, pH 7.0). 1 ml of the sample was loaded onto the column and the flow through fraction collected. The column was washed with 5 CV of the binding buffer before elution was performed. Fractions of all the phases of the chromatography was collected and stored at 4°C before analysis by SDS-PAGE, PSMG ELISA and BCA.

# Chapter 3: Results and discussion

## 3.1 Precipitation steps

### 3.1.1. The precipitation of eCG-spiked equine serum using MPA vs. TCA

TCA is one of the most commonly used acids in the precipitation of proteins and since it is less expensive than MPA we compared the two in the purification of eCG from eCG-spiked equine serum. In this study we compared precipitation using 0.5 M MPA to precipitation with TCA of 15% (v/v) and 25% (v/v) concentrations. 12% SDS-PAGE analysis was performed for the experiment (**Fig 3.1**).

As expected, most of the proteins precipitate out with each acid tested. There are indications of faint bands, at approximately 70 kDa, in the sample containing the supernatant obtained after precipitation with MPA (lanes 4) that is absent in precipitation with the two TCA concentrations. It is important to note that the concentrations of eCG that were used in this experiment is below that which is detectable on a SDS-PAGE gel with Coomassie staining. Therefore, these faint bands are most likely equine albumin.

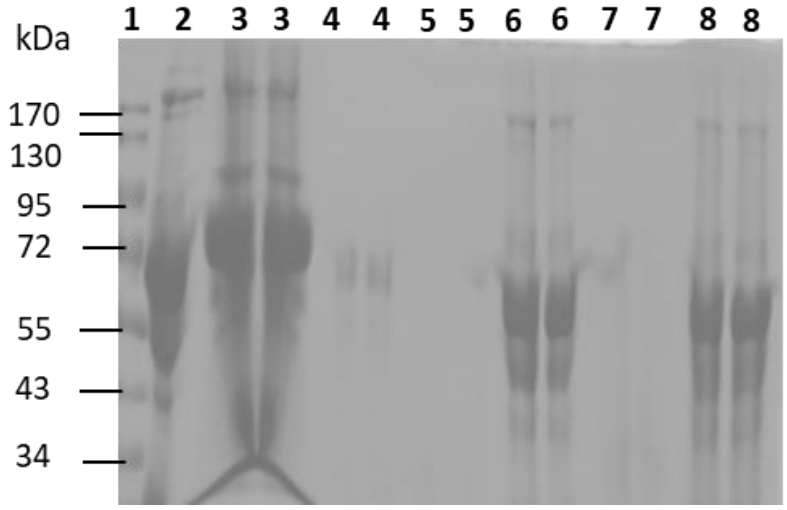


**Figure 3.1:** SDS-PAGE analysis of the precipitation of eCG-spiked equine serum using MPA vs. 15% (v/v) TCA vs. 25% (v/v) TCA. Lane 1 is the Page Ruler™ prestained protein ladder; lane 2 is the eCG-spiked serum; (from lanes 3 samples were analysed in duplicate) lanes 3 are the precipitate obtained after precipitation with MPA; lanes 4 are the supernatant obtained after precipitation with MPA; lanes 5 are the supernatant obtained after precipitation with 15% (v/v) TCA; lanes 6 are the precipitate obtained after precipitation with 15% (v/v) TCA; lanes 7 are the supernatant obtained after precipitation with 25% (v/v) TCA; and lanes 8 are the precipitant obtained after precipitation with 25% (v/v) TCA.

Looking at the quantitative results, it is important to note that the eCG content of the commercially available product was found to be lower than that stated by the manufacturer and also inter-batch inconsistent inconsistencies were evident. The eCG content in the eCG-spiked equine serum was therefore also much lower than expected. This complicated quantitative analysis by PMSG ELISA as the dilution factors had to constantly be adjusted with each new vial used to spike the equine serum.

Quantitative analysis, by BCA and PMSG analysis (**Table 3.1**), show that precipitation with 25% (v/v) TCA results in the highest eCG losses (61% of the starting amount of eCG). This could be due to the fact that the pI for TCA is much lower, at 0.77, than that of 0.5M MPA. There is also a total eCG decrease of 43% and 60% in the samples that were precipitated with 15% TCA (v/v) and 0.5M MPA respectively. Precipitation with 15% (v/v) TCA results in the highest eCG yield (57% of the original amount of eCG) between the three compared precipitation methods. However, when looking at the purification of eCG, 0.5M MPA is more efficient with a purification factor of 4.7 compared to 1.2 for 15% TCA and 1.03 for 25% TCA.

As previously mentioned the pI of eCG is 1.8 but due to the addition of an ethanol (EtOH) precipitation step (Gospodarowicz and Papkoff 1967) most of the glycoprotein hormone is expected to precipitate out after the complete process. Although results (**Table 3.1**) do show more eCG in the precipitate, high amounts of eCG remain in solution in the supernatant as shown for instance by the precipitation using 0.5M MPA where only 40% of the eCG is retained in the precipitate. Precipitation with 0.5M MPA shows the biggest difference in eCG content between the supernatant and the precipitate.

**Table 3.1:** Results of quantitative analysis, by BCA and PMSG ELISA, of the precipitation of eCG-spiked equine serum using 0.5M MPA, 15 (v/v) TCA and 25% (v/v) TCA.

| Sample | Total protein (mg) | Total eCG (mIU) | % Yield | Purification factor |
|---|---|---|---|---|
| eCG spiked equine serum | 46.4 | 14040 | 100 | NA |
| Supernant MPA | 2.9 | 1003 | 7 | 1.1 |
| Precipitate MPA | 4.0 | 5682 | 40 | 4.7 |
| Supernatant 15% TCA | 2.0 | 5523 | 39 | 9.1 |
| Precipitate 15% TCA | 21.2 | 7966 | 57 | 1.2 |
| Supernatant 25% TCA | 2.8 | 4222 | 30 | 5 |
| Precipitate 25% TCA | 17.3 | 5413 | 39 | 1 |

NA: Not Applicable

### 3.1.2 The precipitation of eCG-spiked equine serum with 15% TCA

Taking a more in depth look at the precipitation with 15% TCA, it can be seen in our 12% SDS-PAGE results (**Fig 3.2**) that the precipitation process is effectively reducing total proteins in the sample as the decrease in protein bands from left to right indicates. eCG is expected to remain in solution (lane 4) during the acid precipitation step and then precipitate out (lane 6) during the EtOH precipitation step. This can, however, not be confirmed by SDS-PAGE analysis alone as eCG does not show as a protein band at these concentrations, as previously mentioned.

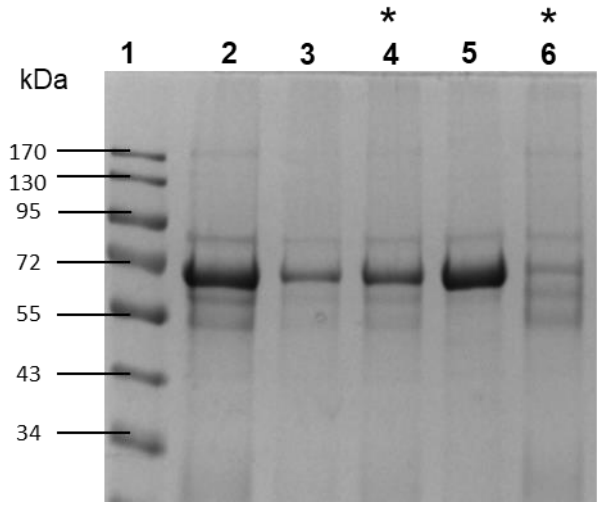


**Figure 3.2:** SDS-PAGE analysis of the precipitation of eCG-spiked equine serum using 15% TCA. Lane 1 is the Page Ruler™ prestained protein ladder; lane 2 is the eCG-spiked equine serum; lane 3 is the precipitate obtained after the acid precipitation step of the process; lane 4 is the supernatant obtained after the acid precipitation step of the process; lane 5 is the supernatant obtained after the EtOH precipitation step of the process; lane 6 is the precipitate obtained after the EtOH precipitation step of the process; (*) indicates where we expect to find the most eCG content.

We performed quantitative analysis by PMSG ELISA (**Table 3.2**). During the acid precipitation step approximately half of the eCG is lost with other precipitating proteins such as equine serum albumin. With the addition of 75% EtOH for precipitation, the process becomes even more ineffective at eCG retention as a 62% protein loss from the previous step is observed and, in the end, only retain 29% of the starting eCG. Most of the eCG remained in solution during this last step of the precipitation process which is contrary to what Gospodarowicz and Papkoff found in their study (Gospodarowicz and Papkoff 1967).

There is also a difference between the amounts of eCG found in the precipitate when the starting acid used was TCA versus MPA (**Table 3.1**). Since the last step (result generating step) in the precipitation process is an EtOH precipitation step this begs the question of the difference in the interaction of TCA with EtOH vs. MPA with EtOH.

**Table 3.2:** Results of quantitative analysis, by PMSG ELISA, of the precipitation of eCG-spiked equine serum using 15% TCA. (*) indicates where we expect to find the most eCG content.

| | Sample | Total eCG (mIU) | % Yield |
|---|---|---|---|
| | eCG spiked serum | 38005 | 100 |
| | Precipitate after acid precipitation step | 23100 | 61 |
| * | Supernatant after acid precipitation step | 28223 | 74 |
| | Supernatant after EtOH precipitation step | 17635 | 46 |
| * | Precipitation after EtOH precipitation step | 11179 | 29 |

Looking firstly at the action of EtOH in protein precipitation we see that, as the concentration of the organic solvent increases, it disrupts more and more hydrogen bonds between water molecules and polar amino-acid residues of the protein thereby disrupting and replacing the hydration shell around the protein molecules. The proteins then tend to aggregate and precipitate out. Now focusing on TCA and MPA, TCA is found to be the most polar acid of the two. This is due to structural differences in the molecules that cause TCA (**Fig 1.16**) to show a greater net dipole moment than MPA (**Fig 1.15**) (The cyclic structure of MPA cancels out most of its net dipole moment).

Therefore, looking at Table 3.2, when adding an equal volume of 75% cold EtOH to the acid solution at the second step in the precipitation process (Gospodarowicz and Papkoff 1967), it is possible that TCA favours more interaction with the hydroxyl group of EtOH compared to MPA (also see **Table 3.1**). This then inhibits EtOH, to a degree, from interacting with the water molecules in the hydration shell around the proteins and results in more proteins remaining in solution at this step than when using MPA as the first step in the precipitation process.

### 3.1.3 The precipitation of eCG-spiked equine serum using MPA vs. isopropanol containing 1% (v/v) TCA.

Using a slightly different approach, Liu *et al.* precipitated human serum albumin with isopropanol containing 1% TCA. In their study they tested a few different combinations of organic solvent and acids, one of which was formic acid (Liu et al. 2014). Since formic acid is less polar than MPA it would be interesting to see, in future research, if using this acid would result in more eCG precipitating out during the EtOH step of the process than using MPA or TCA as the first step in the precipitation process.

Lui and colleagues also found that using isopropanol containing 1% TCA precipitated 95% of the total albumin in human plasma while retaining more than 80% of three low abundance, therapeutic proteins that they tested for (Liu et al. 2014). We compared their method to precipitation with MPA.

12% SDS-PAGE analysis was performed (**Fig 3.3**). In the precipitation with isopropanol containing 1% TCA (v/v) (hereafter just isopropanol) more protein bands are visible in the supernatant (lanes 3) than the precipitate (lanes 4) and these seem to be approximately the size of equine serum albumin. This is due to the fact that unlike in the precipitation with EtOH, isopropanol precipitation should result in most of the eCG remaining in solution after the complete process. The quantitative results (**Table 3.3**) confirm this. Unfortunately, precipitation with isopropanol results in even higher eCG losses than that of MPA (87% compared to 60%). The comparison of the precipitation with isopropanol vs. MPA, results in a purification factor of 0.7 for isopropanol and 0.6 for MPA but due to excessive eCG loss with the Isopropanol method it was found inferior to the MPA method.

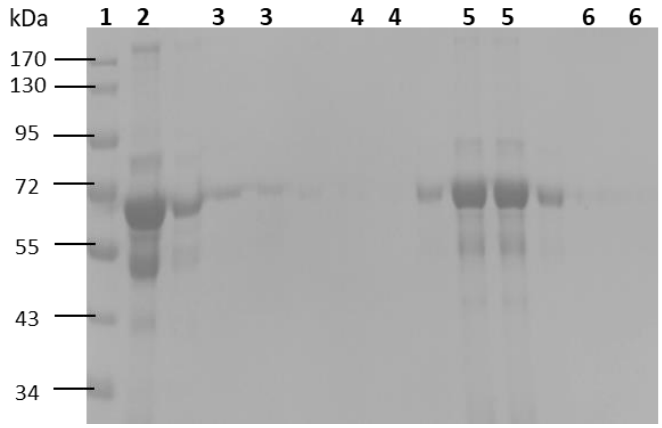


**Figure 3.3:** SDS-PAGE analysis of the precipitation of eCG-spiked equine serum using MPA vs. isopropanol. Lane 1 shows the Page Ruler™ prestained protein ladder; lane 2 is the eCG-spiked serum; (from lanes 3 samples were analysed in duplicate) lanes 3 are the supernatant obtained after

precipitation with isopropanol; lanes 4 are the precipitant obtained after precipitation with isopropanol; lanes 5 are the precipitate obtained after precipitation with MPA; and lanes 6 are the supernatant obtained after precipitation with MPA.

**Table 3.3:** Results of quantitative analysis, by BCA and PMSG ELISA, of the precipitation of eCG-spiked equine serum using MPA vs. isopropanol containing 1% (v/v) TCA.

| Sample | Total Protein (mg) | Total eCG (mIU) | % Yield | Purification factor |
|---|---|---|---|---|
| eCG spiked equine serum | 46.4 | 14040 | 100 | NA |
| Supernatant Isopropanol | 8.6 | 1870 | 13 | 0.7 |
| Precipitate Isopropanol | 8.7 | 425.8 | 3 | 0.2 |
| Supernatant MPA | 2.8 | 1445 | 10 | 1.7 |
| Precipitate MPA | 28.5 | 5562 | 40 | 0.6 |

NA: Not Applicable

## 3.2 Albumin affinity chromatography

As previously mentioned albumin remains the biggest challenge in the purification of eCG from equine serum. We investigated the efficiency of using an albumin affinity column in the purification of eCG from equine serum. 12% SDS-PAGE analysis was performed on fractions obtained through the chromatography process (**Fig 3.4**). It is clear that the affinity column does bind albumin, as claimed by the manufacturers, but no conclusion about its interaction with eCG can be drawn from the SDS-PAGE results.

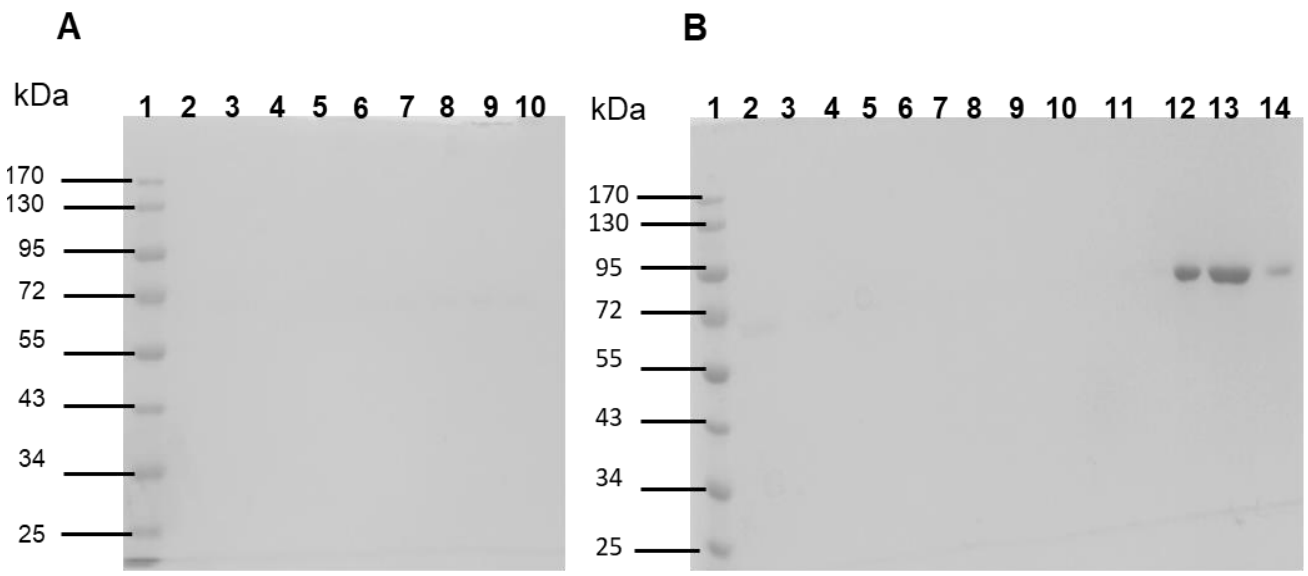


**Figure 3.4:** Results of 12% SDS-PAGE analysis after albumin affinity chromatography. Gel **(A)** and **(B)** show the Page Ruler™ prestained protein ladder in lane 1. **(A)** Lanes 2-10 show wash fractions 1-9. **(B)** Lanes 2-10 show post elution wash fractions 1-9; lanes 11-14 show elution fractions 1-4.

The quantitative results (**Table 3.4**) unfortunately shows that the column also binds eCG as none of the protein is found in the wash and post elution wash fractions.

**Table 3.4:** Results of quantitative analysis, by BCA and PMSG ELISA, for albumin affinity chromatography performed on eCG-spiked serum.

| Sample | Total Protein mg | Total eCG mIU |
|---|---|---|
| Pooled Wash | ND | ND |
| Pooled Eluate | 559.6 | 1918.2 |
| Pooled post elution | ND | ND |

ND: Not Detected

Therefore, both albumin and eCG bind the albumin affinity column and it can be assumed that either the resin also displays an affinity for eCG or albumin and eCG might interact in some way.

It is well known that albumin reversibly binds a great number of exogenous and endogenous compounds. Albumin also acts as a carrier for thyroid hormones (Oppenheimer 1968). The conformational adaptability of albumin plays an important role in its binding capacity as cooperativity and allosteric modulation takes place among its binding sites causing the

protein to act more like a multimeric protein. Under physiological conditions albumin maintains a strong negative charge, however it binds reversibly to both cations and anions (Varshney et al. 2010). Keeping the above mentioned facts in mind it the possibility of an eCG-albumin interaction cannot be excluded. Since the albumin affinity column that was used binds albumin by ionic -and hydrophobic interactions it would also be possible to also bind eCG as both eCG and albumin contain a net positive charge at pH 7.

Elution by continuous salt gradient was not performed as by the time we performed this experiment it was already clear from ion exchange experiments that using a salt gradient was ineffective at separating the elution albumin and eCG.

## 3.3 Ultrafiltration

It is important to note that the eCG-spiked serum was first precipitated with TCA and then run on the albumin affinity chromatography column resulting in a clear homogenous solution on which we performed ultrafiltration. UF units from Amicon with a MWCO of 30 kDa was used to investigate the efficiency of including this step in the purification of eCG from equine serum.

Table 3.5 provides a summary of the quantitative results. Unfortunately, high eCG losses is seen from the results as the 60 kDa protein is expected to remain in the retentate when using a MWCO of 30 kDa. Only 3% of the glycoprotein hormone is retained.

**Table 3.5:** Results of quantitative analysis, by BCA and PMSG ELISA, for ultrafiltration performed on eCG-spiked equine serum.

| Sample | Total protein (mg) | Total eCG (mIU) | % Yield |
|---|---|---|---|
| eCG spiked serum | 89.5 | 143001 | 100 |
| Retentate | 7.2 | 3906 | 3 |
| Filtrate | 2.1 | ND | NA |

NA: Not Applicable

The possible reasons for poor separation obtained in protein ultrafiltration were reviewed by Nakatsuka and Michaels (1992). They suggested that the formation of a protein deposit on the retentate surface of the membrane and also protein-protein interactions in the bulk

solution could contribute to membrane fouling which is a major problem impairing effective separation in ultrafiltration. Another of their reasons included protein adsorption within the porous structure of the membrane (Nakatsuka and Michaels 1992). Previous purification work by a colleague reminded us about eCG's affinity for membranes (N. Lekena, personal communication). Therefore, we propose that perhaps a combination of the three reasons mentioned above might be responsible for the high total protein and eCG losses that our results show.

Research has shown that it is possible to effectively separate proteins with fairly similar molecular weight, via ultrafiltration, by adjusting the pH and salt concentration of the solution. This is due to the electrostatic interactions between the proteins and membrane as well as a so-called electrostatic self-rejection effect. (Saksena and Zydney 1994; Ehsani et al. 1997; Li et al. 1997; Ghosh and Cui 1998). It would be interesting to see if adjusting the pH and/or salt concentration could firstly result in higher eCG content for the retentate and then also separate eCG and albumin.

## 3.4 Selected chromatography methods

### 3.4.1 Ion exchange chromatography

a) Cation exchange

The pI of BSA is 5; therefore at a pH below 5 the protein should contain a net positive charge and bind a cation exchange column, when loaded in a binding buffer of pH 3.2, as was confirmed by 12% SDS-PAGE results (**Fig 3.5**). A protein band, of approximately 70 kDa, is seen at the fractions eluted with a citrate buffer at pH 5.2. There are also similar sized, faint bands visible at the fractions eluted with a citrate buffer at pH 6.2.

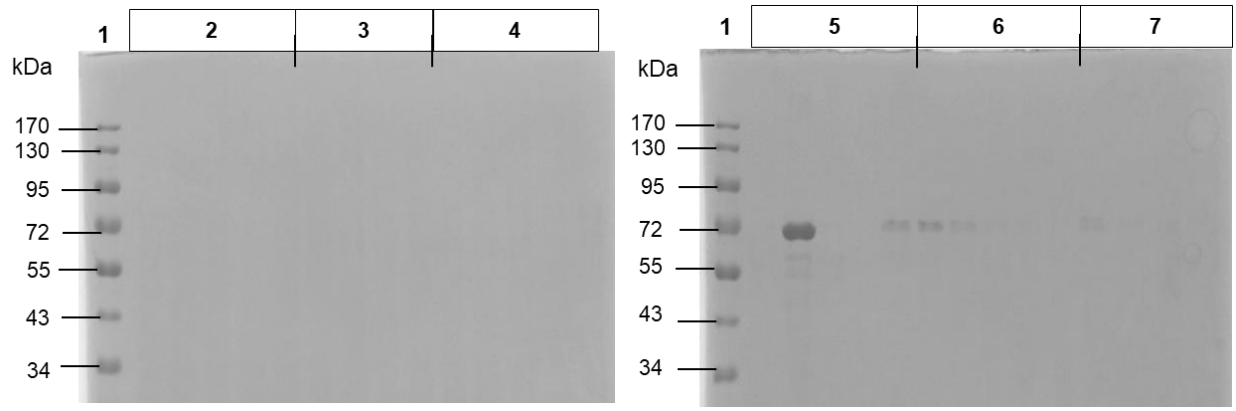


**Figure 3.5:** Results of 12% SDS-PAGE analysis after cation exchange chromatography on BSA eluted with a stepwise pH gradient. Lanes 1 are the Page Ruler™ prestained protein ladder; lanes 2

are the flow through fractions; lanes 3 are the wash fractions; lanes 4 show elution with pH 4.2; lanes 5 show elution with pH 5.2; lanes 6 show elution with pH 6.2 and lanes 7 show elution with pH 6.2 containing 1M NaCl.

12% SDS-PAGE analysis was performed on fractions obtained after cation exchange on commercially available eCG (**Fig 3.6 A**) and on a mixture of eCG+BSA (**Fig 3.6 B**). As previously mentioned, no visible protein bands for eCG were found at the concentrations that were used. Quantitative analysis (**Table 3.6**), by BCA, show that although there are no bands present we see most of the eCG in the flow through with no other fractions containing the protein. Therefore, a conclusion can be made that commercially available eCG did not bind the cation exchange column. This is as expected for the glycoprotein hormone with its pI of 1.8.

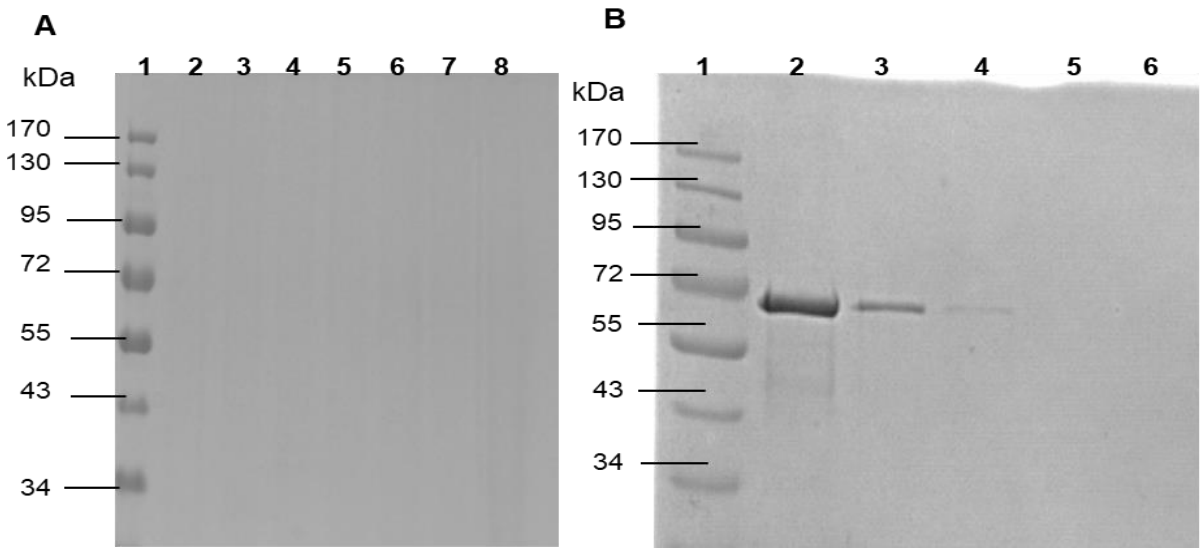


**Figure 3.6:** Results of 12% SDS-PAGE analysis after cation exchange on eCG **(A)** and eCG+BSA **(B)**. Lanes 1 in **(A)** and **(B)** show the Page Ruler™ prestained protein ladder. **(A)** Lane 2 is commercially available eCG; lane 3 is the flow through; lane 4 is the wash fraction; lane 5 shows elution with pH 4.2; lane 6 shows elution with pH 5.2; lane 7 shows elution with pH 6.2; lane 8 shows elution with a citrate buffer pH 6.2 containing 1M NaCl. **(B)** Lane 2 is the flow through; lane 3 is the wash fraction; lane 4 shows elution with pH 5.2; lane 5 shows elution with pH 6.2 and lane 6 shows elution with pH 6 and 1M NaCl.

It is interesting that when a mixture of eCG and BSA is run on the same cation exchange column (**Fig 3.6 B**) we now see a band, similar to the molecular weight of BSA, in the flow through and wash fractions. No conclusion can be made about eCG by only looking at the SDS PAGE results, the quantitative analysis (**Table 3.7**) shows that eCG also behaves different and is present in every fraction in approximately equal amounts.

Therefore, when mixing commercially available eCG and BSA this resulted in altered column binding properties for both proteins (**Fig 3.6 B**). Albumin is known to exhibit allosteric changes when binding proteins (Varshney et al. 2010) and Fenton and Hutchinson showed that these allosteric changes can be regulated by pH (Fenton and Hutchinson 2009). Therefore the reverse is also possible, that allosteric changes in proteins could affect their pI but without further investigation we cannot be sure of the exact interaction that takes place between eCG and BSA in solution.

**Table 3.6:** Results of quantitative analysis, by BCA, after cation exchange on eCG.

| Sample | Total protein (mg) |
|---|---|
| eCG | 0.5 |
| Flow through | 0.6 |
| Wash | ND |
| pH 4.2 | ND |
| pH 5.2 | ND |
| pH 6.2 | ND |
| pH 6.2 & NaCl | ND |

ND: Not Detected

**Table 3.7:** Results of quantitative analysis, by PMSG ELISA, after cation exchange of eCG+BSA.

| Sample | eCG (mIU) |
|---|---|
| Flow through | 28.5 |
| Wash | 23.6 |
| pH 5.2 | 28.7 |
| pH 6.2 | 29.7 |
| pH 6.2 + 1M NaCl | 28.3 |

b) Anion exchange

Manual anion exchange chromatography was performed using a stepwise pH gradient to elute the proteins. 12% SDS-PAGE analysis was performed on fractions obtained after running pure commercially available BSA on the column (**Fig 3.7 A**). One band is present in lane 3, the flow through, which corresponds to the proteins' size. A fainter band of the same size is also marked in lane 4, the wash fraction, with no other visible bands. Therefore, BSA did not bind the column at pH 6.2.

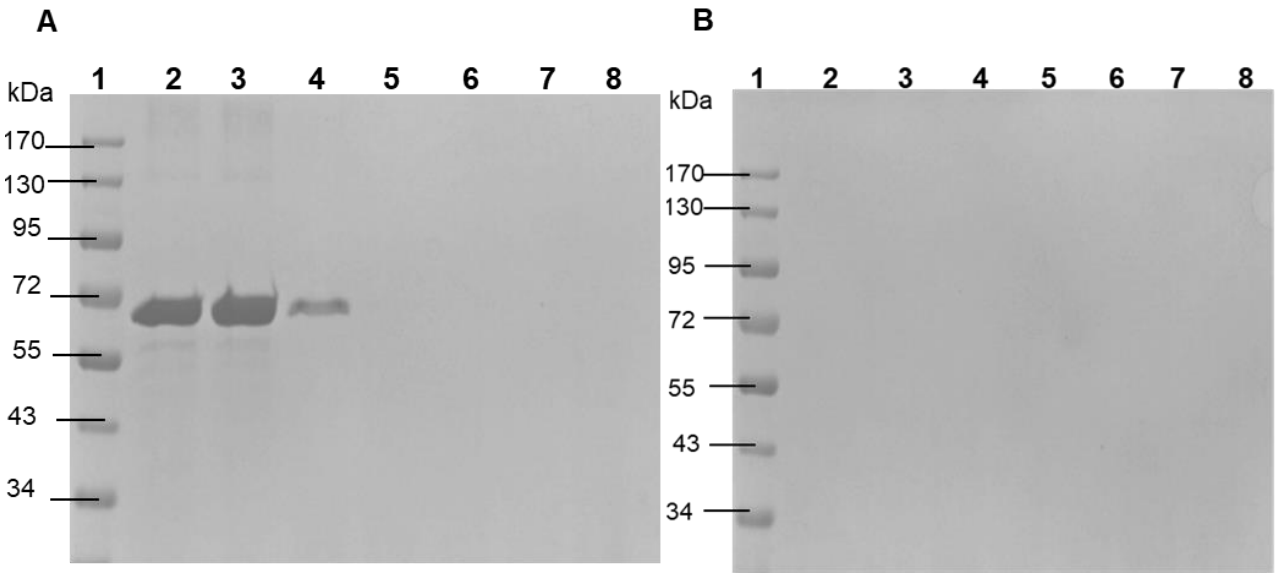


**Figure 3.7:** Results of 12% SDS-PAGE analysis after anion exchange on BSA **(A)** and eCG **(B)**. Lanes 1 in both **(A)** and **(B)** show the Page Ruler™ prestained protein ladder. **(A)** Lane 2 shows commercially available BSA; **(B)** lane 2 shows commercially available eCG; for both **(A)** and **(B)** lane 3 shows the flow through; lane 4 shows the wash; lane 5 shows elution with pH 5.2; lane 6 shows elution with pH 4.2; lane 7 shows elution with pH 3 and lane 8 shows elution with pH 3 & 1M NaCl.

**Table 3.8:** Quantitative results, via BCA, after anion exchange on BSA.

| Sample | Total protein (mg) |
|---|---|
| BSA | 2.8 |
| Flow through | 2.1 |
| Wash | 0.1 |
| pH 5.2 | ND |
| pH 4.2 | ND |
| pH 3.2 | ND |
| pH 3.2 & 1 M NaCl | ND |

ND: Not Detected

This is quantitatively confirmed as there is no protein present in any of the elution fractions (**Table 3.8**). 12 % SDS-PAGE analysis was also performed on fractions obtained after running commercially available eCG on the same anion exchange column (**Fig 3.7 B**). Once again no bands can be seen at these concentrations of eCG but the quantitative results (**Table 3.9**) show only protein content in the flow through fraction, indicating that eCG also didn't bind the column at pH 6.2.

**Table 3.9:** Quantitative results, via BCA, after anion exchange on eCG.

| Sample | Total protein (mg) |
|---|---|
| eCG | 0.7 |
| Flow through | 0.6 |
| Wash | ND |
| pH 5.2 | ND |
| pH 4.2 | ND |
| pH 3.2 | ND |
| pH 3.2 & 1M NaCl | ND |

ND: Not Detected

In this experiment BSA as well as eCG was expected to bind the column, with the BSA eluting first when applying a elution buffer with a pH lower than 5 and lastly it was planned to elute eCG with the addition of salt. However, results (**Tables 3.8 & 3.9**) showed that neither of the proteins bound the column when applied separately though column regeneration was performed multiple times. The experiment was then repeated but this time eluting with a continuous salt gradient. When using the Äkta prime plus both proteins were seen binding the column. It is possible that too much pressure was applied during the manual anion exchange chromatography causing the inhibition of protein binding.

In chromatograms 1-5 that follows, the blue graph indicates the UV absorbance measured at 280 nm. The red graph shows the conductivity of the solution. The green graph shows the concentration of $NaCl/H^+$. In chromatograms 2-5 the size of the black dot indicates the amount of eCG in the fractions, so that a big dot shows a large amount of eCG and a small dot shows a small amount of eCG. Fractions with no dots contain no eCG. In chromatogram 4 the pink graph shows the pH.

Chromatogram 1 shows pure BSA on the column starting to elute just as the conductivity of the solution (red graph) starts to rise. Similarly, in Chromatogram 2, eCG also elutes at the start of the conductivity increase however is shows a double peak profile with most of the eCG in the higher of the two. No other fractions tested contained eCG. Chromatogram 2 shows that the commercially available eCG is not completely pure as the first peak seems very similar to that of Chromatogram 1 where we ran only albumin. It is also possible that the different peaks in Chromatogram 2 represent different isoforms of the protein.

When running a mixture of eCG and BSA on the column (**Fig 3.11)** we see another double peak profile however this time the highest peak is much higher than in Chromatogram 2. Once again this highest peak contains most of the eCG with smaller amounts of the protein detected in the small peak. Therefore the addition of BSA results in a superimposed peak and we can rule out the possibility that the two peaks of Chromatogram 2 could be different isoforms of eCG. It is also clear from this chromatogram that eCG co-elutes with BSA, rending this elution method ineffective at separating the protein.

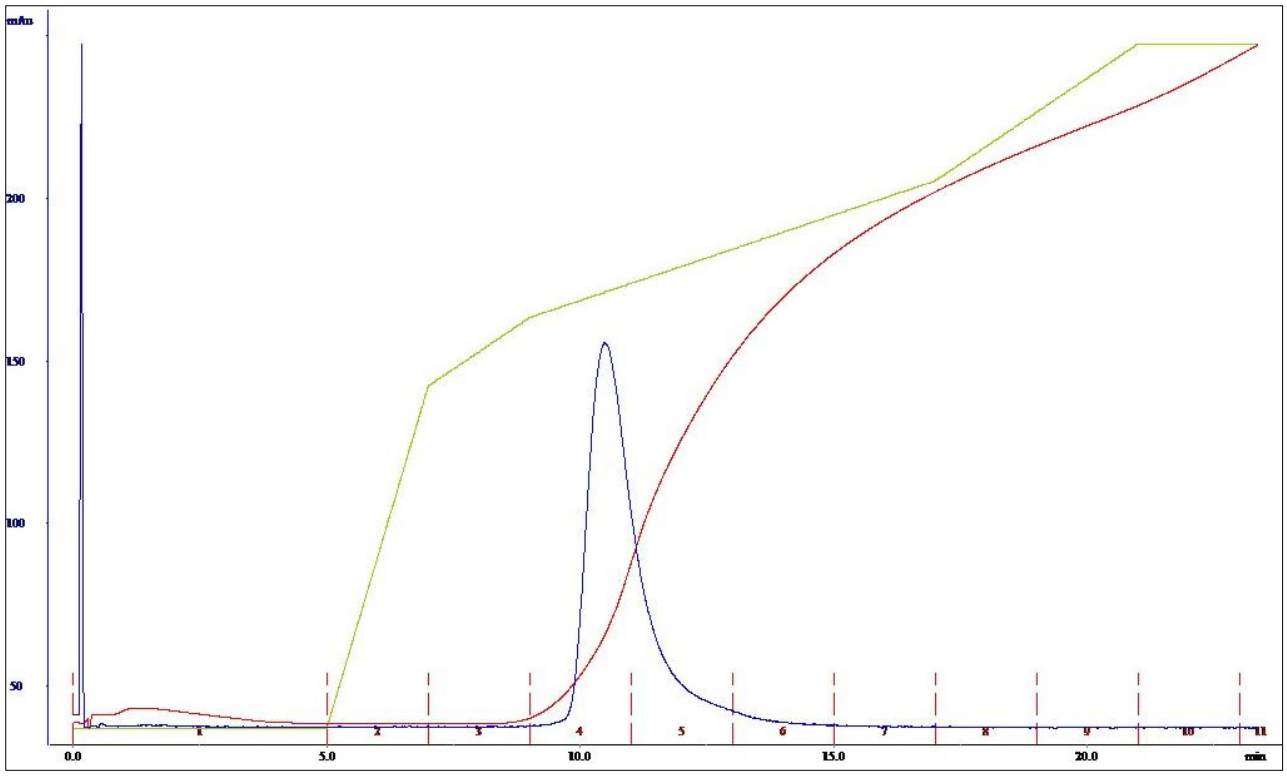


**Figure 3.9:** BSA on an anion exchange column eluted with a salt gradient.

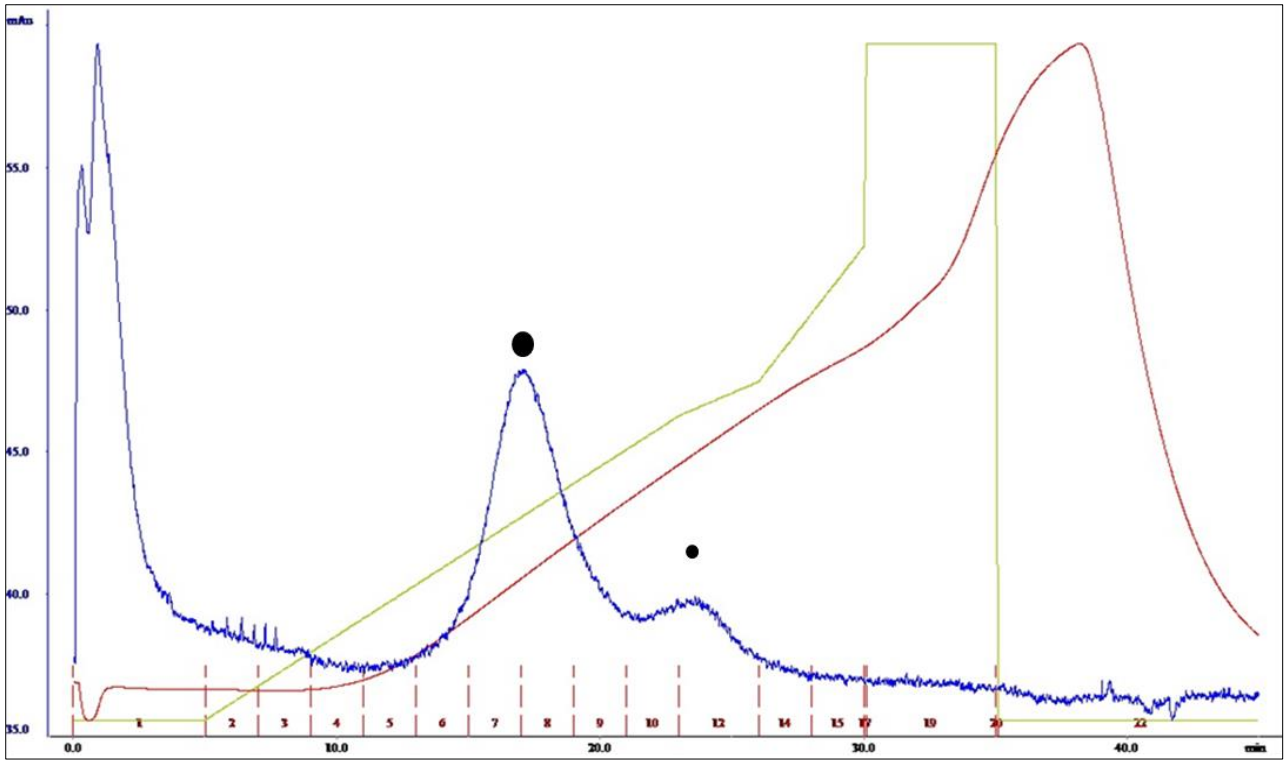


**Figure 3.10:** eCG on an anion exchange column eluted with salt gradient.

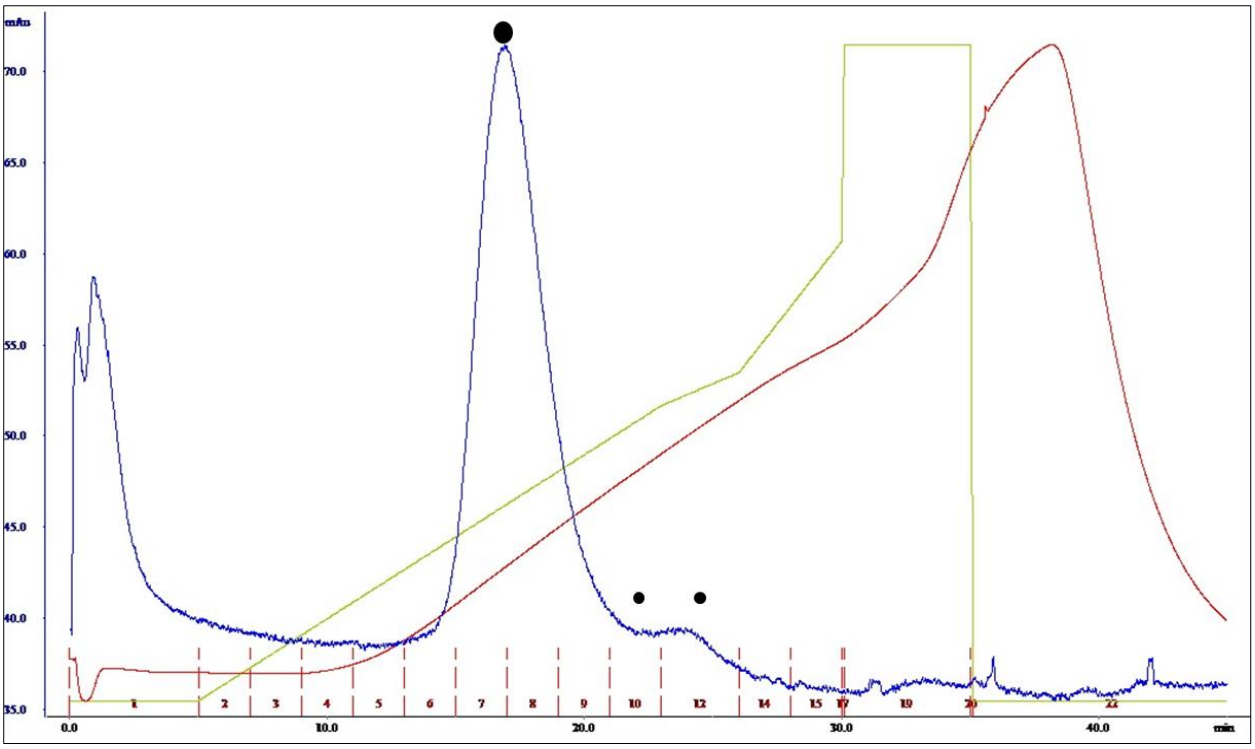


**Figure 3.11:** A mixture of eCG and BSA on an anion exchange column eluted with salt gradient.

We wanted to see what effect the presence of various other proteins, in equine serum, would be on the eCG and albumin profile. Figure 3.10 shows a very similar profile to running a mixture of commercially available BSA and eCG albeit much smaller peaks. The eCG content of the two peaks are also approximately equal in contrast to that of the peaks in Figure 3.11.

Chromatogram 5 shows the results for running a mixture of BSA and eCG on an anion exchange column eluting with a continuous pH gradient. A double peak profile is once again generated, around the point where the pH of the solution is 5, with the highest peak containing most of the eCG. Therefore it seems that once again, when in combination, the proteins exhibit some form of interaction which alters their column binding properties. In this case eCG now elutes from the column early, at a pH of 5. This is very interesting and future research on the exact interaction between the two proteins would be helpful.

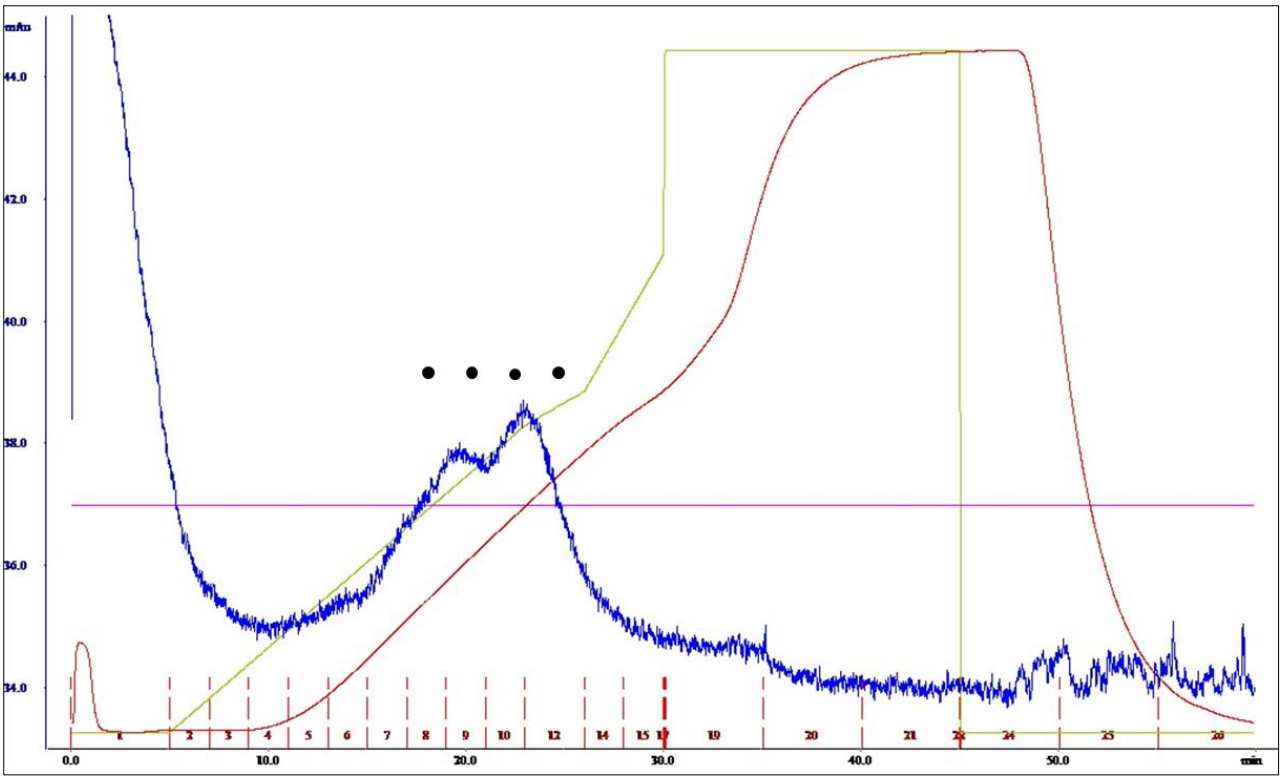


**Figure 3.12:** eCG-spiked equine serum on an anion exchange column eluted with a salt gradient.

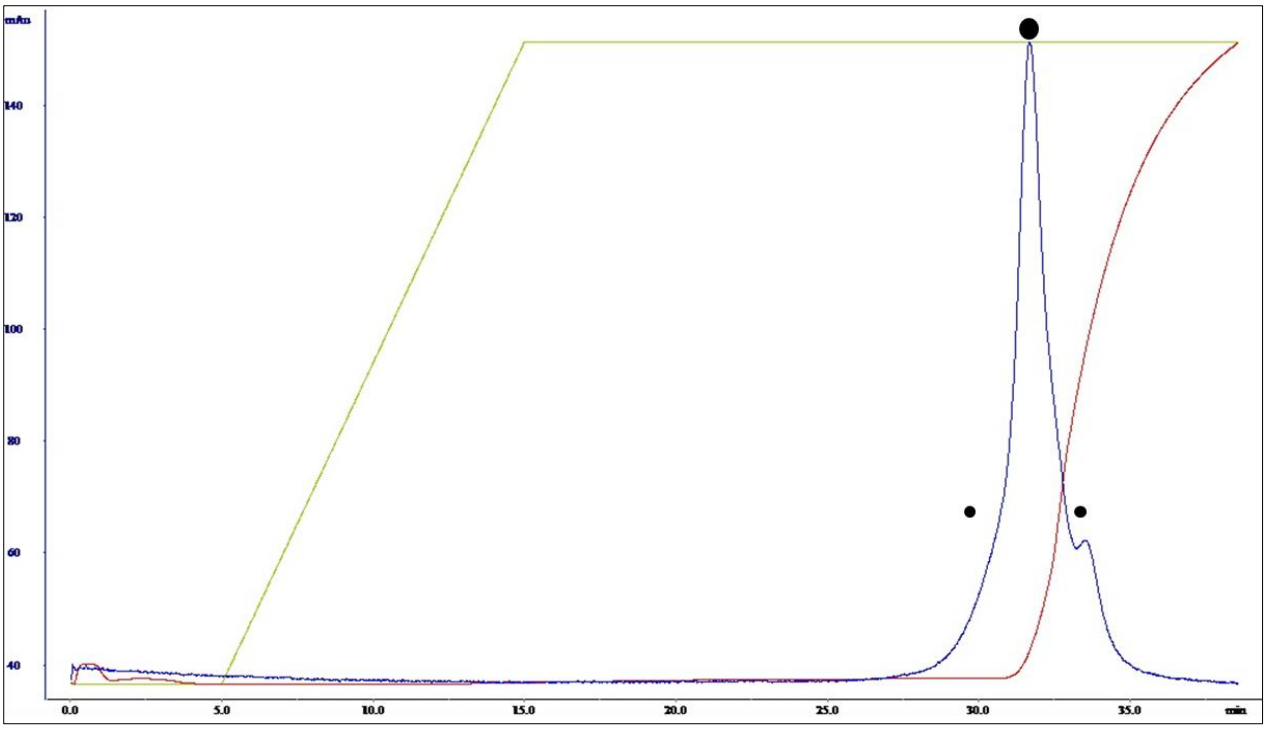


**Figure 3.13:** A mixture of eCG and BSA on an anion exchange column eluted with a pH gradient.

### 3.4.2 Lectin affinity chromatography (LAC)

Since eCG is a highly glycosylated protein we performed lectin affinity chromatography in order to isolate the glycoprotein hormone. 12% SDS-PAGE analysis was performed on fractions obtained in the experiment (**Fig 3.14**). Multiple bands can be seen in the flow through fractions (lanes 2) as un-glycosylated proteins are not expected to bind the column. Bands around the 70 kDa and 52 kDa mark can be seen in the first 3 wash fractions (lanes 3). No bands are visible in either the elution (lanes 4) –or post elution wash fractions (lanes 5), leaving the question as to whether the proteins are still bound to the column or none of the proteins bound the column.

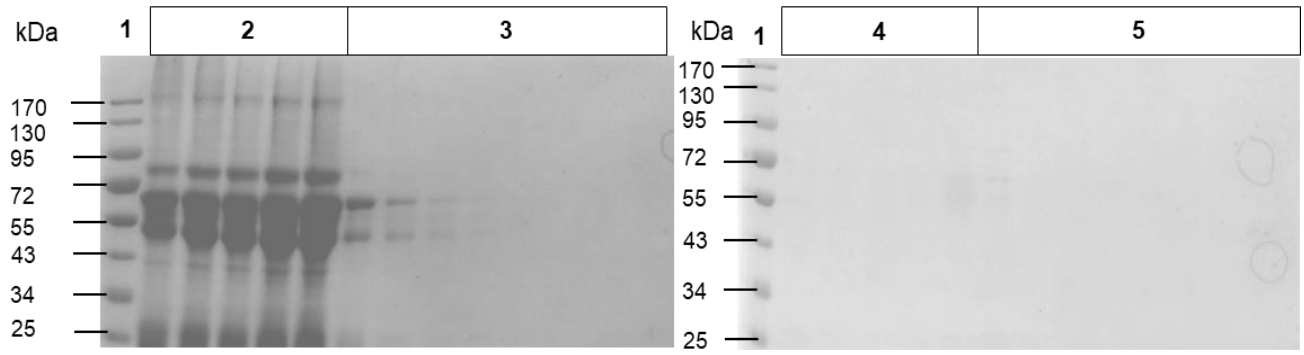


**Figure 3.14:** Results of 12% SDS-PAGE after LAC on eCG-spiked equine serum. Lanes 1 show the Page Ruler™ prestained protein ladder; lanes 2 show the flow through fractions; lanes 3 show the wash fractions; lanes 4 show the elution fractions and lanes 5 show the post elution wash fractions.

However, this question is answered in the quantitative analysis (**Table 12**) as high eCG losses is seen. The sum of the eCG found in the flow through, wash, eluate and post elution wash fractions do not add up close to that of the starting sample. This suggests that the eCG is still bound to the column. Most of the total protein also did not bind the column however the second highest amount of eCG is found in the eluate fraction, indicating that eCG did bind the column. This is the first chromatography method investigated that seems to separate the two proteins. The reason for its success being the fact that albumin is a non-glycosylated protein (Varshney et al. 2010). Unfortunately the inability to elute the eCG results in excessive losses of the protein. It would be interesting to try and further optimize this chromatography method in future work.

**Table 12:** Results of quantitative analysis, by BCA and PSMG ELISA, after LAC on eCG-spiked serum.

| Sample | Total Protein (mg) | Total eCG (mIU) | % Yield | Purification factor |
|---|---|---|---|---|
| eCG spiked serum | 6.1 | 793.3 | 100 | NA |
| Flow through | 7.5 | 190.3 | 24 | 0.2 |
| Wash | 1.9 | 76.5 | 10 | 0.3 |
| Eluate | 1.0 | 112.3 | 14 | 3.7 |
| Post Elution wash | 0.8 | 55.1 | 7 | 0.6 |

NA: Not Applicable

### 3.4.3 Hydrophobic interaction chromatography (HIC)

When performing HIC eCG-spiked serum was firstly precipitated with MPA of a pH 2 or pH 2.8. The SDS-PAGE results are displayed in Figure 3.9. The multitudes of protein in the undiluted lanes 2, 3 and 4 containing the eCG-spiked serum, sample obtained after precipitation with MPA at a pH of 2 and a pH of 2.8 formed a protein smear on the gel. However, protein bands can be seen of approximately albumin's size (70 kDa), in the wash, elution and post wash fractions. It is also noted that the bands in lanes 7 and 8 (elution fractions for both pH's) are more distinct than those in lanes 5, 6, 9 and 10.

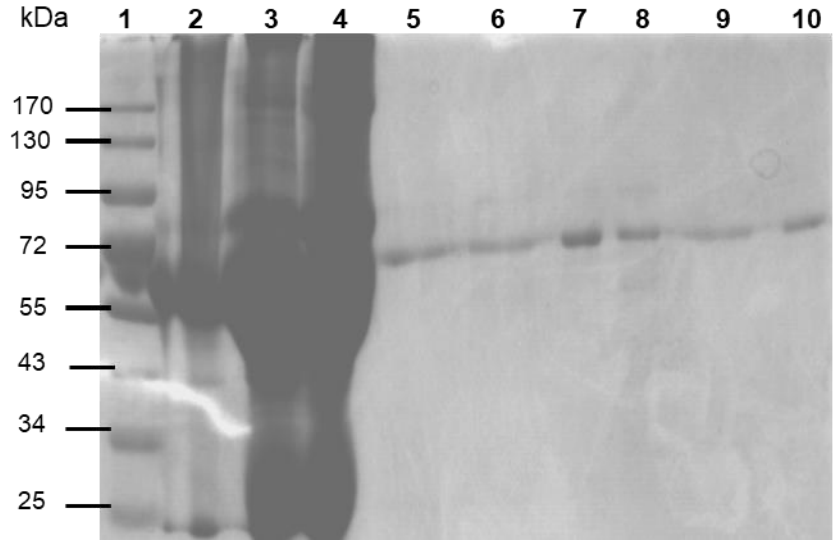


**Figure 3.15:** Results of 12% SDS-PAGE analysis after HIC on eCG-spiked serum. Lane 1 shows the Page Ruler™ prestained protein ladder; lane 2 shows eCG-spiked serum; lane 3 shows sample

obtained after precipitation with MPA at a pH of 2; lane 4 shows sample obtained after precipitation with MPA at a pH of 2.8; lane 5 shows the wash fraction (of sample precipitated with pH 2 hereafter mentioned just as pH 2); lane 6 shows the wash fraction (of sample precipitated with pH 2.8 hereafter mentioned just as pH 2.8); lane 7 shows the elution fraction (pH 2); lane 8 shows the elution fraction (pH 2.8); lane 9 shows the post elution wash fraction (pH 2) and lane 10 shows the post elution wash fraction (pH 2.8).

As previously mentioned it was found that the eCG content in the commercially available products varied greatly and that is the reason for the very low eCG concentration in this experiment. The results of the BCA and PSMG ELISA analysis (**Table 13**) show that most of the total protein as well as a high amount of the eCG is found in the eluate and post elution wash fractions. The highest amount of total protein is found in the eluate where the highest amount of eCG is in the post elution wash fraction suggesting that the column might bind both albumin and eCG but elute albumin before eCG. Once again this chromatography method seems to separate albumin and eCG (**Table 13**) and even provide a higher purification factor (9.43) compared to LAC (3.7). Future work should focus on repeating the experiment with greater eCG concentrations and optimization.

**Table 13:** Results of quantitative analysis, by BCA and PSMG ELISA, after HIC on eCG-spiked serum.

| Sample | Total protein (mg) | Total eCG (mIU) | Purification factor |
|---|---|---|---|
| eCG spiked serum | 15.9 | 0.596 | NA |
| Wash | 2.9 | 0.16 | 1.49 |
| Eluate | 6.5 | 0.65 | 2.7 |
| Post elution wash | 4.3 | 1.50 | 9.43 |

NA: Not Applicable

# Chapter 4: Conclusion

eCG is a glycoprotein hormone widely used in agriculture and animal breeding programs because of its dual hormonal activity in non-equine species which promotes ovulation and the onset of oestrous.

Since eCG is still mostly obtained from the serum of pregnant mares it necessitates highly efficient purification strategies. During our investigation of several of these purification strategies we found that for the precipitation steps, MPA remained the most effective precipitation agent although this resulted in high eCG losses. Equine serum albumin remains the biggest problem in the isolation of eCG from serum due to its similar molecular weight as well as possible protein-protein interactions between the two proteins resulting in the alteration of their ionic properties. These changes prevent the use of ligand affinity and ion exchange in the separation of the two proteins. Lectin affinity chromatography displays positive results for the separation of eCG and albumin but future research should focus on methods to better elute the eCG from the resin. Hydrophobic interaction chromatography showed good separation of eCG and albumin on small scale.

# References


Acikara BO (2013) Ion-Exchange Chromatography and Its Applications. Column Chromatogr. https://doi.org/10.5772/55744

Adams BA, Holmes EL (1935) Adsorptive properties of synthetic resins. J Chem Soc 54:1–6

Aggarwal BB, Farmer SW, Papkoff H, Stewart F, Allen WR (1980) Purification and characterization of the gonadotropin secreted by cultured horse trophoblast cells. Endocrinology 106:1755–1759. https://doi.org/10.1210/endo-106-6-1755

Allen WR (1969a) The immunological measurement of pregnant mare serum gonadotrophin. J Endocrinol 43:593–598. https://doi.org/10.1677/joe.0.0430593

Allen WR (1969b) Factors influencing Pregnant Mare Serum Gonadotropin production. Nat Publ Gr 223:64–66

Allen WR, Short R V. (1997) Interspecific and extraspecific pregnancies in equids: Anything goes. J Hered 88:384–392. https://doi.org/10.1093/oxfordjournals.jhered.a023123

Alvarez RH, Natal F, Almeida B, Oliveira J, Melo A, Ribela M, Bartolini P (2017) Biological Activity of Different Batches of Equine Chorionic Gonadotropin as Determined by Reversed-phase High-performance Liquid Chromatography and in vivo Assay. J Adv Med Pharm Sci 12:1–9. https://doi.org/10.9734/jamps/2017/31956

Amoroso EC, Hancock JL, Rowlands IW (1948) Ovarian activity in the pregnant mare. Nature 161:355. https://doi.org/10.1038/161355a0

Anderson NL, Anderson NG (2002) The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 1:845–867. https://doi.org/10.1074/mcp.R200007-MCP200

Andersson H (2008) Clinical Reproductive Endocrinology. In: Clinical Biochemistry of Domestic Animals. Elsevier, pp 635–662

Animal Welfare Foundation (2022) Production of PMSG in South America. https://www.animal-welfare-foundation.org/service/dossiers/production-of-pmsg-in-south-america. Accessed 20 Oct 2022

Arakawa T (1986) Thermodynamic analysis of the effect of concentrated salts on protein interaction with hydrophobic and polysaccharide columns. Arch Biochem Biophys 248:101–105. https://doi.org/10.1016/0003-9861(86)90405-4

Bahrdt A, Anal Z (1927) The volumetric determination of sulfuric acid (sulfate son) in drinking water and in industrial waters. Chem Ind 70:109–119

Baieli MF, Urtasun N, Hirsch DB, Miranda MV, Cascone O, Wolman FJ (2020) Single-step purification of equine chorionic gonadotrophin directly from plasma using affinity chromatography. J Biotechnol 323:174–179. https://doi.org/10.1016/j.jbiotec.2020.08.004

Baieli MF, Urtasun N, Martinez MJ, Hirsch DB, Pilosof AMR, Miranda M V., Cascone O, Wolman FJ (2017) Affinity chromatography matrices for depletion and purification of casein glycomacropeptide from bovine whey. Biotechnol Prog 33:171–180. https://doi.org/10.1002/btpr.2404

Bhardwaj A, Nayan V, Sharma P, Kumar S, Pal Y, Singh J (2017) Molecular characterization, modeling, in silico analysis of equine pituitary gonadotropin alpha

subunit and docking interaction studies with ganirelix. Silico Pharmacol 5:5. https://doi.org/10.1007/s40203-017-0025-1

Bohme HJ, Schulz G, Hoffman E (1972) Affinity chromatography of phosphofructokinase using Cibacron blue F3G-A. J Chromatogr A 69:209–214

Bousfield GR, Butnev VY, Butnev VY (2001) Identification of Twelve O-Glycosylation Sites in Equine Chorionic Gonadotropin β and Equine Luteinizing Hormone β by Solid-Phase Edman Degradation1. Biol Reprod 64:136–147. https://doi.org/10.1095/biolreprod64.1.136

Bousfield GR, Liu WK, Sugino H, Ward DN (1987) Structural studies on equine glycoprotein hormones. Amino acid sequence of equine lutropin beta-subunit. J Biol Chem 262:8610–20

Bousfield GR, Sugino H, Ward DN (1985) Demonstration of a COOH-terminal extension on equine lutropin by means of a common acid-labile bond in equine lutropin and equine chorionic gonadotropin. J Biol Chem 260:9531–9533. https://doi.org/10.1016/s0021-9258(17)39266-9

Briggs DR (1940) the Metaphosphoric Acid-Protein Reaction. J Biol Chem 134:261–272. https://doi.org/10.1016/s0021-9258(18)73268-7

Burn JH, Finney DJ, Goodwin LG (1950) Biological standardization, 2nd Ed. O.U.P., London, 1950, London

Cartland GF, Nelson JW (1937) the Preparation and Purification of Extracts Containing the Gonad-Stimulating Hormone of Pregnant Mare Serum. J Biol Chem 119:59–67. https://doi.org/10.1016/s0021-9258(18)74431-1

Chalkley RJ, Thalhammer A, Schoepfer R, Burlingame AL (2009) Identification of protein O-GlcNAcylation sites using electron transfer dissociation mass spectrometry on native peptides. 1–6

Chandran YA, Hermadi HA, Hestianah EP (2017) The Application of Equine Chorionic Gonadotropin (Ecg) and Prostaglandin F2α to Increase the Rate of Pregnancy in Bali Cattle at Buleleng, Bali. KnE Life Sci 3:734. https://doi.org/10.18502/kls.v3i6.1204

Chemistry (2017) What is the difference between metaphosphoric acid and phosphonic acid? https://chemistry.stackexchange.com/questions/71030/what-is-the-difference-between-metaphosphoric-acid-and-phosphonic-acid. Accessed 28 Oct 2022

Chopineau M, Maurel MC, Combarnous Y, Durand P (1993) Topography of equine chorionic gonadotropin epitopes relative to the luteinizing hormone and follicle-stimulating hormone receptor interaction sites. Mol Cell Endocrinol 92:229–39. https://doi.org/10.1016/0303-7207(93)90013-a

Christakos S, Bahl O (1979) Mare Serum Gonadotropin. 254:4253–4261

Christy C, Vermant S (2002) The state-of-the-art of filtration in recovery processes for biopharmaceutical production. Desalination 147:1–4. https://doi.org/10.1016/S0011-9164(02)00562-3

Cole HH (1938) High gonadotrophic hormone concentration in pregnant ponies. Proc Soc Exp Biol Med 38:193–194

Cole HH, Hart GH (1930) The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. Am J Physiol Content

93:57–68. https://doi.org/10.1152/ajplegacy.1930.93.1.57

Combarnous Y (1992) Molecular Basis of the Specificity of Binding of Glycoprotein Hormones to Their Receptors. Endocr Rev 13:670–691. https://doi.org/10.1210/edrv-13-4-670

Crabtree JR (2022) A review of oestrus suppression techniques in mares. Equine Vet Educ 34:141–151. https://doi.org/10.1111/eve.13405

Cuatrecasas P, Wilchek M, Anfinsen CB (1968) Selective enzyme purification by affinity chromatography. Proc Natl Acad Sci U S A 61:636–643. https://doi.org/10.1073/pnas.61.2.636

Cui Z (2005) Protein separation using ultrafiltration — an example of multi-scale complex systems. China Particuology 3:343–348. https://doi.org/10.1016/s1672-2515(07)60213-9

Cummings JN (1942) A Study of Estrus and Ovulation in the Mare. J Anim Sci 1:309–313. https://doi.org/10.2527/jas1942.14309x

DAMM JBL, HÅRD K, KAMERLING JP, van DEDEM GWK, VLIEGENTHART JFG (1990) Structure determination of the major N- and O-linked carbohydrate chains of the β subunit from equine chorionic gondotropin. Eur J Biochem 189:175–183. https://doi.org/10.1111/j.1432-1033.1990.tb15474.x

Dan X, Liu W, Ng TB (2016) Development and Applications of Lectins as Biological Tools in Biomedical Research. Med Res Rev 36:221–247. https://doi.org/10.1002/med.21363

Darula Z, Sarnyai F, Medzihradszky KF (2016) O-glycosylation sites identified from mucin core-1 type glycopeptides from human serum. Glycoconj J 33:435–445. https://doi.org/10.1007/s10719-015-9630-6

Day FT, Rowlands IW (1947) Serum gonadotrophin in Welsh and Shetland ponies. J Endocrinol 5:1–8

De Rensis F, López-Gatius F (2014) Use of equine chorionic gonadotropin to control reproduction of the dairy cow: A review. Reprod Domest Anim 49:177–182. https://doi.org/10.1111/rda.12268

Dean PD., Middle F.A., Johnson W. (1985) Affinity Chromatography (A Practical Approach), 1st edn. Irl Press Ltd., Washington, D.C.

Denizli A, Pişkin E (2001) Dye-ligand affinity systems. J Biochem Biophys Methods 49:391–416. https://doi.org/10.1016/S0165-022X(01)00209-3

Durham M, Regnier FE (2006) Targeted glycoproteomics : Serial lectin affinity chromatography in the selection of O -glycosylation sites on proteins from the human blood proteome &. 1132:165–173. https://doi.org/10.1016/j.chroma.2006.07.070

Ebeler M, Pilgram F, Wellhöfer T, Frankenfeld K, Franzreb M (2019) First comprehensive view on a magnetic separation based protein purification processes: From process development to cleaning validation of a GMP-ready magnetic separator. Eng Life Sci 19:591–601. https://doi.org/10.1002/elsc.201800183

Ehsani N, Parkkinen S, Nyström M (1997) Fractionation of natural and model egg-white protein solutions with modified and unmodified polysulfone UF membranes. J Memb Sci 123:105–119. https://doi.org/10.1016/S0376-7388(96)00207-4

Farrugia A (2010) Albumin Usage in Clinical Medicine: Tradition or Therapeutic? Transfus Med Rev 24:53–63. https://doi.org/10.1016/j.tmrv.2009.09.005

Fausnaugh JL, Regnier FE (1986) Solute and mobile phase contributions to retention in hydrophobic interaction chromatography of proteins. J Chromatogr A 359:131–146. https://doi.org/10.1016/0021-9673(86)80068-1

Fenton AW, Hutchinson M (2009) The pH dependence of the allosteric response of human liver pyruvate kinase to fructose-1,6-bisphosphate, ATP, and alanine. Arch Biochem Biophys 484:16–23. https://doi.org/10.1016/j.abb.2009.01.011

Folin O, Bell RD (1917) Applications of a new reagent for the separation of ammonia. I. The colorimetric determination of ammonia in urine. J Biol Chem xxix:329

Gans R (1905) Zeolites and similar compounds, their constitution and meaning for technology and agriculture. Jahrb der Königlich Preuss Geol Landesanstalt 26:179

Ghosh R, Cui ZF (1998) Fractionation of BSA and lysozyme using ultrafiltration: Effect of pH and membrane pretreatment. J Memb Sci 139:17–28. https://doi.org/10.1016/S0376-7388(97)00236-6

Ginther OJ (1998) Equine Pregnancy: Physical interactions between uterus and conceptus. AAEP Proc 44:73–104

Gonzalez A, Wang H, Carruthers TD, Murphy BD, Mapletoft RJ (1994) Superovulation in the cow with pregnant mare serum gonadotrophin: Effects of dose and antipregnant mare serum gonadotrophin serum. Can Vet J 35:158–162

González G, Castro B, Massaldi H (1998) Extraction of equine chorionic gonadotrophin from pregnant mare plasma by direct adsorption on chromatographic media. Biotechnol Bioeng 57:22–25. https://doi.org/10.1002/(SICI)1097-0290(19980105)57:1<22::AID-BIT3>3.0.CO;2-X

Gospodarowicz D, Papkoff H (1967) A Simple Method for the Isolation of Pregnant Mare Serum Gonadotropin. Endocrinology 80:699–702. https://doi.org/10.1210/endo-80-4-699

Goss H, Cole HH (1940) Further studies on the purification of mare gonadotropic hormone. Endocrinology 26:244–249. https://doi.org/10.1210/endo-26-2-244

Goumenou A, Delaunay N, Pichon V (2021) Recent Advances in Lectin-Based Affinity Sorbents for Protein Glycosylation Studies. Front Mol Biosci 8. https://doi.org/10.3389/fmolb.2021.746822

Grodzki AC, Berenstein E (2010) Antibody purification: ion-exchange chromatography. Methods Mol Biol 588:27–32. https://doi.org/10.1007/978-1-59745-324-0_4

Hashim OH, Jayapalan JJ, Lee CS (2017) Lectins: An effective tool for screening of potential cancer biomarkers. PeerJ 2017:1–30. https://doi.org/10.7717/peerj.3784

Hendrickson OD, Zherdev A V. (2018) Analytical Application of Lectins. Crit Rev Anal Chem 48:279–292. https://doi.org/10.1080/10408347.2017.1422965

Hirabayashi J, Tateno H, Shikanai T, Aoki-Kinoshita KF, Narimatsu H (2015) The lectin frontier database (LfDB), and data generation based on frontal affinity chromatography. Molecules 20:951–973. https://doi.org/10.3390/molecules20010951

Hofmeister F (1988) On regularities in the albumin precipitation reactions with salts and their

relationship to physiological behaviour. Arch Exp Pathol Pharmacol 24:247–260

Hofstee BHJ (1979) Non-ionic adsorption chromatography and adsorptive immobilization of proteins. Pure Appl Chem 51:1537–1548. https://doi.org/10.1351/pac197951071537

Janson J (1998) Principles, high-resolution methods, and applications. In: Protein purification, 3rd edn. John Wiley & Sons Inc., pp 1–522

Kajal, Pathania AR (2021) Chemistry behind Serum Albumin: A Review. E3S Web Conf 309:01086. https://doi.org/10.1051/e3sconf/202130901086

Kane RK (1942) The O. F. F. Public Opin Q 6:204. https://doi.org/10.1086/265545

Kara E, Dupuy L, Bouillon C, Casteret S, Maurel M-C (2019) Modulation of Gonadotropins Activity by Antibodies. Front Endocrinol (Lausanne) 10:1–12. https://doi.org/10.3389/fendo.2019.00015

Kaşikçi G, Cirit Ü, Gündüz MC, Bacinoğlu S, Sabuncu A (2011) Effects of halving intravaginal sponges and eCG dose on estrus response and fertility in Tahirova ewes during the breeding season. Turkish J Vet Anim Sci 35:193–199. https://doi.org/10.3906/vet-1101-748

Koontz L (2014) TCA precipitation. Methods Enzymol 541:3–10. https://doi.org/10.1016/B978-0-12-420119-4.00001-X

Kopperschläger G, Diezel W, Freyer R, Liebe S, Hofmann E (1971) Wechselwirkungen der Hefe-Phosphofructokinase mit Dextranblau 2000. Eur J Biochem 22:40–45. https://doi.org/10.1111/j.1432-1033.1971.tb01512.x

Kopperschläger G, Freyer R, Diezel W, Hofmann E (1968) Some kinetic and molecular properties of yeast phosphofructokinase. FEBS Lett 1:137–141. https://doi.org/10.1016/0014-5793(68)80041-9

Kumar S, Jain S (2013) History, introduction, and kinetics of ion exchange materials. J Chem 2013. https://doi.org/10.1155/2013/957647

Legardinier S, Duonor-Cérutti M, Devauchelle G, Combarnous Y, Cahoreau C (2005) Biological activities of recombinant equine luteinizing hormone/chorionic gonadotropin (eLH/CG) expressed in Sf9 and Mimic insect cell lines. J Mol Endocrinol 34:47–60. https://doi.org/10.1677/jme.1.01624

Lekena N (2019) Recombinant production of equine chorionic gonadotropin. MSc. Dissertation, UFS

Li QY, Cui ZF, Pepper DS (1997) Fractionation of HSA and IgG by gas sparged ultrafiltration. J Memb Sci 136:181–190. https://doi.org/10.1016/S0376-7388(97)00159-2

Lin FY, Chen WY, Ruaan RC, Huang HM (2000) Microcalorimetric studies of interactions between protein and hydrophobic ligands in hydrophobic interaction chromatography: effects of ligand chain length, density, and the amount of bound protein. Prog Biotechnol 16:59–62. https://doi.org/10.1016/S0921-0423(00)80013-1

Liu G, Zhao Y, Angeles A, Hamuro LL, Arnold ME, Shen JX (2014) A novel and cost effective method of removing excess albumin from plasma/serum samples and its impacts on LC-MS/MS bioanalysis of therapeutic proteins. Anal Chem 86:8336–8343. https://doi.org/10.1021/ac501837t

Lucy CA (2003) Evolution of ion-exchange: From Moses to the Manhattan Project to Modern Times. J Chromatogr A 1000:711–724. https://doi.org/10.1016/S0021-9673(03)00528-4

Lunenfeld B (2004) Historical perspectives in gonadotrophin therapy. Hum Reprod Update 10:453–467. https://doi.org/10.1093/humupd/dmh044

Majorek KA, Porebski PJ, Dayal A, Zimmerman MD, Jablonska K, Stewart AJ, Chruszcz M, Minor W (2012) Structural and immunologic characterization of bovine, horse, and rabbit serum albumins. Mol Immunol 52:174–182. https://doi.org/10.1016/j.molimm.2012.05.011

Martinez-Ros P, Rios-Abellan A, Gonzalez-Bulnes A (2019) Influence of progesterone-treatment length and eCG administration on appearance of estrus behavior, ovulatory success and fertility in sheep. Animals 9:1–9. https://doi.org/10.3390/ani9010009

Martinuk SD, Manning AW, Black WD, Murphy BD (1991a) Effects of carbohydrates on the pharmacokinetics and biological activity of equine chorionic gonadotropin in vivo. Biol Reprod 45:598–604. https://doi.org/10.1095/biolreprod45.4.598

Martinuk SD, Manning AW, Black WD, Murphy BD (1991b) Effects of Carbohydrates on the Pharmacokinetics and Biological Activity of Equine Chorionic Gonadotropin in Vivo1. Biol Reprod 45:598–604. https://doi.org/10.1095/biolreprod45.4.598

Matsui T, Mizuochi T, Titani K, Okinaga T, Hoshi M, Bousfield GR, Sugino H, Ward DN (1994) Structural Analysis of N-Linked Oligosaccharides of Equine Chorionic. Biochemistry 14039–14048

McCue JT (2009) Chapter 25 Theory and Use of Hydrophobic Interaction Chromatography in Protein Purification Applications, 1st edn. Elsevier Inc.

McDonnell SM (2017) Behavior problem: ovaries or not? Proc 63rd Annu Conv Am Assoc Equine Pract San Antonio, Texas, USA, 17-21 Novemb 2017 63:131–134

Melander W, Horváth C (1977) Salt effects on hydrophobic interactions in precipitation and chromatography of proteins: An interpretation of the lyotropic series. Arch Biochem Biophys 183:200–215. https://doi.org/10.1016/0003-9861(77)90434-9

Melander WR, Corradini D, Horváth C (1984) Salt-mediated retention of proteins in hydrophobic-interaction chromatography. Application of solvophobic theory. J Chromatogr A 317:67–85. https://doi.org/10.1016/S0021-9673(01)91648-6

Merck (2022) Purification or removal of albumin. https://www.sigmaaldrich.com/ZA/en/technical-documents/protocol/protein-biology/protein-purification/purification-or-removal-of-albumin?gclid=CjwKCAjwh4ObBhAzEiwAHzZYU7SOp-4_-GEle4bJ3rRQWGVs48IOEH5JKsfkEBsSV9rZcAHz_M3BBRoCWtcQAvD_BwE&gclsrc=aw.ds. Accessed 1 Nov 2022

Min KS, Hattori N, Aikawa J, Shiota K, Ogawa T (1996) Site-directed mutagenesis of recombinant equine chorionic gonadotropin/luteinizing hormone: differential role of oligosaccharides in luteinizing hormone- and follicle-stimulating hormone-like activities. Endocr J 43:585–93. https://doi.org/10.1507/endocrj.43.585

Min KS, Hiyama T, Seong HH, Hattori N, Tanaka S, Shiota K (2004) Biological activities of tethered equine chorionic gonadotropin (eCG) and its deglycosylated mutants. J Reprod Dev 50:297–304. https://doi.org/10.1262/jrd.50.297

Montel MC, Seronie MP, Talon R, Hebraud M (1995) Purification and characterization of a

dipeptidase from Lactobacillus sake. Appl Environ Microbiol 61:837–839. https://doi.org/10.1128/aem.61.2.837-839.1995

Moore WT, Ward DN (1980) Pregnant mare serum gonadotropin. Rapid chromatographic procedures for the purification of intact hormone and isolation of subunits. J Biol Chem 255:6923–6929

Moudgal NR, Papkoff H (1982) Equine Luteinizing Hormone Possesses Follicle-Stimulating Hormone Activity in Hypophysectomized Female Rats. Biol Reprod 26:935–942. https://doi.org/10.1095/biolreprod26.5.935

Müller C, Preußer-Kunze A, Wagner K, Franzreb M (2011a) Gonadotropin purification from horse serum applying magnetic beads. Biotechnol J 6:392–395. https://doi.org/10.1002/biot.201000380

Müller C, Wagner K, Frankenfeld K, Franzreb M (2011b) Simplified purification of equine chorionic gonadotropin (eCG)-an example of the use of magnetic microsorbents for the isolation of glycoproteins from serum. Biotechnol Lett 33:929–936. https://doi.org/10.1007/s10529-010-0512-5

Nagel AK, Schilling M, Comte-walters S, Berkaw MN, Ball LE, The ON (2013) Identification of O -Linked N -Acetylglucosamine ( O -GlcNAc ) -modified Osteoblast Proteins by Electron Transfer Dissociation Tandem Mass Spectrometry Reveals Proteins Critical for Bone Formation * □. 945–955. https://doi.org/10.1074/mcp.M112.026633

Nakatsuka S, Michaels A. (1992) Transport and separation of proteins by ultrafiltration through sorptive and non-sorptive membranes. J Memb Sci 69:189–211

Nath LC (2014) Reproductive management of the athletic horse. In: Equine Sports Medicine and Surgery. Elsevier, pp 1171–1180

Odaba M, Garipcan B, Dede S, Denizli A (2001) Methacrylamidohistidine in affinity ligands for immobilized metal-ion affinity chromatography of human serum albumin. Biotechnol Bioprocess Eng 6:402–409. https://doi.org/10.1007/BF02932321

Okouyi MWM, Hanzen C (2016) Effects of equine chorionic gonadotropin (ECG) on the sexual behaviour and ovulatory characteristics of female african N'Dama cattle. Rev Med Vet (Toulouse) 167:211–215

Oppenheimer JH (1968) Role of Plasma Proteins in the Binding, Distribution and Metabolism of the Thyroid Hormones. N Engl J Med 278:1153–1162. https://doi.org/10.1056/NEJM196805232782107

Påhlman S, Rosengren J, Hjertén S (1977) Hydrophobic interaction chromatography on uncharged sepharose® derivatives. J Chromatogr A 131:99–108. https://doi.org/10.1016/S0021-9673(00)80924-3

Park JJ, JarGal N, Yoon JT, Min KS (2010) β-Subunit 94~96 residues of tethered recombinant equine chorionic gonadotropin are important sites for luteinizing hormone and follicle stimulating hormone like activities. Reprod Dev Biol 34:33–40

Peters T (1985) Serum Albumin. Adv Protein Chem 37:161–245. https://doi.org/10.1016/S0065-3233(08)60065-0

Porath J, Sundberg S, Fornstedt N, Olsson I (1973) Salting-out in Amphiphilic Gels as a New Approach to Hydrophobia Adsorption. Nature 245:465–466

Queiroz JA, Tomaz CT, Cabral JMS (2001) Hydrophobic interaction chromatography of

proteins. J Biotechnol 87:143–159. https://doi.org/10.1016/S0168-1656(01)00237-1

Rajalingam D, Loftis C, Xu JJ, Kumar TKS (2009) Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. Protein Sci 18:980–993. https://doi.org/10.1002/pro.108

Raoufinia R, Mota A, Nozari S, Aghebati Maleki L, Balkani S, Abdolalizadeh J (2016) A methodological approach for purification and characterization of human serum albumin. J Immunoass Immunochem 37:623–635. https://doi.org/10.1080/15321819.2016.1184163

Röschlau P, Hess B (1972) Affinity Chromatography of Yeast Pyruvate Kinase with Cibacronblau Bound to Sephadex G-200. Hoppe-Seyler´s Zeitschrift für Physiol Chemie 353:441–443. https://doi.org/10.1515/bchm2.1972.353.1.441

Rossdales Laboratories (2022) Proteins (Total protein, Albumin, Globulin). https://www.rossdales.com/laboratories/tests-and-diseases/proteins-total-protein-albumin-globulin. Accessed 28 Oct 2022

Sá Filho MF, Ayres H, Ferreira RM, Marques MO, Reis EL, Silva RCP, Rodrigues CA, Madureira EH, Bó GA, Baruselli PS (2010) Equine chorionic gonadotropin and gonadotropin-releasing hormone enhance fertility in a norgestomet-based, timed artificial insemination protocol in suckled Nelore (Bos indicus) cows. Theriogenology 73:651–658. https://doi.org/10.1016/j.theriogenology.2009.11.004

Saksena S, Zydney AL (1994) Effect of solution pH and ionic strength on the separation of albumin from immunoglobulins (IgG) by selective filtration. Biotechnol Bioeng 43:960–968. https://doi.org/10.1002/bit.260431009

Satih S, Hoshi S (1932) A study of reproduction in the mare (Report 1). J Japanese Soc Vet Sci 11:257–268. https://doi.org/10.1292/JVMS1922.11.257

Schams D, Papkoff H (1972) Chemical and immunochemical studies on pregnant mare serum gonadotropin. BBA - Protein Struct 263:139–148. https://doi.org/10.1016/0005-2795(72)90168-7

Sharifi MA, Kohram H, Zare Shahneh A, Zolfagharian H, Abedi Kiasari B, Hedayati M (2014) Production and Purification of Equine Chorionic Gonadotropin Hormone Using Polyclonal Antibody. Iran J Biotechnol 12. https://doi.org/10.5812/ijb.15137

Sherman GB, Wolfe MW, Farmerie TA, Clay CM, Threadgill DS, Sharp DC, Nilson JH (1992) A single gene encodes the β-subunits of equine luteinizing hormone and chorionic gonadotropin. Mol Endocrinol 6:951–959. https://doi.org/10.1210/mend.6.6.1379674

Sivaraman T, Kumar TKS, Jayaraman G, Yu C (1997) The mechanism of 2,2,2-trichloroacetic acid-induced protein precipitation. J Protein Chem 16:291–297. https://doi.org/10.1023/A:1026357009886

Small H, Stevens TS, Bauman WC (1975) Novel Ion Exchange Chromatographic Method Using Conductimetric Detection. Anal Chem 47:1801–1809. https://doi.org/10.1021/ac60361a017

Smith PL, Bousfield GR, Kumar S, Fiete D, Baenziger JU (1993) Equine lutropin and chorionic gonadotropin bear oligosaccharides terminating with SO4-4-GalNAc and Siaα2,3Gal, respectively. J Biol Chem 268:795–802. https://doi.org/10.1016/s0021-9258(18)54004-7

Stančić I, Radović I, Apić J, Stančić B, Božić A, Apić I (2015) Estrus induction in sows with

eCG injection 24h after weaning within warm and cool season. J Microbiol Biotechnol food Sci 4:105–107. https://doi.org/10.15414/jmbfs.2015.4.special2.105-107

Štrop P, Čechová D, Tomášek V (1983) Model study of hydrophobic interactions of α- and β-trypsin and α-chymotrypsin. J Chromatogr A 259:255–268. https://doi.org/10.1016/S0021-9673(01)88006-7

Svedberg T, Sjögren B (1930) THE Ph-STABILITY REGIONS OF SERUM ALBUMIN AND OF SERUM GLOBULIN. J Am Chem Soc 52:2855–2863. https://doi.org/10.1021/ja01370a044

Thompson HS (1850) On the Absorbent Power of Soils. J R Agric Soc Engl 11:68–74

Trinidad JC, Barkan DT, Gulledge BF, Thalhammer A, Sali A, Schoepfer R, Burlingame AL (2012) Global identification and characterization of both O-GlcNAcylation and phosphorylation at the murine synapse. Mol Cell Proteomics 11:215–229. https://doi.org/10.1074/mcp.O112.018366

Tyner T, Francis J (2017) Phosphoric Acid, Meta-. ACS Reag Chem. https://doi.org/10.1021/acsreagents.4268.20201102

Ulloa-Aguirre A, Espinoza R, Damian-Matsumura P, Chappel SC (1988) Immunological and biological potencies of the different molecular species of gonadotrophins. Hum Reprod 3:491–501. https://doi.org/10.1093/oxfordjournals.humrep.a136734

Vakhrushev SY, Steentoft C, Vester-Christensen MB, Bennett EP, Clausen H, Levery SB (2013) Enhanced mass spectrometric mapping of the human GalNAc-type O-glycoproteome with simplecells. Mol Cell Proteomics 12:932–944. https://doi.org/10.1074/mcp.O112.021972

van Oss CJ, Good RJ, Chaudhury MK (1986) Nature of the antigen-antibody interaction. Primary and secondary bonds: Optimal conditions for association and dissociation. J Chromatogr B Biomed Sci Appl 376:111–119. https://doi.org/10.1016/S0378-4347(00)80828-2

Varshney A, Sen P, Ahmad E, Rehan M, Subbarao N, Khan RH (2010) Ligand binding strategies of human serum albumin: How can the cargo be utilized? Chirality 22:77–87. https://doi.org/10.1002/chir.20709

Vilhanová Z, Novotný F, Valocký I, Hura V, Horňáková P, Karamanová M (2021) Effect of seasonal environmental changes on selected reproductive parameters in mares. Bulg J Vet Med 24:208–218. https://doi.org/10.15547/BJVM.2019-0083

Villarraza CJ, Antuña S, Tardivo MB, Rodríguez MC, Mussio P, Cattaneo L, Fontana D, Díaz PU, Ortega HH, Tríbulo A, Macagno A, Bó GA, Ceaglio N, Prieto C (2021) Development of a suitable manufacturing process for production of a bioactive recombinant equine chorionic gonadotropin (reCG) in CHO-K1 cells. Theriogenology 172:8–19. https://doi.org/10.1016/j.theriogenology.2021.05.013

Wan Y, Vasan S, Ghosh R, Hale G, Cui Z (2005) Separation of monoclonal antibody alemtuzumab monomer and dimers using ultrafiltration. Biotechnol Bioeng 90:422–432. https://doi.org/10.1002/bit.20416

Way JT (1850) On the power of soils to absorb manure. J R Agric Soc Engl 11:313–379

Weis WI, Drickamer K (1996) Structural Basis of Recognition Lectin-Carb Ohydrate. Annu Rev Biochem 65:441–73

Whitehorn JC (1923) Separation of some amino acids. J Biol Chem 56:751

Yang H, Wang G, Zhang T, Beattie JH, Zhou S (2020) Establishing an optimized method for the separation of low and high abundance blood plasma proteins. PeerJ Anal Chem 2:e6. https://doi.org/10.7717/peerj-achem.6

Yon RJ (1978) Recent developments in protein chromatography involving hydrophobic interactions. Int J Biochem 9:373–379. https://doi.org/10.1016/0020-711X(78)90048-4

Zhao Q, Tao C, Pan J, Wei Q, Zhu Z, Wang L, Liu M, Huang J, Yu F, Chen X, Zhang L, Li J (2021) Equine chorionic gonadotropin pretreatment 15 days before fixed-time artificial insemination improves the reproductive performance of replacement gilts. Animal 15:100406. https://doi.org/10.1016/j.animal.2021.100406