An Alternative to Triton X-100 for use in Viral Inactivation for Recombinant Factor VIII

By

Mark Dalton

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Name: Mark Dalton

ID Number: S00166758

Supervisor: Dr. Mary Garvey

Declaration:

"I hereby declare that this project is entirely my own work and that it has not been submitted for any other academic award, or part thereof, at this or any other education establishment".

Mark Dalton

Abstract

Solvent detergent combinations for viral inactivation have long been used in the manufacture of Biopharmaceutical products due their proven safety record for inactivating viruses. Triton X-100, a non-ionic detergent, has been the detergent of choice within the modern Biopharma industry. However, this detergent, when broken down has environmental hazards proving it toxic to aquatic species. Because of this, Triton X-100 has been assessed by the European Chemicals Agency and has formally been placed on the REACH authorisation list.

This has resulted in companies who employ Triton X-100 within their manufacturing process to perform an analysis of alternatives in an attempt to move away from the use of Triton X-100. The basic aim of this research was to identify an alternative chemical to Triton X-100 for use in viral inactivation of recombinant factor FVIII. This research project was based on an extensive literature review followed by a series of practical research studies.

From this research, 6 alternative candidates were identified and evaluated through bench scales studies for impact to a recombinant factor VIII protein. This allowed for the elimination of candidates based on their bench scale results and also the progression of 2 candidates to further design of experiments (DOE) studies. The DOE studies showed that the 2 candidates had no negative impact to product quality or stability. It also allowed for optimal process conditions to be determined using the 2 candidates which were carried forward to a proof of concept virus inactivation study at a third party laboratory. This viral inactivation study employed the use of two model viruses, MuLV and PRV. Both candidates showed their robustness at inactivating the two viruses with a LRV >5 log, providing sufficient certainty that the two candidates are viable alternatives to Triton X-100.

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And to my wife Jennifer, you are no longer a single parent…..I'm back!!

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1. Introduction

The European Chemical Agency (ECHA) is an agency within the European Union (EU) that manages and controls all technical and regulatory legislation pertaining to chemical manufacture and usage. One of the main European regulations that is in the forefront and applies to chemical usage currently is the REACH regulation.

REACH is the Registry, Evaluation, Authorisation and Restriction of Chemicals and is mandated solely through the ECHA. The main aim of this legislation is to improve and ensure the protection of human health and the environment from all risks that may occur through the use of chemicals (The European Chemical Agency, 2019). Going forward however, the ultimate goal is to replace all hazardous chemicals with a safer alternative. The REACH regulation applies to all chemicals but mainly focuses on usage quantities in excess of 1 ton per annum. Therefore, the responsibility lies solely on the manufacturers and downstream users of such chemicals to identify their current demands and to justify its safe use and control through the appropriate risk assessments.

The initial process commences by identifying all chemicals currently in use. At this stage it is important that manufacturers and downstream users collaborate and communicate effectively to enable an efficient and accurate registration. As well as this, all data collated during this stage can be accessed by other third party companies as part of their application which will speed up the process.

It is recommended that companies perform a formal detailed risk assessment of the chemicals used which allow them to collate all information pertaining to the chemical in question, the hazards posed to both human and environmental safety and the current control in place to mitigate any potential hazards. This also applies to chemicals that are used within a solution mixture.

All collated data is submitted to the ECHA in the form of a dossier where a member states committee (MSC) review board will either approve the current use of the chemical or recommend that it is identified as a Substance of Very High Concern (SVHC) and added to the candidate list prior to authorisation.

Applicants have the opportunity to engage with the MSC and provide more information if possible or available. From this the MSC will provide the ECHA with their final opinion on whether the chemical is a SVHC and if so, it will become part of the candidate list requiring authorisation for use in the EU market.

The European Chemicals Agency (ECHA) have granted a sunset date of January 2021 to afford the industry time to assess the impact that this change will have on specific manufacturing processes. It also provides the opportunity to think of the future and commence next generation process changes in order to identify an alternative chemical to use within their manufacturing process. Alternatives identified must have lower toxicity, with a good safety record and also provide the same end result. After this sunset date, companies must either have authorisation to manufacture with the caveat that they can demonstrate their willingness and active participation into seeking an alternative chemical or cease manufacturing using the SVHC chemical.

It is worth nothing that this regulation only applies to companies who are based and trading within the EU. Manufacturers who export products containing a restricted substance into the EU can do so freely. The onus is on the importer of the chemical or product, within the EU, to ensure all necessary evaluations have been conducted and authorisations have been granted. This could have significant impact on business trading between the EU and the Rest of the World (ROW) especially if alternative chemicals are not readily available or due to lack of co-operation to adopt changes in a jurisdiction that the REACH regulation does not impact.

Within both the Biopharmaceutical and Pharmaceutical sector the impact of the REACH regulation has begun to surface with 43 substances now being added to the authorisation list. One in particular is Triton X-100 also known as Octoxynol-9. Triton X-100 is a non-ionic detergent that has many applications within the biopharmaceutical industry. It has the ability to act as a disruptor on a macromolecule level towards various protein and lipid associations (Colavita et al., 2017), and has long showed robustness in viral reduction in comparison to other alternatives (Roberts, 2008).

However, according to the ECHA, Triton X-100 has been classed as a SVHC. This is due to the degradation properties that occur to the molecular structure when released into the environment. Triton X-100 is believed to have slow rate of degradation which results in the accumulation of the degradative agent, 4-tert-octylphenol. This molecule is said to possess endocrine disruptive properties and is therefore potentially an endocrine disruptive chemical (EDC) and as such warrants the SVHC designation.

Alternative products on the market have been deemed not as effective and have raised concerns regarding viral safety of products for the future. This regulation has the potential to cause significant impact to the industry as a change of chemical within a manufacturing process could pose significant impact to product quality, safety and supply if further marketing authorisations are required.

In the manufacturing process of recombinant factor VIII (Product X), Triton X-100 is a major component that is associated with 3 modes of action: product stabilising, viral inactivation and a cleaning agent. In order to adhere to the REACH regulation, an alternative to Triton X-100 must be identified. Therefore the main aim of this research is to identify an alternative chemical to Triton X-100 used in the manufacture of recombinant factor VIII but also be mindful that it must be compatible within the manufacturing process and with the factor VIII molecule.

The elimination of Triton X-100 from the manufacturing process will progress in stages, such that the main focus of this research project will be to seek an alternative chemical for use in the viral inactivation unit operation only.

In order to reach the desired conclusion, a number of objectives will need to be met. The research involved will be carried out in a number of phases. The objective of this project are as follows:

- Identify, through an extensive literature review, various methods of viral inactivation and also a panel of potential detergent candidates that could be progress to laboratory scale viral inactivation studies in order to assess product quality impact. Process knowledge already gained on the product in question will allow most methods for viral

inactivation to be eliminated due to incompatibility with the molecule and will most likely focus on solvent detergent viral inactivation.

- Evaluate, critically, from initial results which candidates successfully showed no product quality impact and should progress to Design of Experiment (DOE) studies.
- From the DOE, provide a recommendation of candidates to undergo third party viral inactivation validation studies.
- Post third party viral inactivation studies, assess the viral inactivation kinetics of each prospective candidate to allow future validation work to be considered.

Further details of the research to be carried out will be documented within the research methods section.

2. Literature Review

It has been several decades since biologics using monoclonal antibodies (MAB) and recombinant proteins have been developed. Since this time they have gifted patients with an increased quality of life through their efficaciousness to treat human disease. Whilst bacterial cell lines were once the host of choice, biopharmaceutical products today have become too complex in structure that only a mammalian cell line could provide the various human like posttranscriptional mechanisms required (Martin Remington, 2015a). Because of this mammalian cell lines have surpassed microbial or bacterial cell lines as the preferred choice of host. Chinese Hamster Ovary (CHO) cells are probably the most commonly used mammalian cell line for the manufacture of recombinant proteins. This is down to a number of factors. The cells are extremely robust in that they have the ability to grow readily in a variety of media types. Their genome is rather unstable and shows susceptibility to mutation. This can be seen as a positive characteristic especially when it comes to the development of Mab. Reliability and knowledge of the cell line are also other reasons CHO cells are chosen. CHO cells have been well characterised and are well understood as a host cell in the Biopharmaceutical industry. They are free of any known infectious retrovirus and provide a good rate a resistance towards adventitious viral infection (Kerr and Nims, 2010).

Manufacturing recombinant protein through a mammalian cell line is an extremely complex process. This is due to the fact the cell is a living system which possess intrinsic characteristics, all of which need to be nurtured in order to produce a product that is of high quality, safe and reproducible (Martin Remington, 2015a).

Even though recombinant proteins are well established and hold a good safety record, they are derived from mammalian cell lines therefore presenting a constant inherent risk of introducing potential viral agents whether endogenously or adventitiously. The presence of viral contamination within cell lines is widely known hence why there are such stringent measures mandated from the regulatory bodies to evaluate and mitigate the risks of viral contamination. When dealing with biopharmaceutical products, viral safety is one of the most important factors to consider as early as conception to full scale production.

A viral contamination event can cause substantial disruption to a company. From significant financial costs, suspension of manufacturing license and worst case facility shutdown.

Although the manufacturing process of recombinant proteins has become more complex, it is encouraging to be aware that there has been no transmission of infectious viruses to any patients through the use of recombinant medicinal products (Martin Remington, 2015a). In fact, (Kerr and Nims, 2010) have reported that in a 10 year review period of adventitious virus screening carried out on bulk harvest samples, there has only be a few occasions of viral contamination which were within CHO cell lines. In all cases reported, the most likely root cause of contamination was raw materials.

Raw Materials are classified as all process inputs ranging from the production cell line to disposable systems. Within a manufacturing process raw material are sourced from a variety of different vendors which ultimately elevates the risk of contamination. This is quite evident as most historical viral contamination events within the industry were as a direct result of contaminated raw materials.

It is no surprise then that raw materials are a strong focal point for both regulatory bodies and biopharmaceutical manufacturers. According to (Garnick, 1998) viral contamination can be non-homogenously distributed throughout any raw material. In these instances, low titres of viral contaminants would result in minimal success in detection. This theory is further supported by (Wisher, 2013) who discusses these raw material contamination events of recent years. In these specific cases no viruses were detected as a result of either assay sensitivity or the quantity of material screened.

The industry has proactively tried to mitigate against re-occurrence of these types of events by moving away from animal derived raw materials where possible, although one cannot provide complete assurance that those raw materials have not been exposed to animal derived components at some stage of the supply chain. These past instances have provided learnings in the industry and reinforce the importance of a well characterised and understood supply chain.

With all good intentions the presence of viruses can still exist endogenously or adventitiously and go undetected. Therefore it is of utmost importance that virus safety of biotech and plasma derived therapeutics is ensured through complementary measures that include the control and monitoring of raw materials, robust validation and implementation of viral clearance technology (Kern and Krishnan, 2006). Through effective viral clearance steps the risks of undetected viruses being transmitted to the product are greatly reduced.

Ensuring viral safety can be as equally complex as the host cell itself. The evolution of new and emerging viruses can cause the technology to become quickly out dated. (Pastoret, 2010) has stated that there are potentially an estimated number of 150,000 viruses in existence with only 2300 detected. Detection of new virus sometimes only occurs due to advances in technology which leaves unanswered questions regarding the viral safety of biopharmaceutical products. Furthermore, viruses are prone to antigenic drift and antigenic shift meaning that new viral strains may emerge quickly which are undetected and unrecognized.

Viral safety of products for the purpose of clinical trials is a priority. For this reason viral clearance studies are required at all stages of product development through to product licencing. Even though products in early stage development have limited characterisation carried out, there should be an active consciousness regarding viral safety. The regulatory bodies have acknowledged the limitations of product characterisation during development and as such have allowed for a reduced viral safety testing programme in comparison to what is expected for a product ready for market authorisation (European Medicines Agency, 2008). Regardless of the lifecycle of a product, a risk based approach should be adopted when designing a viral safety programme (International Conference on Harmonisation, 2005). Performing a risk assessment will enable the user to understand more about the product from a viral safety perspective. This will therefore enhance their ability to thoroughly assess all potential risks and identify mitigation to the risks that could possibly affect the quality of the product (British Standard, 2003). Common risk evaluation tools used would be Hazard and Operability Analysis (HAZOP) and Failure Mode Effect Analysis (FMEA).

There appears to be a noticeable disconnection between regulatory guidelines and recommendation regarding viral safety programmes. The onus is clearly on the industry to select and implement the most up to date solutions (Yuval Shimoni et al., 2016). Instead, regulatory bodies and industry should collaborate in order to collectively establish the appropriate measures to ensure product safety.

Viruses can possess various characteristics, all of which add to their complexity. Therefore various viral clearance methods need to be evaluated accordingly. As well as this, the integration of such virucidal methods into a manufacturing process needs to be assessed as it can determine the overall design of the process (Kim et al., 2008).

Typically in a downstream manufacturing process of a biopharmaceutical product there are a number of unit operations all aimed at purifying the final drug substance. When developing a downstream manufacturing process for a recombinant protein the main aim is to produce a high yielding product.

As per (European Medicines Agency, 2008) guidelines, one needs to ensure there are at least 2 complementary orthogonal virus reduction methods incorporated within the downstream purification train. Generally these are in the form of a viral inactivation method and a viral filtration method.

Therefore when designing a viral reduction step one needs to be cognicant of the impact that the reduction step may have on the product quality or yields. For example, non-enveloped viruses are more difficult to inactivate and conditions that do are generally not suited to recombinant proteins (Martin Remington, 2015b). Protein stability is as important as viral safety. The location of the viral clearance step needs to be considered in order to achieve a high rate of log reduction and high yields.

1.1 Low pH

It is long known the low pH treatment (pH 3.0-4.0) of recombinant proteins has been a successful method for inactivation of enveloped viruses (A Shukla and Aranha, 2015). If the purification process train permits this mode of viral inactivation, it can integrate well at the end of an affinity chromatography step as the elution pH is generally enough

to cause viral inactivation or complementary to commencing such step. The collected eluate can be pH adjusted further to adhere to the required pH value through the addition of a weak acid. From here the process solution undergoes incubation as determined.

A regulatory concern with this type of incubation process is what is known as the "magic droplet". This implies that droplets that may have been displaced onto the dome area of a process vessel may not be exposed to the lower pH conditions. For this reason, it is recommended that the intermediate is transferred to an incubation hold vessel to ensure all the contents are guaranteed to be at the low pH environment.

This is a point of regular discussion among the biopharmaceutical industry and regulatory bodies but the reluctance between both parties to prove or disprove the theory has resulted in a conservative approach been taken for the sake of viral safety.

Low pH viral inactivation is performed in the range of pH 3.0-4.0. Studies carried out by (Brorson et al., 2003) has shown that the solution pH is deemed a critical process parameter (CPP) and that inactivation appears to be more robust when carried out in pH conditions of 3.8 or below. (Brorson et al., 2003) carried out viral inactivation studies on solutions containing a high degree of impurities and aggregation. Using both an infectivity assay and a quantitative Polymerase chain reaction (Q-PCR) method they found that, even in these non-ideal conditions, at pH of 3.8, the virus log reduction was ≥ 4.6 logs.

A publication by (Chen, 2014) discusses a number of studies where pH was also the determining factor for ensuring complete viral inactivation. The studies determined that at pH ≤ 3.6, robust viral inactivation of ≥ 5 logs was achieved across various process parameters such as viral inactivation temperature, product concentration, salt concentration and inactivation duration. When the pH values above 3.6 were tested, temperature, product concentration, salt concentration and even the type of recombinant protein used did influence the virus log reduction value. These studies further corroborate (Brorson et al., 2003) where they concluded that the pH set point is the determining factor in a robust viral inactivation step.

Whilst these studies reveal and further confirm the success and efficacy of low pH viral inactivation , we need to be mindful of the kinetics surrounding the viral inactivation process specific to the recombinant protein within the manufacturing process as aggregation and conformational stability can pose a problem at low pH for some recombinant proteins causing loss of activity and yield. To account for this possibility or known issue, a more conservative pH set point may need to be determined at the expense of another parameter such as inactivation time.

Human plasma derived products play an important role in the health of receiving patients. Such products are delivered in the form of albumin, coagulants and Immunoglobulins (Igs). All these products undergo various manufacturing technologies to deliver the required product. Even though the safety record for such types of products has improved, emergence of new blood pathogens continue to cause concern (Nazari et al., 2017). To help mitigate these concerns viral inactivation is an established step within the manufacturing process of human derived blood products.

Immunoglobulin G (IgG) has the ability to withstand low pH environments. This is known because acid treatment was originally development as a way to reduce aggregation of the IgG molecule. Adoption of this method has led to the procedure evolving over time with the efficacy of the procedure being questioned (World Health Organization, 2004).

(Omar et al., 1996) conducted studies on the IgG molecule at pH 4.0 conditions demonstrating that at that pH set point all other factors such as temperature, IgG concentration and time do influence the rate of viral inactivation. If we compare this study to that reported by (Chen, 2014), we can see that the same influences affect viral inactivation whether the molecule is recombinant or plasma derived.

Factor VIII is plasma derived anti hemophilic protein that contributes significantly to the blood coagulation cascade. It serves as a cofactor to Factor IXa which proceeds further along the cascade. The factor VIII molecule itself is large and complex, comprising of a dimer conformation consisting of a heavy and light chain. The activity of the factor VIII molecule is dependent on the stable binding between the two chain subunits (Wakabayashi et al., 2006). Physiologically, calcium and other metal ions

serve as stabilisers which also contribute to the association of the heavy the light chains. (Wakabayashi et al., 2006) carried out studies to understand the effects of pH on the association of the heavy and light chains. They revealed that the affinity between the two chains were greatest between pH 6.5-6.75. Significant activity loss was observed at pH 6.0.

The findings of this study contribute to already gained knowledge on the factor VIII molecule. (Hoyer, 1981) had previously reported that the activity of factor VIII is affected by pH and calcium concentration, with the molecule being most stable between pH 6.9-7.2. Further to this, it has been noted that inactivation of the factor VIII has been seen at pH 5.0-5.5 (World Health Organization, 2004).

1.2 Pasteurisation

Non-enveloped viruses are generally more difficult to inactivate due to their outer lipid membrane being more physio chemically resistant. Conditions that are known to achieve robust inactivation rates are generally incompatible with recombinant proteins (Martin Remington, 2015b). Due to this chemical resistance, a more physical means of inactivation is required.

Heat is a common and effective method for sterilisation and is relatively effective for viral inactivation also. It can be applied in various forms such as wet heat, dry heat or as a hot vapour; all possessing their own advantages and disadvantages. The success of pasteurisation is well known and has been used to virally inactivate plasma derived products such as albumin for years (Chandra et al., 2002).

In liquid solution, heat transfer is distributed evenly providing uniformity across the treated substance. The confidence in the efficacy of this method by some regulatory agencies has given rise to this method itself not requiring validation. But rather, it is the parameters that affect the efficacy, such as time and temperature, which are scrutinised in order to demonstrate control and reproducibility (World Health Organization, 2004). Because pasteurisation is performed on the liquid process solution, further downstream operations are still required to bring the product to its final state. This gives rise to the

risk of contamination post inactivation. Good GMP practices should be in place through all stages to mitigate these risks.

Protein stability is limited at high temperatures. Therefore added precautions need to be taken when dealing with proteins that are considered labile in nature to ensure their stability during pasteurisation. Various salts such as Calcium Chloride and Sodium Chloride are effective at inhibiting protein aggregation caused by heat in human albumin (Nazari et al., 2017). Formulation buffers containing sucrose or glycerine have also been used. The addition of stabiliser adds to the complexity of the manufacturing process, as the stabilisers now become a process impurity which must be adequately removed. This can be achieved through downstream purification methods such as chromatography or diafiltration. These stabilisers can also have a negative effect as they have the ability to interact with the proteins and potential viruses. This can result in added protective properties to viruses reducing viral susceptibility to sterilisation methods. It is this reason perhaps that transmission of Hepatitis A and other viruses occurred in the past through the use of blood derived products.

Apart from albumin, pasteurisation has been shown to work well for other plasma derived blood products such as immunoglobulins, thrombin, and coagulation factors. Early studies by (Horowitz et al., 1985b) on Factor VIII concentrate showed complete inactivation of the Sindbis virus and EMC virus through pasteurization for 10 hours at 60°C with the addition of both sucrose and glycerin as a stabiliser. The addition of the stabilisers appeared to yield a success as the recovery rate for the Factor VIII protein was more than 90 percent.

A review carried out by (Chandra et al., 2002) on the success of pasteurization as a mode of viral inactivation has shown that pasteurisation has the ability to reduce a complement of enveloped and non-enveloped viruses. The log reduction values reported were in the range of 5.7 to 9.2 log reduction. Specific to Factor VIII, pasteurisation has shown to inactivate a panel of model viruses as seen in Table 1. Whilst the data available shows pasteurisation as an effective viral inactivation method, it should be noted that the panel of viruses used were mainly that of enveloped origin. The smaller non enveloped viruses still remain a challenge for inactivation.

Table 1: Viral reduction achieved through pasteurisation of a Factor VIII concentrate (ref. Chandra et al., 2002)

Virus	Total virus reduction (log_{10})
HIV	>7.1
BVDV	5.8
PRV	5.3
Sindbis virus	>7.3
VSV	>7.5
Vaccinia virus	>6.1
Poliovirus	>7.3

(Hilfenhaus and Nowak, 1994) investigated the inactivation of Hepatitis A virus (HAV) and the Poliovirus using pasteurisation at 60°C for 10 hours on a purified Factor VIII protein. A similar protocol used by many. The results showed complete inactivation of the poliovirus and substantial inactivation of the HAV. The concern raised was that residual infectious agents belonging to HAV were still present after the 10 hour duration. It is thought that the use of human serum albumin as a stabiliser for the Factor VIII also served as a protectant to the HAV, slowing down its inactivation. As a single element this finding would naturally be of concern. However, combined with a full purification train which would include another orthogonal method for viral clearance, the safety factor for the product still exists.

(Chandra et al., 2002) has referenced more recent studies that suggest that HAV can be effectively inactivated using pasteurisation (ref Table 2). The parameters used in these studies have not been disclosed but it is worth nothing that the lowest viral log reduction of 3.8 achieved was using a Factor VIII product. In comparison to other plasma derived products this value is quite low. This may be due to the stability of the Factor VIII product when exposed to higher pasteurisation temperatures. In order to maintain optimal recovery of the Factor VIII protein after this inactivation step, the temperature applied may not have been sufficient enough to completely inactivate HAV.

Table 2: Viral reduction of HAV achieved through pasteurisation of various plasma derived products (ref. Chandra et al., 2002)

Plasma-derived product	Log reductions in HAV
Monoclate P (antihemophilic factor)	5.6
Beriate P (factor VIII concentrate)	3.8
Haemate P, Humate P (antihemophilic factor/von Willebrand factor complex)	4.2
Berinin P (factor IX + antithrombin)	4.1
Prothrombin complex concentrate	4.0
Haemocomplettan P (fibrinogen)	4.1
Fibrogammin P (human factor XIII concentrate)	4.2
Thrombin P (thrombin)	4.0
Kybernin P (antithrombin III)	5.4
API	5.4
Albumin—20% (human albumin)	6.6–6.8

Dry heat treatment has been effectively applied to biopharmaceutical products, as it can easily be integrated into the end of a lyophilisation cycle (Nazari et al., 2017). Because lyophilisation is the last stage of a manufacturing process the risk of further contamination is reduced. However, stabilisers if added, must part of the formulation buffer as they can no longer be removed at this stage. In contrast, if no stabilisers have been added, one must ensure that the protein solution is more thermally stable than the theoretical viruses being inactivated (Nazari et al., 2017). Since the 1980's dry heat treatment has been used to inactivate HIV in coagulation factors. The efficacy of this method is determined through the residual moisture content, protein concentration and formulation (Chandra et al., 2002).

(Roberts et al., 2007) has studied these parameters to quantify their role in the efficacy of dry heat treatment for viral inactivation. Overall, the investigators reported successful inactivation of enveloped viruses that previously were known to be transmitted in Factor VIII. When it came to non-enveloped viruses that were of concern; such as Bovine Parvovirus (BPV), Porcine Parvovirus (PPV) and an animal model of human B19 virus all appeared to show a level of resistance. Log reduction values were all below 4 logs. As part of this study each parameter was assessed at the extreme of each range with the relative moisture content showing the most correlation

to efficacy. However this correction was dependent on what virus was being studied. The HAV was consistently reduced by ≥5.4 logs at various residual moisture content percentages of between 0.3-2.7 %, similar to the HIV-1. Whereas the BPV showed a stronger log reduction when the residual moisture content increased. Whilst it may be reassuring to have effective viral inactivation across a range of residual moisture content values, the stability of the lyophilized product may be impacted in higher moisture conditions.

To date there has been a significant amount of data collated on the virucidal efficacy of heat as an application for viral inactivation. However, one detail that appears to be shortcoming in a lot of studies carried out is the physiochemical impact that heat application has on plasma derived products but more specifically to the Factor VIII molecule.

(Kim et al., 2008) evaluated the impact of dry heat treatment on the Factor VIII concentrate activity. Lyophilised Factor VIII was dry heated at 100°C for 30 mins, 80°C for 72 hours and 60°C for 144 hours with loss of activity reported to be 5 percent, 32 percent and 28 percent respectively. There are a few caveats that could be attributed to these results. The initial purification of the Factor VIII concentrate had a solvent detergent inactivation step incorporated into the manufacturing process. The reduction of enveloped viruses may have reduced the overall challenge to the dry heat step, delivering more optimal results than a concentrate manufactured without a solvent detergent step. The activity value of Factor VIII was also measured pre and post lyophilisation which showed a 5 percent loss when using stabilisers in the formulation buffer. However, it is not known whether this activity loss was incorporated into the overall activity loss value at the end of the dry heat treatment. This stand-alone study simply highlights the lability of Factor VIII concentrate. With knowledge widely known of the efficacy of heat treatment for viral inactivation, attention should now be focused on evaluating the product impact of these methods on the specific protein molecules.

1.3 UVC

In the biopharmaceutical sector, heat treatment and chemical treatment are generally the 'go to' method for viral inactivation. However, even after applying these viral inactivation treatments, transmission of viruses such as HAV and B19 have been reported in patients receiving recombinant Factor VIII (Caillet-Fauquet et al., 2004). Parvovirus B19 has always presented risk of transmission and for this reason most purification processes will incorporate a nanofiltration clearance step into the process. Factor VIII is a relatively large protein and because of this a nanofilter that can ensure passage of Factor VIII also has the capacity of allowing passage of small viruses such as B19 and Minute Virus of Mice (MVM). A method that has the ability to target heat or chemically resistant viruses regardless of its presence or absence of an envelope would be widely welcomed in the biopharmaceutical industry.

Ultraviolet irradiation was used on blood products prior to the 1950's, but is still classified as a new technology for the biopharmaceutical industry. This is due to the limited data on the efficacy of this method for viral inactivation (Bergmann, 2014) but also due to its failed attempt at preventing HAV transmission in the 1950's.

UVC treatment operates at a wavelength of 254nm. At this wavelength the main target is nucleic acids, therefore it is in-discriminatory and can inactivate viruses irrespective of their presence or absence of a lipid envelope (World Health Organization, 2004). In general single stranded DNA or RNA type viruses are easier to inactivate. This is because of the mechanism of repair that exists in double stranded genome virus are more efficient along with single stranded genomes being more photo chemically reactive (Chin et al., 1995). Based on this knowledge, one would expect UVC irradiation to be effective at inactivating single stranded genomic viruses such as HAV and parvovirus B19.

Early studies carried out by (Hart et al., 1993) on immunoglobulins were encouraging at the time. The immunoglobulins were exposed to various UVC dosages for a period of 30 minutes. A series of both single stranded and double stranded genomic viruses were used as viral spikes. The results showed that at 5100 J/m^2 there was a $\geq$ 6.6 log reduction of the double stranded genome Vaccinia virus and $\geq$ 6.4 log reduction of the

singe stranded RNA Polio 2 virus. The Semiliki forest virus (SFV) which is a single stranded genome model toga virus in the Hepatitis C family showed a 3.4 log reduction with infectivity still being detected after the 30 minute duration. Even though these results showed acceptable viral log reduction, there is variability within the results which leaves unanswered questions regarding the parameters that can affect the efficacy of UVC treatment. UV light has a tendency to overheat so the prolonged UV exposure may have inherently caused an increase in temperature affecting the stability of the proteins.

One of the issues pertaining to UVC treatment is the fact that UV light damages proteins. It is suggested that the photochemical damage that occurs in proteins exposed to UVC is facilitated by Reactive Oxygen Species (ROS) (Chin et al., 1995). The addition of quenchers has shown to support viral inactivation by UVC treatment. (Chin et al., 1995) used a flavonoid known as rutin to act as a quencher to ROS and also to protect the Factor VIII protein during their investigation into UVC treatment. The investigators were able to demonstrate that not only did UVC treatment deliver acceptable viral log reduction against HAV and Parvovirus but the activity of Factor VIII was retained.

Upon review of these results it is clear to see that UVC is a promising method of viral inactivation. It is worth nothing however that the Factor VIII used in this study was highly purified by means of ion exchange chromatography and also underwent solvent detergent viral inactivation. These treatments would have clarified the process solution reducing the optical density and reducing the overall inherent viral load challenge present prior to purification. Based on this it would appear that UVC treatment may be more of a supplemental treatment rather than a stand-alone orthogonal method for viral inactivation.

As time has progressed since the Chin et al study, so has the technology. This has brought forward the introduction of continuous flow UVC treatment. This apparatus consists of a helical shaped coil that surrounds the UV bulb. This arrangement allows the dosage to be controlled by the intensity and the flow rate.

(Caillet-Fauquet et al., 2004) adopted this new technology in a study investigating protein activity and viral inactivation. They applied a low UV dosage of up to 240 J/m^2 which showed activity of Factor VIII remaining around 90 percent. For single stranded genome viruses MVM and EMC, a ≥7 log and 5 log reductions were achieved respectively. However for Bovine Herpes Virus (BHV) the log reduction was < 3 log. Like previous studies the starting material used was pre- treated with viral inactivation chemicals and highly purified. Considering the consequences that a MVM contamination can present to a biopharmaceutical company, being able to deliver a ≥7 log reduction through UVC treatment could give adequate justification for the implementation of this method into a purification process.

(Bergmann, 2014) has reported wide spectra of viral log reductions through continuous UVC treatment using a full complement of model viruses. No correlation has been seen between the UV sensitivity of some viruses and the presence or absence of an envelope, genome type or strandedness.

A lot of factors need to be considered when assessing UVC as a mode of viral inactivation. It has proven to be effective and inactivating a range a viruses particularly the smaller non-enveloped viruses. Variability seen across other viruses such as retrovirus confirms that UVC treatment is not a stand-alone orthogonal method. Even though at 254nm nucleic acids are the main target, proteins can be absorbed causing damage. One can either assess the compatibility of addition of quenchers or simply maintain a lower dosage. This is a balance of requirements between viral log reduction and protein recovery. The flow through mode of this technology shows that it could integrate well into a purification process. However, optical density is a factor that must be considered as it will determine if UVC can physically integrate into a manufacturing process. Process solutions that may contain high quantities of nucleic acids or are of high concentration could cause a reduction in absorbance leading to an ineffective UV dose (Bergmann, 2014). Overall UVC is an effective viral inactivation treatment that could easily be used in a large scale manufacturing process. Even though there are variations in its efficacy against all known model viruses, the technology represents a step forward in ensuring viral safety and would serve well as a combination type viral inactivation method. However, there are known risks associated with the use of UV

light which includes damage to DNA. This may present a challenge from an operator safety perspective when trying to integrate the method into a manufacturing process that involves manual personnel interactions.

1.4 Solvent Detergent

For labile proteins that exhibit physiochemical sensitivity, viral inactivation through solvent detergent treatment can sometimes be the most reliable method. Solvent detergent treatment was initially developed in the 1980's for use in blood product derivatives in order to inactivate enveloped viruses. "The development of the solvent detergent treatment by the New York Blood Centre provided an effective and simple technology for inactivating enveloped viruses even at large production scale" (Dichtelmuller et al., 2009).

Detergents are amphipathic, meaning the molecule contains both hydrophilic and hydrophobic groups. In an aqueous solution detergents will cluster into what is known as micelles. These micelles can cause inter membrane proteins to become mobilised and engulfed within the detergent micelle. Because viral phospholipid membranes also have amphipathic properties, they can interact with the detergent micelles. At what is known as the Critical Micelle Concentration (CMC), the phospholipid membrane can become saturated with detergent resulting in membrane solubilisation and disruption. This essentially will lead to the inactivation of the virus.

The CMC is a critical parameter that must be understood when selecting the appropriate detergent for viral inactivation. It is suggested that at low concentrations detergents can exist as a monomer being absorbed into the viral lipid membrane but without the ability to inactivate (Conley et al., 2017). At too high a concentration problems could arise in its removal further downstream in the purification train. Therefore the solvent detergent composition will vary and will be dependent on the specific manufacturing process to which its efficacy will need to be proven (Seitz et al., 2002).

Solvent detergent treatment takes advantage of the natural characteristics of a detergent and disrupts the lipid membrane of enveloped viruses, rendering them inactive. Hence

the main limitation of this type of treatment is its inability to successfully inactivate non-enveloped viruses.

Since this solvent detergent is non-selective, it only target lipids or its derivatives and generally does not impact the activity of recombinant proteins (Martin Remington, 2015b). However, this specificity to lipids can affect the viral inactivation kinetics especially in process solutions with high lipid concentrations. Therefore it is important that the validation of this viral inactivation step is carried out using worst case conditions (World Health Organization, 2004).

(Chandra et al., 2002) have reported on past studies carried out an evaluation on solvent detergent treatment on non-enveloped viruses such as HAV and B19. All findings resulted in infectivity being detected, demonstrating that this treatment is ineffective against non-enveloped viruses. This appears to be the main limitation to this form of treatment as all other reports that will be discussed further indicate that solvent detergent treatment is an excellent form of viral inactivation.

As this method only has the ability in inactivate enveloped viruses, the threat viral threat for non-enveloped viruses remains a concern. For this reason a viral removal step is also required to be incorporated within a purification process provided separate orthogonal viral clearance methods (International Conference on Harmonisation, 1999).

The physical operations associated with the solvent detergent method are similar to that of low pH treatment. The process solution is incubated in the presence of solvent detergent at a set temperature and duration. With the addition of a process impurity such as a solvent detergent to a pharmaceutical product brings about concerns over toxicity. In this type of treatment the compounds are interacting with lipids and proteins and can have the potential to have an adverse effect at a cellular level. As well as this, if the compounds have the ability to interact with nucleic acids it could present genotoxicity and/or carcinogenicity to a perspective patient. For this reason the solvent detergent must be removed to a level where toxicity is not presented (Pelletier et al., 2006). This can be done through chromatographic means, precipitation or oil extraction.

It is well known that Triton X-100 is employed in solvent detergent treatment due to its high degree of efficacy towards inactivating enveloped viruses. However, in order to align with the main aim and objective of this project, Triton X-100 will not be evaluated independently as part of this literature review. It will be used as a comparative, where required, in order to determine the efficacy of other alternative detergents.

At the initial stages of solvent detergent development, ethers were used as the solvent in combinations with Polysorbate 80. This combination showed explosive characteristics which resulted in a move towards a Tri-N-Butyl Phosphate (TNBP) and Sodium Cholate combination (Dichtelmuller et al., 2009). This progressive development was carried out by (Horowitz et al., 1985a), where they initially evaluated the rate of viral inactivation when using TNBP/Tween 80 (also known as Polysorbate 80) at the same conditions as the current treatment method using Ether/Tween 80. Here they showed the rate of inactivation at 4ºC for 6 hours was more rapid using the TNBP/Tween 80 combination. The log reduction values of VSV, Sindbis and Sendai virus were 4.0, ≥5.8 and 3.5 logs respectively. The researchers noted that the EMC virus, a non-enveloped virus was unaffected by the ether treatment, which would be anticipated given the mode of action of this type of treatment. (Horowitz et al., 1985a) further evaluated other alternative detergents at different concentrations whilst using TNBP at a concentration of 0.3% and the same time examined the recovery rates of the anti-haemophilic factor (AHF) protein used. The results showed that the degree of viral inactivation reduced concurrently when the concentration of Tween 80 was decreased from 1% to as low as 0.01%. After a 4 hour incubation at 1% Tween 80 ≥4.2 log reduction of all 3 model viruses was obtained along with a recovery percentage of 114%. Sodium Cholate and Sodium Deoxycholate were also evaluated. The rate of inactivation appeared promising although at higher concentrations, 0.2% and 1%, Sodium Deoxycholate and sodium cholate respectively inactivated the AHF molecule.

As mentioned. These detergents can possess toxic characteristics and must be removed from the process below acceptable limits. When investigated, (Horowitz et al., 1985a) discovered that TNBP was easily removed through gel filtration but due to the large micelle formation, Tween 80 was prevented. Tween 80 was reduced by 90% using a precipitation method. The challenge that is faced in this scenario is adjustments in

Tween 80 concentration in order to improve removed rates could adversely affect the rate of viral inactivation. On the other hand, an additional process step to ensure removal of 1% Tween 80 could result in a decrease in product recovery. Sodium Cholate forms smaller micelles and was easily removed through gel filtration with a slight reduction in recovery (80%).

This early study marked the start of the evolution of solvent detergent treatment of blood products and began to provide the industry with option to alternative detergents. The temperature conditions used in this study were quite static and because of this, many unknown factors regarding inactivation kinetics were presented.

This study was further followed up by (Horowitz et al., 1992), using both plasma and blood derived coagulation factor concentrate. The same virus panel as previous was also used. Figure 1 shows the results of this study. Here we can see that the VSV and Sindbis virus were completely inactivated after 2 hours when using either Tween 80 or Sodium Cholate, although the rate of inactivation for Tween 80 is more rapid.

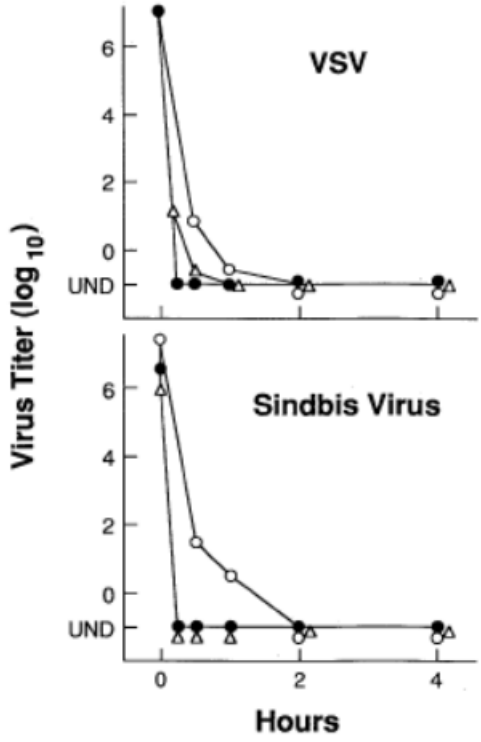

Figure 1: Rate of viral inactivation against VSV (upper panel) and Sindbis (lower panel) in human plasma when treated with TNBP/Triton x-100 (solid circle) at 30°C, TNBP/Sodium Cholate (open circles) at 30°C or TNBP/Tween 80 (triangles) at 24°C.

The results of this study have shown that temperature plays an important role in the kinetics of viral inactivation. In previous studies Sodium Cholate (0.2%) incubated for 4 hours at ambient conditions delivered 2.3 and 3.7 log reduction of VSV and Sindbis respectively. In this current study, by increasing the incubation temperature to 30°C the log reduction values were > 6 logs after 2 hours. Results for Tween 80 were comparatively the same in both instances at 24°C. For both studies carried out by Horowitz et al, an infectivity assay was used to calculate viral log reduction for the Sindbis virus,

It is difficult to get a true comparative picture of the efficacy of the different detergents as not all conditions were the same for each experiment. Rate of recovery studies were only carried out on a process solution exposed to TNBP/Triton X-100. Therefore it cannot be discounted that the increase in temperature used for the Sodium Cholate

experiment may have affected the recovery rate. Also the TNBP/Triton X-100 combination was only trialled against plasma and not the coagulation factor. This prevents a direct comparison of all detergents involved. However, taking a holistic approach, the results showed excellent inactivation rates which enabled the use of both Tween 80 and Sodium Cholate in solvent detergent treatment of blood products.

(Piet et al., 1990) also investigated various TNBP/detergent combinations. Given the concerns over virus transmission in blood products at the time, the investigators also used a HIV and Hepatitis B (HPVB) model virus in their experimental work. In these studies various conditions were evaluated. A 2% TNBP solution at 37°C, 1% TNBP/1% Polysorbate 80 at 30°C and 1% TNBP/1%Triton X-45 at 30°C were used with a range of coagulation factors. As can be seen in Table 3, viral inactivation of VSV and Sindbis occurred rapidly (>4LRV) with no detectable viruses after the 6 hour incubation period using all detergent combinations. The HIV model was successfully inactivated under the set out conditions with ≥3.1 log reduction after 15 minutes using 1% TNBP/1% Polysorbate 80 at 30°C.

Table 3: Viral inactivation rates of viruses added to plasma and treated with a variety of TNBP detergent combinations (ref. Piet et al., 1990)

	Amount of virus killed (log_{10} decline)							
	2% TNBP 37° C		1% TNBP/1% Tween-80 30°C			1% TNBP/1% Triton X-45 30° C		
Virus	1 hour	6 hours	1 hour	4 hours	6 hours	1 hour	4 hours	6 hours
VSV*	4.2	≥5.0†	5.0	≥5.2	≥5.2	≥5.2	≥5.2	≥5.2
Sindbis	≥5.2	≥5.2	≥5.5	≥5.5	≥5.5	≥5.5	≥5.5	≥5.5
HIV‡	≥3.6	≥4.2	≥3.1§	NT‖	NT	NT	NT	NT
NANBHV¶	≥5.0	NT	NT	NT	NT	NT	NT	NT
HBV**	≥6.0	NT	NT	NT	NT	NT	NT	NT

*Vesicular stomatitis virus.
†Determined at 30° C (VSV is not stable at 37° C in plasma).
‡Human immunodeficiency virus.
§After 15 minutes.
‖Not tested.
¶Non-A, non-B hepatitis virus.
**Hepatitis B virus.

Animal testing in chimpanzees was used to detect infectivity of the HPBV model virus treated with 2% TNBP at 37°C. At 6 and 9 month time points no viral antibody markers were detected in the animal models.

The activity of Factor VIII among the other coagulation factors was assessed for impact. With 100% activity in untreated solutions, the results showed near comparability when treated with solvent detergents.

This study is positive in that it shows that even though Factor VIII is a known labile protein, at an elevated temperature of 30°C the biological activity is maintained. In plasma derived coagulation factor such as Factor VIII, the bound Von Willebrand factor (VWF) serves as a stabiliser and protectant at an elevated temperature. This VWF is absent in recombinant Factor VIII and therefore incubation temperature is a factor that needs to be considered when dealing with this type of recombinant protein. Apart from this, the study has evaluated Triton X-45 as another detergent option which could be investigated further.

As part of an efficiency programme to try implement disposable bag technology into the plasma collection process, (Burnouf et al., 2006) carried out trial work within these disposable bag systems. Similar conditions used by (Piet et al., 1990) were employed using 2% TNBP at 37°C and 1% TNBP/1% Triton X-45 at 31°C. The main objective of this experiment was to evaluate product recovery as appose to viral reduction. The treated material was assayed according to the specific blood product that was used and was compared to the starting untreated material. The results showed recovery of Factor VIII was 99 % 104% with 2% TNBP and 1% TNBP/1% Triton X-45 respectively. Whilst this small study was more focused on feasibility and improving the overall receipt and solvent detergent treatment of plasma pools, the results showed the robustness of Factor VIII again at elevated temperatures whilst using Triton X-45.

It is clear from the literature that Polysorbate 80 is a popular and robust choice for solvent detergent treatment. Polysorbate 80 can be derived from both animal and plant sources. As was the case with many process components, Polysorbate 80 has mainly been used from animal derived sources. With the need to move away from materials of animal origin due to fears of Bovine Spongiform Encephalopathy (BSE), researchers

have investigated the use of plant derived Polysorbate 80 as a replacement to animal derived for use in viral inactivation.

(Seitz et al., 2002), compared both origins of Polysorbate 80 against a panel of viruses such as VSV, PRV, SFV and BVDV. Critical process parameters such as temperature, Solvent detergent concentration and protein concentration were variables that were also evaluated. In a 5% human albumin solution both vegetable and animal Polysorbate 80 at 0.3% TNBP/1% Polysorbate 80 were incubated at 25°C for 90 minutes. Further testing was carried out up to 6 hours incubation. Figure 2 shows the inactivation rates in parallel using the standard protocol conditions. It can be seen that the kinetics of both runs using both Polysorbate 80 origins are clearly aligned and show no significant difference. Inactivation of viruses in all cases is complete by 60 minutes if not sooner.

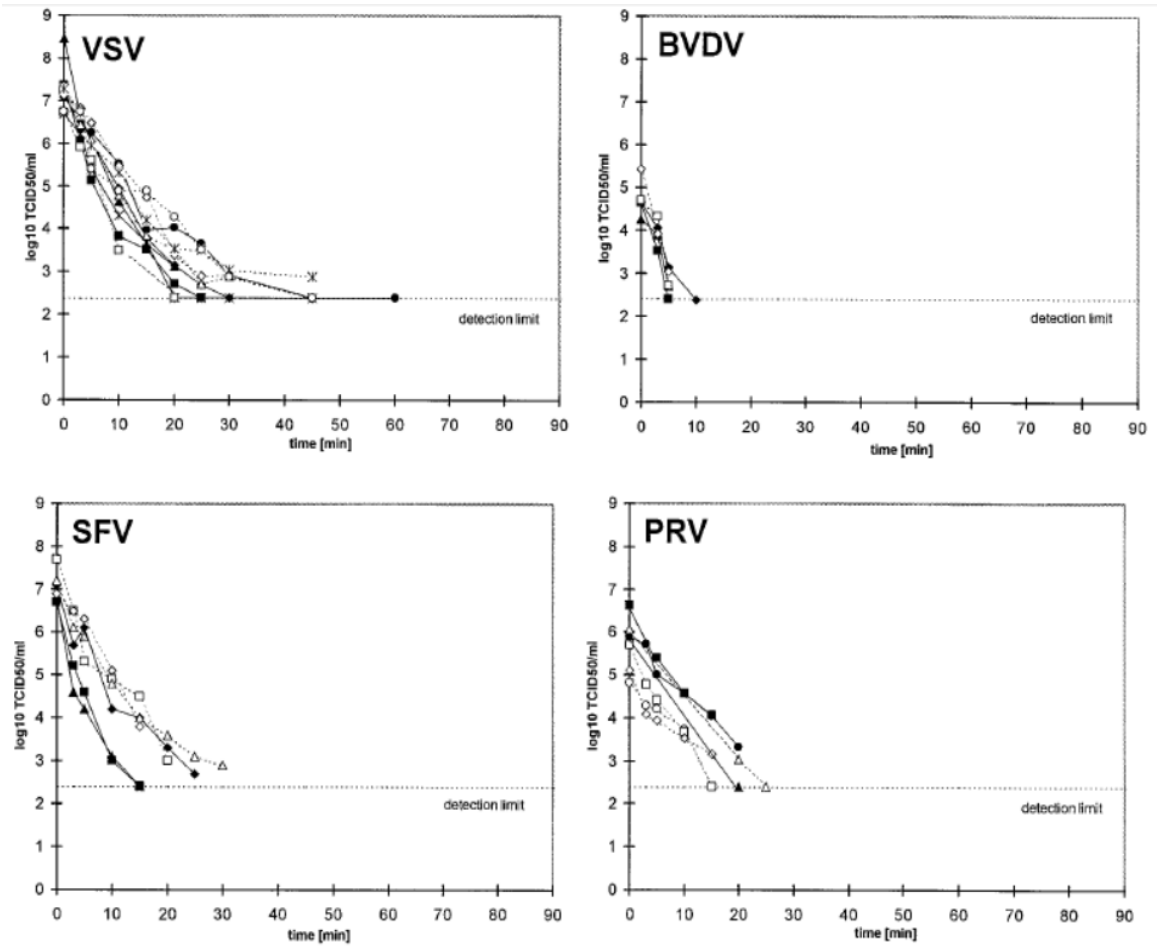

Figure 2: Viral inactivation rates of VSV, BVDV, SFV and PRV using Tween 80 of animal origin (solid markers) and plant origin (open markers)

As part of the robustness studies an incubation temperature of between 6-27°C, protein concentrations of 5 and 10%, and solvent detergent concentrations ranging 25, 50 and 75% the standard concentration was used. 75% solvent detergent concentration appears to be the optimal set point that inactivated all viruses (>4LRV). Whilst the BVDV virus showed high inactivation at 50% concentration, high infectivity was still present at 25% concentration.

One focal point with using plant derived material is phytovirus exposure. Phytoviruses are prevalent in all plant species and because of this human are exposed to these viruses on an ongoing basis. Although phytoviruses are not considered to be pathogenic towards human they have the potential to elicit an immune response in some cases (Balique et al., 2015). However, in a biopharmaceutical industry, it is expected that Good Manufacturing Practice (GMP) would result in thorough characterisation of all raw materials within a manufacturing process before being qualified for use. Therefore all known contaminants would be well documented and controlled accordingly.

Temperature is a known critical process parameter for solvent detergent viral inactivation so this was investigated using both Polysorbate 80 origins. The results appear to show that temperature correlates to the rate of viral inactivation. At 27°C, near complete viral inactivation was achieved using both Polysorbate 80 origins within 30 minutes whereas infectivity was present at the lower temperature of 6°C. These findings simply reconfirm previous conclusions drawn by Horowitz and Piet surrounding the more optimal inactivation temperature prescribed.

In higher product concentration solutions there was again a negligible difference in viral inactivation rates. Collectively the results demonstrate the robustness of Polysorbate 80 as a viral inactivation detergent but also highlight the efficacy regardless of the origin. The product solution used in this study was human albumin and may not be comparable to a coagulation factor such as Factor VIII due to the lability of the AHF. However the robustness conditions used in this study could be considered less extreme to those carried out previously by other investigators when studying the effect on AHF. All of which results were positive.

In an effort to seek alternative detergents to the commonly used detergent Triton X-100 and to optimise a current manufacturing process, (Roberts et al., 2009), investigated the use of Polysorbate 20, Pluronic F68 and F127 in combination with TNBP. In this study they used a Factor VIII/vWF concentrate product alongside a range of commonly used model viruses. In the initial study using a concentration of 0.3% TNBP and 1% detergent against the Sindbis virus, rapid inactivation was seen (>6LRV) after 2 minutes. This was equally comparable to Triton X-100. In contrast, the 2 Pluronic detergents were essentially ineffective at inactivating the Sindbis virus. Poly sorbate 20 was carried forward into more detailed studies. Against a panel of viruses >4.1 LRV was achieved after 1 minute and ≥6LRV after 60 minutes. Vaccinia virus showed some resistance as expected but was fully inactivated after the 60 minute period. Such log reduction claims have also been corroborated through studies carried out by Norling reported by (Miesegaes, 2014), who also achieved ≥5.7 LRV at 20°C after 1 hour using Polysorbate 20.

During manufacturing, variability can occur within the process. To understand the effects this variability may have (Roberts et al., 2009), examined those parameters that may exhibit variation i.e. Solvent detergent concentration, protein concentration and temperature at extreme ranges. Independently, all parameters tested at the high and low end of their ranges all inactivated the Sindbis virus by > 5LRV within 2 minutes. However when combined into a worst case process scenario, that being high protein concentration, low solvent detergent concentration and low temperature, a slight shift in the inactivation kinetics occurred resulting in > 6 LRV between 10-30 minutes. These studies give a good indication not only of the robustness of Polysorbate 20 but also the tolerance that could be applied within a manufacturing process. However, the worst case temperature (20°C) that was applied represents more of a worst case condition in routine manufacturing rather than a worst case condition for a robustness study, since other studies have used temperatures as low as 6°C. Even though product recovery percentage was not calculated, assurances of good recovery would be anticipated based on Polysorbate 80's usage within the Pharmaceutical industry currently.

In the last number of decades since the first approval of solvent detergent as a form of viral inactivation treatment a lot of data has been generated thanks to the dedication and

perseverance of investigators past and present. (Dichtelmuller et al., 2009), courtesy of 7 Plasma Protein Therapeutic Association (PPTA) members, has merged their viral inactivation data on a range of blood products such as Factor VIII and immunoglobulins. This has resulted in over 300 different studies being published detailing the various conditions employed.

In summary, all solvent detergent combinations evaluated delivered effective viral inactivation rates with mean values exceeding >4 LRV. In robustness studies carried out, pH, temperature and protein concentration did not appear to affect viral inactivation kinetics. This appears to contradict previous studies where inactivation rates declined in parallel with decreasing temperature. However these previous studies were only carried out using Tween 80 and may not reflect the inactivation kinetics of other detergents. (Dichtelmuller et al., 2009) reports that the robustness studies at temperatures below 10°C were only performed on TNBP/Triton X-100 combination.

The only variable that showed some influence to viral inactivation was the concentration of the TNBP solvent. In concentrations of 10% of the standard concentration (3g/L) infectivity was still detected in some model viruses. This is not surprising as both the solvent and detergent are intrinsically linked. Detergent is required in order to help solubilise the solvent in an aqueous solution. Therefore any decrease in solvent concentration would directly decrease the concentration of detergent and overall affect the log reduction values.

These combined studies give confidence in the solvent detergent treatment under a variety of detergent combinations. Although Factor VIII was not subjected to Sodium Cholate in any of the mentioned studies, previous data from initial and subsequent development of the solvent detergent method has ensured its efficacy in Factor VIII solutions albeit plasma derived and not recombinant based.

Unsaturated fatty acids have been noted to display virucidal characteristics (Horowitz et al., 1988). Such fatty acid Sodium Caprylate has been used as a stabiliser in the heat treatment of Factor VIII to inactivate enveloped viruses (Korneyeva et al., 2002). In the manufacture of IGIV by Bayer Corp., they have included a solvent detergent treatment step using 0.3%TNBP/0.2% Sodium Cholate. An increase in manufacturing time and

decrease in yields reported incentivised the investigation into an alternative form of viral inactivation. (Korneyeva et al., 2002) identified Sodium Caprylate as a potential candidate and examined it with the aim of evaluating its viral inactivation efficacy. A performance comparison to the current solvent detergent process using Sodium Cholate was also performed with the hope of reducing the manufacturing processing time.

At a low concentration of Sodium Caprylate (0.15%), log reductions of ≥4.7 and ≥ 4.2 were reported against HIV and PRV virus respectively. The BVDV virus showed some resistance so further studies on this virus was performed using wider concentration range and time as a variable. The virus became rapidly inactivated after 30 minutes in a 0.2% concentration. As the concentration increased, the rate of inactivation also increased, as expected.

The TNBP/Caprylate combination was evaluated against the TNBP/Sodium Cholate at a 0.3% concentration. Sodium Cholate required 4 hours to completely inactivate the BVDV virus whereas the Caprylate detergent inactivated BVDV below a level of detection after 3 minutes. The incubation temperatures for this study were carried out at 28°C and 24°C for Cholate and Caprylate respectively.

The results of this study show effective viral inactivation when using Sodium Caprylate as a detergent. Even though Factor VIII wasn't he protein of choice in the study (Korneyeva et al., 2002) have indicated that the use of Sodium Caprylate may well be of benefit when treating other proteins.

(Durno and Tounekti, 2015) have reported, from a PDA conference held, that the concerns around detergents and their environmental impact were discussed. These concerns have led to the establishment of the REACH list and have initiated the pursuance of alternative, more eco-friendly detergents. (Durno and Tounekti, 2015) reports the findings of experiments carried out by Fisher and Norling to seek alternative detergents to Triton X-100. Table 4 and 5 shows the full list of detergents evaluated along with the log reduction values obtained. It can be seen that > 4LRV has been obtained using a wide range of eco-friendly detergents. Polysorbate 20 and 80 both had delayed inactivation rates when used without a solvent.

Table 4: Inactivation of XMuLV in Cell Culture Fluid of Mab 1 with detergent ± solvent

Time (h)	Detergent with and without Solvent (LRV of XMuLV)					Detergent (LRV of XMuLV)					
	1% Poly-sorbate 20	1% Poly-sorbate 20 + 0.3% TnBP	1% Poly-sorbate 80	1% Poly-sorbate 80 + 0.3% TnBP	1% TNBP	0.5% SafeCare 1000	1% SafeCare 1000	1.4% Sodium Caprylate	1% Decyl Glucoside (mixture)	1% Ecosurf EH-6	1% Ecosurf EH-9
1	1.4	≥5.7	3.3	5.2	2.9	≥5.1	≥5.5	≥6.0	≥5.1	≥4.7	≥5.1
3	2.3	NT	5.7	≥5.5	3.1	5.7	≥5.5	≥6.0	≥5.1	≥4.7	≥5.1

NT: not tested.

Table 5: Inactivation of XMuLV in Cell Culture Fluid of Mab 2 with detergent only

Time (h)	0.5% Ecosurf EH-9	1% Ecosurf EH-6	0.5% APG 325N	1% APG 325N	0.5% Lutensol XP90	1% Lutensol XP90	0.5% Triton CG110	1% Triton CG110	0.5% Tomadol 900	1% Tomadol 900
1	≥4.9	≥4.8	≥5.5	≥5.4	≥5.2	≥5.1	≥5.7	≥5.6	≥4.9	≥4.8

Impact to product quality of the 3 Mab used were assessed using 1% detergent only solution and incubated for 18 hours. No product impact was observed using these detergents except for Lutensol XP90 which showed a slight increase in acidic variants. Further robustness studies at a lower incubation temperature of 12°C using 0.3% detergent was carried out with the addition of 2 other viruses, PRV and BVDV. Eco-Surf EH-9 and Lutensol XP90 both failed to inactivate the PRV virus. Whereas APG 325N and Triton CG110 both inactivated all 3 viruses to ≥5 LRV.

This study gives some insight into viral inactivation of recombinant proteins and is positive to note that product quality was not affected. Both detergents were easily removed by a Pro A Chromatography step, which commonly proceeds viral inactivation step. So their integration in a purification process would seem to be straight forward. However, foaming was reported to be an issue with the two candidates. Even though antifoam may alleviate this issue, the presence of another process impurity is not desirable and would have to be evaluated separately.

Another study was carried out by (Conley et al., 2017), to also try and replace Triton X-100 for a more eco-friendly detergent. An initial study carried out on a Fc Fusion protein using 4 zwitterionic detergents, Sulfobetadine (SB) 3-12,3-14, 3-16 and Lauryldimethylamine N-oxide (LDAO) and also a non-ionic BRI J-35 detergent. Bioactivity assays conclude that all 5 detergents did not have an adverse effect on the

protein structure. However, after a 120 minute incubation period, only the 4 zwitterionic detergents were capable of virus inactivation. All 4 completely inactivated the MuLV after a 20 minute period (see Figure 3). Based on these results and a favourable environmental assessment LDAO was carried forward for further kinetic studies.

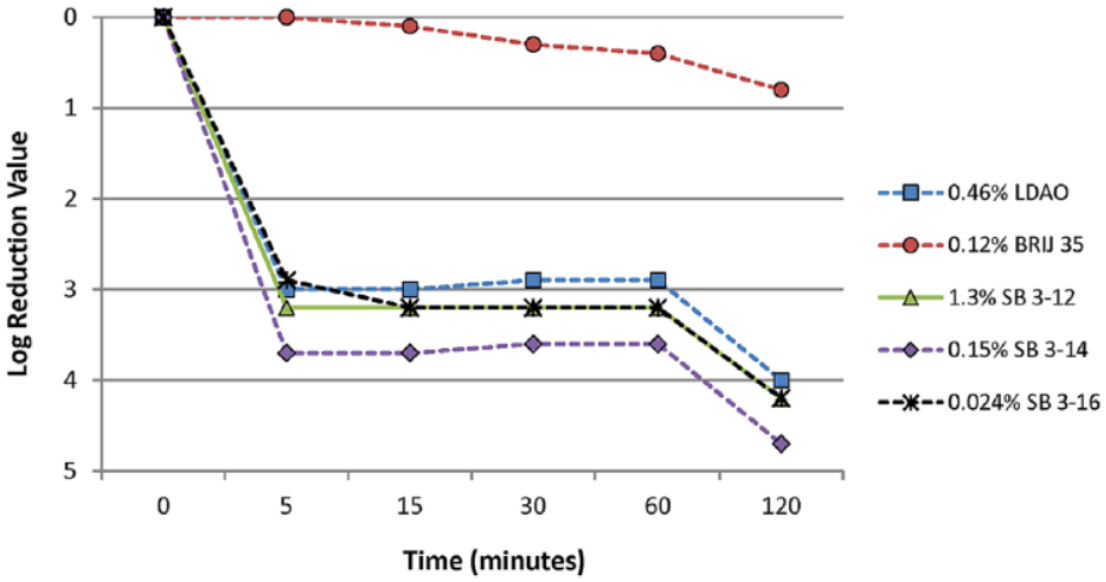

Figure 3: Viral inactivation of MuLV using various detergent alternatives

Out of all the process variables that may affect the kinetics of viral inactivation, temperature is the one known variable that has a direct impact i.e. the higher the temperature, the quicker the rate of inactivation (Bertrand et al., 2012). (Conley et al., 2017), also determined that detergent concentration plays a critical role in viral inactivation. LDAO concentrations of 0.012%-0.138% were run using Mab and the FC fusion protein at a temperature range of 2-8°C. They found that at concentration below 0.023% viral infectivity was still detected. pH, conductivity, lipid concentration and protein concentration were also evaluated which showed no impact to viral inactivation rates, achieving > 4 LRV.

The data confirms that LDAO is a viable alternative to Triton X-100. The robustness studies carried out showed viral inactivation is only affected by detergent concentration. Given that the CMC of a detergent plays an important role in membrane disruption, as long as the concentration validated is above the CMC successful viral inactivation should be achieved using LDAO in recombinant proteins.

The study of viral inactivation literature has revealed that due diligence has been taken with respect to ensuring the safety of blood derived products. This is backed up by the high safety record when it comes to preventing the transmission of viruses in blood products. The innovation and development within the pharmaceutical industry has allowed multiple modes of viral inactivation to become available and evaluated within a manufacturing process. Granted, not all methods of viral inactivation are practical or feasible within certain manufacturing processes. However, there appears to be methods available that can be applied to all manufacturing processes of blood derived products. One point to note is that modern research and focus appears to be lacking overall in this area which is evident by the limited number of published research articles within the last decade. However this merely validates the rationale behind this research project.

To align with the aim of the project, the literature review also focused on alternatives to the detergent Triton X-100 within the solvent detergent method of viral inactivation. Since the first development of the solvent detergent method in the 1980's to the present day, many alternatives of solvent detergent combinations and detergents alone have been successfully evaluated. Polysorbate 80 has shown its robustness since its first use in the 1980's and continues to do so. However, the review has shown that there is still a strong reliance on the use of Triton X-100 due to its efficacy in viral inactivation.

Obviously, with Triton X-100 being added to the REACH authorization list this reliance on Triton X-100 needs to conclude and the evaluation of more eco-friendly detergents needs to be performed. This is an area that appears to be lacking in research.

To further improve patient safety and diverge from the reliance of blood derived medicinal products, recombinant proteins have entered the market, more specifically recombinant Factor VIII. The development of recombinant Factor VIII was seen as a welcome introduction to the market as it greatly reduced the underlying risks of virus

transmission in hemophiliac patients. Although recombinant proteins are well established and hold a good safety record, they are derivatives of mammalian cell lines therefore the risk of viral contamination is still present. The presence of viral contamination within cell lines is widely known hence why there are such stringent measures mandated from the regulatory bodies to evaluate and fully characterise cell lines to mitigate the risks of viral contamination.

The review of the literature has highlighted a few disparities within the realm of virus inactivation. Firstly, in a significant number of studies, the protein of interest used was either an Immunoglobulin or an albumin. Given the resilience of these types of blood products in comparison to the labile protein Factor VIII, it was difficult to correlate a relationship. In studies where coagulation factors were used, they were of high purity. Since viral inactivation generally occurs at the start of a purification train, the process solution is of low purity and contains many process impurities. However the research failed to recognise this by employing the use of highly purified coagulation factors.

Secondly, there appears to be a lack of detailed research involving recombinant Factor VIII protein. Recombinant Factor VIII products have been on the market for a number of decades, therefore it was surprising to see the majority of viral inactivation studies still being performed on blood derived Factor VIII. Blood derived Factor VIII also contains the vWF, which helps stabilise the molecule. Therefore recovery rates reported on blood derived Factor VIII may not be equivalent to recombinant Factor VIII which is void of vWF.

In the case of solvent detergent virus inactivation, there have been a number of recent studies carried out on alternatives to Triton X-100 which aligns with the aim of this research project. These studies were carried out on MAb proteins and showed positive results of which invites the opportunity for these alternative detergents to be carried forward and evaluated on recombinant Factor VIII.

Overall, the review of literature has stressed the need for research data on recombinant Factor VIII protein specific to viral inactivation as a means to find an alternative to the use of Triton X-100. Given the strong use of Triton X-100 in the industry, I would be confident that more data on alternatives to Triton X-100 will become available in the

future. However, this project will progress and evaluate all possible alternatives to the use of Triton X-100 for viral inactivation in recombinant Factor VIII, generating sufficient research data to enable a solid definitive conclusion. The proceeding stages of this project will detail the research methods to be employed to gather the research data.

3. Research Methodology

To obtain the relevant data to test the hypothesis, experimental research was the most suited choice of methodology to engage with. This type of research provided non biased qualitative data that allowed statistical analysis to be performed. This has determined what virus inactivation detergents had a direct quality impact on the recombinant factor VIII molecule and what detergents could possible progress to further proof of concept viral inactivation studies.

Preliminary screening

A thorough literature review has been carried out above which has reviewed various methods of viral inactivation. It has also identified a number of detergents that have undergone viral inactivation studies that may be compatible to progress for evaluation within the remit of this research.

Since the current manufacturing process of the recombinant Factor VIII protein incorporates a solvent detergent step using Triton X-100, the main focus of this research involved identifying an alternative detergent within the manufacturing process. Evaluations of other modes of viral inactivation were, at a minimum, carried out through a paper based technical assessment. Small bench scale studies were performed, where feasible, as process knowledge on the recombinant Factor VIII molecule suggested other forms of viral inactivation are incompatible.

Each identified candidate underwent a paper based technical assessment. The criterion for assessment was as follows:

- The amount of available literature present on the specific detergent.
- The detergent's history with Factor VIII molecules.
- Potential impact to the manufacturing process downstream of the viral inactivation step.
- Ability to remove the detergent from the manufacturing process.
- Ability for detection of residual detergent.
- The supply chain availability

- Overall safety profile of the detergent.

Upon narrowing down the candidates, each remaining potential candidate was involved in small scale bench and analytical studies to evaluate the process performance and product quality attributes. This concluded whether or not the candidates had an adverse impact on the product itself. Based on these results, the chemicals that were shown to have the least impact on product quality were identified and carried forward as part of the factorial based Design of Experiment (DOE).

Characterisation Design of Experiment

The purpose of this study was to characterise the impact of different detergent or solvent/detergent concentrations, operating temperatures and inactivation times on performance of the viral inactivation step which was assessed through a statistical design of experiment (DOE). The impact of operating within these conditions on process performance and product quality was evaluated by comparing results between characterisation and control runs. The product quality and process performance has identified optimal viral inactivation conditions which may permit further proof of concept viral inactivation studies at an external third party laboratory.

The process parameters for each potential candidate were investigated via a screening study with experimental factorial design using the Design Expert 9.0.2 application. A factorial design was used to assess the main effects of each condition. Each condition was ran either singly or in duplicate at a level determined through the Design Expert 9.0.2 application. Center point runs were also included in the study. The particular number of viral inactivation experimental runs determined is displayed in the relevant appendix which is referenced within the results and discussion section. Results from this screening study may be augmented with follow on studies to further investigate response surfaces of significant parameters and their interactions. Significant parameters may be included in worst case runs using values at the extremes of their proven acceptable range. The impact of different detergents and different operating conditions were evaluated by comparing results between the test (characterisation) runs and the control Triton X-100 runs.

Stock Detergent

In order to obtain the target conditions specific for each experimental run, a stock solution of each candidate at specific concentrations was formulated. These stock detergent solutions were added to the process pool in order to achieve the overall target viral inactivation concentration.

Process Intermediate Pool

Process material was needed in order to carry out the multiple viral inactivation experiments. This material was obtained from a number of cell culture batches which were generated as per the standard process conditions followed by an initial capture chromatography step. The only exception to this was that all buffers used were free of Triton X-100. The eluate material was prepared by 0.22µm filtration and was either used immediately in the DOE or frozen at 80°C until required.

Viral Inactivation Experiments

The viral inactivation procedure was carried out as per the current process procedure. Amendments to this procedure based on the DOE are outlined within the results and discussion. 20ml aliquot samples were taken and placed in both incubators and refrigerators to ensure temperature control with a gentle agitation performed. A timer was used to monitor the inactivation time of each sample which were then removed from the incubator/fridge when the designated incubation time was met.

After incubation with each solvent/detergent combination, 15 mL of each viral inactivation pool were purified immediately using an affinity chromatography step to facilitate additional product quality testing analysis (SEC, RP-HPLC).

Third Party Viral Inactivation Experiments

The purpose of this study was to evaluate the efficacy of the two potential alternative candidates at inactivating two model viruses. The two model viruses used in this study were the Xenotropic Murine Leukaemia Virus (x-MuLV), which is a single stranded RNA virus, and the Pseudorabies Virus (PRV), which is a double stranded DNA virus. Viral detection was done through an in-vitro quantitative infectivity assay that employed the use of PG4 indicator cells. Both Candidate A and D were formulated to

specific target concentrations for use in the study (see Table 6). Duplicate runs were performed for both candidate experiments.

Table 6: Candidate target concentrations used in viral inactivation experiments

Candidate Name	**Detergent Target Conc. (w/v %)**	**Solvent Target Conc. (w/v %)**
Candidate A	0.7	0.25
Candidate D	0.05	N/A

Prior to conducting the viral inactivation studies, the influence of the test solution on cell growth and virus replication needed to be discounted through a cytotoxicity assay and interference assay.

For the cytotoxicity assay, a target dilution, of the test solution, which was non-cytotoxic was determined through microscopic methods using the cell culture medium for the PG4 indicator cells. The impact of the non-cytotoxic dilution on the virus titre was then analysed through an interference assay. This involved cultivating the model viruses and determining the overall virus titre. After addition of the test solution, the virus titre is again determined and compared to the original titre to ensure no significant changes.

For the viral inactivation study, the test solution was spiked with virus stock solution at a spike ratio of 5%. A control sample of the spiked test solution was taken pre viral inactivation and held for the duration of the experiment. The sample was analysed at initial sample procurement and at the end of the viral inactivation period to evaluate if incubation time or other test solution components had an impact on the model viruses used.

The spiked test solution was incubated at 12 °C and the detergent was added. Samples were taken at specific time points and diluted with cell culture medium before being

analysed for virus titre. Since the limit of detection of a sample is dependent on the volume of sample incubated with the indicator cells, large volume plating was performed on samples for time points 60 mins and 120 mins.

4. Results and Discussion

4.1. Analysis of Alternatives

The initial assessment involved evaluating the various methods of viral inactivation that were identified through the literature review as well as potential detergent candidates.

Low pH

Since the setup of low pH viral inactivation is relatively straight forward using primarily low pH buffers a small scale bench study was performed on the recombinant Factor VIII molecule. Low pH buffers of 3.5 to 4.1 were incubated with the process solution for a period of up to 2 hours. After this time high turbidity was noted. Following a subsequent filtration step loss of protein up to 47% was observed due to precipitation of the protein (data not shown). Based on these findings, it was concluded that low pH viral inactivation was an unsuitable replacement for the solvent/detergent method.

Pasteurisation

The literature review has shown that pasteurisation is an effective mode of viral inactivation particularly with blood derived products such as albumin and immunoglobulins. However, (Hilfenhaus and Nowak, 1994) has shown that stabilisers used in Factor VIII process solutions had caused reduced viral inactivation towards some viruses particularly the HAV virus. Further studies have shown that the activity of Factor VIII is reduced at higher pasteurisation temperatures, simply highlighting how labile Factor VIII is towards heat treatment.

Another consideration is that the Factor VIII molecule used in any of the reviewed literature was blood derived and not recombinant based. Therefore the impact of pasteurisation on recombinant Factor VIII is unknown. The process solutions used in

experimental works were highly purified. Since the viral inactivation step for the recombinant Factor VIII occurs at the start of purification it contains many process and product impurities that could affect the efficacy of this type of method.

From the knowledge known on the impact of heat to the Factor VIII molecule, as well as the lack of research carried out on recombinant Factor VIII, pasteurisation was eliminated as a potential alternative treatment.

UV Irradiation

UV irradiation at 254nm has the ability to target nucleic acids regardless of their presence of a lipid envelope. Although results reviewed showed variability, this method can deliver acceptable log reduction values against certain viruses.

A number of concerns present itself with this method:

- UV irradiation can cause protein damage leading to aggregation. Although Chin et al, 1995 demonstrated protection of Factor VIII protein using a flavonoid known as rutin, the Factor VIII molecule was highly purified and was blood derived.
- UV irradiation can generate heat which with prolonged exposure could inherently lead to loss of Factor VIII activity.
- From a safety perspective UV irradiation present a risk to the operator

Upon investigation of the marketed systems for UV irradiation, both flow UVC systems were found to be discontinued. Due to the lack of availability of equipment as well as product impact concerns this technology for viral inactivation was not considered any further.

Solvent Detergent

Arising from the extensive literature review, several potential alternative detergents were identified that could possibly be used in various solvent detergent combinations. Where no data was available for some detergents, an independent evaluation was performed. Viral inactivation studies can amount to a significant cost due to the time

and resources required. Therefore the initial evaluation was a paper based technical assessment in order to reduce the number of potential candidates prior to proceeding to any experimental work.

The initial evaluation was based on a number of criteria. These were:

- Whether the detergent supplier was qualified and approved to supply to the manufacturing facility.
- The type of detergent i.e. Ionic, non-ionic and zwitterionic.
- Health and safety hazard as per the detergent Material and Safety Data Sheet (MSDS).
- The toxicity of the detergent.
- The detergents solubility within an aqueous solution.
- The detergent's Critical Micelle Concentration (CMC).

From the initial evaluation only 6 candidates met all the required criteria. These were carried forward to bench scale experiments where impact to the recombinant Factor VIII molecule was assessed. At the time of experimentation only 4 candidates became available for evaluation due to supplier availability. These are presented in Table 7

Table 7: Detergent candidates selected for experimental work

Detergent	**Detergent Type**
Candidate A	Non-Ionic
Candidate B	Non-Ionic
Candidate C	Ionic
Candidate D	Zwitterionic

3.2 Bench Scale Studies on Selected Candidates

The detergent used in the solvent detergent step is an integral part of the treatment and therefore it must not adversely affect the integrity of the final product with respect to protein structure or function.

To determine whether the 4 selected candidates adversely affected the Factor VIII protein, each detergent or solvent detergent combination was added to the process solution containing the Factor VIII protein and incubated. The concentration of the detergents and solvent detergent combinations used were aligned with the same protocols highlighted within the literature review (see Table 8).

Table 8: Bench scale solvent detergent combination concentrations

Detergent	**Solvent**	**Literature Detergent Concentration %**	**Literature Solvent Concentration %**
Candidate A	TNBP	1	0.3
Candidate B	TNBP	1	0.3
Candidate C	TNBP	0.2	0.3
Candidate D	N/A	0.023	N/A

The process performance attributes, being turbidity, pH and conductivity, of each pool were initially analysed followed by product quality attribute analyses for potency and protein concentration. A pre-inactivated process solution sample containing the recombinant Factor VIII molecule was ran as an assay control. Triton X-100 was also run in parallel to serve as a comparative at a concentration currently used in the manufacturing process.

As can be seen in Table 9, the turbidity values reported for Candidate B were significantly higher than the other candidates. The average reported turbidity for Candidate B was 446 Nephelometric Turbidity Units (NTU), whereas the other candidates reported turbidity values less than the Triton X-100 control. The low

concentration used for Candidate D may have contributed to the variability among the results reported due to variability in the dispensing weights of the detergent. However all results were still below the control value indicating no immediate adverse effects. pH and conductivity values for all 4 candidates were within the specification range of 6.1-6.5 and 28-51 mS/cm respectively.

Table 9: Process Performance Attribute Analyses

Detergent	Sample Number	Turbidity (NTU)	pH (-)	Conductivity (mS/cm)
Pre-Inactivated Pool	#1	23	6.29	38.0
Triton X-100	Comparison	174	6.30	34.1
Candidate A	#1	45	6.28	35.8
	#2	49	6.28	35.7
Candidate B	#1	422	6.28	35.8
	#2	470	6.28	35.8
Candidate C	#1	29	6.37	34.5
Candidate D	#1	27	6.34	36.9
	#2	26	6.40	37.1

When the product quality attributes (potency and protein concentration) were analysed the results were more variable, specifically towards the potency assay (ref. Table 10). Potency results ranged from 2516-3871 IU/ml against the control result of 2855 IU/ml. In theory, the addition of a detergent should lower the protein concentration of a sample due to the overall dilution factor taking affect. However, the potency assay has known inherent variability which the high potency result for Candidate A can be attributed to. If we consider the potency result of Triton X-100 which was reported as 2929 IU/ml, it can be seen that this result is also higher than the pre-inactivated pool potency of 2855 IU/ml. Given that Triton X-100 is the currently used detergent in the manufacturing process, a degree of assurance is obtained that the high potency results, even if

attributed to assay variability, are acceptable and provide no inherent risk to the product. For the protein concentration results no candidate showed any apparent impact.

Table 10: Product Quality Attribute Analyses

Detergent	**Sample Number**	**Potency (IU/ml)**	**Protein Concentration (mg/ml)**
Pre-Inactivated Pool	#1	2855	1.34
Triton X-100	Comparison	2929	1.42
Candidate A	#1	3074	2.18
	#2	3872	2.11
Candidate B	#1	2516	1.59
	#2	2573	1.58
Candidate C	#1	2719	1.25
Candidate D	#1	2740	1.39
	#2	2723	1.35

From a review of the results above and from physical observations during the studies several irregularities were noted. During the incubation period, it was observed that for Candidate C there were signs of immiscibility between the solvent detergent and the process solution. For this reason further analysis on this candidate was not progressed and was therefore eliminated as a potential alternative detergent candidate. Concentrating on Candidate B, it can be seen that overall the results look promising with the exception of turbidity. In the case of turbidity, the results were greatly higher than any of the other potential candidates as well as the Triton X-100 detergent. The significant increase in turbidity may be an indication of the occurrence of protein aggregation. Based on these results Candidate B was also eliminated from the study.

The remaining 2 candidates, candidate A and D have shown to have had no adverse effect to both product and process quality attributes. Candidate A is a detergent that is widely used within the Biopharmaceutical industry and is currently used further downstream in the current manufacturing process. All historical data reviewed indicated that Candidate A was a promising alternative detergent. This positive data has allowed for the endorsement of Candidate A to progress to further DOE studies. Candidate D has shown to have a good safety profile and is ecologically friendly as it rapidly bio-degrades in water systems. Recent studies carried out using Candidate D as a viral inactivation detergent has shown it to be an effective virucidal agent. As well as this, the recent studies were carried out on recombinant MAb proteins showing no impact to the stability of the molecule. Even though the protein molecule was not that of recombinant Factor VIII, it is thought that Candidate D would equally serve well as a virucidal detergent for proteins other than MAbs. From the analysis of the results obtained for process performance and product quality attributes, it can be seen that all results were favorable and showed no apparent impact to product quality allowing for Candidate D to progress to further DOE studies.

3.3 Design of Experiment

From knowledge obtained through past research the critical parameters that can potentially affect viral inactivation kinetics are detergent concentration, incubation temperature and time. The literature has shown the impact temperature can have on viral inactivation kinetics, with higher temperatures increase the rate of inactivation. Spoken about previously, the detergent CMC is critical to achieving effective viral inactivation. Too low a concentration will provide inadequate inactivation where as too high a concentration can impact the purification process further downstream.

The DOE evaluated these critical parameters, temperature, time and alternative solvent/detergent combinations, on the product quality and process performance of the viral inactivation step. The DOE study assessed the following product quality and process performance outputs: product isoforms, aggregates and fragments, potency, pH, conductivity, turbidity and protein concentration. There were no acceptance criteria for this study as it is for characterisation purposes only. However, all parameters were investigated in order to determine ranges of conditions suitable for the viral inactivation (VI) step. Any parameters that indicated statistical significance were assessed for practical significance.

3.3.1 Candidate A

The DOE was performed to scope suitable conditions for the viral inactivation step using Candidate A. The parameter ranges tested within the DOE are outlined in Table 11 below.

Table 11: DOE Parameter Ranges for Candidate A

Parameter	**Range**
Time (mins)	0 - 120
Temperature (^{0}C)	5 - 30
Candidate A Concentration (% w/v)	0.7 – 1.3
TNBP Concentration (% w/v)	0.25 – 0.35

Statistical Approach

Four process parameters were investigated in this study with experimental statistical design using the Design Expert 9.0 application. An irregular fraction Res V design was selected to execute the study. This is a special resolution V design with fewer runs than a normal fractional factorial to estimate main effects and two-factor interactions. Each condition was run singly at 2 levels and 2 centre points were included in the study resulting in 14 run conditions (Appendix 1).

Responses were fitted individually for all of the outputs. The significance of the contribution of each input parameter to the model was examined using analysis of variance and removed from the model if the p value was greater than 0.05. Effects where the p value was between 0.05 and 0.1 were included where appropriate. Plots of the residuals from the reduced models were examined to see if there was any violation of the assumptions of the analysis of variance model used to fit the response surface. The response surfaces were graphed in Design Expert 9.

Evaluation of Process Attributes

pH

pH samples were measured post viral inactivation and prior to the affinity chromatography step. A statistical review of the data has shown that the detergent concentration and time were significant input parameters for the model with an overall p value of < 0.0001. Detectable curvature can be seen in the model due to the average of the centre points giving a p-value of 0.045 which is slightly below the limit of significance (0.05). However, this does not affect practical performance or product quality so therefore not practically significant.

The effect of increasing levels of detergent resulted in a decrease in pH (Figure 4). Extending inactivation time from 0-120 minutes resulted in an increase in pH of 0.07 (Figure 5). The observed range of pH values was only 0.07 pH units over the ranges of the input parameters tested. The viral inactivation step has a pH acceptance criteria ranging from 6.1-6.5. Data from all runs fell within the acceptance criteria (6.29-6.36).

Therefore the concentration of detergent and TNBP, time and temperature did not have a significant practical effect on the pH of the viral inactivation solution.

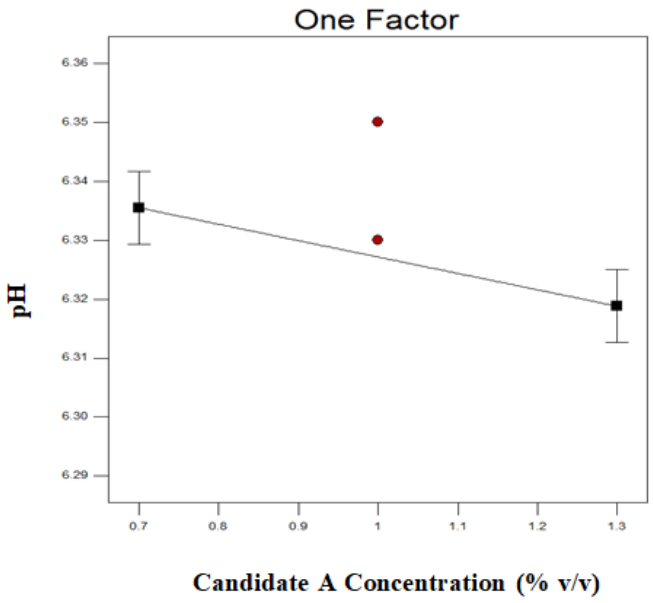

Figure 4: Candidate A concentration influence on pH

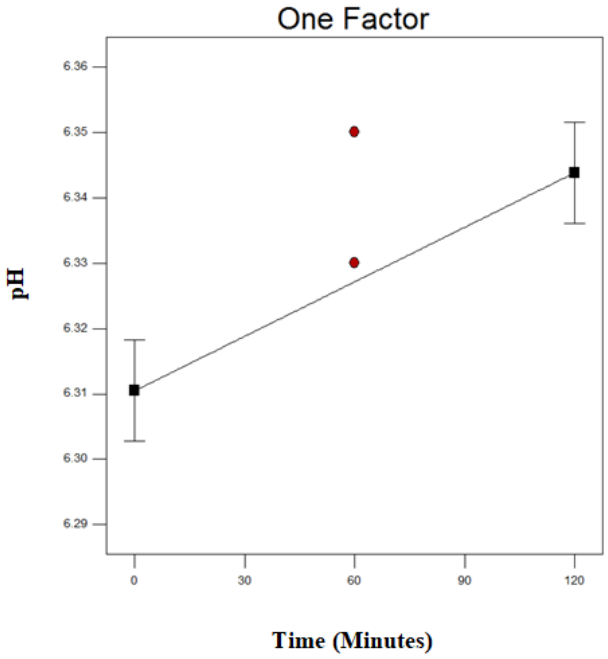

Figure 5: Influence of inactivation time on pH of process solution

Conductivity

Conductivity of the viral inactivation solution was measured after viral inactivation had been performed. This was monitored for information only. The change in temperature on conductivity is shown in Figure 6.

The p-value for this output parameter is reported at < 0.0001 suggesting the model is significant but as the observed conductivity values fall within process parameter ranges the variation in conductivity under all the conditions tested was not practically significant. It is also well established that the conductivity of a solution is affected by temperature due to the mobility of ions so the observed relationship in the model of increased conductivity with increased temperature is to be expected.

After viral inactivation the inactivated pool is processed by an affinity chromatography step. The conductivity range at the equilibration step is 28-51 mS/cm. The viral inactivated solution has to fall within this range in order to meet process parameters for the chromatography step. Conductivity data for all the runs fell within this range (34.8-38.7 mS/cm).

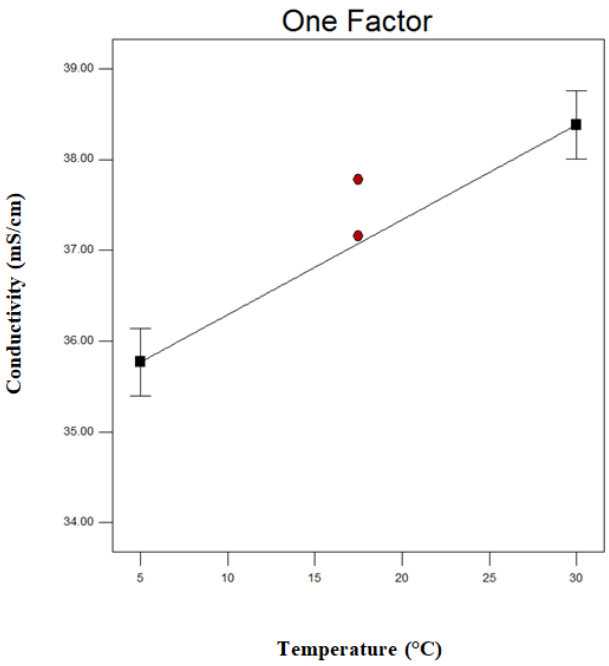

Figure 6: Influence of temperature on conductivity

Turbidity

The turbidity of each sample was measured after viral inactivation and before loading onto the affinity chromatography column. A statistical analysis of the data has shown significance (p-value of 0.0001) for an interaction between candidate A concentration and TNBP concentration. The interaction is presented in Figure 7. There is detectable curvature in the model due to one of the centre points giving a p-value of 0.0299. The lack of fit within the model was statistically significant at a p value of 0.0093 which shows issues with linearity. However, this does not affect practical performance or product quality so therefore not practically significant.

The results have shown high concentrations of TNBP (0.35 % w/v) and low concentrations of detergent (0.7 % w/v) produced a significant increase in turbidity ranging between 500 -1000 NTU. Low levels of turbidity were found with higher levels of candidate A (1-1.3 % w/v). Turbidity was monitored for information only. The observation of increased levels of turbidity was noted as it may indicate a loss of material due to precipitation. However, it is known that solvent and detergents are intrinsically linked. Sufficient detergent is required in order to help solubilise the solvent in an aqueous solution. Therefore excess solvent in a solution may result in insolubility of the component attributing to the increase in turbidity.

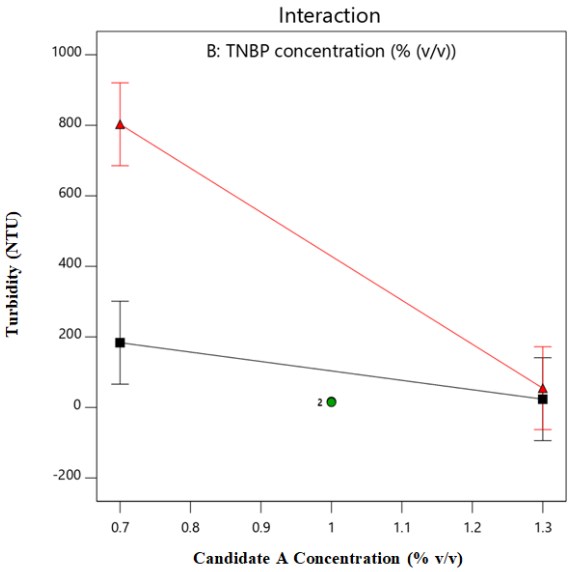

Figure 7: Influence of TNBP concentration and candidate A concentration on turbidity

Total Protein (Bradford Assay)

A review of the data was showed no statistical significance, with a p value of 0.0692. The Bradford results gave a range of 0.601 mg/mL to 0.810 mg/mL during runs 1-14 (ref. Figure 8). The lowest result of 0.601 mg/mL was observed when candidate A concentration is at the midpoint range of 1.0 (% w/v). The duplicate sample gave a value of 0.759 mg/mL. The protein concentration of the Triton X-100 control was 0.953 mg/mL before inactivation and 0.776 mg/mL after inactivation, therefore it was concluded that this result (0.601 mg/mL) was an outlier. As the acceptance criteria ranges from 0.3-1.1 mg/mL this was not practically significant.

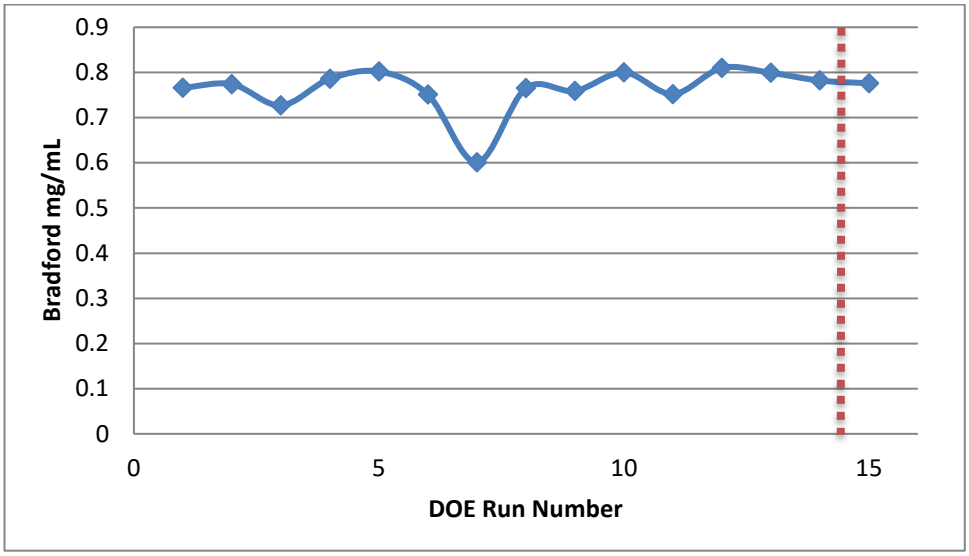

Figure 8: Control chart of total protein across 15 runs incl. Triton X-100 control (run15)

Potency

Potency was determined by an analytical assay based on a two-stage principle; the first activates the intrinsic pathway where factor VIII acts as a co-factor. The reaction is then determined by the use of a synthetic chromogenic substrate. The reaction is then stopped and the activity, which is proportional to the release of pNA (para-nitroaniline), is determined photometrically at 405 nm against a reagent blank. This is then reported in IU/mL.

Potency of the solution was measured post the affinity chromatography. With a p value of 0.0735, the data showed no statistical significance. The raw data showed the potency ranged between 4758 – 6834 IU/mL. Historical ranges for this assay is 2194-12066 IU/mL at this step, so all the study data lies within the historical process range.

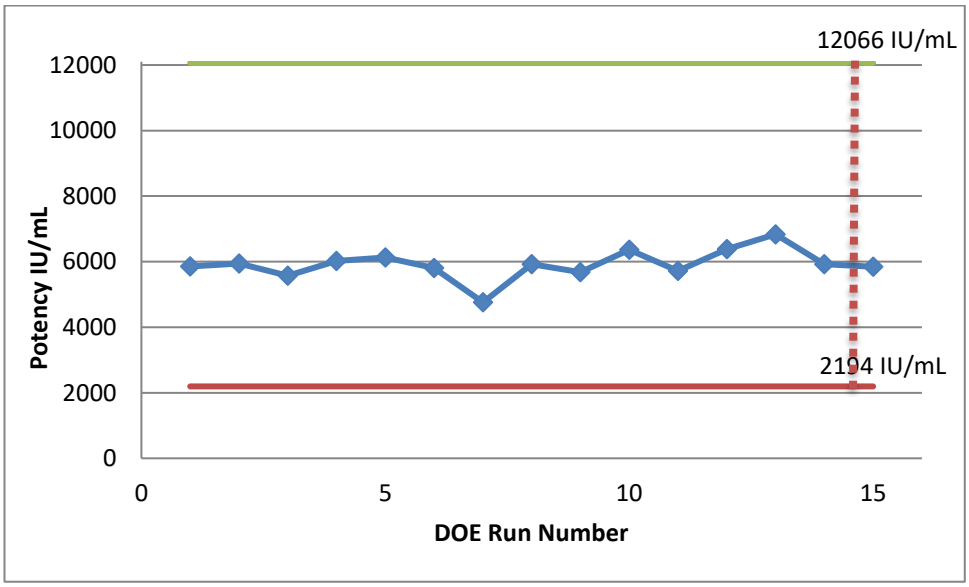

Figure 9: Control chart of potency across 15 runs incl. Triton X-100 control (run15)

SE-HPLC

Size exclusion chromatography (SEC) is designed to separate aggregates and fragments from the monomer peak. The results are based on a percentage referencing the area of that peak.

Fragments

The raw data shows little effect on the level of fragments, under all study conditions (Figure 10, data range 10-15 %). This data is not routinely collected at commercial scale but was tested for information only. A control sample, using Triton X-100 (current process conditions), was run along with the DOE for comparison and resulted in a fragment percentage of 15 %. During pilot model qualification this sample point was tested by SEC for 3 batches. The level of fragments detected for each batch was 15, 14 and 13 % respectively. Therefore there was no statistical or practical effect on the level of fragments.

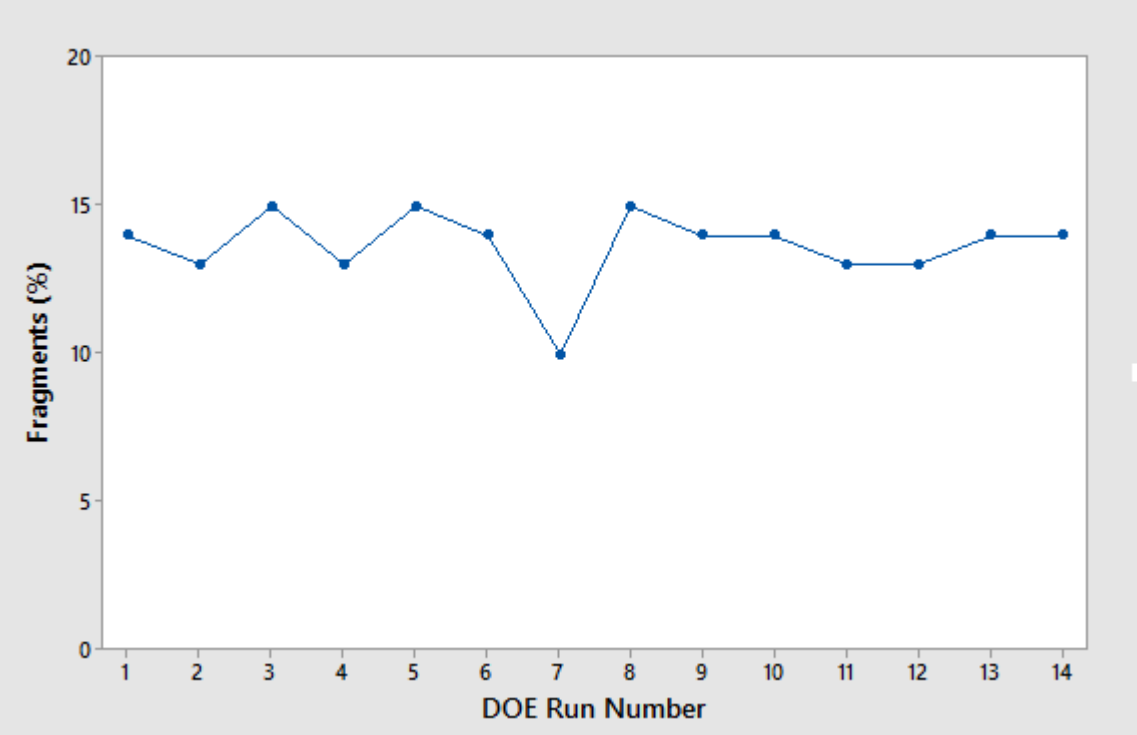

Figure 10: Control chart of percent fragments across the 14 DOE runs

Aggregates

The SEC data for aggregates was analysed but a model could not be constructed. The raw data shows little effect on the level of aggregates under all study conditions (range of 1-2 %). Figure 11 shows the percentage aggregates detected for each run. This data is not routinely collected at commercial scale but was tested for information. A control sample, using Triton X-100 (current process conditions), was run along with the DOE for comparison and resulted in a fragment percentage of 1 %. During pilot model qualification this sample point was tested by SEC for 3 batches. The level of aggregates detected for each batch came in at ≤1 % for all samples. Therefore there was no statistical or practical effect on the level of aggregates.

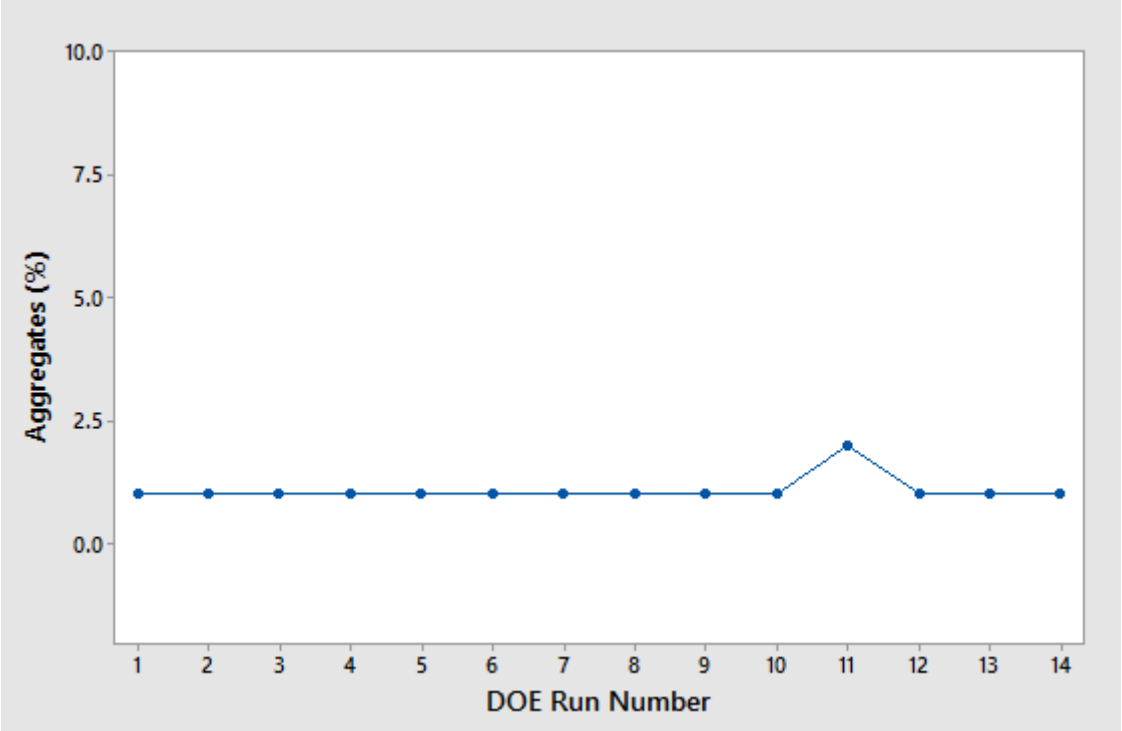

Figure 11: Control chart of percent aggregates across the 14 DOE runs

RP-HPLC

RP-HPLC is an analytical method performed to distinguish between the different isoforms of the recombinant factor VIII protein. The factor VIII molecule has 3 isoforms. Each isoform is 170 kDa in size but is excreted from the cell as a full 170 kDa chain, an 80 kDa chain and a 90 kDa chain attached via a methylated ion bridge. The analytical assay is designed to break the methylated ion bridge and the output determines the percentage ratio of 170 kDa, 80 kDa and two forms for 90 kDa known as 90:1 and 90:2. This test is not performed routinely at commercial scale but provides information that can determined whether the factor VIII molecule is stable and physio chemically active.

Samples of the affinity chromatography pool were analysed by the RP-HPLC assay. Each of the isoform species were modelled individually.

80 kDa Species

The data was analysed using a model with no transformations. The p-value obtained of 0.0005 for the temperature parameter indicates that this is a significant parameter with regards to the 80 kDa species (high temperature results in a decrease in 80 kDa species, Figure 12). Samples analysed from the DOE study resulted in a range between 54-57 %. Although the DOE showed a highly significant model, the species is known to decrease further during downstream purification.

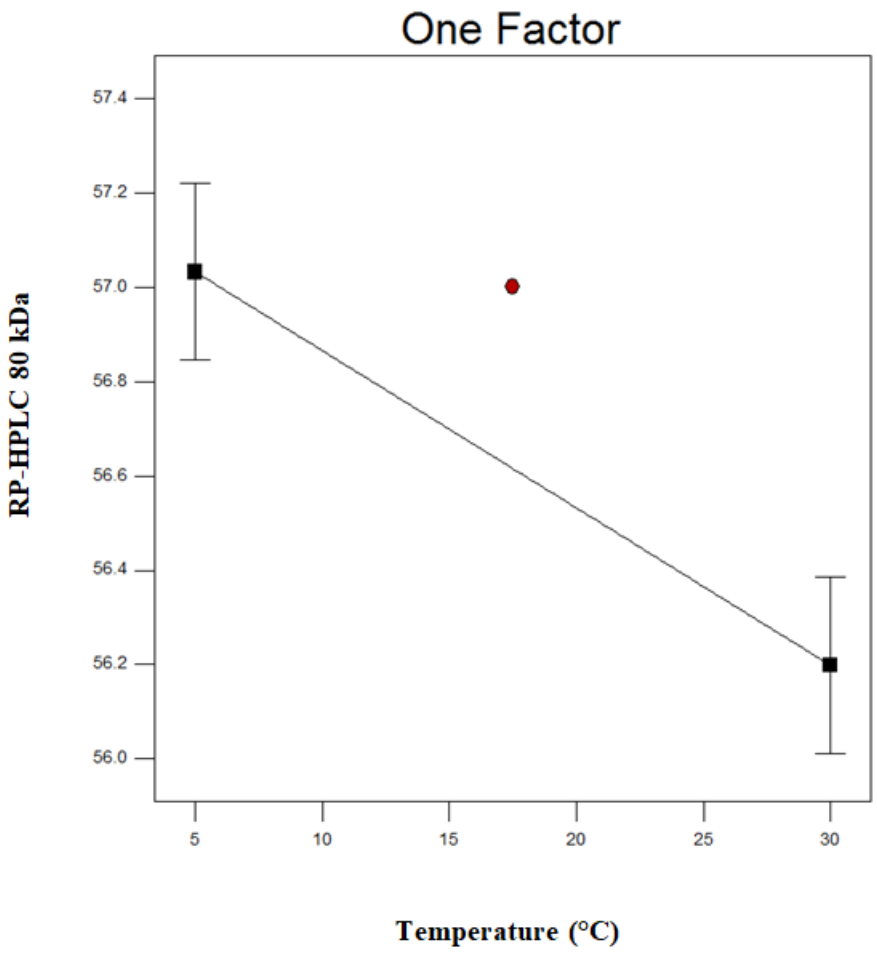

Figure 12: Influence of temperature on the 80 kDa species

170 kDa Species

The data was analysed using a model with no transformations. The p-value of 1.0000 indicates no parameters are significant. Results for the 170 kDa species from the DOE study samples were 3 % for all samples tested. Therefore none of the study factors had a significant effect on the level of 170 kDa species.

90:1 kDa Species

The data was analysed using a model with no transformations. The p-value was determined as 0.0069 (Figure 13) for the temperature parameter which indicates that this is a significant parameter (low temperature equals low 90:1 species). The DOE

resulted in a range between 19-20 %. Although the DOE indicates a significant model, the comparisons to the pilot model qualification and with variability being only ≤1 %, this is not practically significant.

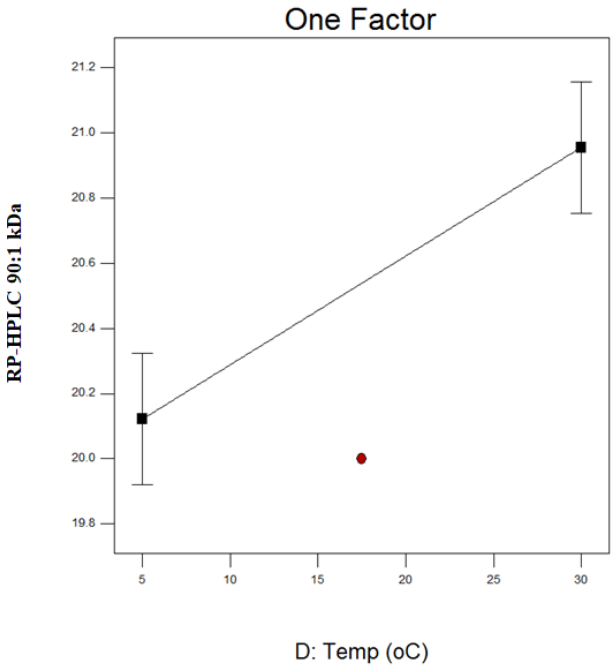

Figure 13: Influence of temperature on the 90:1 kDa species

90:2 kDa Species

The data was analysed using a model with no transformations. The p-value was determined as 0.0005 for the temperature parameter which indicated that this is a significant parameter with low temperature resulting in a decrease in 90:2 species (Figure 14). The DOE resulted in a range from 20-21 %. Although the DOE shows a significant model, the variability is ≤1 %. This can be deemed as not practically significant for this study given the wide range at the end of the process of (6-22%).

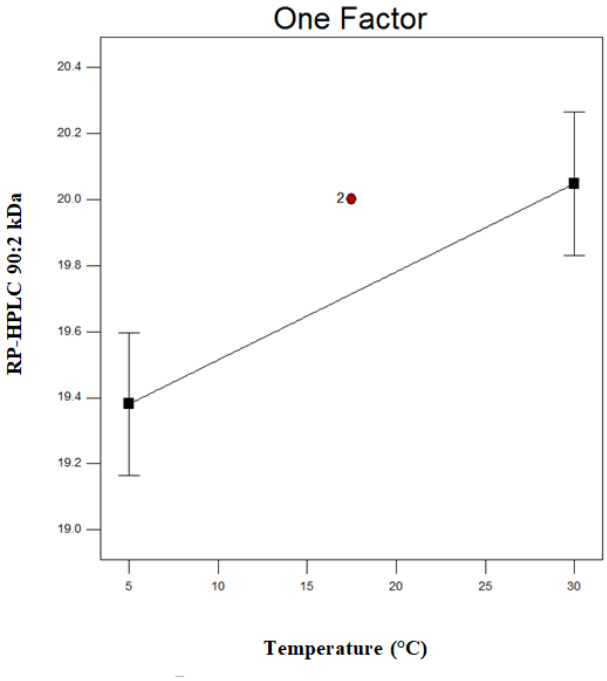

Figure 14: Influence of temperature on the 90:2 kDa species

Specific Activity

No model could be constructed from the data for specific activity. The specific activity of the product is calculated on the potency and total protein concentration and is expressed in IU/mg. This information is not routinely calculated at this stage. As a comparison, the specific activity of the affinity chromatography eluate during the pilot qualification ranged from 5171 – 7659 IU/mg. The DOE results ranged from 7487-8553 IU/mg (Figure 15). Although the DOE results are slightly higher, the values overlap. As well as this, the drug substance release specification is 6700 – 9900 IU/mg for reference.

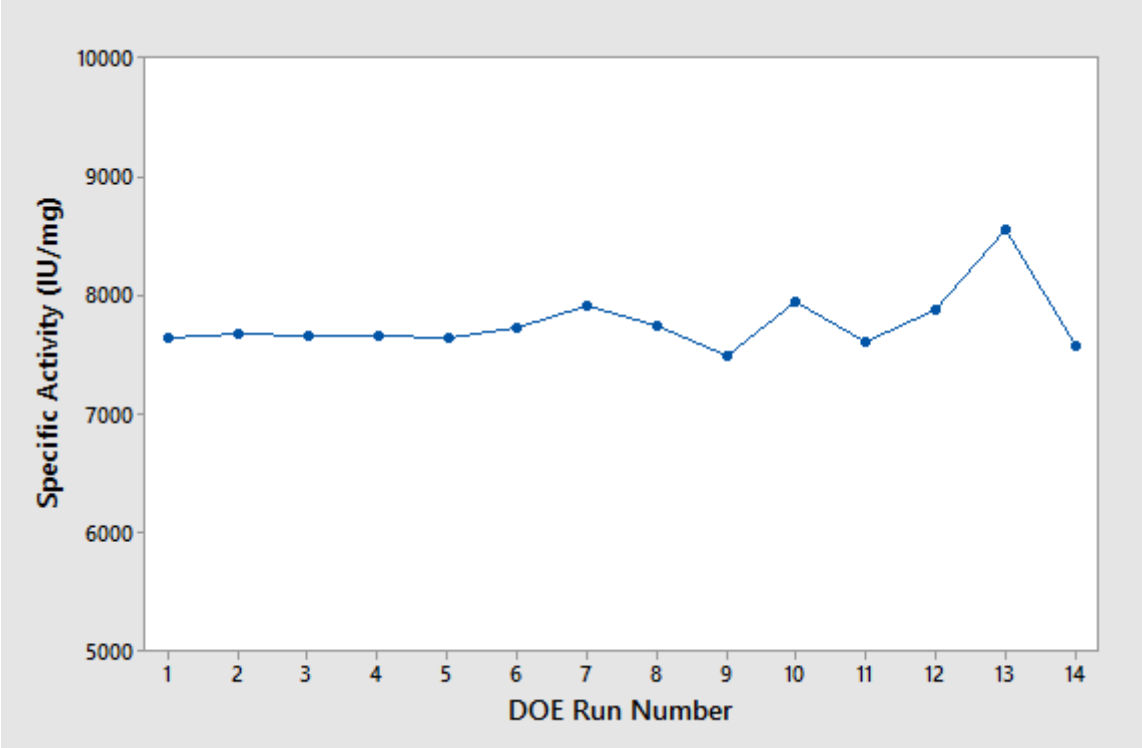

Figure 15: Control chart of specific activity across the 14 DOE runs

3.3.2 Candidate D

DOE

The DOE was performed to scope suitable conditions for the viral inactivation step using candidate D. The parameter ranges tested within the DOE are outlined in Table 12 below.

Table 22: DOE Parameter Ranges

Parameter	**Range**
Time (mins)	0 - 120
Temperature (^{0}C)	5 - 30
Candidate D Concentration (% v/v)	0.2 - 0.6

Statistical Approach

Three process parameters were investigated in this study with experimental statistical design using the Design Expert 9.0.2 application. A 2 level factorial design was selected to execute the study. Each condition was run singly at 2 levels and 2 centre points were included in the study resulting in 18 run conditions (Appendix 2).

Responses were fitted individually for all of the outputs. The significance of the contribution of each input parameter to the model was examined using analysis of variance and removed from the model if the p value was greater than 0.05. Effects where the p value was between 0.05 and 0.1 were included where appropriate. Plots of the residuals from the reduced models were examined to see if there was any violation of the assumptions of the analysis of variance model used to fit the response surface. The response surfaces were graphed in Design Expert 9.0.2.

Evaluation of Process Attributes

pH

A pH measurement was taken after viral inactivation. A statistical review of the data determined that Candidate D concentration displayed statistical significance with a p value of < 0.0001. Temperature was also found to be significant with a higher p value of 0.0463 but overall the output is dominated by the concentration input parameter.

The physical effect of increasing the concentration of candidate D resulted in an increase in pH (Figure 16). An increase of 0.7 pH units was observed over the concentration ranges of 0.2 % to 0.6 % v/v at 5 degrees. Increasing the inactivation temperature from 5-30 degrees resulted in a decrease in pH at 0.6% v/v concentration and a slight increase at 0.2 % v/v concentration. The viral inactivation step has a pH acceptance criteria ranging from 6.1-6.5. Data from all runs fell outside the acceptance criteria starting at the lowest pH of 6.53 to the highest recorded pH of 7.26. Therefore, the concentration and temperature of candidate D had a significant practical effect on the pH of the viral inactivation solution.

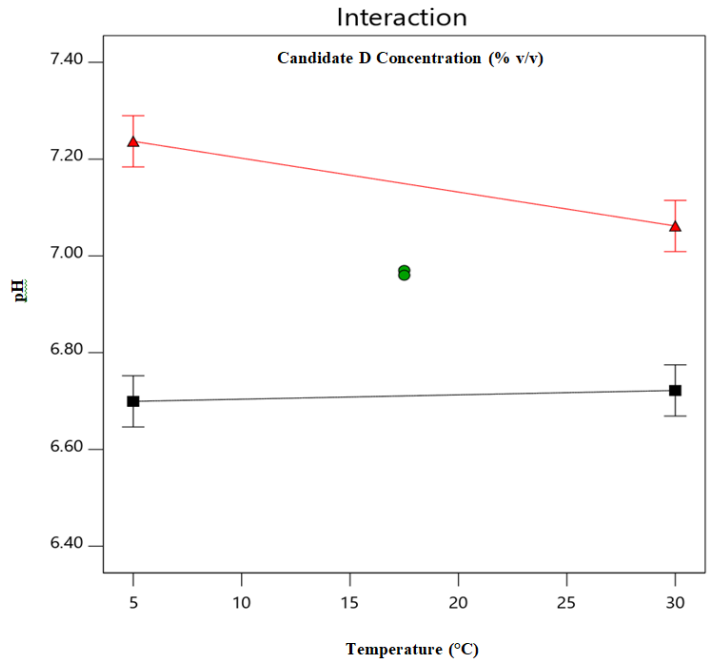

Figure 16: Influence of temperature and candidate D concentration on pH

Conductivity

The interaction of time and temperature on conductivity is shown in Figure 17. The p-value of < 0.0001 for temperature suggests the model is significant but as the observed conductivity values fall within process parameter ranges the variation in conductivity under these conditions tested was not practically significant. It is well established that the conductivity of a solution is affected by temperature due to the mobility of ions so the observed relationship in the model of increased conductivity with increased temperature is not unexpected.

The data for time and concentration also reported p values less than 0.05 (0.0040 and 0.0396 respectively) indicating significant parameters also. The interaction between time and temperature showed that at 5 degrees the conductivity decreased over the 120 minutes. Conductivity of the viral inactivation solution was measured after viral inactivation had been performed. This was monitored for information only. After viral inactivation the inactivated pool is processed by affinity chromatography. The conductivity range at the equilibration step is 28-51 mS/cm. The viral inactivated process solution must fall within this range in order to meet process parameters for the chromatography step. Conductivity data for all the runs fell within this range (31.52-37.44 mS/cm).

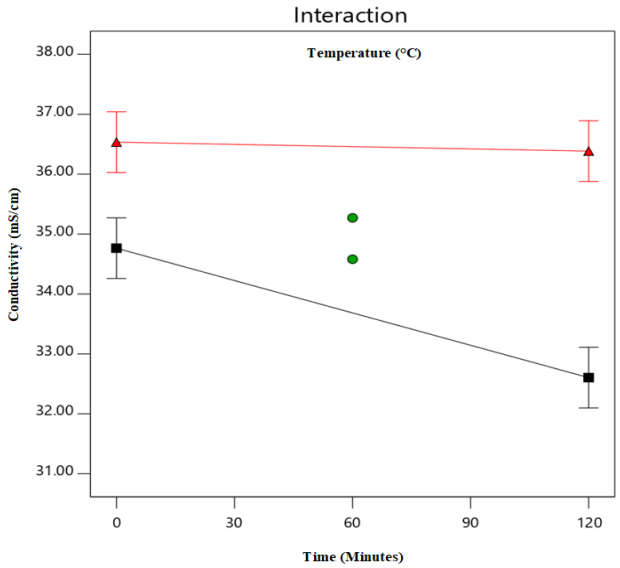

Figure 17: Influence of inactivation time and temperature on conductivity

Turbidity

The turbidity of each sample was measured after viral inactivation and before loading onto the affinity chromatography column. The model was found to be significant (p-value of 0.0420) for an interaction between concentration and time. The interaction is presented in Figure 18. The model was also found temperature to have a significant effect obtaining a p value of 0.0076.

Low concentrations of candidate D (0.2 % v/v) produced a significant increase in turbidity ranging from 61-110 Nephelometric Units (NTU) at longer time (120 mins). Low levels of turbidity were also found with higher concentrations of candidate D (0.6 % v/v). Turbidity was monitored for information only. The observation of increased levels of turbidity was noted as it may indicate a loss of material due to precipitation or insolubility of the solvent/detergent components.

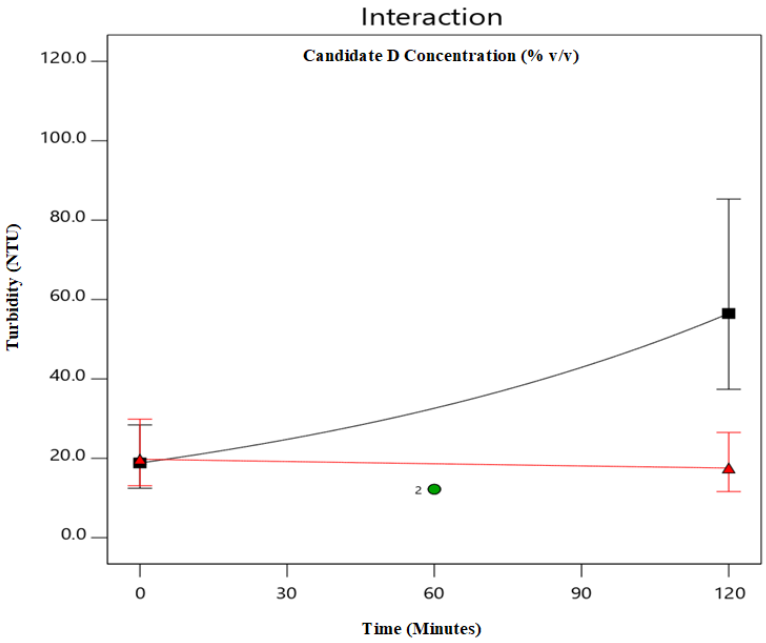

Figure 18: Influence of candidate D concentration and inactivation time on turbidity

Total Protein (Bradford Assay)

Total protein content (Bradford assay) was measured post the affinity chromatography step. A review of the data deemed the input parameters, time, temperature and concentration, statistically significant with p values of 0.0018, 0.0036 and <0.0001 respectively (Figure19). An interaction was observed in the model between the concentrations of candidate D and temperature (Figure 19). The results from the Bradford assay were reported within the range 0.038 mg/mL to 0.664 mg/mL for all 18 runs. The lowest result of 0.038 mg/mL was observed when candidate D concentration is at the highest range of 0.6 (% v/v). An increase in temperature also gave a decrease in total protein. This decrease was seen in both low and high concentrations although it is more extreme at the higher concentration of LDAO.

The protein concentration of the Triton X-100 control was 0.7617 mg/mL after inactivation. As the acceptance criteria ranges from 0.3-1.1 mg/mL the results were deemed practically significant.

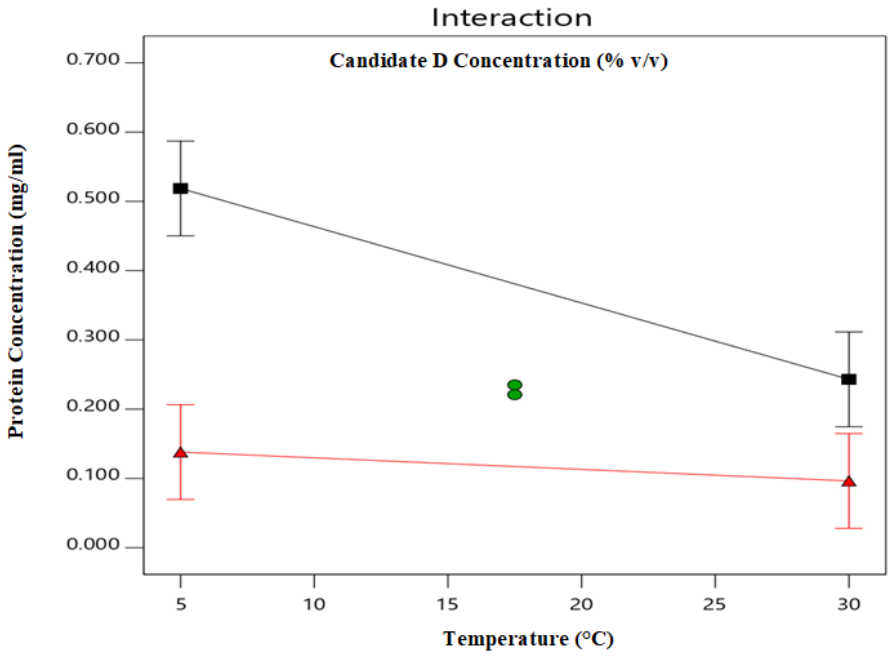

Figure 19: Influence of candidate D concentration and temperature on total protein

Potency

Potency was determined by an analytical assay based on a two-stage principle; the first activates the intrinsic pathway where factor VIII acts as a co-factor. The reaction is then determined by the use of a synthetic chromogenic substrate. The reaction is then stopped and the activity, which is proportional to the release of pNA (para-nitroaniline), is determined photometrically at 405 nm against a reagent blank. This is then reported in IU/mL.

Potency of the solution was measured post the affinity chromatography step. Statistically it was determined that time, temperature and concentration were significant (p values 0.0004, 0.0010 and <0.0001 respectively). The model also found an interaction of temperature and concentration to be a contributing factor. This is shown in Figure 20 below. The interaction shows that the lower the concentration of candidate D the higher the potency value and the higher the temperature the lower the potency value across the both the concentrations of candidate D. The raw data showed the potency ranged between 148 – 5119 IU/mL. The historical range for this assay is 2194-12066 IU/mL at this step, showing the majority of the data for this study lying outside the expected process range. Of the 6 out of 18 runs that were within the process range all had the lower concentration of 0.2 % v/v.

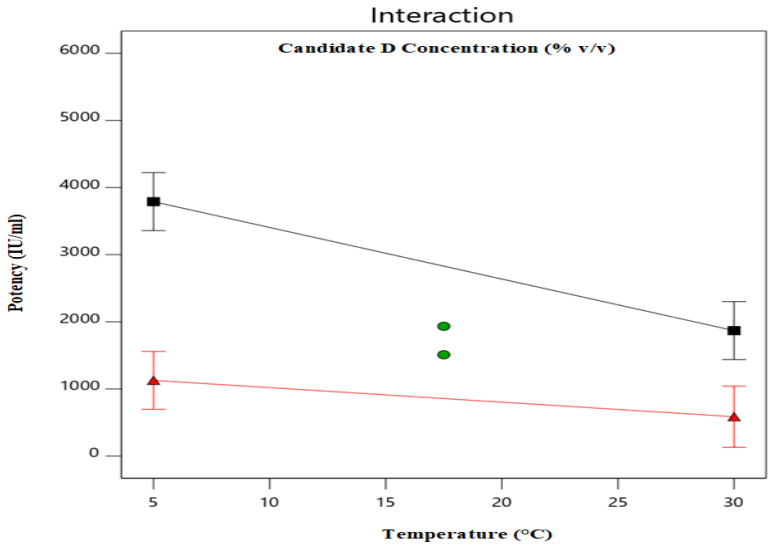

Figure 20: Potency interaction between LDAO Concentration and Temperature

SE-HPLC

Size exclusion chromatography (SEC) is designed to separate aggregates and fragments from the monomer peak. The results are based on a percentage referencing the area of that peak.

Fragments

The model was found to be significant for LDAO concentration with a p-value of <0.0001. The model graph shown in Figure 21 shows that an increase in concentration leads to an increase in fragmentation. Two of the input parameters, time and temperature, tested separately were not found to be significant. However, the interaction between both parameters has been shown to be significant with a p-value of 0.0024. The graph in Figure 22 shows at the mid-point of the concentration range the lower temperature for longer time will produce higher fragmentation.

The raw data ranged from 8 – 29 %. A control sample, using Triton X-100 (current process conditions), was run along with the DOE for comparison and resulted in a fragment percentage of 9 %. During pilot qualification this sample point was tested by SEC for 3 batches. The level of fragments detected for each batch was 15, 14 and 13 % respectively. The data from this analysis was gathered for information purposes but does display a practical impact in comparison to historical data.

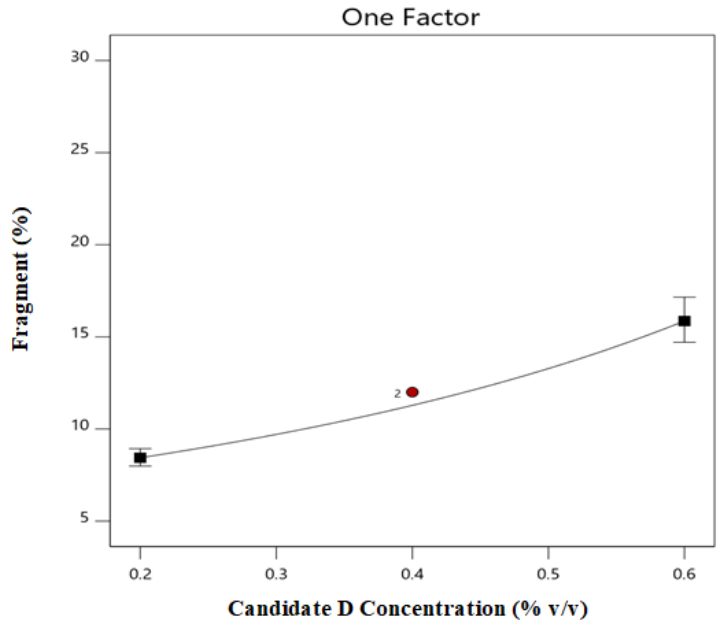

Figure 21: Influence of candidate D concentration on percent fragments

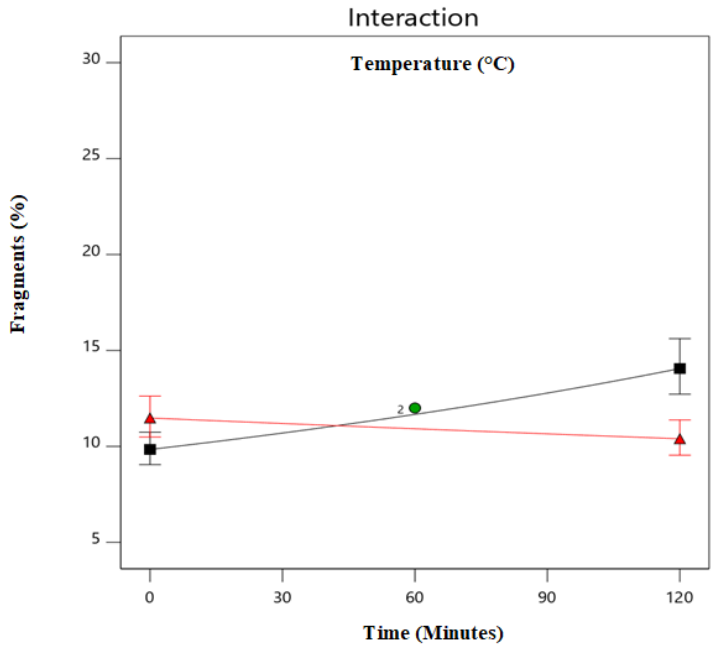

Figure 22: Influecnce of inactivation time and temeprature on percent fragments

Aggregates

The interaction between temperature and concentration was found to be significant Figure 23. At the highest concentration of 0.6 % v/v, the rate of aggregation appeared to increase with an increase in temperature. However, at the lowest concentration (0.2 % v/v) there was a decrease in aggregation at a higher temperature of 30°C.

The raw data shows the level of aggregates under all study conditions ranged of 1-9 %. A control sample, using Triton X-100 (current process conditions), was run along with the DOE for comparison and resulted in a fragment percentage of 1 %. During qualification of the pilot model this process point was tested by SE-HPLC on 3 batches. The level of aggregates detected for each batch was reported as ≤1 % for all samples. Therefore there was a statistical and practical effect on the level of aggregates.

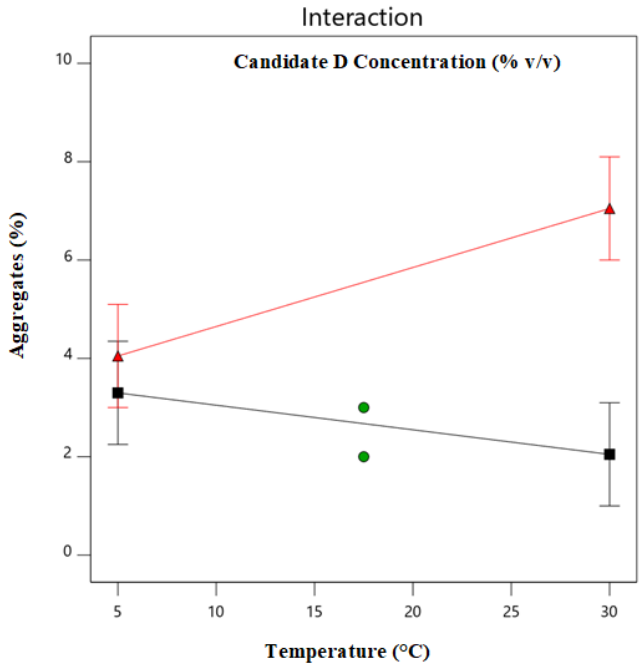

Figure 23: Influence of candidate D concentration and temperature on percent aggregates

RP-HPLC

RP-HPLC is an analytical method performed to distinguish between the different isoforms of the recombinant factor VIII protein. The factor VIII molecule has 3 isoforms. Each isoform is 170 kDa in size but is excreted from the cell as a full 170 kDa chain, an 80 kDa chain and a 90 kDa chain attached via a methylated ion bridge. The analytical assay is designed to break the methylated ion bridge and the output determines the percentage ratio of 170 kDa, 80 kDa and two forms for 90 kDa known as 90:1 and 90:2. This test is not performed routinely at commercial scale but provides information that can determined whether the factor VIII molecule is stable and physio chemically active.

Samples of the affinity chromatography pool were analysed by the RP-HPLC assay. Each of the isoform species were modelled individually.

80 kDa Species

All 3 input parameters indicate significance with regards to the 80 kDa species. The interaction between time and temperature was significant with a p-value of 0.0011 (Figure 24). As the time increased the 80 kDa isoform increased.

The interaction between time and concentration was significant with a p-value of <0.0001 (Figure 25). As the time increased the 80 kDa species increased. This had a greater impact at the higher concentration of 0.6 % (v/v).

The interaction between concentration and temperature was significant with a p-value of 0.0060 (Figure 26). This interaction follows a similar trajectory as the previous 2 interactions. An increase in temperature gave an increase in 80 kDa species.

Samples analysed from the DOE study resulted in a range between 54-89 %. During qualification of the ReFacto pilot model this process point was tested by RP-HPLC for 3 batches (ReF-135-001, ReF-135-002 and ReF-135-004). The level of 80 kDa species detected for these batches ranged from 55-56 % for all samples. Therefore there was a statistical and practical effect on the level of aggregates.

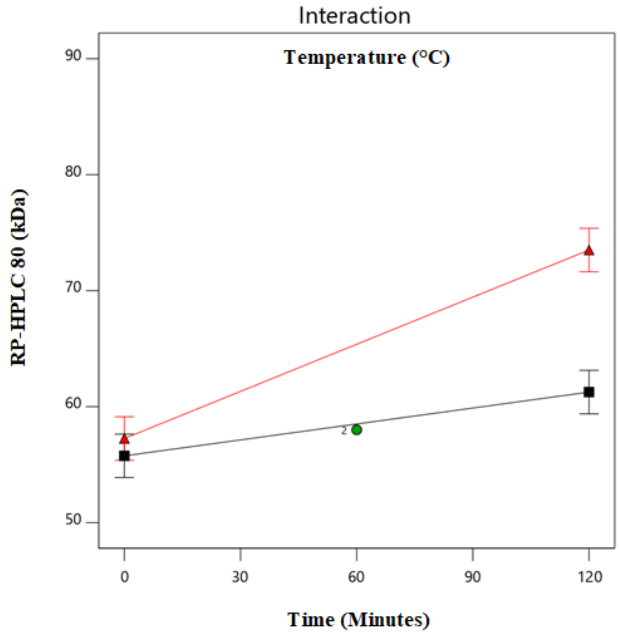

Figure 24: Influence of inactivation temperature and time on 80 kDa species

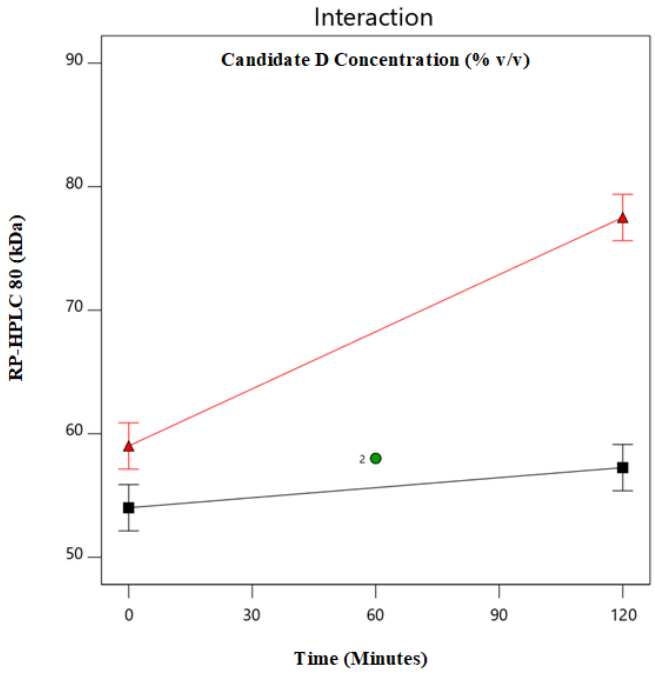

Figure 25: Influence of candidate D concentration and time on 80 kDa species

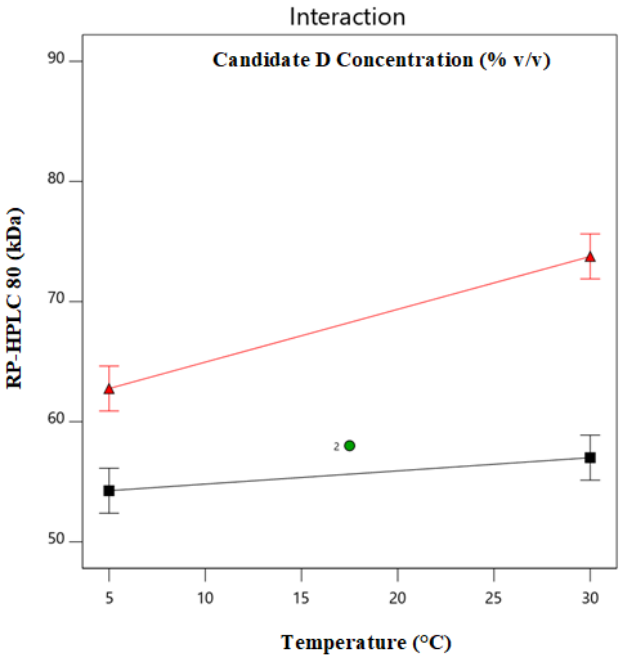

Figure 26: Influence of candidate D concentration and inactivation temperature on 80 kDa species

170 kDa Species

The p-value above 0.0500 indicates no single parameters are significant. However the interaction between time and concentration reported a p-value of 0.0003 (Figure 27). An increase in concentration gave a decrease in the 170 kDa isoform over a time period of 120 mins. At the lowest concentration (0.2 % v/v) the 170 kDa isoform increased over time.

The interaction between temperature and concentration was also found to be significant with a p-value of 0.0009 (Figure 28). This interaction results in a similar trajectory as the interaction between concentration and time in which an increase in concentration gave a decrease in percentage isoform. This is also seen for the lower concentration resulting in an increase in isoform at a temperature of 30°C. Due to these observations, an interaction between all 3 input parameters was found to be significant. Results for the 170 kDa species from the DOE study samples ranged from 3-7 % for all samples tested. These values extended beyond those seen during initial pilot qualification where results ranged from 3 – 5%. Therefore all of the study factors had a significant effect on the level of 170 kDa species.

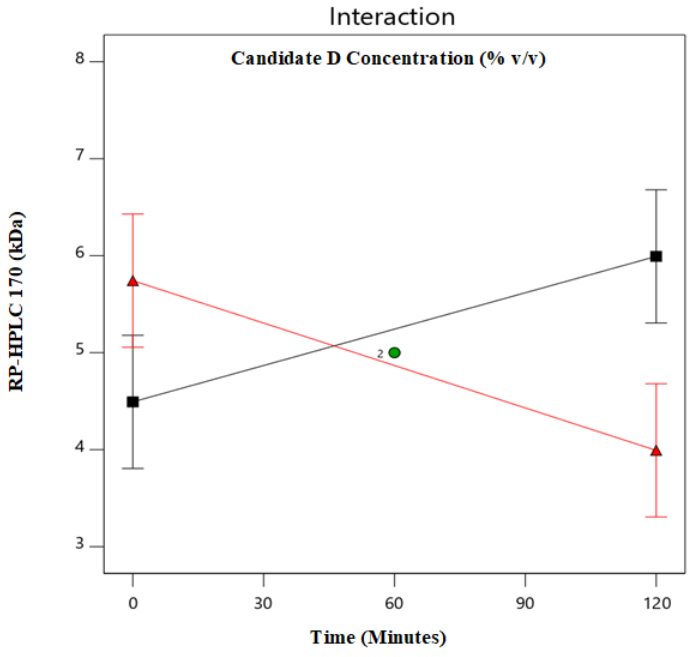

Figure 27: Influence of candidate D concentration and time on 170 kDa species

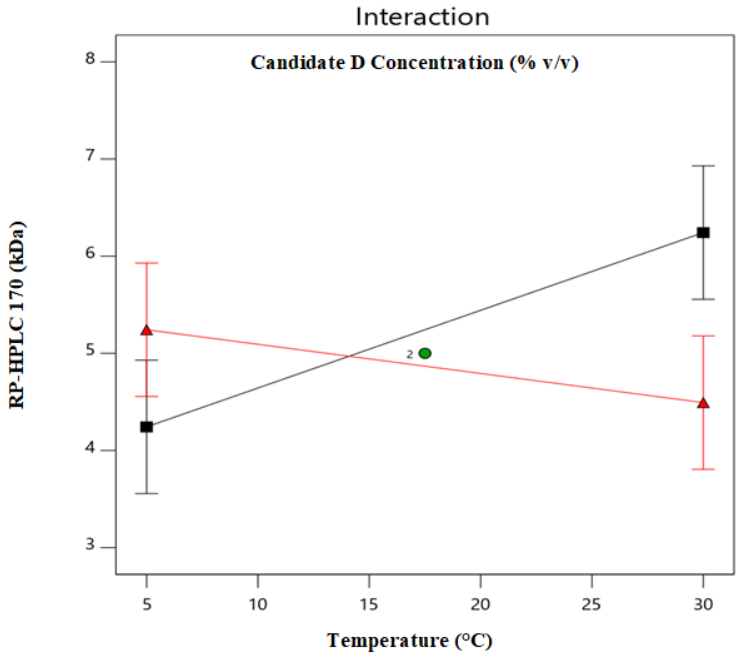

Figure 28: Influence of candidate D concentration and inactivation temperature on 170 kDa species

90:1 kDa Species

The p-value was determined as <0.0001 for all 3 input parameters. Two interactions were also found to be significant; Time and temperature with a p-value of 0.0013 (Figure 29) and time and concentration with a p-value of 0.0390 (Figure 30). Both interactions show a decrease in 90:1 kDa species over the time period of 120 mins. The DOE resulted in a range between 0-21 %.

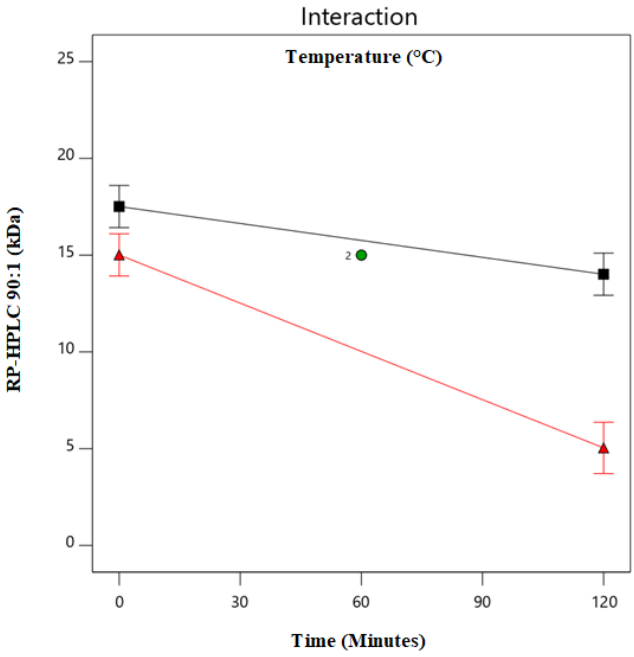

Figure 29: Influence of inactivation temperature and time on 90:1 kDa species

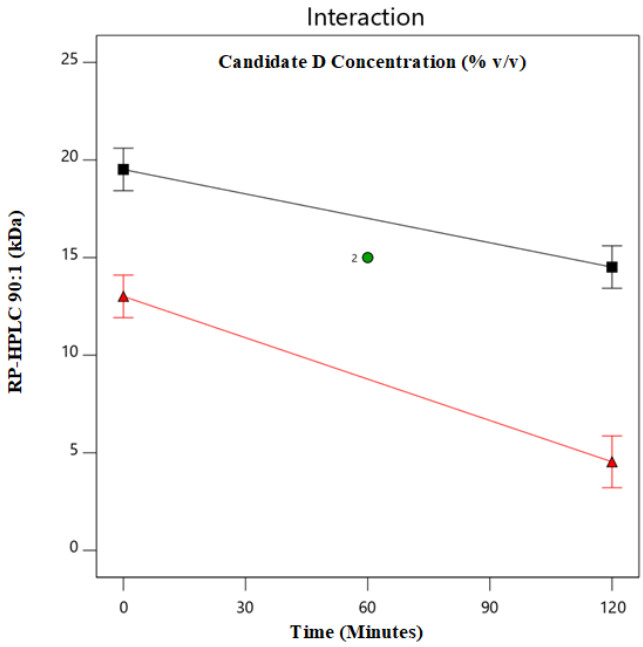

Figure 30: Influence of candidate D concentration and time on 90:1 kDa species

90:2 kDa Species

The model p-value was determined as 0.0003. Both time and concentration was found to have a significant effect on the output result for the 90:2 kDa species. (p-values 0.0078 and 0.0010 respectively). As both of these parameters gave a statistical significance the interaction between them also was found to be significant. (Figure 31) Regarding a higher concentration (0.6 % v/v) the 90:2 kDa species decreased over time. The lower concentration showed an increase in the isoform. The DOE resulted in a range from 9-23 %.

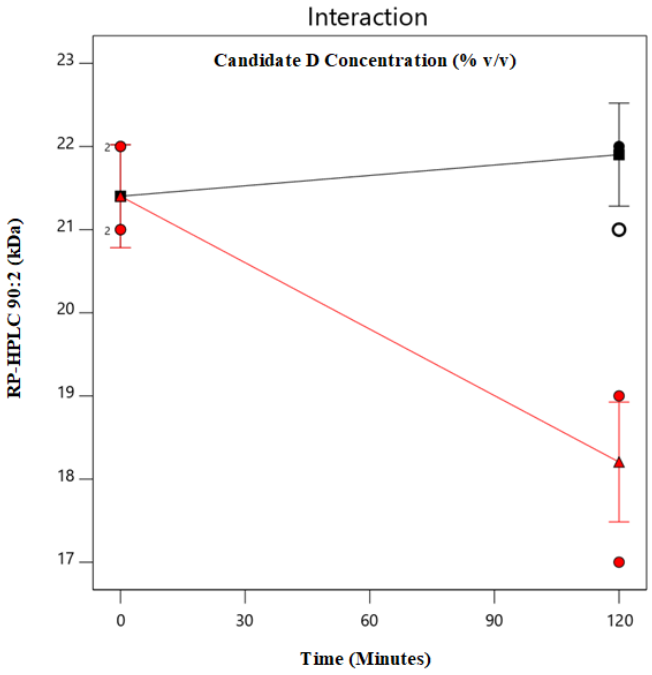

Figure 31: Influence of candidate D concentration and time on 90:2 kDa species

Specific Activity

No model could be constructed from the data for specific activity. The specific activity of the product is calculated on the potency and total protein concentration and is expressed in IU/mg. This information is not routinely calculated at this stage. The DOE results ranged from 1421-14077 IU/mg. In comparison to the results obtained during pilot qualification (5171 – 7659 IU/mg) the DOE results show wide variability. Figure 32 shows a control graph for the specific activity with the DS release specification limits included for reference. Although the specific activity graph below is based on the DS release criteria. The results from the DOE show an inconsistent trend in the raw data.

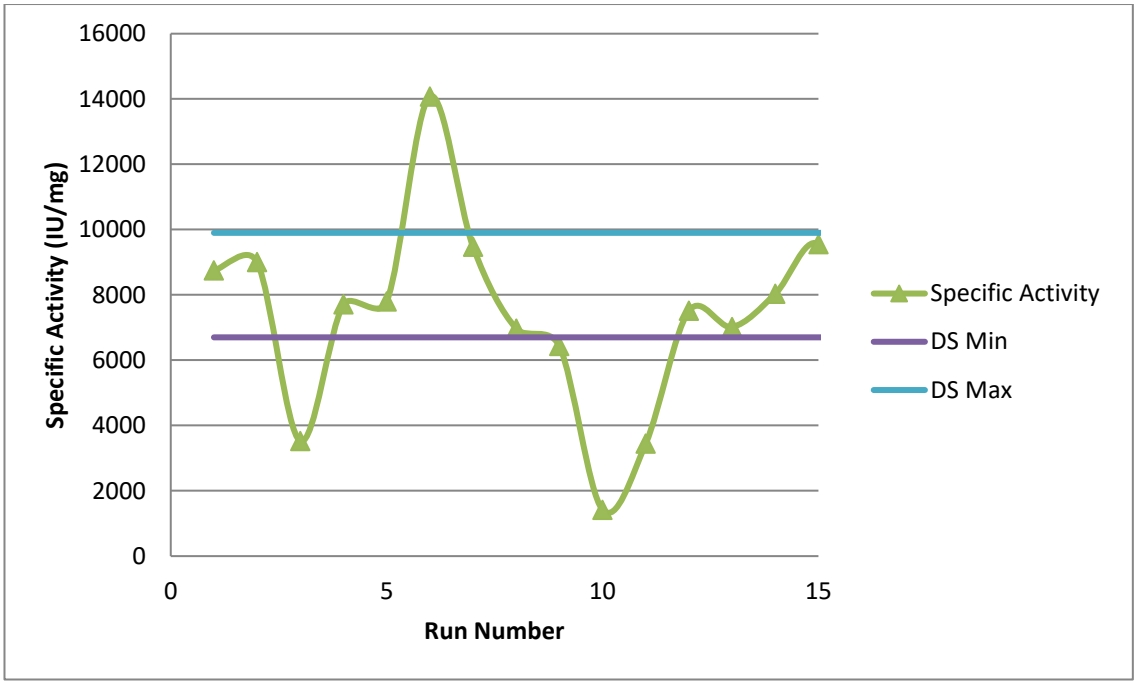

Figure 32: Control chart of specific activity across the 15 DOE runs incl. Triton x-100 control run (run 15)

3.4 Additional Studies

The DOE carried out for the study examined a number of parameters for the use of candidate D as a replacement for Triton X-100. These parameters were based on the current viral inactivation conditions as well as conditions mentioned within the literature review.

On completion of the DOE, it was found that the concentration of candidate D was statistically significant in all outputs. On review of these results, additional studies were performed to gain a better understanding of individual factors. These studies were conducted as OFAT studies (One Factor At a Time).

OFAT Study 1 -Lower Candidate D Concentrations

The first OFAT study was a decrease in the concentration previously used within the DOE. The parameters for the study are found in Table 13. The two concentrations tested were 0.06% v/v and 0.13 % v/v. The viral inactivation was performed at the stated concentrations at room temperature for 60 mins. A control was performed with these studies (Triton X-100/ TNBP) for comparability.

Table 13: OFAT Study 1 parameter list

Parameter	**Range**
Time (mins)	60
Temperature (^{0}C)	Room Temperature
Candidate D Concentration (% v/v)	0.06 and 0.13

Potency

The graph below, Figure 31, shows the DOE potency values followed by the OFAT potency values. The potency was tested post TN8.2 batch bind column. Potency data was consistent and comparable to the Triton X-100 control. For 0.13% v/v of candidate D the measured potency was significantly lower to that of 0.06% v/v, indicating that the lower the concentration of candidate D the higher the potency value.

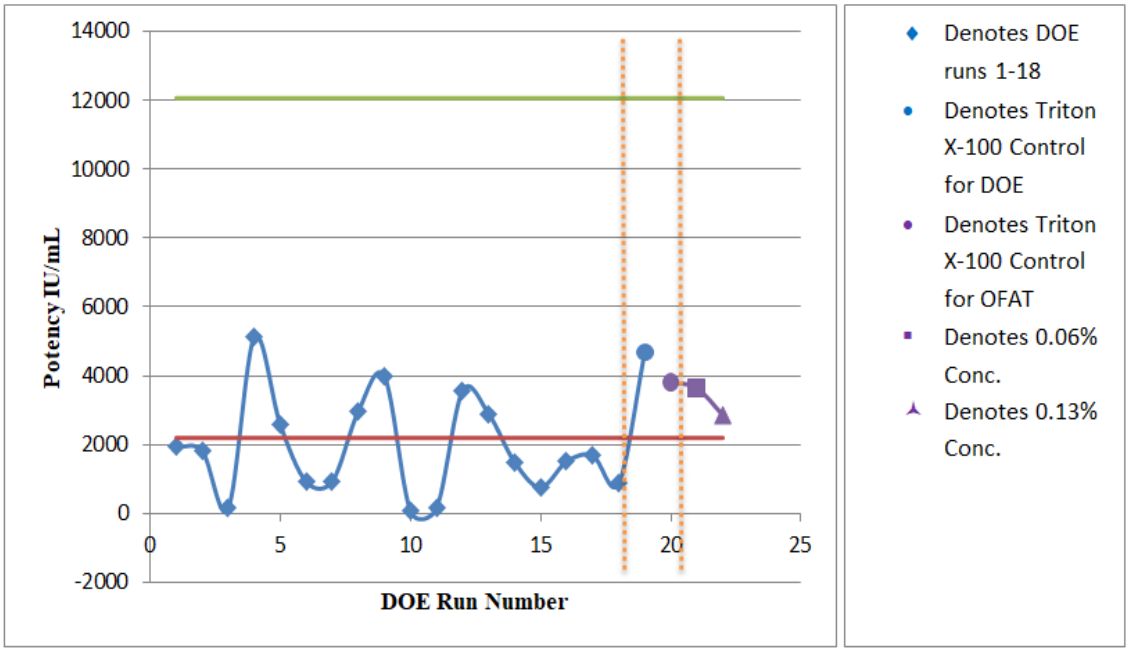

Figure 31: Potency control chart displaying DOE runs (blue markers) and OFAT runs using lower concentrations of candidate D (purple markers)

Bradford (Total Protein)

Bradford (protein concentration) data showed that 0.06% v/v candidate D was comparable to the control (Figure 32). The protein concentration was significantly lower for 0.13% v/v candidate D. This result is similar to the potency values in which the 0.13% v/v of candidate D reported a lower value than 0.06 % v/v. This indicates that the lower the concentration of candidate D the higher the Bradford value.

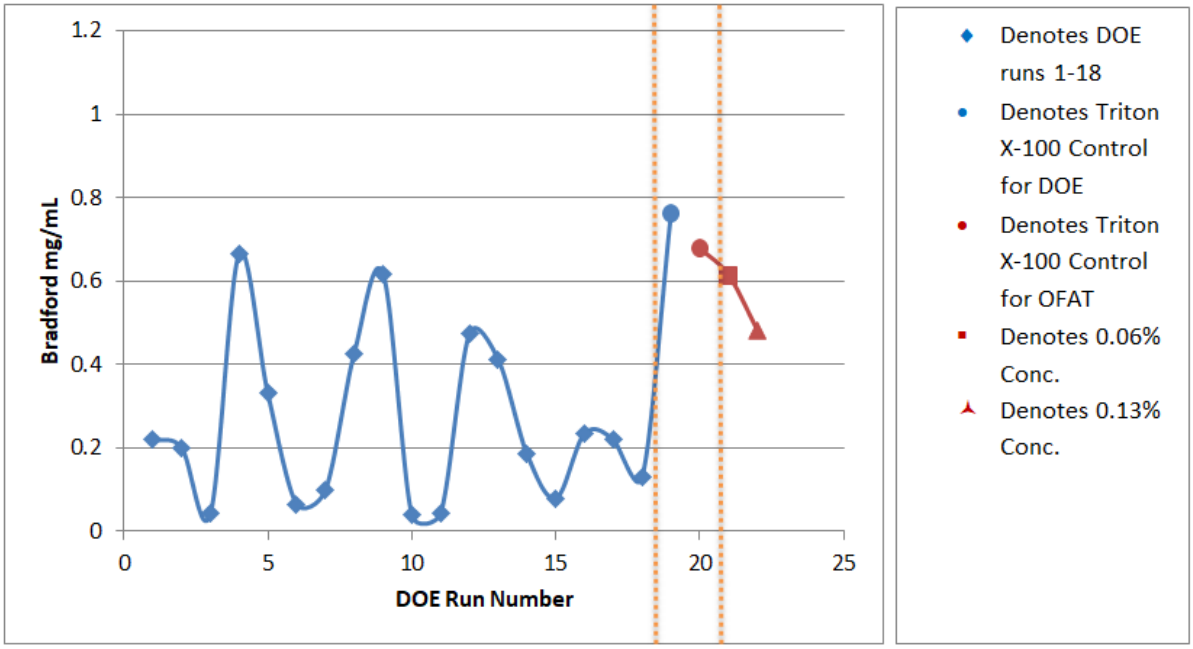

Figure 32: Total protein control chart displaying DOE runs (blue markers) and OFAT runs using lower concentrations of candidate D (red markers)

RP-HPLC

The RP-HPLC (isoform) analysis data was consistent and comparable to the control for both concentrations of candidate D. Both the 170 kDa (2 - 11%) and 90:2 kDa (6 – 21%) species were within DS release specification. 80 kDa (42-50 %) and 90:1 kDa (27-41%) were out of the acceptance criteria for both concentrations. It has been noted previously that the 80 kDa decreases and the 90:1 species increases over the anion exchange chromatography step within the downstream process. The graph below, Figure 33, showed that the concentrations of candidate D had no detrimental effect on the isoform distribution.

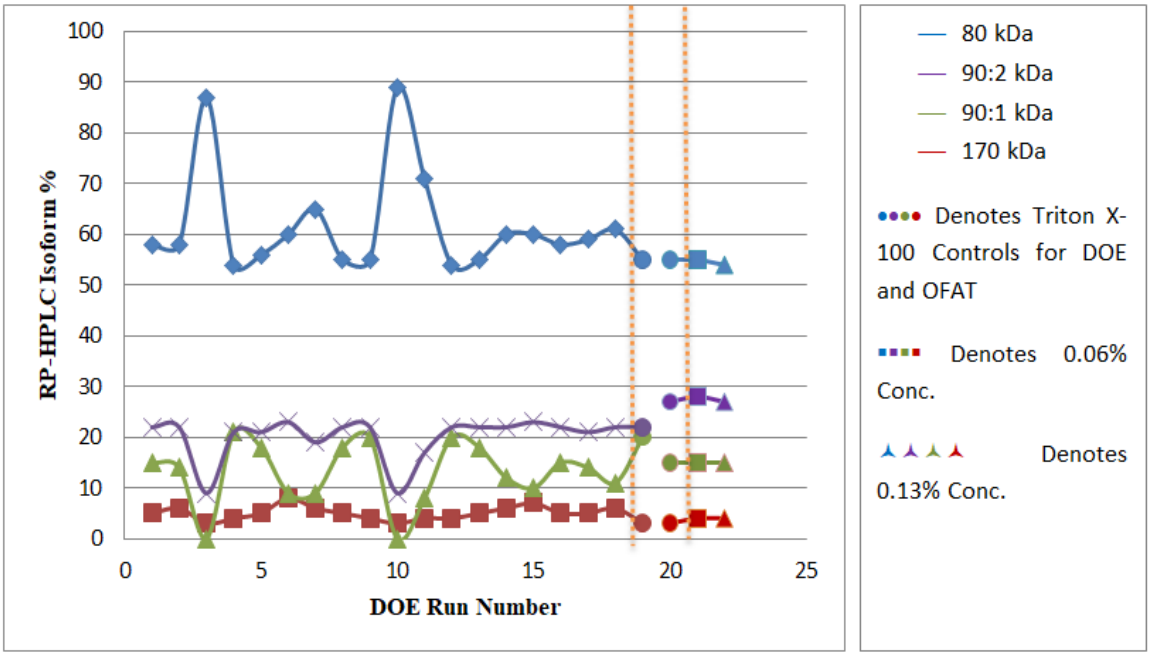

Figure 33: Isoform control chart displaying DOE runs and OFAT runs using lower concentrations of candidate D

SE-HPLC

SE-HPLC is tested after the affinity chromatography step. The data showed a decrease in the formation of fragments versus the control when using both concentrations of LDAO (Figure 34). The data also shows a similar value of aggregates to the control with only a 1% variation. The level of fragments is shown to decrease throughout the downstream process.

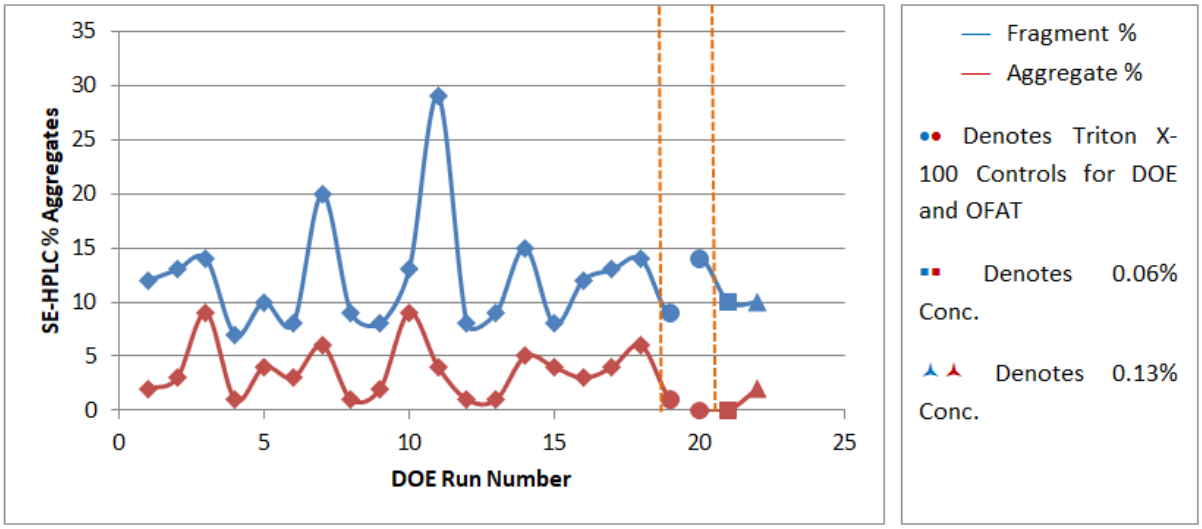

Figure 34: Percent aggregate control chart displaying DOE runs and OFAT runs using lower concentrations of candidate D

3.5 Third Party Viral Inactivation Experimentation

The viral inactivation efficacy of the two chosen alternatives was assessed. Both candidates reported effective viral log reduction values after the 120 minute incubation period (Figure 35 & 36). Candidate A showed a slower rate of viral inactivation kinetics overall with some low level of infectivity for PRV being detected after the 120 minute time point using the large volume plating assay. However the log reduction value at this point was reported to be 5.32. Candidate D also showed successful results with rapid inactivation being observed. After 1 minute > 3 LRV was obtained with complete inactivation of both model viruses after the 60 minute time point.

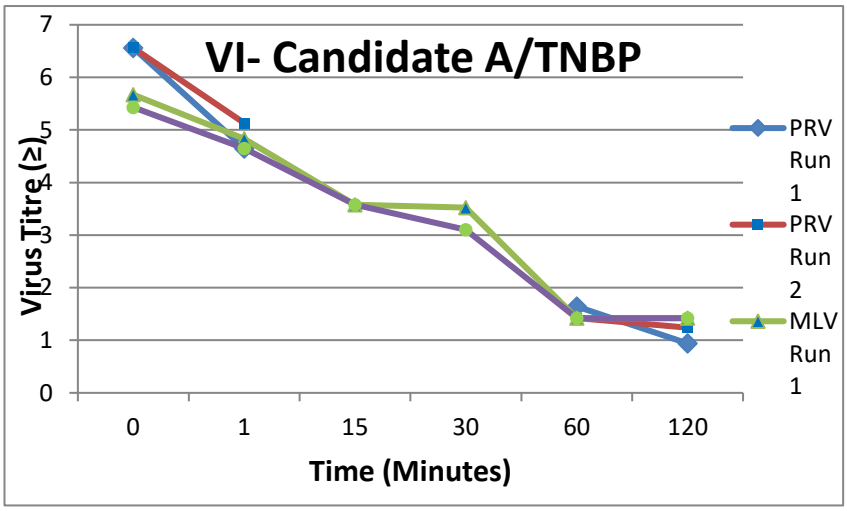

Figure 35: Viral inactivation rates of PRV and MLV using candidate A/TNBP combination

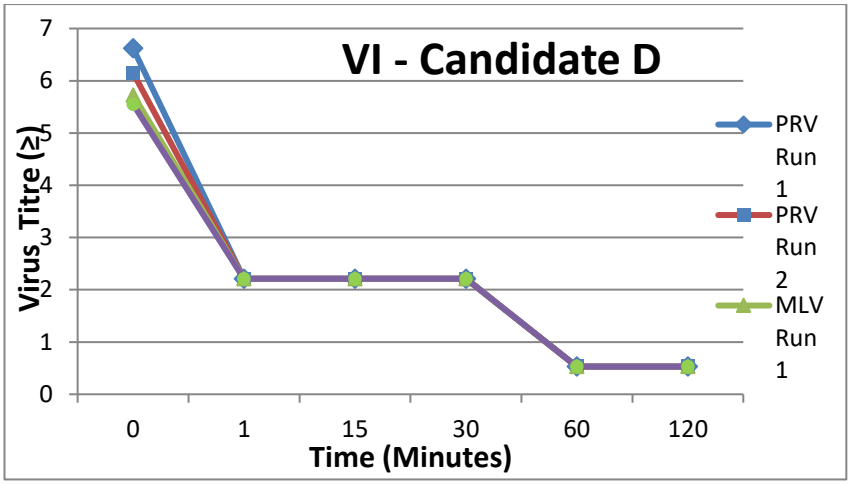

Figure 36: Viral inactivation rates of PRV and MLV using candidate D

The current viral inactivation process conditions using Triton X-100/TNBP was also ran for comparative purposes. The results of this experiment showed log reduction values of ≥3.24 and ≥ 4.90 for MuLV and PRV respectively.

The preliminary viral inactivation study carried out has shown that the two potential candidates have both delivered satisfactory results indicating that both candidates can successfully inactivate the two model viruses used in this study at the process conditions called out. Table 14 outlines the results reported for the 2 alternative detergents.

Table 14: Overall viral log reduction using both candidates and Triton X-100 control against MLV and PRV

VI	MLV	PRV
Candidate A/TNBP	≥4.01	5.32
Candidate D	≥5.02	≥5.62
Triton X-100/TNBP	≥3.24	≥4.90

3.6 Additional studies post third party viral inactivation experiments

Upon concluding the literature review 4 potential alternate solvent/detergent combinations were identified. Physical and chemical properties in combination with initial lab studies allowed 2 alternatives to be investigated via DOE's and also progress to viral inactivation studies at a third party laboratory. During this time, another alternative (Candidate E) that was originally eliminated from the testing due to availability became available for testing. Even though the viral inactivation studies had been completed using candidate A and D, upon receipt of candidate E it was included as part of additional studies to take place mainly for information purposes.

3.6.1 Alternative reagent (Candidate E)

The second OFAT study completed was to investigate a range of Candidate E concentrations. The parameters for the study are found in Table 15. The following product quality data is post TN8.2 batch bind columns. The 3 concentrations tested were 0.23% v/v, 0.40 % v/v and 0.53 % v/v. The viral inactivation was performed at the stated concentrations at room temperature for 60 mins. A control was performed with these studies (Triton X-100/ TNBP) for comparability. These studies were performed using concentrations based on literature findings to investigate any adverse effects of the detergent on the FVIII product.

Table 15: OFAT Study 2 parameter list

Parameter	**Range**
Time (mins)	60
Temperature (^{0}C)	Room Temperature
Candidate E Concentration (% v/v)	0.23 - 0.53

Potency

The graph below, Figure 37, shows the potency values for all 3 concentrations of Candidate E tested including a control run for comparison with Triton X-100 currently used within the viral inactivation process. There was no correlation found between the 3 different concentrations of Candidate E in terms of potency of the Factor VIII molecule and no trend observed.

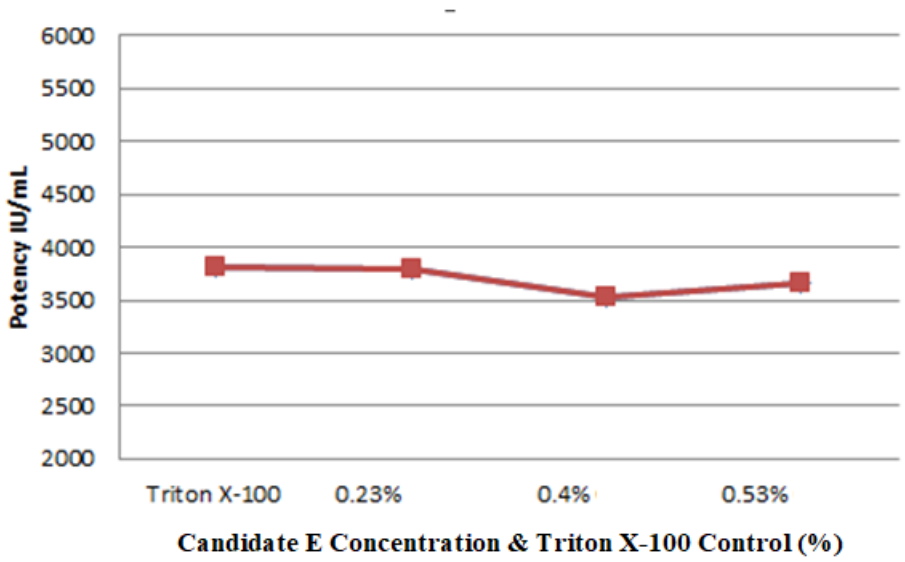

Figure 37: Potency control chart for Candidate E (OFAT Study 2)

Bradford (Total Protein)

Figure 38 shows the Bradford assay results (mg/mL) for all 3 concentrations of Candidate E tested and the control run with Triton X-100. There was no correlation found between the 3 different concentrations of Candidate E and no trend observed. The results are within the acceptance criteria for DS (0.3-1.1 mg/mL).

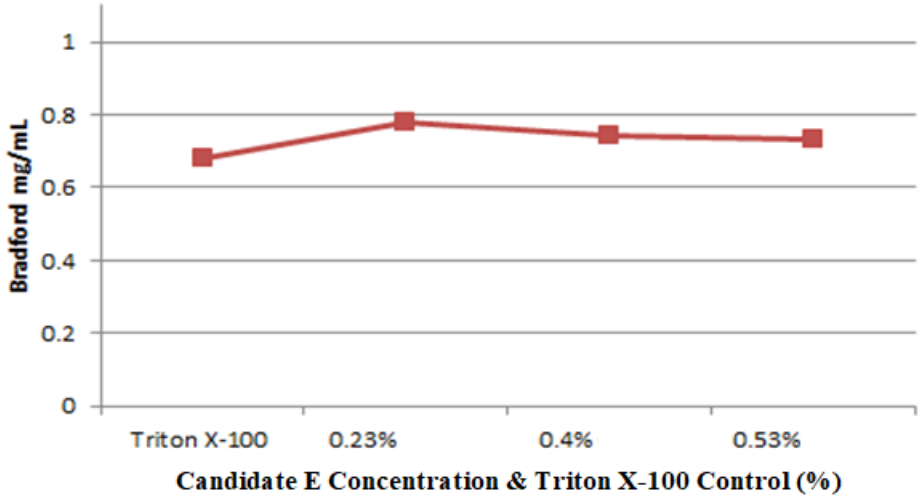

Figure 38: Bradford control chart for Candidate E (OFAT Study 2)

RP-HPLC

The RP-HPLC (isoform) analysis data was consistent and comparable to the control for all concentrations of Candidate E. Both the 170 kDa (2 - 11%), 90:1 kDa (27-41%) and 90:2 kDa (6 – 21%) species were within DS release specification. 80 kDa (42-50 %) was of the acceptance criteria for all concentrations tested. It has been seen before that the 80 kDa decreases over Q Sepharose chromatography step within the downstream process. The graph below Figure 39 showed that the concentrations of Candidate E had no detrimental effect on the isoform distribution.

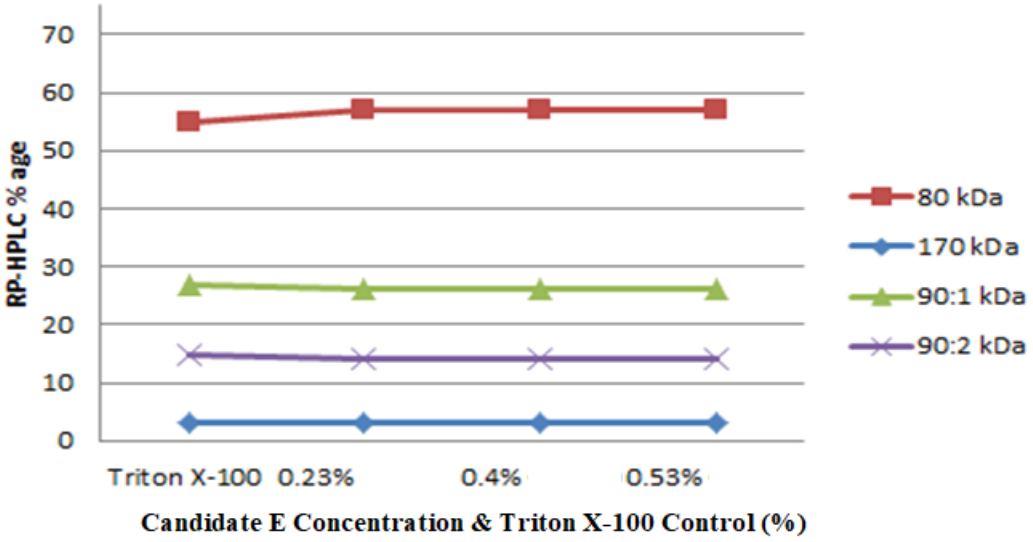

Figure 39: RP-HPLC control chart for Candidate E (OFAT Study 2)

SE-HPLC

SE-HPLC is tested after the TN8.2 batch bind column. The data showed an increase in the formation of fragments from 13 % for the control to 17 % when using Candidate E (Figure 40). The data shows a similar value of aggregates to the control at 0 % with no variation. The percentage of both fragments and aggregates do not change over the increase in concentration of Candidate E. Historical data indicates that the level of fragments is known to decrease throughout the downstream process.

Candidate E was found to be comparable for all product quality tested. Bradford, Potency, SE-HPLC and RP-HPLC indicated no trend in concentration versus assay output.

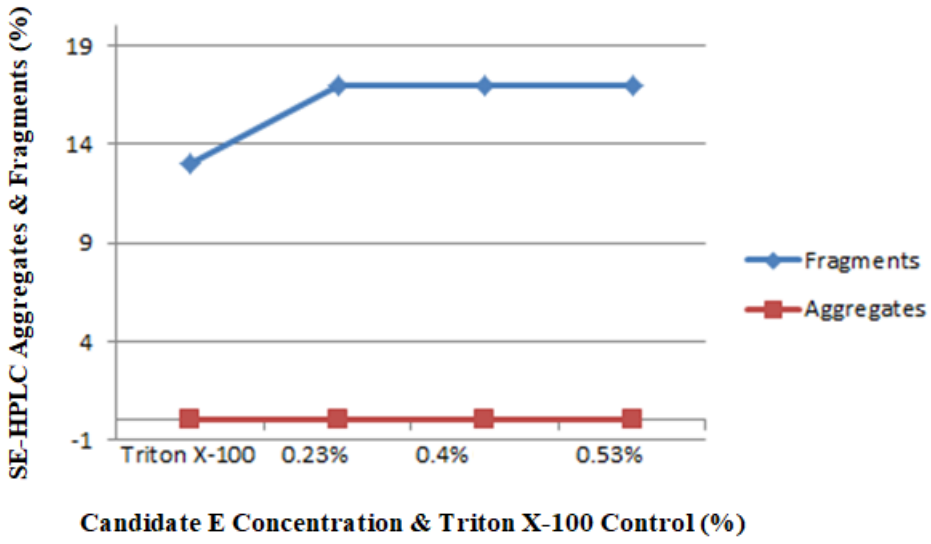

Figure 40: SE-HPLC control chart for Candidate E (OFAT Study 2)

5. Conclusion

The main aim of this research project was to identify an alternative detergent to Triton X-100 for viral inactivation of recombinant Factor VIII. In order to deliver on this aim a number of objectives were required to be met.

These were:

- Identify, through an extensive literature review, a panel of potential candidates that could be progress to laboratory scale viral inactivation studies in order to assess product quality impact.
- Evaluate, critically, from initial results which candidates successfully showed no product quality impact and should progress to Design of Experiment (DOE) studies.
- From the DOE provide a recommendation of candidates to undergo third party viral inactivation validation studies.
- Post third party viral inactivation studies, assess the viral inactivation kinetics of each prospective candidate to allow future validation work to be considered.

Here, we will re-examine the research objectives outlined above summarizing the findings. It will allow conclusions to each objective to be presented in a way which demonstrates that each objective has been met. Based on these conclusion recommendations in how to progress future work will be discussed. Additionally, the importance of the work carried out to date will be highlighted.

The literature review has shown that there has been substantial innovation and development within the realm of virus inactivation over the last number of decades. This innovation has led to the development of many modes of viral inactivation becoming available such as low pH, solvent detergent inactivation, UV irradiation and pasteurisation. Whilst not every mode is feasible or suitable for all biopharmaceutical manufacturing processes, it has shown there are many options available for evaluation. Solvent detergent was the main focus of this objective as knowledge already gained has

shown the recombinant factor FVIII is a labile protein result in incompatibility with most other modes of viral inactivation.

A significant body of viral inactivation research carried out using a variety of alternative detergents has been demonstrated. All of which reported viral reduction values greater than 4 logs. However, there still appears to be a continued reliance on the use of Trion X-100 within the research. With Triton X-100 being added to the REACH authorization list the research now needs to sway in the direction of evaluating eco-friendly detergents. Concluding from the review, all alternative detergents identified where technically evaluated under parameters such as toxicity, solubility, CMC, detergent type. This reduced the potential candidate number to 6 candidates with 4 being available at the time testing was scheduled. Each of these candidates progressed to bench scale viral inactivation studies where the impact to product quality was evaluated.

The bench scale viral inactivation studies carried out evaluated the impact of the 4 candidates on process performance attributes (pH, conductivity, turbidity) and product quality attributes (potency and protein concentration) under defined process conditions. Concerns relating to immiscibility and turbidity were noted during this stage with 2 of the candidates (candidate B and C). Immiscibility may be an indication of incompatibility with the process solution whereas turbidity can be indication of protein aggregation. Because both characteristics can ultimately affect product quality attributes neither candidates were carried forward to DOE studies. However, favorable product quality results that compared well against the control detergent (Triton X-100) partnered with historical usage within the Biopharmaceutical industry allowed for candidate A and D to progress to DOE studies.

The DOE carried out for this study examined the ranges of a number of parameters for use of candidate A and candidate D as a potential replacement for the current viral inactivation solution of Triton X-100. This study assessed the process performance and whether the recombinant factor VIII molecule would stay active and stable using either candidate

Candidate A showed no detrimental effects on the Factor VIII molecule under the process conditions tested in the DOE. Product quality was comparable to pilot model qualification and commercial historical data. Although a number of input parameters were found to be statistically significant, there were none that were judged to be practically significant. In contrast, candidate D showed significant quality impact on the Factor VIII molecule under the DOE process conditions. The data was not comparable to any of the historical norms previously demonstrated. If the potency and protein concentration data for candidate D pertaining to the initial lab scale studies and the DOE are compared, a significant difference can be seen. For the lab scale study a detergent concentration of 0.023% reported an average protein concentration value of 1.37 mg/ml and an average potency value of 2732 IU/ml, all of which were within specification. However the DOE results show statistical and practical significance for candidate D concentration whereby an increase in concentration delivered a decrease in both protein concentration and potency values.

Due to these irregular results additional studies were carried out. The additional studies were investigated using mid-point ranges for time and temperature and only varied in concentration of the detergents. The OFAT study found SE-HPLC (fragments and aggregates) and RP-HPLC (all isoforms) for both concentrations of candidate D (0.06 and 0.13 % v/v) were comparable to the control run. These studies concluded that the increase in candidate D concentration gave a decrease in total protein concentration and potency.

The results of this DOE study demonstrated that candidate A was comparable to Triton X-100 having no impact to product quality. Although the DOE for candidate D reported adverse results, it appears these results can be attributed to the higher detergent concentrations used. The follow on OFAT study carried out on candidate D reaffirms that a lower concentration delivers improved product quality results albeit only 2 data points were obtained. These studies enabled the establishment of appropriate process conditions to be used in the viral inactivation validation study which investigated the effects of candidate A and D on inactivation of model viruses.

Two models viruses, MuLV and PRV, were used as part of the viral inactivation studies. A fixed concentration and incubation temperature were used with both candidates. Both Candidate A and D demonstrated effective ability to inactivate both virus, each delivering > 4 log reduction values. Even though at the endpoint of incubation there were some levels of infectivity of PRV detected using candidate A, the overall log reduction was determined to be 5.32 log. The rate of kinetics displayed by candidate D was rapid, with > 3 LRV obtained after 1 minute and complete inactivation of both model viruses after the 60 minute time point.

Based on the research carried out it can be concluded that 2 alternative detergents in replacement for Triton X-100 have been identified. Both detergents have shown to have effective viral inactivation properties and no product quality impact towards the recombinant factor FVIII molecule.

As mentioned previously Triton X-100 is used within many unit operations within the manufacture of recombinant factor FVIII (Product X). To successfully eliminate Triton X-100 from all areas of the manufacturing process, a substantial body of research work is required. This was the main limitation of this research project as the time frame involved would not have permitted enough of robust data to be generated. Therefore the main scope of this research project was focused on an identifying an alternative detergent to Triton X-100 within the viral inactivation unit operation.

Although a significant body of research has been conducted as part of this project which will indeed form part of the REACH assessment of alternatives application process, there are further avenues that also need to be investigated from a manufacturing and regulatory perspective. These include clearance and removal studies which need to be carried out to assess the current manufacturing purification process' ability to remove and clear the alternative detergents below a specified limit. The viral inactivation studies that were carried out at the third party laboratory were conducted using 2 model viruses. Further validation runs need to be performed as part of an official viral clearance validation programme. This study will involve a larger number of model viruses with various physiochemical properties. It is foreseen that this study would again involve the 2 alternative detergents identified with 1 serving as the lead candidate.

The literature has revealed that, to date, viral inactivation studies that have been carried out by researchers have been performed using a blood derived protein of interest that was generally highly purified. This research has attempted to fill a void by performing viral inactivation studies on a recombinant factor VIII protein whilst combining learnings from previous research using various viral inactivation detergents. In the modern biopharma industry where recombinant proteins play a vital role, it is surprising to see this disparity. However, this research has provided an invaluable insight at a time where the replacement of Triton X-100 is in the spotlight whilst in parallel achieving the main project objective.

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7. Appendix 1

Table 16: DOE for Candidate A/TNBP Viral Inactivation(Run Order for Factorial Design)

Std.	Run	Factor 1 A: Candidate A Concentration % (v/v)	Factor 2: TNBP Concentration % (v/v)	Factor 3: Time (Minutes)	Factor 4: Temperature (˚C)
1	1	0.7	0.25	0	5
7	2	0.7	0.25	0	30
3	3	0.7	0.25	120	5
9	4	0.7	0.35	0	30
4	5	1.3	0.25	120	5
10	6	1.3	0.35	0	30
13	7	1.0	0.30	60	17.5
6	8	1.3	0.35	120	5
14	9	1.0	0.30	60	17.5
8	10	1.3	0.25	0	30
12	11	0.7	0.35	120	30
11	12	1.3	0.25	120	30
5	13	0.7	0.35	120	5
2	14	1.3	0.35	0	5

8. Appendix 2

Table 17: DOE for Candidate D Viral Inactivation (Run Order for Factorial Design)

Std.	Run	Factor 1: Time (Minutes)	Factor 2: Temperature (˚C)	Factor 3: Candidate D Concentration (% v/v)
17	1	60	17.5	0.4
9	2	0	5	0.6
16	3	120	30	0.6
1	4	0	5	0.2
4	5	120	5	0.2
8	6	120	30	0.2
12	7	120	5	0.6
6	8	0	30	0.2
3	9	120	5	0.2
15	10	120	30	0.6
11	11	120	5	0.6
2	12	0	5	0.2
5	13	0	30	0.2
14	14	0	30	0.6
7	15	120	30	0.2
18	16	60	17.5	0.4
10	17	0	5	0.6
13	18	0	30	0.6