Production, concentration and purification strategies for Bluetongue virus

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Resumo

Para o tratamento e prevenção de várias doenças, é indispensável o uso de produtos bio farmacêuticos para a síntese de medicamentos e vacinas. Mas, enquanto avanços em processos a montante têm aumentado, de modo, a obter elevados rendimentos e volumes de colheitas de vírus para vacinas, o processamento a jusante tem sofrido um estrangulamento nos vários processos de fabrico, representando a maioria dos custos globais de produção, sendo por isso uma questão importante a ser resolvida. Nos últimos anos, tem sido dedicado um enorme esforço à procura de novos métodos de "downstream processing" para a purificação de bioprodutos de forma economicamente viável e com maiores rendimentos de pureza sem perdas da actividade biológica.

Desta forma, o objetivo deste trabalho é abordar estes desafios atuais, desenvolvendo e otimizando novas tecnologias alternativas para a concentração e purificação do vírus da língua azul. A purificação de partículas virais para a remoção de contaminantes é conseguido por uma combinação de diferentes operações unitárias, tais como precipitação, ultracentrifugação, ultrafiltração, cromatografia hidrofóbica (HIC) e a cromatografia monolítica (CIM DEAE). No que respeita aos ensaios cromatográficos, o pH neutro revelou ser ideal para a estabilidade do vírus. Em HIC a melhor recuperação do vírus foi obtida utilizando a coluna com o ligando fenil e 2,5M de sulfato de amónio com 10 mM de fosfato. No que diz respeito à CIM DEAE o melhor resultado foi conseguido com 10 mM de NaH_2PO_4 como tampão de adsorção e de 20 mM NaH_2PO_4 com 1 M NaCl como tampão de eluição, ambos a pH 7. Nos sistemas aquosos de duas fases, os melhores resultados foram obtidos usando os sistemas de 15% de PEG6000 e 25% de sulfato de amónio e o sistema de 25% de PEG6000 e 15% de sulfato de amónio ambos a pH 7, com o vírus a particionar para a fase rica em sal.

Palavras Chaves: Vírus da Língua Azul (BTV), Sistema aquoso de duas fases (ATPS), cromatografia hidrofóbica (HIC), cromatografia Monolítica

Abstract

For the treatment and prevention of various diseases, it is indispensable to use biopharmaceutical products for the production of vaccines and medicines. Nevertheless, while advances in upstream procedures have allowed to obtain high tiers and harvest volumes for viruses vaccines, downstream processing (DSP) has been considered the bottleneck in various manufacturing processes, being, therefore, an important issue to be solved. In recent years, there has been a tremendous effort devoted to the search for new methods for the purification of bioproducts in a more economical and efficient way that allow higher purity yields without loss of biological activity.

The goal of this thesis is to address these current challenges, by developing and optimizing new alternative technologies for the concentration and purification of Bluetongue virus. The purification of virus particles with removal of impurities was achieved by a combination of different unit operations, which included precipitation, ultracentrifugation, ultrafiltration, hydrophobic interaction chromatography (HIC) and monolithic chromatography (CIM DEAE). Concerning the chromatographic assays, neutral pH revels to be the best for virus stability. In HIC, the best recovery of virus was obtained using phenyl ligands and 2.5M of ammonium sulphate in 10 mM phosphate. Regarding to CIM DEAE the best result was achieve by using 10 mM phosphate as adsorption buffer and 20 mM NaH_2PO_4 with 1 M NaCl as elution buffer. Regarding ATPS, the best results were obtained using 15% PEG6000 and 25% ammonium sulphate and 25% PEG6000 and 15% ammonium sulphate systems, pH 7.0, with BTV partitioning to the salt-rich phase.

Keywords: Bluetongue Virus (BTV), Aqueous two-phase systems (ATPS), Hydrophobic interaction chromatography (HIC), Monolithic chromatography

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List of Abbreviations

AEX Anion exchange chromatography

ATPS Aqueous two-phase systems

BHK Baby-hamster-kidney

BSA Bovine serum albumin

CIM® Convection interaction media

CV Column volume

DEAE Diethyl amine

DTT Dithiothreitol

$E_{(n)}$ Elution (number of the elution peak)

ELISA Enzyme linked immunoadsorbent assay

FBS Fetal bovine serum

FT Flow through

HIC Hydrophobic interaction chromatography

HPLC High pressure liquid chromatography

Kp Partition coefficient

IEX Ion exchange chromatography

MW Molecular weight

MWCO Molecular weight cut-off

PBS Phosphate buffered saline

PEG Polyethylene glycol

PEEK Polyetheretherketone

pI Isoelectric point

SDS-PAGE Sodium dodecyl sulphate-polyacrylamid gel electrophoresis

TEMED Tetramethylethylenediamine

TNE buffer Tris-HCl, NaCl and EDTA buffer

1. Introduction

Nowadays, viral particles are widely applied in medicine and scientific research. Their therapeutic usage has been of great interest in the development of vaccines, which are administrated to treat or prevent viral diseases. These vaccines are based on attenuated viruses, inactivated viruses, virus-like particles, virus membrane fractions or recombinant viral proteins expressed in different hosts. It is, therefore essential to have an efficient downstream process for the purification of viral particles. [1][2]

Over the last few years, ultracentrifugation and chromatography have represented the main processes used for purification of these bioparticles, mainly due to its simplicity and high resolution power. However, due to the high costs of operation, low yields and difficult scale up, some new alternative methods to tackle the traditional methods' disadvantages have appeared in the market. [3][4]

The liquid-liquid extraction using aqueous two-phase systems (ATPS) has recently been considered to be an alternative method to chromatography for the recovery of biomolecules, showing excellent purity and yield on a large scale and using conditions that prevent denaturation or loss of biological activity. ATPS has applications in the field of biotechnology being an excellent option for the purification of proteins, enzymes, nucleic acids, virus, antibodies and cell organelles.[3][5]

Regarding chromatography, in the last decades this technique has been considerably improved by the development of new supports like membranes and monoliths. These new supports offer flow-independent performance and can run over a range of flow rates, which would be unachievable with a conventional resin column. Besides that, it presents high advantages in binding capacity for large molecules such as virus. [6][7]

1.1 Viruses: General remarks

The viruses are pathogen agents that depend on their hosts to survive and replicate by using the cellular machinery and energy sources of the infected host, which makes them obligate intracellular parasites. The infectious form of the virus will depend upon the entry of genetic material into the host cell. [8]

Viruses have their genetic material, DNA or RNA, and other components such as enzymes essential for replication within a protein layer, called the capsid. Viruses can be classified into three general classes based on the symmetry of the protein arrangement within the capsid. The first class has a helical symmetry where the subunits are wrapped around a central axis. The second class is icosahedral, with a spherical particle with 2, 3 and 5 fold axis of symmetry. The third class consists in more complex virion structures. Some of the viruses are surrounded by a membrane associated to proteins and lipids, a structure called the viral envelope (Figure 1) .[8][9][10]

Figure 1- Examples of an icosahedral enveloped virus and a helical non-enveloped virus.

The mechanism of the virus entry in cells is different from the non-enveloped and enveloped viruses. In non-enveloped viruses, entering into cells occurs by binding to cell surface receptors and consequently being internalized by invagination of the plasma membrane and intracytoplasmic endocytosis. On the other hand, the enveloped virus entry involves the attachment of the virus, by the approximation of the viral and cellular membranes. Then, the two membranes fuse by an energetically unfavourable process involving the destabilization of the membranes to form a fusion pore. The opening of this pore is maintained by viral exposure of a hydrophobic fusion peptide, which will allow the entry of viral capsid within the cell. [11]

1.2 Bluetongue Virus: Virology, Epidemiology and Pathogenesis

Bluetongue virus is a member of the *Orbivirus* genus within the family *Reoviridae* and transmitted by *Culicoides* biting midges, encompassing 26 known serotypes, is transmitted to ruminants (mainly in sheeps and less frequently in cattle, goats, buffalos, deers and dromedaries) and causes thrombo-hemorrhagic fevers. [12][13][14]

Bluetongue virus was first recorded in the late 19th century in South Africa on a sheep wool importation. In 1933, it has also been diagnosed in cattle. During the 20th century, there started to appear occasional records of BTV in Europe. However, over the past 10 years, new strains of BTV (serotypes 1, 2, 4, 8, 9, 16) have appeared in Europe leading to a devastating disease in sheep and bovine, being the serotype 8 the most virulent one. This strain of BTV serotype 8 also crosses the ruminant placenta causing fetal infections, which is unusual compared with other strains of BTV. [10][13]

Climate change may explain the partial extension of the distribution of the virus to geographical regions further north from the epicentre of the disease since the change in temperature favours the vector distribution for these regions. [12][14]

For these reasons, the BTV has become a preoccupation since a wide dissemination of bluetongue would have a serious economic impact on the amount of meat and animal products available on the market. Therefore, bluetongue is a potential biological weapon. [11]

Bluetongue virus is a non-enveloped virus with 90 nm in diameter and has a triple-layered icosahedral symmetry and also a genome of approximately 19 200 base pairs composed of ten linear segments of double-stranded RNA responsible for encoding seven structural proteins (VP1 to VP7) and three non-structural proteins (NS1, NS2, and NS3). (Figure 2) [9][10][13]

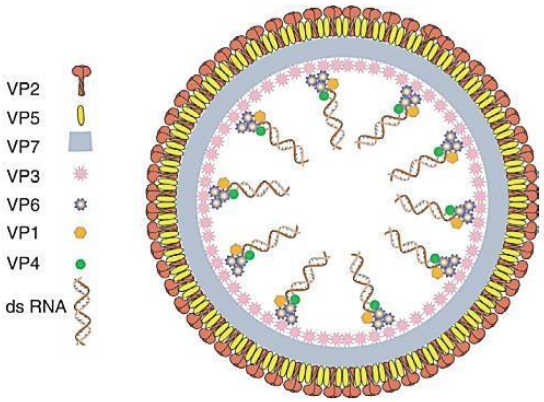

Figure 2- Representative scheme of BTV structural proteins and dsRNA

The outer shell is composed of two structural proteins, 60 trimers of VP2 (111 kDa) and 120 trimers of VP5 (59 kDa). The VP2 protein establishes the serotype and is responsible for receptor binding, haemagglutination and eliciting host specific immunity. The VP5 protein interacts with the host's cell endosomal membrane and plays a minor role in inducing an antibody response. The intermediate layer is composed by a major immunodominant VP7 structural protein (38 kDa) organized in 260 trimers covering the subcore, which is the responsible for the serogroup's specificity providing an epitope in ELISA tests for detection of antibodies against BTV. The subcore consists of the 12 decamers of the VP3 protein (100 kDa), which houses the viral genome segments and three minor proteins involved in transcription and replication, namely the RNA polymerase (VP1, 149 kDa), the RNA capping enzyme (VP4, 76 kDa) and the dsRNA helicase (VP6, 36 kDa). Non-structural proteins (NS1, NS2, NS3 and NS3A) probably participate in the control of BTV replication, maturation and export from the infected cell.[12][13][15]

Bluetongue is transmitted by biting midges of the genus Culicoides and therefore outbreaks depend on the presence of insect vectors and susceptible ruminants. Nowadays, the genus Culicoides includes 1300 to 1400 species but only about 30 of them are capable to transmit the BTV. [12] The transmission of BTV by midges is influenced by ambient temperature, air humidity, and total seasonal rainfall. The virus vectors can replicate at a temperature above 15°C, with the intensity of replication growing with the increasing of temperature. Recently, with the global warming, midges

activity occurs for longer periods of time during which they are capable of BTV transmission. In addition, the higher temperatures recently experienced in northern Europe have increased the competence of indigenous Culicoides species to transmit BTV. [12] [16]

The pathogenesis of BTV infection is similar in cattle and sheep, and most probably all ruminant species. However, there are differences in the severity of the disease occurring in different species of ruminant animals, and in the same species infection with different strains of virus. After skin inoculation of BTV (by inoculation or through the bite of an infected Culicoides BTV vector), the virus travels to the regional lymph node, where there is the initial replication. Then the virus is spread to the blood circulation inducing viraemia, which seeds a variety of tissues throughout the secondary sites, principally lungs and spleen, where it replicates in endothelium and mononuclear phagocytes (Figure 3). [12][13][14]

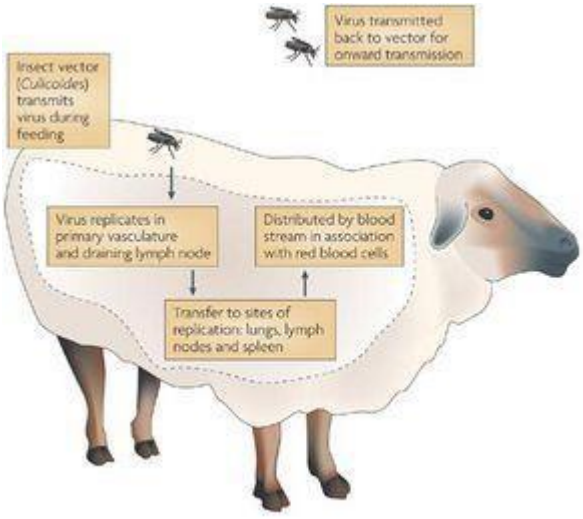

Figure 3- Summary of BTV transmission root (Nature Reviews, Microbiology).

An incubation period of four to eight days is followed by fever, apathy and hyperaemia of the lips and nostrils with excessive salivation are the common symptoms. Blue tongues are found in rare cases. In acute cases occurs the inflammation of the respiratory tract, hypertrophy of endothelial capillaries and heart and skeletal muscles

have haemorrhage and necrosis which, in chronic cases, results in fibrosis and infiltration with mononuclear cells (Figure 4).[12][13][14]

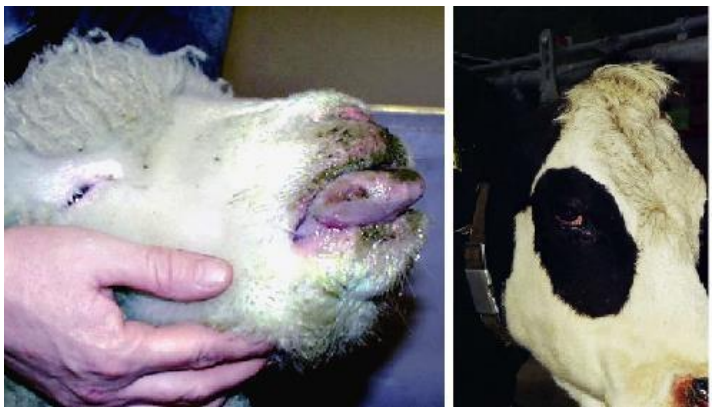

Figure 4- Lesions of bluetongue in sheep and cattle. [12]

1.3 Virus manufacture

Much effort has been devoted to the development of vaccines against BTV. In the manufacture of these types of vaccines, the protective activity is serotype-specific, probably due to the key role of the outer protein VP2 in the B and T cells mediated protective immunity. Inactive vaccines are a good option for the prevention of BTV because they can induce reliable and protective immunity. Nevertheless, they are expensive to produce and require large amounts of highly pure materials.[17]

Therefore, having a manufacturing capacity capable of meeting the demands of viral vectors production is a challenge. In addition, market speed is critical to deliver health benefits quickly and to achieve business successfully. So for the production of viruses there are two different methods: egg-based manufacture and cell-culture-based manufacture.[17]

1.3.1 Egg based manufacture

Each year, vaccine manufacturers use millions of fertilized eggs as a culture to grow viruses that, after numerous steps become a commercial vaccine. This method provide safe and effective vaccines for the near future, although, new and more advanced technologies are arriving on the scene. The novel methods for producing vaccines use mammalian or insect cell lines, instead of eggs to grow the virus. [17]

The egg-based manufacture developed in 1950, and has been used to produce seasonal influenza vaccines for more than 30 years. Since it was developed for the first

time, it has gone from a manual operation to an almost completely automated process. [18]

For the manufacture of vaccines based on embryonated eggs, it is required eggs with 9 to 12 days after fertilization. Then, the virus is inoculated into the allantoic sac with a syringe under aseptic conditions and the hole is then sealed with wax. Then, the inoculated eggs are incubated for three days. At this time, they are transferred to an environment at the temperature of 4 °C, which will kill the embryos. Finally, the top of the egg is cut, the membrane is perforated with a pipette and the allantoic fluid is removed. After that, it is carried out a centrifugation so as to proceed with the removal of cell debris. [19]

This method manipulates virus in a way of infecting embryonic eggs to produce recombinant proteins. These egg recombinant technologies are designed to increase the harvested yields and reduce the costs and reduce the time of full-scale vaccine production. The advantages of using embryonic eggs are the safety and effectiveness of the vaccines produced. However, this manufacturing process is labour intensive, time consuming (3–6 months) and unable to produce vaccines in sufficient quantities to meet e global demand.[2]

1.3.2 Cells based manufacture

New technologies based on the use of cells for vaccine production have been increasing with the aim of improving the market's arrival rate as well as the vaccine's potency. However, these vaccines undergo many of the same critical processes as the methods used for the egg-based vaccines such as its isolation, extraction and purification. For the development of this kind of processes, it is used roller bottles, incubators and bioreactors to grow cell cultures. Virus particles generated via animal cells allow more complex post-translational modifications. Nevertheless, this cell-based technologies, have some disadvantages such as the fact that the use of animal cells has a greater risk of contamination.[20]

For the production of based-cells' vaccines, it is vital for the seed virus to be carried under carefully specified conditions. After that, the cell lines must be expanded to the desired quantity after which they must be inoculated with a small amount of virus and placed into a cell factory, with the addition of an appropriate medium, to allow the

multiplication of the virus. The chosen medium will depend on the type of virus desired to multiply. Furthermore, the incubation time, the temperature and the pH of the mixture must be monitored for each cell line and each type of virus. It is only by doing so that the multiplication of the virus can be successful. After incubation, the virus is separated from the cells, and concentrated using ultracentrifugation or ultrafiltration. For higher amounts of cell culture, the scale up is done by using bioreactors able to provide the conditions required for cell proliferation. [19]

1.4 Downstream processing

After the production of the virus, it is essential to choose a downstream process for the purification and removal of cell debris. The traditional methods used for virus purification include ultrafiltration, precipitation by polyethylene glycol (PEG), or density gradient centrifugation. However, other methods have emerged as an alternative to these ones with the advantage of being able to purify on a larger scale. Examples of those new methods are for example, chromatographic techniques and aqueous two-phase systems. [2][21][22]

The three main steps of general downstream processing (DSP) for virus particles are clarification, concentration/purification and polishing (Figure 5). [23][24]

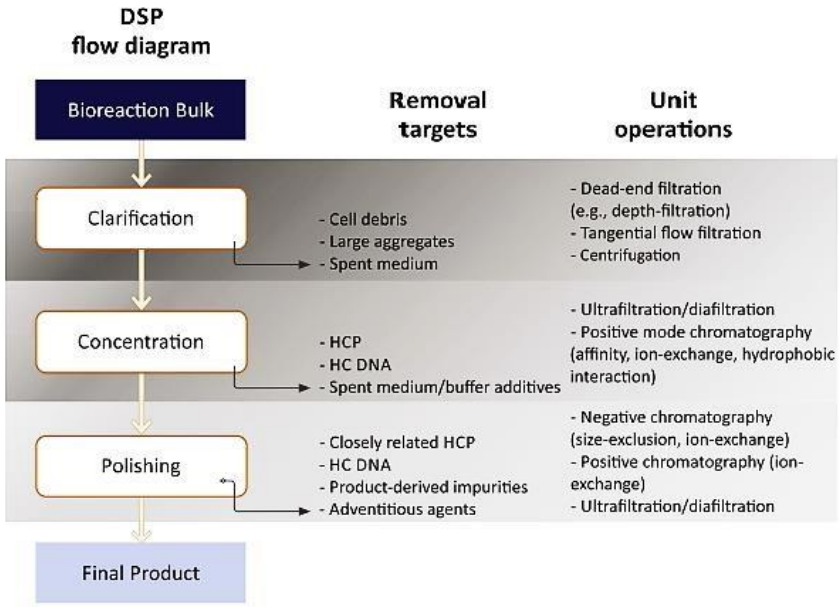

Figure 5- Typical platform downstream process for virus purification with removal targets and correspondent unit operations. [4]

1.4.1 Virus clarification methods

1.4.1.1 Density Gradient Centrifugation

Density Gradient Centrifugation is the method used for both clarification and separation of macromolecules, or subcellular fractions according to their sedimentation coefficient differences, most often according to their mass differences.

There are two ultracentrifugation methods that can be used. The first one, called differential centrifugation, consists in centrifuging a homogeneous solution of macromolecules. At the time where the most rapidly sedimenting molecules are pelleted at the bottom of the tubes, part of the more slowly sedimenting ones will still be in solution. The second method uses density gradients that can be continuous or discontinuous. Continuous gradients are used when the virus needs to be separated from other particles with similar density. These have a gradual transition from high to low density so that each particle-type can band at its own specific density. In discontinuous gradient the particles band at the interface between the density gradient layers, which makes harvesting easier (Figure 6). [25][26]

The gradient ultracentrifugation can still be divided into zone or isopycnic centrifugation. Zone centrifugation consists on a very thin layer of a macromolecular solution being layered on top of an appropriate medium, with a sharp interface between them where all biological material, with a specific density, between the high and low solutions concentrates. During centrifugation, macromolecules with the same sedimentation speed move through this medium in a single zone. Then, there will appear as many zones as the number of macromolecules present in the initial layer, and each of those zones of sediments have as a characteristic a different spinning speed. The content of the tube is then fractionated into layers perpendicular to the direction of the centrifugal force field, and the macromolecular content of each fraction is measured. In isopycnic separation, particles are separated on the basis of their density. Particle size only affects the rate at which particles move until their density is the same as the surrounding gradient medium. [25] [27]

Many different substances can be used for creating the density differential like sugars, salts and polymers even though, for most virus purification purposes, sucrose or caesium chloride (CsCl) gradients are adequate. [25]

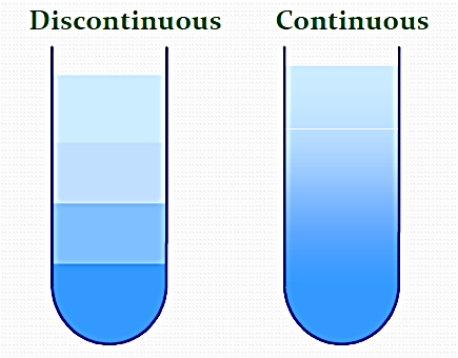

Figure 6- Differences between a continuous and a discontinuous method.

Ultracentrifugation is an important tool in biochemical research, which, through rapid spinning, imposes high centrifugal forces on suspended particles, or even molecules in solution, and causes separations of such matter based on differences in weight. For this type of centrifugation, the use of swinging bucket rotors is required. These rotors allow the density gradient to be always parallel to the force which is submitted and thus, the zones do not suffer any major distortion. The choice of a particular rotor depends basically on the amount of macromolecules to be centrifuged, on the resolving power, and finally on the centrifugation time (Figure 7). [25]

Figure 7- Swinging bucket rotor

The rotor is essentially characterized by a set of buckets, which hang in the vertical when the rotor is at rest. Tubes containing the solution for clarification are put

inside of each bucket, which is then submitted to a force parallel to their axes. Once the spin starts, the buckets come to the horizontal position.

The purpose of centrifugation for concentration of virus particles according to their specific density has demonstrated to be highly useful at both an industrial and small scale. However, these methods require high investment costs and, in some cases, suffer from losses of infectivity, leading to an increased necessity of the usage of ultrafiltration techniques particularly outside of laboratory scales. Two advantages of centrifugation methods is, their potential to separate assembled virus from the empty capsids and their sensibility in relation to viral strain differences. In the case of ultracentrifugation-based procedures, the major disadvantage is the fact that it is not feasible to scale up. [1][21]

1.4.1.2 Ultrafiltration

Virus filtration is one of the emerging markets for membranes, because it is considered to be a robust and effective virus clearance technology and a common unit operation in various industries. The work in this field dates back to 1971 when virus retention using membranes was first attempted. Among various filtration processes available, ultrafiltration is emerging as a powerful tool for virus filtration. [1][28]

As an alternative to centrifugation to remove cell debris and organelles, ultrafiltration has been commonly used for clarification in biotechnological production processes. Moreover, a number of works demonstrate ultrafiltration being employed for virus purification/concentration studies. [1][21]

Ultrafiltration is a tangential flow filtration technique, which uses membranes with pore sizes in the range of 0.001 to 0.1 µm, and it is used to remove high molecular-weight substances, such as virus, colloidal materials, and organic and inorganic polymeric molecules. In this process, the fluid is ran tangentially along the surface of the membrane, under a certain pressure drop, which forces the fluid to pass through the membrane to the filtrated side. [1][28] The fluid flow dynamics in a typical tangential flow filtration system used for virus filtration is controlled by the pressure gradients,

flowrate, fluid properties and, if applicable, the polarization of the membrane by solute or solids.[29]

There are two types of membranes available: tubular membranes and flat sheets (cassettes), which are commonly used in the biopharmaceutical industry. The choice of the type of membrane depends on the viscosity, solid content and volume of the feed as well as the product stability. [2]

Ultrafiltration when compared to non-membrane processes like liquid-liquid extraction and centrifugation is far gentler to the molecules being processed, it does not require an organic extraction, which may denature proteins, is a rapid and a relatively inexpensive method. Nevertheless, this method presents some disadvantages with virus losses, mainly attributed to mechanical disruption, pore obstruction, membrane entrapment as well as unspecific virus adsorption. [1][28]

1.4.1.3 Precipitation

PEG was first used by Albertson, Frick and Philipson [30] to precipitate viruses between two immiscible aqueous polymer phases. Since then, PEG has been used, usually in combination with salts such as NaCl, to recover various viruses in different growing medium.

Precipitation and flocculation purification protocols that were and, in some cases, still are the standard purification step, are rarely used in downstream processes for purification of certain virus particles because this type of methods when require low pH may compromise the viability as well as represent losses in biological activity. [1][30] [31]

However, PEG is a widespread polymer used as a fractional precipitating agent for the purification of proteins from a variety of sources due to its non-denaturing qualities, like being chemically inert, nontoxic, water-soluble and a synthetic polymer as well as being functional for a wide range of different molecular weights. [31][32] Moreover, it is known that the molecular weight of PEG influences the conditions of precipitation. The higher the molecular weight of PEG used, the lower the concentration required for precipitation. [33]

The proteins precipitate in the presence of PEG due to the quantity of the excluded volume depends only on the polymer concentration and it is independent of particle concentration or any other factor.[34] Therefore, the precipitation process occurs when an insoluble substance is formed in the solution due to an excluded volume or when the solution is supersaturated by the presence of a compound as salt. In most situations, the precipitate settles on the bottom of the solution, although it can fluctuate if it is less dense than the solvent. The deposition of the precipitate on the vessel bottom can be achieved by sedimentation, decantation, or centrifugation.

The use of this technique despite its disadvantages, has the following advantages: its gentle effect on viruses and the ability to obtain precipitation at neutral pH with high ionic concentrations and absence of other organic materials. [30][32]

1.4.2 Virus Polishing Methods

Polishing steps are performed for a final removal of trace contaminants from the solution in order to achieve acceptable concentrations of these contaminants and to obtain the final solution required. The polishing steps depend on the nature of the product and the impurities present in it. So most purification processes are used for reducing high molecular weight aggregates, charge-variants, residual DNA and host cell proteins. [35]

The scientific community has made a huge effort to move away from ultracentrifugation and density gradient-based purification methods that were, and in some cases, still are the standard purification methods used for purification of virus particles. Since the major concern of downstream processing is to guarantee the product stability, new strategies have emerged in the way to improve the purity, potency and quality of the bio products. As a result, chromatography and liquid-liquid extractions appeared as an alternative purification technique to the conventional methods for virus purification. [1][2]

1.4.2.1 Chromatography

Chromatography is the most popular methodology for large-scale purification of virus and virus-like particles. Currently, there are five different chromatographic separation methods for virus particles: size-exclusion chromatography (SEC), ion exchange chromatography (IEC), affinity chromatography (AC), hydrophobic interaction chromatography (HIC) and mixed mode chromatography. [1]

Recently, chromatographic matrices based on porous membrane layers or monoliths have been the focus for downstream processing, being particularly used for the purification of viral particles. [21][36][37]

Viruses possess different biological and biochemical properties and therefore purification conditions must be established specifically for each virus. Consequently, chromatographic separation is based on differences in the interaction of target viruses and other components to the applied stationary phase. The principles of each chromatography are dependent of the stability of the individual viruses. Also, the processes optimization is limited by the extreme running conditions such as pH, osmolarity, ion compositions and organic solvents that might influence the efficiency of the partition of the virus. The main advantage of this type of separations is high resolution with high purity degrees that are required for biopharmaceutical industry. [38]

Principles

Chromatography is a downstream process used for separating organic and inorganic compounds, which involves a sample being dissolved in a mobile phase, which can be a gas, a liquid or a supercritical fluid. Then, the mobile phase is forced to move through an immiscible stationary phase that can be either a solid or a liquid. [38]

After the chromatography, the analysis of the chromatogram will allow to identify which compounds are present in the sample, which makes the chromatography a great physical method for observing mixtures and solvents. Depending on the type of chromatography that is used it is possible to choose different ligands, which may need to be carefully selected taking into account the binding capacity and the affinity of the virus we want to purify. [39][40]

Hydrophobic Interaction Chromatography (HIC)

Hydrophobic interaction chromatography (HIC) is performed to separate and purify biological molecules based on their degree of hydrophobicity. In 1949, Shepard & Tiselius used, for the first time, the term “salting out chromatography”, being followed by Shaltiel & Erel in 1973, who introduced the term “hydrophobic chromatography”. In the same year, Hjerten described this technique as “hydrophobic interaction chromatography”, based on the retention of proteins on weakly hydrophobic matrices in presence of salt. [38]

In HIC, the mobile phase contains a high-concentration of salting-out agents like ammonium sulphate, which increase the hydrophobic interaction between the solute and the stationary phase. Therefore, to ensure the proper binding of the proteins, high concentrations of salt are needed in order to expose, by salting out, the hydrophobic regions of proteins that would otherwise be turned to the inner parts. However, this may also cause the aggregation of proteins, which is a disadvantage in this kind of purification method. [41][42]

The most used salts in HIC are ammonium sulphate ($(NH_4)2SO_4$) and sodium sulphate (Na_2SO_4) that are also known to have a stabilizing influence on protein structure. As a consequence of that, the ammonium sulphate pH has to be below 8 because it is unstable and forms ammonia gas when under basic conditions. In the case of sodium sulphate, it is suitable as a salting-out agent, but it often causes solubility problems at high concentrations.[38] Two other salts very often used in HIC are sodium phosphate (NaH_2PO_4) and potassium phosphate (KH_2PO_4). HIC has been applied for separating homologous proteins , receptors, antibodies, recombinant proteins, nucleic acids and viruses. [43]

Hydrophobicity can be defined as the repulsion of a non-polar molecule present in a polar environment, which can be conferred by water, methanol and other polar solvents. Consequently, when two hydrophobic molecules are located in a polar environment, they tend to minimize the contact with the polar solvent by coming in contact with each other, and thereby minimizing the molecular surface that is exposed to the solvent. This phenomenon is known as "hydrophobic interaction", and is an

example of what happens in HIC between the selected column and the sample to be purified during the passage of the adsorption buffer [38][43][44]

The retention of biomolecules in HIC is due to the hydrophobic interactions between the hydrophobic ligands immobilized on a stationary phase and the hydrophobic regions of the surface of the biomolecule. There are a variety of stationary phases used in HIC, which correspond to organic polymers or silica, among which, the most commonly used are, polyacrylamide (BiogelP TM), cellulose (Cellulafine TM), dextran (Sephadex TM) and agarose (Sepharose TM). These supports have been further modified so as to the linkers into hydrophobic ligands that permit the hydrophobic interaction with the biomolecule to be separated from a solution (Figure 8).[38] [41][43][44]

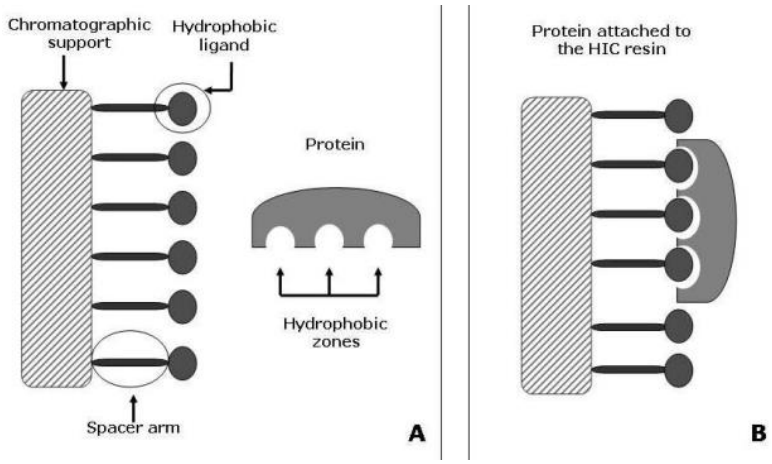

Figure 8 - Representation of HIC retention mechanism (A) the structure of a HIC resin and (B) how protein contacts with the ligands of the resin.

The nature of the hydrophobic ligand determines the performance of a HIC process. The most widely used ligands for HIC are linear chain alkanes in which the hydrophobic interaction is directly proportional to the length of the alkyl chain. Sometimes it can be advantageous to use aryl ligands like phenyl, which provide some aromatic (π-π) interactions, since aromatic groups on protein surfaces can interact specifically with the aromatic ligands. The most commonly used ligands in HIC resins are butyl, octyl and phenyl, in the following order in terms of relative interaction strength: Phenyl > Octyl > Butyl[44]

Once the biomolecule of interest is attached to the stationary phase, it is necessary to detach it in order to recover and separate it from the other molecules that might be attached to the ligands. Desorption is accomplished by reducing the ionic

strength in the mobile phase, by building a decreasing gradient of salt concentration, which results in the weakening of the interaction between the biomolecule and the ligand. The desorption depends on the physicochemical properties of the biomolecule, so when a specific salt concentration is reached, the biomolecule is desorbed. In this way, HIC can be used to selectively detach different biomolecules in a solution and, as so, become a powerful separation process. [38][43]

Chromatography using monolithic supports

Chromatography is a commonly used method in the purification of biomolecules, with the market dominated by conventional particle based resins. However, these type of resins have been evidencing low capacities for the purification for nanoparticles, with diameters between 20 and 750 nm, such as viruses. Besides that, these resins show long processing times. So, in order to improve the limited capacity of large particles, companies produce resins with smaller bead sizes. However, the problem is these new resins have a higher resistance to the flow of the mobile phase. [6][45]

In 1990, solid phases for purification of nanoplexes were developed based on membranes and monoliths for viruses and plasmid DNA. Since then, monolithic ion exchange chromatographic purification has been proving to be a powerful method for the purification of virus particles with high yields and maintaining its infectivity. This technology is based on large pore sized monolithic anion exchangers, quaternary amine (QA) or diethyl aminoethyl (DEAE), which are applied to membranes containing icosahedral bacteriophage PRD1, which bind specifically to both matrices.[46] The first commercial columns were from BIA separations called CIM (Convective Interaction Media) disks and were on the market in 1998 (Figure 9). [46][47]

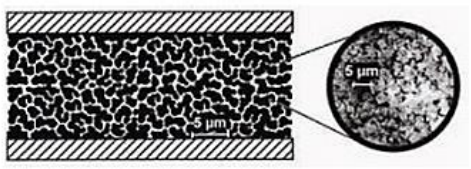

Figure 9- External and internal aspect of monolithic chromatographic column and CIM disk. CIM® disks (small disk with blue ring around) are constitute by white monoliths placed in the middle of the disk of a non-porous self-sealing ring which can have different colours according with their ligand chemistry. The CIM disk is inserted in the CIM housing (right) and used as a chromatography column.

The monoliths are hollow sponge like structures with high porosity allowing mass transport by convection with pore sizes of 1.2 µm or more, and the structure of stationary phase can be in the shape of disks or columns. These supports can be a single piece of organic highly porous material (like CIM disks mentioned above) or inorganic (like silica). Most of the generic discs are prepared from reactive monomers (glycidyl methacrylate) that are subsequently being modified to provide the desired interacting functionalities, which allow a various number of separation modes, such as, reversed-phase, ion-exchange, hydrophobic interaction and affinity chromatography.[46]

Monoliths exhibit flow-independent performance and can run over a range of flow rates, unlike conventional resin columns, indicating that the adsorption is not mass--transfer limited. These characteristics allow fast separations and low pressure drops at high flow rates. Besides being a rapid and simple procedure, the monolithic chromatography has the key of preserving the virus infectivity during the purification process and to obtain pure viruses at high concentrations without any additional concentration steps. [6][45]

Ionic interactions are the basis for purification of proteins by monolithic ion exchange chromatographic. The separation occurs due to competition between proteins with different surface charges for oppositely charged groups present in ion exchanger support. In cation exchange chromatography, positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged

solid support. For IEC, two strategies can be used: bind the target molecule to the column support with opposite charged and then wash away non-bound contaminants with same charged of the column or bind the impurities, and in that case the protein of interest should be found in the flow through.

The charge of the protein depends on the ratio of amino acid residues in its structure. Positive charges are usually provided by arginine, lysine and histidine and N-terminal amine. Aspartate and glutamate residues and the C-terminal carboxyl group provide negative charges. At the isoelectric point (pI) the net charge of the protein is zero. The charge of the protein is affected by the pH of the adsorption buffer. So at values of pH below the pI, the protein charge is positive because all the carboxyl groups are protonated and N-terminal are ionized, which makes the protein to be attracted to the negatively charged solid support. At values of pH above the pI, the protein is negatively charged due all the carboxyl groups that are deprotonated and thus ionized. Besides pH, for a good adsorption the conductivity of the running buffer has to be controlled. Low conductivity gives the protein an optimal charge.

The elution of bound proteins is mediated by a change in the pH of the eluting buffer or by increasing the ionic strength through the addition of NaCl. More weakly charged proteins are eluted at lower salt concentrations while the more strongly charged proteins are eluted at higher salt concentrations.

Nowadays, monolithic chromatography has applications at different scales: applications in capillary and microfluidic channels of a chip, and also in macroscale with columns of up to 8 L are being produced for a commercial plasmid DNA process. Therefore, monolithic supports have showed many applications for isolation, concentration and purification of different types of viruses but also for other macromolecular complexes like proteins, immunoglobulins and nucleic acids. [6][47]

1.4.2.2 Liquid-liquid extractions: Aqueous two-phase system (ATPS)

Lately, liquid-liquid extractions in aqueous two-phase systems have shown a great potential for the downstream processing of biopharmaceutical products, however, despite its advantages it has not gained wide industrial recognition in the field of biotechnology. This alternative technique seeks to avoid problems associated with most chromatographic supports, such as high cost, limited capacity and diffusional limitations. [1][2]

In 1958, Per-Åke Albertsson reported that the ATPS could be a good method for the purification of biomolecules. Albertsson also reported the partition of some proteins and virus particles between two aqueous phases by using phase system of dextran and methylcellulose. Since then, aqueous two-phase extraction has been successfully used for the purification and separation of other types of biomolecules, such as proteins, virus particles, nucleic acids and inorganic compounds, using different components for the two-phase system, such as polyethylene glycol (PEG) with potassium phosphate buffer.[3][48][49][50][51]

Principles

Aqueous two-phase systems (ATPS) are formed spontaneously upon mixing two aqueous solutions of different components, such as two polymers or one polymer and a salt prepared in an aqueous solution above a certain critical concentration. [23][49] The mixture will then separate into two immiscible phases and form an interphase between both phases, where the light phase (top phase) is rich in one polymer and the heavy phase (bottom phase) is rich in the second polymer or in salt. [52]

The partition for the proteins is dependent on their physico-chemical properties. The most soluble biomolecules usually partition to the lower phase which is the more polar one, while the proteins prefer the top one which is less polar and is also more hydrophobic. [51][53]

The partition coefficients (Kp) of the proteins in ATPS can be expressed as:

$$Kp = \frac{C_T}{C_B} \qquad \text{(Equation 1)}$$

Where CT and CB represent the equilibrium concentrations of the partitioned proteins in the top and bottom phases, respectively.

To determine the effects of polymer size and polymer and salt concentrations in ATPS, phase diagrams are calculated. These type of diagrams are the fingerprint of a specific system under certain conditions, such as pH, temperature, salt concentration and polymer molecular weight. The phase diagrams present a binodal curve (Figure 10), which divides a region of component concentrations that will form two immiscible aqueous phases (concentrations of components are above the curve) from those that will form one phase (concentrations of components are at and below the curve). [5][54][55]

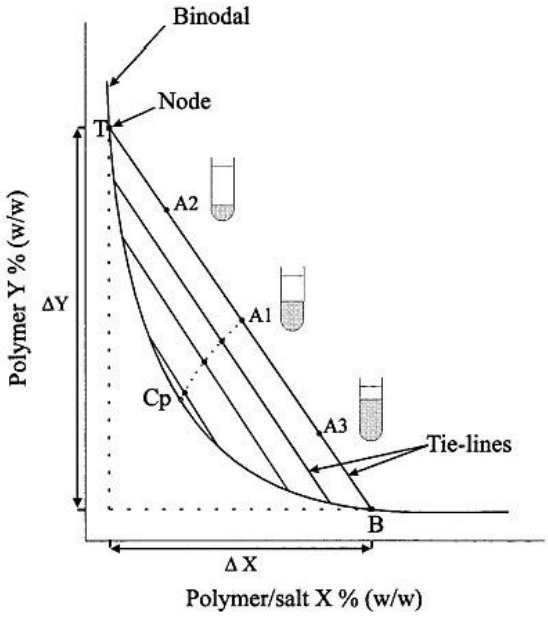

Figure 10 - Illustration of the phase diagram.[32]

Tie lines have the same units as the component concentrations (% w/w) and are often used to express the effect of system composition on the partitioned material. This means that moving along the tie line indicates the points where the final concentration of the components in the top phase and bottom phase are the same, but with different compositions and different volume ratios. [50][55]

Two phase formation

Phase separation in aqueous two-phase systems is a result of entropic repulsion, thermoseparation and electrochemical driving forces. The driving forces are the enthalpy associated with the interaction of the components during phase separation.[54]

The presence and the concentration of salts will also influence the phase behaviour due to the presence of ions in solution, which will cause the formation of an

electrochemical potential. [54] An increase in salt concentration, can result in an increase in partition coefficients of biomolecules to the top phase or to the interface due to salting out. [56]

The phase separation also occurs due to high molecular weight of the polymers combined with the interaction of the components during phase separation, which is also associated to the enthalpy of the system. [55] The increase in polymer weight causes the reduction of free volume on the top phase, resulting in partitioning of the biomolecules to the bottom phase and the partitioning coefficient decreases. [52][56]

Factors influencing partitioning

In order to form two immiscible phases capable of separating the target product it is necessary to select the parameters related to the desired system, such as pH, concentration, molecular weight of the polymers to be used, the ionic strength, the density, the viscosity and the interfacial tension, etc. [3][51]

Intrinsic properties of the target product also contributes to the partition, such as size, molecular weight, electrochemical properties, biospecific affinity, stereo chemical conformation and hydrophobic and hydrophilic regions. It is through the intrinsic and extrinsic properties that will determine the partition of the target product. [5][37][57] [58]

Applications

The main application of ATPS in biotechnology has been the isolation and purification of proteins but can be also applied to other molecules, for example: (i) separation of membrane proteins, cholesterol oxidase and bacteriorhodopsin[59]; (ii) structural analysis of the biological membranes such as thylakoid membranes[59]; (iii) concentration and purification of viruses.[60] Furthermore, the applications of ATPS have been extended also to the extraction of biocatalysts in order to avoid inactivation by the product. [61]

A high variety of high-value biomolecules have been recovered from various plants and fruits such as papain, α- and β-amylases and serine proteases. Other bioproducts that were recovered are the recombinant human serum albumin from Pichia pastoris broths, luciferase and immunoglobulin G.[56]

Nowadays, ATPS has been extensively used for purification of valuable biopharmaceuticals such as monoclonal antibodies, growth factors and hormones. It also finds applications in novel techniques like extractive fermentation for the removal of a product from the broth; membrane supported ATPS which combines liquid-liquid extraction with a membrane; and aqueous two phase floatation (ATPF) which combines the principles of ATPS and solvent sublation. [5]

Therefore, ATPS seem to be an attractive technique for the recovery of biological active molecules with applications in food and pharmaceutical industries.[62]

Advantages and disadvantages

Partitioning of biomaterials in ATPS is used for purification and analytical studies on cell constituents of several sizes, by providing the means to the stabilization of the biomolecules without affecting their biochemical activities. [52]

ATPS presents advantages over density gradient centrifugation and chromatography such as the easy removal of cell debris and other types of impurities, low cost of chemicals, and short processing time.[3] Furthermore, this process is easy to scale-up since the equipment used for this process can be adapted from the equipment used in chemical industry for liquid–liquid extraction, such as mixer-settlers, column contactors and centrifugal contactors. [63]

ATPS is a valuable tool in biotechnology for the extraction of specific proteins with commercial interest, concentration of viruses and removal of impurities, but also for studies of physical properties and behaviours of macromolecules in solutions.[52] ATPS also provides a friendlier environment due to the high content in water, which facilitates the partition of bioproducts without affecting their chemical or biological characteristics. [3] It is however important to make the right choice of polymers or/and salts as well as the concentration used. [64] The disadvantages presented by the use of ATPS are the difficulties to predict the behaviour of certain proteins in ATPS and the need to keep the characteristics of the proteins that are affected by the presence of high concentrations of salts or polymers. Environmental aspects are also a concern, so recycling polymers should be a hypothesis to be considered. [56]

2. Materials and Methods

2.1 Culture expansion and virus inoculation

BHK-21 (Baby Hamster Kidney) cell lines were cultured in Glasgow (Gibco®, CA, USA) medium supplemented with 10% fetal calf serum and 1% of antibiotics (Gibco®). After two days in culture, the cells formed a monolayer and the culture was expanded for higher capacity flasks (175 cm^2) by replacing the medium and using trypsin for cells detachment suspension.

After three days, there were enough cells for virus inoculation. Consequently, the growth medium (Glasgow) was removed and 2 mL x 10^5 $TCID_{50}$ of BTV were inoculated for previous incubation for 30 min at 37°C in a 5% CO_2 atmosphere. After 3 days of incubation, when extensive cytopathic effect was observable the culture medium was removed and clarified by centrifugation 1500 x g for 15 min. The virus was inactivated by heating to 60° C for 12h, and then the supernatant was stored at 4˚C until further experiments were carried out.

2.1.1 $TCID_{50}$

Bluetongue virus (BTV) was obtained by BHK21 cell culture inoculated with a strain of BTV-4 supplied by INIAV. The determination of cytopathic effects (CPE) of virus infection can be quantified by using the $TCID_{50}$ Assay. $TCID_{50}$ is the median tissue culture infective dose, which represents the amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated and it is expressed as $TCID_{50}$/ml. The results from viral infection can then be observed by optical microscope where CPE can be confirmed. [65]

Viral samples were titrated in 96-well microtiter plates. Ten fold dilutions of the virus stock (10^{-2} to 10^{-10}, 1:200) were prepared in Glasgow medium and added to the plates (eight wells/dilution) with an equal volume (100 µL) of BHK-21 cells suspension. The plates were incubated at 37˚C in a 5% CO_2 atmosphere during 4 days. The cells were checked on the optical microscope for specific viral cytopathic changes such as cell rounding and detachment.

2.2 BTV concentration

2.2.1 BTV pre-purification by polyethylene glycol-NaCl precipitation

BTV was precipitated from the supernatant (300 ml) using 6% (w/v) polyethylene glycol 6000 (Sigma-Aldrich) and 0.4 M NaCl (Sigma-Aldrich) with overnight stirring and centrifuged at 10 400 × g for 30 min at 4 ˚C in a Sorvall GSA rotor and resuspended in 1 mL of phosphate buffered saline (PBS) (Sigma-Aldrich).

2.2.2 Supernatants ultracentrifugation

The clarified supernatants were centrifuged in a SW-41 rotor (Beckman Coulter) for 90 min at 100 000 x g at 4°C. The virus pellet was suspended in 1 mL of one of the three buffers: PBS, 10 mM phosphate or ammonium sulphate and then were stored at 4˚C until being used in aqueous two-phase system and in chromatography experiments.

2.2.3 Supernatants ultrafiltration

Due to the high volume, the supernatant from cell culture was subjected to a ultrafiltration step utilizing an Amicon Ultra 15 mL (Millipore, Bedford, MA, USA) centrifugal filters containing a 10 kDa molecular weight cut-off (MWCO) regenerated cellulose membrane. Approximately 12 mL of supernatant solution was placed in the filter unit and centrifuged (Eppendorf, Hamburg, Germany) at 14 000 x g for 1 h. After this first centrifugation the retentate solution was collected and analysed.

2.3 BTV concentration and purification by Aqueous two-phase

2.3.1 Polymers and Salts

Polyethylene glycol (PEG) was obtained from Sigma-Aldrich and the molecular weights used in these experiments were 1500 and 6000. Potassium phosphate monobasic anhydrous (KH_2PO_4), potassium phosphate dibasic anhydrous (K_2HPO_4), sodium phosphate monobasic anhydrous (NaH_2PO_4) and sodium phosphate dibasic anhydrous (K_2HPO_4) were obtained from Sigma-Aldrich.

2.3.2 Phase Diagrams

The turbidometric titration method was used to determine the binodal curves of the PEG 1500/ammonium sulphate systems. In the turbidometric titration method,

water was added drop-wise to several biphasic systems with different compositions, until one phase systems were formed, after vortex mixing.

Tie-lines were determined by calculating the composition of both top and bottom phase of selected systems. Salt concentration in both phases was determined by conductivity measurements using a conductivimeter from Oaklon Instruments (Hacienda Heights, CA, USA). PEG concentration was determined by refractive index measurements using a 30PX refractometer from Mettler Toledo (Greifensee, Switzerland). [55]

2.3.3 BTV concentration and purification

The necessary amount of phase-forming chemicals for a 3 g ATPS were constructed on a % w/w basis using the quantities predetermined from stock solution of PEG 6000 (Sigma-Aldrich) and ammonium sulphate (Sigma-Aldrich). Aqueous solutions of 40% (w/w) sulphate were prepared and buffered at pH 6.

The virus was added in the end and consisted of a remaining percentage of the total ATPS weight. Partition assays were set up in 15 mL graduated centrifuge tubes. In the case of PEG/Salt systems, the pH was assumed to be the same as the original ammonium sulphate stock solution, pH 7. The phase components were thoroughly mixed on a vortex agitator (Ika, Staufen, Germany). The formation of two-phase system was accomplished within 2 minutes. After the phase separation, the volumes of each phase were measured and finally the top and bottom phases were carefully separated with a syringe and taken for further electrophoresis and chromatography analysis.

2.4 BTV concentration and purification by chromatography

2.4.1 Hydrophobic Chromatography

Three different chromatographic columns were used: HiTrap Octyl FF, Butyl HP and Phenyl HP (GE Healthcare Biosciences, Uppsala, Sweden). These columns are prepacked with Sepharose media (Fast Flow for the FF and High Performance for the HP), they have 1 ml of bed volume and bed dimensions of 0.7 cm × 2.5 cm. The Fast Flow (FF) and High Performance (HP) columns differ in the particle size (45-165 µm for the FF and 24-44 µm for the HP) and on the amount of ligand bound to the matrix which is

approximately 50 µmol butyl/ml gel for HiTrap Butyl HP, 5 µmol octyl/ml medium HiTrap Octyl FF, and 25 µmol phenyl/ml gel for HiTrap Phenyl HP.

All the chromatographic separations were performed on an Äkta TM Purifier system from GE Healthcare. All runs were performed at a flow rate of 1 ml/min. Each column was first washed then equilibrated with 5 column volumes of the adsorption buffer before the injection of the ATPS bottom phase. For HIC experiments were used three different concentrations of adsorption buffer: a solution of 1M, 1.5M and 2 M of ammonium sulphate in 10 mM of phosphate at pH 7. The samples were recovered from the bottom phase of an ATPS with a 5 ml syringe with no previous treatment and were injected using a 1 ml sample loop. The unbound compounds were washed with 2 column volumes of adsorption buffer. The elution was provoked by a decrease in the salt concentration due to the use of the 10 mM phosphate elution buffer at pH 7 with a 15 column volumes elution. During this operation the column pressure, UV absorbance at 280 nm and the conductivity were continuously monitored. The column flowthrough was recovered in 1 ml fractions and the eluate in 1 ml fractions using a FRAC 950 fraction collector (GE Healthcare).

2.4.2 Monolithic Chromatography

Chromatography experiments were performed in an Äkta TM Purifier system from GE Healthcare. CIM® disk monolithic column (BIA Separations, Ljubljana, Slovenia) of diethylamineethyl (DEAE) was used during the experimental work. CIM® monolithic column is a 3 mm×12 mm disk-shaped highly porous polyglycidyl methacrylate-co-ethylene dimethacrylate matrix and its bed volume and porosity is 0.34 mL and 62%, respectively. The channels within the column have a diameter of 1500 nm. The disk is stuck in a polyetheretherketone (PEEK) housing (BIA Separations).

During the procedure, the absorbance was measured at 280 nm and 214 nm and the flow rate was 1 mL/min. Virus suspension volumes applied to the monolithic column were 1.5 mL. Equilibration of a disk monolithic column was carried out with 5 CVs of the adsorption buffer, which was 10 mM of phosphate. All buffers were filtered prior to the chromatography procedure through a 0.45 µm filter (Millipore, Bedford, MA, USA).

Elution was undertaken by using a linear gradient with 5 CV of 1 M of NaCl with 20 mM of phosphate. After loading, washing and elution, an aliquot of each fraction or

fraction pools were analysed. All runs were performed at room temperature. Samples were stored at 4°C until further analysis. Cleaning in Place of disk monolithic column was carried out after 20 column volumes of 20 % (v/v) ethanol for the remove of bound hydrophobic proteins or lipids. The column flow-through was recovered in 1 ml fractions and the eluate in 1 ml fractions using a FRAC 950 fraction collector (GE Healthcare).

2.5 Analytical Methods

2.5.1 SDS-PAGE electrophoresis:

SDS–PAGE was performed to evaluate the purity of each phase collected (bottom and top) from ATPS systems and each elution fraction from chromatography experiments. Samples were diluted in a loading buffer containing 62.5 mM Tris–HCl (Sigma-Aldrich), pH 6.2, 2% SDS (Biorad, CA, USA), 0.01% bromophenol blue (Bio-Rad) and 10% glycerol (Sigma-Aldrich) and denatured in reducing conditions with 0.1 M of dithiothreitol (DTT) (Sigma-Aldrich) at 100˚C for 5 min. Samples were applied in a 12% acrylamide gel prepared from 40% acrylamide bis stock solution (29:1) from Bio-Rad (Hercules) and run at 200 mV using a running buffer containing 192 mM glycine, 25 mM Tris and 0.1% SDS, pH 8.3. Every gel was loaded with a Precision Plus Protein Dual-color standard from Bio-Rad. Gels were stained by soaking gels in Coomassie PhastGel™ Blue R, from Pharmacia (Uppsala, Sweden) solution for 1 hour. Several washing steps were made for the destaining of the gels with a solution containing 30% (v/v) ethanol and 10% (v/v) acetic acid for 30 min. Gels were then stored in Milli-Q water.[66][67]

2.5.2 Silver Staining

When the intensity of the bands was unsatisfying, the gels were silver stained due to method's sensitivity (in the very low ng range) according to the steps described in Table 1. The rationale of silver staining is quite simple. Proteins bind silver ions, which can be reduced under appropriate conditions to build up a visible image made of finely divided silver metal. [68]

The silver staining of gels stained with Coomassie should proceed to step 2 from the protocol without having to pass through the fixation step with 40% methanol / 10% acetic acid, because the gels were already stored in 30% methanol/10% acetic acid.

Table 1- Steps used in the silver-staining protocol

Step	Description
Fixative	Several washes with 40% methanol/10% for 30 minutes minimum.
2. Oxidizer	Incubation in an oxidizer solution, for 5 minutes.
3. Water Washes	Several washes with large volumes of water for 15 minutes maximum.
4. Silver Reagent	Incubation with silver reagent solution for 20 minutes.
5.Quick Water Rinse	Washing with Milli-Q water for 30 seconds maximum.
6. Developer	Incubation with a developer solution for 30 seconds or until a brown or smokey precipitate appears. Quickly pour off the solution and add fresh developer.
7. Stop	Incubation in a stop solution composed by 5% acetic acid (v/v) 15 minutes.

3. Results and Discussion

Ultracentrifugation, PEG precipitation and ultrafiltration were used in order to find the best clarification and concentration method for BTV, while PEG/salt systems were developed to find the best system composition for the purification of BTV. Also for purification of BTV, hydrophobic and anion exchange chromatography were tested.

3.1 Clarification results

As previously stated, BTV was produced from BHK-21 cell cultures. After three days of incubation, the culture medium was removed and clarified by centrifugation. After that, the pellet containing mostly cells was discarded and the virus in supernatant was heat inactivated before stored at 4˚C until used in different techniques of purification.

The three pre-purification methods tested aim to find the best feedstock resulting that will be used for the chromatography and aqueous two-phase systems experiments, in order to concentrate the BTV and decrease the load of impurities. Represented in figure 11 are the protein profile correspondent to the initial feedstock, which is the supernatant collected after the centrifugation of cell culture, and the three different feedstocks resultant from the three different pre-purification methods.

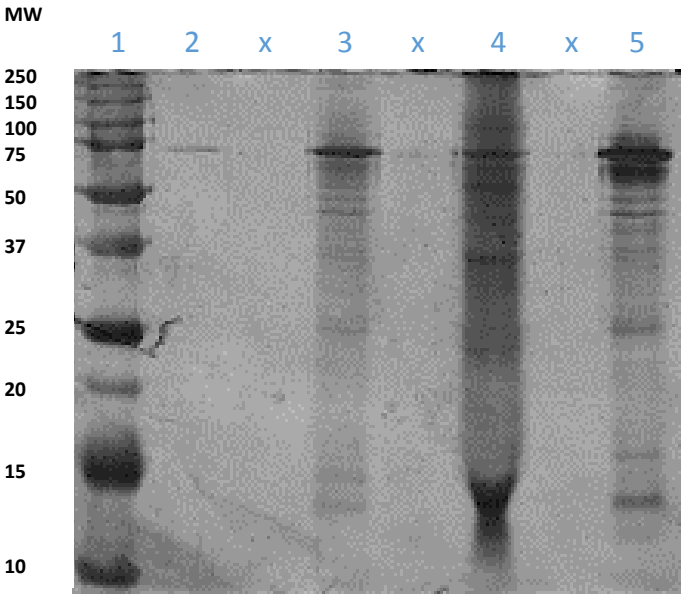

Figure 11 - Silver stained reducing SDS-PAGE analysis of the feedstocks. Lane 1: Precision Plus Protein™ Dual Colour Standards, molecular weight (in kDa); Lane 2: BTV clarified by ultrafiltration; Lane 3: BTV 100x concentrated by ultracentrifugation; Lane 4: BTV 100 x concentrated by PEG precipitation; Lane 5: Clarified BHK-21 cell supernatant (initial feedstock).

By SDS-PAGE results, it is possible to verify the presence of several impurities from the culture medium supplemented with FBS such as, bovine serum albumin (BSA, with an apparent MW around 66 kDa), the major component of FBS.

The supernatant filtrated by ultrafiltration with a 10 KDa MWCO Amicons® Ultra centrifugal filter unit (lane 2, Figure 11) was only able to concentrated one protein. The presence of other proteins were impossible to observe for being in solution in very low concentrations. The impossibility of observe viral proteins may also be due to the fact of BTV being adsorpted to the membrane and not present in the retentated which was collected in the end of the ultrafiltration.

The BTV was precipitated from the cell cultured supernatant by using polyethylene glycol and NaCl (Lane 4, Figure 11) and consequently, become insoluble (precipitate). Although if the concentration of particles in suspension is low, the particles may precipitate but do not aggregate to form large pellets. Thereafter, the pellet obtained by PEG precipitation was resuspended in TNE buffer and analysed by SDS-PAGE. This method presents good results for concentration of proteins, however, is not a very selective method, because it involves not only the precipitation of viral proteins but also proteins that we wish to eliminate.

The ultracentrifugation of BTV shows a clearer protein profile (Lane 3, Figure 11), with well-defined bands that allow the identification of viral proteins as well as some impurities, but in less amount as compared with the initial supernatant. Moreover, is possible to observe two low molecular weight bands that may correspond to viral RNA, which also proves the presence of the virus.

Thus, the two methods capable to concentrate the viral proteins were PEG precipitation and ultracentrifugation. The precipitation proved to be a method with a higher concentration yield but with less selectivity, as it concentrates not only the viral proteins but also other proteins that we wish to eliminate. In the case of ultracentrifugation, it exhibits a lower concentration yield but a greater selectivity for viral proteins, whereby the following experiments were carried out with the BTV feedstock from this method.

The viral proteins of BTV have different molecular weights, which if there were no impurities could be very well distinguished. The three minor components are VP1, VP4 and VP6 with MW of 149 kDa, 76 kDa and 36 kDa, respectively. The core is composed of two major proteins VP3 with 100 kDa and VP7 with 38 kDa, which is in turn enclosed by the two outer virion proteins VP2 with 111 kDa and VP5 with 59 kDa, which forms the complete virus particle.

3.2 Aqueous two-phase system

The objective of using ATPS is to find an aqueous biphasic system that can be used for BTV purification with high purification yields. For protein separation, PEG/salt and PEG/dextran systems are the most commonly used. Therefore, in order to form two immiscible phases capable of separating the BTV it is necessary to select some parameters related to the desired system, such as pH, concentration of polymers and salts, molecular weight of the polymers to be used, since ATPS is strongly influenced by psychochemical properties. [55]

PEG-ammonium sulphate systems are not the most used systems in ATPS but show interesting features such as a high salting out capacity of ammonium sulphate; a fast formation of the two phases using relatively low concentrations and usually present high extraction yields.[69] Because of these advantages, in this work the partition of BTV was made by testing three different conditions where PEG molecular weight and the concentrations of PEG and ammonium sulphate compositions were varied (Table 2).

Table 2- ATPS conditions tested for BTV purification.

PEG MW (Da)	PEG concentration (% w/w)	Ammonium Sulphate concentration (% w/w)	Name
6000	25%	15%	System 1
	15%	25%	System 2
	5%	30%	System 3

The molecular weight of PEG influences the partition of proteins by steric exclusion effects since larger polymers tend to form more aggregated structures making it harder for large proteins to partition to their phase. [70] Based on this, the choice made was PEG 6000. The selection of the concentration was made based on phase diagrams, from where distinct points were chosen and all the systems were tested at pH 7.

To determine for which phases have the virus preferentially partitioned, samples from the top and bottom phase of each tested system were collected, and subsequently, analysed by SDS-PAGE followed by Coomasie staining. In some cases due to the lack of intensity of the bands, the gels were restained with silver because it represents a more sensitive method. The samples from conditions 1, 2 and 3 were run in a gel, which is represented in Figure 12.

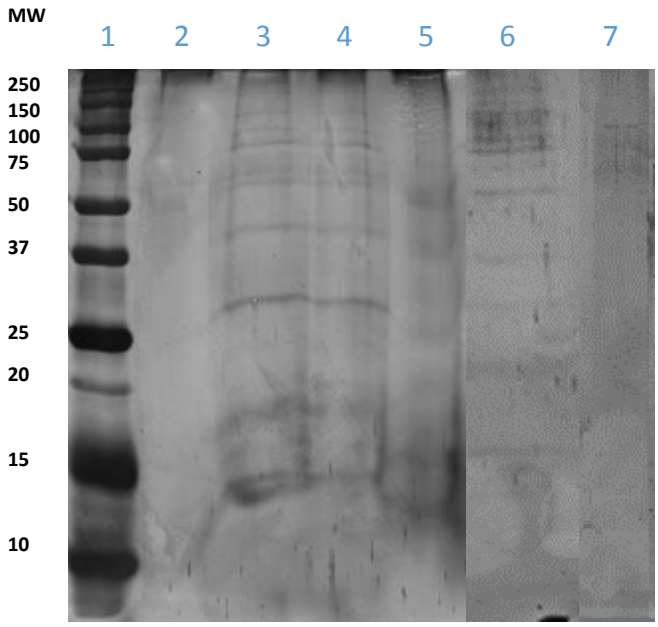

Figure 12- Silver stained reducing SDS-PAGE analysis of the feedstocks. Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 2: Top Phase of system 2; Lane 3: Bottom Phase of system 2; Lane 4: Top Phase of system 3; Lane 5: Bottom Phase of system 3; Lane 6: Bottom Phase of system 1; Lane 7: Top Phase of system 1.

By observing Figure 12, it is possible to observe that the lanes containing top phases from system 1 and system 2 present almost no visible bands or just some smears. This gives the hypothesis of the partition of BTV particles in this aqueous two-phase systems is occurring to bottom phase, which is proved by the presence of several bands in lanes 3 and 6, which may be viral proteins in the presence of many impurities.

However, this does not happen for system 3 as it can be seen in lanes 4 and 5. In system 3, proteins are partitioning mostly to the top while just a few proteins are partition to the bottom phase, which is confirmed by the presented of just a few smear bands in lane 5. Moreover, top phase from this system presents a protein pattern very similar to the bottom phase of system 2, which comes to confirm the inverse partition. The reason for viral proteins are partioting to top phase in system 3 is due to the decreasing of the PEG concentration which leaves more free volume in the top phase that allowed the partition of viral particles.

Since, the objective of ATPS is to use a system where the virus particles partition to salt phase, and then collected and used for chromatography; results from system 3 were excluded for not showing any advantage for it.

The conditions tested in system 1 and 2 showed again the presence of the major impurity protein, bovine serum albumin (66 kDa). The presence of several impurities difficult the confirmation of the presence of the viral particles in bottom phases in both systems. Although, it is possible to identify some bands with molecular weight around, 100 kDa, 76 kDa, 60 kDa, 40 kDa that might belong to BTV proteins.

In summary, bottom phases from systems 1 and 2 can be used directly for hydrophobic interaction chromatography experiments even though there are several impurities present from the culture medium where the cells were expanded. Since the goal of ATPS was to concentrate the virus, the presence of such impurities we can infer that this method only yields partial purification. Consequently, to improve the purification of BTV, samples from bottom phase of both systems were collected and purified by chromatography.

3.3 Hydrophobic chromatography

As it was previously mentioned, in hydrophobic interaction chromatography, the retention is enhanced by the presence of an osmotropic salt, and its concentration is the most important parameter to assure the binding of biomolecules. From a process point of view, it is essential to choose the right type of salt and the right concentration in order to avoid future problems like clogging of chromatographic columns induce by the precipitation of the biomolecules at high salt concentrations. However, if the

concentration of salt is too low, exposure of hydrophobic groups will not occur preventing their binding to the hydrophobic ligands, which leads to sample losses.

The system with higher salt content of 30% ammonium sulphate induce the partitioning of the BTV to the top phase, which means to PEG phase, and therefore it could not be further processed by chromatography and be injected in the column. Thus, the systems composed by 15% and 25% of ammonium sulphate were chosen for the further HIC studies in three different columns and at three different concentrations of elution buffer.

3.3.1 Bottom phases processing by HIC under different concentrations of adsorption buffer

Since there are no reported data describing the concentration and purification of BTV by HIC chromatography, in order to select the most suitable conditions for the virus downstream processing, initial studies were performed at neutral pH to find an appropriate salt concentration that would permit the binding of virus particles to the column without inducing the precipitation of the viral proteins.[38] For that, three different concentrations of ammonium sulphate were tested for the adsorption of the BTV particles present in the bottom phases of systems 1 and 2. The column used for this experiments was phenyl HP and for the elution was used a 10 mM phosphate buffer pH 7.

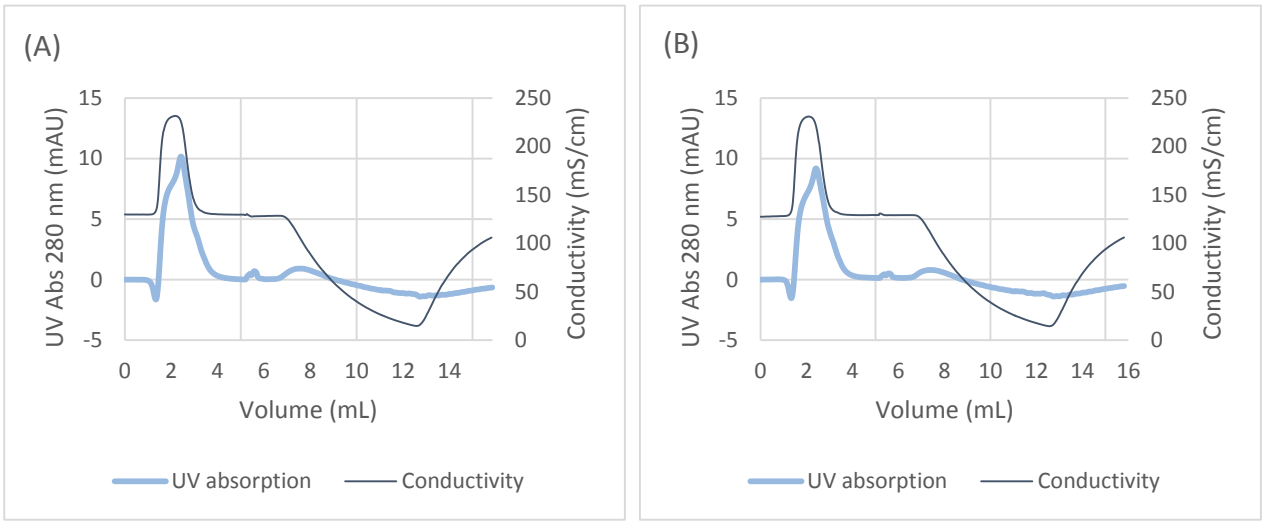

Figure 13- HIC runs with a HiTrap phenyl HP column for the purification of BTV from the ATPS bottom phases of (A) a 25% PEG 6000-15% Ammonium Sulphate (A-system 1) and a 15% PEG 6000-25% Ammonium Sulphate (B-system 2) using 1 M of ammonium sulphate with 10 mM of phosphate adsorption buffer and 10mM phosphate buffer for elution.

The expected results were that in presence of salts, hydrophobic regions of the virus will be exposed to the phenyl ligands and protein impurities will be eluted in flow through (FT), while the elution peak would have the BTV.

In Figure 13, is possible to observe the increase of the conductivity when the sample is injected which may represent a problem since if the concentration of ammonium sulphate of the adsorption buffer is inferior to the present in the sample, the hydrophobic regions will be exposed not only from viral particles but also from other proteins that we intended to eliminate. Other case that may happen is the precipitation of BTV and in this case, it will not bind to the column, and will appear in FT peak, but in this situation, the pressure would increase and the column would go to waste. In order to confirm, in which fraction peak the BTV was eluted, a SDS-PAGE was performed (Figure 14).

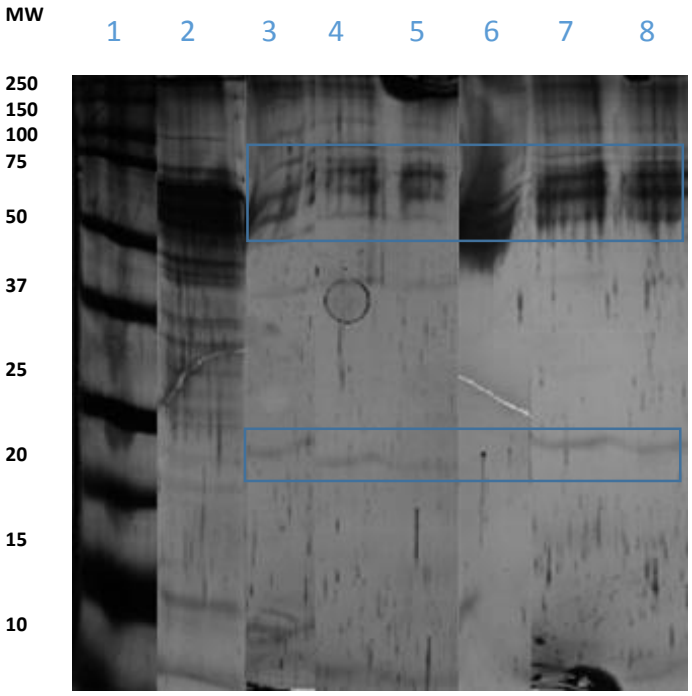

Figure 14- Silver stained reducing SDS-PAGE analysis of chromatography samples. Lane 1: Precision Plus Protein™ Dual Colour Standards, molecular weight (in kDa); Lane 2: BTV virus after ultracentrifugation; Lane 3: Bottom phase from ATPS of 25% PEG6000-15% Ammonium Sulphate (system 1); Lane 4: Flow through from sample containing bottom 1; Lane 5: Elution peak from sample containing bottom 1; Lane 6: Bottom phase from ATPS of 15% PEG 6000-25% Ammonium Sulphate (Bottom 2). Lane 7: Flow through from sample containing bottom 2; Lane 8: Elution peak from sample containing bottom 2.

The SDS PAGE, allows us to identify a similar pattern of bands in lanes corresponding to the FT and the elution peak when compared to lanes containing samples from the bottom phases of ATPS systems. Even so, is possible to observe that

samples from ATPS and chromatography experiments present less impurities when compared with BTV feedstock from ultracentrifugation. However, the fact that the pattern of bands in the FT peak is being similar to elution peaks means that the virus binds non-specifically to the column, reaching a possible equilibrium. Thus, in the injection of sample, some viral particles bind to the column while others are immediately eluted in the FT along with other impurities that also did not bind to the column. With the decreasing of the ionic strength by changing the buffer, the viral particles that were before attached to the column are then eluted, being this the explanation for the presence of a same band pattern. Another explanation could be at the level of the support that may be barely functional and thus the interaction between the virus and the ligand will not be strong enough to assure good purification results.

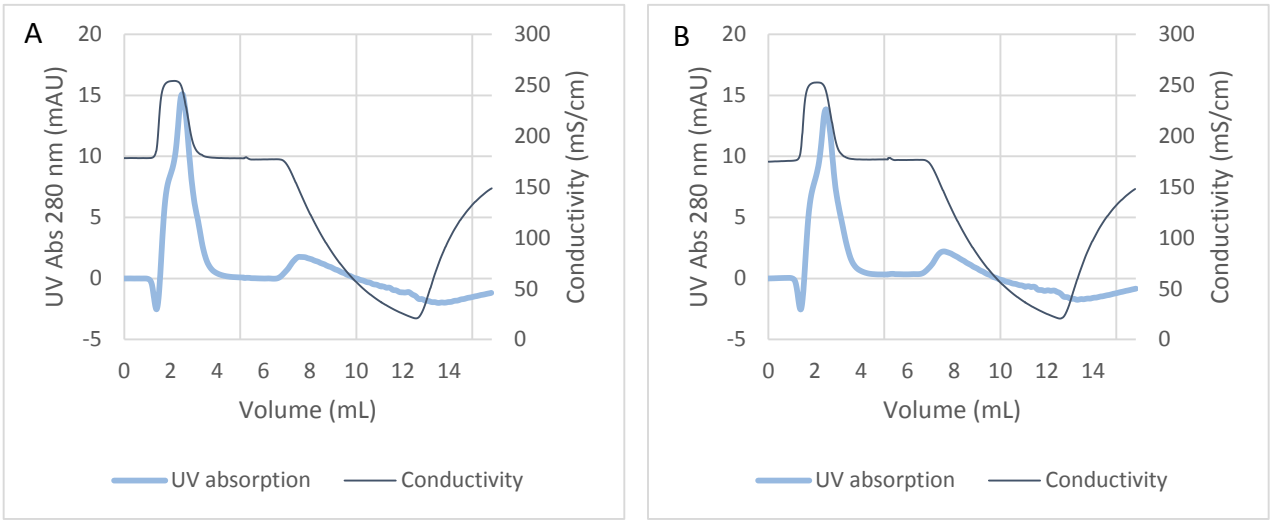

Figure 15- HIC runs with a HiTrap phenyl HP column for the purification of BTV from the ATPS bottom phases of a 25% PEG6000-15% Ammonium Sulphate (A-system 1) and a 15% PEG 6000-25% Ammonium Sulphate (B-system 2) using 1,5 M of ammonium sulphate with 10 mM of phosphate adsorption buffer and 10 mM phosphate buffer for elution.

The ionic strength of the adsorption buffer used in Figure 13 (1 M Ammonium sulphate) could not be sufficient to promote the binding of all the viral particles, and in a subsequent experiment, it was increased to 1.5 M. Once again, it is possible to verify an increase in the conductivity after sample injection, although not as pronounced as in Figure 13. There are two peaks in each chromatogram representing the proteins recovered in the flow-through and elution fractions. The first peak with a residence time of three column volumes (CV) corresponds to all unbound molecules. The second peak with a higher residence time, around eight CV containing the bound proteins. For

confirmation in which peak fraction BTV was eluted a SDS-PAGE was performed (Figure 16).

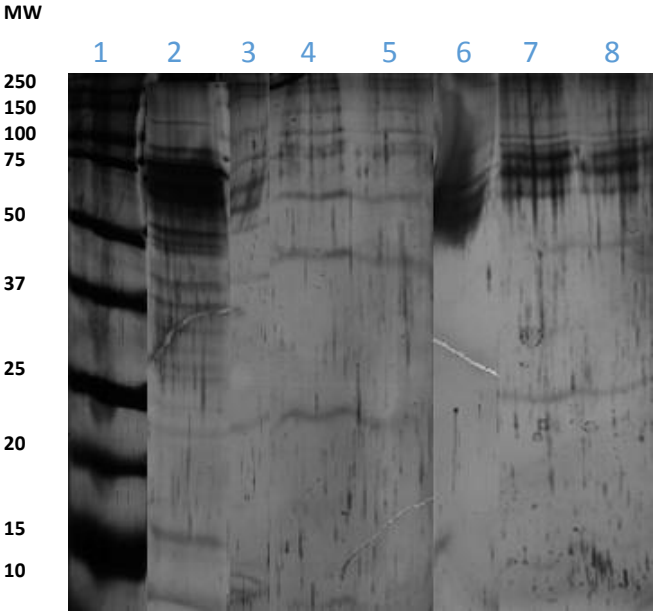

Figure 16- Silver stained reducing SDS-PAGE analysis of chromatography samples. Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 2: BTV virus after ultracentrifugation; Lane 3: Bottom phase from ATPS of 25% PEG6000-15% Ammonium Sulphate (system 1); Lane 4: Flow through from sample containing bottom 1; Lane 5: Elution peak from sample containing bottom 1; Lane 6: Bottom phase from ATPS of 15% PEG 6000-25% Ammonium Sulphate (Bottom 2). Lane 7: Flow through from sample containing bottom 2; Lane 8: Elution peak from sample containing bottom 2.

Once again, BTV is not binding to the column specifically, which can be seen by the comparison of band patterns corresponding to samples collected during the chromatography in the peaks of flow through and elution (lanes 4 and 5; and lanes 7 and 8, Figure 16).

As the concentration of a salt increases, the amount of protein bound will increase almost linearly up to a specific salt concentration. Thus, ammonium sulphate often gives best resolution when compared to other salts and can be used at concentrations up to 3 M. [44] Therefore, new experiments were performed for this concentration of salt (Figure 17).

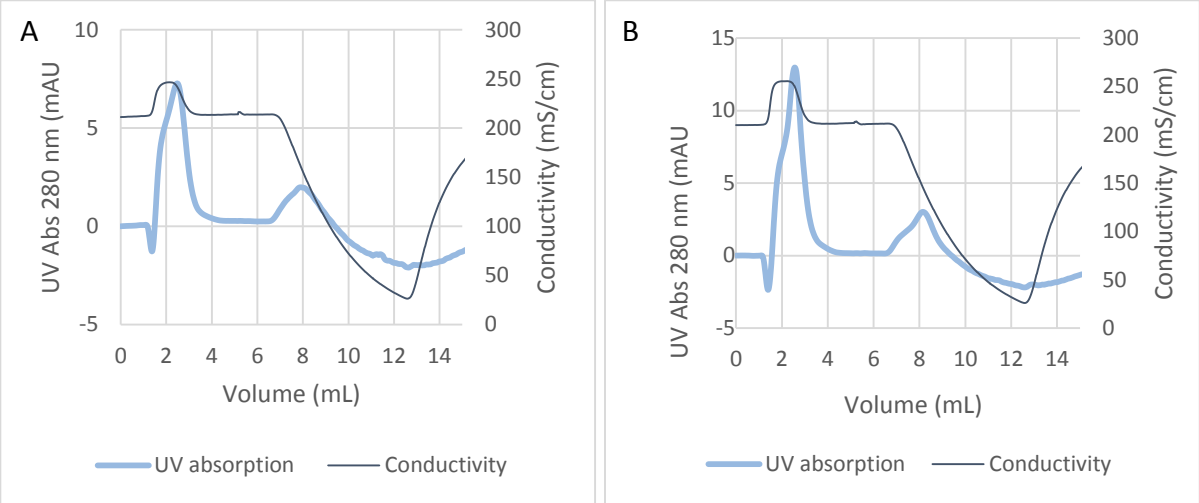

Figure 17- HIC runs with a HiTrap phenyl HP column for the purification of BTV from the ATPS bottom phases of a 25% PEG6000-15% Ammonium Sulphate (A-system 1) and a 15% PEG 6000-25% Ammonium Sulphate (B-system 2) using 2 M of ammonium sulphate with 10 mM of phosphate adsorption buffer and 10 mM phosphate buffer for elution.

Like in the previous chromatograms, in figure 17 there are two peaks for each sample injected and an increase in the conductivity during injection. In order, to verify the presence of the virus in the fractions of each peak, a SDS PAGE was performed (Figure 18).

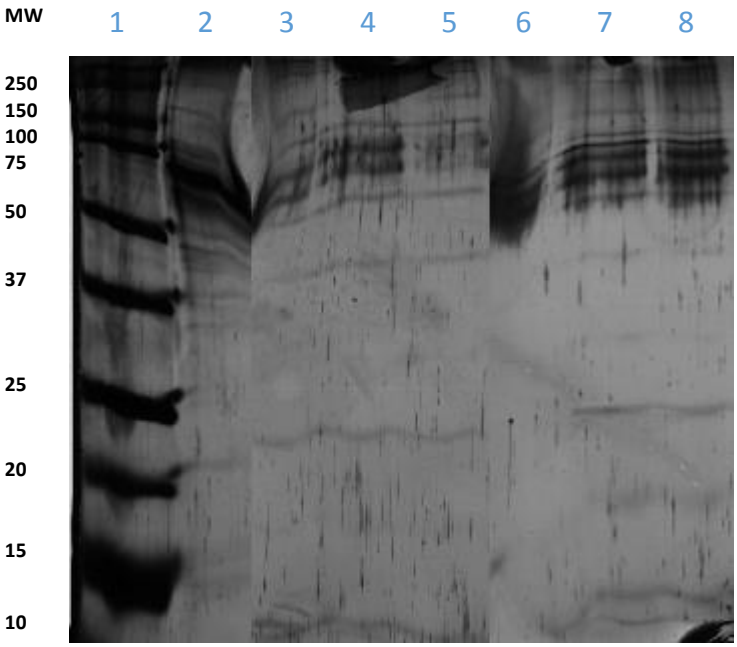

Figure 18- Silver stained reducing SDS-PAGE analysis of chromatography samples. Lane 1: Precision Plus Protein™ Dual Colour Standards, molecular weight (in kDa); Lane 2: BTV virus after ultracentrifugation; Lane 3: Bottom phase from ATPS of 25% PEG6000-15% Ammonium Sulphate (system 1); Lane 4: Flow through from sample containing bottom 1; Lane 5: Elution peak from sample containing bottom 1; Lane 6: Bottom phase from ATPS of 15% PEG 6000-25% Ammonium Sulphate (Bottom 2). Lane 7: Flow through from sample containing bottom 2; Lane 8: Elution peak from sample containing bottom 2.

The presence of the virus in both peaks has been confirmed in all SDS PAGE performed which means one of the following causes:

- The binding to the column does not specifically occurs because the support that is being used is not the most suitable for the purification or its conditions are not well function.
- It is known that with the increasing of ammonium sulphate concentrations, aggregates bound to the column can appear and, which might be happening with the viral particles when concentrations of 2 M are used.
- Flow through peaks longer than elution peaks are an evidence that failure of binding or non-specific binding are occurring, which leads to product losses along with impurities. However, additives can be used to improve virus purification, but of course, when used at high concentrations, the risk of inactivating or denaturing of the virus maintains. Additives can influence a separation by improving protein solubility, modifying protein conformation and promoting elution of bound proteins. [44]
- Other explanation, but very unlikely, is column saturation by higher amounts of virus particles.

Thus, in order to know if the problem was from the chosen support, we proceeded to further studies where two columns containing different ligands, butyl and octyl, were tested. The ammonium sulphate concentrations tested were 2M and 2.5M and this time, the injected samples did not undergo a pre-purification by ATPS, being only clarified by ultracentrifugation. The resulting pellet from ultracentrifugation was resuspended in adsorption buffer to be used in chromatography assays.

3.3.2 Feedstock from ultracentrifugation processed by HIC: effect of the ligand

The type of ligand and the nature of the target protein are highly significant parameters in determining the selectivity of a HIC medium. Consequently, to determine the most suitable ligand screening experiments were performed with phenyl, octyl and butyl ligands.

In general, HIC ligands fall into two groups, depending on their interactions with sample components. Straight alkyl chains (butyl, octyl, isopropyl) show a hydrophobic character, while aryl ligands (phenyl) show a mixed-mode behaviour, aromatic and hydrophobic interactions.[43] The binding capacity increases with increased ligand density up to a certain level, in the case of alkanes the hydrophobicity and strength of interaction increase with the n-alky chain length. A column with a phenyl ligand is typically an interesting starting point as it is the stronger of the ligands and therefore, should assure the stronger binding and consequently the highest recovery. [43]

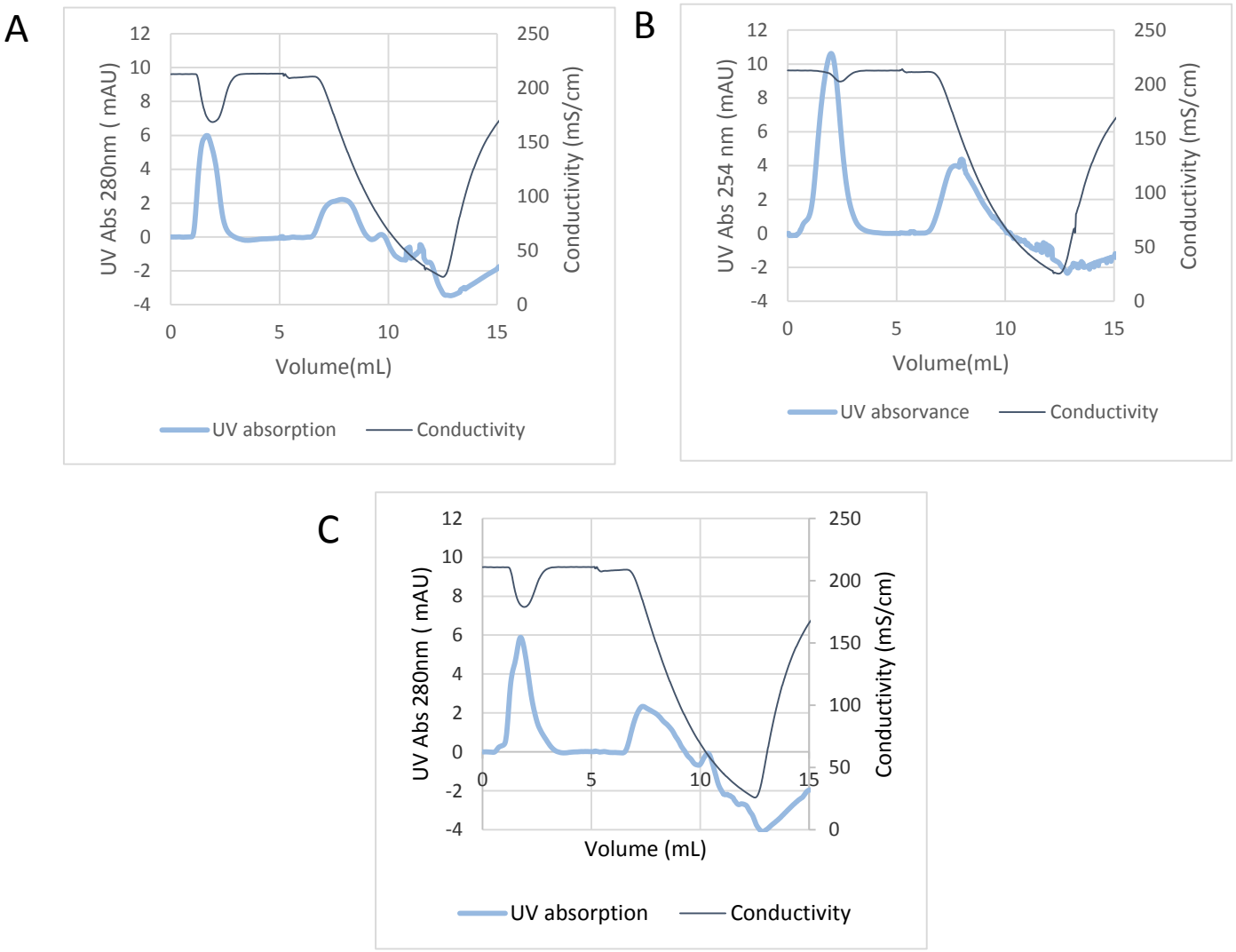

Figure 19- Chromatograms from experiments testing BTV purification by using (A) phenyl HP, (B) octyl FF and (C) butyl HP columns and 2 M of ammonium sulphate with 10mM of phosphate at pH 7 as adsorption buffer and 10mM of phosphate as elution buffer.

To test the effect of three ligands in purification of BTV, a sample with 1.5mL containing a feedstock from ultracentrifugation suspended in 2 M of ammonium sulphate was prepared to be used in HIC. After injection of the sample, a decrease in conductivity was observed, being less pronounced when octyl ligand was used (Figure 19). Thus, if the concentration of ammonium sulphate is too low the salting out of viral proteins will not occur preventing their binding to the hydrophobic ligands.

When the ten CV are reached, the absorbance becomes negative, probably because of refractive index effects, and therefore have not been analysed as eluting peaks. SDS PAGE was performed in order to verify which species are being eluted and in each fraction collected.

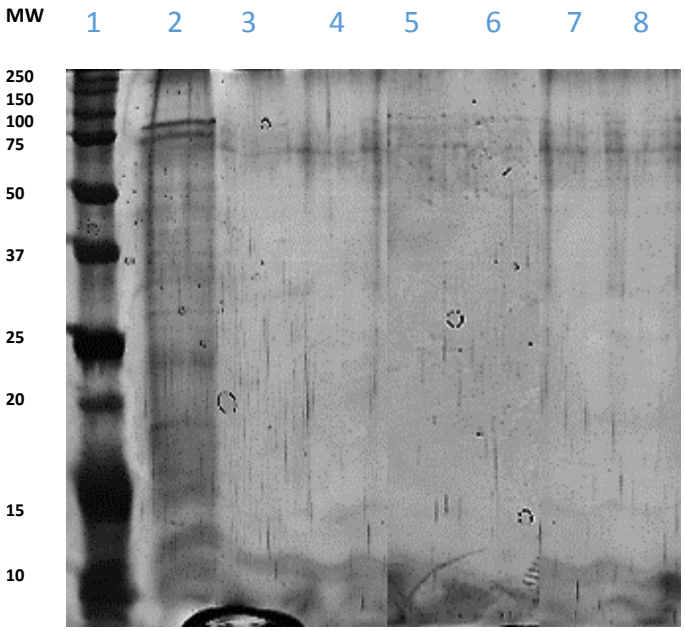

Figure 20- Silver stained reducing SDS-PAGE analysis of chromatography samples. Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 2: BTV virus after ultracentrifugation; Lane 3: Flow through of HIC experiments using octyl column; Lane 4: Elution peak of HIC experiments using octyl column; Lane 5: Flow through of HIC experiments using phenyl column; Lane 6: Elution peak of HIC experiments using phenyl column. Lane 7: Flow through of HIC experiments using butyl column; Lane 8: Elution peak of HIC experiments using butyl column.

The results presented in the gel of SDS-PAGE do not allow todraw many conclusions since the bands are not very clear. The band with molecular weight around the 75kDa is present and very concentrated in all lanes, which might be the protein BSA, one of the many impurities. Although they are unclear, other bands with molecular weight around 100kDa are present, which may be one of the viral proteins VP2, VP3 or

VP6. In the case of being the viral proteins, the problem maintains: BTV is being eluted in both peaks, flow through and elution peak, independent of the ligand that is used.

Experiments using the same column ligands were repeated but this time using 2.5M of ammonium sulphate with 10 mM of phosphate pH 7 as adsorption buffer. Once again, the feedstock from ultracentrifugation was diluted in the same concentration of salt as the adsorption buffer.

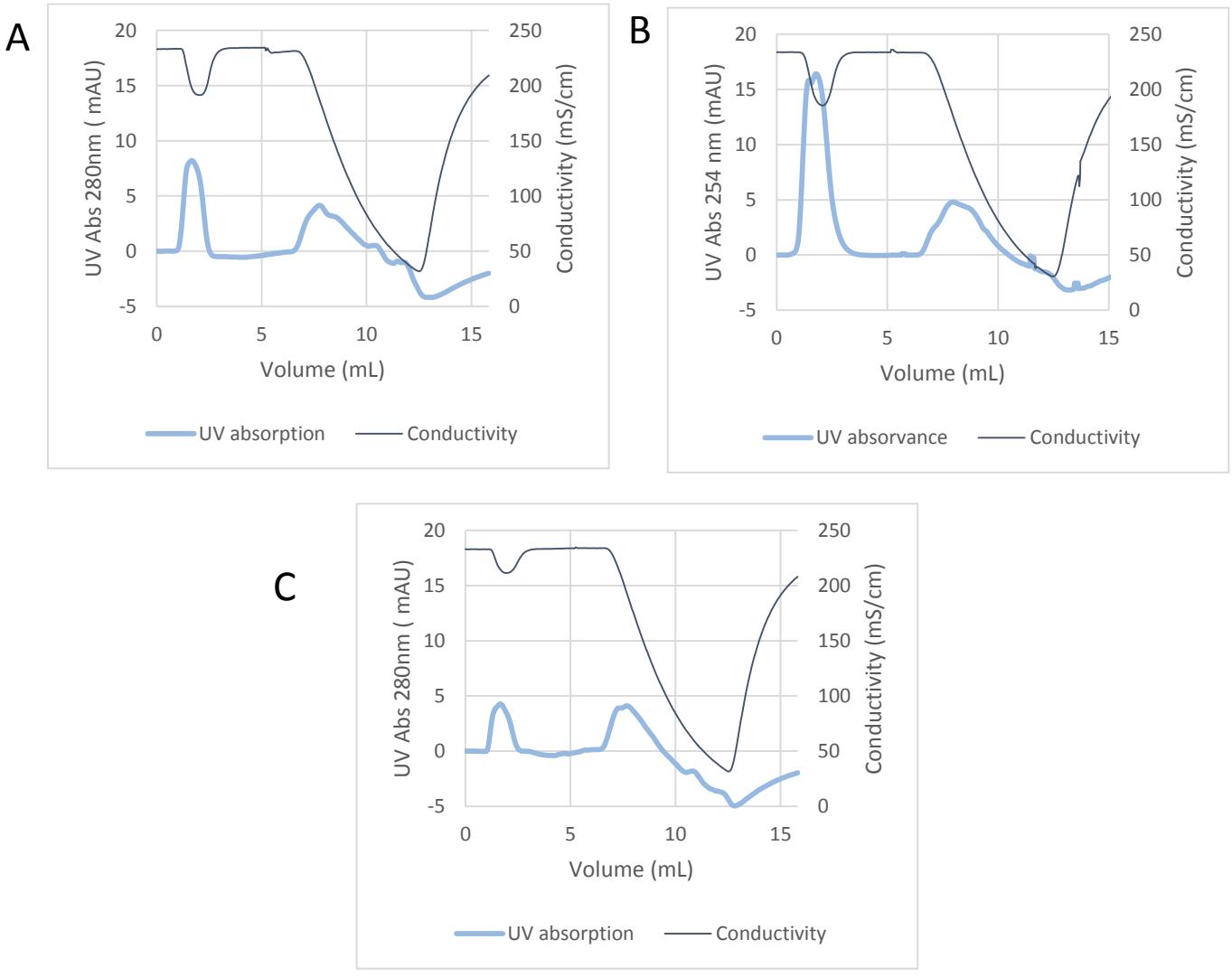

Figure 21- Chromatograms from experiments testing BTV purification by using (A) phenyl HP, (B) octyl FF and (C) butyl HP columns using 2,5 M of ammonium sulphate with 10 mM of phosphate as adsorption buffer and 10 mM phosphate as elution buffer.

By the observation of Figure 21, all the chromatogram show a decrease in conductivity as the sample is injected which means the samples are at a lower concentration of ammonium sulphate that the adsorption buffer. The decrease is lower for the experiment performed using column ligand butyl. Also the absorbance is very low in all chromatograms especially in this one. The low absorbance can be explained by the precipitation of the virus and therefore it was not injected or because the virus was irreversibly adsorbed on the column. Once again, samples were analysed by SDS PAGE (Figure 22).

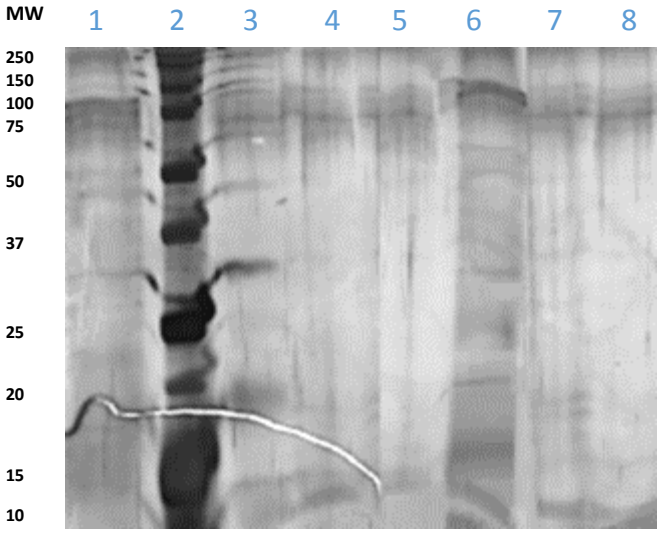

Figure 22- Silver stained reducing SDS-PAGE analysis of chromatography samples. Lane 1: BTV virus after ultracentrifugation; Lane 2: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 3: Flow through of HIC experiments using octyl column; Lane 4: Elution peak of HIC experiments using octyl column; Lane 5: Flow through of HIC experiments using phenyl column; Lane 6: Elution peak of HIC experiments using phenyl column. Lane 7: Flow through of HIC experiments using butyl column; Lane 8: Elution peak of HIC experiments using butyl column.

In the gel from SDS-PAGE (Figure 22), lane 3 might be contaminated with the sample from lane 2, molecular weight marker that makes these unreliable results. In the case of lane didn't being contaminated, lane 3 and lane 7, both flow through peaks from experiments with octyl and butyl, respectively, have showed a band pattern similar to lane 1, which works as a control, since the sample that is injected for HIC is the same from lane 1. So the objective is to see a similar pattern in samples collected during chromatography, but with less impurities, which will correspond too few less bands. In lane 6, corresponds to the fraction collected from elution peak in chromatography

experiment using phenyl column and seems to show similar bands with lane 1. This result, is very promising, since the objective is to purify and collect the virus in the elution peak without seeing its presence in flow through samples, which means that virus particles have bound to the column and were eluted with the decreasing of the ionic strength.

After the experiments done with phenyl ligand, the expectation for better results and successful purification was going to octyl and butyl ligands, since octyl is the second strongest ligand, right after phenyl; and butyl is the less hydrophobic of the available ligands and was developed to provide a medium that was less hydrophobic than the standard Phenyl to avoid problems in elution step when target protein is very hydrophobic and binds strongly to the column. Thus, the ligand phenyl has proved to be the most suitable for the purification of BTV when an adsorption buffer of 2.5 M of ammonium sulphate with 10 mM of phosphate is used at neutral pH.

3.4 Chromatography using monolithic supports

In CIM DEAE chromatography, the working conditions are different from HIC, namely the type and concentration of the salts in the buffers, pH and working flow rate. The purification of BTV by anion-exchange chromatography using a monolithic bed was first studied using a weak anion exchanger diethylaminoethyl (DEAE). The sample used in following experiments, was the pellet resultant from supernatant ultracentrifugation that was suspended in adsorption buffer, 10 mM of phosphate at different pH values.

The goal for this technique is to find the better conditions for BTV purification while preventing the binding of impurities to the monolith, by changing the work pH of the adsorption buffer.

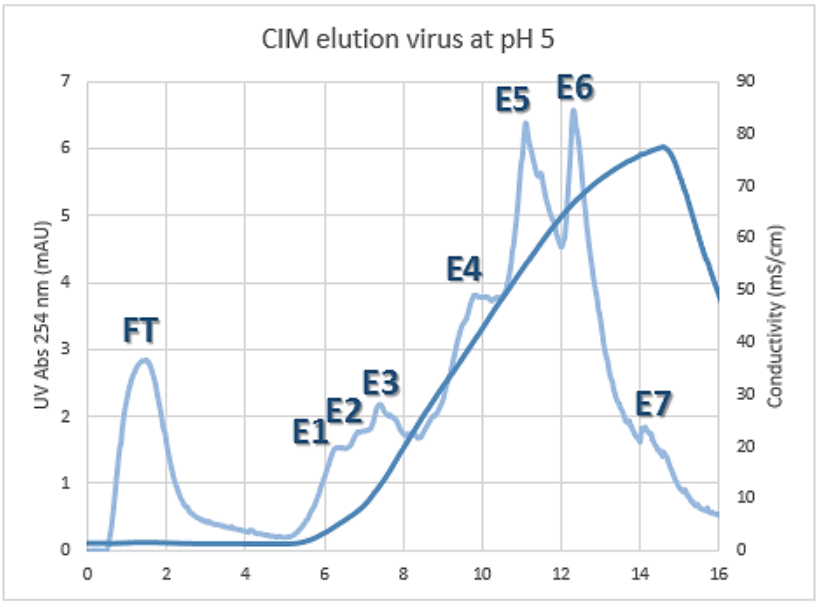

Figure 23-CIM DEAE® chromatography profile of BTV purification from clarified BHK-21 cell supernatant, using as adsorption buffer 10 mM NaH_2PO_4 at pH 5 and elution buffer 20 mM NaH_2PO_4 with 1 M NaCl at pH 7.

The first chromatography using monolithic supports was performed at pH 5. In this chromatogram is possible to identify eight peaks, the flow through (FT), where unbound species are washed out from the column, and seven elution peaks (E1, E2, E3, E4, E5, E6, E7) where species that have bound to the column are removed by changing the buffer for elution conditions unfavourable for ionic binding of the molecules. In this way, it is expected as a good result that the flow through (FT) peak should only have impurities whereas one of the elution peaks would have the BTV. In order to evaluate the virus presence in the previous peaks an SDS gel was performed which is shown in the Figure 24.

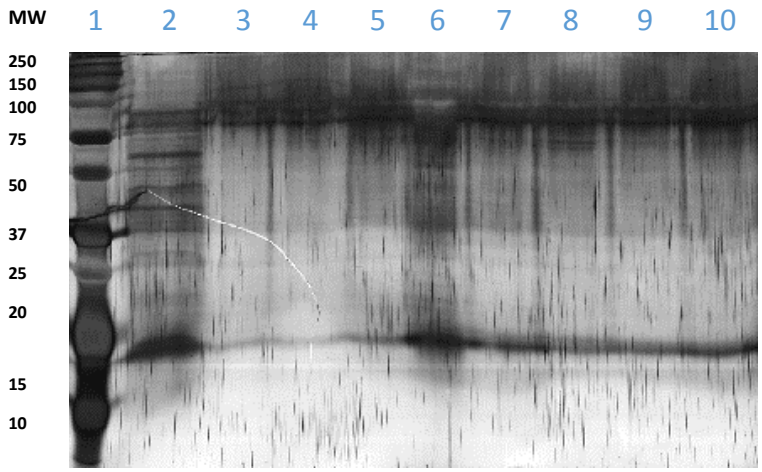

Figure 24- Silver stained reducing SDS-PAGE analysis of Monolithic chromatography samples. Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa; Lane 2: Initial sample for CIM DEAE: BTV ultracentrifuge; Lane 3: Flow through of CIM DEAE experiments; Lane 4: Elution peak E1 of CIM DEAE experiments at pH 5; Lane 5: Elution peak E2 of CIM DEAE experiments at pH 5; Lane 6: Elution peak E3 of CIM DEAE experiments at pH 5; Lane 7: Elution peak E4 of CIM DEAE experiments at pH 5; Lane 8: Elution peak E5 of CIM DEAE experiments at pH 5; Lane 9: Elution peak E6 of CIM DEAE experiments at pH 5; Lane 10: Elution peak E7 of CIM DEAE experiments at pH 5.

Figure 24 shows a possibility of BTV being eluted in the elution peak E3, by the presence of similar bands between lane 6 and lane 2. Although it is possible to observe the presence of several impurities. Another effect that is present in the gel, is the drag and smeared protein.

At pH 6, the chromatogram shows, five peaks well define (Figure 25), although the SDS PAGE analysis did not help in the evaluation of BTV purification because of the lack or the much smeared bands, the reason why the gel is not showed.

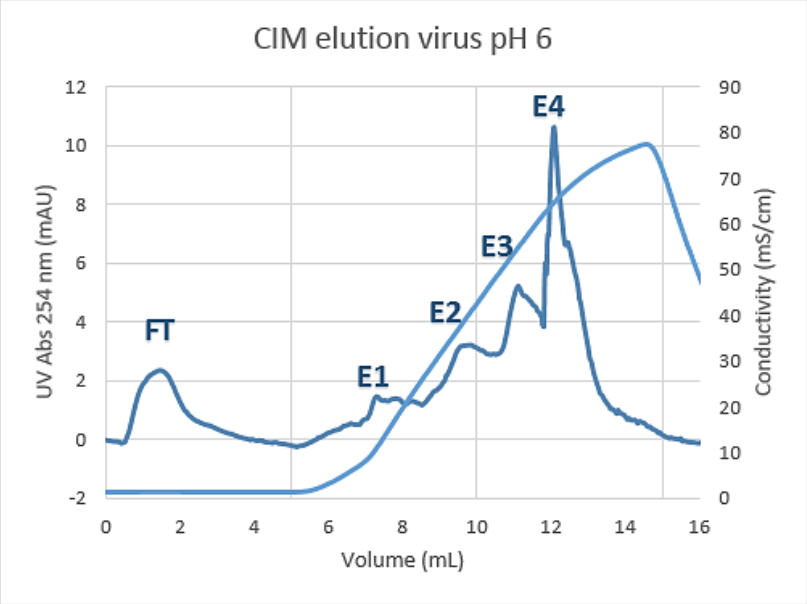

Figure 25 - CIM DEAE® chromatography profile of BTV purification from clarified BHK-21 cell supernatant, using as adsorption buffer 10 mM NaH_2PO_4 at pH 6 and elution buffer 20 mM NaH_2PO_4 with 1 M NaCl at pH 7.

The chromatography of BTV at pH 7 revealed five peaks with higher absorbance than the previous chromatograms (Figure 23 and 25). The purity of the samples collected and analysed by SDS-PAGE indicated the presence of BTV in peak E3, even though the bands are a little blurred.

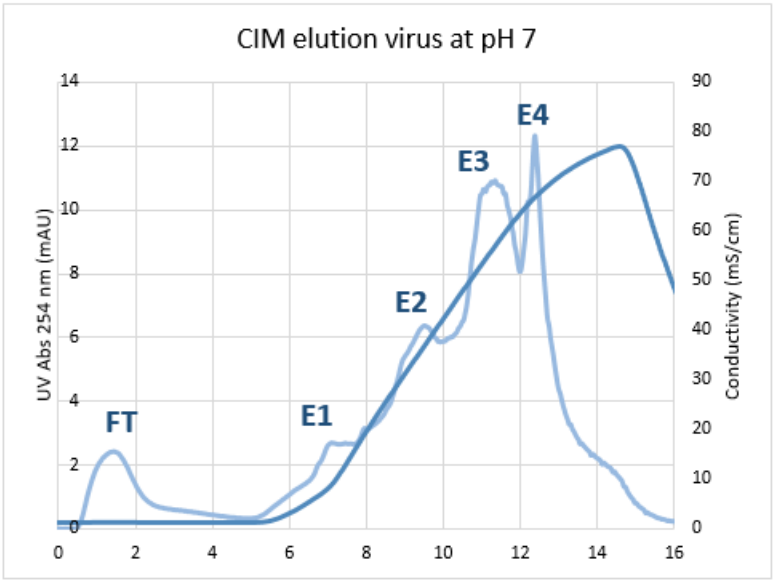

Figure 26- CIM DEAE® chromatography profile of BTV purification from clarified BHK-21 cell supernatant, using as adsorption buffer 10 mM NaH_2PO_4 at pH 7 and elution buffer 20 mM NaH_2PO_4 with 1 M NaCl at pH 7.

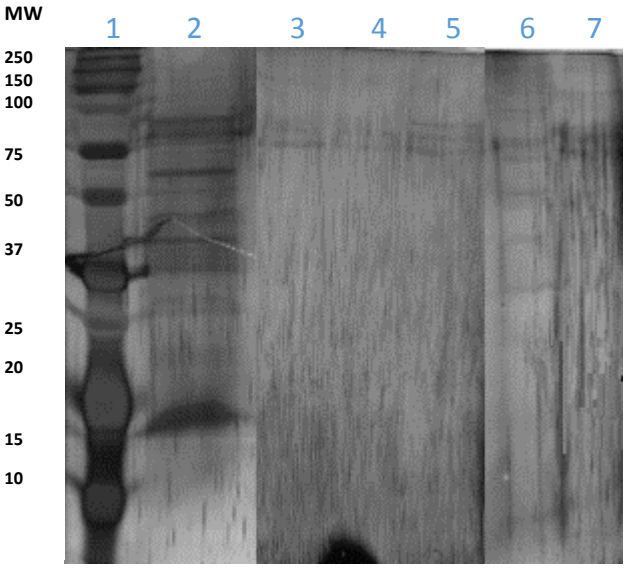

Figure 27- Silver stained reducing SDS-PAGE analysis of Monolithic chromatography samples. Lane 1: Precision Plus Protein™ Dual Color Standards, molecular weight (in kDa); Lane 2: Initial sample for CIM DEAE: BTV ultracentrifuge; Lane 3: Flow through of CIM DEAE experiments at pH 7; Lane 4: Elution peak E1 of CIM DEAE experiments at pH 7; Lane 5: Elution peak E2 of CIM DEAE experiments at pH 7; Lane 6: Elution peak E3 of CIM DEAE experiments at pH 7; Lane 7: Elution peak E4 of CIM DEAE experiments at pH 7.

Therefore, from the three ion exchange experiments performed, the one that showed better results was the one performed at neutral pH for presenting a better resolution, which means the separation between the peaks eluted from the column was better performed with less peaks that means less impurities and which come to be

proved by SDS PAGE gel (Figure 27). Nevertheless, the absorbance in all chromatograms is very low.

3.5 Results of virus titration

For a more reliable verification of the presence of virus in the fractions collected from the chromatography, in the samples of each phase of the ATPS and the feedstocks from ultracentrifugation and from medium culture, were all subjected to a cytopathic test. Virus titration allowed determining the efficiency of concentration and purification process by observing the cytopathic effect of the virus in cells. Therefore, if BTV is present in the samples collected it should be observed by optical microscope structural changes in BHK-21 cells.

$TCID_{50}$ assays have some problems regarding the presence of some solvents that can have negative impact upon BHK-21 cells since cells had presented a different aspect namely, cells shrinking, that may be confused with cytopathic effect provoked by virus presence. Therefore, some results were considered suspicious due to the presence of high concentrations of salt that may harm the cells and induce a similar cytopathic effect in the cells even if the virus is not present.

The results for flow-through and eluted fractions from HIC chromatography were analysed by $TCID_{50}$ experiments and it was possible to acknowledge that BTV was mainly present in almost all the flow-through and elution fractions of both chromatographies, indicating that it did not interact specifically with the ligands probably due to the high ionic strength of the adsorption buffers or the lack of affinity. However, in CIM DEAE chromatography at neutral pH confirmed the presence of BTV in elution peak E3 fraction, through cytopathic effect visible in the cells.

For samples from ATPS only samples from bottom phases of system 1 and 2 show cytopathic effect, which proves that BTV is partitioning to the salt phase, while system 3 show the presence of cytopathic effect in both phases. Feedstock obtain from ultracentrifugation was also analysed and showed cytopathic effect in the cells as also the supernatant from culture medium.

Several $TCID_{50}$ assays were performed and sometimes there were some discrepancies for similar samples. A possible reason for these discrepancies is the fact of this test being a biological assay which depend on many factors: the age of the BHK-21 culture, number of subcultures, the time between the collection of the samples and day of $TCID_{50}$ assays were performed, the presence of cell debris which interferes during the infection, small variations in time of contact during the infection.

4. Conclusions and Future Perspectives

The aim of this work is to find the most suitable method for clarification/concentration and purification of the BlueTongue Virus. The intention of clarification/concentration is to remove the maximum amount of impurities or solid matter and concentrate the virus in the less volume of solution possible to be used in purification experiments. There are many challenges in the downstream processing for virus purification due to the complexity of the molecules, cost and time consumption of the standard unit operations. Therefore, biotech companies have been developing novel and appealing techniques to produce and recover these bioproducts. For a successful purification of virus particles with a high removal of contaminants, it is necessary to combine different unit operations, in order to maintain the virus infectivity and high purification yields.

In the present work, PEG precipitation, ultra centrifugal filtration using Amicons® and ultracentrifugation were analysed as possible methods to concentrate and separate BTV from the supernatant of culture medium. For the purification of BTV, tests were made for the following techniques: aqueous two-phase system, monolithic chromatography and hydrophobic chromatography.

From the three clarification methods used, the feedstocks from PEG precipitation and ultracentrifugation were the ones that showed the best results, with bands that were more concentrated and with fewer impurities present. Thus, the aqueous two-phase systems were performed using samples from these two feedstocks. The ultra centrifugal filtration using Amicons® proved to be a less efficient method than the others. PEG precipitation was the method that concentrated more the proteins although, during the precipitation step, not only BTV was precipitated but also some other proteins present in the medium. In addition, ultracentrifugation as a clarification method proved also to be a good option for further processing through ATPS and chromatography as it appears to present fewer impurities and a better separation of proteins in the SDS-PAGE, being this BTV feedstock chosen for ATPS and Chromatography experiments. The SDS-PAGE gel present in Figure 11 showed that, in the three methods, the resulting viruses were not totally free of host contaminants since

the conditions, which favour the recovery of BTV, can also favour the recovery of impurity proteins.

Aqueous two-phase systems can be effectively used for the separation and purification of proteins, by using PEG and salts systems. In this work, three PEG/ammonium sulphate systems were studied and the results reported that BTV partitions to the bottom phase when the (% w/w) concentration of PEG is not too low (more than 10%). The systems tested with conditions 1 (25% (w/w) PEG_{6000}/15% (w/w) ammonium sulphate) and 2 (15% (w/w) PEG_{6000}/25% (w/w) ammonium sulphate) seem to show the appropriate conditions for the purification of the virus, even though, impurities presente in the culture medium partitioned to the same phase as the virus, which is a problem to be solved with the goal of preserving the virus infectivity and to obtain the pure virus. Other problems are the presence of the same molecular weight of bands, corresponding to the viral proteins and impurities like proteins present in the culture medium that were required for optimal growth of cells, as well as smeared protein gels due to the presence of salts and polymers. Despite the ATPS advantages of being a scalable method, with low cost chemicals in the case of PEG/salt systems and shorter processing time, this method can only be suitable as a partial purification. For further work, new protocols must be set in order to test new components and new concentrations so that the virus and its impurities partition to different phases.

In addition, in order to confirm the proteins authenticity, a Western immunoblotting could be performed using anti-BTV4 serotype antisera, which unfortunately, even though it was performed, the results obtained were not shown in this work because they were not conclusive.

Regarding chromatography, two different techniques were used based on different interactions between the mobile and the stationary phase. In HIC chromatography different ligands were also tested with different concentrations of salt in the adsorption buffer. From the results testing phenyl, butyl and octyl ligands the best result was achieved with the phenyl ligand at 2,5 M of ammonium sulphate for the efficient adsorption of BTV to the column. Regarding the results obtained with the other columns and the other salt concentrations it was verified an unspecific binding of BTV

to the columns, being possible to visualize in SDS-PAGE analyses the presence of the virus both in the flow through and elution fractions.

In ion exchange chromatography, three working pHs were tested, being the neutral pH the one that presented the best results for BTV purification, since it was able to separate BTV from the other proteins contained in the initial feedstock.

CIM chromatography seemed to be more efficient for BTV purification since its affinity to it was higher when compared with HIC chromatography and the objective is to isolate the virus and purify it in only one elution peak with the less impurities possible in the fraction collected. In conclusion, in both chromatography experiments, neutral pH is suitable for the purification of the virus. In future, other cation exchangers could be used for CIM, to test the efficiency of the binding by using weak or strong exchangers. Moreover, the use of other alternative buffers that might lead to differences regarding virus stability and a decrease in the overall costs.

Anion chromatographic exchange represents an alternative to the conventional methods for BTV concentration and purification, even though other types of chromatography can be studied as an alternative separation technique, like affinity chromatography.

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