Design of a Scalable Strategy for the Initial Recovery and Capture of Bacteriophages: Nuclease Digestion and Phenyl Boronate Chromatography

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Preface

The work presented in this thesis was performed at Institute for Bioengineering and Biosciences (iBB) of Instituto Superior Técnico, Universidade de Lisboa (Lisbon, Portugal) in the BioEngineering Research Group (BERG), during the period January-July 2021, under the supervision of Prof. Ana Azevedo (BERG-iBB).

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Resumo

As bactérias resistentes a antibióticos são uma preocupação para a saúde, dado não possuírem tratamentos eficazes. Os bacteriófagos poderão ser uma solução para este problema. Estes são vírus que infectam bactérias, impedindo a sua replicação e diminuindo a concentração pela indução à lise celular. Terapias com base de fagos requerem uma suspensão altamente purificada, o que não é alcançado pela infeção natural. A purificação atualmente utilizada, precipitação com polietilenoglicol seguida por ultracentrifugação em gradiente de CsCl, é inadequada para a purificação de grandes quantidades de fagos.

Esta dissertação teve como objetivo remover proteínas e DNA bacterianos (*E. coli*) de uma suspensão de fagos T4 sem diminuição da infectividade. As técnicas estudadas foram a digestão com Denarase® e a Cromatografia de Fenil Boronato. Na digestão foram testados quatro tempos de incubação (30 minutos, 1 hora, 1 hora e 30 minutos e 2 horas), paragens de atividade enzimática (72 ºC e gelo) e comportamento na presença/ausência do cofator enzimático. A digestão ao lisado apresenta os melhores resultados, com rendimento (69,33%) e remoção de DNA bacteriano (99,96%), para os 30 minutos de incubação, sendo a remoção de proteínas bacterianas, 95,91%, superior para 1 hora e 30 minutos de incubação. Diferentes condições de adsorção e eluição foram testadas na cromatografia, sendo os melhores resultados (49,49% de rendimento, 96,61% e 47,60% remoção de proteína e DNA, respectivamente) obtidos usando 15 mM Tris-HCl, pH 7 como adsorção, e 1,5 M Tris-HCl, pH 8,5 como eluição.

Palavras-Chave: Fagos, Cromatografia de Fenil Boronato, Denarase®, Proteínas, DNA

Abstract

Antibiotic-resistant bacteria are a health concern as they do not have effective treatments. Bacteriophages could be the answer to these concerns. These infect bacteria, preventing replication and decreasing concentration by inducing cell lysis. Phage research and therapy requires a highly purified suspension, which is not achieved by natural infection. The currently used purification, polyethylene glycol precipitation followed by CsCl gradient ultracentrifugation is inadequate for purification of large amounts of phage.

This master's thesis aimed to remove bacterial proteins and DNA (*E. coli*) from a suspension of T4 phages without decreasing infectivity. The studied techniques were the digestion with Denarase® and the Phenyl Boronate Chromatography. In the digestion, four incubation times (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours), enzymatic activity stopping methods(72 ºC and ice) and behavior in the presence/absence of the enzymatic cofactor were tested. The lysate digestion presents the best results, with yield (69.33%) and bacterial DNA removal (99.96%) for the 30 minutes of incubation, being the removal of bacterial proteins, 95.91%, higher for the 1 hour and 30 minutes incubation. Different adsorption and elution conditions were tested in chromatography, with the best results (49.49% yield, 96.61% and 47.60% removal of protein and DNA, respectively) obtained using 15 mM Tris-HCl, pH 7 as adsorption, and 1.5 M Tris-HCl, pH 8.5 as elution.

Key-Words: Phages, Phenyl Boronate Chromatography, Denarase®, Protein, DNA

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Abbreviations

DNA - Deoxyribonucleic acid

EPS - Exopolysaccharide

PDE - Polysaccharide Depolymerase Enzymes

LPS - Lipopolysaccharides

KDO - 3-deoxy-D-manno-2-octulosonic acid

ICTV - International Committee on Taxonomy of Viruses

E. coli - Escherichia coli

TSB - Tryptic Soy Broth

TSA - Tryptic Soy Agar

OD_{600} - Optical Density measured at 600 nm

MOI - Multiplicity of Infection

USA - United States of America

PBS - Phosphate Buffered Saline

CFU - Colony Forming Units

PFU - Plaque Forming Units

PB - Phenyl Boronate

NMWC - Nominal Molecular Weight Cutoff

BCA - Bicinchoninic acid

BSA - Albumin Standard

USA - United States of America

TEMED - N, N, N', N' - Tetramethyl-ethylenediamine

v/v - volume/volume

APS - ammonium persulfate

SDS-Page - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

MOI - Multiplicity of Infection

Introduction

Phage Particles

Bacteriophages or simply phages are viruses that infect bacteria and archaea, replicating within them. These organisms have an important role both in molecular biology and in bacterial genetics, as phage can confer phenotypes to their host, transforming into a pathogenic strain, one previously non-pathogenic strain, and regulate bacterial population.[1] An interesting fact is that phage-bacteria relationships can vary a lot, meaning that while some relationships have the simple model predator-prey, others, more complex, can show a symbiotic relationship between them, promoting the survival and evolutionary success of both.[1] A single phage particle can be specific for just one bacterium species or for a subset of the same species. It is noted that phages are also the most diverse and abundant organisms found in the biosphere and are thought to exist in every ecosystem, from the extremely hot environments to the extremely cold ones.[1]

Brief History of Discovery and Research

The first phage identification occurred in 1896 by Ernest Hankin who noticed something taking action against cholera in the waters of Indian rivers. In 1915, William Twort found a small agent that infected and killed bacteria.[2] He believed that the agent could be a stage in the life cycle of the bacteria, an enzyme produced by the bacteria themselves, or a virus that destroyed the bacteria.[2] However, due to World War I, only in 1917 was this organism's potential to kill bacteria as a virus proposed by Felix d'Herelle, who named it bacteriophage.[3] Still, after their peak in the pre-antibiotic era, bacteriophages were dismissed as important therapeutic agents in the West, mainly due to Alexander Fleming's discovery of penicillin in 1928, which widespread the use of antibiotics in the 1940s that were easier to be administered.[3] Yet, in some countries such as Georgia, the research and the use of these organisms to treat various diseases was and still is done.[3]

The misuses of antibiotic drugs have resulted in antibiotic-resistant bacteria and several attempts to turn this problem around were made by the discovery and development of novel antibiotics. However, in the last few years, antibiotic discovery has slowed, increasing the rates of antibiotic resistance, and compelling scientists to find new ways to control bacteria growth.[4]

Research in bacteriophages was, primarily, focusing on a model of phages that infected *Echerichia coli*, providing the base for modern molecular biology. Some of the impacts that this research has made are the use of phages to identify the basis of genetic material, the 3 nucleotides code for an amino acid, or even the identification of restriction enzymes.[5] However, for years, only a small percentage of phages was studied with detail, resulting in an irregular knowledge of phage biology. The growing perception of the number of phages in bacteria environments and the scientists

observations on the dictating of phages in many aspects of Bacterial/Archaeal biology, have reinvigorated interest in bacteriophages studies and applications.[5]

Phage Morphology and Structure

Phages can show diverse and complicated structures (Figure 1). The tailed phages have two main features in their structure: a capsid, where the genetic information is maintained, and a tail which can vary its size according to the different bacteriophages.[6] The capsid head is attached to the tail by the collar.[6]

The tail is divided in the collar, which helps to attach capsid head to the tail and the sheath which, when contracted, drives the tail tube through the outer membrane of the host cell (creating a channel for genome delivery).[6] At the end of the sheath there is another structure, the baseplate. This structure attaches the long tail fibers (needed in the attachment to the host cell) to the phage particle during the virus assembly. It is also this structure that initiates infection when the tail fibers bind to a host cell.[6] The last part of the phage structure is the spikes (pins, teeth or short tail fibers) that are responsible for creating an opening in the host cell surface to enter the phage genome.[6]

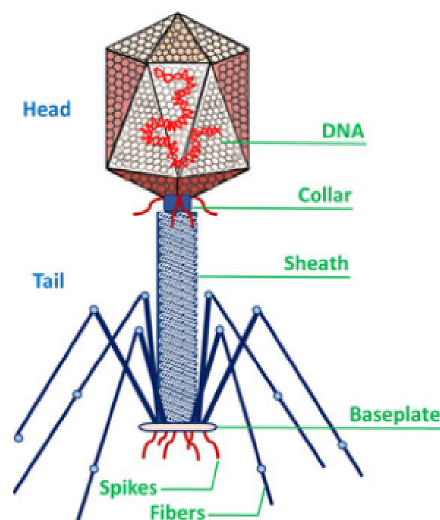

Figure 1. Schematic representation of a bacteriophage structure. From the top to bottom: capsid head evolving the nucleic acid (DNA or RNA), tail divided in the collar and the sheath, baseplate, spikes and tail fibers. 3D phage structure.[7]

Due to all the differences that phages can show within themselves, their classification is based on some specific characteristics: morphology, type of nucleic acid, replication mode, host organism and type of disease.[8] In the morphology classification, bacteriophages can be divided into filamentous phages, phages with a lipid-containing envelope and phages with lipids in the particle shell. For the type of nucleic acid, phage's genome can be either DNA or RNA, single or double stranded, and include information about proteins responsible for constituting the particles (switch cell molecular metabolism in favour of viruses).[8] Phages genome can, also, be found in only one fragment, or various ones (multipartite), but in either way, the genome is found inside the capsid, which can have

different, simpler or more complex, organisations according to the size of the genome.[8] Phages can also have different replication modes according to their life cycles, which can be lytic or lysogenic. According to the host organism, phages can be divided into three types: phages that infect archaea, phages that infect bacteria and phages that infect both archaea and bacteria.[8]

It is important to note that phage's length can vary widely, being, usually, in the range of 24 to 200 nm. One of the largest known phages is the T4 phage, used in this study, which is approximately 200 nm in length and 80 to 100 nm wide.[8]

Phage Taxonomy

With the enormous amount of different phages known, there is the need to decipher taxonomic characterization for all. This can be a challenging task for the nano-sized phage particles. The first phage classification occured in 1967 by Bradley, where 111 phages were classified and listed, getting approval from the International Committee on Taxonomy of Viruses (ICTV).[9] Bradley's classification, however, only projected taxonomy for tailed phages, dividing them into six basic morphological types (morphotypes). Due to this and to the discovery of new phages, the phage taxonomy has had various alterations and suggestions.[9] For example, one of the most recent suggestions was to replace the word "phage" in prokaryotic virus taxon names with the word "virus" in order to avoid confusion related to phage action on specific bacterial hosts.[9] Let's note that the majority of phages are tailed and carry dsDNA, approximately 96%, yet, they can still vary in shape, being this cubic, filamentous or pleomorphic. The genomic relationship was, also, one of the polyphasic taxonomy revised and emphasised.[9]

Phage Life Cycles

In order to understand phage interactions with their hosts is necessary, to take a look at this organisms life cycles, as this, together with the interaction phages have with the physical environment, dictate their role in bacterial/archaic biology.[10]

Phages have different life cycles, the lytic cycle and the lysogenic cycle (Figure 2). In the lytic cycle, phages, named virulent phages, infect their host by attaching themselves to a specific receptor found on the host surface, such as lipopolysaccharides, teichoic acids, proteins or flagella[10] (Figure 3). After the recognition of the receptor, the absorption is made, allowing for the phage penetration and DNA injection. In its turn, the host cell will provide all the needs, molecular building blocks and enzymes, for the phage to replicate its genetic material and produce progeny phage.[6] Due to phage-encoded proteins, like endolysin and holin, the host cells will suffer lyses from the inside. Holin proteins are small and pile up at the host membrane, which allows endolysin to digest peptidoglycan.[6] Note that the host cell's death is fast, which shapes their population dynamics as the lytic phage can infect and destroy all neighboring cells.[5] It is important to note that the bacteria type, Gram-negative or

Gram-positive, its growth conditions and virulence can influence the phage attachment to the host surface.[10]

In contrast, in the lysogenic cycle, phages, named temperate phages, may not immediately lyse their host cells, but, instead, integrate the host genome by inserting their own genome into the host chromosome at specific sites.[6] Yet, they can also just exist as a plasmid within the host cell. Here, there are two new concepts to understand, the prophage and the lysogen.[6] The prophage corresponds to the phage DNA inserted in the host genome and the lysogen to the host cell containing the prophage.[6] In this cycle, a stable relationship is established as the prophage is replicated together with the host genome. This cycle can be stable indefinitely, only suffering modifications if the host cells are under stress or adverse conditions, such as antibiotic treatment, oxidative stress or DNA damage.[6] When this happens, prophage is normally induced by the host's SOS responses and the lysogenic cycle may stop, which, due to the pursuit of the expression of phage DNA, may start the lytic cycle.[6]

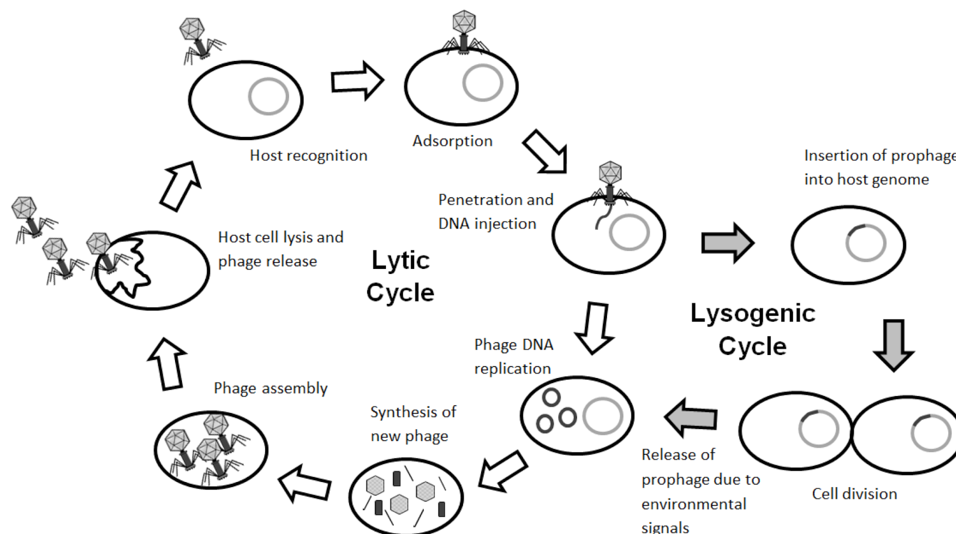

Figure 2. Phages two life cycles: lytic (on the left) and lysogenic cycle (on the right). Both cycles pass through the phage DNA replication step, however, in the lysogenic cycle the phage genome is incorporated into the host genome and suffers cell division first. In the lytic cycle, the host is lysed and the phages are released to the environment.[6]

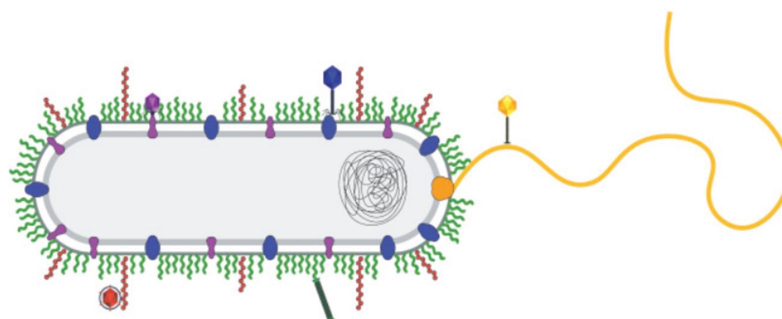

Figure 3. Schematic example of bacterial receptors in the cell surface available for phage binding. As shown, phages are attached to their specific receptor, lipopolysaccharides, teichoic acids, proteins or flagella.[4]

In the lytic cycle, the large number of progeny produced is an advantage that can be used in phage therapy or investigation work.[6] However, lytic phage normally infect specific host species due to the host's receptor. The lysogenic cycle, on the other hand, is a cycle that may be stable for generations, but it's biggest disadvantage is that some of the phage populations that insert their genome can lay dormant or alter the phenotype of the host by expressing genes not usually observed in the usual infection process.[6] Even so, both cycles can be interconnected, as the phage may rely on small molecules named "arbitrium" to communicate and make lysis–lysogeny decisions.[6] This means that when a large number of host cells are available to infect, the lytic cycle is activated, however, with the decrease of the host numbers, the lysogenic cycle is prefered by the phage.[6]

Applications

After the assembling of phage's potential, these organisms have been studied and used in different areas such as food industry, agriculture and aquaculture industry, wastewater treatments, biotechnology or even pollution remediation.[11] However, one of the most studied and important applications is in health problems, mainly in the fight against multidrug-resistant bacteria. Thus, the use of phages as therapeutic agents, phage therapy, allows an alternative to antibiotic-based strategies.[11]

Food Industry

Phages are an antimicrobial alternative that can prevent pathogenic bacterial contaminations at the commercial food chain. Food bacterial contamination is one of the major complications of the food industry and health care system, especially with the cause of foodborne illness and death.[12] It also brings high economical problems. Although the hygienic conditions, particularly in the western world, have improved, and the intensive use of antibiotics helped for a period of time, the emergence of bacterial resistance is a reason for the foodborne disease outbreaks not decreasing.[12] Being phages highly specific for their bacterial targets, and only being able to replicate if the host is available and in its surrounding, they are considered a useful resource in the food industry. A cocktail of phages is recommended in order to limit the phage resistance and to improve phage efficacy.[13] However, even though phages can be used in the food industry especially for food safety, their use can also be a threat to food processing.[13]

Due to reutilization of bacterial cultures in successive batches under non-sterile conditions, the fermentation failures may be related with the use of phages at industrial scale. In order to inactivate microorganisms, for example in raw milk, heat is often used. However, the thermal stability of many dairy phages, which can survive pasteurization, reduces the level of phages in whey which can be challenging.[13] [14]

Agriculture

Agriculture is an area closely related to climatic conditions, often adverse, natural disasters and even product diseases. With plant conditions came a lot of different parasites and pathogens, especially bacterial plant pathogens, also known as phytopathogens.[15] Plant pathogens are responsible for huge losses in cultivated and stored crops, being one of the biggest impediments to effective food distribution. Note that these losses are bigger in countries where the lack of food is more noticeable, that is, in developing countries.[15] However, also in cattle it is possible to observe the impact of phytopathogens, since the animal's diet is based, essentially, on plants. Among the various pathogens of plants, viruses, fungi and bacteria, almost 200 different species of bacteria are known, being these microorganisms common in humid and hot environments and easily spread by rain, wind, animals and even humans as they can adapt to various environmental conditions.[15]

Nevertheless, these bacterial pathogens, as many other bacteria in different areas, have started to show some antibiotics resistance, being the first report on phytopathogens resistance published in 1954. The use of chemicals was then approved as an alternative to antibiotics, but several concerns about pesticide toxicity soon arose.[16] Thus, and due not only to bacterial resistance, but also to chemical contamination of soils, there was a need to look for new ways to combat these pathogens. Therefore, phages have been studied and considered a natural and possibly safer alternative to bacterial plant diseases, being that one of its greatest advantages is its propagation followed by cell lysis.[17]

Aquaculture Industry

More recently, mainly in the last three decades, the aquaculture industry has shown considerable growth as fish-based products are gaining relevance especially as a cheap source of protein.[9] However, this is an industry where economical losses are seen mostly due to uncontrolled microbial diseases, threatening the industry development and sustainability.[9] It is easy to note the increasing contamination of fish, crustaceans and water related with bacteria causing human diseases due to the consumption of contaminated aquaculture products.[18] Although this type of bacteria can cause human diseases, they are also fatal for the animals in fisheries, marine, estuarine and even freshwater. An example is vibriosis, a common disease, capable of up to 100% mortality in fish larvae and significant mortality in fish.[18]

A solution for this problem was, and has been antibiotic treatments, however, these treatments have shown a reduction in its efficiency mainly due to bacteria resistance. In some cases of infection, as is the one caused by *Vibrio anguillarum*, phage treatment has proven its efficiency.[19] Even though phage treatments can show success to the bacterial problem, aquaculture phage therapy is dependent on two main factors. The first one is the number of phages that each host cell is able to produce before its lysis and the second one is the latency time between a new infection in a new host by phage particles. What is wanted in an aquatic condition is for phage therapy to have an abundant number of phages and a short latency period, however, this is still a challenge for future work.[9]

Wastewater Treatments

In aquatic environments bacteriophages can be highly abundant, with a range that can go from 10^4 to 10^8 per mL, thus phage numbers can be 3 to 10 times higher than the bacterial ones. When concentrating viral particles, the bacterial population shows a tendency to decrease by 20-40%. This relationship with the bacterial count and activity demonstrates that most aquatic viruses may be in fact phages.[20] This knowledge, of the importance of phages in aquatic environments, has led to an increased interest in their use in environmental applications.[20]

Nowadays, phages used in wastewater treatments are mainly as indicators for bacterial presence.This is essentially due to the poor understanding of phages' role in microbial communities. Also the instability of most treatments, such as phosphorus removal and nitrification, poses a challenge to phages in wastewater treatments.[20] However, phages do have potential to be used in this type of environmental treatments, not only in pathogen control, but also in improving dewaterability or digestibility, in controlling filamentous bacteria in activated sludge and in controlling non-phosphate accumulating bacteria.[20]

Sludge dehydration is an important process in the treatment of wastewater, since it reduces the volume of sludge, which promotes both the saving of equipment capacity and decreases downstream treatment costs.[21] The formation of sedimentation in water is often influenced by the microbial exopolysaccharides (EPS), which are responsible for binding cells to particulate material. The levels of microbial EPS can be very high which decreases the dehydration capacity of the activated sludge.[21] One of bacteriophage characteristics that has aroused interest in this area is the fact that these microorganisms have polysaccharide depolymerase enzymes (PDE). These enzymes are necessary when binding the phage to its host bacteria, and are also able to degrade the bacterial EPS capsule.[21]

In order to produce clean liquid effluents, it is essential to settle the sludge that forms during the activated sludge processes. However, due to the overproliferation of filamentous bacteria, there is the occurrence of foam formation, which constitutes an operational problem. The high surface area of the filamentous flakes, formed by filamentous bacteria, produces sedimentary properties in poor sludge, which increases the volume of the formed foam.[22] However, foaming is promoted by the stability of bubbles that is caused by hydrophobic cell surfaces and by the production of extracellular polymers. The bacteriophage's potential to control foam formation in activated sludge plants has been demonstrated by Thomas J. A. and his collaborators, who were able to isolate 17 phages from 6 activated sludge samples. These phages show the capacity of lysing foam-forming bacterias.[23]

However, even though phages show great potential to be useful in wastewater treatments, they also have their limitations. Some of those limitations stand in the host specificity as it is necessary to know the host present in the water to choose the phage to use.[24] Also phage isolation and production can constitute a problem for this type of treatment. It is important to note that in phage production and isolation a decay and loss of phage infectivity can happen.[24] This may be due to the ingestion of viral

particles by the bacteria, to the environmental stress, mainly associated with solar radiation as the sunlight penetration may be limited by the water turbidity, making it difficult to oligotrophic conditions to occur.[24]

Phage therapy

One of the biggest challenges to human health nowadays is the evolution and selection of antibiotic resistance in bacterial populations. One of the major contributions to this resistance is the continuous and sometimes abusefull use of antibiotics, which allowed the bacteria to select the resistance genes.[25] However, phages are being rediscovered and proposed as new antibacterial, being an alternative to antibiotics. Phage therapy has some advantages when compared to antibiotic therapy.[6] Some of these are the fast isolation, the continuous infective work even under tough conditions, the relatively high level of specificity for their host and ineffectiveness of infection in eukaryotic cells, reducing the risk of damaging the natural human microbiota.[6] Also the increase of phage resistance is almost ten times slower than antibiotic resistance. However, the fact that bacteriophages are a natural threat to bacteria, and are able to adapt in order to keep up with their host evolution is, probably, the most important advantages phages show against antibiotics.[6]

Even though phage therapy shows several advantages, it also has some drawbacks. Once phages are unable to enter eukaryotic cells, these organisms can not be used in therapy against intracellular pathogens as these are inaccessible.[6] Just because phages are not a threat to eukaryotic cells, this does not mean that the human immune system will not recognise them as foreign antigens and produce phage-neutralizing antibodies. Although phages are part of all ecosystems being natural components in them, the concentration used in phage therapy is likely to be greater than the one found in nature, meaning that the use of this type of therapy can make a huge impact in ecological communities.[6] Another problem with phage therapy, which is commun to some antibiotics, stands with the lysis of bacteria, especially gram-negative bacterias as they release compounds such as endotoxins. When there is the release of a large amount of endotoxins, the body can show some reactions as fever or septic shock, which could lead to death.[6]

Due to all this, phage therapies need to be studied and improved during the manufacturing process, both at laboratory and industrial scales.

Biosensor Development

In order to detect chemical compounds, either by electrical, either by thermal or even optical signals, the use of a device that uses specific bio-chemical reactions mediated by isolated enzymes, immune-systems, tissues, organelles or whole cells may be needed.[9] This device is called a “biosensor”. Biosensores have the potential to be used in different applications, from defense security to environmental monitoring or even pharmaceutical science. They normally have a standard composition made of bio-based recognition transducer components and electronic systems, which are responsible for signal amplification, processing and display.[9] The unique biological, geometrical and

mechanical phage characteristics are positive technical features to be exploited in different fields such as bacterial identification, pathogen detection and even biocontrol. The increasing development of biosensors is due to their numerous advantages going from their sensitivity to their specificity, fast and easy preparation, passing through their accurate detection.[9]

Although the development of biosensor surfaces by adsorption of the phage surface can result in inconsistencies related to the instability of the immobilization density, this process is very simple. A consistent improvement in the density and detection of the phage can be observed when phages are chemically anchored to a detection platform.[9] Still, in order to use chemically functionalized phage-based biosensors, it is necessary to pay attention to some criteria, the main one being the phage suspension selection purity. This means that biosensors must be free of contaminating agents such as carbohydrates, proteins and lipids. Yet, wild-type phages are responsible for the lyse of their host bacteria, which may contribute to losing the reduction of signal on a biosensor platform.[9]

Phage Purification

The use of phages for its applications requires that these organisms are purified to a high level in order to preserve a high infectivity.[26] This purity is normally obtained by polyethylene glycol precipitation and subsequent CsCl gradient ultracentrifugation. However, a relatively low yield may be achieved and this process may not work for some phages as they can either be damaged by the centrifugal forces or interact with CsCl and lose their infectivity. Also the high cost and time consumption of the process are not an advantage to it. So the need to find an alternative method of purification is real. One of the methods being considered is chromatography, which is a frequent method of choice when high purity products are wanted.[27] Another method considered is a nuclease digestion to increase the DNA removal.

Chromatography

Chromatography is a purification method where the mixture wanted to be separated is in a fluid called mobile phase. This phase carries the substances through a system, normally a chromatographic column filled with a stationary phase, but may also be a capillary tube for example. The substances present on the mobile phase present different affinity to the stationary phase, staying for longer or shorter periods of time attached to this phase according to their interactions with it.[28] The interactions between the different sample components and the stationary phase can be based on their charge (ion-exchange chromatography), size (size-exclusion chromatography), polarity (hydrophobic interaction chromatography) or even on selective non-covalent interactions (affinity chromatography).[28] Due to phages being negatively charged, anion-exchange chromatography (one of the divisions of ion-exchange chromatography) stands as a promising separation method.[28]

Anion-exchange chromatography is a chromatographic method with the objective of separating substances according to their charges, being the resin positively charged. This means that negative

charged particles will have affinity to the resin present in the column.[29] This type of chromatography is a very common technique in the purification of peptides, proteins, oligonucleotides, nucleic acids and charged molecules. The popularity of this purification method is due to its high resolving power, high capacity, simplicity and controllability.[30] This type of chromatographic process was proposed in 1953 as a useful method to purify and concentrate phages and has proven its utility with numerous studies being published and described over the years. One advantage of this process is the relatively high phage recovery, typically in the range of 35-70% of the amount of phages loaded.[31]

However, it also has its disadvantages, mainly related with the removal of other negatively charged molecules such as RNA/DNA and endotoxins, which are large molecules of a lipid and a polysaccharide composed of O-antigen found in the outer membrane of Gram-negative bacterias, also known as lipopolysaccharides (LPS).[31] In order to separate these components from the phages some other methods may be required. For the removal of endotoxins, an affinity chromatography based for example on a bacteriophage-derived protein or on phenyl boronate may be a solution. An incubation with detergents in order to avoid the formation of endotoxin micelles can also be considered.[31] However, one of the most used methods to remove endotoxins is precipitation. On the other hand, for the removal of DNA/RNA molecules, a nuclease incubation may do the trick as the molecules will be smaller, thus having a much lower charge density which will impair their adsorption to the anion-exchange chromatography resin.[31]

Phenyl Boronate Chromatography

Phenyl boronate chromatography is a chromatographic method, for the removal of *E. coli* proteins and DNA, that uses boronic acid or boronates as ligands. The use of this method for the separation of nucleic acid compounds and carbohydrates was reported for the first time in 1970 by Weith and colleagues. This chromatography is inserted in affinity chromatography being one of the first reported ways to determine glycohemoglobin for long-term diabetes management. Most boronate derivatives are able to form covalent bonds with compounds that have cis-diol groups, having been widly exploited for a variety of cis-diol-containing compounds, such as sugars (glucose), nucleotides, nucleic acids and carbohydrates, boronates have demonstrated its value for the removal of these compounds.[32] The esterification between boronate ligands and cis-diols is the basic interaction for this chromatography. In order for the boronate/cis-diol esterification to happen there is a structural requirement. This requirement consists of the two hydroxyl groups of a diol to be present on adjacent carbon atoms in an nearly coplanar configuration (1,2-cis-diol).[32] However, it is important to note that boronate interactions with 1,3-cis-diol may also accour in a weaker boronate ester bond. Under basic conditions, boronate, which presents a trigonal coplanar geometry, is hydroxylated, producing a tetrahedral boronate anion. This anion is able to form esters with cis-diols. To reverse the reaction, the cyclic diester is hydrolyzed under acidic conditions. Still, phenyl boronate is able to bind to cis-diol free proteins and gDNA fragments that interact by charge transfer interactions.[32]

Nuclease Digestion

DNA can be difficult to remove by chromatography, however, there are some methods that can help with this issue. One of those methods consists in doing a nuclease digestion.[33] A nuclease is an enzyme which has the ability of cleaving the phosphodiester bonds between nucleotides of nucleic acids. These cuts may occur in different DNA positions, only at the ends of DNA molecules (exonucleases) or in the middles of the DNA chain (endonucleases), according to the DNAse used. DNAses can also be very specific about the sequence which they cut, being theses restriction enzymes.[33] It is also important to note that nucleases may be categorized as deoxyribonucleases or ribonucleases, meaning the first one takes action in the DNA and the second one in the RNA.[33]

These enzymes have a huge natural role, mainly in the DNA repair within the cells. Their importance is due not only to the error prone process of DNA replication, but also to the vulnerability of the DNA molecules to modification associated with metabolic and environmental stressors.[33]

Precipitation

Precipitation in water or in a solvent happens by changing the form of the dissolved materials into solid particles, meaning that by adding counter-ions, which reduce the solubility, ionic constituents are removed from the solvent. Not only salts can be used in the process but also enzymes or even detergents.[34] Due to the addition of counter-ions, most metals, when precipitated, are as hydroxides, being, sometimes, necessary to use other precipitation methods as sulfide or carbonate. It may also be necessary to oxidize or reduce the components to be removed. This means that major precipitation processes may need water softening or component stabilization.[34]

Precipitation can be associated with several advantages and disadvantages. Some of the advantages focus on the low capital cost of the process, its simple operations and low energy costs for the operation. However, their disadvantages are related to, mostly, environmental issues as the disposal of the precipitated sludge that is produced may be a problem. Also this process shows high levels of operating costs.[34]

Endotoxins

Gram-negative bacteria show in their membrane constitution endotoxins, which are lipopolysaccharides (LPS) responsible for the cells organization and stability. These components are present inside the cell wall, however they are continuously liberated into the environment.[35] Being this release during cell death or even cell growth and division. Endotoxins can be found almost everywhere as bacteria are able to grow in nutrient poor media (water, saline, and buffers). It is important to understand that one single *E. coli* cell can contain about 2 million LPS molecules.[35]

Endotoxins can be complicated to remove due to its physicochemical characteristics and to their ability to form micelles because of their 10-20 kDa hydrophobic region. These molecules can also associate

themselves with protein products which makes phage purification a challenge.[35] To achieve a successful purification it is necessary to focus on the properties that differ between phages and endotoxins. One of these properties is the size of these molecules, phages and endotoxins, which results in a precipitation at different salts and conditions.[36] With precipitation, the phages will precipitate and most endotoxins stay in the supernatant, which is removed from the samples.[36]

Aim of Studies

The current phage purification technique in use, polyethylene glycol precipitation followed by CsCl gradient ultracentrifugation, as stated, is expensive and difficult to perform. However the biggest disadvantage of this technique is its unfitness for the purification of large amounts of phage.

This Master Thesis study has the aim of purifying a phage suspension (T4 phages specific for *E. coli*) by removing the bacterial (*E. coli*) proteins and the DNA from it while maintaining the phage infectivity. This is accomplished by resorting to a Phenyl Boronate Chromatography and a Nuclease Digestion (Denarase®). The process should be suitable for a scalable downstream process.

Materials and Methods

T4 Phage Sample Preparation

Escherichia coli (*E. coli*) (DSM 613) (DSMZ, German Collection of Microorganism and Cell Cultures, Germany) was pre-inoculated overnight in 30 mL of Tryptic Soy Broth (Biokar Diagnostic, Allonne, France) medium (TSB medium) and grown at 37 ºC under agitation (250 rpm) in the incubator (Aralab Agitorb 200, Sintra, Portugal). The TSB medium (2.5% w/v TSB) was prepared according to the instructions of the manufacturer, being the powder measured in a lab scale (Mettler Toledo, Ohio, USA). The pre-inoculum Optical Density (OD) at 600 nm was measured in a spectrophotometer (Hitachi U2000, USA) and the volume needed to incubate 500 mL of TSB medium (2.5% w/v TSB) was calculated by performing a dilution where the final concentration is the OD_{600} wanted in the beginning of the inoculum, around 0.1, and the initial concentration is the OD_{600} measured. The incubation was done in the incubator. When the OD_{600} is 0.20-0.25, meaning a bacteria concentration of 1.52×10^{7} cfu/mL, phage T4 (DSM 4505) (DSMZ, German Collection of Microorganism and Cell Cultures, Germany) from stock culture (with a concentration of 1.52×10^{10} pfu/mL, previously obtained through a plaque assay) and 5 mL of 1 M $MgCl_2$ (Sigma Aldrich, Missouri, USA) were added to the suspension. The volume of stock culture added was calculated in order to present a Multiplicity of Infection (MOI) of 0.1, which corresponds to the number of phages per number of bacteria. After adding the T4 phages, lytic phage, an incubation at 37 ºC with 250 rpm agitation was done, in the incubator, until the OD_{600} was equal or below 0.1. This was followed by a 20 min centrifugation 8000×g at 4 ºC in a centrifuge (Eppendorf Centrifuge 5810 R, Hamburgo, Alemanha). The suspension was then filtered in a 0.22 µm pore size bottle top filter, obtaining the lysate.[37] The lysate was stored until further use at 4 ºC.

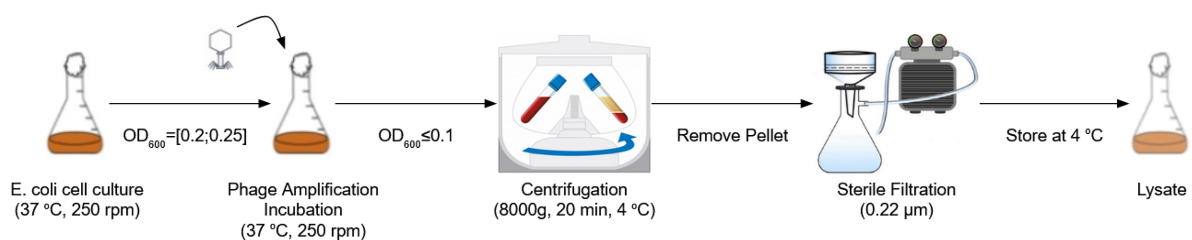

Figure 4. Amplification process for the T4 phage, from the inoculum to the lysate. Heat and rotation conditions for incubation; heat, velocity and time conditions for centrifugation, in which the phages are present in the supernatant being the pellet remains of the host cell; filter porosity for sterile filtration; heat conditions to storage.

Bacterial Growth Curve

E. coli cells were pre-inoculated overnight in 10 mL TSB medium (2.5% w/v TSB) and grown at 37 ºC under agitation (250 rpm) in the incubator. The TSB medium (2.5% w/v TSB) was prepared according to the instructions of the manufacturer. The OD_{600} of the pre-inoculum was measured and the volume to incubate 100 mL of TSB medium (2.5% w/v TSB) was calculated according to equation 1. The final concentration corresponds to the OD_{600} wanted in the beginning of the inoculation, around 0.1, and the initial concentration is the OD_{600} measured. The final volume is 100 mL of TSB medium where the inoculum will happen. The bacteria were put in incubation at 37 ºC and 250 rpm, being taken 3 samples every 15 minutes in order to do 3 different bacteria growth curves.

The OD_{600} was measured for the first sample. The second sample was successively diluted in Milli-Q water and plated in Tryptic Soy Agar (Biokar Diagnostic, Allonne, France) medium (TSA medium) (4% w/v TSA), which was then put in incubation overnight at 37 ºC, being the colony forming units (CFU) counted. The third sample was centrifuged at 8 000 rpm for 10 minutes, being the pellet washed with Milli-Q water and centrifuged at 8 000 rpm for 10 minutes.[38] The pellet was weighed and put on the incubator at 4 ºC for 24 hours to evaporate the water.[39] After the sample was taken from the incubator, it was weighed again in order to calculate the grams of bacteria.

The petri dishes with TSA medium (4% w/v TSA) were prepared in advance, being the TSA medium (4% w/v TSA) prepared as TSB medium (2.5% w/v TSB) and poured in the plates to solidify. It was then stored at 4 ºC until needed.

Phage Bank

From the lysate obtained in the T4 phage amplification, 5 samples with 60 µL were taken, being added 10 µL of SM buffer 10x (0.1 M Tris-HCl pH 7.4, 0.1 M NaCl, 10 mM $MgSO_4.7H_2O$, 0.01% w/v gelatine) and 30 µL of glycerol 50%. Both of the solutions were available at the lab.

Cell Bank

In order to do a cell bank, *E. coli* cells were pre-inoculated in 5 mL TSB medium (2.5% w/v TSB) overnight at 37 ºC and 250 rpm. The pre-inoculum OD_{600} was measured and the volume needed to be taken to another 5 mL TSB medium (2.5% w/v TSB) was calculated with equation 1, where the final concentration is the OD_{600} wanted at the beginning of the inoculation, around 0.1, the initial concentration is the OD_{600} measured and the final volume was 5 mL. Cells were incubated at 37 ºC and 250 rpm until the OD_{600} was 1. At that time,10 samples with 70 µL were taken. To these samples, 30 µL of glycerol 50% available in the lab was added, respectively.

Phenyl Boronate Chromatography

Phenyl Boronate Chromatography was performed at room temperature in columns packed with PB (Phenyl Boronate) resin, which were connected to an Äkta start (GE Healthcare, Uppsala, Sweden). The column with 5 mm of diameter was packed up to 3.8 cm in height. Several different equilibration, adsorption and elution buffers were tested. The first experience (Condition 1) was made with Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The second test (Condition 2) had 15 mM of Tris-HCl, pH 8.5 as equilibration and adsorption buffers, maintaining the elution buffer (1.5 M Tris-HCl, pH 8.5). Another attempt (Condition 3) was done with 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffers and also maintaining the elution buffer (1.5 M Tris-HCl, pH 8.5). The last experience (Condition 4) had 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffers and 1.5 M Tris-HCl, pH 7 as elution buffer. All trials were done with the lysate (before buffer changes) and with a phagestock, obtained after buffer changes and nuclease digestion.

In order to use the column, it is necessary to do an equilibration. The column was equilibrated with 5 column volumes of equilibration buffer. After equilibration, 1 mL of sample was injected into the column. The unbound material was washed with 5 column volumes of adsorption buffer and the bound material was eluted with the elution buffer. All buffers were run at a constant flow rate of 1 mL/min.[40]

The absorbance of the eluate was monitored at 260 nm[41] and the fractions were pooled in twenty final samples for further analysis. The runs were done in triplicate, as three independent assays.

Nuclease Digestion

In an attempt to decrease the amount of nucleic acid present in the lysate, a nuclease incubation was done. For this, the nuclease Denarase®, c-LEct, Germany, was used. To use this enzyme, it was necessary a defined concentration of $MgCl_2$ at a defined pH, a nuclease buffer was previously prepared (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8).

In order for the samples used to be incubated with Denarase®, a change to the nuclease buffer was made using 20 mL Amicons (membrane module with a Nominal Molecular Weight Cut-off, NMWCO, of 100 kDa, so that the phages would be retained) from GE Healthcare, Vivaspin 20, United Kingdom, with the support of a centrifuge (10 minutes, 4000 rpm, 4 ºC). The Amicons were first washed with Milli-Q water and then a concentration of the lysate was performed, until the volume of sample was half than the volume in the beginning (20 mL). The volume removed was equivalent to the one inserted in the nuclease buffer. This was repeated until a 4 diafiltration volume exchange was completed. The samples were tested for amount of DNA, protein concentration and phage infectivity.

Denarase® was diluted in equal proportion of nuclease and nuclease buffer. The concentration of nuclease in each sample was 1 U/mL. The samples with denarase® were incubated at 37 ºC for different time periods (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours). Two different methods

were studied in order to stop the digestion. The first one was a 10 minutes incubation at 72 ºC and the second one consisted in saving the samples in ice until further use (only incubation times of 30 minutes and 1 hour and 30 minutes were studied for ice incubation). The samples were tested for the amount of DNA, protein concentration and phage infectivity. In order to understand the impact the 72 ºC and ice incubations have in the phages, the incubation was performed to a sample, after exchanging to the nuclease buffer, but without the nuclease incubation. The samples were used in a phenyl boronate chromatography, with the condition previously decided as the most suitable for the experiment, Condition 3 (15 mM Tris-HCl, pH 7 as adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The chromatography was accomplished for each viable sample and triplicates (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours), being the fraction also tested for DNA and protein quantity and phage infectivity.

In order to understand salt interference (2 mM $MgCl_2$) in the phenyl boronate chromatography, a change of buffer (to the adsorption buffer 15 mM Tris-HCl, pH 7) was accomplished, by using Amicon Ultra 0.5 mL (membrane module with a NMWC of 10 kDa) from Merck Millipore, Ireland. The procedure was the same as for the exchange for the nuclease buffer, except for the centrifuge conditions (14 000g, 10 minutes, room temperature). A Phenyl Boronate Chromatography was then accomplished for each viable sample and triplicates (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours). It was also used condition 3 to perform the chromatography.

In an attempt to study the enzyme action without previously doing a buffer exchange, Denarase® was, again, diluted in equal proportion of nuclease and nuclease buffer, being the concentration of enzyme in each sample 1 U/mL. The lysate samples were incubated at 37 ºC for different time periods (30 minutes and 1 hour and 30 minutes). It is important to note that the digestion was not stopped by heat nor cold. Right after the digestion time ended, the samples were used in a phenyl boronate chromatography (it was used condition 3 to perform the chromatography). The samples were, also, done in triplicate and tested for DNA and protein quantity and phage infectivity.

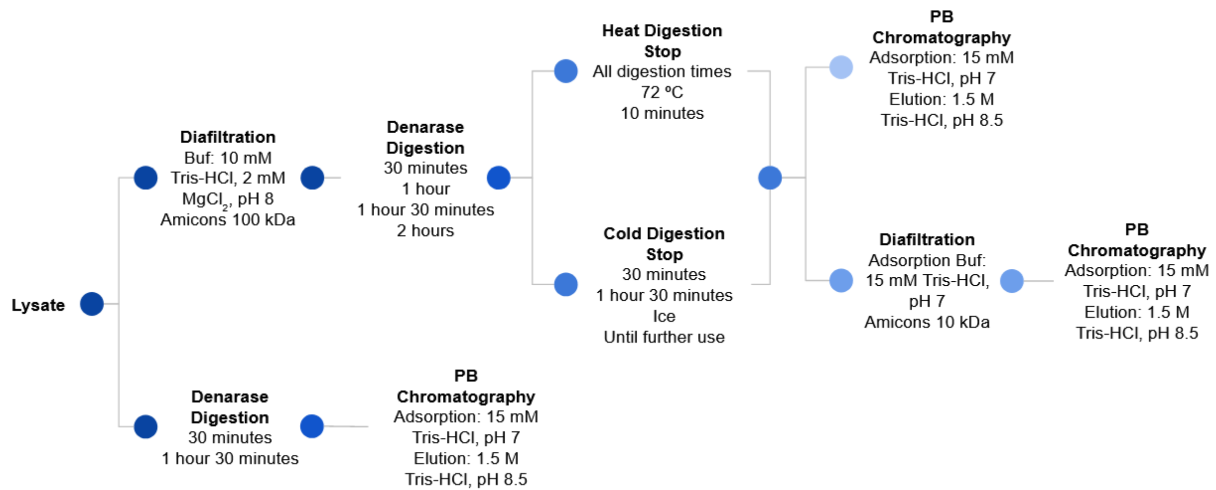

Figure 5. Digestion process steps. There are two routes starting from the lysate. The first one begins with the first Amicons (100 kDa) buffer exchange into the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8), followed by the Denarase® digestion with the four different digestion times in study. The digestion was stopped by two different methods, heat (72 ºC for 10 minutes with all digestion times) and cold (ice until further use with digestion time 30 minutes and 1 hour and 30 minutes). After the digestion stop either a Phenyl Boronate Chromatography was performed to the samples (15 mM Tris-HCl, pH 7 as adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer) or another Amicons (10 kDa) buffer exchange into the adsorption buffer (10 mM Tris-HCl, pH 7). The last step was a Phenyl Boronate Chromatography (15 mM Tris-HCl, pH 7 as adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The second route consists in a Denarase® digestion with the 30 minutes and 1 hour and 30 minutes times, which is followed by a Phenyl Boronate Chromatography (15 mM Tris-HCl, pH 7 as adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

Plaque Assay

In order to determine the titer of phages, a plaque assay technique was done. Firstly, *E. coli* was pre-inoculated in 5 mL TSB medium (2.5% w/v TSB) overnight at 37 ºC and 250 rpm. The OD_{600} of the pre-inoculum was measured and the volume taken to another 5 mL TSB medium (2.5% w/v TSB) was calculated according to equation 1. The final concentration corresponds to the OD_{600} wanted in the beginning of the inoculation, around 0.1, and the initial concentration is the OD_{600} measured. The final volume is 5 mL. The bacteria were put in incubation until the OD_{600} was 0.2 at 37 ºC and 250 rpm. In the meanwhile, successive dilution in SM buffer (0.1 M Tris-HCl pH 7.4, 0.1 M NaCl, 10 mM $MgSO_4.7H_2O$, 0.01% w/v gelatine), were made to the samples containing the T4 phage. When bacteria OD_{600} is as expected, 50 µL of 1 M $MgCl_2$ was added and 200 µL of the new solution was mixed with 100 µL of diluted T4 phage samples.

Top agar (0.7% w/v agar-agar, 2.5% w/v TSB) was prepared in advance by adding 12.5 g of TSB medium (2.5% w/v TSB) and 3.5 g of Agar-Agar (Biokar Diagnostic, Allonne, France) at 500 mL of Milli-Q water. It was melted and thermostated at 64 ºC. 1:100 mL of 1 M $MgCl_2$ was added to the top

agar, being 3 mL of this solution added to the phage/cells mixture previously described. The solution was mixed gently and poured onto TSA medium in petri dishes, which were prepared as described in Bacteria Growth Curve. The plates were incubated overnight at 37 ºC. The plaque forming units were counted (30-300 pfu) and the concentration in pfu/mL was calculated according to the equation:

$$C = N_p \times 10^d \times 10 \quad (1)$$

Where C is the concentration in pfu/mL, Np is the number of plaques and d is the dilution factor.

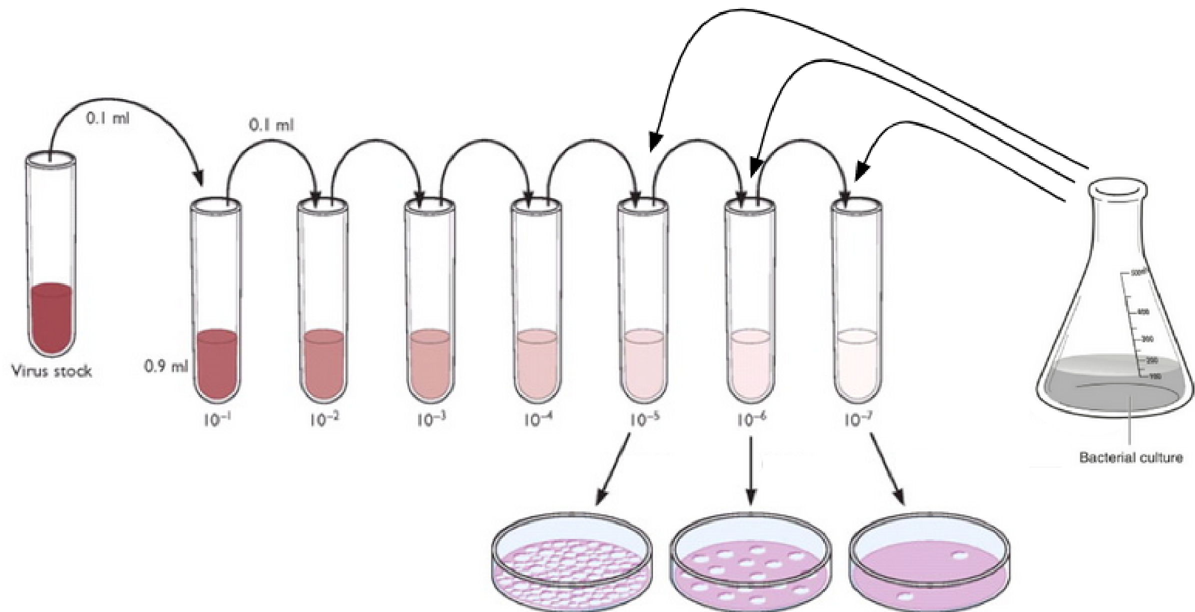

Figure 6. Scheme of the plaque assay process. Successive dilutions in SM buffer; addition of E. coli to the samples to put in the petri dishes. Phages in petri dishes after the overnight incubation at 37 ºC.

BCA Assay

In order to quantify the proteins in a sample, it is necessary to do a BCA assay. A Pierce BCA (Bicinchoninic acid) Protein Assay Kit from Thermo Scientific was used.

First a curve of protein standards was done. To do it, 9 points (A, B, C, D, E, F, G, H, I) of diluted Albumin (BSA) Standard were prepared, where the diluent was Phosphate Buffered Saline (PBS), from Sigma. PBS was prepared by adding 1 pill of product to 200 mL of water. Point A was prepared only by adding 200 µL of stock (2 mg/mL BSA) to 800 µL of buffer (PBS). Point B was obtained by adding125 µL of PBS to 375 µL of sample A. Also sample C was prepared with 325 µL of sample A and 325 µL of PBS. Point D was achieved by adding 175 µL of vial B to 175 µL of PBS. From sample C, a total of 325 µL was taken and added to 325 µL of PBS in order to prepare point E. Pois F was obtained by taking 325 µL of point E and mixing it with 325 µL of PBS. From sample F was prepared point G, by adding 325 µL of sample F to 325 µL of PBS. Point H was achieved by adding 100 µL of vial G and adding it to 400 µL of PBS. Finally, sample I was prepared to be the blank point, meaning it

was done by only 400 µL of PBS. Every sample has its concentration already determined in order to do the standard curve. From point A to I the concentration goes 400, 300, 200, 150, 100, 50, 25, 5 and 0 mg/L, respectively.

The samples were analysed in a microplate. To each well was added 25 µL of sample and 200 µL of reagent. The reagent was prepared by adding 1:50 µL of Reagent A and Reagent B, available in the kit. Take notice that the quantity prepared should be according to the number of wells used. The microplates were then put in incubation at 37 ºC and 300 rpm for 30 minutes. Absorbance at 562 nm was measured in a SpectraMax 340 pc, USA, equipment.

PicoGreen Assay

A PicoGreen Assay is used in order to quantify the DNA in a sample. An invitrogen (Quant-iT™ PicoGreen™ dsDNA Assay Kit) from Thermo Fisher Scientific was used.

First a standard curve was done. For this 10 µL of 100 µg/mL λ DNA was added to 490 µL of 1x TE, previously prepared from a dilution of 20x TE in MilliQ water. A total of 100 µL of 1x TE and 100 µL of sample or standard is added per well, being the standard diluted 1:2 (take notice that the standard curve dilution is made in the wells by taking 100 µL of the previous well and discarding the 100 µL of the last one). It is also necessary to do a blank well, only with 1x TE. With the lights off, the PicoGreen 1:200 was prepared in 1x TE (example 60 µL of PicoGreen s added to 11940 µL of 1x TE). 100 µL of the prepared PicoGreen is, then, added to each well. The plate is incubated for 5 minutes at room temperature, but kept from the light. The fluorescence is measured using Tecan.

It is important to refer that for this assay, 2 pools (Pool 1 and Pool 2) were done corresponding to the samples from each chromatographic peak, being the first peak (fractions T2, T3, T4, T5 and T6) corresponded to pool 1 and the second peak (fractions T17, T18 and 19) to pool 2.

SDS-Page

When doing a nuclease digestion (stopped by heat or cold), followed by a chromatography, it is important to confirm the amount of protein present at the end of the analysis, if these quantities are according to the chromatogram and which proteins do still remain in samples. In order to do this an SDS-Page (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was performed.

The samples used in the SDS-Page were the same Pools prepared for the Picogreen Assay. The samples are prepared by adding 25 µL of loading buffer 2x, 312.5 mM Tris-HCl, 10% SDS, 50% glycerol, 0.05% bromophenol, pH 6.8, (Bio-Rad Laboratories, USA), 5 µL of 1 M DTT, previously available in the laboratory and 20 µL of protein sample. The sample is heated at 100 ºC for 10 minutes.

In order to perform an SDS-PAGE, it is necessary to prepare first an electrophoresis gel. This gel consists of two parts, the resolving gel and the stacking gel. The first one is done by mixing 3 mL of Acrylamide/bis-acrylamide 40% (Bio-Rad Laboratories, USA), 2 mL of Resolving buffer 4x (1.5 M Tris-HCl, 0.4% SDS, pH 8.8), available in the laboratory, 4.445 mL of Milli-Q water, 0.005 mL of N, N, N', N' - Tetramethyl-ethylenediamine, TEMED, (Sigma, Aldrich, USA) and 0.05 mL of ammonium persulfate, APS, previously available in the laboratory. The second one is done by mixing 0.25 mL of acrylamide, 0.625 mL of stacking buffer, 0.5 M Tris-HCl, 0.4% SDS, pH 6.8, previously available in the laboratory, 1.61 mL of Milli-Q water, 0.0025 mL of TEMED and 0.0125 mL of APS.

After mixing the resolving gel, this is placed and left to solidify, however, in order to prevent bubbles from happening, 2-Methyl-2-butanol is placed above. When solid, the 2-Methyl-2-butanol is removed and the stacking gel is placed above the resolving gel and left to solidify too. After solidification, the samples, previously prepared, are put in the gel. The Precision Plus Protein™ Dual Color Standards ladder is also put in one or two wells of the gel.

The gel is prepared for running by being covered with a running buffer (250 mM Tris-HCl, 1.92 M glycine, 1% SDS, pH 8.3), available in the laboratory, and then it is run at 100 mV until the samples reach the bottom of the gel. Afterwards, the gel is covered with Coomassie Blue, available in the laboratory, and left for 24 hours.

After staining with Coomassie, the gel is washed with Milli-Q water and covered with destaining solution (30% v/v ethanol, 10% v/v acetic acid), until the solution is blue. Once the destaining solution is blue, the gel is, again, washed with Milli-Q water and covered with more destaining solution. This procedure is repeated until the gel is clear, and the bands are visible. The gel is then stored in Milli-Q water and ethanol.

Results

T4 Phage Sample Preparation

In order to obtain the quantity needed of phages to study the purification process, an amplification of the phages available is needed. This amplification began with the inoculation of *E. coli* bacteria cells. A typical bacterial growth curve is shown in Figure 7, where there is the first phase of growth, the lag phase, during the first 55 minutes of incubation. At 60 minutes, the concentration of bacteria had already started to increase, and cells entered in the exponential phase. It was at this point, 60 minutes, that the phage infection (T4 phage) was normally performed. Bacteria concentration did not start to decrease immediately after the infection, in fact cells continued to grow, but at a smaller rate then the non-infected ones. At some point, the phage's multiplication started to affect all the bacteria, lising them, as is seen in the last result, point at 150 minutes where the OD_{600} is 0.09. The OD_{600} correspondent to each point is available in the Appendix.

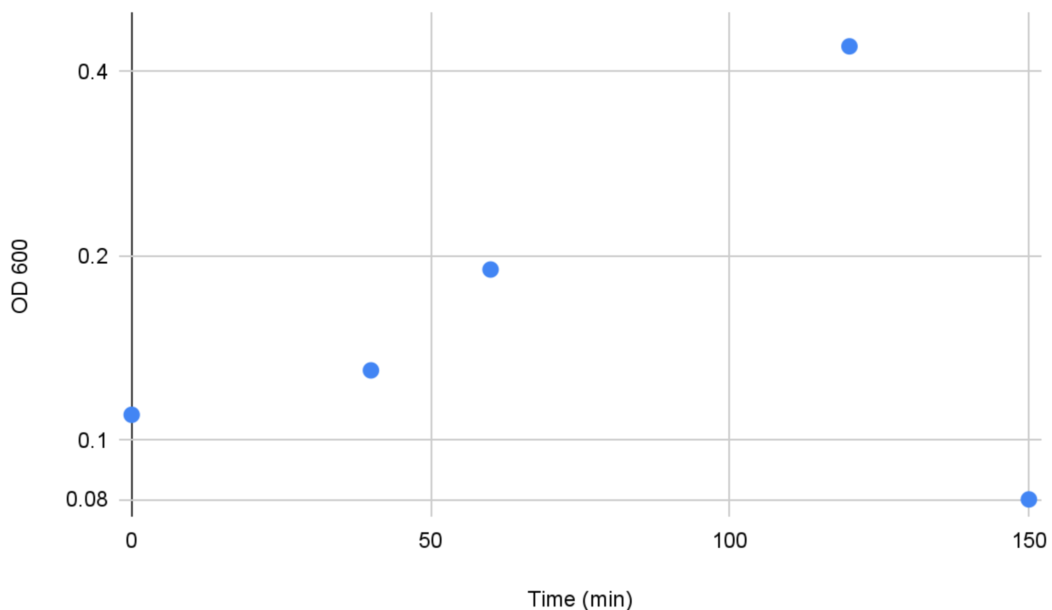

Figure 7. Amplification of T4-like phage (DSM 4505) on *E. coli* strain DSM 613 in a 500 mL TSB culture medium. The time course, in minutes, after inoculation of the broth with bacteria corresponds to the first 4 points. The infection with phage was done at the fourth point, 60 minutes. The growth of the bacterial culture was followed by reading the OD_{600} and is presented in log (OD_{600}).

After the decrease in bacteria concentration, a centrifugation was made, followed by a sterile filtration, which produced the phage-containing clarified lysate. A plaque assay method was done to this lysate in order to determine the amount of infective phages after the amplification. A concentration of 4.9×10^9 PFU/mL was obtained.

Bacterial Growth Curve

In an attempt to understand how the phage infection affects the bacteria growth, the bacterial growth curve was determined (Figure 8). This curve was obtained as the one shown in Figure 7, with the inoculum of *E. coli* starting the curve at an OD_{600} of 0.08. Several samples were taken at specific time points (Appendix). As it is possible to observe in Figure 8, the lag phase corresponds approximately to the first 60 minutes of incubation. However, a phage infection was not performed so when the bacteria entered the exponential phase (from minute 60 until at least, 150) they continued their growth, being this the main difference between a normal growth curve and the one for phage amplification. It should be noticed that, as the objective of this curve was to identify the difference between a normal growth curve and an infected one, there were no measurements on the stationary phase or death phase. The OD_{600} was only measured in the first 150 minutes of incubation as this was the time bacteria showed a decrease in the phage amplification.

Other bacterial growth curves obtained by the bacterial dry weight and colony forming units (CFU) are available in the Appendix.

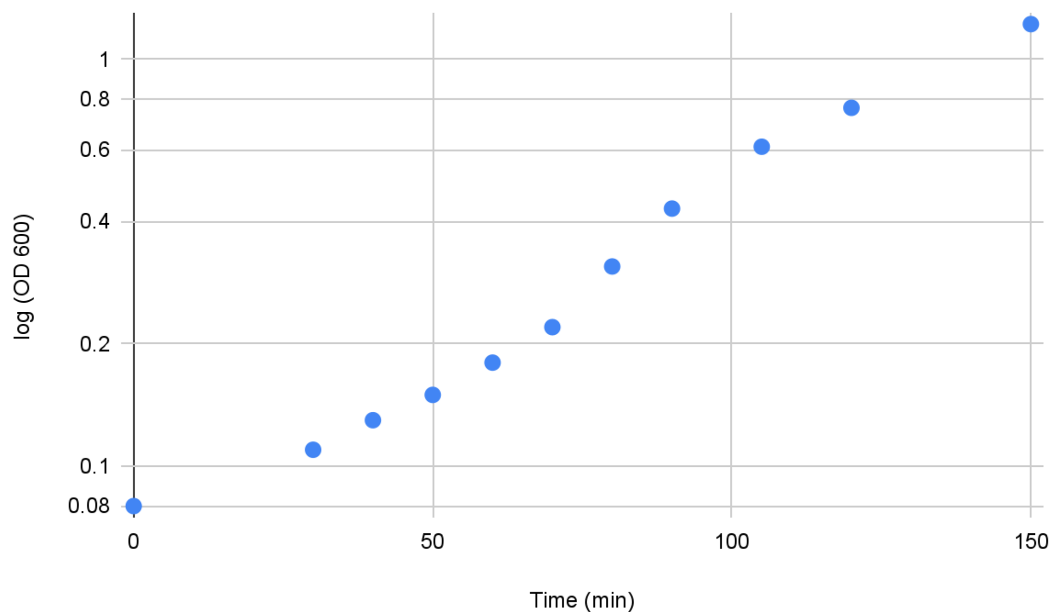

Figure 8. Bacterial Growth Curve of *E. coli*, strain 613 in a 100 mL TSB culture medium. The time, in minutes, corresponds to the time after inoculation of the broth with bacteria. The growth of the bacterial culture was followed by reading the OD_{600} and is presented in log (OD_{600}).

Phenyl Boronate Chromatography

Purification of phages by phenyl boronate chromatography was performed directly from the lysate using 4 different conditions. Condition 1 corresponds to using Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. Condition 2 is the use of 15 mM of Tris-HCl, pH 8.5 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as the elution buffer. Condition 3 uses 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffers and 1.5 M Tris-HCl, pH 8.5 as the elution buffer. At last, condition 4 corresponds to the use of 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffers and 1.5 M Tris-HCl, pH 7 as elution buffer. Each condition was performed in triplicate and for every run, a plaque assay, a BCA assay and a PicoGreen Assay were done in order to characterise the phage purification. The phage yield, percentage of protein and DNA removal for each condition studied is shown in Table 1.

Table 1. Average phage yield, percentage of protein and DNA removal, and respective standard deviations, in the elution pool, for each condition tested in the Phenyl Boronate Chromatography.

Chromatography	Yield (%)	Protein Removal (%)	DNA Removal (%)
Condition 1	55.99 ± 4.22	96.26 ± 0.60	49.09 ± 2.39
Condition 2	33.41 ± 8.57	96.73 ± 0.82	53.69 ± 1.59
Condition 3	49.49 ± 7.88	96.61 ± 0.25	47.60 ± 0.57
Condition 4	53.45 ± 8.77	96.85 ± 0.51	46.67 ± 7.50

Condition 1

In Figure 9 is shown the chromatogram of condition 1. Even though the conditions were tested in triplicates, Figures below correspond to the first triplicate (being the other two available in the Appendix).

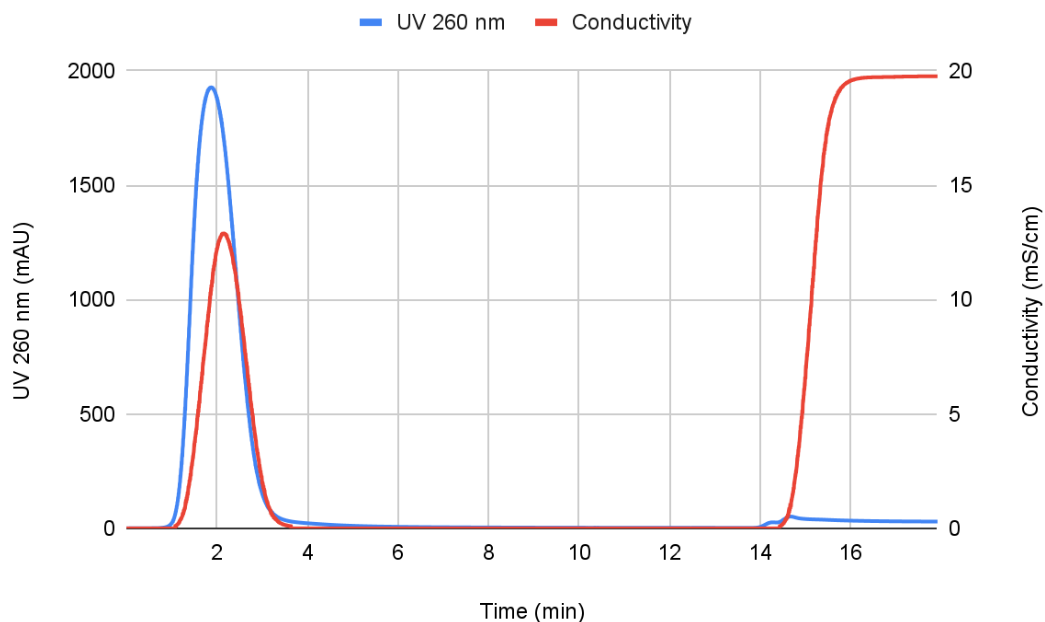

Figure 9. Chromatogram of the first triplicate of Condition 1 (Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

Figure 10 shows the infectivity of phages, quantity of protein after phenyl boronate chromatography per fraction collected during the chromatography.

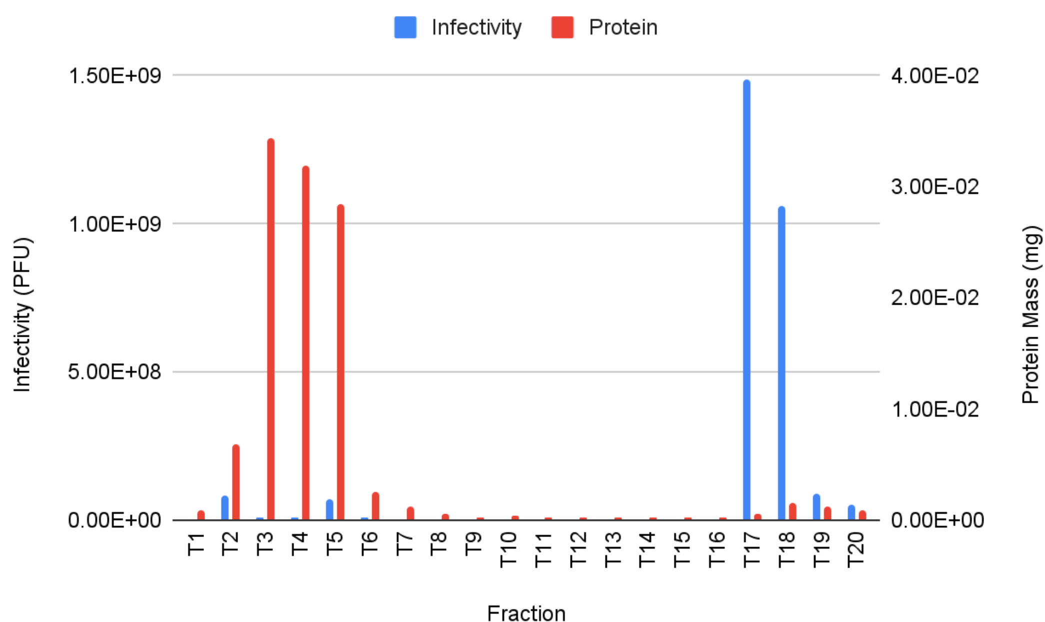

Figure 10. Phages infectivity (PFU) and Protein Mass in mg, for the first triplicate of condition 1 (Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

In an attempt to help visualize the corresponding infectivity and protein quantities to the chromatogram, a combination of Figure 9 and 10 was performed and is shown in Figure 11.

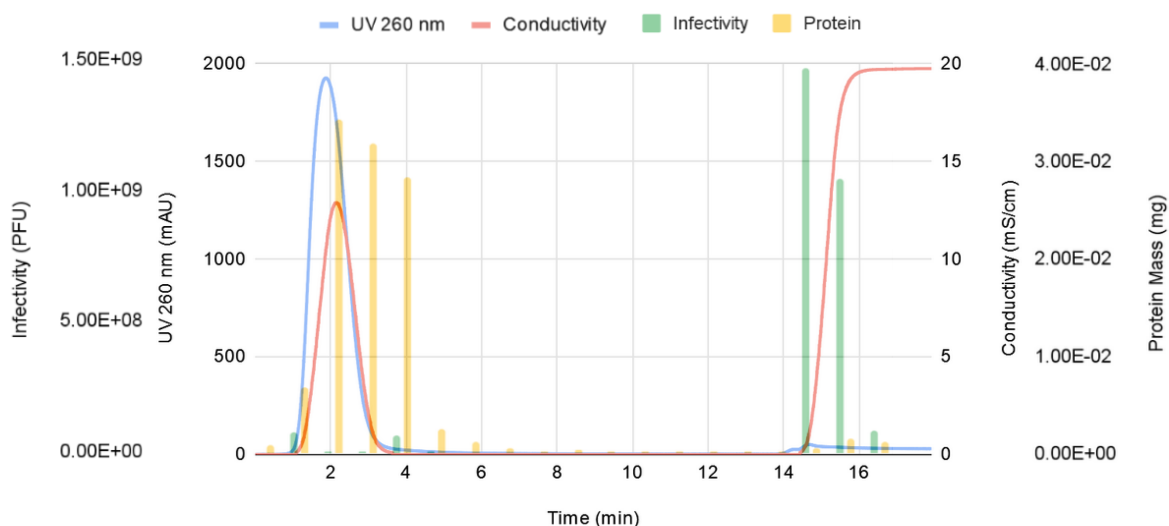

Figure 11. Phages infectivity (PFU) and Protein Mass in mg, for the first triplicate of condition 1 (Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer) corresponding to the chromatogram of the first triplicate of condition 1. The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min). As fractions correspond to 1 mL, and the flow rate is 1 mL/min, the fractions are also per retention time (min).

A PicoGreen Assay was also performed to two pools of samples, flowthrough and elution, in order to determine the amount of DNA present in each pool . These quantities are available in Table 2. The first pool was done with fractions T2, T3, T4, T5 and T6. The second pool was obtained with fractions T17, T18 and T19.

Table 2. DNA quantity in µg for the initial lysate, flowthrough pool and elution pool, for the three replicates done for Condition 1 (Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	0.7256	0.7256	0.7256
Pool FT	0.3364	0.3525	0.4557
Pool Elution	0.3641	0.3520	0.3922

Condition 2

Condition 2 chromatogram is available in Figure 12. The results of the second and third triplicates of this condition are shown in the Appendix.

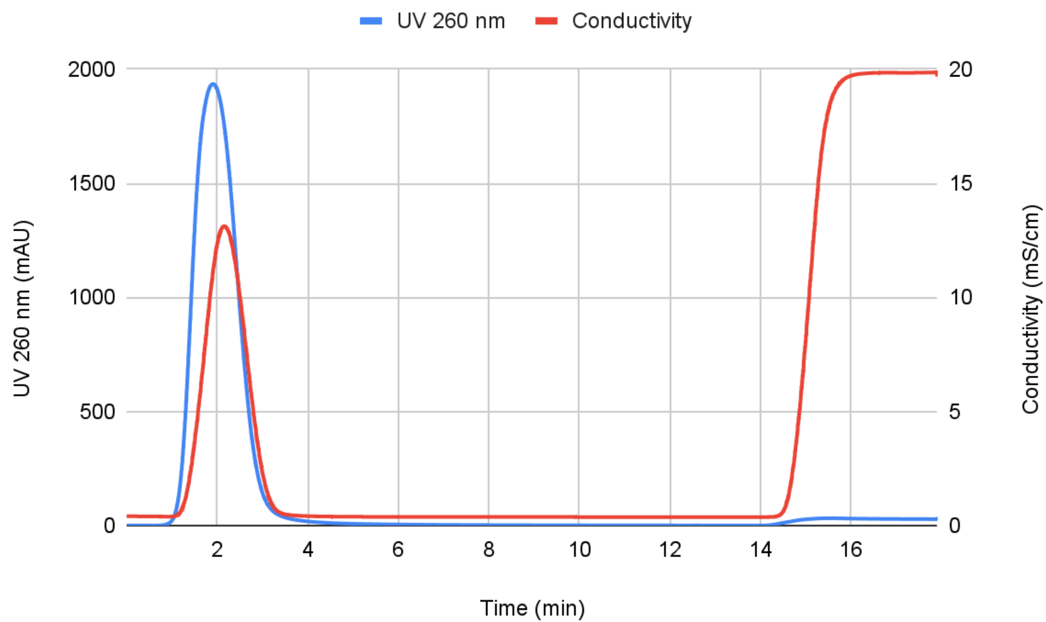

Figure 12. Chromatogram of the first triplicate of Condition 2 (15 mM of Tris-HCl, pH 8.5 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

After phenyl boronate chromatography, the phage infectivity and protein quantity (Figure 13) were measured, and presented below.

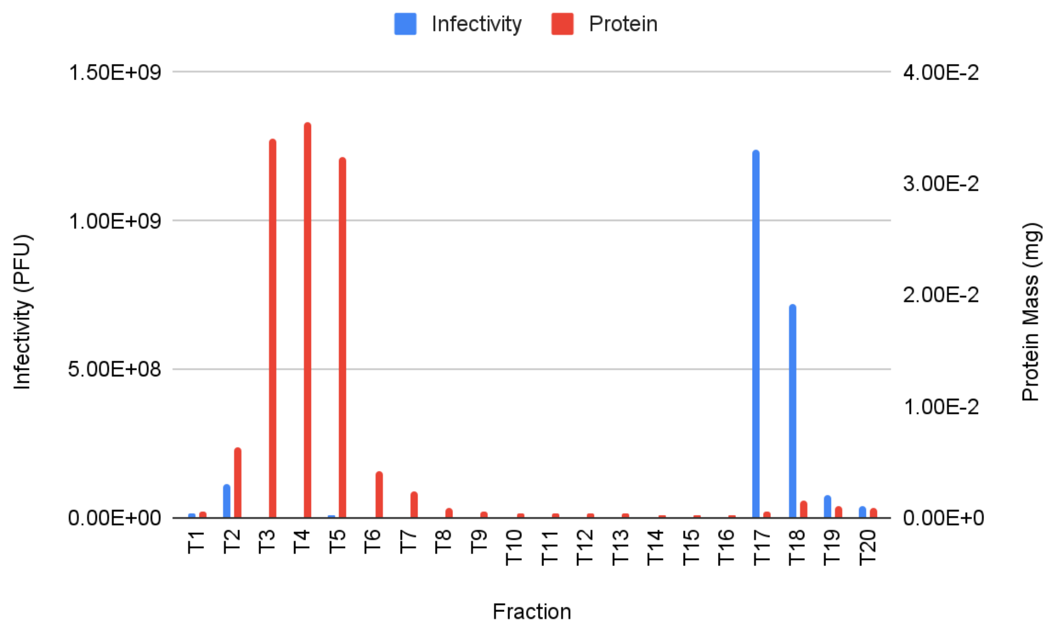

Figure 13. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of condition 2 (15 mM of Tris-HCl, pH 8.5 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

The DNA present in the chromatographic peaks was quantified using a PicoGreen Assay. The DNA quantities are available in Table 3.

Table 3. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three replicates done for Condition 2 (15 mM of Tris-HCl, pH 8.5 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	0.7256	0.7256	0.7256
Pool FT	0.8861	0.8665	0.8730
Pool Elution	0.3267	0.3325	0.3489

Condition 3

The chromatogram for tests of condition 3 is presented below. As in condition 1 and 2, the second and third triplicates results are available in the Appendix.

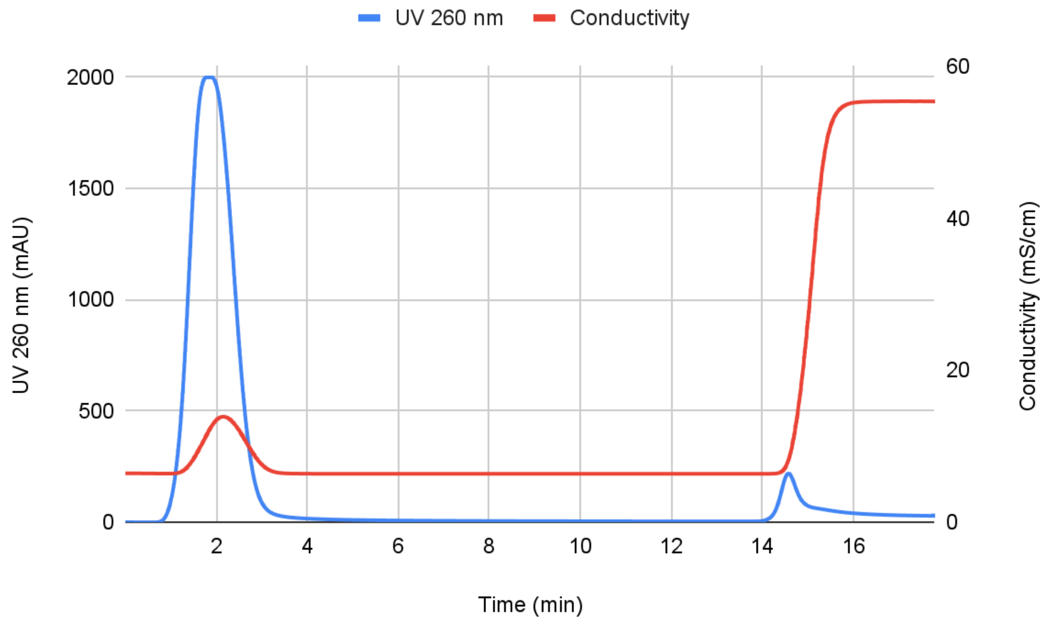

Figure 14. Chromatogram of the first triplicate of Condition 3 (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV, in mAU, measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

For each fraction collected during the chromatography, phage infectivity and protein quantity was measured, being the results available in Figure 15.

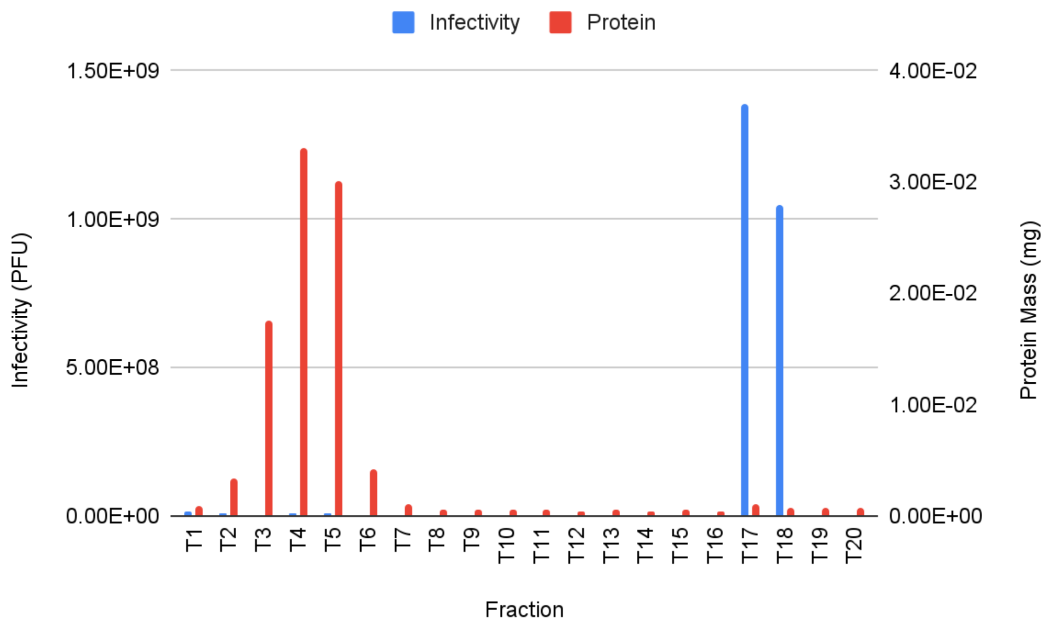

Figure 15. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of condition 3 (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

Two pools were done with the factions which correspond to the chromatographic peaks, being the DNA quantities measured by using a PicoGreen Assay, being this quantification presented in Table 4.

Table 4. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three replicates done for Condition 3 (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	0.7256	0.7256	0.7256
Pool FT	0.3019	0.4212	0.4776
Pool Elution	0.3819	0.3755	0.3833

Condition 4

Below (Figure 16) is shown the chromatogram for the first triplicate of condition 4. As happened with other conditions, the results of the second and third triplicates are available in the Appendix.

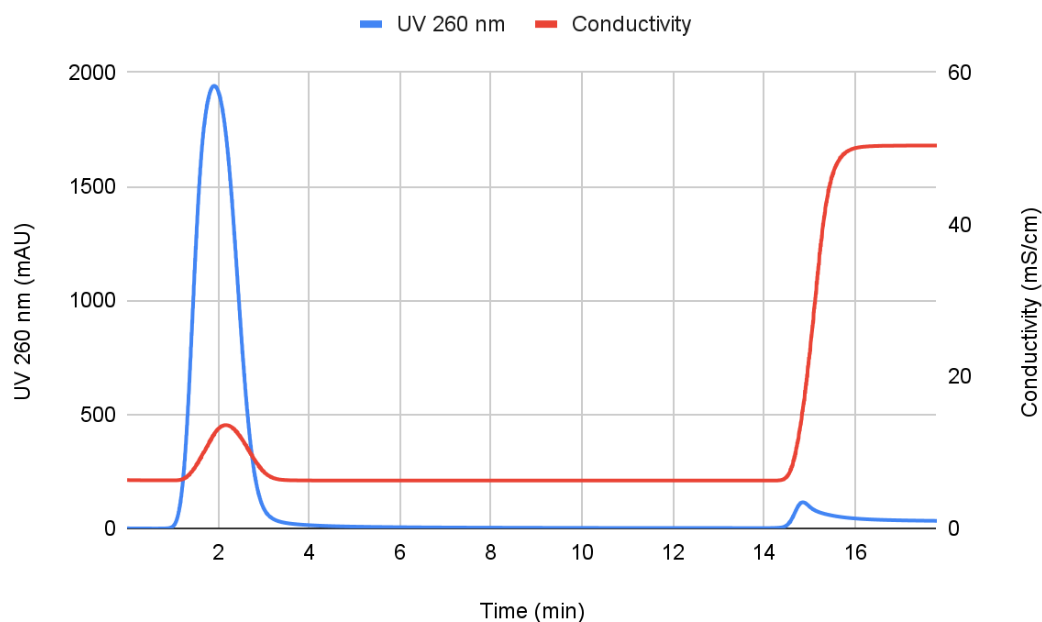

Figure 16. Chromatogram of the first triplicate of Condition 4 (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 7 as elution buffer). The UV, in mAU, measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

The phage infectivity and protein quantity was measured for each fraction collected in the phenyl boronate chromatography, being the results presented in Figure 17.

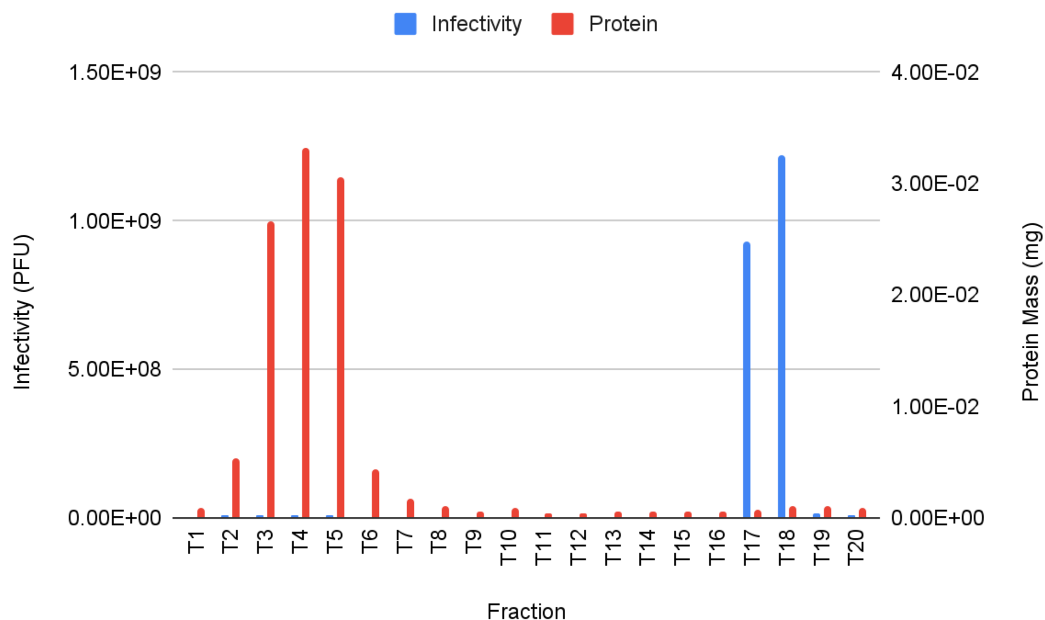

Figure 17. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of condition 3 (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 7 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

In order to measure the DNA quantities, two pools were done with the chromatographic peaks fractions. The measurement was achieved through a PicoGreen Assay. These results are available in Table 5.

Table 5. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three replicates done for Condition 4 (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 7 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	0.7256	0.7256	0.7256
Pool FT	0.1953	0.2127	0.3374
Pool Elution	0.3389	0.3759	0.4461

Nuclease Digestion - Activity Stop by Heat

The DNA quantification on samples from condition 1, 2, 3 and 4, shows that the majority of the remaining DNA stays in the pools which contain the phages. So, in order to decrease DNA quantity, a nuclease digestion was done. To do this, a buffer exchange was performed in order to make sure that the optimal digestion conditions are met, i.e., pH 8 and 2 mM MgCl2.

Nuclease Buffer Exchange

After buffer exchanging the lysate into the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8), using Amicon® centrifugal filters, the samples, in the new buffer, were tested for amount of DNA, protein quantity and phage infectivity. The results are presented in Table 6.

Table 6. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate and the samples present in the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8).

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.96	4.64E-02	7.20E+09
Nuclease Buffer	1.39	1.47E-02	2.34E+09

In this step (nuclease buffer exchange), the phage yield is 32.50%. The DNA and protein removal (in %) is 29.08% and 68.32%, respectively. After having the samples in the nuclease buffer, it is then possible to proceed with digestion.

Nuclease Digestion

For the nuclease digestion, four different nuclease incubation times (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) were tested in triplicates. After each incubation step the mixture is heated to 72 ºC to stop the enzymatic reaction by nuclease inactivation. Each triplicate was tested for phage infectivity, DNA and protein quantity. The results of the first triplicate are shown in Table 7. The results for triplicate 2 and 3 are available in the Appendix.

Table 7. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate, sample present in the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8), sample present in the nuclease buffer after the 72 ºC incubation and for the samples of the first triplicate with nuclease digestion times 30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours, heat stopped.

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.960	4.64E-02	7.20E+09
Nuclease Buffer	1.395	1.47E-02	2.34E+09
Nuclease Buffer (72 ºC)	1.308	1.41E-02	2.25E+08
30 min	0.037 ± 0.005	1.29E-02 ± 6.40E-04	6.45E+07 ± 3.77E+06
1 h	0.038 ± 0.016	1.43E-02 ± 1.68E-03	1.61E+08 ± 3.88E+07
1 h 30 min	0.167 ± 0.033	1.02E-02 ± 4.88E-04	1.73E+08 ± 2.49E+7
2 h	0.059 ± 0.096	1.51E-02 ± 2.72E-03	2.31E+08 ± 1.28E+07

In this step (nuclease digestion), there are 2 different yields to consider, the yield of the nuclease digestion step (relation between the amount of phages after and before nuclease digestion) and the total yield (relation between the amount of phages after the nuclease digestion and the lysate), which also accounts for phage losses during the buffer exchange step. Both yields are available in Table 8. It is important to note that the 72 ºC incubation also presents 2 different yields, the heat incubation one, which is 9.61%, and a total yield, 3.13%, which include the incubation and the buffer exchange. Note that this incubation was performed for 10 minutes to the sample present in the nuclease buffer, without nuclease digestion.

Table 8. Average Phage Digestion and Total Yields for each incubation time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) in the heat stopped nuclease digestion.

	Digestion Yield (%)	Total Yield (%)
30 min	27.11 ± 0.02	0.85 ± 0.00
1 h	86.67 ± 0.17	2.71 ± 0.01
1 h 30 min	66.44 ± 0.11	2.08 ± 0.00
2 h	96.22 ± 0.06	3.01 ± 0.00

There are, also, 2 different DNA and protein removal to consider, the removal during the nuclease digestion step (relation between the amount of DNA/protein after and before nuclease digestion) and the total removal (relation between the amount of DNA/protein after the nuclease digestion and the lysate). Both DNA/protein removals are available in Table 9.

Table 9. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) heat stopped tested in the nuclease digestion.

	Digestion DNA Removal (%)	Total DNA Removal (%)	Digestion Protein Removal (%)	Total Protein Removal(%)
30 min	97.34 ± 0.00	98.23 ± 0.00	8.31 ± 0.04	70.90 ± 0.01
1 h	98.22 ± 0.01	98.81 ± 0.01	10.46 ± 0.11	71.59 ± 0.04
1 h 30 min	84.37 ± 0.03	89.57 ± 0.02	27.19 ± 0.03	76.90 ± 0.01
2 h	90.57 ± 0.07	93.71 ± 0.05	18.71 ± 0.19	74.20 ± 0.06

These samples were further processed by Phenyl Boronate Chromatography. The results obtained after each incubation time are presented below.

Phenyl Boronate Chromatography - 30 Minutes Digestion

The chromatogram corresponding to the 30 minutes digestion is presented in Figure 18. The results of the second and third triplicates are shown in the Appendix.

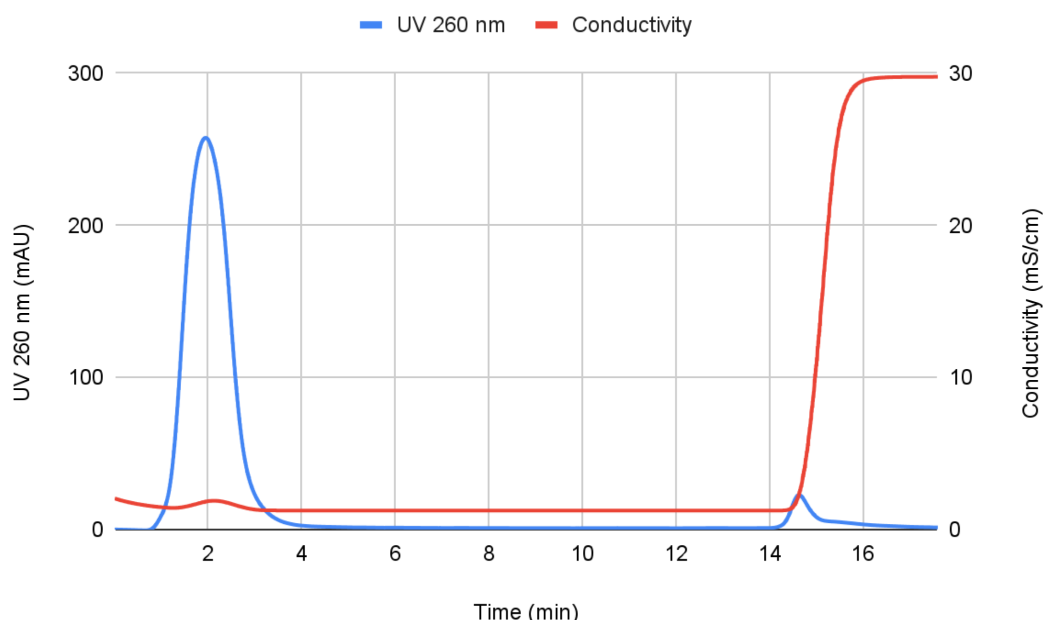

Figure 18. First triplicate of the 30 minutes digestion heat stopped chromatogram, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

Phages infectivity and protein quantity after the Phenyl Boronate chromatography per fraction collected during this process is presented in Figure 19.

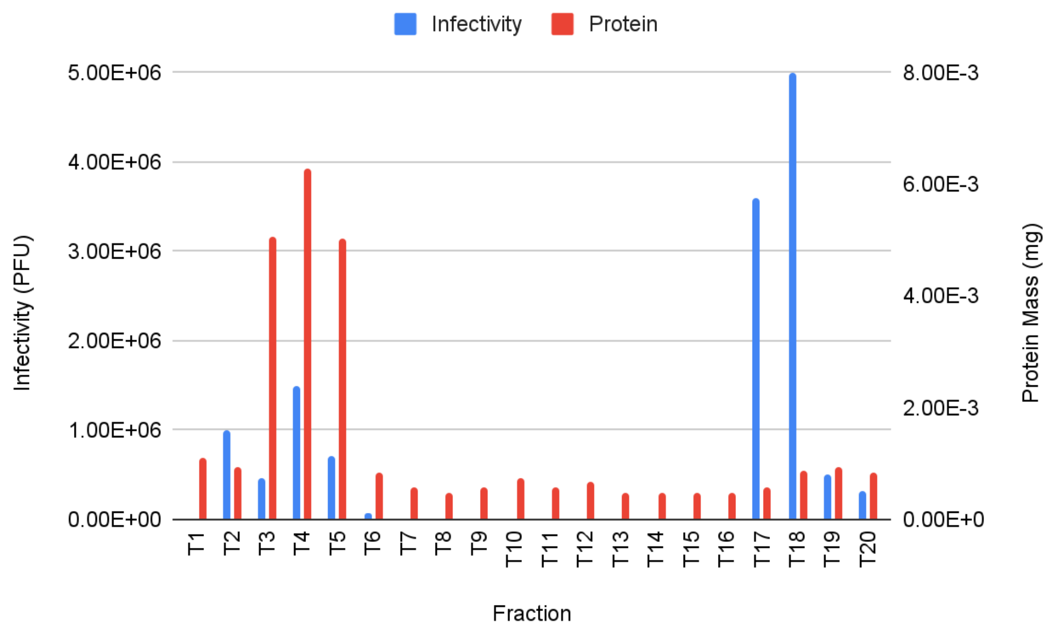

Figure 19. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 30 minutes digestion heat stopped, after the nuclease digestion, in each fraction (20 fractions total) collected during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

To quantify the DNA present in the samples, two pools of the fractions samples were prepared (the first pool was done with fractions T2, T3, T4, T5 and T6 and the second with fractions T17, T18 and 19). A PicoGreen Assay was performed on both pool samples and the DNA quantities are available in Table 10.

Table 10. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 30 minutes digestion heat stopped, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00	1.96E+00
Pool FT	1.30E-01	1.67E-01	9.95E-02
Pool Elution	3.75E-02	4.06E-02	3.15E-02

Phenyl Boronate Chromatography - 1 Hour Digestion

The first triplicate chromatogram of the 1 hour digestion is available in Figure 20. The results of the second triplicate are present in the Appendix.

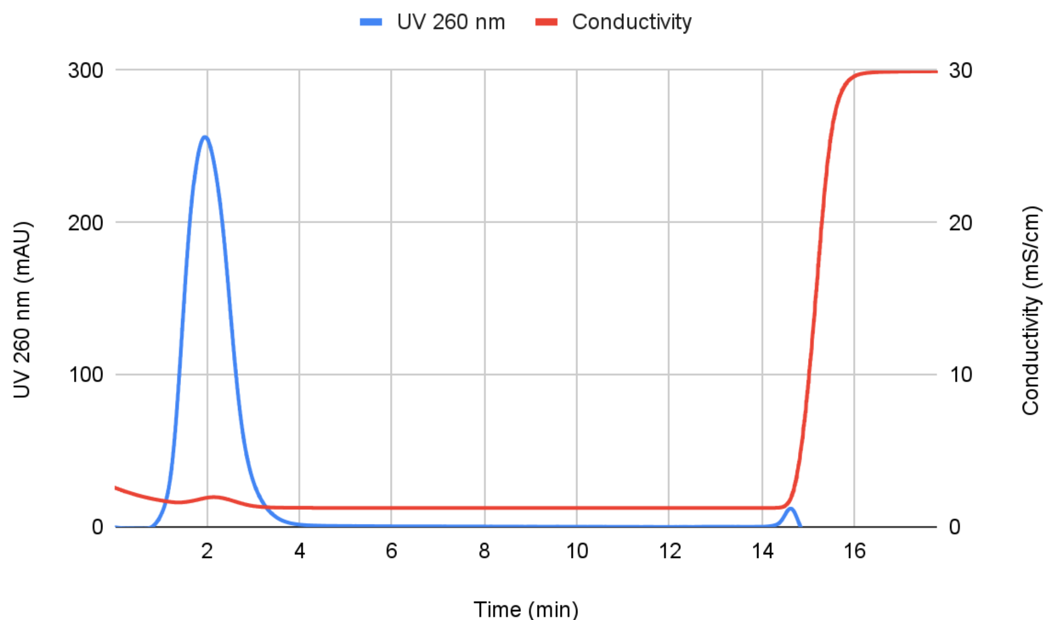

Figure 20. First triplicate of the 1 hour digestion heat stopped chromatogram, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

Phage infectivity and protein quantity were measured after the chromatography by a plaque assay and a BCA assay, respectively. The results of the first triplicate are shown in Figure 21, the results of the second and third triplicates are available in the Appendix.

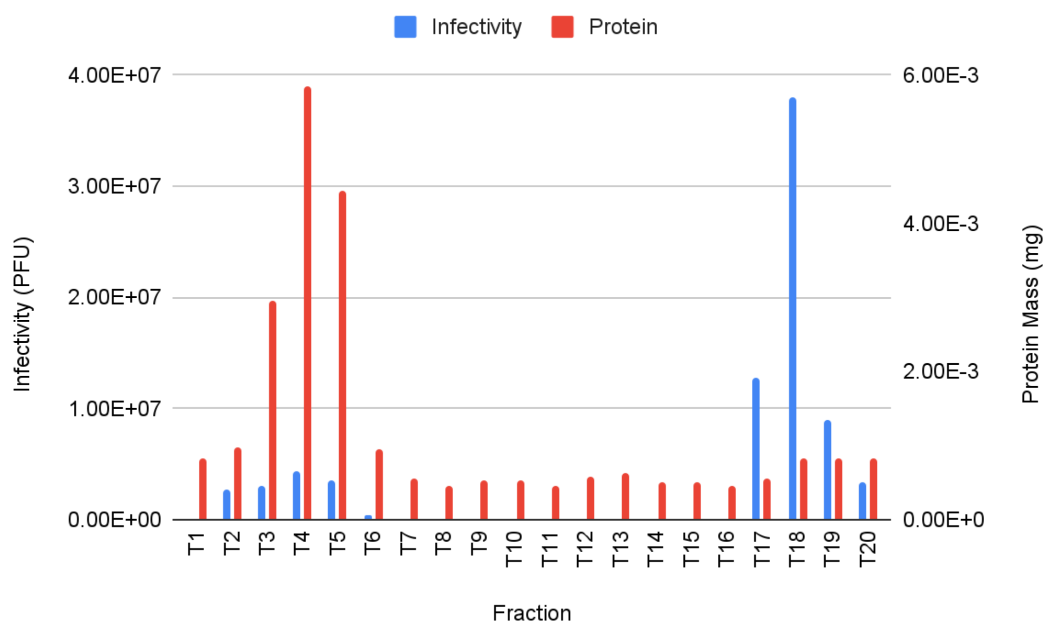

Figure 21. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 1 hour digestion heat stopped, after the nuclease digestion, in each fraction (20 fractions total) collected during phenyl

boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

A PicoGreen Assay was used in order to quantify the DNA present in the sample Pools. The quantities for each pool is presented in Table 11.

Table 11. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 1 hour digestion heat stopped, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00	1.96E+00
Pool FT	2.52E-02	1.65E-02	1.47E-02
Pool Elution	3.96E-03	5.78E-03	1.14E-02

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

The chromatogram for the first triplicate of tests of a 1 hour and 30 minutes digestion is presented below. The results of the second and third triplicates are shown in the Appendix.

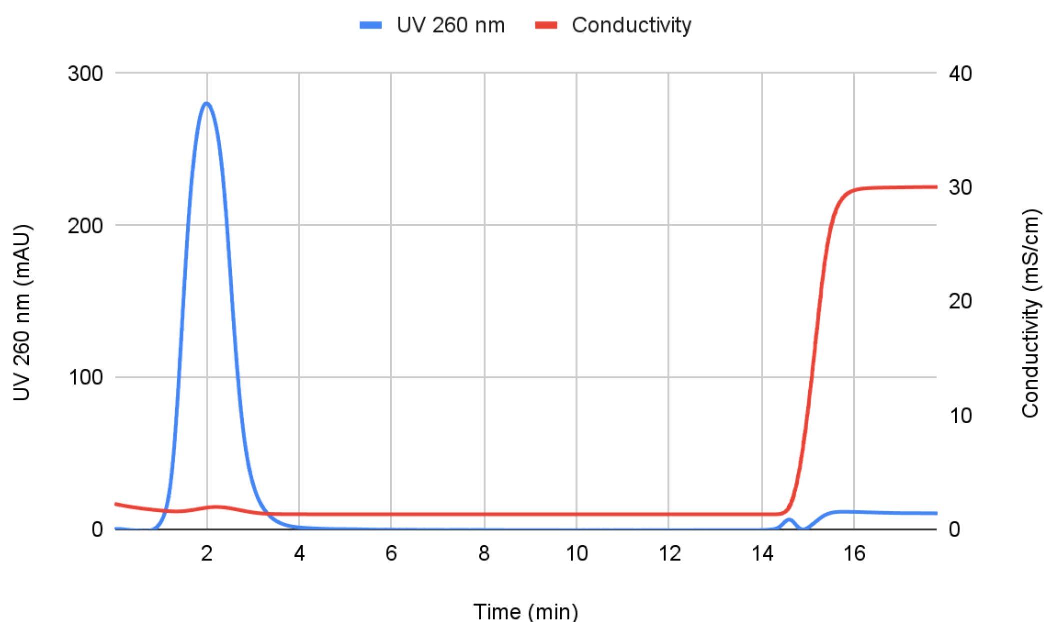

Figure 22. First triplicate of the 1 hour and 30 minutes digestion heat stopped chromatogram, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time.

For each chromatographic fraction collected, the phage infectivity and the protein quantity was measured, being the results available in Figure 23.

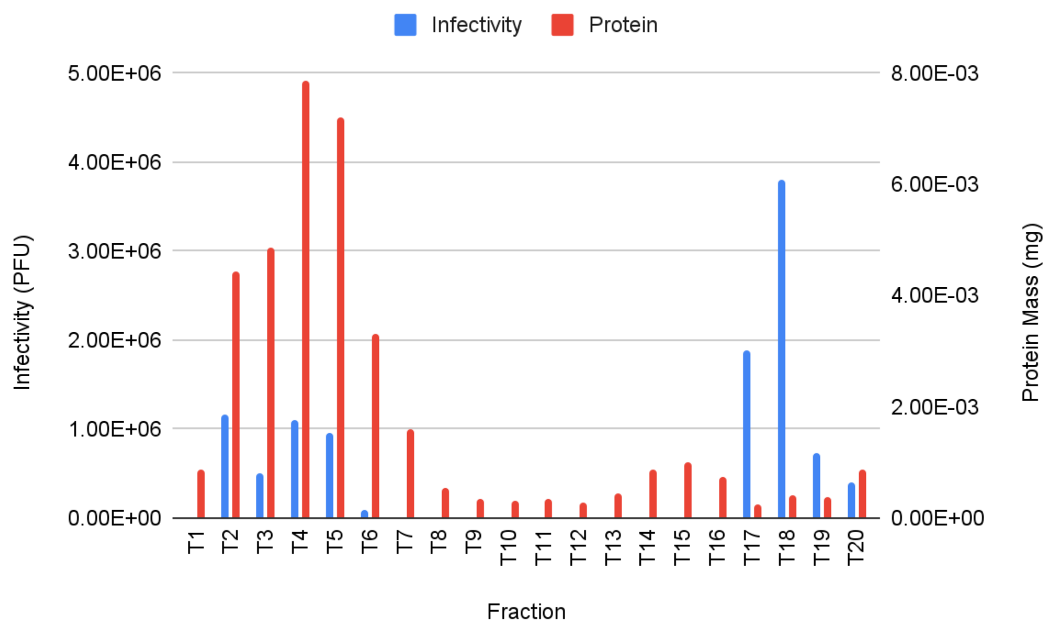

Figure 23. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 1 hour and 30 minutes digestion heat stopped, after the nuclease digestion, in each fraction (20 fractions total) collected during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Two pools were done with the fractions which correspond to the chromatographic peaks, being the DNA quantities measured by using a PicoGreen Assay. This quantification is presented in Table 12.

Table 12. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 1 hour and 30 minutes digestion heat stopped, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00	1.96E+00
Pool FT	5.38E-03	9.05E-03	4.67E-03
Pool Elution	1.01E-03	4.25E-03	2.40E-03

Phenyl Boronate Chromatography - 2 Hours Digestion

In Figure 24 is shown the chromatogram for the first triplicate of the 2 hours digestion. The results of the second and third triplicate are available in the Appendix.

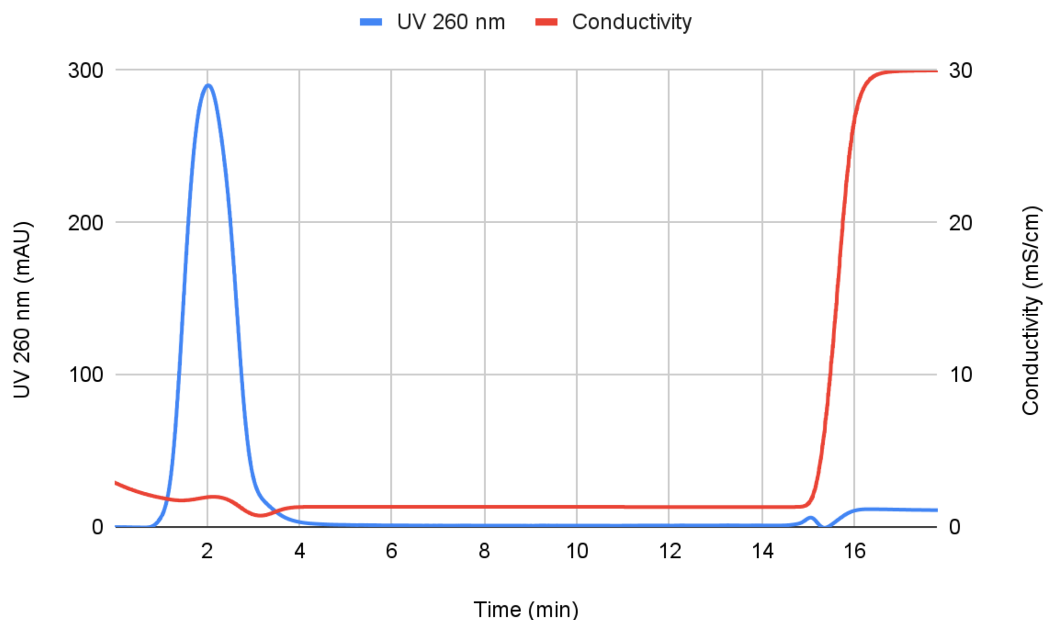

Figure 24. First triplicate of the 2 hour digestion heat stopped chromatogram, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

The phage infectivity and protein quantity was measured for each fraction collected in the phenyl boronate chromatography. The results are presented in Figure 25.

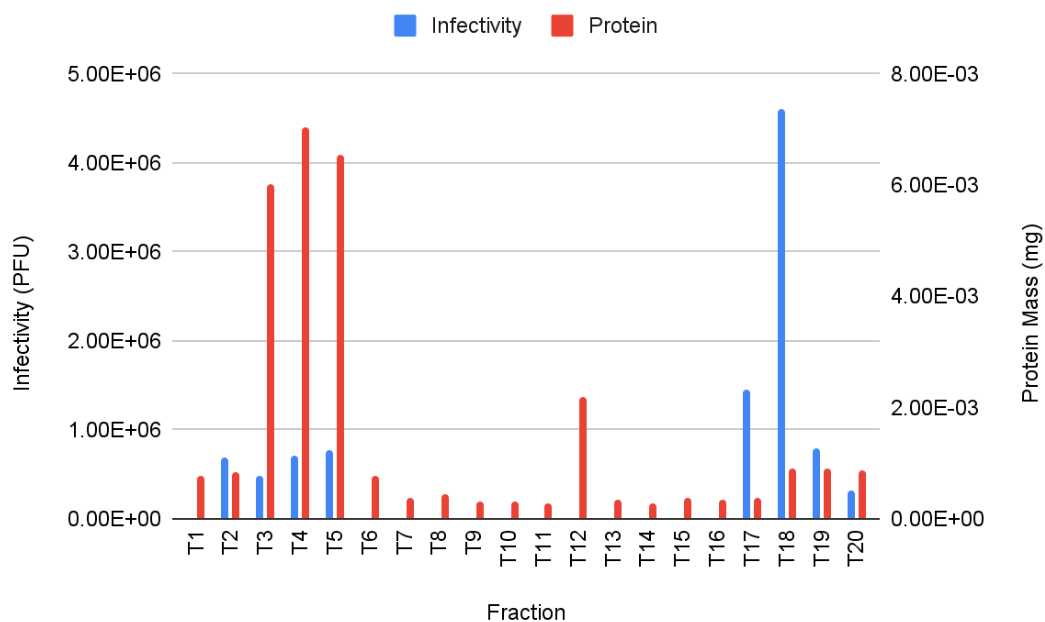

Figure 25. Phages infectivity, PFU, and Protein Mass in mg, for the first duplicate of 2 hours digestion heat stopped, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption

buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

In order to measure the DNA quantities, two pools were done with the chromatographic peaks fractions. The measurement was achieved through a PicoGreen Assay. These results are available in Table 13.

Table 13. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 2 hour digestion heat stopped, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00	1.96E+00
Pool FT	6.23E-03	1.19E-02	1.32E-02
Pool Elution	5.58E-03	7.32E-03	3.23E-03

Phenyl Boronate Chromatography - Yields

For the chromatographic steps, there are also 2 different yields to consider, the chromatographic yield itself (relation between the amount of phages after and before chromatographic step) and total yield (relation between the amount of phages after the chromatographic step and the lysate).

Table 14. Average Phage Yield for each Digestion time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) heat stopped, after the nuclease digestion, tested in the Phenyl Boronate Chromatography.

	Chromatographic Yield (%)	Total Yield (%)
30 min	9.16 ± 4.36	0.14 ± 0.02
1 h	31.15 ± 7.14	1.31 ± 0.17
1 h 30 min	9.37 ± 6.14	0.29 ± 0.14
2 h	3.16 ± 0.98	0.15 ± 0.03

Again, as in the previous steps, there should be considered 2 different DNA and protein removal, the chromatographic removal and the total removal. Both the removals are available in Table 15.

Table 15. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) heat stopped, after nuclease digestion, tested in the Phenyl Boronate Chromatography.

	Chromatography DNA Removal (%)	Total DNA Removal (%)	Chromatography Protein Removal (%)	Total Protein Removal(%)
30 min	15.20 ± 9.35	98.14 ± 0.24	82.52 ± 0.86	94.92 ± 0.14
1 h	52.20 ± 38.70	99.64 ± 0.20	82.31 ± 2.58	95.03 ± 0.21
1 h 30 min	99.49 ± 0.09	99.87 ± 0.08	90.27 ± 0.32	97.75 ± 0.09
2 h	89.08 ± 2.47	99.73 ± 0.10	81.04 ± 4.25	95.25 ± 0.54

Adsorption Buffer Exchange

In order to understand the impact of salt concentration in the Phenyl Boronate Chromatography, an exchange of buffers into the adsorption buffer (15 mM of Tris-HCl, pH 7) was performed after the digestion step and before the chromatography. Due to inconsistencies in values, the triplicates 30 minutes 3, 1 hour 3 and 2 hours 3, were discarded. The samples were tested for phage infectivity, DNA and protein quantity, being the results of the first duplicate/triplicate shown in Table 16. The results for duplicates and triplicates are available in the Appendix.

Table 16. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate, sample present in the adsorption buffer (15 mM of Tris-HCl, pH 7) and for the samples of the first duplicate/triplicate with nuclease digestion times 30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours, heat stopped.

	Duplicate/Triplicate 1		
Sample	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.96E+00	4.64E-02	7.20E+09
Adsorption Buffer	1.01E+00	4.13E-03	6.30E+06
30 min	2.61E-03 ± 9.74E-03	1.81E-04 ± 1.94E-05	6.90E+06 ± 3.54E+06
1 h	2.33E-03 ± 2.41E-03	1.63E-05 ± 3.87E-05	9.75E+05 ± 9.55E+04
1 h 30 min	3.48E-04 ± 1.14E-02	1.63E-05 ± 1.58E-05	2.15E+06 ± 4.59E+06
2 h	6.37E-03 ± 6.76E-02	4.37E-05 ± 5.81E-05	2.36E+06 ± 7.11E+05

Again, in this step (adsorption buffer exchange), there are 2 different yields to consider, buffer exchange yield (relation between the amount of phages after and before buffer exchange) and the total yield (relation between the amount of phages after the buffer exchange and the lysate). Both yields are available in Table 17.

Table 17. Average Phage Buffer Exchange and Total Yields for each Digestion time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) stopped by heat tested in the buffer exchange.

	Buffer Exchange Yield (%)	Total Yield (%)
30 min	7.01 ± 0.05	0.06 ± 0.00
1 h	0.48 ± 0.00	0.01 ± 0.00
1 h 30 min	9.27 ± 0.03	0.19 ± 0.00
2 h	0.84 ± 0.04	0.03 ± 0.00

As in the nuclease digestion, in this step there are 2 different DNA and protein removal to consider, the nuclease digestion removal and the total removal. Both the removals are available in Table 18.

Table 18. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) stopped by heat tested in the buffer exchange.

	Buffer Exchange DNA Removal (%)	Total DNA Removal (%)	Buffer Exchange Protein Removal (%)	Total Protein Removal(%)
30 min	67.90 ± 0.36	99.56 ± 0.00	93.92 ± 0.01	99.64 ± 0.00
1 h	53.43 ± 0.57	99.81 ± 0.00	89.78 ± 0.09	99.91 ± 0.00
1 h 30 min	93.98 ± 0.05	99.38 ± 0.01	93.60 ± 0.03	99.95 ± 0.00
2 h	90.33 ± 0.01	99.41 ± 0.00	69.61 ± 0.03	99.94 ± 0.00

These samples were used in a Phenyl Boronate Chromatography, being the results presented below.

Phenyl Boronate Chromatography - 30 Minutes Digestion

In Figure 26 is shown the 30 minutes digestion chromatogram. Even though this digestion time was tested in triplicates, Figures below correspond to the first one (being the other, second one, available in the Appendix). The third triplicate was discarded due to inconsistencies in the results.

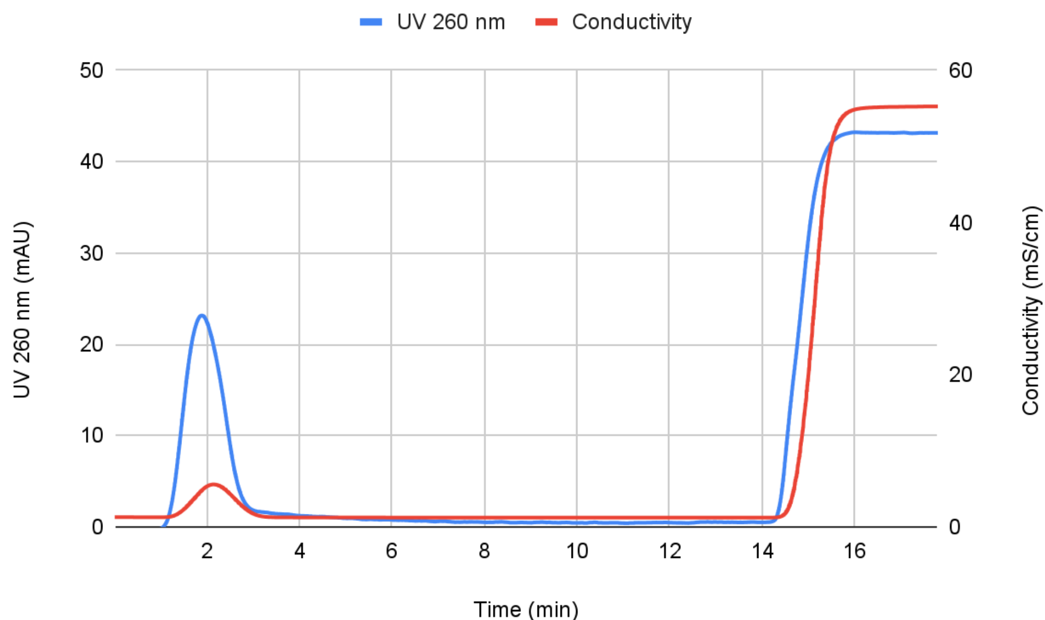

Figure 26. Chromatogram of the first duplicate of 30 minutes digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

Figure 27 presents the infectivity of phages and the quantity of protein after phenyl boronate chromatography per fraction collected during the chromatography.

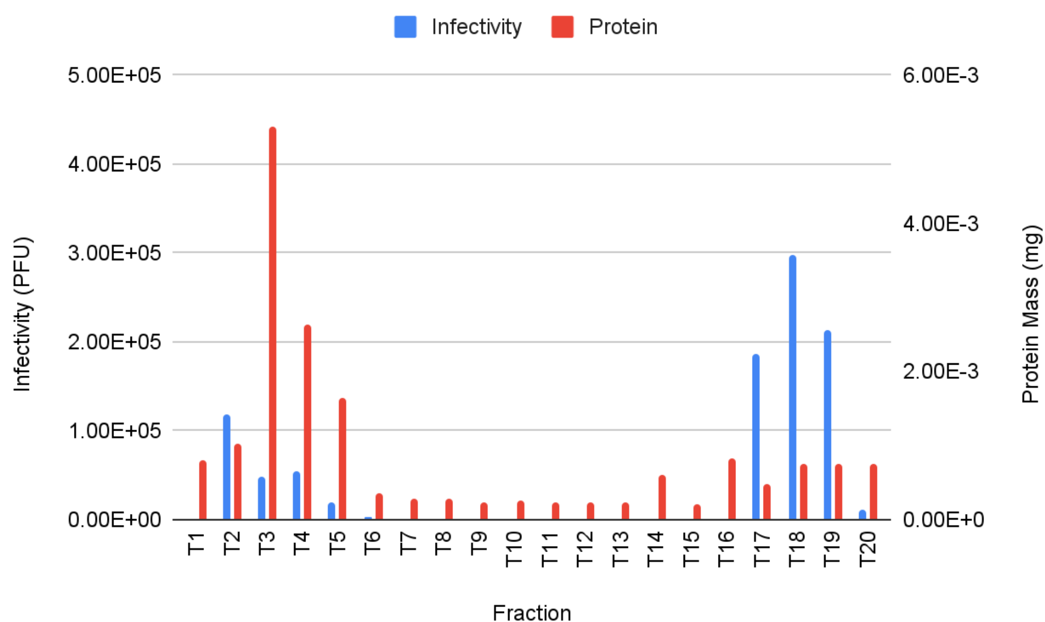

Figure 27. Phages infectivity, PFU, and Protein Mass in mg, for the first duplicate of 30 minutes digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as

equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

A PicoGreen Assay was performed on two Pools of samples in order to understand the DNA quantity present in the chromatographic peaks. These quantities are available in Table 19. Note that the first pool was done with fractions T2, T3, T4, T5 and T6. The second pool was obtained only by fractions T17, T18 and 19.

Table 19. DNA quantity in µgfor the Lysate, Pool FT and Pool Elution, for the two duplicates done for the 30 minutes digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Duplicate 1	Duplicate 2
Sample	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00
Pool FT	2.56E-03	5.69E-03
Pool Elution	2.82E-03	5.47E-03

Phenyl Boronate Chromatography - 1 Hour Digestion

The chromatogram corresponding to the first triplicate of the 1 hour digestion is available in Figure 28. The results of the second triplicate are present in the Appendix.

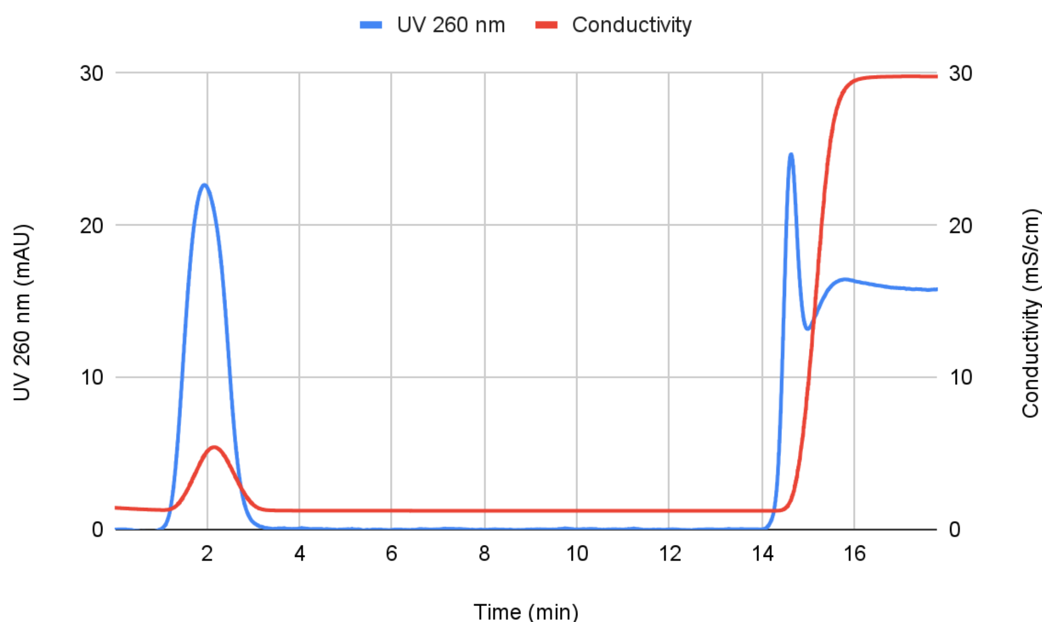

Figure 28. Chromatogram of the first duplicate of 1 hour digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

After the chromatography, the phage's infectivity and protein quantity were measured, being presented in Figure 29.

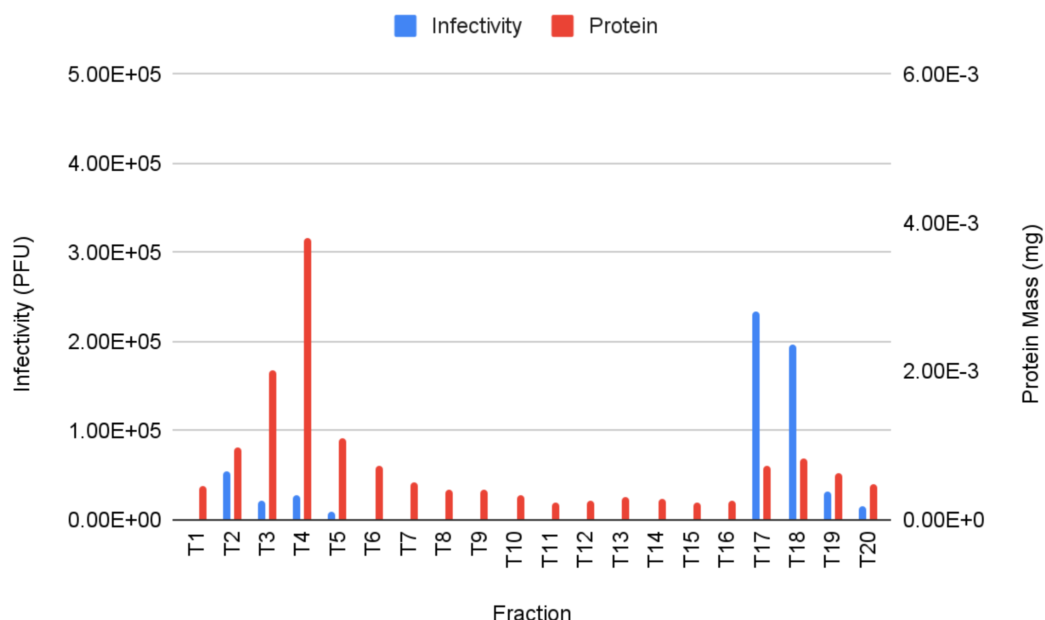

Figure 29. Phages infectivity, PFU, and Protein Mass in mg, for the first duplicate of 1 hour digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

The DNA present in the chromatographic peaks was quantified using a PicoGreen Assay. The DNA quantities are available in Table 20.

Table 20. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the two duplicates done for the 1 hour digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Duplicate 1	Duplicate 2
Sample	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00
Pool FT	2.02E-03	2.92E-03
Pool Elution	2.32E-03	5.00E-03

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

The chromatogram for the first triplicate of tests of a 1 hour and 30 minutes digestion is presented below. The results of the second and third triplicates are shown in the Appendix.

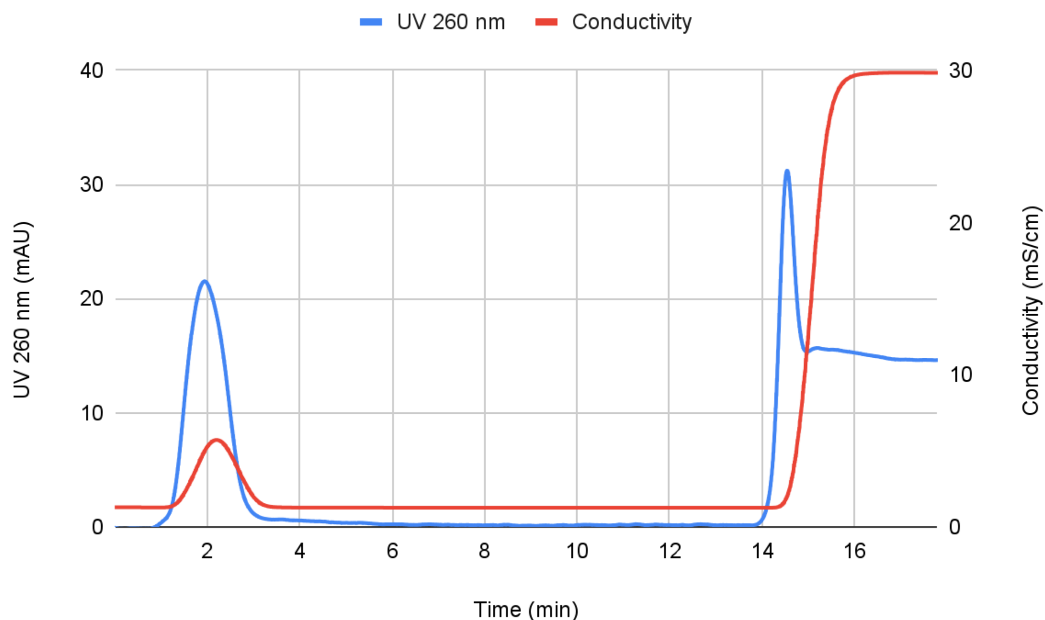

Figure 30. Chromatogram of the first triplicate of 1 hour and 30 minutes digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

For each fraction collected during the chromatography, phage infectivity and protein quantity was measured, being the results available in Figure 31.

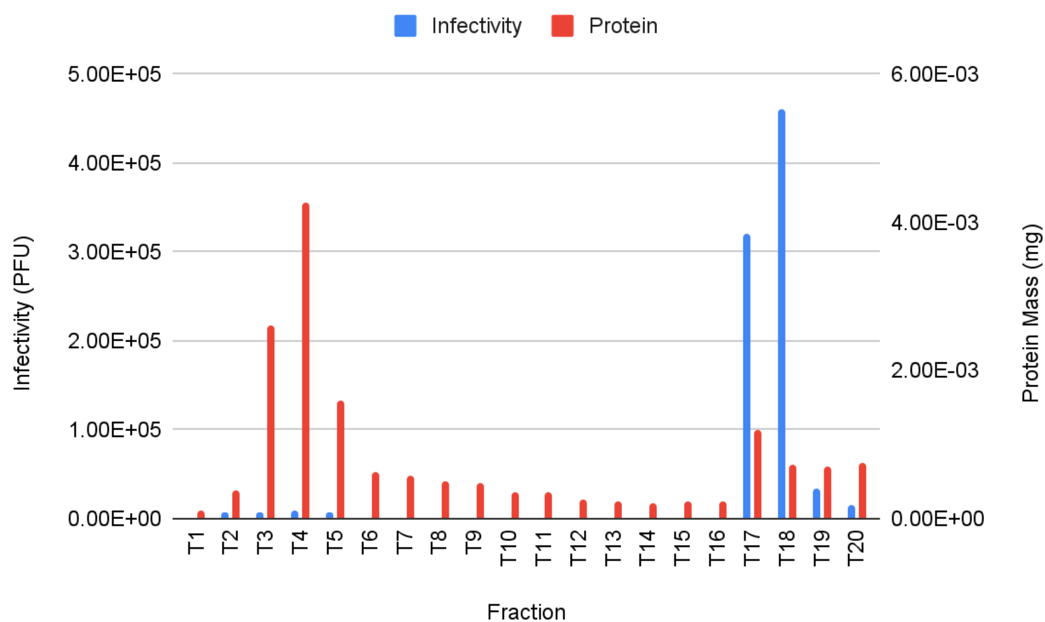

Figure 31. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 1 hour and 30 minutes digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as

equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

Two pools were done with the fractions which correspond to the chromatographic peaks, being the DNA quantities measured by using a PicoGreen Assay, being this quantification presented in Table 21.

Table 21. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three replicates done for the 1 hour and 30 minutes digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00	1.96E+00
Pool FT	2.33E-04	1.22E-03	1.17E-03
Pool Elution	2.39E-03	3.97E-03	5.22E-03

Phenyl Boronate Chromatography - 2 Hours Digestion

Below (Figure 32) is presented as the chromatogram for the first duplicate of a 2 hours digestion. The results of the second duplicate are available in the Appendix.

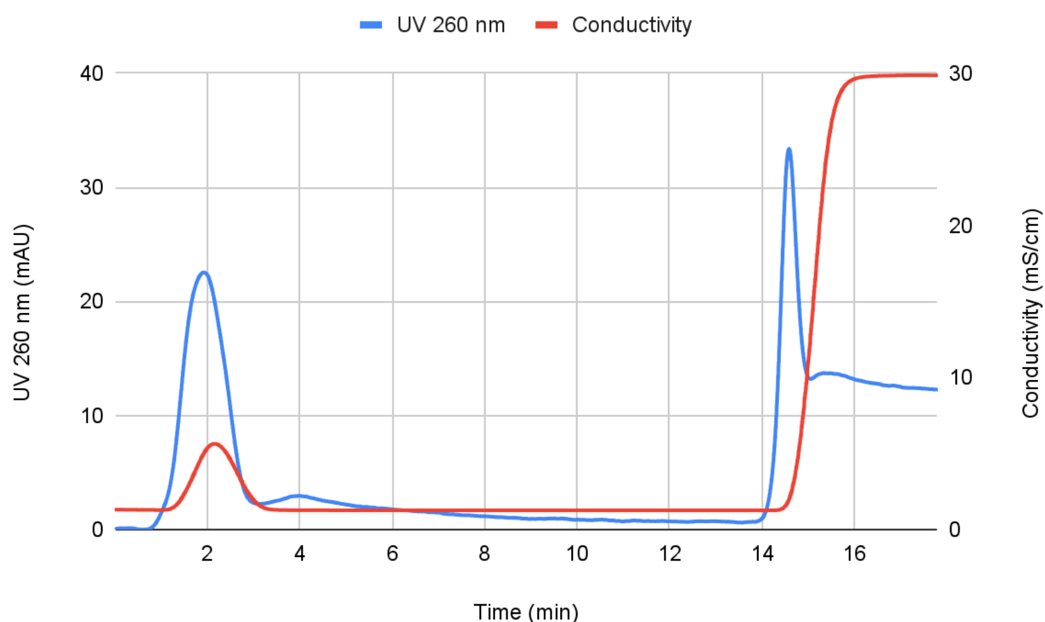

Figure 32. Chromatogram of the first duplicate of 2 hours digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

The phage infectivity and protein quantity was measured for each fraction collected in the phenyl boronate chromatography, being the results presented in Figure 33.

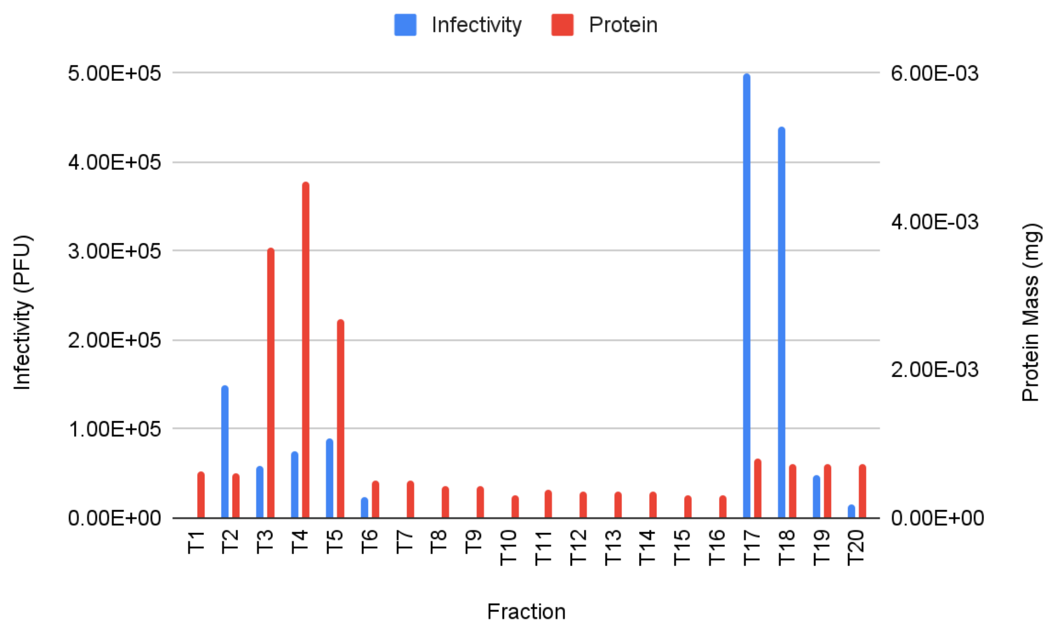

Figure 33. Phages infectivity, PFU, and Protein Mass in mg, for the first duplicate of 2 hours digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction (20 fractions total) collected during phenyl boronate chromatography.

In order to measure the DNA quantities, two pools were done with the chromatographic peaks fractions. The measurement was achieved through a PicoGreen Assay. These results are available in Table 22.

Table 22. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the two duplicates done for the 2 hours digestion stopped by heat, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Duplicate 1	Duplicate 2
Sample	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.96E+00	1.96E+00
Pool FT	1.40E-03	1.71E-03
Pool Elution	3.10E-03	7.30E-03

Phenyl Boronate Chromatography - Yields

In the chromatographic step there are, also, 2 different yields to consider, chromatographic yield (relation between the amount of phages after and before chromatographic step) and total yield (relation between the amount of phages after the chromatographic step and the lysate).

Table 23. Average Phage Yield for each Digestion time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours), after the adsorption buffer exchange, tested in the Phenyl Boronate Chromatography.

	Chromatographic Yield (%)	Total Yield (%)
30 min	19.15 ± 11.41	0.03 ± 0.02
1 h	7.56 ± 4.84	0.01 ± 0.00
1 h 30 min	23.44 ± 10.53	0.03 ± 0.01
2 h	12.25 ± 4.84	0.02 ± 0.01

Again, as in the previous steps, there should be considered 2 different DNA and protein removal, the chromatographic removal and the total removal. Both the removals are available in Table 24.

Table 24. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours), after the adsorption buffer exchange, tested in the Phenyl Boronate Chromatography.

	Chromatography DNA Removal (%)	Total DNA Removal (%)	Chromatography Protein Removal (%)	Total Protein Removal(%)
30 min	29.42 ± 52.61	99.79 ± 0.10	51.88 ± 0.00	99.93 ± 0.00
1 h	8.28 ± 8.91	99.81 ± 0.10	47.88 ± 1.41	99.93 ± 0.13
1 h 30 min	31.53 ± 6.61	99.80 ± 0.07	39.38 ± 5.22	99.92 ± 0.46
2 h	72.10 ± 29.33	99.73 ± 0.15	46.88 ± 2.83	99.93 ± 0.25

Nuclease Digestion - Activity Stop by Cold

The infectivity analysis of samples from digestion stopped by heat show a huge loss of infectivity, especially during the 10 minute heating (72 ºC) step. In an attempt to decrease the loss of phages, the digestion was stopped using an ice incubation. Again, to do this, a buffer exchange was necessary.

Nuclease Buffer Exchange

Amicon® centrifugal filters were used to exchange the sample buffer into the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8). After this procedure the samples, in the new buffer, were tested for amount of DNA, protein quantity and phage infectivity. The results are presented in Table 25.

Table 25. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate and the samples present in the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8).

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.21	5.94E-02	1.61E+10
Nuclease Buffer	1.04	1.99E-02	1.02E+10

The nuclease buffer exchange stepp presents a phage yield of 63.55%. In this step DNA and protein removal (in %) are 14.05% and 66.50%, respectively. After having the samples in the nuclease buffer, it is possible to proceed with digestion.

Nuclease Digestion

In this nuclease digestion step, two different nuclease incubation times (30 minutes and 1 hour and 30 minutes) were tested in triplicates. Each triplicate was tested for phage infectivity, DNA and protein quantity. The results of the first triplicate are shown in Table 26, being the other triplicate available in the Appendix.

Table 26. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate, sample present in the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8), sample present in the nuclease buffer after the ice incubation and for the samples of the first triplicate with nuclease digestion times 30 minutes and 1 hour and 30 minutes, cold stopped.

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.211	5.94E-02	1.61E+10
Nuclease Buffer	1.036	1.99E-02	1.02E+10
Nuclease Buffer (Ice)	1.001	1.99E-02	9.30E+09
30 min	0.336 ± 0.032	1.67E-02 ± 8.90E-04	8.55E+09 ± 2.44E+09
1 h 30 min	0.549 ± 0.024	1.93E-02 ± 1.04E-04	4.80E+09 ± 9.18E+08

In this nuclease digestion step, as has happened in the previous one, there are 2 different yields to consider, the nuclease digestion yield and the total yield, being both in Table 27. It is important to note that the ice incubation also presents 2 different yields, the ice incubation one (during 30 minutes), which is 91.18%, and a total yield, 57.76%.

Table 27. Average Phage Digestion and Total Yields for each Time (30 minutes and 1 hour and 30 minutes) cold stopped tested in the nuclease digestion.

	Digestion Yield (%)	Total Yield (%)
30 min	56.42 ± 0.24	35.86 ± 0.15
1 h 30 min	47.94 ± 0.09	30.47 ± 0.06

Again, as before, there are 2 different DNA and protein removal to consider, the nuclease digestion removal and the total removal. Both DNA/protein removals are available in Table 28.

Table 28. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes and 1 hour and 30 minutes) cold stopped tested in the nuclease digestion.

	Digestion DNA Removal (%)	Total DNA Removal (%)	Digestion Protein Removal (%)	Total Protein Removal(%)
30 min	66.26 ± 0.03	71.13 ± 0.03	11.23 ± 0.04	70.21 ± 0.01
1 h 30 min	44.59 ± 0.02	52.59 ± 0.02	2.61 ± 0.01	67.32 ± 0.00

These samples were, then, used in a Phenyl Boronate Chromatography. The results obtained from this chromatography are presented below.

Phenyl Boronate Chromatography - 30 Minutes Digestion

In Figure 34 is presented the chromatogram corresponding to the sample which was digested for 30 minutes. The results of the second and third triplicates are shown in the Appendix.

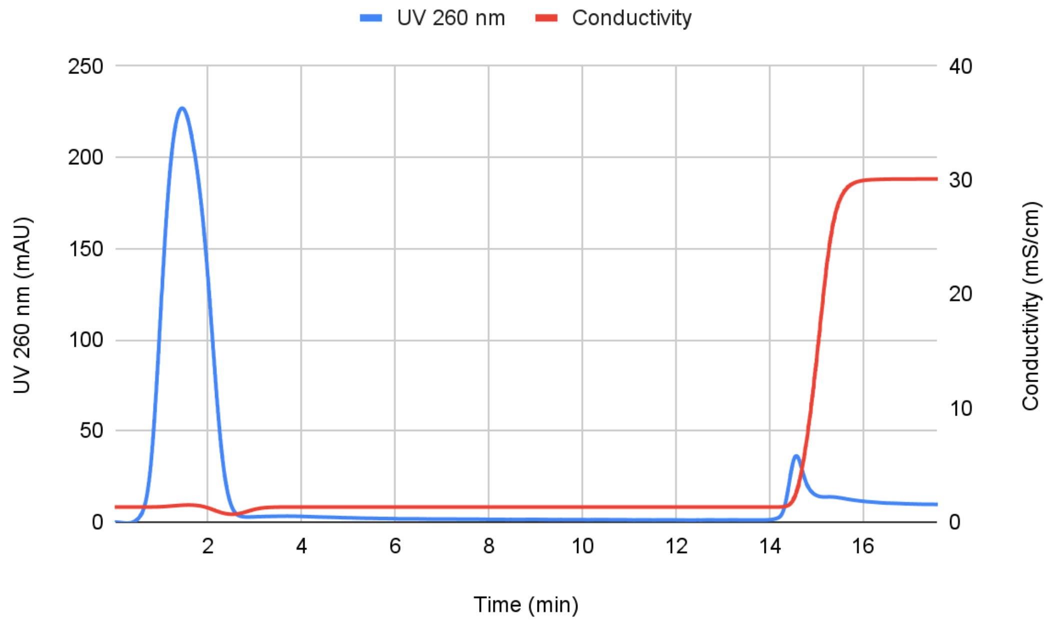

Figure 34. First triplicate of the 30 minutes digestion chromatogram stopped by cold, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

Figure 35 presents the infectivity of phages and the quantity of protein which were tested after phenyl boronate chromatography per fraction collected during the chromatography.

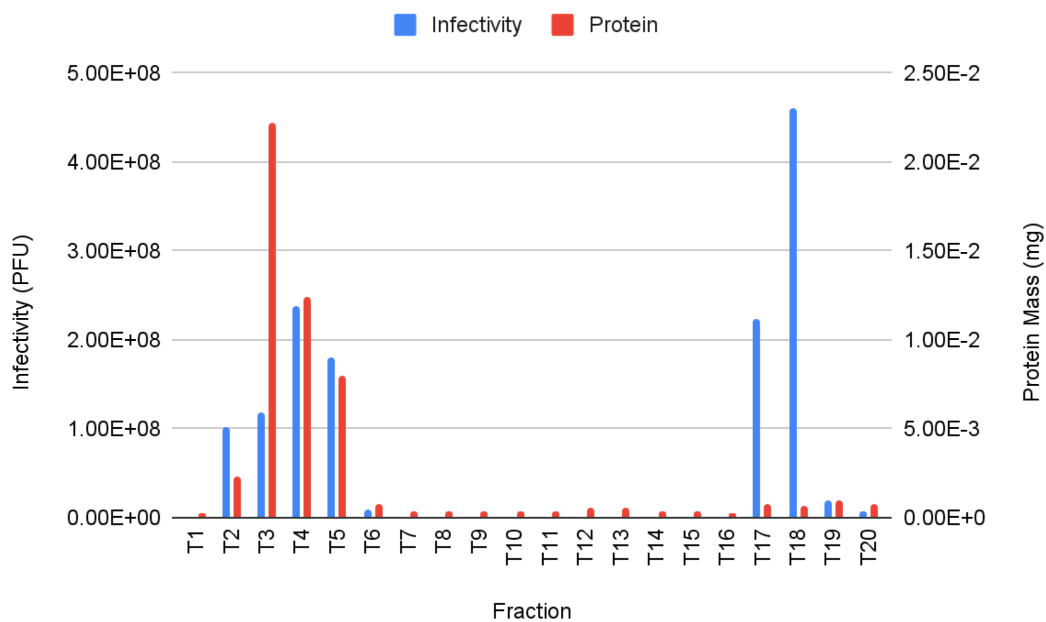

Figure 35. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 30 minutes digestion stopped by cold, after the nuclease digestion, in each fraction (20 fractions total) collected

during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Two pools of the fractions samples were prepared (the first pool was done with fractions T2, T3, T4, T5 and T6 and the second with fractions T17, T18 and 19) in order to quantify the DNA present in those samples. A PicoGreen assay was performed on the pools. The DNA quantities are available in Table 29.

Table 29. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 30 minutes digestion stopped by cold, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.21E+00	1.21E+00	1.21E+00
Pool FT	1.45E-01	1.70E-01	1.59E-01
Pool Elution	5.59E-02	5.57E-02	6.29E-02

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

The first chromatogram triplicate of digestion time 1 hour and 30 minutes is shown in Figure 36. The results of the second and third triplicates are shown in the Appendix.

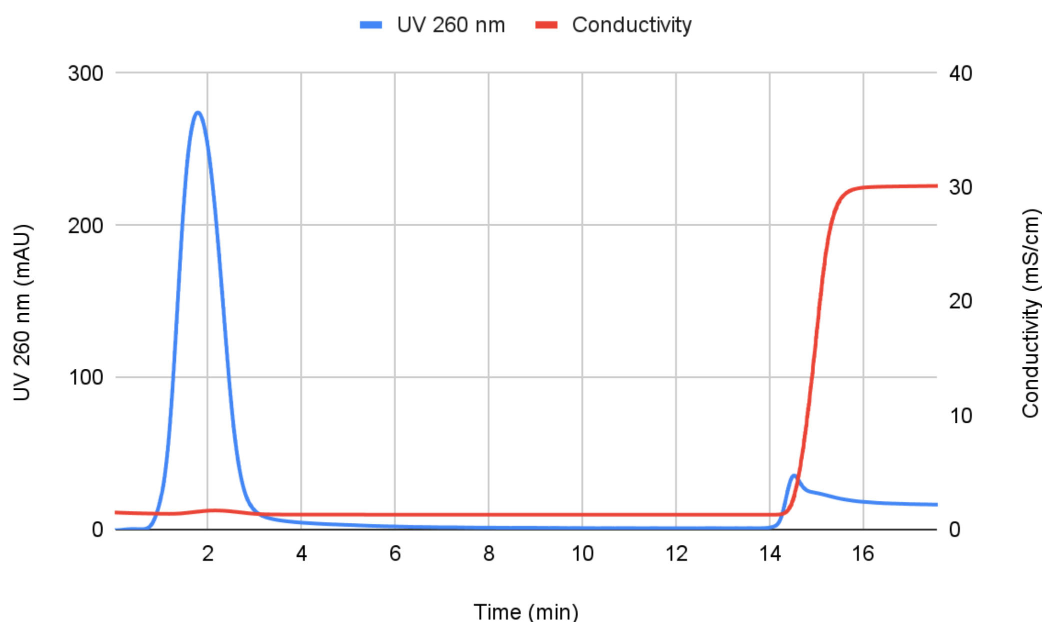

Figure 36. First triplicate of the 1 hour and 30 minutes digestion cold stopped chromatogram, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

For each chromatographic fraction collected, the phage infectivity and the protein quantity was measured, being the results available in Figure 37.

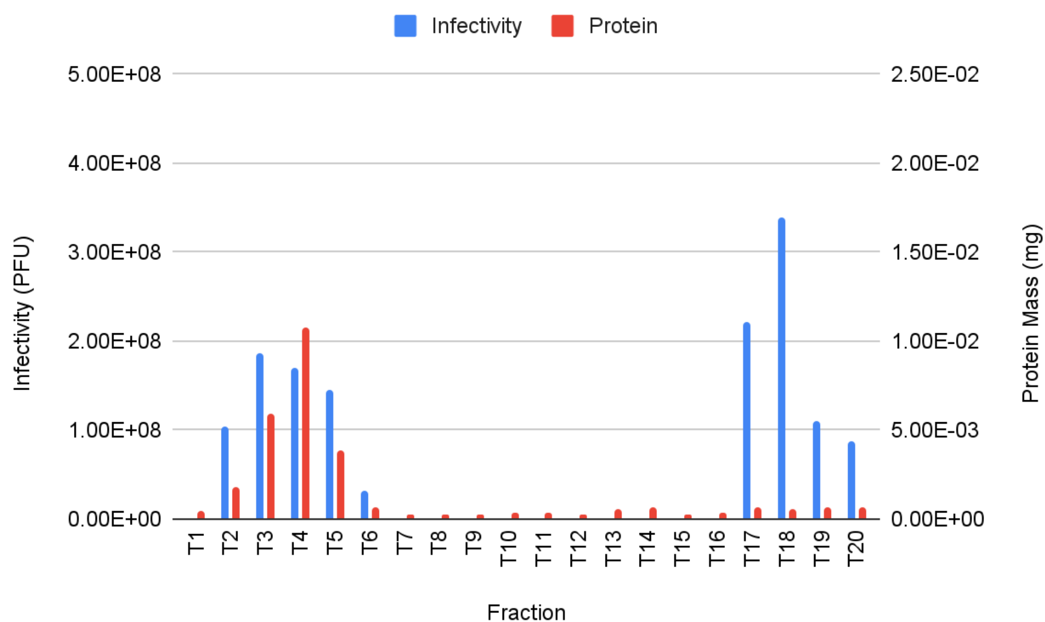

Figure 37. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 1 hour and 30 minutes digestion, stopped, after the nuclease digestion, in each fraction (20 fractions total) collected during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

As for the 30 minutes digestion, two pools were done with the fractions which correspond to the chromatographic peaks, being the DNA quantities measured by using a PicoGreen Assay. This quantification is presented in Table 30.

Table 30. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 1 hour and 30 minutes digestion cold stopped, after the nuclease digestion, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.21E+00	1.21E+00	1.21E+00
Pool FT	8.61E-02	1.54E-01	1.20E-01
Pool Elution	2.19E-02	2.53E-02	2.54E-02

Phenyl Boronate Chromatography - Yields

In the chromatographic step there are 2 different yields to consider, chromatographic yield and total yield. Both are available in Table 31.

Table 31. Average Phage Yield for each Digestion time (30 minutes and 1 hour and 30 minutes) cold stopped, after the nuclease digestion, tested in the Phenyl Boronate Chromatography.

	Chromatographic Yield (%)	Total Yield (%)
30 min	13.44 ± 4.52	4.36 ± 0.25
1 h 30 min	58.79 ± 40.18	18.09 ± 14.96

As in the previous steps, there are 2 different DNA and protein removal to be considered, the chromatographic removal and the total removal. Both the removals are available in Table 32.

Table 32. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes and 1 hour and 30 minutes) cold stopped, after the nuclease digestion, tested in the Phenyl Boronate Chromatography.

	Chromatography DNA Removal (%)	Total DNA Removal (%)	Chromatography Protein Removal (%)	Total Protein Removal(%)
30 min	83.34 ± 0.40	95.20 ± 0.34	87.38 ± 1.75	96.26 ± 0.32
1 h 30 min	95.79 ± 0.21	98.00 ± 0.17	90.37 ± 0.18	96.86 ± 0.05

Adsorption Buffer Exchange

An exchange of buffers into the adsorption buffer (15 mM of Tris-HCl, pH 7) before chromatography was done in an attempt to study the salt concentration interference to the Phenyl Boronate Chromatography. After the buffer exchange, the samples were tested for phage infectivity, DNA and protein quantity, being the results of the first triplicate shown in Table 33. The results for the 30 minutes and 1 hour and 30 minutes triplicate, are available in the Appendix.

Table 33. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate, sample present in the adsorption buffer (15 mM of Tris-HCl, pH 7) and for samples of the first duplicate/triplicate with nuclease digestion times 30 minutes, and 1 hour and 30 minutes, cold stopped.

	Duplicate/Triplicate 1		
Sample	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.21E+00	5.94E-02	1.61E+10
Adsorption Buffer	9.97E-01	6.11E-03	2.22E+09
30 min	1.28E-02 ± 7.60E-04	5.30E-03 ± 9.45E-05	2.19E+08 ± 6.24E+07
1 h 30 min	2.21E-02 ± 3.67E-03	5.26E-03 ± 5.05E-04	1.58E+08 ± 8.53E+06

In this step (adsorption buffer exchange), as previously, there are 2 different yields to consider, buffer exchange yield and the total yield. Both yields are available in Table 34.

Table 34. Average Phage Buffer Exchange and Total Yields for each Digestion time (30 minutes and 1 hour and 30 minutes) stopped by cold, tested in the buffer exchange.

	Buffer Exchange Yield (%)	Total Yield (%)
30 min	6.38 ± 0.00	1.39 ± 0.00
1 h 30 min	7.96 ± 0.01	1.45 ± 0.00

As in the nuclease digestion, in this step there are 2 different DNA and protein removal to consider, the nuclease digestion removal and the total removal. Both the removals are available in Table 35.

Table 35. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes and 1 hour and 30 minutes) stopped by cold, tested in the buffer exchange.

	Buffer Exchange DNA Removal (%)	Total DNA Removal (%)	Buffer Exchange Protein Removal (%)	Total Protein Removal(%)
30 min	34.98 ± 0.00	96.55 ± 0.00	70.53 ± 0.02	91.24 ± 0.00
1 h 30 min	34.48 ± 0.01	95.49 ± 0.00	73.66 ± 0.02	91.39 ± 0.01

These samples were used in a Phenyl Boronate Chromatography, being the results presented below.

Phenyl Boronate Chromatography - 30 Minutes Digestion

In Figure 38 is presented the chromatogram corresponding to the 30 minutes digestion. The results of the second and third triplicates are shown in the Appendix.

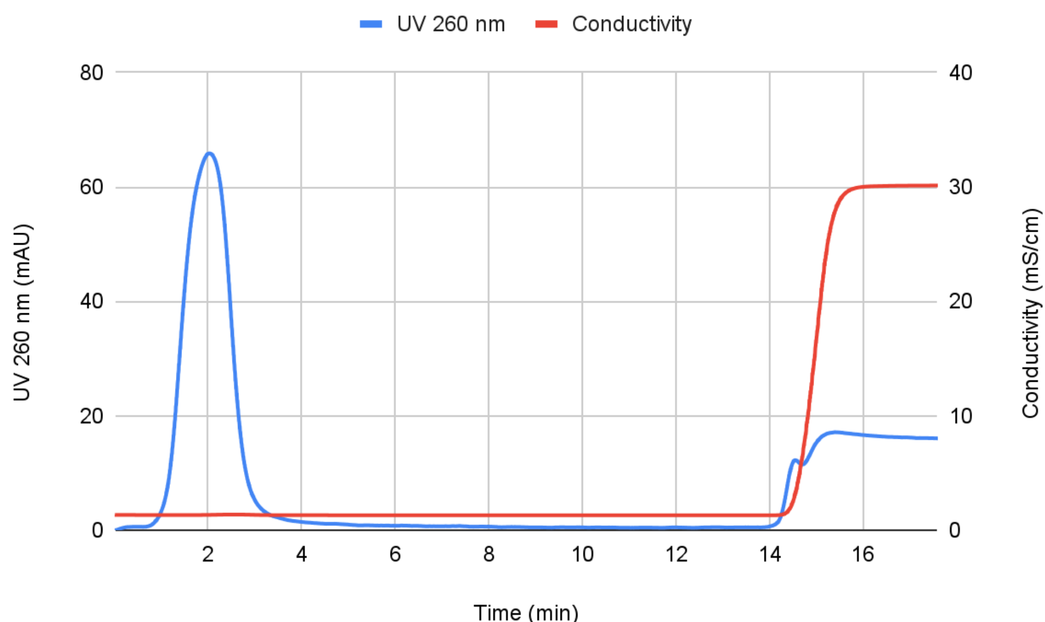

Figure 38. First triplicate of the 30 minutes digestion chromatogram stopped by cold, after the adsorption buffer exchange. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and

1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

After Phenyl Boronate Chromatography, the samples were tested for phages infectivity and protein quantity. Being the results, per fraction collected, available in Figure 39.

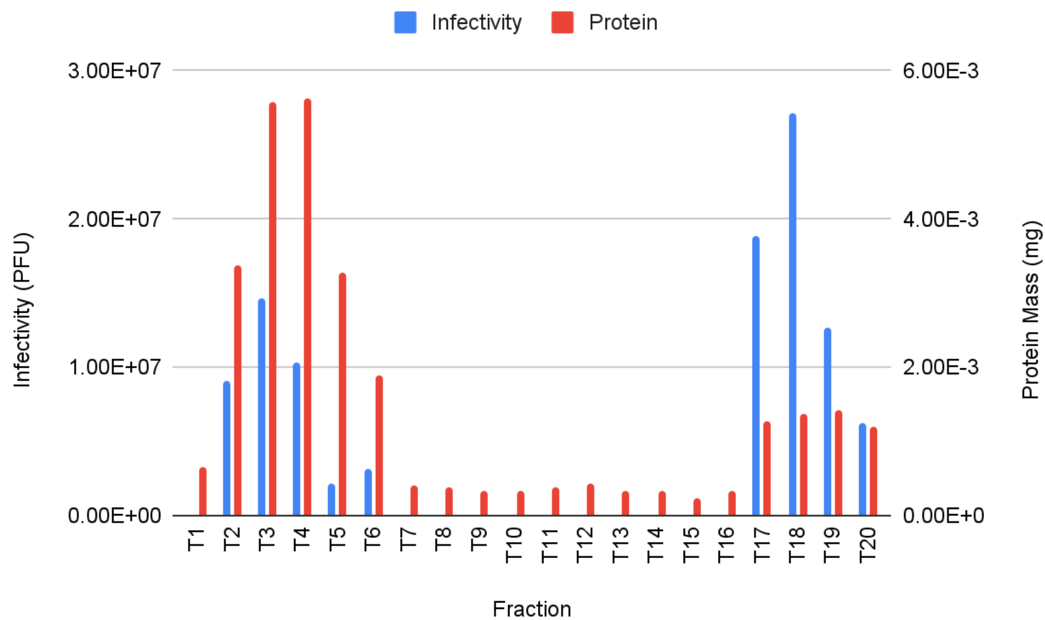

Figure 39. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 30 minutes digestion stopped by cold, after the adsorption buffer exchange, in each fraction (20 fractions total) collected during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Two Pools of samples were prepared and a PicoGreen Assay was performed in order to understand the DNA quantity present in the chromatographic peaks. These quantities are available in Table 36.

Table 36. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 30 minutes digestion stopped by cold, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.21E+00	1.21E+00	1.21E+00
Pool FT	1.14E-02	9.13E-03	1.06E-02
Pool Elution	6.69E-03	6.87E-03	6.27E-03

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

The first chromatogram triplicate of digestion time 1 hour and 30 minutes is shown in Figure 40. The results of the second and third triplicates are shown in the Appendix.

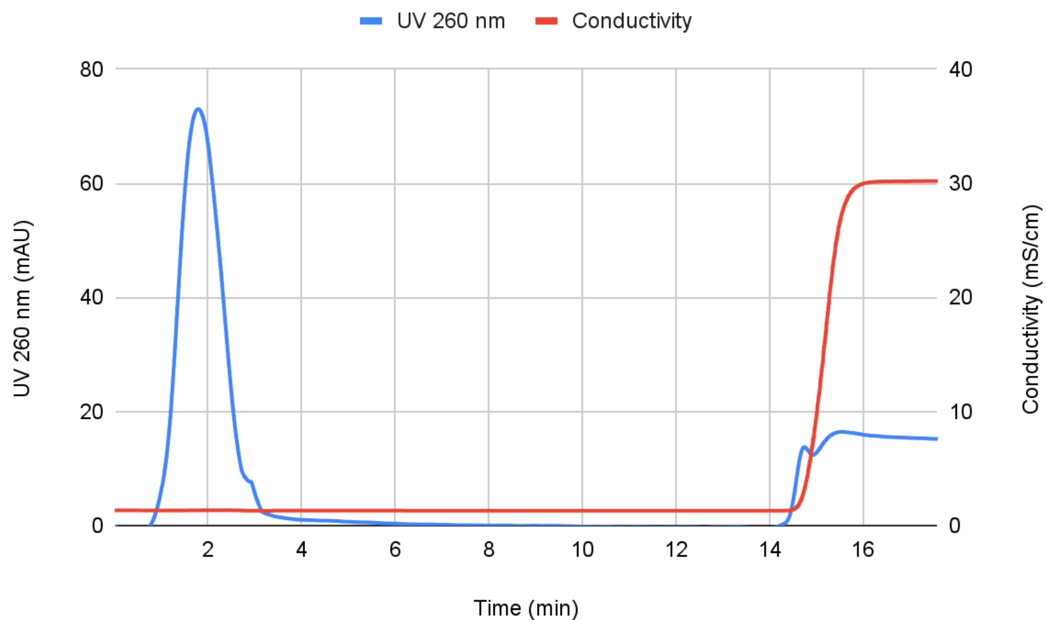

Figure 40. First triplicate of the 1 hour and 30 minutes digestion cold stopped chromatogram, after the adsorption buffer exchange. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

For each fraction collected during the chromatography, phage infectivity and protein quantity was measured. The results obtained are available in Figure 41.

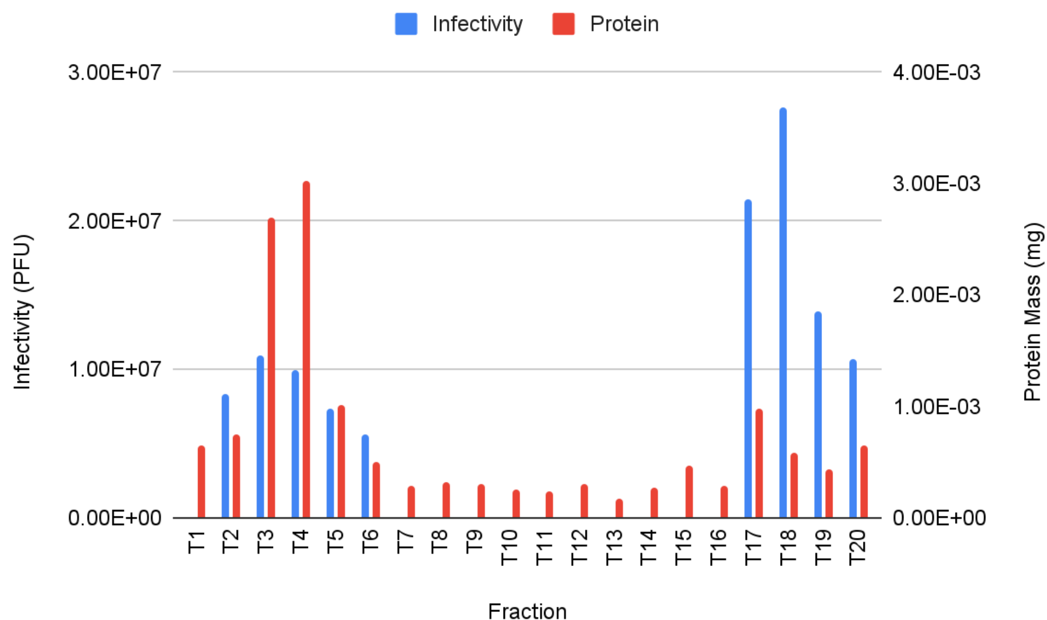

Figure 41. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 1 hour and 30 minutes digestion, stopped by cold, after the adsorption buffer exchange, in each fraction (20 fractions total) collected during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

As for the 30 minutes digestion, the fractions which correspond to the chromatographic peaks were organized in two pools, being the DNA quantities measured by using a PicoGreen Assay. This quantification is presented in Table 37.

Table 37. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 1 hour and 30 minutes digestion cold stopped, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.21E+00	1.21E+00	1.21E+00
Pool FT	1.73E-02	2.80E-02	2.10E-02
Pool Elution	1.34E-02	1.70E-02	1.35E-02

Phenyl Boronate Chromatography - Yields

Also, in the chromatographic step 2 different yields are to be considered, chromatographic yield and total yield. Both are available in Table 38

Table 38. Average Phage Yield for each Digestion time (30 minutes and 1 hour and 30 minutes) cold stopped, after the adsorption buffer exchange, tested in the Phenyl Boronate Chromatography.

	Chromatographic Yield (%)	Total Yield (%)
30 min	43.70 ± 16.24	0.36 ± 0.01
1 h 30 min	41.60 ± 1.54	0.34 ± 0.05

Again, in this step, there should be taken in consideration 2 different DNA and protein removal, the chromatographic removal and the total removal. Both the removals are available in Table 39.

Table 39. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes and 1 hour and 30 minutes) cold stopped, after the adsorption buffer exchange, tested in the Phenyl Boronate Chromatography.

	Chromatography DNA Removal (%)	Total DNA Removal (%)	Chromatography Protein Removal (%)	Total Protein Removal(%)
30 min	44.68 ± 5.08	99.45 ± 0.03	63.52 ± 4.53	96.80 ± 0.39
1 h 30 min	47.73 ± 7.60	98.79 ± 0.17	67.98 ± 12.35	97.31 ± 0.81

Nuclease Digestion in the Lysate

In an attempt to reduce the amount of phages lost, and therefore to increase the yield, a study of the nuclease digestion directly in the lysate, without previously doing a buffer exchange, was performed. The results obtained are presented below.

Nuclease Digestion

Here two different nuclease incubation times (30 minutes and 1 hour and 30 minutes) were tested, in triplicates. Each triplicate was, then, tested for phage infectivity, DNA and protein quantity. The results of the first triplicate are shown in Table 40, being the other triplicate available in the Appendix.

Table 40. Phage infectivity (PFU), DNA quantity (μg) and Protein quantity (mg) for the Lysate and for the samples of the first triplicate with nuclease digestion times 30 minutes and 1 hour and 30 minutes.

	DNA Quantity (μg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	1.009	5.53E-02	8.70E+08
30 min	0.008 ± 0.001	4.89E-02 ± 1.04E-03	6.75E+08 ± 3.12E+07
1 h 30 min	0.029 ± 0.007	4.84E-02 ± 4.48E-04	6.60E+08 ± 3.97E+07

This nuclease digestion step presents a phage yield and a DNA and protein removal. These values are presented in Table 41.

Table 41. Average Phage Yield (%), DNA Removal (%) and Protein Removal (%) for the Lysate and for the samples of the first triplicate with nuclease digestion times 30 minutes and 1 hour and 30 minutes.

	Phage Yield (%)	DNA Removal (%)	Protein Removal (%)
30 min	73.56 ± 0.04	99.37 ± 0.00	13.43 ± 0.02
1 h 30 min	72.41 ± 0.05	97.87 ± 0.01	13.16 ± 0.01

These samples were, then, used in a Phenyl Boronate Chromatography, being the results obtained from this chromatography presented below.

Phenyl Boronate Chromatography - 30 Minutes Digestion

In Figure 42 is presented the chromatogram corresponding to the 30 minutes digestion. The results of the second and third triplicates are shown in the Appendix.

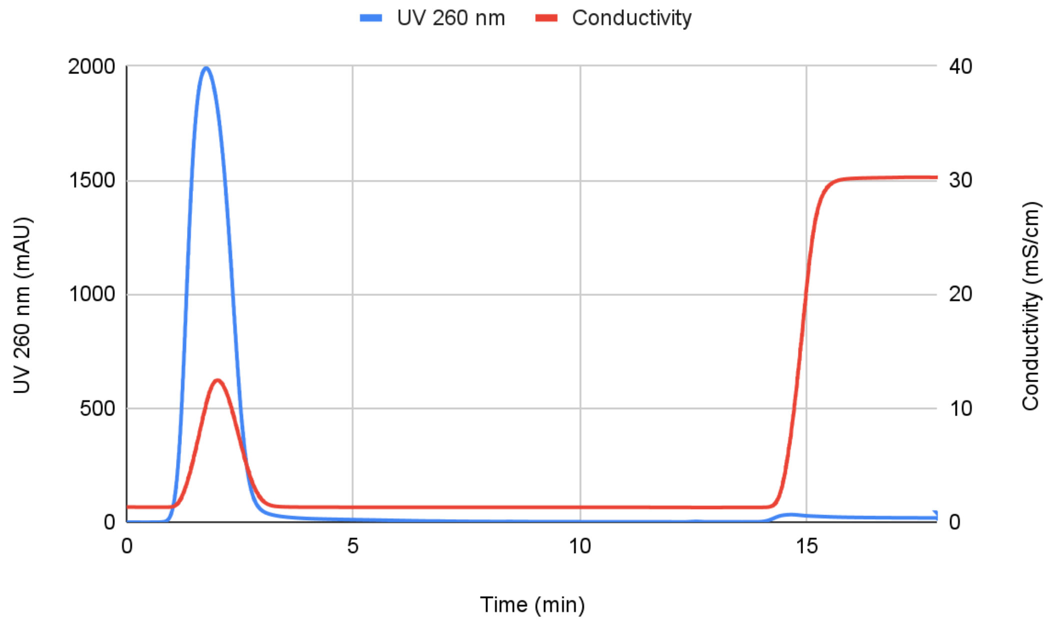

Figure 42. First triplicate of the 30 minutes digestion chromatogram, after the nuclease digestion to the lysate . It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

Figure 43 presents the phage infectivity and the protein quantity tested after phenyl boronate chromatography per fraction collected during the chromatography.

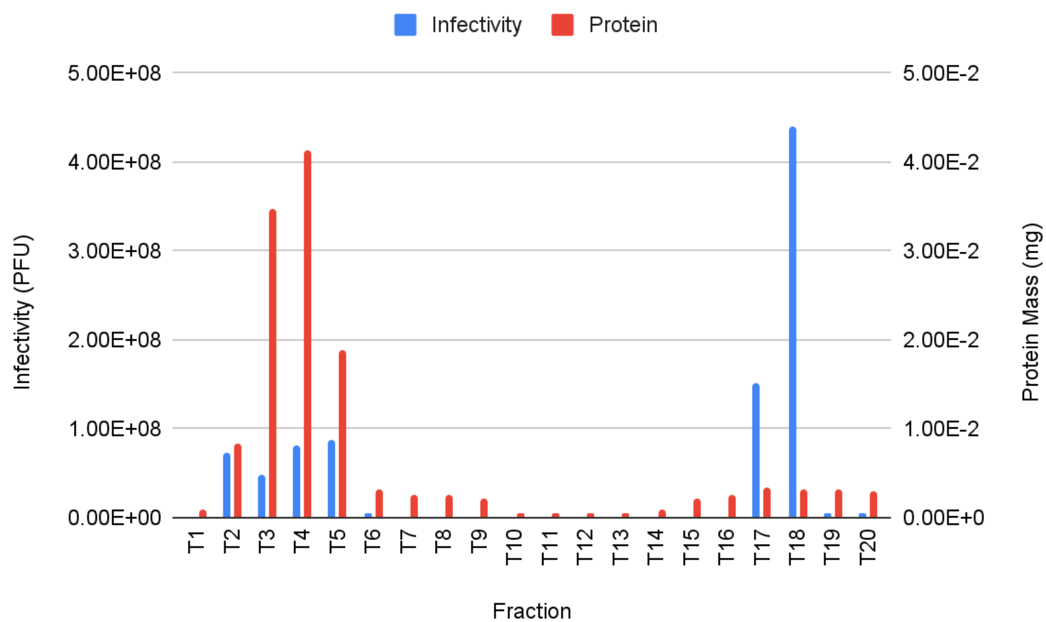

Figure 43. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 30 minutes digestion after the nuclease digestion to the lysate, in each fraction (20 fractions total) collected during

phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Two pools were prepared from the fractions, being the first one obtained with fractions T2, T3, T4, T5 and T6 and the second one with fractions T17, T18 and 19. These pools were done to quantify the DNA present in those samples. A PicoGreen Assay was performed on the pools. The DNA quantities are available in Table 42.

Table 42. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 30 minutes digestion, after the nuclease digestion to the lysate, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.01E+00	1.01E+00	1.01E+00
Pool FT	9.34E-05	9.26E-05	9.30E-05
Pool Elution	4.22E-04	4.22E-04	4.22E-04

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

The first chromatogram triplicate of digestion time 1 hour and 30 minutes is shown in Figure 44. The results of the second and third triplicates are shown in the Appendix.

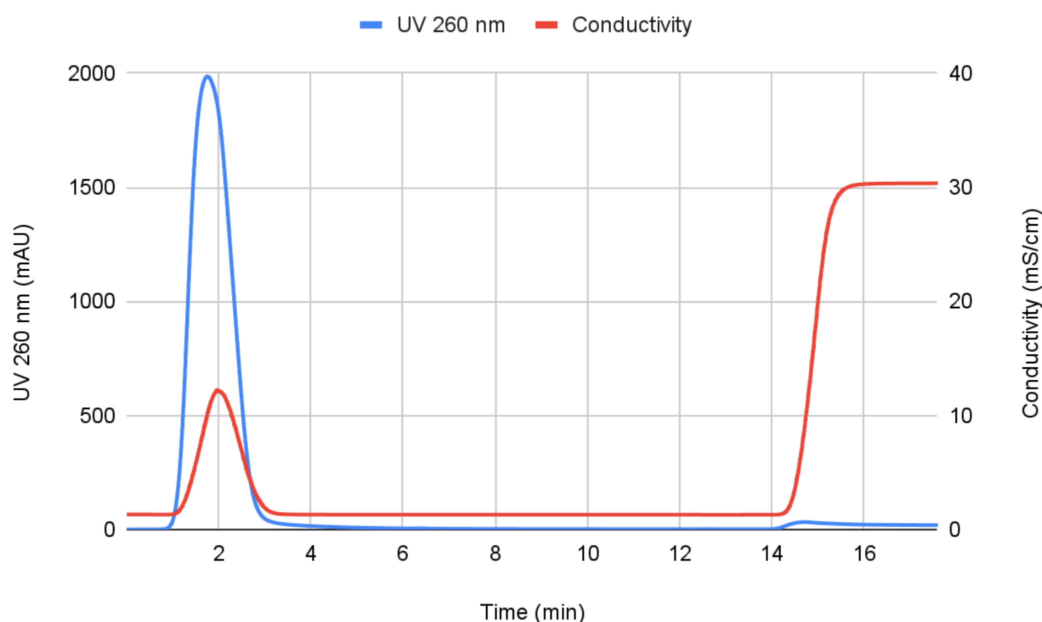

Figure 44. First triplicate of the 1 hour and 30 minutes digestion chromatogram, after the nuclease digestion to the lysate. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

For each chromatographic fraction collected, the phage infectivity and the protein quantity were measured, being the results available in Figure 45.

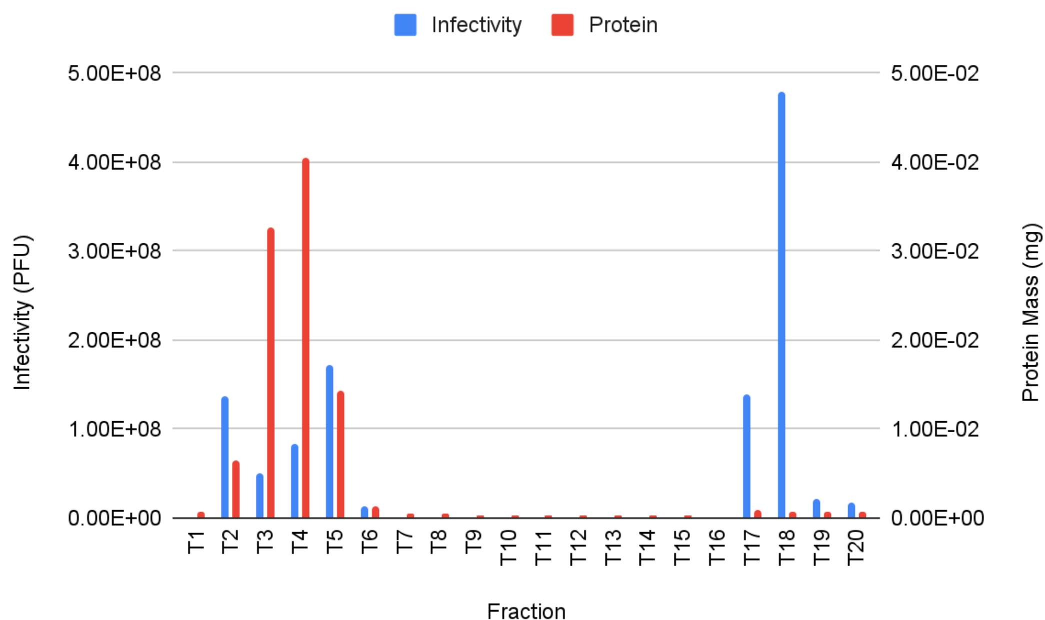

Figure 45. Phages infectivity, PFU, and Protein Mass in mg, for the first triplicate of 1 hour and 30 minutes digestion, after the nuclease digestion to the lysate, in each fraction (20 fractions total) collected during phenyl boronate chromatography. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

As for the 30 minutes digestion, two pools were done with the fractions which correspond to the chromatographic peaks. The DNA quantity was, then, measured using a PicoGreen Assay, being the quantification presented in Table 43.

Table 43. DNA quantity in µg for the Lysate, Pool FT and Pool Elution, for the three triplicate done for the 1 hour and 30 minutes digestion, after the nuclease digestion to the lysate, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer).

	Triplicate 1	Triplicate 2	Triplicate 3
Sample	Quantity DNA (µg)	Quantity DNA (µg)	Quantity DNA (µg)
Lysate	1.01E+00	1.01E+00	1.01E+00
Pool FT	6.37E-04	7.33E-04	6.85E-04
Pool Elution	8.75E-03	4.93E-03	6.84E-03

Phenyl Boronate Chromatography - Yields

In the chromatographic step there are 2 different yields to consider, chromatographic yield and total yield. Both are available in Table 44.

Table 44. Average Phage Yield for each Digestion time (30 minutes and 1 hour and 30 minutes), tested in the Phenyl Boronate Chromatography for the lysate digestion.

	Chromatographic Yield (%)	Total Yield (%)
30 min	94.42 ± 5.34	69.33 ± 0.78
1 h 30 min	95.78 ± 1.54	67.76 ± 4.05

As in the previous steps, there are 2 different DNA and protein removal to be considered, the chromatographic removal and the total removal. Both the removals are available in Table 45.

Table 45. Average DNA and Protein Digestion and Total Removals for each Time (30 minutes and 1 hour and 30 minutes) tested in the Phenyl Boronate Chromatography for lysate digestion.

	Chromatography DNA Removal (%)	Total DNA Removal (%)	Chromatography Protein Removal (%)	Total Protein Removal(%)
30 min	93.09 ± 1.59	99.96 ± 0.00	79.61 ± 0.87	82.36 ± 0.37
1 h 30 min	67.78 ± 1.89	99.32 ± 0.19	95.29 ± 0.14	95.91 ± 0.16

SDS-Page

In order to confirm and visualize the removal of proteins a SDS-Page gel was performed to the samples from the nuclease digestion in the lysate (first and the second triplicates digestion time 30 minutes and 1 hour and 30 minutes). Note that not all fractions collected during the chromatography were used, but the two pools obtained for the Picogreen Assay. The results obtained are present in Figure 46.

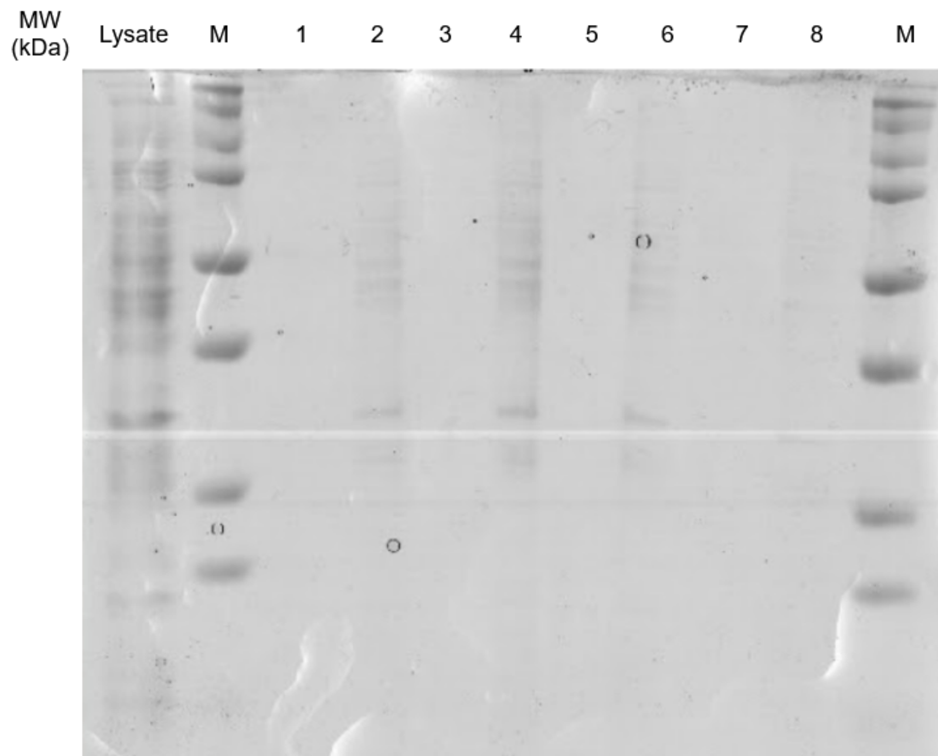

Figura 46. SDS-PAGE of the results of the nuclease digestion to the lysate followed by a Phenyl Boronate Chromatography for two different incubation times (30 minutes and 1 hour and 30 minutes) in duplicate. The gel was stained with Coomassie. The samples from the chromatography are organized in two pools according to the chromatographic peaks. The lysate well consists of a sample of lysate previously before the digestion and the chromatography. M wells correspond to the Precision Plus Protein™ Dual Color Standards ladder. Wells 1 and 3 present the elution pool of the 1 hour and 30 minutes digestion to the lysate (duplicate). Wells 2 and 4 consist of the FT pool of the 1 hour and 30 minutes digestion to the lysate (duplicate). In wells 5 and 7 it is possible to see the elution pool of the 30 minutes digestion to the lysate (duplicate). In wells 6 and 8 it is observable the FT pool of the 30 minutes digestion to the lysate (duplicate). In the FT pool wells some bands can be seen.

Discussion

In this study, different purification steps (Phenyl Boronate Chromatography, Nuclease Digestion and Buffer Exchange) for the downstream processing of bacteriophages were evaluated. A bacteriophage is a virus responsible for bacteria and archaea infection by replicating within these organisms. The bacteria and phage strains used in the study show a predator-prey relationship.

In order to understand the difference between phage infected bacteria (Figure 7) and normal bacteria (Figure 8) cell growth the OD_{600} was followed throughout the growth time. It is also possible to notice that when infected with bacteriophages (at 60 minutes after initiating the growth) (Figure 7) the bacteria concentration (OD_{600}) decreases, becoming even lower than when it started. This happens due to phage infection which leads to the bacteria lyses and therefore death. When comparing this behaviour with the bacterial growth in Figure 8 it is possible to see that when not infected, the bacteria continues to grow. The OD_{600} for normal bacteria cells was only followed for the same period of time as the infected bacteria, so it did not reach the predictable, stationary phase, where the number of dying cells equals the number of dividing cells. This is due, normally, to a growth-limiting factor (lack of essential nutrients, formation of inhibitory products for example).

Phages applications require a high purification while still preserving a high infectivity. In order to achieve this purification, Phenyl Boronate Chromatography, under different binding and elution conditions,was thoroughly evaluated (Condition 1: equilibration and adsorption buffer - Milli-Q water, elution buffer - 1.5 M Tris-HCl, pH 8.5. Condition 2: equilibration and adsorption buffer - 15 mM of Tris-HCl, pH 8.5, elution buffer - 1.5 M Tris-HCl, pH 8.5. Condition 3: equilibration and adsorption buffer - 15 mM Tris-HCl, pH 7, elution buffer - 1.5 M Tris-HCl, pH 8.5. Condition 4: equilibration and adsorption buffer - 15 mM Tris-HCl, pH 7, elution buffer - 1.5 M Tris-HCl, pH 7). For every condition, a chromatogram was obtained and is presented in Figure 9, Figure 12, Figure 14 and Figure 16, for condition 1, condition 2, condition 3 and condition 4, respectively. All chromatograms present a high intensity peak (2000 mAU) around a retention time of 2 min (first peak), and a smaller peak at 14 min (second peak). Condition 3 and 4 chromatograms show a second peak with a higher intensity (250 mAU and 150 mAU, respectively) when compared with the other conditions, however, the third condition presents the highest intensity for the second peak.

Each chromatogram was collected in 20 fractions, being these fractions tested for phage infectivity and protein quantity (Figure 10, 13, 15 and 17 for condition 1, 2, 3 and 4 respectively). From the plaque essay, it is possible to see that there are more infectable phages in the second peak than in the first peak, for all the chromatographic runs performed. With the BCA assay, the protein quantity was obtained, allowing the observation that the quantity of protein is higher in the first peak. This shows that the chromatography allows a separation from the proteins and the infectable phages. According to these results, the Phenyl Boronate Chromatography may be a good purification method to remove the proteins.

From the 20 fractions collected, two pools were done. The first pool, flowthrough (FT), was obtained with the fractions collected from the first peak (fraction T2, T3, T4, T5 and T6). The second pool, elution, was done by using the fractions from the second peak (T17, T18 and T19). These pools were used in order to quantify the DNA present (Table 2, 3, 4 and 5 for condition 1, 2, 3 and 4, respectively). From the Tables, it is possible to see that the DNA quantity in the elution pool is around 0.3-0.4 μg for all the conditions tested. However, the DNA quantity for the FT pool can vary for all the conditions, being the highest for condition 2 (0.87-0.89 μg) and the lowest (0.19-0.33 μg) for condition 4. As the second peak presents a higher infectable phage than peak one, it is important that the DNA and protein quantities are the lowest in this pool/fractions. This does not happen for the DNA quantity (except for condition 2), as the DNA present in the first peak is similar or lower than in the second one. This was predictable as it is known that the DNA forms bonds with the boronates present in the chromatographic column. Due to this, it was decided that a nuclease digestion should be done previously to the Phenyl Boronate Chromatography in an attempt to remove the bacterial DNA.

In order to decide which adsorption/elution condition should be used after the nuclease digestion, allowing to study different digestion conditions, the yield and percentage of protein and DNA removed (Table 1) were analysed. The higher yield is achieved in condition 1 (55.99%), followed by condition 4 (53.45%), being the lowest in condition 2 (33.41%). As the yield is one of the major concerns, condition 2 is excluded from the following studies. Condition 2 presents the higher DNA removal (53.69%), however, this condition was already excluded. The second condition with the higher DNA removal is the first one (49.09%), followed by the third one (47.60%). Due to this, condition 4 is excluded from the next steps. As condition 3 shows a higher protein removal (96.91%) than condition 1 (96.26%), condition 3 was then chosen for the studies with the nuclease digestion.

To perform the nuclease digestion decided above it was used Denarase®, an enzyme whose incubation, according to the manufacturers, requires the presence of a cofactor, Mg^{2+}. Due to this, a buffer exchange step to the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8) was performed using Amicon® centrifugation filters. As the filters used have an NMWC of 100 kDa, some proteins will be removed. This is verified as the removal of proteins in this step is 68.32% for the first attempt (Table 6) and 66.50% for the second assay (Table 25). Also, some phages may be lost, or lose their infectivity due to this step, as is shown by the 32.50% and 63.55% yields on the first and second assays, respectively. There was also a small DNA removal in this step, 29.08% (first assay) and 14.05% (second assay).

With the samples present in the nuclease buffer, the nuclease digestion was performed. The samples were incubated for 4 different times (30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours) at 37 ºC. After the incubation, it is necessary to stop the digestion as the Denarase® remains active in temperatures between 15 ºC and 50 ºC. There were 2 methods used to achieve this, a heat stop with an incubation at 72 ºC for 10 minutes, and a cold stop, by storing the samples on ice. As shown in

Table 7 and Table 26, the phage's infectivity in the samples present in the nuclease buffer after stopping the digestion, decreases immensely when the stop in by heat. This is proven by the 9.61% yield for the incubation at 72 ºC versus the 91.18% yield for the ice storage.

The digestion itself does not show a big effect on the phage's infectivity. In fact, for the heat stopped digestion, only the 30 minutes incubation shows a yield lower than 65% (Table 8), being the 2 hours digestion the incubation with the higher yield, 96.22%. For the cold stopped digestion, both the incubation times tested (30 minutes and 1 hour and 30 minutes) show a lower yield (Table 27) when compared to the heat stopped, being the 1 hour and 30 minutes the lower between the two with a yield of 47.94%. This is due to the variability of the digestion process. The protein removal (Table 9 and 28) in the digestion step is not significant, as expected. However, the DNA removal in the heat stopped digestion (Table 9) is quite high, being the 1 hour and 30 minutes incubation the lower with a removal of 84.37%. The highest DNA removal is the 1 hour incubation with 98.22%. When compared with the DNA removal in the cold storaged digestion (Table 28), it is possible to see that this second method presents a lower removal, with the 30 minutes incubation showing a 66.26% removal and the 1 hour and 30 minutes incubation a 44.59% removal. It is important to note that the heat may also favor DNA degradation, increasing the DNA removal for this digestion stopping method.

After the nuclease digestion, the protein quantity is still high in the samples, being a Phenyl Boronate Chromatography performed for the heat stopped digestion (Figures 18, 20, 22 and 24) and for the cold stopped digestion (Figures 34 and 36). For all the chromatograms obtained, there is a first peak around a retention time of 2 min and a smaller peak after around 14 min (second peak). After being tested for phage infectivity and protein quantity, it is possible to see that the first peak presents a higher protein quantity and the second a higher phages quantity (Figures 19, 21, 23 and 25 for the heat stopped digestion and Figures 35 and 37 for the cold stopped digestion). The samples were also tested for the DNA quantity (Tables 10, 11, 12 and 13 for the heat stopped digestion and Tables 29 and 30 for the cold stopped digestion), being two pools done with the peaks fractions. The results show that the DNA quantity present in the elution pool is lower than the one present in the FT pool, for all the assays studied. When comparing the heat and cold stopped digestion chromatographic yields (Tables 14 and 31, respectively), it is possible to understand that the cold stopped presents higher yields (13.44% for the 30 minutes digestion and 58.79% for the 1 hour and 30 minutes digestion) than the heat stopped, which higher yield is 9.27% for the 1 hour and 30 minutes digestion. This happens as the heat interferes with the phages' infectability. The protein removal during the chromatographic step after the nuclease digestion is very high for both heat stopped (Table 15) and cold stopped digestion (Table 32), being always higher than 80%. However the removal in the cold stopped digestion is a little higher (87. 38% for the 30 minutes digestion and 90.37% for the 1 hour and 30 minutes digestion) when compared to the heat stopped digestion, which the higher removal is 90.27% for the 1 hour and 30 minutes digestion and the lower 81.04% for the 2 hours digestion. In terms of DNA removal in the chromatographic step, the 1 hour and 30 minutes digestion presents the higher removal, both in the heat stopped, 99.49% (Table 15), and the cold stopped, 95.79%, digestion (Table

32). When comparing the total removal in the heat stopped digestion, the 1 hour and 30 minutes shows the higher value for both the protein removal, 97.75%, and the DNA removal, 99.87%, which is the reason this digestion time was chosen for the cold stopped digestion. The 30 minutes was also chosen as is the time recommended by the manufacturers.

The salt concentration (2 mM $MgCl_2$) present in the nuclease buffer may interfere with the Phenyl Boronate chromatography as in acidic conditions the reaction between the boronate and the cis-diols is reversed, as the cyclic diester is hydrolyzed undoing the tetrahedral boronate anion. In an attempt to study this interference, a new change of buffer was accomplished right after the nuclease digestion. As the samples from the second buffer exchange are going to be used in the chromatography, the buffer chosen was the adsorption buffer (15 mM Tris-HCl, pH 7). After exchanging the sample buffers to the adsorption buffer, phage infectivity and DNA/protein quantities were determined (Table 16 for the heat stopped digestion and Table 33 for the cold stopped). With these values it was possible to calculate the phage yield (Table 17 and 34 for the heat and cold stopped digestion, respectively). Cold stopped digestion samples present higher yields than the heat stopped ones, being the higher yield (1.45%) the 1 hour and 30 minutes digestion. This value corresponds to the total yield, as the heat seems to interfere with the phages' infectability, this was expected.In the heat stopped digestion the higher yield is shown also in the 1 hour and 30 minutes digestion (0.188%), and the lower in the 1 hour digestion, 0.013%. For the DNA removal, the heat stopped digestion presents higher removals, for both the buffer exchange step and the total removal. The higher DNA removal belongs to the 1 hour digestion (99.81%) for the total removal, however, for the buffer exchange step the 1 hour and 30 minutes digestion presents the higher removal (93.98%), even though this being the lower total DNA removal (99.38%) in the heat stopped digestion. The higher DNA removal shown for the cold stopped digestion is in the 30 minutes digestion (96.55%). For the protein removal, the heat stopped digestion presents the higher removals when comparing the heat and the cold stopped digestion. In the heat stopped, for all digestion times, the removal is higher than 99%, being the lower total removal for the 30 minutes digestion (99.64%), regardless of this showing the higher protein removal in the buffer exchange step, 93.92%. The cold stopped digestion shows a protein removal around 91% for both the digestion times studied, 91.24% for the 30 minutes digestion and 91.39% for the 1 hour and 30 minutes digestion.

With the samples present in the adsorption buffer (15 mM Tris-HCl, pH 7), a Phenyl Boronate Chromatography was done. From the heat stopped chromatograms (Figures 26, 28,30 and 32) it is observable that the first peak is present as in the previously studied chromatograms, with a lower intensity, however, the second peak shows some differences. First for the 30 minutes digestion (Figure 26), a second peak does not seem to be present. Secondly, for the other digestion times studied, the second peak is divided in two, being this more distinguished in the 1 hour digestion chromatogram (Figure 28). This is also visible in the cold stopped digestion chromatograms (Figures 38 and 40), however the second peak does not stand out as much as in the heat stopped digestion. After being tested for phage infectivity and protein quantity (Figures 27, 29, 31 and 33 for the heat stopped

digestion and Figures 39 and 41 for the cold stopped), it is possible to see that the first chromatographic peak corresponds to the proteins and the second one to the infectable phages. For the DNA quantities two pools were done with the fraction from the two prominent chromatographic peaks. The heat stopped digestion shows a higher quantity of DNA in the elution pool than in the FT one (Tables 19, 20, 21 and 22), however, as these values are quite small, they may be out of the method calibration. Nevertheless, in the cold stopped digestion the DNA quantities present in the elution pool are lower than in the FT one (Tables 36 and 37).

After testing the samples from the Phenyl Chromatography for phage infectivity and DNA and protein quantities, the yield and DNA/protein removals were calculated. The chromatographic step presents a higher yield for the cold stopped digestion (Table 38), than for the heat stopped (Table 23). The same happens with the total yield. The heat stopped digestion shows a higher yield in 1 hour and 30 minutes digestion, 0.034%, being the lowest the 1 hour digestion yield (0.011%). However, the cold stopped digestion presents the higher yield for the 30 minutes digestion, with a value of 0.36%, 10 times higher than the heat stopped highest yield. For the DNA removal (Table 24 for the heat stopped digestion and Table 39 for the cold stopped), all the conditions studied, heat and cold stopped digestion with different digestion times, present a similar amount of DNA removed, however, the cold stopped still presents smallish lower values, being the lowest for the 1 hour and 30 minutes digestion stopped by cold, with a DNA removal of 98.79%. The highest DNA removal, 99.81%, corresponds to the 1 hour digestion heat stopped. The heat stopped digestion also presents higher values for the protein removal, being all higher than 99.90%, with a lowest in the 1 hour and 30 minutes digestion of 99.92%. Even so, for the cold stopped digestion it is possible to see a higher protein removal in the chromatographic step (63.52% for the 30 minutes digestion and 67.98% for the 1 hour and 30 minutes digestion).

When analysing all the results obtained, yields, DNA and protein removal it is seen that, in general, the heat stopped digestion presents higher removals (DNA and protein) and the cold stopped presents higher yields. As this is a first step in the phage purification, the biggest concern is to maintain the most infectable phage possible. Consequently, the cold stopped digestion should be the one to prevail for future studies. As for the digestion times, the 30 minutes digestion, can be the one chosen, as recommended by the manufacturers.

As previously said, Denarase® activity requires, according to the manufacturers, the presence of a cofactor (Mg^{2+}), however, in this study, it was, also, tested the enzyme action on samples that did not suffer a buffer exchange in order to present the ideal cofactor concentration. Still, Denarase® was diluted in equal proportion in the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8), before incubating directly with the lysate. It is important to note that this digestion was not stopped by heat nor cold, as the samples were tested immediately after the incubation.

For this assay, two different incubation times were tested, 30 minutes and 1 hour and 30 minutes. From Table 41, it is possible to see that the nuclease digestion to the lysate presents a high yield,

more than 70%, for both times studied, being the highest yield obtained for the 30 minutes digestion. In terms of DNA, the digestion shows more than 95% removal, with the highest one also observed for the 30 minutes digestion, 99.37%. This removal is higher than the digestion removal with the digestion stopped by cold or heat. As expected, the protein removal in this step is low (around 13% for both digestion times), as proteins are not the target of the nuclease enzyme.

As soon as the digestion was done, the samples underwent a Phenyl Boronate Chromatography. As before, the chromatograms (Figure 43 and 45) show two peaks, however the first peak, around 2 min of retention time, has a higher intensity than with other nuclease digestion assays. In fact this peak shows an intensity similar to the ones from the buffer tests (Figure 10, 12, 14, and 16). Due to the higher intensity of the first peak, the second one is more difficult to see,but it is still visible after the 14 min of retention time. As previously, the infectivity study and the protein quantification (Figure 43 and 45) confirm that the first peak corresponds to a higher protein quantity and the second shows a higher quantity of infectable phages. In order to quantify the DNA, two pools, corresponding to the peak fractions, were done. This quantification, Table 42 and 43, shows that the amount of DNA in the elution pool is higher than the amount in the FT one, for both digestion times studied. This is not concerning as the DNA already removed from the digestion was high, in fact the total DNA removal is higher than 99% for both digestion times, Table 45. From these results it is, also, possible to see that the protein removed in the chromatographic step was high, 19.61% for the 30 minutes digestion and 95.29% for the 1 hour and 30 minutes digestion. Meaning the total protein removal is also high, being higher for the 1 hour and 30 minutes digestion, 95.91%. In terms of yield (Table 44), the chromatographic step shows a bigger yield, 94.42% and 95.78% for the 30 minutes and the 1 hour and 30 minutes digestion, respectively, when the digestion is done to the lysate than to samples present in the nuclease buffer. This translates into the total yield being higher than 65% for both digestion times studied (69.33% for the 30 minutes digestion and 67.76% for the 1 hour and 30 minutes digestion).

The two pools obtained from the nuclease digestion to the lysate were used in a SDS-Page in order to confirm if the protein's presence is mainly on the FT pool. From the results obtained (Figure 46) it is possible to see that the lanes corresponding to the FT pool of the 30 minutes digestion (lanes 6 and 8) and the 1 hour and 30 minutes digestion (lanes 2 and 4) present some bands which confirms the presence of proteins in these samples. The lanes regarding the elution pools (laness 5 and 7 and lanes 1 and 2 for the 30 minutes digestion and the 1 hour and 30 minutes digestion, respectively) do not present a high amount of bands, confirming the less protein quantity in these samples. The lysate lane presents the highest amount of bands, which confirms the protein removal.

When comparing the digestion to samples in the nuclease buffer (Table 14 and 15 for the heat stopped digestion and Table 31 and 32 for the cold stopped digestion) to the lysate ones (Table 44 and 45), it is possible to understand that the amount of protein removed is higher when the samples undergo buffer exchanges. The DNA removal, however, presents similar values for samples in the nuclease buffer and in the lysate. On the other hand, the phage infectivity is much higher in samples where the

digestion was done directly to the lysate. As this consists in the first purification step, the biggest concern is to maintain the highest infectivity possible, being that the digestion to the lysate should be the one to be considered for further studies.

Conclusion

Phages, viruses which infect bacteria,must present a high purification and infectability in order to be used for further applications. In this work, different purification methods and strategies were successfully used in order to purify phages. These methods included a Phenyl Boronate Chromatography step where different adsorption, equilibration and elution buffers (mainly different Tris concentrations and pH) were tested. All samples were tested for phage infectivity, protein and DNA removal. Condition 3 which employed an equilibration and adsorption buffer composed of 15 mM Tris-HCl, pH 7 and an elution buffer composed of 1.5 M Tris-HCl, pH 8.5 showed the most promising results, originating a yield of 49.49%, a DNA removal of 47.60% and a protein removal of 96.91%. Further studies were performed with these adsorption/elution conditions.

When tested for the amount of DNA, it was possible to see that the DNA removal was lower than 50% when using a Phenyl Boronate Chromatography as a purification step. In order to decrease the amount of DNA, an incubation with Denarase®, at 37ºC, was performed under four different incubation times (30 minutes, 1 hour, 1 hour and 30 minutes, 2 hours). It was also important to ensure that the digestion stopped after finishing the incubation time, so two different conditions were successfully studied, heat stopped and cold stopped digestion. It was also important to understand that Denarase® requires a cofactor (Mg^{2+}), which implies that a buffer exchange to nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8) could be needed. However, an incubation to the lysate was also tested.

The nuclease buffer exchange presents as a strong point the high protein removal (above 65%), however some phages were lost or became not infectable. The Denarase® digestion showed that it could be effective to decrease the DNA quantities when it is stopped by heat in the samples with a DNA removal above 84%. However, when only tested the heat in the samples, this showed a huge decrease in infectable phages, 9.61% yield. Even though the DNA removal in the cold stopped digestion, may not show the same effectiveness, it is still high enough (above 44%) to be considered as it presents a yield of 91.18% for the cold tested samples. In order to remove more DNA, but mainly more proteins, the nuclease digestion was followed by a Phenyl Boronate chromatography.

With the chromatography done, the samples were tested and the heat stopped digestion ones showed a yield between 0.14% and 1.31%, a DNA removal above 98% and a protein removal above 94%. The cold stopped digestion samples in its turn, showed a yield varying from 4.38% an 18.09%, a DNA removal above 95% and a protein removal higher than 96%.

In an attempt to understand the effect of $MgCl_2$ in the chromatography, the salt concentration was decreased by buffer exchanging the samples to the adsorption buffer (15 mM Tris-HCl, pH 7) before the chromatography. Even though after the Phenyl Boronate chromatography, the amount of DNA and protein removed for both heat and cold stopped digestion was pretty high (being the lower DNA and protein removal 98.79% and 96.87%, respectively), the amount of phages lost was too high (0.034%

and 0.36% were the higher yields obtained for the heat and cold stopped digestions, respectively) for this second buffer exchange to be recommended for further studies.

In order to understand the Denarase® activity without the added cofactor, the digestion of the lysate, followed by a Phenyl Boronate chromatography was accomplished. The digestion itself showed a phage recovery yield higher than 70% and a DNA removal of more than 95%. After performing the chromatography, the DNA and protein removal values increase to more than 99% and 80%, respectively, being the protein removal confirmed by the SDS-Page. However, the yield does not show a big decrease, being the total yield higher than 65%. As this consists in a first purification step, maintaining a high yield is one of the biggest concerns, and with the high DNA removal and good protein removal, this variable (digestion directly to the lysate) should be the one considered for further studies.

As this Master's Thesis studies consists in a first purification step, the main goal is to maintain the highest infectability possible. Having this in mind, for further investigations, it recommended the use of the digestion to the lysate process. Some future works for this Master's Thesis topic may pass by quantifying and studying endotoxins removal, either by precipitation or a new chromatographic step specific for these molecules removal. Performing a competitive chromatography may also be something to consider in order to elute the bacterial DNA and the phages at different retention times.

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Appendix

T4 Phage Sample Preparation

Table 46. Time, in minutes, of *E. coli*, strain 613 inoculation in a 500 mL TSB culture medium and corresponding OD_{600}. The phage infection occurred at the fourth point, 60 minutes. The correspondent OD_{600} was measured in order to follow the bacteria growth.

Time (min)	OD 600
0	0.11
40	0.13
55	0.13
60	0.19
120	0.44
150	0.08

Bacterial Growth Curve

Table 47. Time, in minutes, of *E. coli*, strain 613 inoculation in a 100 mL TSB culture medium and corresponding OD_{600}, dry weight, in mg, and concentration, in CFU/mL. The measurements were made in order to follow the bacteria growth. Due to incorrections in the dry weight, this method was not considered in the comparison.

Time (min)	OD_{600}	Dry Weight (mg)	CFU/mL
0	0.08	0.32	4.40E+07
30	0.11	-29.65	2.33E+08
40	0.13	-39.63	3.10E+08
50	0.15	-49.61	4.90E+08
60	0.18	-59.58	5.50E+08
70	0.22	-69.54	9.80E+08
80	0.31	-79.45	1.31E+09
90	0.43	-89.33	1.76E+09
105	0.61	-104.15	2.09E+09
120	0.76	-119	2.71E+09
150	1.22	-148.54	3.40E+09

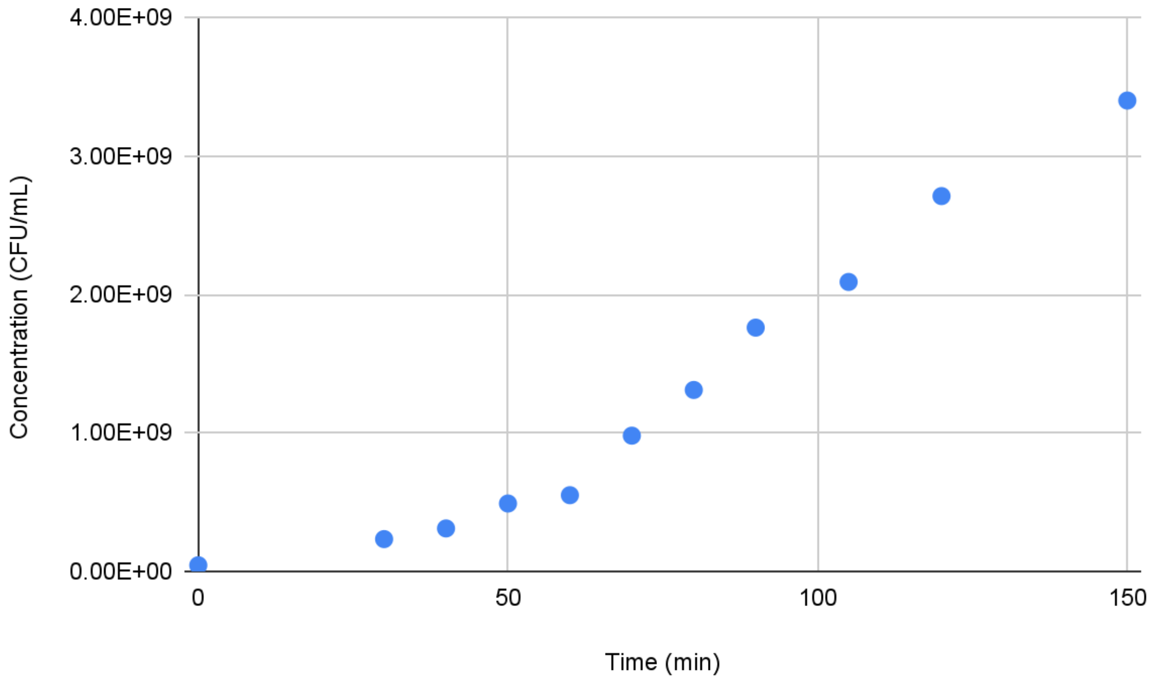


Figure 47. Bacterial Growth Curve of *E. coli*, strain 613 in a 100 mL TSB culture medium. The time, in minutes, corresponds to the time after inoculation of the broth with bacteria. The growth of the bacterial culture was followed by the concentration in CFU/mL.

Phenyl Boronate Chromatography

Condition 1

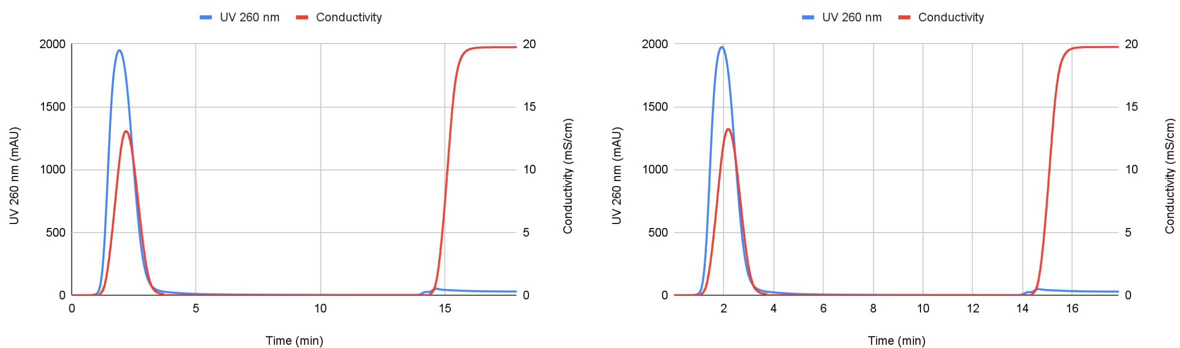


Figure 48. Chromatogram of the second (left) and third (right) triplicates of Condition 1 (Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

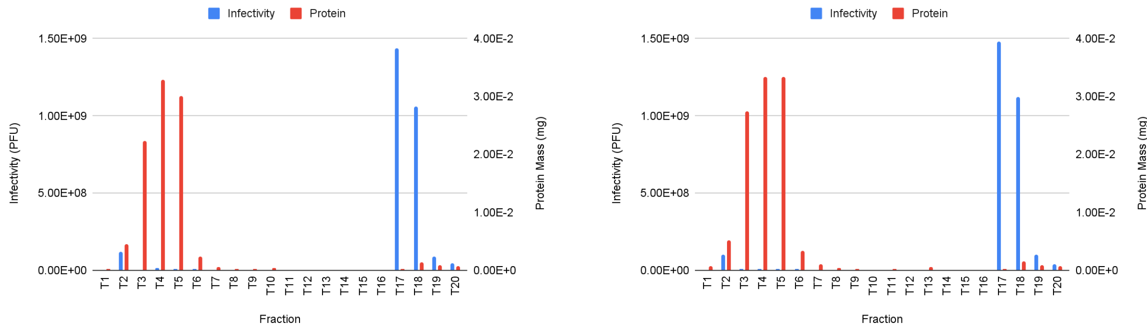


Figure 49. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of condition 1 (Milli-Q water as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction collected during phenyl boronate chromatography.

Condition 2

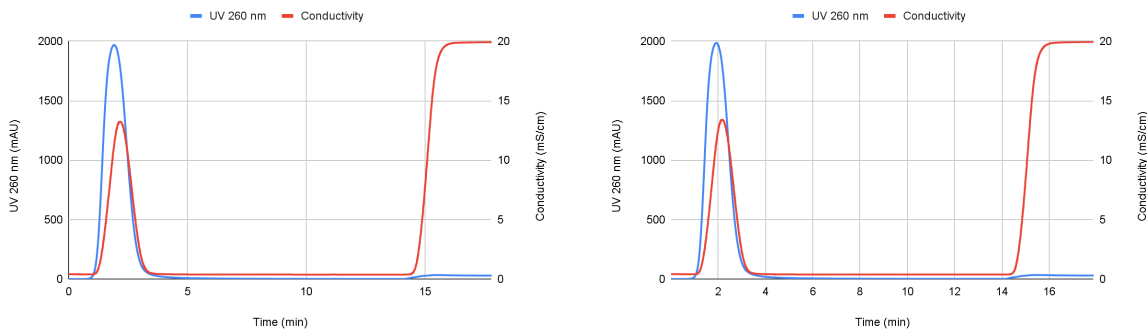


Figure 50. Chromatogram of the second (left) and third (right) triplicates of Condition 2 (15 mM Tris-HCl, pH 8.5 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

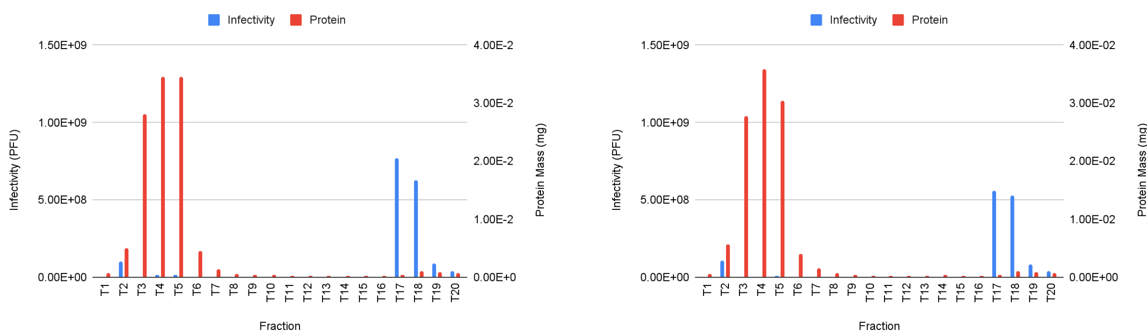


Figure 51. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of condition 2 (15 mM Tris-HCl, pH 8.5 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction collected during phenyl boronate chromatography.

Condition 3

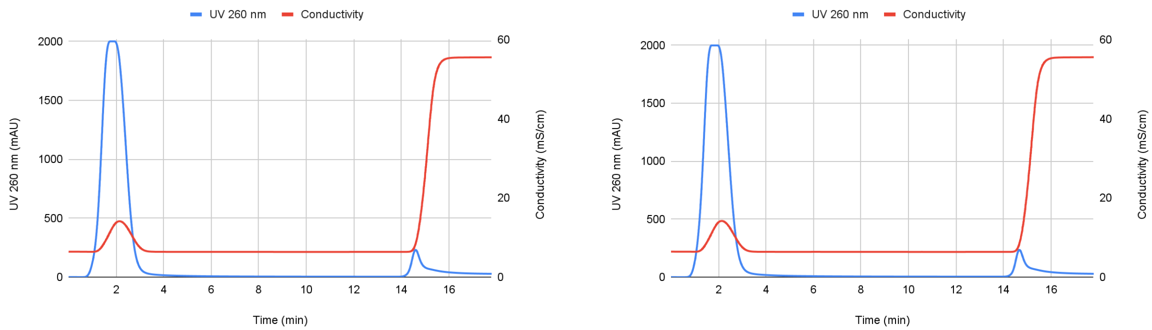


Figure 52. Chromatogram of the second (left) and third (right) triplicates of Condition 3 (15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time.

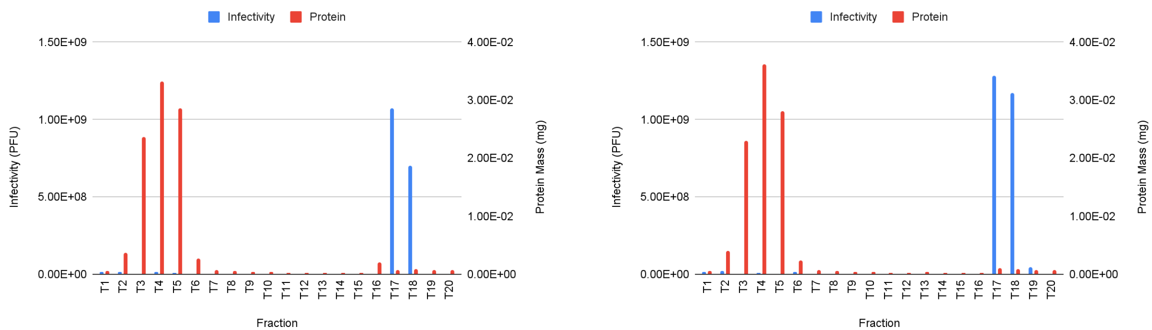


Figure 53. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of condition 3 (15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction collected during phenyl boronate chromatography.

Condition 4

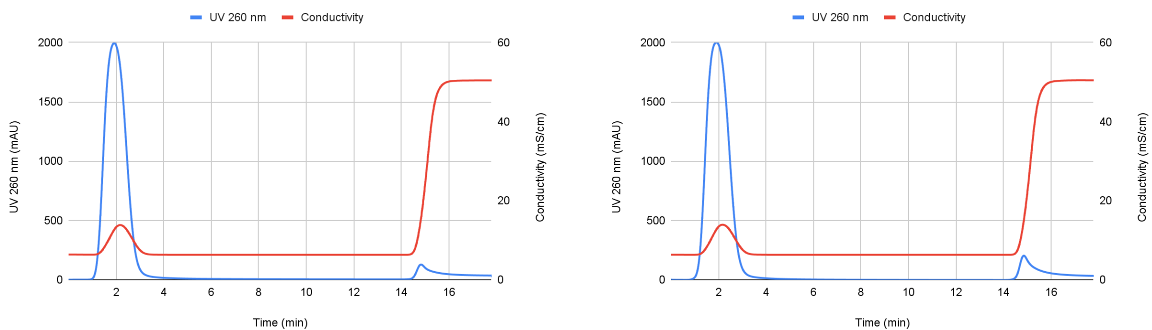


Figure 54. Chromatogram of the second (left) and third (right) triplicates of Condition 4 (15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 7 as elution buffer). The UV, in mAU, was measured at 260 nm per retention time (min). The conductivity, in mS/cm, was also measured per retention time (min).

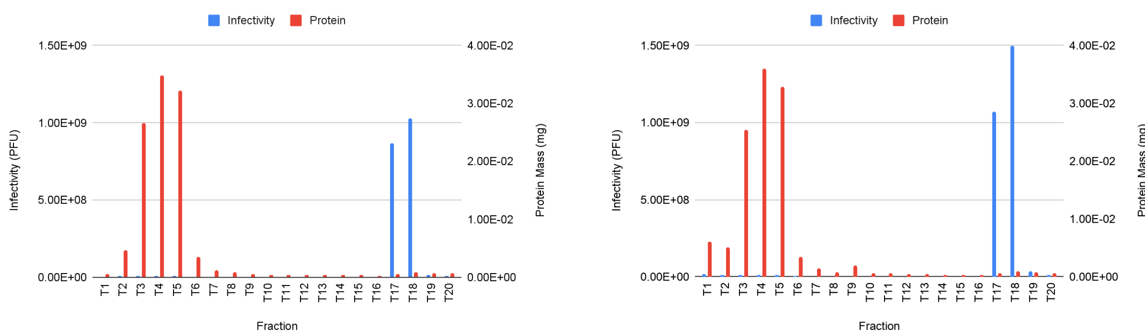


Figure 55. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of condition 4 (15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 7 as elution buffer), in each fraction collected during phenyl boronate chromatography.

Nuclease Digestion - Activity Stop by Heat

Nuclease Digestion

Table 48. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the samples of the second triplicate with nuclease digestion times 30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours, heat stopped.

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
30 min	0.029	1.34E-02	5.70E+07
1 h	0.007	1.39E-02	2.37E+08
1 h 30 min	0.230	1.08E-02	1.23E+08
2 h	0.076	9.97E-03	2.07E+08

Table 49. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the samples of the third triplicate with nuclease digestion times 30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours, heat stopped, present in the nuclease buffer (10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8).

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
30 min	0.038	1.42E-02	6.15E+07
1 h	0.025	1.12E-02	1.88E+08
1 h 30 min	0.216	1.11E-02	1.53E+08
2 h	0.234	1.08E-02	2.12E+08

Phenyl Boronate Chromatography - 30 Minutes Digestion

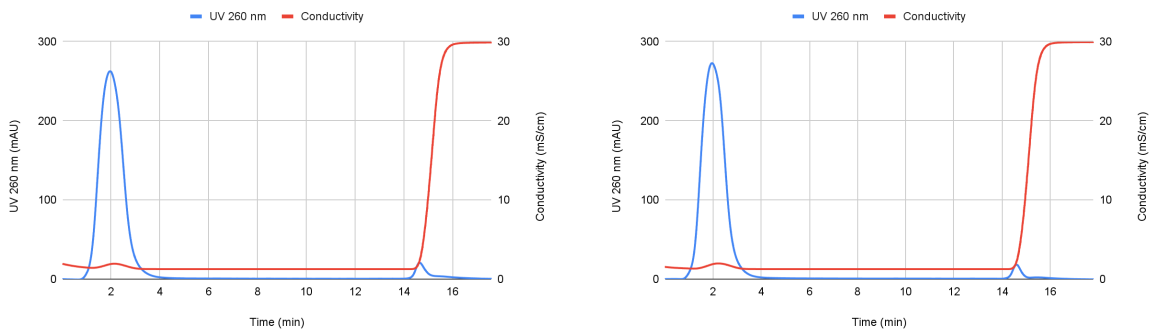


Figure 56. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 30 minutes digestion, heat stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

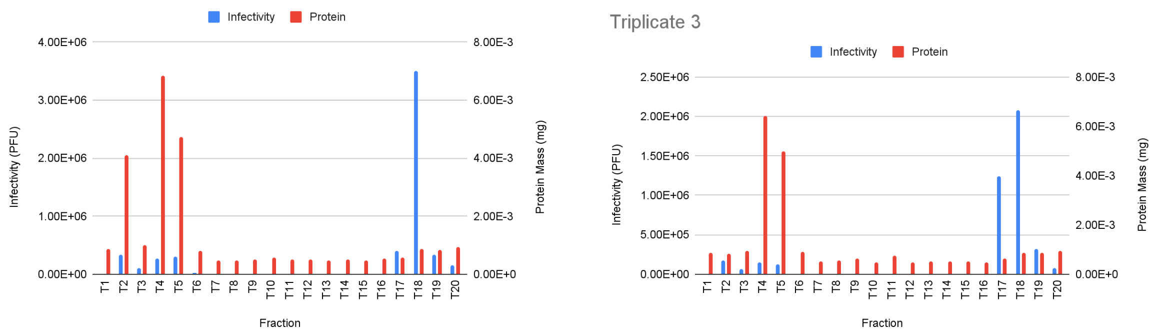


Figure 57. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 30 minutes of digestion, heat stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Phenyl Boronate Chromatography - 1 Hour Digestion

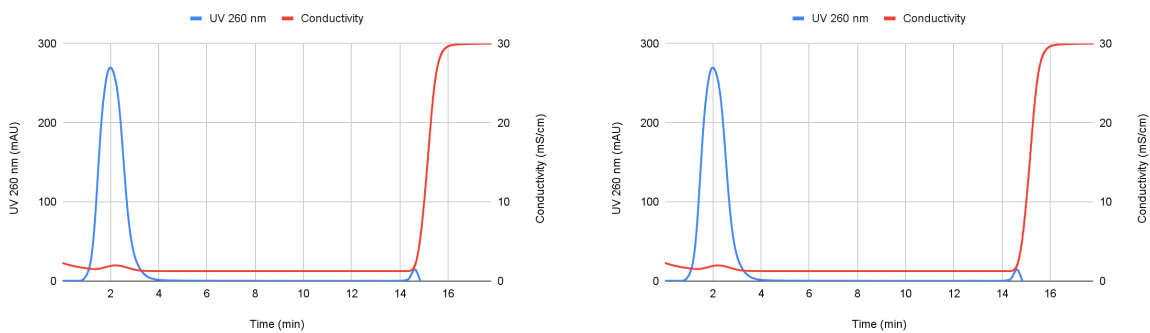


Figure 58. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 1 hour digestion, heat stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as

equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

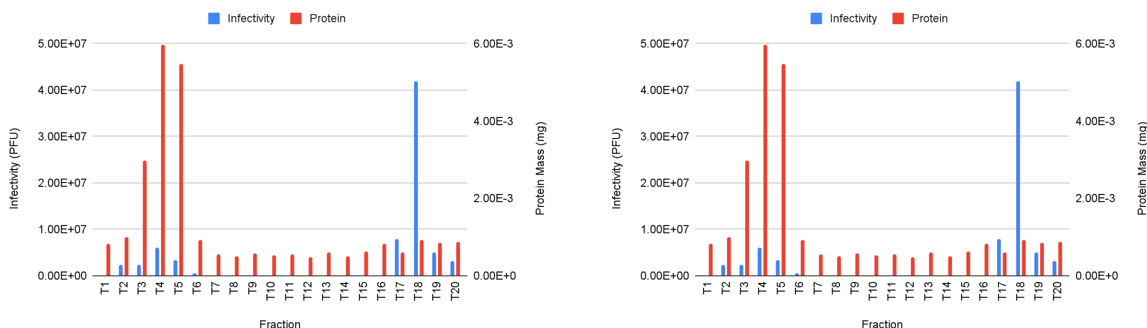


Figure 59. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour of digestion, heat stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

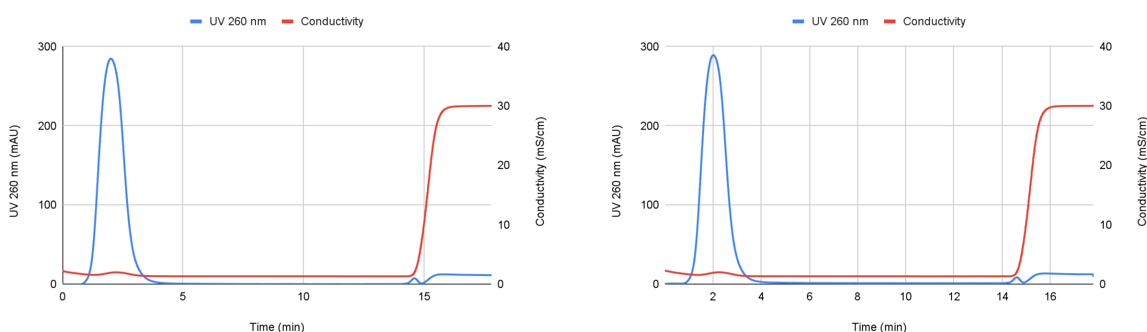


Figure 60. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 1 hour and 30 minutes digestion, heat stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

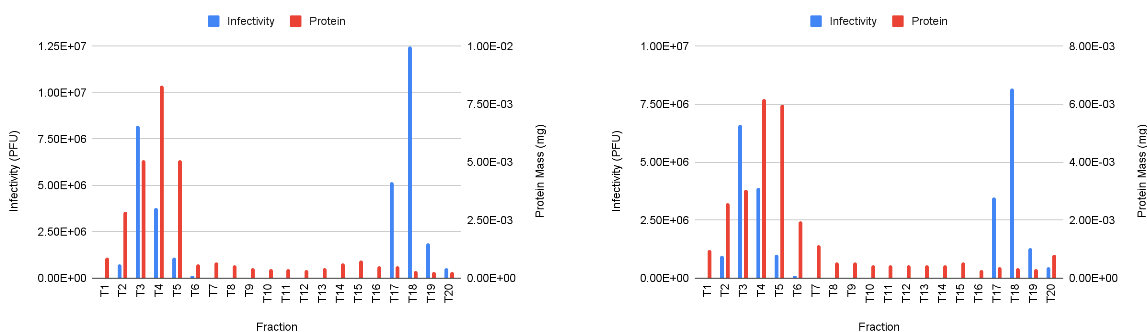


Figure 61. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour and 30 minutes of digestion, heat stopped, after the nuclease digestion in each

fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Phenyl Boronate Chromatography - 2 Hours Digestion

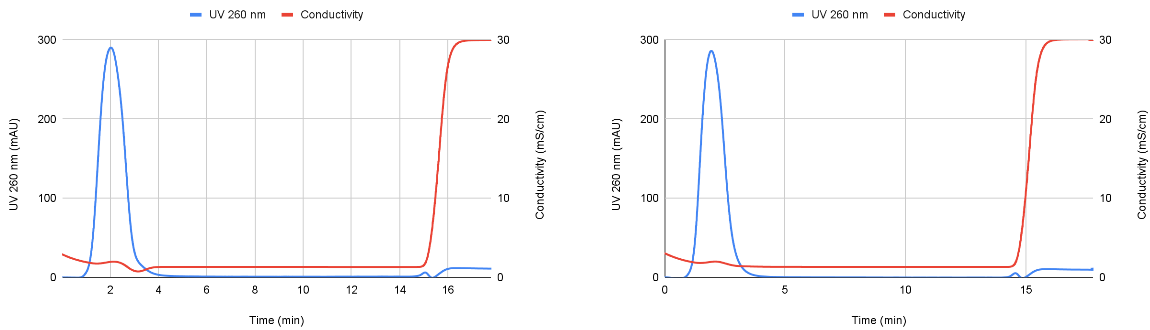


Figure 62. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 1 hour and 30 minutes digestion, heat stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

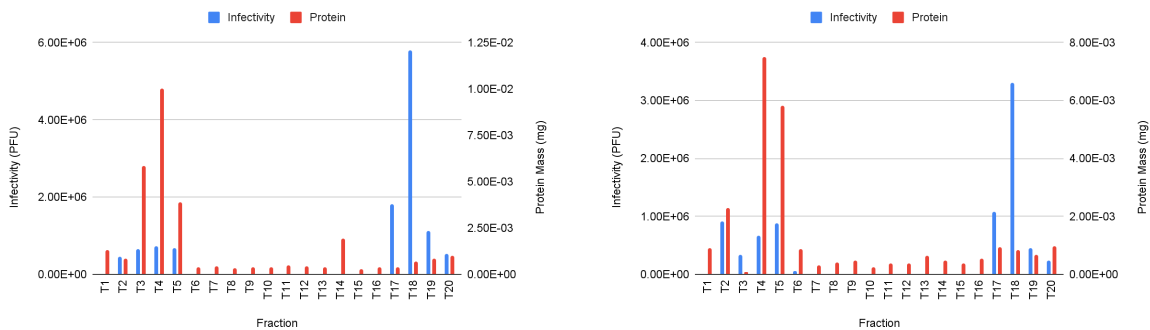


Figure 63. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour and 30 minutes of digestion, heat stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Adsorption Buffer Exchange

Table 50. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate, sample present in the adsorption buffer (15 mM of Tris-HCl, pH 7) for the samples of the second and third duplicate/triplicate with nuclease digestion times 30 minutes, 1 hour, 1 hour and 30 minutes and 2 hours, heat stopped.

	Duplicate/Triplicate		
Sample	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
Lysate	2.18E+00	4.64E-02	7.20E+09
Adsorption Buffer	1.01E+00	1.81E-04	6.30E+06
30 min	1.64E-02	1.53E-04	1.89E+06
1 h	5.74E-03	7.11E-05	8.40E+05
1 h 30 min (2)	2.13E-02	4.37E-05	7.65E+06
1 h 30 min (3)	1.86E-02	1.63E-05	1.13E+07
2 h	1.02E-01	1.53E-04	1.35E+06

Phenyl Boronate Chromatography - 30 Minutes Digestion

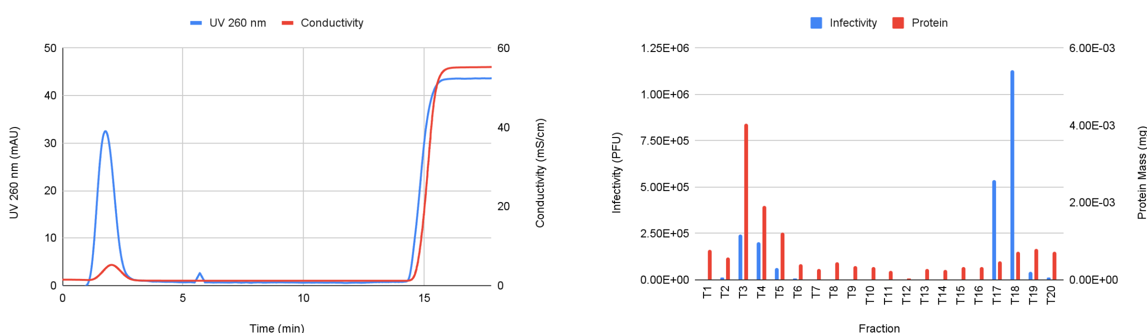


Figure 64. On the left, chromatogram of the second duplicate of 30 minutes digestion, heat stopped, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min). On the right Phages infectivity (PFU) and Protein Mass in mg, for the second duplicate of 30 minutes digestion, after the adsorption buffer exchange, in each fraction collected during phenyl boronate chromatography.

Phenyl Boronate Chromatography - 1 Hour Digestion

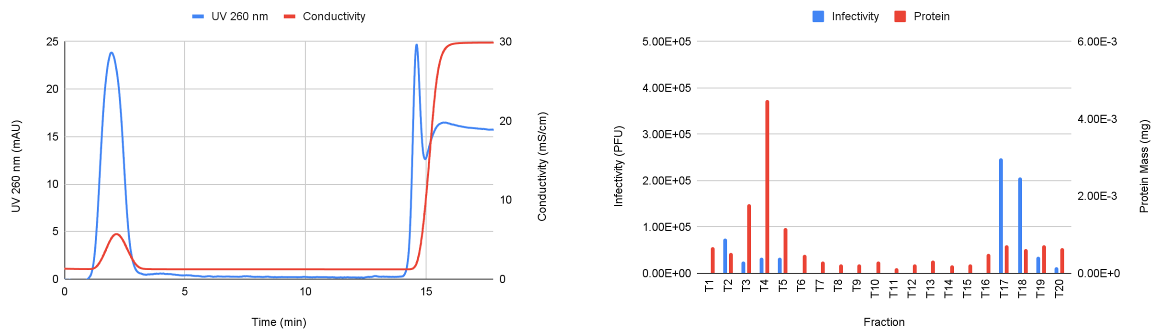


Figure 65. On the left, chromatogram of the second duplicate of 1 hour digestion, heat stopped, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min). On the right, Phages infectivity (PFU) and Protein Mass in mg, for the second duplicate of 1 hour digestion, after the adsorption buffer exchange, in each fraction collected during phenyl boronate chromatography.

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

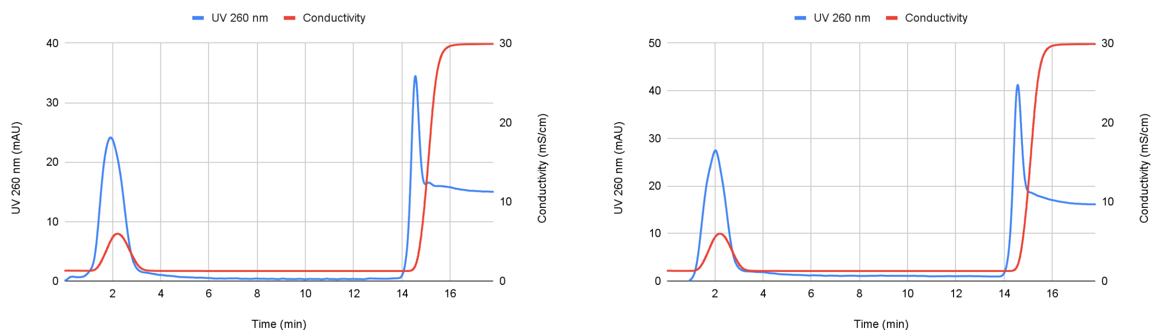
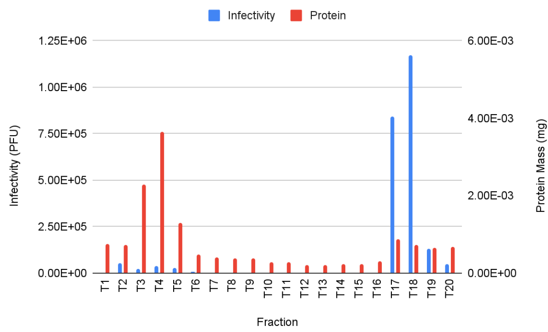


Figure 66. On the left, chromatogram of the second triplicate and on the right of the third triplicate of 1 hour 30 minutes digestion, heat stopped, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).



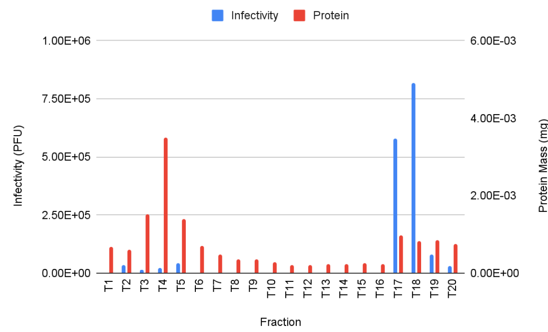
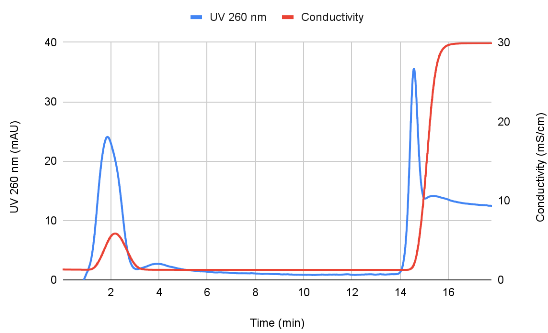


Figure 67. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour and 30 minutes of digestion, heat stopped, after the adsorption buffer exchange, (15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer), in each fraction collected during phenyl boronate chromatography.

Phenyl Boronate Chromatography - 2 Hours Digestion



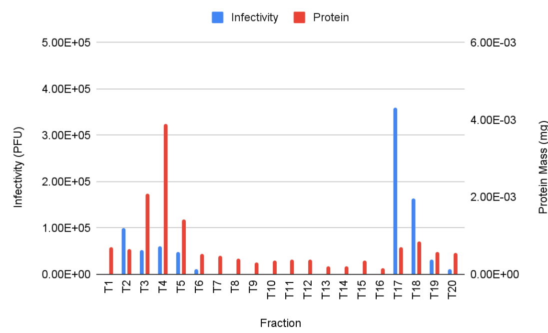


Figure 68. On the left, chromatogram of the second duplicate of 2 hours digestion, heat stopped, after the adsorption buffer exchange, (15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer). The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min). On the right, Phages infectivity (PFU) and Protein Mass in mg, for the second duplicate of 2 hours digestion, after the adsorption buffer exchange, in each fraction collected during phenyl boronate chromatography.

Nucleo Digestion - Activity Stop by Cold

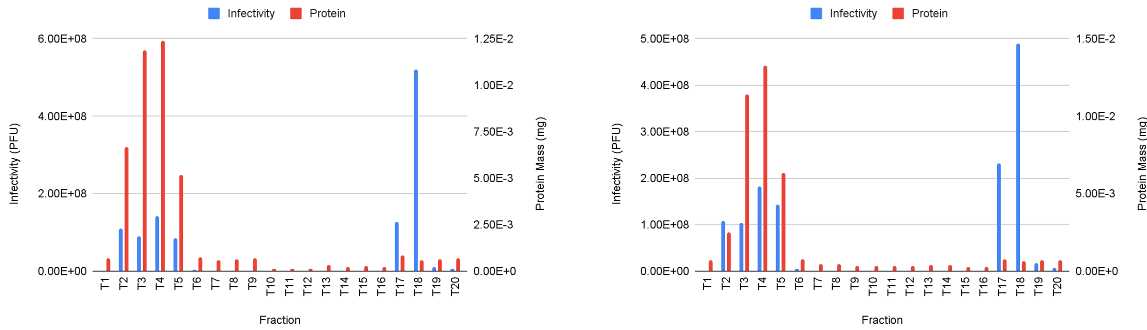


Figure 70. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 30 minutes of digestion, cold stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

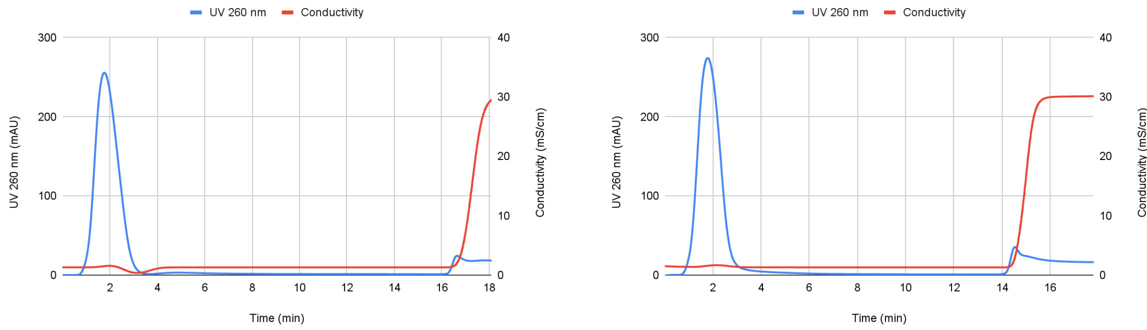


Figure 71. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 1 hour and 30 minutes digestion, cold stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

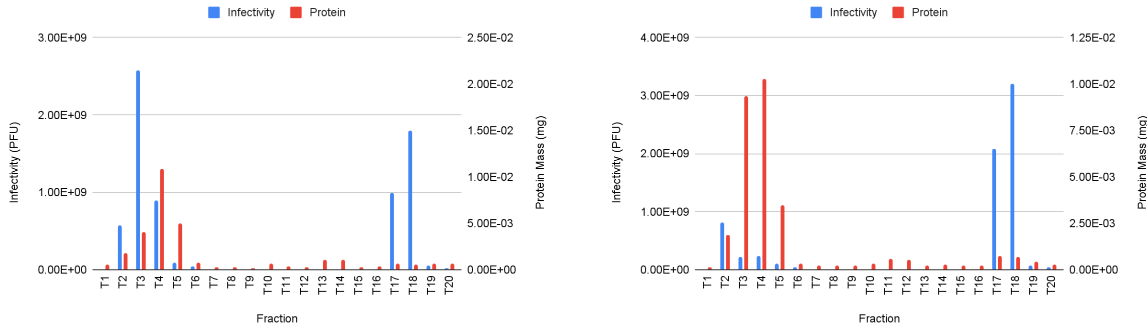


Figure 72. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour and 30 minutes of digestion, cold stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Adsorption Buffer Exchange

Table 53. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the samples of the second triplicate with nuclease digestion times 30 minutes, and 1 hour and 30 minutes, cold stopped.

Sample	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
30 min	1.12E-02	5.19E-03	1.26E+08
1 h 30 min	2.62E-02	5.53E-03	1.62E+08

Table 54. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the samples of the third triplicate with nuclease digestion times 30 minutes, and 1 hour and 30 minutes, cold stopped.

Sample	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
30 min	1.20E-02	5.11E-03	1.01E+08
1 h 30 min	2.95E-02	5.26E-03	1.58E+08

Phenyl Boronate Chromatography - 30 Minutes Digestion

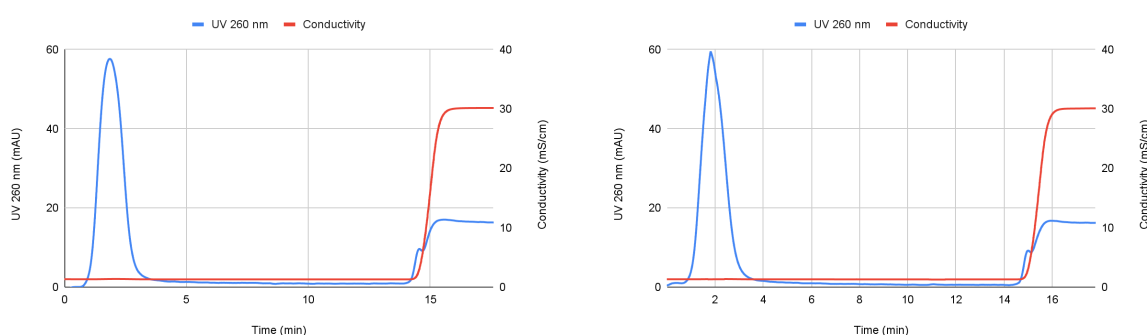


Figure 73. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 30 minutes digestion, cold stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

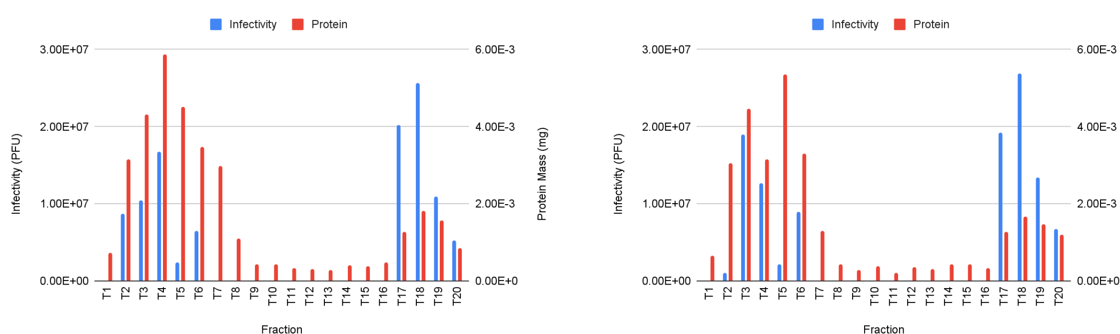


Figure 74. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 30 minutes of digestion, cold stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

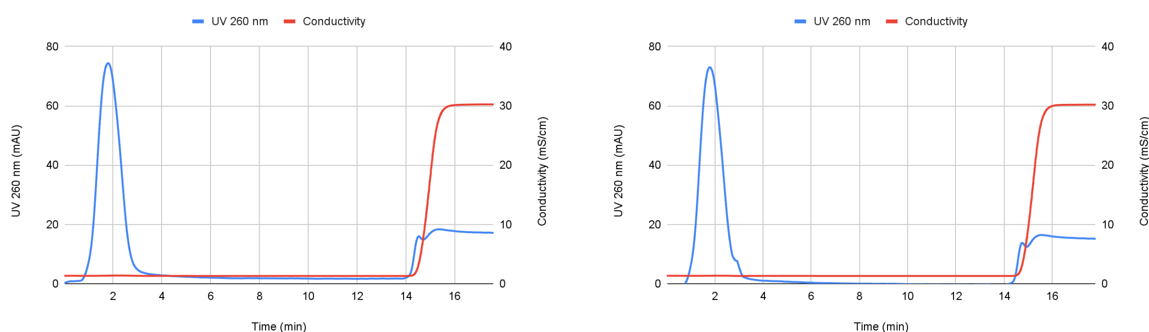


Figure 75. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 1 hour and 30 minutes digestion, cold stopped, after the nuclease digestion. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

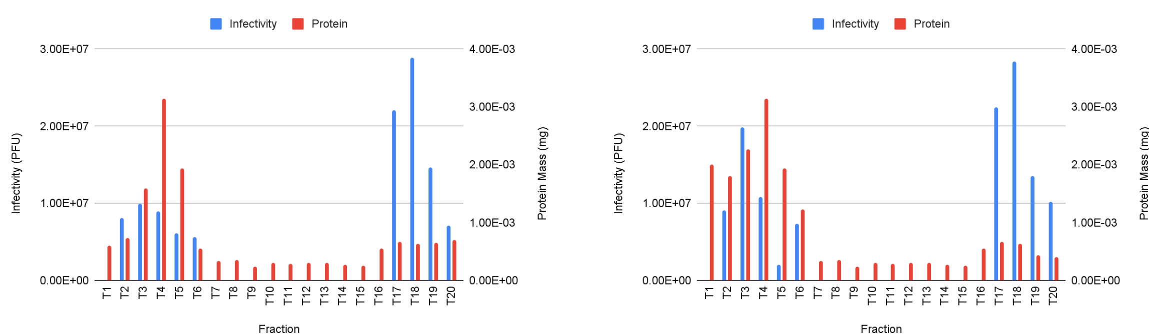


Figure 76. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour and 30 minutes of digestion, cold stopped, after the nuclease digestion in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Nuclease Digestion to the Lysate

Nuclease Digestion

Table 55. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate and for the samples of the second triplicate with nuclease digestion times 30 minutes and 1 hour and 30 minutes.

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
30 min	0.005	4.68E-02	6.15E+08
1 h 30 min	0.014	4.75E-02	6.45E+08

Table 56. Phage infectivity (PFU), DNA quantity (µg) and Protein quantity (mg) for the Lysate and for the samples of the second triplicate with nuclease digestion times 30 minutes and 1 hour and 30 minutes.

	DNA Quantity (µg)	Protein Quantity (mg)	Infectivity (PFU)
30 min	0.006	4.80E-02	6.30E+08
1 h 30 min	0.022	4.81E-02	5.85E+08

Phenyl Boronate Chromatography - 30 Minutes Digestion

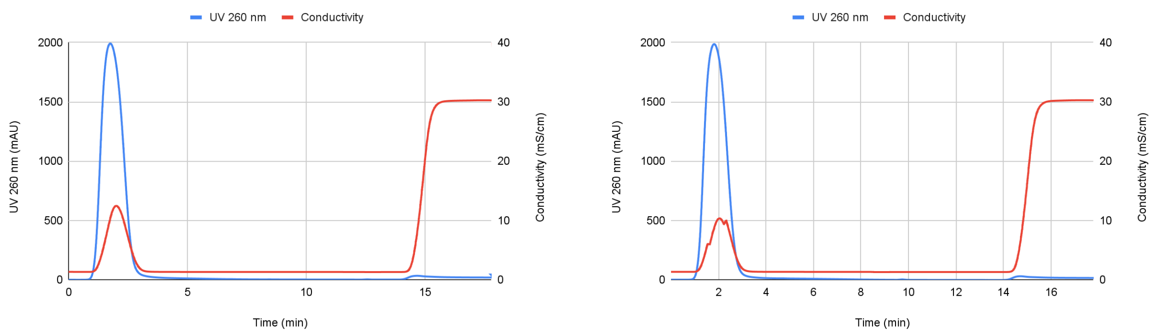


Figure 77. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 30 minutes digestion, after the nuclease digestion to the lysate. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

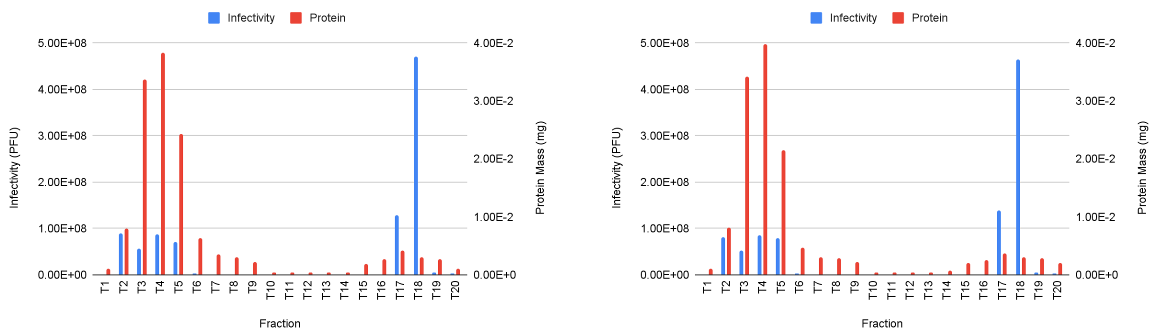


Figure 78. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 30 minutes of digestion, after the nuclease digestion to the lysate in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.

Phenyl Boronate Chromatography - 1 Hour and 30 Minutes Digestion

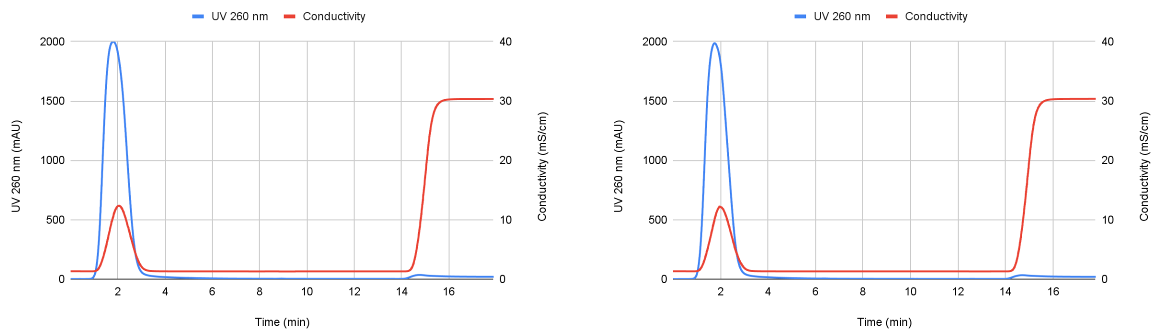


Figure 79. On the left, chromatogram of the second triplicate and on the right of the third triplicate of the 1 hour and 30 minutes digestion, after the nuclease digestion to the lysate. It used 15 mM of Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer. The UV at 260 nm, in mAU, and the conductivity, in mS/cm, were measured per retention time (min).

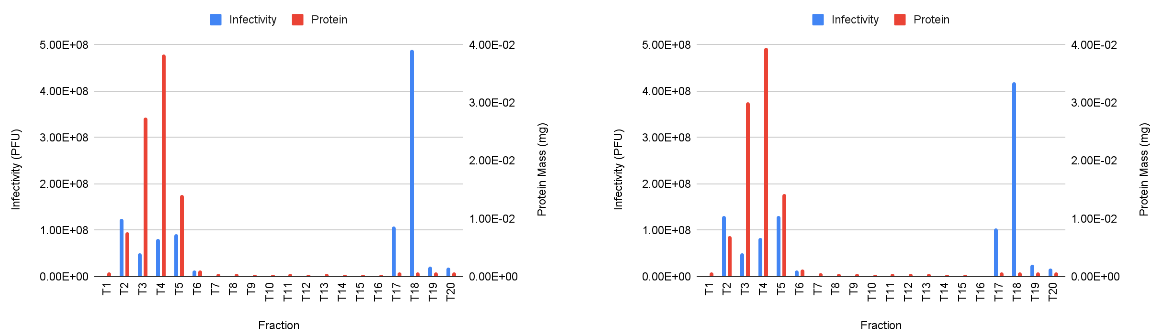


Figure 80. Phages infectivity (PFU) and Protein Mass in mg, for the second (left) and the third (right) triplicates of 1 hour and 30 minutes of digestion, after the nuclease digestion to the lysate in each fraction collected during phenyl boronate chromatography. Chromatography performed using 15 mM Tris-HCl, pH 7 as equilibration and adsorption buffer and 1.5 M Tris-HCl, pH 8.5 as elution buffer.