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Exploring hydrophobic interactions for the purification of the T4 bacteriophage: Phenyl and Phenyl Boronate Chromatography

André Trigo Moutinho Gomes Nº98470

iBB – Institute of Bioengineering and Biosciences, Instituto Superior Técnico

Abstract

The world is on the verge of entering once again a "pre-antibiotic era", associated with the emergence of antibiotic-resistant bacteria. Bacteriophage therapy poses as one of the most promising tools to combat this threat. However, there is yet an established bacteriophage purification scheme that considers the complete removal of host impurities. Various chromatography modes were tested in this work as this technique should be at the center of a solution. Phenyl Boronate Chromatography (PBC) was successful at adsorbing bacteriophages via hydrophobic interactions, resulting in a yield of 54.5% and a removal of proteins, dsDNA, and endotoxins of 94.9%, 95.8%, and 98.1%, respectively. Based on these optimistic results, Hydrophobic Interaction Chromatography (HIC) was tested. Different ammonium sulfate concentrations in the equilibration phase were tested, with 0.75 M attaining the highest bacteriophage recovery (93.0%) and a removal of proteins, dsDNA, and endotoxins of 98.3%, 88.3%, and 93.3%, respectively. A linear gradient served as a foundation for the development of multi-step gradients more appropriate for an industrial setting. With 0.75 M ammonium sulfate in the equilibration buffer and five elution steps, a bacteriophage recovery of 70.2% was attained, with a removal of proteins, dsDNA, and endotoxins of 96.8%, 92.5%, and 98.8%, respectively. PBC and HIC looked to be innovative tools for the purification of T4 bacteriophages. What is yet to be answered is how these operations would perform with other bacteriophages, along with the further optimization of HIC in terms of ligands and salt selection.

Keywords: Bacteriophage; Antibiotic-resistant bacteria; Phenyl Boronate Chromatography; Hydrophobic Interaction Chromatography; Host impurities removal

1. Introduction

Antibiotic use is on the verge of becoming idle, as the human population dangerously takes a step closer to a "pre-antibiotic era".[1] In the last 30 years, only two new antibiotic classes have been approved for use.[2] Pharmaceutical companies are not interested in developing new antibiotics, mainly due to the complexity behind novel biological activity against bacteria.[3] Bacteriophage therapy poses as a simple and efficient alternative to antibiotics.[4] The downstream processing of bacteriophages is historically based on a CsCl density gradient ultracentrifugation. However, this process is not a viable option for large-scale manufacturing under cGMP conditions, due to its limited productivity. Additionally, some phages become inactive due to interaction with CsCl, osmotic shock, or exposure to high-shear stress.[5] Nowadays, alternatives to ultracentrifugation may be found in the literature with chromatography being primarily selected due to its scalability and high purification factors. Anion-exchange chromatography (AEC) is the most used system for viral particle purification.[6]

Affinity chromatography results in high purification factors however, no specific ligands are known for phages.[7] Despite this, phenyl boronate chromatography (PBC) was successfully used for T4

bacteriophage purification.[8] The biological affinity behind PBC is bedded in the reversible esterification between phenyl boronate and 1,2-*cis*-diol groups.[9–11] Boronic acid (BA) ligands have a pKa of 8.8 and depending on the medium's pH, present two distinct conformations: the tetrahedral conformation (pH > 8.8) and the trigonal planar conformation (pH < 8.8).[9–11] PBC's use in phage purification is not associated with the referred "primary" interaction in these columns but with secondary, interactions, such as charge-transfer or hydrophobic interactions.[10,11] Charge-transfer interactions are associated with the trigonal planar BA conformation, where the boron atom works as an electron acceptor due to the existence of an empty orbital. Hydrophobic interactions are based on aromatic π-π interactions between the phenyl ring of BA ligands and hydrophobic moieties.[9,10]

Hydrophobic interaction chromatography (HIC) exploits the interaction between immobilized hydrophobic ligands and hydrophobic surface regions of proteins. Little scientific knowledge associating HIC to virus purification is available.[12] The adsorption step of this chromatography is based on the salting out effect, where the addition of salts to a solution enhances protein-protein hydrophobic interactions.[13–15] Generally speaking, ammonium sulfate is the most used solution for HIC adsorption as it is highly soluble and stable, also offering a high salting out effect.[16] HIC-based processes are usually problematic due to the initially applied high salt concentrations, which may instantly inactivate the virus or lead to their precipitation.[12] The stationary phase of HIC is based on linear chain alkanes ligands or aromatic ligands. The hydrophobicity of the stationary phase increases with the increase of the length of the alkyl-chain, while the adsorption selectivity decreases.[15]

This work aims to design an integrated purification process for bacteriophages with complete host impurities quantification and that can be scaled up to industrial use. Viúla[8] proposed PBC as a novel alternative chromatography mode for the purification of bacteriophages but did not explain how bacteriophages adsorb to this column. The findings in PBC motivated the use of HIC for the purification of bacteriophages. Three operation modes were tested: single-step gradient, linear gradient, and multi-step gradient. Various ammonium sulfate concentrations in the equilibration buffer were tested.

2. Methods

2.1. Materials

Escherichia coli DSM 613 and T4 bacteriophage DSM 4505 were acquired from DSMZ (Braunschweig, Germany). Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were purchased from Biokar Diagnostics (Pantin, France). Tris-HCl was bought from Fischer Scientific (Pittsburgh, USA). The impurity quantification kits were purchased from Thermo Fischer (Massachusetts, USA). CHES and Sorbitol were purchased from Sigma Aldrich (Missouri, USA). $MgCl_2$ and $(NH_4)_2SO_4^-$ were acquired from Panreac Quimica (Barcelona, Spain). The aminophenylboronate P6XL resin (1 mL) was acquired from ProMetic Biosciences (Montreal, Canada), while the 1 mL HiTrap™ Phenyl Sepharose 6 Fast Flow (High sub) was purchased from Cytiva (Uppsala, Sweden). Centrifugation of the crude phage lysate was done in an Eppendorf Centrifuge 5810 R from Eppendorf (Hamburg, Germany). Filtration of the crude phage lysate was done with a 0.22 µm pore-sized falcon top filter from Labbox (Barcelona, Spain). All chromatography experiments used an ÄKTA start system, associated with the UNICORN 1.1 start software and the Frac30 fraction collector, acquired from GE Healthcare (Uppsala, Sweden). Diafiltration was done with Vivaspin® 6 100 kDa MWCO centrifuge filters purchased from GE Healthcare

(Uppsala, Sweden). The impurity quantification assays utilized both a SpectraMax 340PC spectrophotometer from Molecular Devices (San Jose, USA) and a POLARstar OPTIMA spectrofluorometer from BMG Labtech (Allmendgrün, Germany).

2.2. T4 bacteriophage amplification

The production of the T4 bacteriophage lysate was initiated by the overnight pre-inoculation of *E. coli* K-12 in 30 mL of TSB medium, at 37 ºC and under agitation (250 rpm). On the next day, 200 mL of TSB medium were inoculated with the previously prepared pre-inoculum, knowing that a final OD of 0.1 was intended to attain in the inoculum. Next, the inoculum was grown at 37ºC and under agitation. At the beginning of the exponential phase ($OD_{600\ nm}$ = 0.25-0.30), T4 bacteriophage infection was performed, considering a Multiplicity of Infection of 0.1. Upon infection, 10 mM $MgCl_2$ was also added to the culture. The same incubation conditions were resumed until a final $OD_{600\ nm}$ lower than 0.1 was obtained. The resultant crude T4 bacteriophage lysate was clarified using a centrifugation followed by a microfiltration. Centrifugation was done at 8000 x g, with a temperature of 4ºC for 20 min. The recovered supernatant was microfiltered through a falcon top filter, using a vacuum pump.

2.3. Double-agar plaque assay

Bacteriophage quantification was performed by the double-agar plaque assay (DLPA). Initially, a TSB medium was inoculated with a grown overnight *E. coli* K-12 culture. Bacteria were grown at 37ºC and under agitation until the beginning of the exponential phase. 200 µL of bacteria culture was added to 100 µL of T4 bacteriophage diluted samples in Saline-Magnesium buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8.1 mM $MgSO_4.7H_2O$, 0.01% w/v gelatine). A pre-adsorption step between bacteria and bacteriophages for 15 min at 37ºC, without agitation, followed. In parallel, top agar, prepared with 0.7% w/v agar-agar in a TSB medium, was melted, thermostatized at 64ºC, and supplemented with 10 mM $MgCl_2$. After the pre-adsorption step, 3 mL of the top agar was added to each vial, which was poured onto TSA plates (4% w/v TSA). The plates were incubated overnight, at 37ºC and without agitation. Plaque forming units (PFU) were counted and the bacteriophage concentration (PFU/mL) was determined. Only plates with 30-300 plaques were considered acceptable.

2.4. Phenyl boronate chromatography

A constant flow rate of 1 mL/min and an injection volume of 5 mL were employed in all runs. Equilibration was done with 6 column volumes (CV) of the correspondent washing buffer. The initially tested washing and elution phase conditions were performed with 10 CV of, respectively, 15 mM Tris-HCl pH 7.0 and 1.5 M Tris-HCl pH 8.5.[8] Hydrophobic interactions were promoted by employing a washing phase of 5 CV (15 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$ pH 7.0) and an elution phase of 12 CV (15 mM Tris-HCl pH 7.0). Sample conditioning before loading with 1.5 M $(NH_4)_2SO_4$ was done. Charge-transfer interactions were hampered by employing 5 CV of washing buffer (15 mM CHES pH 9.5) and 10 CV of elution buffer (1.5 M Tris-HCl pH 8.5). Sample diafiltration before loading with centrifuge filters was performed. Fractions of 1 mL were collected and pooled together. Bacteriophage titer in each fraction pool was determined with the DLPA. Additionally, the protein, dsDNA, and endotoxin content were assessed.

2.5. Hydrophobic interaction chromatography

HIC experiments were carried out with the same system configurations as described for the PBC. The equilibration/washing buffer was composed of 15 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$ pH 7.0 (except when

stated otherwise), while the elution buffer was composed of 15 mM Tris-HCl pH 7.0. Column equilibration was done with 6 CV, followed by a loading phase of 5 CV and a washing phase of 5 CV. Three elution operation modes were tested: linear-gradient elution, single-step-gradient elution, and multi-step-gradient elution. In the linear-gradient operation mode, elution was done with 20 CV (0-100% elution buffer). For the single-step-gradient operation mode, elution was done with 7 CV and different ammonium sulfate concentrations (1.5, 1.0, 0.75, and 0.50 M) were tested. Two multi-step-gradient elution schemes were devised: Processes 1 and 2. Process 1 used a five-step-gradient elution operation mode (70%, 75%, 80%, 85%, and 100% elution buffer). Process 2 implemented a five-step-gradient elution operation mode (35%, 50%, 65%, 80%, and 100%) with an equilibration/washing buffer composed of 15 mM Tris-HCl, 0.75 M $(NH_4)_2SO_4$ pH 7.0. All processes used elution steps of 5 CV. Fractioning and content analysis of each defined pool was done as described for the PBC.

2.6. Protein, dsDNA, and endotoxin quantification assays

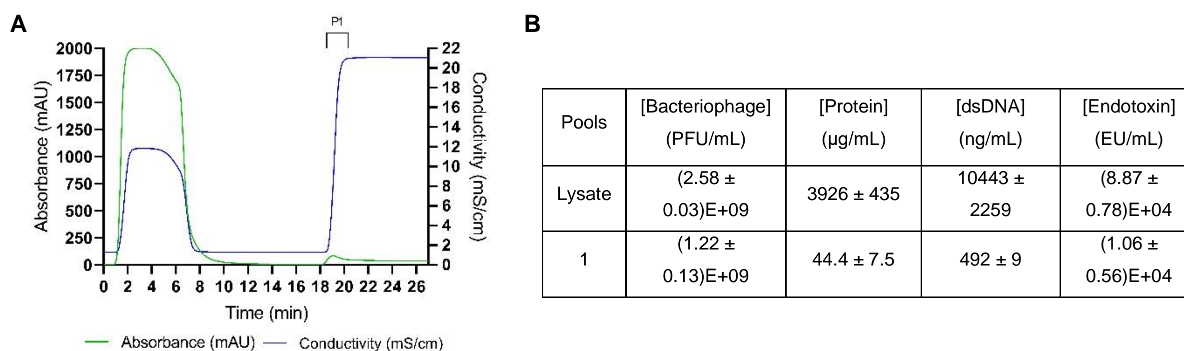
Protein quantification was performed with the Pierce™ BCA protein assay kit, while DNA quantification was performed with the Quant-iT™ PicoGreen™ dsDNA assay kit, both according to the manufacturer's instructions. Endotoxin quantification was performed with the Pierce™ chromogenic endotoxin assay kit, according to the manufacturer's instructions. All procedures took place inside the laminar flow chamber. Besides sample dilution, every procedure was done under pyrogenic-free conditions. Throughout the assay, the 96-well microplate should have been maintained at 37ºC ± 1ºC. However, due to the lack of appropriate equipment, this was not possible to achieve.

3. Results and discussion

3.1. Phenyl boronate chromatography

PBC was the first chromatography operation mode to be tested in this work. Viúla[8] managed to attain a bacteriophage yield of 49%, with 96% and 47% of proteins and DNA removed, respectively. Despite the promising results, no endotoxin quantification was done, nor was any attempt to explain the interactions involved in the adsorption of these viral particles to the chromatographic column. Initially, the optimized buffer conditions determined by Viúla[8] were applied in a PBC run (Figure 1A). The content composition for the loaded lysate and the elution pool is represented in Figure 1B.

According to Figure 1A, two distinct absorbance peaks are observed. The first one is the flow-through which is associated with the column loading and washing and contains all the components that did not interact with the PBC column. The second peak coincides with the elution, triggered by increasing the ionic strength and contains the components that were adsorbed and eluted under the working conditions. The bacteriophage recovery in pool 1 was 37.8%, while the removal of proteins, dsDNA, and endotoxins were, respectively, 99.1%, 96.2%, and 90.4%. Likewise the work from Viúla[8], a high protein removal was registered, likely due to the absence of *cis*-diol moieties in these molecules. Secondary interactions appear to also not contribute to the column adsorption of proteins, despite the presence of amine and carboxyl groups in the side chains of amino acids that could allow adsorption by charge-transfer interactions. DNA removal was much higher in this work compared to that of Viúla[8]. Charge-transfer interactions between the nitrogen atoms of adenine, guanine, and cytosine with the boron receptor in BA ligands could contribute to dsDNA column adsorption. Nonetheless, this effect was not apparent in the developed work. Despite the high endotoxin removal, the final absolute value (10^4



Pools	[Bacteriophage] (PFU/mL)	[Protein] (µg/mL)	[dsDNA] (ng/mL)	[Endotoxin] (EU/mL)
Lysate	(2.58 ± 0.03)E+09	3926 ± 435	10443 ± 2259	(8.87 ± 0.78)E+04
1	(1.22 ± 0.13)E+09	44.4 ± 7.5	492 ± 9	(1.06 ± 0.56)E+04

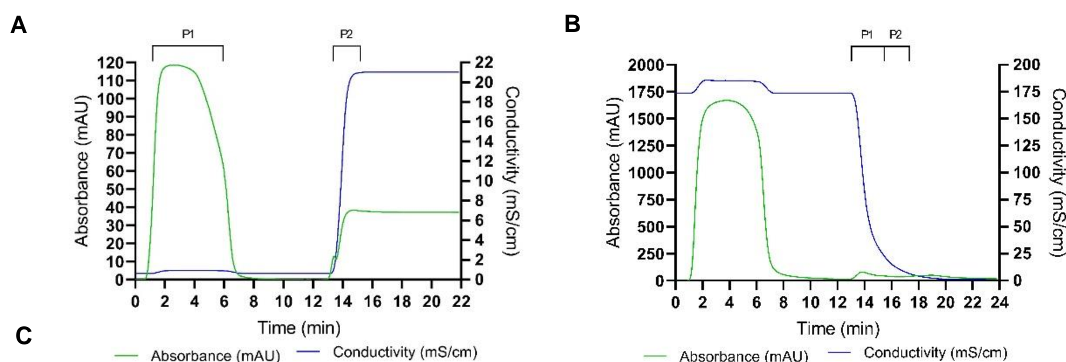
Figure 1- PBC chromatogram using the previously optimized chromatography conditions (A), with the content composition for the defined pools (B). (A) Absorbance (mAU) at 280 nm and conductivity (mS/cm) were measured throughout time (min) at 1 mL/min on the outlet stream of the chromatography column. The equilibration/washing buffer was 15 mM Tris-HCl pH 7.0 and the elution buffer was 1.5 M Tris-HCl pH 8.5. Fractions of 1 mL were collected, with the elution pool (Fraction 21 to 24) being devised and represented in the upper section of the chromatogram by **P1**. **(B)** For the lysate and pool 4, the bacteriophage (PFU/mL), protein (µg/mL), dsDNA (ng/mL), and endotoxin (EU/mL) concentration are represented.

EU/mL) was far beyond the needed endotoxin concentration for therapeutic use, which should be below 10^3 EU/mL.[17]

3.2.1. Charge-transfer and hydrophobic interactions

Bacteriophage adsorption in PBC is most likely associated with secondary interactions with the aminophenyl boronic acid. Among these, charge-transfer and hydrophobic interactions are thought as the most likely to be at the core of this adsorption. To hamper charge-transfer interactions in PBC, its ligand conformation had to be altered, from the trigonal planar structure to the tetrahedral one. Therefore, the working pH of the chromatography equilibration/washing buffer had to be increased above 8.8. Note that the loaded bacteriophage lysate went through a buffer exchange against the PBC equilibration buffer, with a yield of 34.4%. Hydrophobic interactions enhancement was attained by pre-equilibrating the PBC column with a high ammonium sulfate concentration and was then reduced by the complete removal of ammonium sulfate in the elution phase. The PBC chromatograms, as well as the content composition, for the buffer conditions either hampering the charge-transfer interactions or promoting hydrophobic interactions, are represented in Figure 2.

If bacteriophages are adsorbed to a PBC column via charge-transfer interactions, they should be collected in the washing phase of the performed chromatography. This occurs due to the tetrahedral conformation of the BA ligands in the equilibration phase, which does not allow this type of interaction. According to the registered results in Figure 2A, most (48.8%) of the injected bacteriophages in this chromatography were collected in the elution phase (Pool 2), while a reduced fraction (15.1%) was collected in the flow-through peak (Pool 1). This indicates that most bacteriophages were still able to adsorb to the column under the set conditions. Therefore, the elimination of charge-transfer interactions during the loading phase did not prevent the binding of bacteriophages to the column. Regarding the enhancement of hydrophobic interactions (Figure 2B), the combination of pools 1 and 2 resulted in a bacteriophage recovery of 54.5%, with a removal of proteins, dsDNA, and endotoxins of 94.9%, 95.8%, and 98.1%. The increase in the bacteriophage recovery compared to the buffer conditions defined by Viúla[8] indicated that hydrophobic interactions were responsible for the bacteriophage adsorption to a PBC column, either via the conventional hydrophobic interaction or via aromatic π-π interactions with the phenyl ring of BA ligands. The T4 bacteriophage presents a few protein complexes that could explain



C

Pools	Volume (mL)	[Bacteriophage] (PFU/mL)	[Protein] (µg/mL)	[dsDNA] (ng/mL)	[Endotoxin] (EU/mL)
Hampering charge-transfer interactions (A)					
Lysate	5	(1.07 ± 0.10)E+09	420 ± 79	4646 ± 397	1.60E+09
1	7.5	(1.07 ± 0.02)E+08	238 ± 60	2156 ± 479	-
2	2	1.30E+09	-	50.1 ± 9.5	(1.11 ± 0.28)E+03
Promoting hydrophobic interactions (B)					
Lysate	5	(2.63 ± 0.20)E+09	4189 ± 394	6194 ± 343	(6.13 ± 3.45)E+04
1	3	(1.58 ± 0.15)E+09	71.2 ± 12.0	300 ± 75	(7.05 ± 2.27)E+02
2	2	(1.22 ± 0.19)E+09	140	206 ± 33	1.79E+03

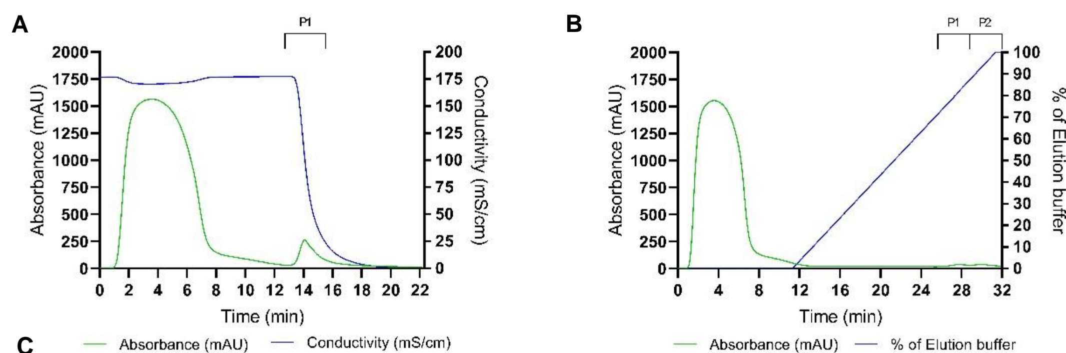
Figure 2- PBC chromatogram hampering the charge-transfer interactions (A) and promoting the hydrophobic interactions (B), with the content composition for the defined pools (C). Absorbance (mAU) at 280 nm and conductivity (mS/cm) were measured throughout time (min) at 1 mL/min on the outlet stream of the chromatography column. **(A)** The equilibration/washing buffer was 15 mM CHES pH 9.5 and the elution buffer was 1.5M Tris-HCl pH 8.5. Fractions of 1 mL were collected and various pools of fractions were considered for further analysis, which are represented in the upper section of the chromatogram by **PX** (with **X** being the pool number). The following pool composition was devised: **Pool 1** (Fraction 2 to 10) and **Pool 2** (Fraction 16 to 17). **(B)** The equilibration/washing buffer was 1.5 M $(NH4)_2SO_4$, 15 mM Tris-HCl pH 7.0 and the elution buffer was 15 mM Tris-HCl pH 7.0. Fractions of 1 mL were collected and various pools of fractions were considered for further analysis, which are represented in the upper section of the chromatogram by **PX** (**X** being the pool number). The following pool composition was devised: **Pool 1** (Fraction 16 to 18) and **Pool 2** (Fraction 19 to 20). **(C)** For the lysate and each defined pool, the bacteriophage (PFU/mL), protein (µg/mL), dsDNA (ng/mL), and endotoxin (EU/mL) concentration are represented.

these interactions. The cell-puncturing device is present at the distal end of the bacteriophage tail tube, in the baseplate hub, and works as a needle that penetrates the periplasmic space of the host for phage DNA translocation.[18] This structure is composed of 6 copies of the gp (gene product) 5-gp27 complex with hydrophobic external surfaces.[19] Surrounding the tail tube, the contractile outer sheath is composed of 138 copies of the tail sheath protein (gp18). Contemplating all three states the outer sheath presents (extended, intermediate, and contracted), between 35% and 42% of surface amino acids are hydrophobic, while only 21-24% are hydrophilic.[20]

3.3. Hydrophobic interaction chromatography

PBC is a type of affinity chromatography however, its most interesting part appears to be the phenyl ring, which was able to efficiently interact with hydrophobic moieties among the bacteriophage's coat proteins. Therefore, the next hypothesis put forward in this work was the use of Phenyl Sepharose for the purification of the T4 bacteriophages. Two different operation modes were tested: a single-step-gradient elution (Figure 3A) and a 20 min linear-gradient elution (Figure 3B). The content composition for the defined pools and lysate is represented in Figure 3C.

In the single-step-gradient elution (Figure 3A), the bacteriophage recovery was 90.7%, with a removal of proteins, dsDNA, and endotoxins of 99.4%, 85.9%, and 96.6%. Regarding the linear-gradient elution (Figure 3B), the bacteriophage recovery was 27.0%, with a removal of proteins, dsDNA, and endotoxins of 99.9%, 94.8%, and 99.6%. These results portray the pros and cons of the single-step-gradient elution versus the linear-gradient elution. While the former offers a high bacteriophage recovery, it fails at efficiently removing dsDNA. The latter offers high impurities removals, but with a significant sacrifice in



Pools	Volume (mL)	[Bacteriophage] (PFU/mL)	[Protein] (µg/mL)	[dsDNA] (ng/mL)	[Endotoxin] (EU/mL)
Lysate	5	(3.23 ± 0.25)E+09	3114 ± 131	6083 ± 890	(6.14 ± 3.05)E+04
Single-step-gradient elution (A)					
1	4	(3.67 ± 0.19)E+09	23.4 ± 8.6	1157 ± 362	(2.61 ± 0.20)+03
Linear-gradient elution (B)					
1	2	(6.10 ± 0.30)E+08	32.0	778 ± 183	(3.28 ± 0.72)E+02
2	2	(2.18 ± 0.07)E+09	6.77	853 ± 159	(5.79 ± 0.12)E+02

Figure 3- Phenyl Sepharose FF chromatogram with a single-step-gradient elution (A) and a 20 min linear-gradient elution (B), with the content composition for the defined pools (C). Absorbance (mAU) at 280 nm and conductivity (mS/cm) were measured throughout time (min) at 1 mL/min on the outlet stream of the chromatography column. The equilibration/washing buffer was 1.5 M $(NH4)_2SO_4$, 15 mM Tris-HCl pH 7.0 and the elution buffer was 15 mM Tris-HCl pH 7.0. Fractions of 1 mL were collected and various pools of fractions were considered for further analysis, which are represented in the upper section of the chromatogram by **PX** (with **X** being the pool number). The following pool composition was devised: **(A) Pool 1** (Fraction 16 to 19); **(B) Pool 1** (Fraction 30 to 31) and **Pool 2** (Fraction 33 to 34). **(C)** For the lysate and each defined pool, the bacteriophage (PFU/mL), protein (µg/mL), dsDNA (ng/mL), and endotoxin (EU/mL) concentration are represented.

the bacteriophage yield. Moreover, it is possible to conclude that bacteriophages appear to strongly beneficiate from the aforementioned hydrophobic surface regions for their efficient adsorption and subsequent elution from the chromatography column. In terms of proteins, these appear to have their hydrophobic residues shielded, not interacting with the phenyl ring and being washed during the initial phase. Endotoxins have a hydrophobic region represented by the lipid A.[21] Therefore, their binding to an HIC column was of no surprise, although it occurred at a shorter extent than expected.

3.3.1. Influence of ammonium sulfate concentration in HIC

For optimization purposes, it is critical to explore the lowest possible ammonium sulfate concentration one must use, without sacrificing product yield. This becomes increasingly important at an industrial scale, where cost saving associated with reagents and wastewater treatment is taken into consideration.[6] From the point of view of the product viability, high salt concentrations may affect virus integrity, reducing virus infectivity and/or leading to their precipitation.[7,12] Employing a single-step-gradient operation mode, three different ammonium sulfate concentrations were tested in the equilibration buffer, namely 1.0, 0.75, and 0.50 M. The correspondent chromatograms and content composition of the defined pools and lysate were not shown in this work. A comparison between these conditions, in terms of bacteriophage recovery and impurities removals, is represented in Table 1.

The lower the concentration of salt during the equilibration phase, the lower will hydrophobic interactions be promoted, resulting in reduced binding of particles during the loading phase.[14] This results in higher removals and lower yields. For instance, the lowest salt concentration in the equilibration buffer resulted in the lowest bacteriophage yield, but the highest removal of endotoxins, proteins, and dsDNA. What was interesting in Table 1 is that the intermediate ammonium sulfate concentration (0.75 M) resulted in the highest bacteriophage yield out of all tested salt concentrations. Compared to 1.5 M ammonium sulfate (Figure 3), the protein and endotoxin removals were lower, but the dsDNA removal was higher.

Table 1- Bacteriophage recovery and impurities removals for the different ammonium sulfate concentrations (1.0, 0.75, and 0.50 M) in the equilibration buffer of a Phenyl Sepharose FF chromatography.

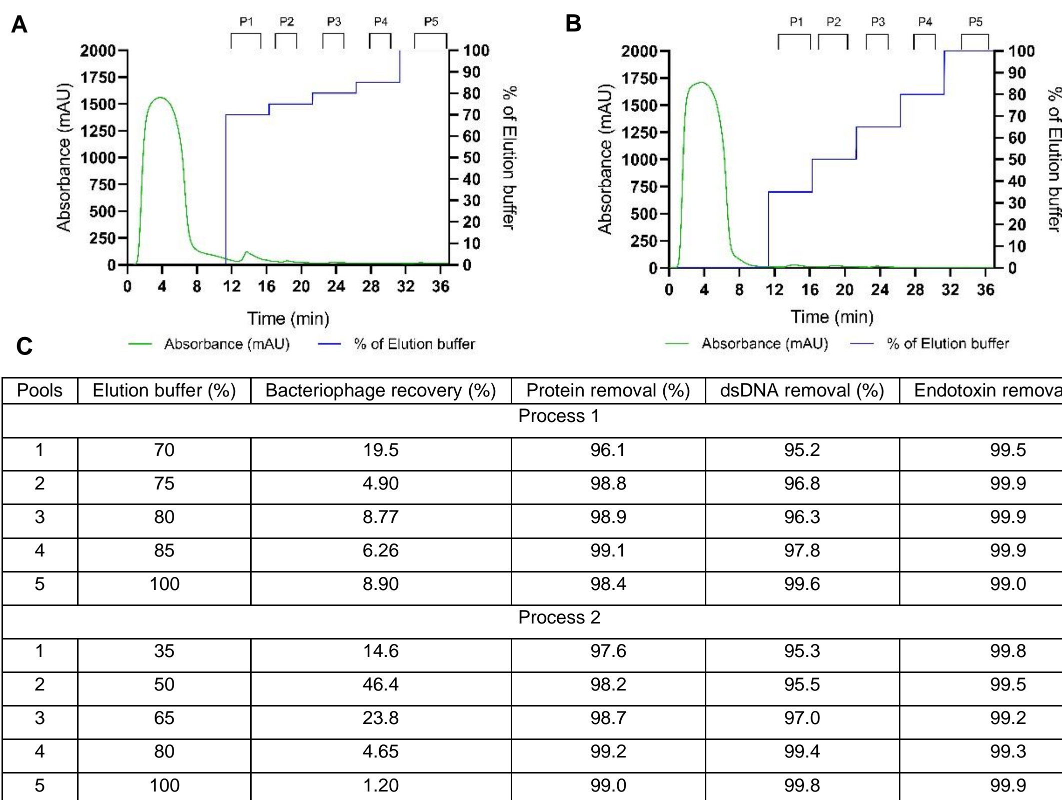
Ammonium sulfate concentration (M)	Bacteriophage recovery (%)	Protein removal (%)	dsDNA removal (%)	Endotoxin removal (%)
1.0	85.8	97.4	91.6	97.4
0.75	93.0	98.3	88.3	93.3
0.50	72.0	99.2	94.9	97.9

By decreasing the salt concentration, fewer particles are expected to bind during the equilibration phase. This leads to an increased number of available ligands for binding. On the other hand, the same decreasing salt concentration reduces the enhancement of hydrophobic interactions and may prevent these available ligands to be occupied. A certain balance is at play. A decrease in salt concentration in the equilibration phase leads to a decrease in competition for the ligands, which may increase bacteriophage binding and yield in the elution phase. However, bacteriophages may not be able to establish hydrophobic interactions due to the reduced hydrophobicity strength. What appears to occur with the salt concentration of 0.75 M, is that the former effect imposes itself compared to the latter. This means that the vacancy of ligands compensates for the reduced hydrophobicity enhancement, leading to a higher bacteriophage yield.

3.3.1. Multi-step-gradient elution

Linear gradients may be considered as preliminary experiments for the identification of optimal buffer composition and at which salt concentration bacteriophages or impurities elute. The identified salt concentrations may then be converted into a multi-step-gradient elution scheme, with a better resolution between peaks when compared to a linear-gradient elution.[13,14] The final section of this work is dedicated to the translation of the linear gradient into a multi-step gradient that could increase the bacteriophage yield whilst maintaining, across the board, the high impurities removals. Two multi-step gradients were devised. Process 1 (Figure 4A) implemented an ammonium sulfate concentration in the equilibration buffer of 1.5 M, whereas Process 2 (Figure 4B) utilized 0.75 M ammonium sulfate. For this case, the elution steps from Process 1 were downscaled, considering the new equilibration conditions. The content composition for the defined pools and lysate is represented in Figure 4C.

According to Figure 4C (Process 1), throughout pools 2-5, the bacteriophage content is somewhat distributed evenly. A certain heterogenicity in the way T4 bacteriophages interact with the phenyl ring is present. For example, by interacting with the column via their tail or head, different hydrophobic strengths are at play, which affects the way these viral particles are eluted. As an alternative, some bacteriophages' tails could be stuck in the column's bead pores, which would result in different times of retention not associated with the presence or not of different hydrophobic strengths. Process 2 comes much closer to the initial set goal for this work section. When considering pool 2 alone, the impurities removals, mainly endotoxins, and proteins, are high. dsDNA removal is much higher compared to the initial single-step-gradient elution (85.9%). While pool 3 presents comparable impurities removals, it contains less bacteriophages and should not be considered alone. Overall, the combination of pools 2 and 3 results in a bacteriophage yield closer to the values observed in the single-step-gradient elution (70.2%), with much higher impurities removals, mainly in terms of dsDNA and endotoxins. Furthermore, the total endotoxin content is below 1000 EU/mL (data not shown). The decrease in ammonium sulfate concentration in the equilibration phase, combined with the tweaked elution steps, resulted in a



Pools	Elution buffer (%)	Bacteriophage recovery (%)	Protein removal (%)	dsDNA removal (%)	Endotoxin removal (%)
Process 1					
1	70	19.5	96.1	95.2	99.5
2	75	4.90	98.8	96.8	99.9
3	80	8.77	98.9	96.3	99.9
4	85	6.26	99.1	97.8	99.9
5	100	8.90	98.4	99.6	99.0
Process 2					
1	35	14.6	97.6	95.3	99.8
2	50	46.4	98.2	95.5	99.5
3	65	23.8	98.7	97.0	99.2
4	80	4.65	99.2	99.4	99.3
5	100	1.20	99.0	99.8	99.9

Figure 4- Multi-step-gradient elution Phenyl Sepharose FF chromatogram of Process 1 (A) and Process 2 (B), with the bacteriophage recovery and impurities removals for the defined pools (C). Absorbance (mAU) at 280 nm and % of elution buffer were measured throughout time (min) at 1 mL/min on the outlet stream of the chromatography column. The equilibration/washing buffer was 1.5 M $(NH4)_2SO_4$, 15 mM Tris-HCl pH 7.0 and the elution buffer was 15 mM Tris-HCl pH 7.0. Fractions of 1 mL were collected and various pools of fractions were considered for further analysis, which are represented in the upper section of the chromatogram by **PX** (with **X** being the pool number). The following pool composition was devised: **(A) Pool 1** (Fraction 16 to 19), **Pool 2** (Fraction 21 to 22), **Pool 3** (Fraction 26 to 27), **Pool 4** (Fraction 31 to 32), and **Pool 5** (Fraction 36 to 39); **(B) Pool 1** (Fraction 16 to 19), **Pool 2** (Fraction 21 to 24), **Pool 3** (Fraction 26 to 28), **Pool 4** (Fraction 31 to 32), and **Pool 5** (Fraction 36 to 38).

purification process capable of improving the impurities removals whilst maximizing bacteriophage yield.

4. Conclusion

Bacteriophages are currently viewed as efficient alternatives to antibiotics in the everlasting fight against antibiotic-resistant bacteria. What prevents these viral particles from overtaking the industry is a standardized, efficient, downstream processing scheme, which contemplates all host impurities. Several novel alternative chromatography modes were tested for the purification of T4 bacteriophages. Phenyl Boronate Chromatography was found to be successful in adsorbing bacteriophages by hydrophobic interactions between surface viral proteins and the aminophenyl boronic acid. The PBC data motivated the use a Hydrophobic Interaction Chromatography for the purification of T4 bacteriophages, which required extensive optimization. HIC attained positive results when implementing a multi-step-gradient elution based on a linear-gradient elution. PBC and HIC appeared to be unrecognized crucial tools for the purification of T4 bacteriophages. Nonetheless, many key points in this work required further studying. It would be narrow-minded to assume that the use of phenyl as the column ligand and ammonium sulfate as the salt is the best choice. Alternative ligands should be tested, such as the linear chain alkaline ligands octyl or butyl. Although ammonium sulfate is generally seen as the best salt for HIC, other alternatives could be better for the product in hand. The use of citrate or ammonium phosphate should be tested. Importantly, the devised processes should also be tested with different bacteriophages, as this is the key to create a standardized bacteriophage purification process.

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