10-9-2020

Optimization of Cation Exchange Chromatography Purification of a Small Peptide: An Industrial and Practical Approach

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Introduction

Ion exchange chromatography (IEX) is a common chemical separation technique used to separate ionizable target molecules from a mixture by adsorption of the target molecule to a resin. A typical ion exchange resin consists of microscopic spherical beads that are coated with a binding ligand that is positively or negatively charged. The ion exchange chromatography process can be divided into four broad categories: strong cation exchange, strong anion exchange, weak cation exchange, and weak anion exchange. Cation exchange involves a negatively charged stationary phase (affinity for *cations),* whereas anion exchange involves a positively charged stationary phase (affinity for *anions)* [8]. In chromatography, the stationary phase is the porous solid that the target molecule and mobile phase flows through, and is commonly referred to as resin or beads. The mobile phase is the liquid solvent which flows through the stationary phase. The target molecule is solvated in the mobile phase and is captured onto the stationary phase while remaining fractions flow through and are discarded. Strong IEX resins are named so because they display the same amount of charge over the entire pH range of possible mobile phases used. While pH resins can vary by distributor, the range is typically 1-14. Weak IEX resins tend to optimally operate over a smaller pH range [12]. This is often a direct result of the binding ligand the resin utilizes. In part, the strength of charge of the binding ligand is used to determine the separation protocol for the target analyte. Strong cation exchange resins typically have analogs of sulfonate groups ($-CH_2CH_2CH_2SO_3^{2-}$) as the binding ligand, and strong anion exchangers typically have analogs of quaternary ammonium groups ($-N^+(CH_3)_3$). Weak cation exchange resins typically use analogs of carboxymethyl groups ($-OCH_2COO^-$) and weak anion exchangers tend to use analogs diethylaminoethyl groups ($-N^+H(CH_2CH_3)_2$) [10]. While these functional groups are generally the most common for each respective class of ion

exchange, vendors slightly modify the basic chemistries of their resins. Furthermore, many resin chemistries are proprietary to the vendor and thus their intricate chemical structures are not common knowledge. Important volumes used in IEX chromatography are bed volume (BV) and column volume (CV). The bed volume is the volume of the resin inside the column, while the column volume is the volume of the column including the resin bed. The void volume is the difference between the resin bed and the column volume.

The ability of IEX to separate target analytes depends on the chemical composition of the stationary phase and the mobile phase, as well as the properties of the target analyte. Comparative studies of commercial ion exchange resins on the basis of their chemical properties [9,14,15] have been reported. In IEX, the resin (stationary phase) is commonly packed into a hollow cylindrical column, over which the liquid buffer mobile phase flows. Proper mobile phase selection is required to equilibrate the resin to the appropriate pH. The target analyte is then added to the column and should adsorb onto the beads, allowing impurities with less affinity to flow through the column. Wash buffer is used to wash the resin of potential adsorbed impurities without causing elution of the target analyte. Elution buffer liberates the target molecule from the resin. Eluate, the fluid exiting the column upon elution, can be collected and analyzed for presence of analyte of interest. The process of wash and elution of the analyte is accomplished through a salt gradient and/or pH gradient. In a salt gradient, elution buffers contain cation or anions that compete with the analyte for resin ligand binding sites. As the concentration of ions in the buffer increase, the analyte is eluted from the column for collection. In a pH gradient, buffers of different pH flow through the column to protonate or deprotonate the resin and analyte, in order to elute the analyte. IEX has many practical purposes, including water softening, separation of organic molecules from a mixture; purification of biological molecules

such as proteins, amino acids, and nucleic acids; as well as other applications [6,9,11,13]. Of particular interest to many biotechnology companies is the purification of proteins from mixtures. IEX may be used at benchtop scale to industrial scale production of biomolecules.

Vestaron Corporation is an industrial biotechnology company that specializes in the development of insecticidal peptides for use in the agricultural industry. The peptides developed are toxic to target crop pests but non-toxic to non-target organisms. VST-6700 is a proprietary peptide being developed as the next potential insecticide active ingredient. Vestaron's current production strategy includes fermentation to produce the peptide, filtration to remove cells, then concentration or purification depending upon the application. IEX is useful for purification due to the ability to scale-up the process from small benchtop to production-scale fermentation. Strategies for ion exchange chromatography scale up have been published, in which mathematical models and experimental data is utilized [16]. The resin that Vestaron currently uses to separate peptides is no longer in large-scale production, and therefore must be replaced with a more modern commercially available resin. The resin to be replaced is SP Sephadex C-25, from GE Healthcare. SP Sephadex C-25 is a strong cation exchange resin with sulphopropyl ligands and a dry particle size of >40µm in diameter [5]. Cation exchange chromatography is the recommended IEX method for the purification of VST-6700 because the isoelectric point (pI) of the peptide is ~7 and thus VST-6700 tends to be positively charged after fermentation at pH ~4.5.

The purpose of the current study was to evaluate, optimize, and compare commercial cation exchange resins of different chemical and physical properties for the small-scale purification of VST-6700 from cell-free fermentation beer. Flow rates, process time, peptide

retention, and recovery were analyzed for the resins tested by HPLC. The optimal resin chosen must balance total process time, peptide retention, and elution efficacy.

Materials and Methods

Resin Selection

Cation IEX is the optimal purification method based on the pI of the VST-6700 molecule. The current IEX resin in use is SP Sephadex C-25 which is a strong cation exchanger. Four cation exchange resins were researched, optimized, and evaluated. Resins tested included: SP Sephadex C-25 (GE Healthcare); Capto S (GE Healthcare); Nuvia HR-S (Bio-Rad); and Macro-Prep CM (Bio-Rad). Capto S is a rigid strong cation exchange resin with a median particle size of ~90µm diameter, with a sulfonate ($–SO_3^-$) binding ligand [4]. Nuvia HR-S is a strong cation exchange resin with a median particle size of 50 ± 10µm diameter and a sulfonate ($–SO_3^-$) binding ligand. [2]. Macro-Prep CM is a weak cation exchange resin with a median particle size of 50µm and a ($–COO^-$) binding ligand [1].

Resin Preparation

Capto S, Nuvia HR-S, and Macro-Prep CM resins were received as slurries. Slurries were gently shaken and stirred until a consistent solution was achieved, then loaded on the column. SP Sephadex C-25 was received as a powder and was reconstituted by suspending 7g of powder in 30mM sodium acetate (NaOAc), pH 4.5 equilibration buffer for 24hr and allowing the beads to swell. The solution was then gently mixed and loaded on the column.

Buffer Preparation

In order to optimize resins, multiple buffers at various concentrations & pH's were used. Buffers used in a given trial for a resin were based off previous trials of that resin. Buffers that were successful in a previous trial were kept for further trials and buffers that were unsuccessful in a certain IEX step were changed in subsequent trials. Changes included pH, NaCl concentration, and/or buffer reagent type. In the case of a preliminary trial for a resin, previous IEX protocols used by other Vestaron researchers were used. This is especially the case in Capto S trial 1. The buffers used in IEX purification of VST-6700 using SP Sephadex C-25 are listed in **Table 1**. All buffers were titrated to desired pH with 5M HCl or 5M NaOH using a pH meter (Thermo Scientific OrionStar A211). No buffer solutions prepared precipitated during titration. All buffers were filter-sterilized through 0.2µm PES vacuum filtration units into 250mL or 500mL bottles for storage at ambient temperature. Solutions provided by Vestaron were stored in glass media bottles.

Table 1: Buffers used for IEX purification of VST-6700 using SP Sephadex C-25 strong cation exchange media.

SP Sephadex C-25 Buffers			
Trial	**Equilibration Buffer**	**Wash Buffer(s)**	**Elution Buffer(s)**
1	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 5.0 + 300mM NaCl
2	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	30mM NaOAc pH 5.0 + 450mM NaCl 30mM NaOAc pH 5.5 + 500mM NaCl
3	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8.0
4	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8.0
5	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8.0
6	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8 + 50mM NaCl
7	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8 + 100mM NaCl
8	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8 + 100mM NaCl
9-1	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8 + 100mM NaCl
9-2	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	50mM Tris-HCl, pH 8 + 100mM NaCl
10	30mM sodium citrate pH 3.0	30mM sodium citrate pH 3.0	100% Acetonitrile

The buffers used in the IEX purification of VST-6700 using Capto S are listed in **Table 2**.

Table 2: Buffers used in the optimization of IEX purification of VST-6700 from a 2mL Capto S resin bed

Capto S Buffers			
Trial	**Equilibration Buffer**	**Wash Buffer(s)**	**Elution Buffer(s)**
1	30mM NaOAc pH 4.5	30mM MES pH 6 30mM MES pH 6 + 100mM NaCl	30mM MES pH 6 + 200mM NaCl
2	30mM NaOAc pH 4.08	30mM MES pH 6	30mM MES pH 6 + 100mM NaCl
3	30mM NaOAc pH 4.5	30mM MES pH 6	30mM MES pH 6 + 200mM NaCl
4	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 200mM NaCl
5	30mM NaOAc pH 4.5	30mM MES pH 6	30mM MES pH 6 + 200mM NaCl
6	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 100, 200,350, 500mM NaCl	NA
7	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 400mM NaCl
8	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 400mM NaCl
9	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 400mM NaCl
10	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 400mM NaCl
11	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 5.0 + 300mM NaCl
12	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 400mM NaCl
13	30mM sodium citrate pH 3	30mM Sodium Citrate, pH 3	30mM NaOAc, pH 5 + 300mM NaCl
14	30mM sodium citrate pH 3	30mM Sodium Citrate, pH 3	30mM Tris-HCl, pH 8.8

Multiple wash buffers of increasing NaCl concentration were used in Capto S trial 6 (see Results)

The buffers used in the IEX purification of VST-6700 with Nuvia HR-S strong cation exchange resin are listed in **Table 3**.

Table 3: Buffers used in the optimization of the purification of VST-6700 from a 2mL Nuvia HR-S strong cation exchange resin.

Nuvia HR-S Buffers			
Trial	**Equilibration Buffer**	**Wash Buffer**	**Elution Buffer**
1	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5	30mM NaOAc, pH 4.5 + 400mM NaCl
2	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 50mM NaCl	30mM NaOAc, pH 5.0 + 450mM NaCl
3	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 50mM NaCl	30mM NaOAc, pH 5.0 + 450mM NaCl
4	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 100mM NaCl	30mM NaOAc, pH 5.0 + 400mM NaCl
5	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 50mM NaCl	30mM NaOAc, pH 5.0 + 200mM NaCl 30mM NaOAc, pH 5.0 + 300mM NaCl
6	30mM NaOAc pH 4.5	30mM NaOAc pH 4.5 + 50mM NaCl	30mM NaOAc, pH 5.0 + 300mM NaCl
7	30mM sodium citrate, pH 3	30mM sodium citrate pH 3 + 50mM NaCl	30mM NaOAc, pH 5.0 + 300mM NaCl
8	30mM sodium citrate, pH 3	30mM sodium citrate, pH 3 + 50mM NaCl	30mM Tris HCl pH 8.8

The buffers used in IEX purification of VST-6700 with Macro-Prep CM weak cation exchange resin are listed in **Table 4**

Table 4: Buffers used in the Ion Exchange purification of VST-6700 on a Macro-Prep CM weak cation exchange column.

Macro-Prep CM Buffers			
Trial	**Equilibration Buffer**	**Wash Buffer**	**Elution Buffer**
1	30mM Sodium Citrate, pH 3.0	30mM Sodium Citrate, pH 3.0	30mM Tris-HCl pH 8.8
2	30mM Sodium Citrate, pH 3.0	30mM Sodium Citrate, pH 3.0	30mM Tris-HCl pH 8.8
3	30mM HEPES, pH 7	30mM HEPES, pH 7	30mM HEPES, pH 7 + 500mM NaCl
4	30mM HEPES, pH 7	30mM HEPES, pH 7	30mM HEPES, pH ~ VST-6700 pI + 500mM NaCl

Note that the pH elution buffer in Trial 4 is reported as an approximate due to the proprietary nature of the peptide.

Test Material Preparation

Starting materials were small aliquots of cell free fermentation beer. Aliquots were titrated with 5M HCl or 5M NaOH as needed with a pH meter or checked with pH paper (Hydrion). In the event of matrix precipitation of the beer nearing pH 3 and 7 (SP Sephadex C-25 Trial 2, Macro Prep CM Trial 3), the aliquot was centrifuged at 10500rpm for 10min (Eppendorf Centrifuge 5810R). The supernatant was then transferred to a syringe (BD) and filtered through a series of 1.2µm, 0.45µm, and 0.2µm polyethersulfone (PES) syringe filters (AcroPrep), and collected into a 50mL or 15mL conical tube, depending on aliquot size.

The starting materials used in the IEX purification of VST-6700 with SP Sephadex C-25 strong cation exchange resin are listed in **Table 5**. The starting materials used in the IEX purification of VST-6700 using Capto S are listed in **Table 6**. The starting materials used in the IEX purification of VST-6700 using Nuvia HR-S are listed in **Table 7**. The starting materials used in the IEX purification of VST-6700 using Macro-Prep CM are listed in **Table 8**.

Table 5: Concentrations, titrated pH values, and batch numbers of VST-6700 cell-free beer starting material used in SP-Sephadex C-25 columns.

VST-6700 Starting Material			
SP Sephadex C-25			
Trial	**Peptide Conc. (mg/mL)**	**pH**	**Batch**
1	2.18	2.18	19-099
2	2.33	3	19-099
3	2.23	3	19-099
4	2.4	3	19-099
5	2.4	3	19-099
6	2.4	3	19-099
7	2.4	3	19-099
8	2.28	3	19-099
9.1	10	4.8	UGA 6700
9.2	10	3	UGA 6700
10	10	3	UGA 6700

Table 6: Concentrations, titrated pH values, and batch numbers of VST-6700 cell-free beer starting material used in Capto S columns

VST-6700 Starting Material			
Capto S			
Trial	**Peptide Conc. (mg/mL)**	**pH**	**Batch**
1	0.125	3.6	NR
2	2.226	4.11	19-063
3	1.971	4.25	19-063
4	1.861	4.5	19-063
5	1.903	4.5	19-063
6	1.65	4.5	NR
7	NR	NR	NR
8	0.612	4.5	NR
9	0.627	4.5	NR
10	3.793	4.5	19-099
11	3.793	4.5	19-099
12	2.23	4.5	19-099
13	3.3	3	19-163
14	3.3	3	19-163

NR = Not Recorded

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Table 7: Concentrations, titrated pH values, and batch numbers of VST-6700 cell-free beer starting material used in Capto S columns.

VST-6700 Starting Material			
Nuvia HR-S			
Trial	**Peptide Conc. (mg/mL)**	**pH**	**Batch**
1	3.780	4.5	NR
2	2.39	4.5	NR
3	3.26	4.5	NR
4	2.42	4.5	NR
5	2.42	4.5	NR
6	2.42	4.5	NR
7	2.56	3	19-163
8	2.56	3	19-163

NR = Not Recorded

Table 8: Concentrations, titrated pH values, and batch numbers of VST-6700 cell-free beer starting material used in Capto S columns.

VST-6700 Starting Material			
Macro-Prep CM			
Trial	**Peptide Conc. (mg/mL)**	**pH**	**Batch**
1	2.801	3	19-163
2	2.936	3	19-163
3	2.993	7	19-163
4	2.661	7	19-163

NR = Not Recorded

IEX Column

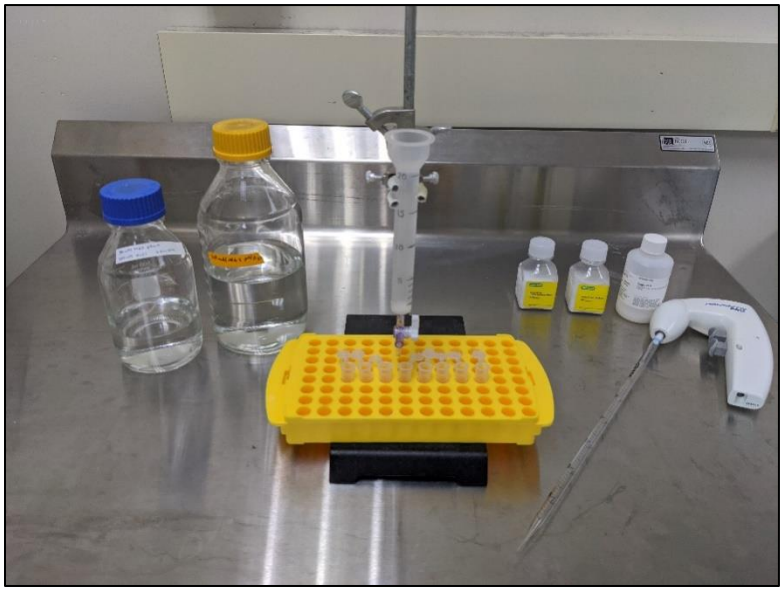

Figure 1: Typical IEX benchtop setup. Pictured from left to right: MES wash and elution buffer, Econo-Pac column and microcentrifuge tubes, Nuvia HR-S resin, Macro-Prep CM resin, Capto S resin, powerpipetter. Econo-Pac columns were used for all resin trials.

Resins were packed into disposable plastic Bio-Rad Econo-Pac® Chromatography columns (**Fig. 1**). The column was fitted with a 3-way stopcock to control flow rates and capped to prevent evaporation or contamination. Resin slurries were transferred to the column via serological pipette with a powerpipetter. All columns had 2mL resin loaded. Columns were equilibrated with at least 6 bed volumes of equilibration buffer. Equilibration buffer was transferred via serological pipette and deposited directly on top of the resin bed, being sure to not disturb the bed. The stopcock was then opened to allow maximum flow rate and equilibration. Flowthrough was discarded. Once equilibrated, the desired volume of cell-free beer containing a known amount of VST-6700 was loaded on the resin bed in a similar manner to equilibration buffer. Load volumes varied depending upon amount of peptide to be loaded. Peptide concentration in starting material and column fractions were determined by HPLC analysis of the beer (see *Sample Processing* and *Mass Balance*). The mobile phase was allowed to flow via

gravity through the resin bed, with the flow rate being controlled as necessitated by each trial. The maximum flow rate varied for each resin and was by gravity. The maximum flow rate by gravity was achieved by fully opening the three-way stopcock on the column (see "Flow Rates" under Results). The volume of equilibration buffer trapped in the resin bed (the void volume) was collected in one fraction, and fractions of VST-6700 flowthrough were collected (**Fig. 2**). The void volume was calculated using the following formula [10]:

$$V_{void} = \frac{0.5Ld_c^2}{1000}$$

$$L = column\ length$$

$$d_c = column\ diameter$$

Once all analyte finished flowing through the resin bed, wash buffer was loaded on top of the resin bed, and the stopcock opened to allow gravity flow. The void volume of peptide and subsequent wash fractions were collected. Based off previous Vestaron research, the volume of wash buffer required was at least 4 column volumes (CV). Once wash buffer stopped flowing through the resin bed, elution buffer was loaded on the resin bed and fractions of eluate collected in a similar manner. Flow rate was controlled with the stopcock as necessitated by the parameters of each trial. Peptide concentration in each fraction was determined by HPLC, and mass balance was performed for each trial. Any changes to the volume of wash or elution buffer needed was determined. The fractions of peptide load, wash and elution were collected in 0.65mL, 1.7mL, or 2mL microcentrifuge tubes (VWR) (**Fig. 2**). Fractions were collected in 15mL centrifuge tubes for larger fractions. Flow rates, when timed, were timed with a VWR stopwatch. Most trials were conducted in this manner, some exceptions are noted below.

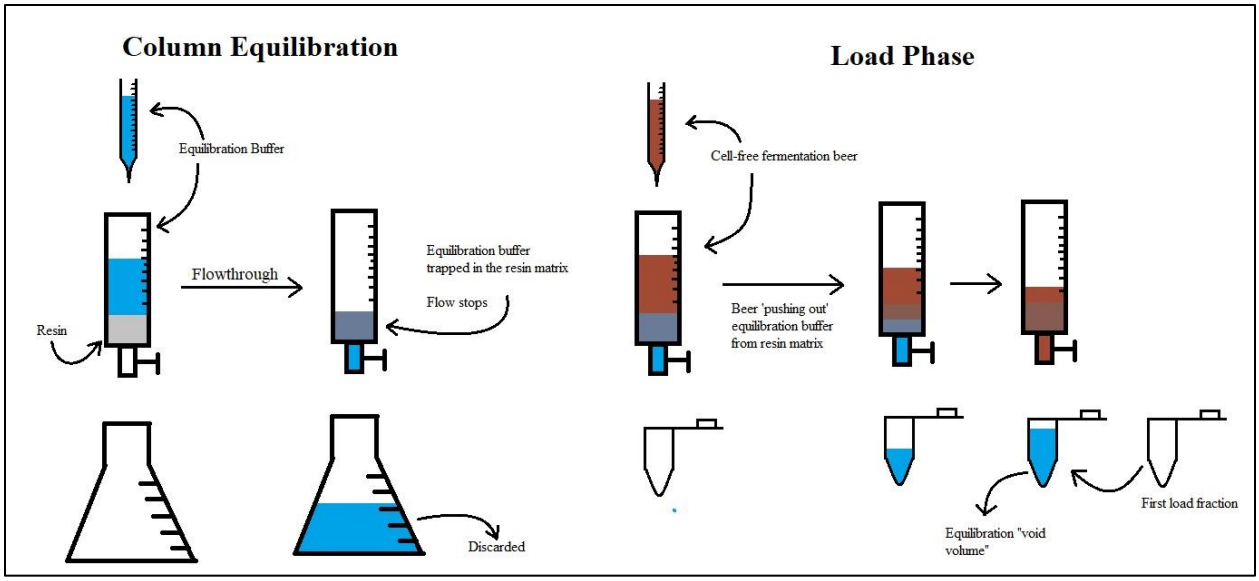

Figure 2: Diagram showing void volume collection procedure.

Observations of the color of the column and column fractions were used in determining the volume of mobile phase used in the column run. Clean resin beds were a white or grey color, and this color was not altered during equilibration. During the peptide load, the column and flowthrough fractions would change color from clear to amber. During the wash phase, collected column fractions would change color from amber to clear, due to impurities being removed from the column. A successful elution with adequate elution buffer resulted in a color change of the column from brown back to white or grey. Furthermore, elution fractions collected would change color from clear to a deep amber color. Fractions then would change from amber to clear once all analyte was liberated from the column. Fractions were then checked for peptide concentration via HPLC, and mass balance was performed.

The peptide load in SP Sephadex C-25 Trial 8 was different than the above procedure (See SP Sephadex C-25 Results). After an initial peptide load, small masses of VST-6700 were sequentially loaded and collected from the column in order to determine the maximum binding capacity of the resin bed.

Capto S trial 6, Capto S Trial 10, and Capto S Trial 12 differ from the above procedure. In Capto S Trial 6, several buffers of 30mM NaOAc pH 4.5 were flown through the column, with different NaCl concentrations in order to determine an adequate NaCl concentration necessary to elute VST-6700 from the Capto S column (see Capto S Results). Two resin bed volumes (4mL) of each buffer was passed through the column in increasing NaCl concentration order. The first and last fraction of each buffer was analyzed for peptide to roughly determine the optimal concentration of NaCl to use in an elution buffer.

Capto S Trial 10 and Trial 12 are replicates. In these trials, multiple column runs were performed, one after the other, in order to get a set of triplicate data for peptide load loss consistency. In Capto S Trial 10, peptide was loaded, washed, and eluted four times successively on one resin bed. In Trial 12, three column runs were performed.

Sample Processing and Analysis

When the column trial was completed, samples of eluate fractions were filtered either through 0.2µm Pall Nanosep spin filter inserts or Pall AcroPrep 96 well filter plates. Nanosep filters were centrifuged at 15000rpm for 30 sec (Eppendorf Centrifuge 5424). 96 well plates were centrifuged at 800rpm for 3min (Eppendorf Centrifuge 5810R). All HPLC samples were prepared in a 2mL glass Phenomenex Verex vial with rubber septa cap. Aliquots of each filtered sample were diluted either 10x or 5x with 0.15µg/µL caffeine solution. For example: in a 10x dilution, 50µL sample was added to 450µL caffeine internal standard. For a 10x dilution and a 100µL aliquot was added to 400µL caffeine standard for a 5x dilution. In all HPLC samples, caffeine concentration was 0.15µg/µL. Caffeine internal standards were prepared by adding

7.5mL of 1mg/mL caffeine stock to 37.5mL of DI water. An Agilent 1100 Series HPLC and/or a Thermo Fischer Ultimate 3000 UHPLC were used. Twenty microliter injections were made onto a Chromolith Performance RP-18e 100-4.6mm column at ambient temperature. A gradient method was used to elute caffeine and peptide. The method is listed in **Table 9**. Typical retention time of caffeine standard was ~2min and typical retention time of VST-6700 was ~7min. Chromeleon Software was used to visualize chromatograms. Microsoft Excel was used to calculate peptide concentrations and perform mass balances. Data recorded included retention time, and peak areas, peak height, injection volume, of VST-6700 and caffeine for each injection.

Table 9: The method used in the program for Agilent 1100 HPLC and Thermo Ultimate 3000 UHPLC.

HPLC Method			
Time [min]	**H_2O + 0.1% TFA [%]**	**Acetonitrile [%]**	**Flow [mL/min]**
0.00	90.0	10.0	2.000
1.00	90.0	10.0	2.000
4.00	77.0	23.0	2.000
9.00	63.0	37.0	2.000
9.10	0.0	100.0	2.500
12.00	0.0	100.0	2.500
12.10	90.0	10.0	2.500
14.00	90.0	10.0	2.500

Solvent A is 0.1% TFA in H20 and Solvent B is 100% ACN.

Determination of Peptide Concentration Using Internal Standard

The mass of VST-6700 in µg for a single 20µL HPLC injection was determined using Beer's Law by the following formula:

$$m_{VST-6700} = \left(\frac{\varepsilon_{caffeine} \times MM_{VST-6700}}{\varepsilon_{VST-6700} \times MM_{caffeine}}\right) \times \left(\frac{A_{VST-6700}}{A_{caffeine}}\right) \times m_{caffeine\ injected}$$

$$m = mass\ (\mu g)$$

$$MM = molar\ mass\ (g.mol^{-1})$$

$$A = peak\ area\ (mAU.sec)$$

$$\varepsilon = molar\ extinction\ coefficient\ (L.mol^{-1}.cm^{-1})$$

Previous research by another Vestaron researcher shows that:

$$\left(\frac{\varepsilon_{caffeine} \times MM_{VST-6700}}{\varepsilon_{VST-6700} \times MM_{caffeine}}\right) = 1.39492$$

Thus, the equation was simplified to:

$$m_{VST-6700} = (1.39492) \times \left(\frac{A_{VST-6700}}{A_{caffeine}}\right) \times\ m_{caffeine}$$

The numerical constant 1.39492 was obtained previously and used for this study. This constant was multiplied by the ratio of the area of VST-6700 and caffeine (in mAU.sec), which was then multiplied by 3µg, the mass of internal standard that was present in each 20µL HPLC injection.

Mass Balance

Once the sample concentration was known, the total amount of peptide in each column fraction was calculated by multiplying the concentration of VST-6700 in that fraction by the volume of that fraction. Mass (mg) of VST-6700 lost and retained in each step (load, wash, elution) was calculated by summation of VST-6700 mass in each fraction for each individual step. Mass balances were expressed as percentages. The percent of VST-6700 lost in the load and wash was calculated by dividing the total mass of VST-6700 lost in each step by the total mass of VST-6700 loaded on the column. Mass recoveries were expressed as percentages and were calculated by dividing the total mass of VST-6700 in eluate fractions by the mass of VST-6700 retained on the resin bed after the wash step.

Results

SP Sephadex C-25

In trial 1 the column was equilibrated with 6 BV 30mM NaOAc pH 4.5. Wash buffer was 30mM NaOAc pH 4.5, and 4.25 BV were used. Elution buffer for trial 1 was 30mM NaOAc pH 4.5 + 300mM NaCl. These buffers were selected based on results from Capto S trial 8 and Nuvia HR-S trial 6 because these resins had minimal peptide losses in the load steps and recoveries of above 90% of retained peptide in the elution steps. All flow rates in trial 1 were the maximum flow rate allowed by gravity, which was ≥ 3mL/min. Flow rates were also based of Capto S trial 8 and Nuvia HR-S trial 6. A VST-6700 load loss of approximately 42% (**Table 11**) was observed. A loss of zero peptide in the wash was assumed because 30mM NaOAc pH 4.5 resulted in no loss of VST-6700 in Capto S trial 8. Loss of VST-6700 in the wash step was also assumed zero for trials 4, 5, and 8. Six BV elution buffer achieved an 82% recovery of retained peptide. The high loss of VST-6700 in the load step in trial 1 indicated that a change of pH was necessary to allow better analyte binding to the column. Furthermore, the peptide recovery in trial 1 suggested that an elution buffer with higher NaCl concentration may result in a higher peptide recovery.

In order to achieve a higher binding capacity of VST-6700 on the SP Sephadex resin bed in trial 2, the column was equilibrated with 6 BV of 30mM sodium citrate pH 3.0. 30mM sodium citrate pH 3 equilibration buffer was used for the remainder of the trials for SP Sephadex C-25. The wash buffer was also changed to 30mM sodium citrate pH 3.0 in order to maintain a pH of 3 on the column while washing off impurities. This wash buffer was also used for the remainder of SP Sephadex C-25 trials. The volume of wash buffer in trial 2 was kept the same as trial 1. An elution buffer of 30mM NaOAc pH 5.0 + 450mM NaCl was used in order to elute more VST-

6700 off the column (**Table 1**). A total of 4.5BV of this elution buffer was initially used. During the trial, the elution buffer was changed to 30mM NaOAc pH 5.5 + 500mM because of the poor elution efficacy of the previous buffer (data not shown). 6BV of this new 30mM NaOAc pH 5.5 + 500mM elution buffer was used. All flow rates were the maximum allowed by gravity (**Table 10**). The column was found to retain all 5.6mg of VST-6700 loaded on the column at a pH of 3. Furthermore, the wash of 30mM sodium citrate pH 3 resulted in no loss of VST-6700 from the column. Both elution buffers used in trial 2 resulted in a combined peptide recovery of 76.5%. The low loss of VST-6700 in the load step indicated that more mobile phase could flow through the resin bed before saturation of analyte. The peptide recovery suggested that an elution buffer of a higher pH was needed, as high salt concentrations proved unable to elute all VST-6700.

Table 10: Flow rates for equilibration, load, wash, and elution steps of VST-6700 purification on a 2mL SP Sephadex C-25 resin bed

SP Sephadex C-25 Flow Rates											
Trial	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9-1**	**T9-2**	**T10**
Equilibration (mL/min)	5.45_m	5.45_m	6.54_m	6.15_m	NR_m	8.57_m	8.39_m	8.33_m	NR_m	9.11_m	NR_m
Load (mL/min)	2.97_m	3.51_m	2.46_m	3.95_m	NR_m	0.89	1.5(3mL) 0.90(5mL)	0.83	0.54	0.73	NR
Wash (mL/min)	4.74_m	3.91_m	NR_m	5.55_m	NR_m	1.43 (1.5mL) 6_m	1.48(1.5mL) $5.71(6mL)_m$	0.91 6.31_m	6.32_m	6.81_m	NR_m
Elution (mL/min)	5.45_m	5.45_m	6.21_m	1.18	5.5(11mL) 1.09(11mL) 1.18 (11mL) NR (11mL)	1.11	1.14	0.76	1.13	NR	NR

Subscript "m" denotes maximum flow rate. NR = not recorded.

In trial 3, the elution buffer was changed to 50mM Tris pH 8.0 based off a previously developed VST-6700 IEX elution protocol by another researcher. All flow rates were maximum allowed by gravity. In trial 3, the peptide load mass was nearly doubled, to 9.122mg with a loss of 1.7% in the load step. No VST-6700 was lost in the wash step. Elution using 4.5 BV 50mM Tris pH 8.0 at maximum flow rate resulted in a peptide recovery of 78.3%, a marginal increase from trial 2. This peptide recovery suggested that a restriction of flow rate could increase elution efficacy of the elution buffer.

In trial 4, the load mass of VST-6700 was kept the same as trial 3. Equilibration, load, and wash flow rates were the maximum allowed by gravity; elution flow rate was restricted to 1.18mL/min to allow the elution buffer more time to liberate VST-6700 from the stationary phase, and thus allow the elution buffer to achieve better elution efficacy on the column. No loss of VST-6700 in the load or wash step was observed. Restriction of elution flow rate resulted in a peptide recovery of 86.1%. Twelve BV of elution buffer was used. The results for this trial indicated that more elution buffer could be used to achieve a higher peptide recovery.

Table 11: Mass balance for VST-6700 on a 2mL SP-Sephadex C-25 resin bed.

SP Sephadex C-25 Mass Balance											
Trial	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9-1[a]**	**T9-2[a]**	**T10**
VST-6700 Load Mass (mg)	5.603	5.600	9.122	9.135	9.134	~18	~18	32	9.494	10.50	20
Mass VST-6700 Lost in Load (mg)	2.365	0	0.157	0	0.059	NR	NR	0.613	1.432	0.264	NR
% Loss in Load	42.2	0	1.7	0	0.6	NR	NR	1.9	15.1	2.5	NR
Mass VST-6700 Lost in Wash (mg)	0*	0	0	0*	0*	NR	NR	0*	0.0658	0.0231	NR
% Loss in Wash	0*	0	0	0*	0*	NR	NR	0*	0.7	0.2	NR
Mass VST-6700 Retained (mg)	3.237	5.600	8.965	9.135	9.075	NR	NR	31.39	8.063	10.23	NR
Mass VST-6700 Recovered (mg)	2.669	4.285	7.022	7.862	7.958	NR	NR	24.24	8.343	9.329	0
% Peptide Recovery	82.5	76.5	78.3	86.1	87.7	NR	NR	77.2	103.5	91.2	0

All numerical values are calculated from tabulated absorbance and retention data from HPLC analysis. Mass balances for trial 6, trial 7, and trial 10 were not performed. Percent Yields of elution are based on retained peptide rather than initial VST-6700 loaded. NR = not recorded. * = assumed zero

[a]After HPLC analysis of the column fractions, it was discovered the Thermo-Fischer UltiMate 3000 UHPLC had a broken mixing valve, which likely altered the absorbance data obtained by the machine.

In trial 5, equilibration, peptide load, and wash step flow rates were not recorded, but were the maximum flow rate by gravity. Peptide load was kept the same as trial 4. The elution flow rate was monitored in a similar manner to trial 4 (**Table 10**), but more elution buffer flowed through the column in order to achieve higher peptide recovery. The first 5.5 BV of elution buffer flowed at a rate of 5.5mL/min due to inadequate control of the stopcock. A total of 22 BV of elution buffer flowed through the column. The final 5.5 BV of elution flowthrough was not timed, but flow rate was restricted. No significant loss of VST-6700 was observed in the load step and wash step. Despite the large amount of elution buffer flowed through the resin bed, a

peptide recovery of 87.7% was achieved, similar to trial 4, in which much less elution buffer was used. The results of trial 4 indicated that a stronger elution buffer was needed, and more peptide could be loaded on the column with minimal peptide loss in the load step.

Due to high peptide retention in trial 5, peptide load was doubled in trial 6 (**Table 11**). Load flow rate was restricted to ≤ 1mL/min in order to increase the residence time of VST-6700 on the stationary phase. 50mM of NaCl was added to the 50mM Tris pH 8 buffer in order to increase elution efficacy on the column. Flow rate for elution step was restricted in order to maximize elution efficacy of the elution buffer. No mass balance for trial 6 was determined; changes to the column were determined based off HPLC results (not shown). However, analysis of peptide presence in the eluate fractions indicated that at least 8 BV of elution buffer was needed. This observation indicated that adding salt to the elution buffer could provide better elution efficacy of the elution buffer, and thus use lower volumes to achieve elution of peptide.

In trial 7, the salt concentration in the 50mM Tris pH 8 elution buffer was doubled from 50mM to 100mM in order to elute more VST-6700 from the column. Flow rates for load and elution were restricted, similar to trial 6. In trial 7, however, mobile phase was allowed to sit in the stationary phase for approximately 1hr in order to achieve a higher peptide binding. There was no observed difference in peptide binding between trial 6 and trial 7 (data not shown) based on analysis of HPLC chromatograms for the presence or absence of peptide. At this point, the binding capacity of the resin was approximately 9mg VST-6700 per mL of resin. The low binding capacity of the resin in trial 7 resulted in a different method to load the peptide on the column in trial 8.

In trial 8, flow rates were controlled in a similar manner to trials 6 and 7. A total of 18mg of VST-6700 was initially loaded onto the column, and then an additional 2mg of VST-6700 was

then loaded on the column; each resulting flowthrough was collected and analyzed on HPLC for presence or absence of VST-6700. This stepwise loading of peptide at a controlled flow rate was continued for a final load of 32mg of peptide. Based on HPLC results obtained, it was determined that 20mg of VST-6700 was an optimal load mass for a 2mL SP Sephadex C-25 resin bed. While a 32mg load resulted a 1.9% loss of peptide as determined by mass balance, the stepwise loading method proved too slow to be optimal. No loss of VST-6700 was observed in the wash step. Elution buffer was the same as trial 7, and elution with 8.25 BV with a restricted flow rate resulted in a 77.2% recovery of peptide.

Trial 9 was split into two separate trials due to a mistake; in trial 9-1, the cell-free fermentation beer was not titrated to pH 3.0 and left at pH 4.8. This mistake was rectified in trial 9-2. In all other aspects, trials 9-1 and 9-2 were performed in the same manner. Load flow rates and elution flow rates were restricted, while wash and equilibration flow rates were the maximum allowed by gravity. A total of 20mg of VST-6700 was loaded onto the column, which was then washed with 3.4 BV of 30mM sodium citrate pH 3. Elution was achieved with 8 BV of 50mM Tris pH 8 +100mM NaCl. Loading beer at a pH of 4.8 was found to result in a weaker binding of VST-6700 on the column than at pH 3.0, as more mass was lost during the load step in trial 9-1. There was no significant loss of VST-6700 from the column during the wash step in trial 9-1 and trial 9-2, and peptide recovery were both above 90% for the trials. However, upon completion and analysis of the column runs, it was found that the mixing valve on the Thermo Fisher Ultimate 3000 UHPLC that the fractions were analyzed on had broken. Thus, the mass balance may not be accurate (see footnote [a] in **Table 11**).

In trial 10, the elution reagent was changed to 100% acetonitrile in order to mimic the elution efficacy of an HPLC gradient to an IEX column. Of 20mg of VST-6700 loaded on the

column, 100% acetonitrile resulted in elution of no peptide from the column. Flow rates were not recorded due to time constraints. Mass balances were not performed due to a lack of chromatograms. Very few HPLC injections of this trial were performed due to time constraint.

Capto S

The initial IEX protocol for trial 1 was provided by another Vestaron researcher. The equilibration buffer was 30mM NaOAc pH 4.5 and 4 BV were used. Two wash buffers were used. Four BV of the first wash buffer, 2-(N-morpholino)ethanesulfonic acid (MES, 30mM, pH 6.0) was used and an equal amount of the second wash buffer, MES with added 100mM NaCl, was used. The elution buffer used in trial 1 was 30mM MES pH 6.0 + 200mM NaCl. The starting material used in trial 1 was a lower concentration than all other starting materials used (**Table 6**). Upon HPLC analysis of the starting material, there were many large peaks from unknown species (not shown), suggesting many impurities in the beer. The cell-free beer also clogged filters, making sample preparation difficult. Flow rates were not recorded but were the maximum flow rate by gravity. Of approximately 1.246mg of VST-6700 loaded on Capto S, 18.9% was lost. In the wash steps with 30mM MES pH 6.0 and 30mM MES pH 6.0 + 100mM NaCl, nearly 50% of bound peptide was desorbed (**Table 13**). Elution buffer desorbed 0.010mg VST-6700 for a peptide recovery of less than 3%. The very low peptide recovery indicated that the wash buffer needed to be changed in order to reduce the amount of VST-6700 lost in the wash. Furthermore, the poor starting material needed to be changed to one of higher VST-6700 concentration and less contamination.

In trial 2 the pH of the equilibration buffer was changed from 4.5 to 4.08 in order to match the pH of new starting material. This should have increased binding of the mobile phase to the stationary phase. The starting material in trial 2 had a higher concentration of VST-6700 and

could be filter-sterilized easily. Flow rates were not recorded but were the maximum flow rate by gravity. The volume of equilibration buffer used was not recorded. 19.593mg of VST-6700 was loaded onto the column, of which 27.2% was lost. The wash buffer in trial 1 of 30mM MES pH 6.0 + 100mM NaCl was changed to a MES wash buffer with no salt, in order to reduce VST-6700 loss during the wash step. Due to a low peptide recovery in trial 1, the elution buffer in trial 2 was changed to 30mM MES pH 6.0 + 100mM NaCl in order to determine optimal NaCl concentration for elution of VST-6700 from Capto S resin bed. Four BV of both wash and elution buffer were used. In trial 2, loss of VST-6700 during the wash step was 26.1%. The peptide recovery in the elution step was 73.5%. The high loss of VST-6700 from the column in the load step indicated that the stationary phase was oversaturated with analyte and the load mass needed to be decreased. A high loss of VST-6700 in the wash step suggested that the pH of the wash buffer needed to be changed. The salt concentration of the elution buffer was increased in trial 3 due to the low peptide recovery in trial 2.

Table 12: Flow rates for equilibration, load, wash, and elution steps of VST-6700 purification on a 2mL Capto S resin bed.

Capto S Flow Rates							
Trial	**T1-T8**	**T9**	**T10**	**T11**	**T12**	**T13**	**T14**
Equilibration (mL/min)	NR_m	2.16_m	NR_m	2.41_m	NR_m	3.41_m	NR_m
Load (mL/min)	NR_m	NR_m	NR_m	0.81_m	NR_m	1.25_m	1.86_m
Wash (mL/min)	NR_m	NR_m	NR_m	1.84_m	NR_m	2.09_m	2.33_m
Elution (mL/min)	NR_m	NR_m	NR_m	1.97_m	NR_m	2.90_m	3.42_m

Subscript "m" indicates maximum flow rate by gravity. NR = not recorded.

The VST-6700 load mass in trial 3 was reduced to 8.871mg in order to reduce the loss of peptide in the load step. The pH of the NaOAc equilibration buffer was changed to 4.5, similar to trial 1. It was decided that the starting material would be titrated as necessary to the pH of the equilibrated column in further trials, starting with trial 3. 30mM NaOAc pH 4.5 equilibration buffer and wash buffer were used until trial 13. Flow rates were not recorded but were the maximum flow rate by gravity. The volume of equilibration buffer used in trial 3 was not

recorded. The wash buffer was kept the same as trial 2, and the elution buffer was changed to 30mM MES pH 6.0 + 200mM NaCl in order to elute more analyte from the column. The volume of wash and elution buffer used in trial 3 was not changed from trial 2. In Trial 3, adsorbed analyte and peptide recovery could not be accurately calculated due to experimental error, in which the load void volume was discarded instead of collected. Thus, it was not possible to obtain an accurate measurement of total peptide bound to the column after the wash step and the peptide recovery in the elution step. It was upon summation of VST-6700 mass in wash fractions that 22% loss of total loaded peptide in the wash step was calculated. This result suggested a wash buffer of different pH was needed.

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Prior to trial 4, it was learned that starting material was potentially unstable in pH conditions at 6+. The load mass was reduced to 5.6mg because the last analyzed load fraction indicated the presence of peptide for that fraction. At that point, no additional VST-6700 was loaded onto the stationary phase. Flow rates were not recorded but were the maximum flow rate by gravity. The volume of equilibration buffer used in trial 4 was not recorded. The wash buffer was changed to 30mM NaOAc pH 4.5 in order to minimize VST-6700 loss in wash fractions; 4 BV was used, with no VST-6700 loss. In trial 4 the elution buffer was changed to 30mM NaOAc pH 4.5 + 200mM NaCl in order to prevent VST-6700 degradation in higher pH. Eight BV of elution buffer was used, which resulted in a peptide recovery of 74.5%. The low peptide recovery indicated that a higher NaCl concentration was necessary in the elution buffer.

Table 13: Mass balance results for VST-6700 on a 2mL Capto S resin bed.

Capto S Mass Balance											
Trial	**T1**	**T2**	**T3**	**T4**	**T5**	**T7**	**T8**	**T9**	**T11**	**T13**	**T14**
VST-6700 Load Mass (mg)	1.246	19.593	8.871	5.582	5.709	NR	5.506	5.896	5.543	16.535	11.07
Mass VST-6700 Lost in Load (mg)	0.236	5.321	0.059	0.063	0.077	NR	1.923	1.495	0.332	0.725	0.434
% Loss in Load	18.9	27.2	0.7	1.1	1.3	NR	34.9	25.4	6.0	4.4	3.9
Mass VST-6700 Lost in Wash (mg)	0.634	5.104	1.948	0	0.553	NR	0*	0*	0	0*	0*
% Loss in Wash	50.9	26.1	22.0	0	9.7	NR	0*	0*	0	0*	0*
Mass VST-6700 Retained (mg)	0.376	9.169	NR	5.519	5.079	NR	3.583	4.401	5.211	15.810	10.634
Mass VST-6700 Recovered (mg)	0.010	6.737	5.822	4.112	4.761	NR	3.728	4.247	5.435	15.971	11.384
% Peptide recovery	2.7	73.5	NR	74.5	93.7	NR	104.1	96.5	104.3	101.0	107.1

All numerical values are calculated from tabulated absorbance and retention data from HPLC analysis. Mass balances for trial 6, trial 10, and trial 12 are not included (See Tables 14 and 15). NR = not recorded. * = assumed zero

Trial 5 was an attempt to obtain a more accurate mass balance for the column conditions in trial 3. The trial was conducted because trials 1-3 did not involve the use of void volumes when changing mobile phases (see *IEX Column* in **Materials and Methods**). Thus, the use of void volumes would result in a more accurate mass balance. Flow rates were not recorded but were the maximum flow rate by gravity. A total of 5.709mg of VST-6700 was loaded on the column, with a loss of 1.3%. The buffers in trial 5 were the same as trial 3. The volume of equilibration buffer used in trial 5 was not recorded. 3.75BV of wash buffer and 5.5 BV of elution buffer were used. There was a 9.7% loss of loaded VST-6700 in the wash step, however a 93.7% peptide recovery was obtained in elution. A better mass balance for trial 5 supported the previous results of trials 1-3, in that the MES wash buffer resulted in a high loss of VST-6700 from the column. While the elution buffer in trial 5 proved to be effective, the pH of the elution buffer was kept lower in trial 6 in order to prevent any potential VST-6700 degradation from higher pH.

Trial 6 was an attempt to determine an optimal NaCl concentration in the pH 4.5 NaOAc elution buffer. The volume of equilibration buffer used in trial 6 was not recorded. Load mass of VST-6700 was 4.94mg. Only the first and last load fractions were analyzed because a 5.709mg

load mass in trial 5 resulted in less than 2% loss, and so the load loss in trial 6 was assumed to be near zero. Similarly, the loss of VST-6700 in the wash step was assumed to be zero for trial 6 because trial 4 showed no loss using NaOAc without NaCl. Four buffers for elution were 30mM NaOAc pH 4.5, with 100mM, 200mM, 350mM, and 500mM NaCl. Two BV wash buffer and each elution buffer were used. Flow rates were not recorded but were the maximum flow rate by gravity. The first and last column fractions of each elution buffer were analyzed for the presence or absence of peptide, and these fractions were analyzed for VST-6700 in the mass balance. Of the four buffers used, 30mM NaOAc pH 4.5 + 350mM NaCl eluted the most peptide (**Table 14**), suggesting that the proper salt concentration for the elution buffer to be used in trial 7 was around 350mM.

Table 14: Mass Balance of the first and last fractions of each wash and elution buffer flowthrough collected for Capto S trial 6.

Capto S Trial 6 Mass Balance						
Mass VST-6700 Loaded (mg)	**Mass VST-6700 lost in load 1 and 5 (mg)**	**Mass VST-6700 lost in NaOAc Wash 1 and 5 (mg)**	**Mass VST-6700 lost in 100mM salt Wash 1 and 4 (mg)**	**Mass VST-6700 lost in 200mM salt Wash 1 and 4 (mg)**	**Mass VST-6700 lost in 350mM salt Wash 1 and 4 (mg)**	**Mass VST-6700 lost in 500mM salt Wash 1 and 4 (mg)**
4.942	0.012	0.024	0.148	0.865	0.983	0.110

The values shown are summations of the mass balance of the first and last fraction for each buffer.

In trial 7, the load mass of VST-6700 was kept the same as trial 5 because there was minimal loss in the load for trial 5. The wash buffer was kept the same as trial 6 because no significant loss of VST-6700 in the wash occurred. In trial 7, 4BV of wash buffer was used. The elution buffer was changed to 30mM NaOAc pH 4.5 + 400mM NaCl to increase VST-6700 recovery in elution fractions with NaOAc. Furthermore, 6 BV of elution buffer was used in order to maximize peptide recovery. Flow rates were not recorded but were the maximum flow rate by gravity. Mass balance of trial 7 was not recorded due to experimental error in which spillage of some column fractions occurred. The trial was repeated in trial 8.

All buffer reagents and volumes were kept the same in trial 8 as trial 7. Flow rates were not timed but were the maximum flow rate by gravity. Significant loss of VST-6700 occurred in the load step, nearly 35%. The starting material was significantly more dilute than previous samples used (**Table 6**). However, it is not known why such a high loss occurred. Only the first and last wash fractions were analyzed in trial 8, and the loss of VST-6700 in the wash step is assumed to be zero because 30mM NaOAc pH 4.5 resulted in no VST-6700 loss in previous trials. Loss of VST-6700 in the wash step is assumed to be zero for trial 9 as well. A peptide recovery of 104.1% was obtained, and thus the elution buffer desorbed all analyte from the stationary phase. The high loss of VST-6700 in the load step resulted in a different loading method in trial 9.

Table 15: Mass balances of load step for the IEX Purification of VST-6700 on a 2mL Capto S resin bed for trial 10 and trial 12.

Capto S Trial 10 and Trial 12 Mass Balance							
Trial	**T10**				**T12**		
Load	Load 1	Load 2	Load 3	Load 4	Load 1	Load 2	Load 3
Mass VST-6700 Loaded (mg)	4.039	4.039	4.039	4.039	5.60	5.60	5.60
Mass VST-6700 Lost in Load	0.166	0.130	0.145	0.153	0.79	0.68	0.76
% Loss in Load	4.1	3.2	3.6	3.8	14.1	12.1	13.6
Average % Loss in Load	3.5				13.3		

The purpose of trial 9 was to starting material onto the column in smaller portions, resulting in a slower flow rate. Equilibration was timed at the maximum flow rate by gravity, resulting in an average flow rate of 2.16 mL/min. 5.5BV of buffer was used. It was observed that the instantaneous flow rate slowed as more equilibration buffer flowed through the stationary phase. Thus, it was hypothesized that smaller load amounts would result in a slower flow rate and would increase the residence time of the analyte in the stationary phase, which would achieve higher binding of analyte to the column. Starting material was loaded in volumes of 1.5 BV, which flowed through the stationary phase until flow stopped. Another 1.5 BV volume of starting material was then loaded on top of the column. Three total 1.5 BV volumes of starting

material were loaded, for a total of 5.896mg of VST-6700. Wash buffer, elution buffer, and volumes were kept the same as trial 8. No flow rates were restricted by closing of the stopcock in trial 9. The loading method in trial 9 resulted in a VST-6700 load loss of 25.4%, a near 10% decrease from trial 8. Only the first and last column fractions of the wash step were analyzed, and thus peptide loss in the wash step is assumed to be zero. Peptide recovery was 96.5%. The high loss of VST-6700 in the load step resulted in the procurement of a different starting material of higher VST-6700 concentration in order to limit VST-6700 loss in the load step.

Trial 10 was conducted in order to obtain a quadruplicate set of results for optimal peptide retention, with the goal of obtaining consistent results. Four column runs were performed sequentially. Initially a target of 5.6mg of VST-6700 was to be loaded on the column for each column run. Due to experimental error, only 4.039mg of VST-6700 loaded on the columns. At the time of Capto S trial 10, it was assumed that the Agilent 1100 was malfunctioning. The current hypothesis is that the error is probably due to starting material injected into the Agilent 1100 HPLC being too concentrated for accurate peptide measurement, though this was not hypothesized until Nuvia HR-S trial 7. In trial 10 and in all previous Capto S trials, starting material was diluted 5x with caffeine and water (see *Sample Processing* in **Materials and** Methods). After the trial 10 was completed, an additional analysis was performed of the starting material, this one diluted 10x instead of 5x. The concentration of VST-6700 calculated in the mass balance of the 10x dilution starting material was verified by another Vestaron Researcher. This new analytical resulted in the determination that only 4.039mg of VST-6700 was initially loaded on the column. The wash buffer was kept the same as trial 9, and 2 BV was used for each of the four column runs in trial 10. Elution buffer was also kept the same as trial 9, but the elution flowthrough was discarded in the four column runs. Mass balance of wash and elution

fractions was not performed. In Capto S trial 10, all four peptide loads of 4.039mg had less than 5% peptide loss, with an average value of 3.5%. After trial 10 was conducted, it was discovered that lower salt concentration in elution buffer was optimal, as NaCl could interfere with further processing of the purified VST-6700. Thus, NaCl concentration in the elution buffer was reduced in trial 11.

In trial 11 the elution buffer was changed to NaOAc (30mM, pH 5.0 + 300mM NaCl) in order to minimize the amount of NaCl necessary for the elution of VST-6700 from Capto S. Elution buffer pH was increased to 5.0 in order to achieve a similar elution efficacy as the elution buffer in trials 7-10. Equilibration buffer, VST-6700 load mass, and wash buffer were kept the same as trial 7-9. Six BV of equilibration buffer was used and 3.75 BV of wash buffer was used. Flow rates were recorded (**Table 12**) and were the maximum flow rate by gravity. VST-6700 load loss was 6% and peptide loss in the wash step was assumed to be zero. The pH increase and NaCl decrease in the elution buffer resulted in a peptide recovery of 104.3%, which suggests that all bound peptide was desorbed from the stationary phase.

Trial 12 was an attempt to obtain a precise set of triplicate data for VST-6700 retention. The purpose of the trial was to obtain load loss results using the buffers of trial 10, and to obtain a more accurate mass balance than trial 10. Column fraction samples were analyzed in the Thermo Fisher Ultimate 3000 UHPLC in trial 12 instead of the Agilent 1100 Series due to suspected Agilent 1100 malfunction. Equilibration, wash, and elution buffer were kept the same as trial 10. Six BV of equilibration buffer, 3.75 BV of wash buffer, and 6 BV of elution buffer were used in trial 12. All flow rates were maximum by gravity. All thee peptide loads of 5.60mg had a less than 88% peptide retention (**Table 15**). No loss of VST-6700 was observed in the

wash step. Elution flowthrough was discarded. The results from trial 12 indicated that column pH should be changed in order to increase binding capacity of Capto S.

The equilibration buffer used in trial 13 was changed to 30mM sodium citrate pH 3.0 in order to improve loading capacity of VST-6700 on the 2mL Capto S resin bed. Initially, 10mg of VST-6700 was loaded on the column, and the resulting flowthrough was analyzed in HPLC for presence or absence of peptide. A total of 2mg was then sequentially loaded and analyzed in HPLC until a final load mass of 16.5mg. Stationary phase and starting material pH were changed to pH 3.0 based off optimized SP Sephadex C-25 trial 9 protocol. The wash buffer was also changed to 30mM sodium citrate pH 3.0 based off optimized SP Sephadex C-25 trial 9 protocol. Six BV of equilibration buffer, 4 BV of wash buffer, and 7 BV of elution buffer were used. Elution buffer was kept the same as trial 11. An equilibration, load, and wash at pH 3 with 30mM sodium citrate resulted in nearly tripling the peptide load to 16.5mg with a greater than 95% peptide retention during loading and no loss of VST-6700 in the wash step. However, the stepwise manner of loading was much slower than previous loading methods. Analysis of HPLC chromatograms for peptide presence or absence determined that the optimal peptide load on Capto S at pH 3.0 was approximately 11mg. Other Vestaron researchers scaled-up the SP Sephadex C-25 IEX protocol and determined that 30mM Tris pH 8.8 was optimal in elution of VST-6700 from the stationary phase. This buffer was tested in trial 14.

In trial 14, the elution buffer was changed to 30mM Tris pH 8.8 in order to minimize the amount of NaCl necessary to elute VST-6700 from Capto S. Equilibration of the stationary phase with 6 BV of 30mM citrate pH 3 was performed. A peptide load of 11.07mg on an equilibrated column at pH 3 resulted in VST-6700 retention of 96.1%. Only the first wash fraction was analyzed in the HPLC. VST-6700 loss in the wash step after 4 BV of wash buffer was assumed

zero because 30mM sodium citrate pH 3.0 resulted in no VST-6700 loss in the wash step of trial 13. Elution buffer of 30mM Tris pH 8.8 resulted in elution of all adsorbed peptide from the column. Seven BV of elution buffer was used. All flow rates were maximum by gravity.

Nuvia HR-S

The volume of resin bed in Nuvia HR-S trials 1-6 was 2.25mL. The equilibration buffer used in trial 1 was 30mM NaOAc pH 4.5 based off Capto S trial 8. A total of 5.33 BV of equilibration was used. 30mM NaOAc pH 4.5 equilibration buffer was used for Nuvia HR-S trials 1-6. The wash buffer of 30mM NaOAc pH 4.5 and elution buffer of 30mM NaOAc pH 4.5 + 400mM NaCl were used based of Capto S trial 8 which indicated no peptide loss during the wash step and ~100% peptide recovery during the elution step. All flow rates for Nuvia HR-S were the maximum flow rate by gravity. Flow rates in trial 1 were not recorded. A total of 11.34mg of VST-6700 was loaded on the 2.25mL Nuvia HR-S resin bed, with a resulting peptide loss of less than 1%. Use of 3.6 BV of wash buffer resulted in no loss of VST-6700 during the wash step, and 4 BV of elution buffer resulted in a 68% mass recovery of adsorbed analyte on the Nuvia HR-S stationary phase (**Table 17**). The very low load loss in trial 1 suggested that more mobile phase could be introduced to the stationary phase and achieve higher binding of peptide. The low mass recovery indicated that the elution buffer pH or salt concentration needed to increase. Furthermore, there were numerous smaller peaks near the VST-6700 peak in the elution fraction chromatograms (not shown), suggesting that added salt to the wash buffer could better remove impurities from the stationary phase.

The concentration of the VST-6700 starting material was not validated before trial 2. A concentration of 2.30mg/mL VST-6700 was provided by another Vestaron researcher. Intended load mass for trial 2 was 20mg. Starting material was analyzed along with column fractions at

the end of the column run. The concentration of VST-6700 in the starting material was calculated to be 2.9mg/mL resulting in a load mass being calculated to be 24.75mg. In trial 2, 50mM NaCl was added to the NaOAc wash buffer in order to better remove impurities from the starting material. Four BV was used. In trial 2 the elution buffer was changed to was 30mM NaOAc pH 5.0 + 450mM NaCl in order to increase elution of VST-6700 from the Nuvia HR-S resin bed. During elution, 5.33 BV of elution buffer was used. Flow rates for trial 2 were not recorded but were the maximum flow rates allowed by gravity. A total of 24.75mg of VST-6700 was loaded onto the stationary phase with a measured 93.6% retention of analyte during loading. Adding salt to the wash buffer resulted in 6.4% loss of VST-6700. A 133.8% peptide recovery was calculated by HPLC mass balance (see footnote [a] in **Table 17**). At the time of the trial, it was thought that the Agilent 1100 Series had malfunctioned and incorrectly analyzed the starting material and elution fractions, resulting in an inaccurate mass balance.

In trial 3 the elution buffer was changed to 30mM NaOAc pH 5.0 + 400mM NaCl in order to minimize the concentration of NaCl required to elute VST-6700 off the column effectively. In trial 3 only the equilibration flow rate was recorded (**Table 16**). The volume of equilibration buffer used was not recorded. Load, wash, and elution flow rates were not recorded but were the maximum flow rate by gravity. A total of 3.6 BV of wash buffer was used. In trial 3, the goal was to load 20mg of VST-6700 onto the stationary phase. Based on starting material concentration provided by another researcher, 8.5mL starting material was loaded for an intended peptide load of 20mg. Starting material was analyzed along with column fractions at the end of the column run. The concentration of VST-6700 starting material was calculated as 3.26 mg/mL. The load mass was then revised to be 27.72mg instead of 20mg. Approximately 5.5% of loaded VST-6700 was lost in the wash step, leaving 24.072mg VST-6700 on the column (**Table**

17). Elution with 5.33 BV of 30mM NaOAc pH 5.0 + 450mM NaCl resulted in elution of all bound analyte from the stationary phase. At the time of the trial it was believed that the Agilent 1100 Series had malfunctioned and incorrectly analyzed the samples for the trial 3. For Nuvia HR-S trials 4-6, all fractions were analyzed on the Thermo-Fisher Ultimate 3000 UHPLC, because analysis of starting material on the UHPLC resulted in comparable mass balances to another Vestaron researcher.

The VST-6700 load in trial 4 was reduced to 23mg in order to maximize peptide load. The concentration of VST-6700 in the starting material mass balance was determined to be 2.84mg/mL, therefore 8.1mL of starting material was loaded. Upon completion of the trial, it was discovered that the Thermo-Fisher Ultimate 3000 UHPLC integrated the peaks for the starting material in a drop perpendicular integration (**Fig. 3**). Valley-to-valley integration was previously used for all samples. This issue was fixed upon manual valley-to-valley integration and it was determined that the concentration was 2.42mg/mL; thus, 19.6mg VST-6700 was actually loaded on the stationary phase. This load resulted in 5.5% loss in the load step, less loss than trial 3. The NaCl concentration in the wash buffer was doubled to 100mM in order to better wash off impurities from the stationary phase. A total of 3.2 BV of wash buffer was flowed through the stationary phase with a loss of 11.8% peptide during in the wash step. In trial 4, the elution buffer was changed to 30mM NaOAc pH 5.0 + 400mM NaCl in order to reduce the

amount of NaCl needed in the elution buffer. After 4.8 BV of elution buffer, a peptide recovery of 100.4%

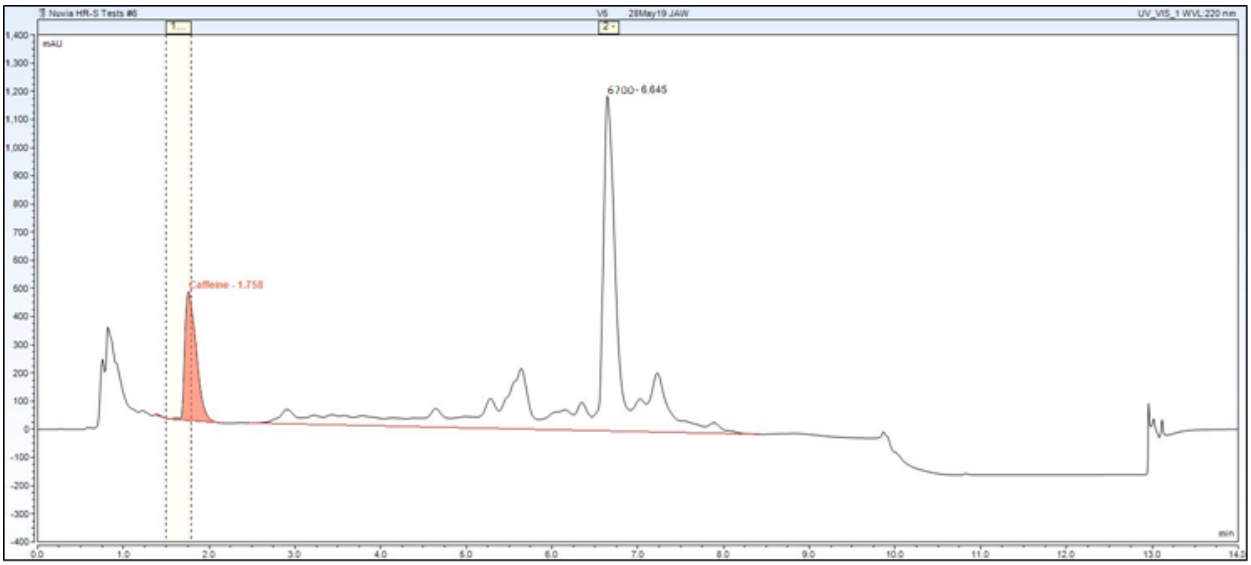

Figure 3: Chromatogram for VST-6700 starting material, Nuvia HR-S trial 4. Typically, valley-to-valley integration was used for all samples. For this trial, integration was drop perpendicular which resulted in an overestimation of peptide.

of bound analyte was calculated. All flow rates were the maximum flow rate by gravity The results of trial 4 suggested that less NaCl could be used in the elution buffer to achieve a high mass recovery. The high load loss indicated that the Nuvia HR-S stationary phase was oversaturated with analyte and that a smaller load was needed. The high loss of VST-6700 in the wash step indicated that the salt concentration in the wash buffer was too high and needed to be reduced.

Table 16: Flow rates for equilibration, load, wash, and elution steps of VST-6700 purification on a 2mL Nuvia HR-S resin bed.

Nuvia HR-S Flow Rates								
Trial	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**
Equilibration (mL/min)	NR_m	NR_m	0.748_m	1.09_m	1.04_m	1.21_m	1.523_m	NR_m
Load (mL/min)	NR_m	NR_m	NR_m	0.54_m	0.63_m	0.60_m	0.77_m	0.68_m
Wash (mL/min)	NR_m	NR_m	NR_m	0.73_m	0.72_m	NR_m	0.69_m	0.78_m
Elution (mL/min)	NR_m	NR_m	NR_m	0.73_m	0.91_m	0.97_m	1.08_m	1.12_m

Subscript “m” indicates maximum flow rates. NR = not recorded.

In trial 5 the load mass of VST-6700 was reduced to 18mg, which resulted in a 2.8% loss in the load step. A total of 5.33 BV of equilibration buffer was used. NaCl concentration in the

NaOAc wash buffer was reduced to 50mM in order to reduce the loss of VST-6700 in the wash step. After 3.2 BV of wash buffer passed through the stationary phase, a VST-6700 loss of 4.9% was calculated. The elution buffer was changed to 30mM NaOAc pH 5.0 + 200mN NaCl in order to reduce the concentration of NaCl needed to achieve high elution efficacy. This buffer resulted in a ~13% mass recovery of bound analyte after 4.8 BV was used. Increasing the concentration of NaCl to 300mM in the same trial resulted in elution of 78.6% of adsorbed analyte after 3.2 BV, for a total mass recovery of 92.3%. All flow rates were the maximum flow rate by gravity. 30mM NaOAc pH 5.0 + 300mM NaCl was proposed in trial 6 to improve elution efficacy compared to 30mM NaOAc pH 5.0 + 200mM NaCl.

Table 17: Mass balance of HPLC absorption data of VST-6700 IEX purification on a 2mL Nuvia HR-S resin bed.

Nuvia HR-S Mass Balance								
Trial	**T1**	**T2[a]**	**T3[a]**	**T4**	**T5**	**T6**	**T7**	**T8**
VST-6700 Load Mass (mg)	11.341	24.75	27.716	19.617	18.15	18.15	32.0	31.622
Mass VST-6700 Lost in Load (mg)	0.025	1.581	2.293	1.073	0.63	0.50	0.122	0.972
% Peptide Loss in Load	0.2	6.4	8.3	5.5	3.5	2.8	0.4	3.1
Mass VST-6700 lost in Wash (mg)	0	1.579	1.351	2.322	0.89	0.79	0.163	0
% Peptide Loss in Wash	0	6.4	4.9	11.8	4.9	4.3	0.5	0
Mass VST-6700 Retained (mg)	11.32	21.59	24.07	16.22	16.62	16.86	31.72	30.65
Mass VST-6700 Recovered (mg)	7.775	28.89	24.34	16.28	2.28 13.07 15.35 (tot.)	15.83	29.85	32.35
% Peptide Recovery	68.7	133.8	101.1	100.4	13.7 78.6. 92.3 (tot.)	93.9	94.1	105.6

[a]In Trial 2 and 3, it was found that the samples ran in the HPLC had a larger absorbance than was typical, resulting in inaccurate mass balances. It was previously thought that the Agilent 1100 was malfunctioning. The samples may have been outside the LDR for the Agilent 1100 Series due to a 5x dilution for the samples, resulting in error. This was not hypothesized until Nuvia HR-S trial 7. Trials 4-7 were analyzed in the Thermo-Fisher Ultimate 3000 in order to obtain accurate mass balances. In trial 7 and 8, starting material, wash, and load fractions were diluted 10x. Elution fractions were diluted 20x.

In trial 6, 5.33 BV of 30mM NaOAc pH 4.5 equilibration buffer was used. The VST-6700 load mass was kept the same as trial 5. The wash buffer and volume of wash buffer used was kept constant to trial 5. Elution buffer used was 30mM NaOAc pH 5.0 + 300mM NaCl in order to optimize the NaCl concentration in the elution buffer. In trial 6, all flow rates were the maximum flow rate by gravity. The wash flowthrough was not recorded. Using 3.2 BV of wash

buffer resulted in a VST-6700 loss of 4.3%. Using 5.33 BV 30mM NaOAc pH 5.0 + 300mM NaCl elution buffer resulted in a peptide recovery of 93.9%.

In trial 7, for HPLC analysis all fractions were diluted 10x rather than 5x in an attempt to stay within the limit of linearity in the Agilent 1100 Series and the Thermo-Fisher Ultimate 3000. Equilibration buffer was changed to 30mM sodium citrate pH 3.0 in order to load more VST-6700 on a 2mL Nuvia HR-S resin bed. A total of 8 BV of equilibration buffer was used. Wash buffer was changed to 30mM sodium citrate pH 3.0 + 50mM NaCl in order to maintain a pH of 3.0 while still washing impurities from the stationary phase, and 4BV was used. The equilibration was chosen based on SP Sephadex C-25 trial 9, in which a column at pH 3.0 allowed the stationary phase to achieve a higher binding capacity for VST-6700. Elution buffer was kept constant to trial 6. Initially, 20mg of VST-6700 was loaded and the resulting flowthrough analyzed by HPLC for presence or absence of peptide. A total of 2mg of VST-6700 was then sequentially loaded and analyzed in HPLC until a final load mass of 32mg was obtained. There was a total peptide loss of 0.3% in the load step. A total of 8.88 BV of elution buffer was used with a corresponding peptide recovery of 94.1%. The results of trial 7 suggested that a pH gradient may improve mass recovery of bound analyte, and that elimination of NaCl from the wash buffer could result in less loss of VST-6700 in the wash step.

In trial 8, the VST-6700 load was kept the same as trial 7, but 32mg was loaded at once, rather than in a stepwise method. This resulted in a peptide loss of 3.1% during the load step. The elution buffer was changed to 30mM Tris pH 8.8 in order to minimize the amount of NaCl necessary to elute VST-6700 from Nuvia HR-S. During elution, 8 BV was used. Equilibration buffer was the same as trial 7. Equilibration volume and flow rate was not recorded. All flow rates were the maximum by gravity. Wash buffer and volume used was kept the same as trial 7.

There was no loss of VST-6700 in the wash step. In trial 8, the column was pre-equilibrated the day before the trial was conducted. In trial 8, the elution buffer of 30mM Tris pH 8.8 resulted in a peptide recovery of 105.6%, suggesting that all analyte was desorbed from the stationary phase.

Macro-Prep CM

The equilibration buffer used in trial 1 was 30mM sodium citrate pH 3 based on SP Sephadex C-25 trial 9, Capto S trial 14, and Nuvia HR-S trial 8. These trials found that column conditions of pH 3 resulted in the highest analyte adsorption to the stationary phase. Seven BV of equilibration buffer was used. The wash buffer used in trial 1 was 30mM sodium citrate pH 3.0 + 50mM NaCl based on Nuvia HR-S trial 7. This buffer had been found to remove impurities left on the stationary phase from the mobile phase after loading. A total of 4 BV of wash buffer was used. The elution buffer was 30mM Tris pH 8.8 based on Nuvia HR-S trial 8 results indicating a peptide recovery of above 90%. In trial 1, all flow rates were the maximum flow rate allowed by gravity (**Table 18**), based off previously optimized Nuvia HR-S and Capto S results. Due to high peptide retention in Nuvia HR-S trial 8, a VST-6700 load mass of 32mg was chosen. Of the 32mg VST-6700 loaded on Macro-Prep CM at pH of 3.0, 30.175mg passed through the column during the load and wash steps for a total peptide loss of 94.2%. Of the 1.844mg VST-6700 still bound to the stationary phase, 0.179mg was desorbed in the elution phase after 10 BV for a peptide recovery of 9.7%. The results of trial 1 suggested that flow rates for the load, wash, and elution phases needed to be restricted to less than 1 mL/min, similar to SP Sephadex C-25. Further, overall load needed to be reduced.

Table 18: Flow rates for equilibration, load, wash, and elution phases of VST-6700 purification on a 2mL Macro-Prep CM resin bed.

Macro-Prep CM Flow Rates				
Trial	**T1**	**T2**	**T3**	**T4**
Equilibration (mL/min)	NR_m	1.704_m	1.57_m	1.60_m
Load (mL/min)	1.19_m	0.84	0.51	0.48
Wash (mL/min)	1.01_m	0.76	0.49(1.34mL) 1.13 (6.66mL)$_m$	0.46(1.5mL) NR_m
Elution (mL/min)	1.82_m	0.90	1.29	0.73

Subscript "m" denotes maximum flow rate by gravity. NR = not recorded.

Seven BV of the equilibration buffer used in trial 1 was used in trial 2. In trial 2, NaCl was eliminated from the Tris wash buffer in order to minimize VST-6700 loss in the wash. A total of 4 BV of the wash buffer was used. The elution buffer was kept the same as trial 1. In trial 2, the load and wash flow rates were restricted to minimize VST-6700 loss. The elution flow rate was also restricted to improve 30mM Tris pH 8.8 elution efficacy. The VST-6700 load mass was reduced to 5mg in trial 2, with a 12% peptide loss. During the wash step, 2.678mg of VST-6700 was lost resulting in a 53% peptide loss (**Table 19**). No peptide was pulled off in the elution phase after 10 BV of elution buffer at restricted flow rate. The high analyte loss from the stationary phase during the load and wash indicated that different buffer and pH conditions were needed on the column. The poor elution efficacy of the 30mM Tris pH 8.8 buffer suggested that a pH gradient was not sufficient to elute the bound analyte from the stationary phase.

Table 19: Mass balance of VST-6700 IEX purification on a 2mL Macro-Prep CM resin bed.

Macro-Prep CM Mass Balance				
Trial	**T1**	**T2**	**T3**	**T4**
VST-6700 Load Mass (mg)	32.02	4.991	9.997	9.315
Mass VST-6700 lost in load (mg)	22.78	0.613	0.259	0.332
% Load Loss	71.1	12.3	2.6	3.6
Mass VST-6700 lost in wash (mg)	7.40	2.678	0	0
% Loss from wash	23.1	53.7	0	0
Mass VST-6700 Retained (mg)	1.844	1.7	9.737	8.983
Mass VST-6700 Recovered (mg)	0.179	0	7.293	7.072
% Peptide recovery	9.7	0	74.9	78.7

All values were calculated from HPLC absorbacnce values.

In trial 3 the equilibration buffer was changed to 30mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0 in order to increase VST-6700 loading on the

2mL Macro-Prep CM resin bed. The wash buffer in trial 3 was changed to 30mM HEPES pH 7 in order to minimize VST-6700 loss with the equilibration buffer and starting material pH change. HEPES was chosen based off an IEX purification procedure from Staby et al. and Calik et al. [3,14]. In trial 3, elution buffer was changed to 30mM HEPES pH 7 + 500mM NaCl in order to elute more VST-6700 from the Macro-Prep CM stationary phase. The VST-6700 loading mass was changed to 10mg in order to determine the binding capacity of the stationary phase at pH 7. Flow rates were restricted as in a similar manner as trial 2. In trial 3, column conditions of pH 7 with HEPES (Table **4**) resulted in a peptide loss of 2.6. No loss of VST-6700 was observed in the wash phase after 4 BV of wash buffer. Elution buffer of 500mM NaCl resulted in a 74.9% peptide recovery after 8 BV. The low load loss in trial 3 suggested that 10mg was an acceptable load mass of VST-6700 on the column at column pH of 7. The low peptide recovery in the elution phase indicated that the elution buffer needed to be changed.

The equilibration buffer, wash buffer, and VST-6700 load mass in trial 4 were kept the same as trial 3. Nine BV of equilibration buffer and 4 BV of wash buffer were used. The elution buffer in trial 4 was changed to 30mM HEPES pH ~ pI, + 500mM NaCl in order to create a net charge of zero on the VST-6700 molecule, which should allow the elution buffer to achieve a higher elution efficacy. Flow rates were restricted as in trial 2 and 3. In trial 4 a similar peptide load of 10mg had a 3.6% loss in the load phase. No loss of VST-6700 was observed in the wash phase. A 78.8% peptide recovery was determined after 8 BV of elution buffer for trial 4, a small increase from trial 3.

Discussion

All optimized resins show different load capacities, recoveries, and process times. For SP Sephadex C-25, the max peptide load was 20mg. Using a pH gradient from 3.0 to 8.0 and a NaCl

gradient from 0 to 100mM (**Table 20**) resulted in a peptide recovery of 77%. The optimized process time for SP-Sephadex C-25 is approximately 20 minutes for a 2mL resin bed volume, excluding cleaning and re-equilibration of columns. Process times were determined using flow rates and buffer volumes for equilibration, load, wash, and elution steps.

Capto S had a maximum load capacity of 11mg. Using a pH gradient from 3.0 to 8.8 during elution corresponded to a 100% recovery of retained peptide. Capto S had the fastest process time of all resins tested, at approximately 13 minutes for a 2mL resin bed. This is due to being able to use maximum flow rates by gravity.

Table 20: Optimal conditions and process times for the four resins evaluated in this project.

Resin	Load Capacity (mg)	Equilibration Buffer	Wash Buffer	Elution Buffer	Est. Recovery	Est. Process Time per mL resin (min:sec)	Est. Process Time per mg loaded VST-6700 (min:sec)
SP Sephadex C-25	20	30mM Citrate, pH 3.0 (6 BV)	30mM Citrate, pH 3.0 (3.4 BV)	50mM Tris, pH 8.0 + 100mM NaCl (8 BV)	77%	9:37	0:58
Capto S	11.07	30mM Citrate, pH 3.0 (6 BV)	30mM Citrate, pH 3.0 (4 BV)	30mM Tris, pH 8.8 (7 BV)	100%	6:40	1:12
Nuvia HR-S	32.0	30mM Citrate, pH 3.0 (8 BV)	30mM Citrate, pH 3.0 (4 BV)	30mM Tris, pH 8.8 (8 BV)	100%	25:37	1:36
Macro-Prep CM	9.315	30mM HEPES, pH 7 (9 BV)	30mM HEPES, pH 7 (4 BV)	30mM HEPES, pH ~pI + 500mM NaCl (8 BV)	78%	23:37	5:04

All bed volumes were a volume of 2mL in optimized IEX protocols for all resins. Bed volumes for VST-6700 load depend on starting material concentration.

Nuvia HR-S had the slowest process times, at ~50 minutes per BV of resin. The slow process time for Nuvia HR-S could be because of the small particle size of the resin. Inversely, Nuvia HR-S had the highest load capacity at 32mg VST-6700. The pH gradient from 3.0 to 8.8

corresponds to a peptide recovery of 100% retained analyte. Maximum flow rates by gravity were used in all trials of Nuvia HR-S.

Macro-Prep CM had a maximum VST-6700 load of 9.3mg using 30mM HEPES pH 7. A combination of a pH gradient and NaCl gradient was used. The pH gradient was from 7 to VST-VST-6700 pI, and the NaCl gradient was from 0 to 500mM. The combination of these two gradients during elution corresponded to a peptide recovery of 78%. Process times for Macro-Prep CM are slower than Capto S and SP Sephadex C-25, at 47 minutes per BV of resin.

Conclusion

Ion exchange chromatography is a common chemical separation technique and the theory and kinetics of ion exchange are well-characterized. In this study, resins were optimized on a trial-by-trial basis. The purpose of the study was initially to optimize and evaluate a commercial IEX cation exchange resin for the replacement of SP Sephadex C-25 for the purification of VST-6700. However, the project was not completed by the time scale-up was required. Thus, it was determined that SP Sephadex C-25 resin was the best resin available for Vestaron, and the resin optimization presented in the current study was performed for the scale-up. Optimized protocols of Capto S, Nuvia HR-S, and Macro-Prep CM will be compared to the optimized protocol of SP Sephadex C-25 for future purification projects.

While SP Sephadex C-25 was the best resin for Vestaron for scale-up, Capto S is the best resin to replace SP Sephadex C-25 for VST-6700 purification. While SP Sephadex C-25 and Nuvia HR-S were found to load more VST-6700 than Capto S, the faster process times of Capto S (due to maximum flow rates by gravity) makes Capto S a more efficient IEX resin for VST-

6700 purification. Furthermore, the elution buffer used in Capto S elutes all VST-6700 from the stationary phase which results in a higher peptide recovery than SP-Sephadex C-25.

Capto S has sulfonate binding ligands attached to dextran extenders. While the exact structure of Capto S is proprietary to GE Healthcare, it is possible that this structure may contribute to the binding affinity of VST-6700 on Capto S. The presence of dextran extenders may affect how proteins interact with the agarose matrix of Capto S [7,15]. A slower flow rate may have allowed a larger peptide load on the 2mL resin bed [7], but it must be stressed that the proteins themselves dictate much of the interaction between analyte and stationary phase.

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