Quantifying Orthogonality and Separability: A Method for Optimizing Resin Selection and Design

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1. Abstract

Recent studies have shown that by combining orthogonal, non-affinity chromatography steps, it is possible to rapidly develop efficient purification processes for molecules of interest. Here, we build upon previous work to develop a flexible framework for identifying resins that remove optimally orthogonal sets of impurities for a wide variety of products. Our approach involves screening a library of proteins on a library of resins and quantifying each resin's ability to separate every set of protein pairs in the library. Orthogonality is then defined as the degree to which two resins separate mutually exclusive sets of protein pairs. We applied this approach to a library of model proteins and a series of strong, salt tolerant, and multimodal ion exchangers and evaluated which resin combinations performed well and which performed poorly. In particular, we found that strong cation and strong anion exchangers were orthogonal, while strong and salt tolerant anion exchangers were not orthogonal. Interestingly, salt tolerant and multimodal cation exchangers were found to be orthogonal and the best resin combination included a multimodal cation exchange resin and a tentacular anion exchange resin. This approach for quantifying orthogonality is valuable in

that it can be used both as a criteria for resin design as well as process design. We envision that, using this framework, it will be possible to design a set of next generation chromatography ligands that are explicitly engineered to optimize separability and orthogonality.

2. Introduction

In recent years, the biopharmaceutical industry has used a platform-based approach to develop downstream processes for the vast majority of commercially available products[1, 2]. Platform-based approaches are advantageous in that they significantly reduce the time and effort required to develop purification processes by constraining the selection of resins and mobile phase conditions [3]. An important downside to using platform processes, however, is that they can be inflexible, such that the platform must be altered or reinvented for every sufficiently different molecule. Thus, for molecules that do not fit into the platform (for example, many non-mAb products) extensive process development efforts are often required [4]. While high throughput screening techniques coupled with rational resin selection have facilitated process development in these cases, the large process design space and the accelerated timelines of many programs require the development of a more efficient alternative to traditional platform process development [5, 6, 7, 8].

A promising alternative to using a platform process is to adopt a platform approach for quickly developing processes. Shukla et al. discuss that a semi-flexible platform approach is able to purify a suite of diverse mAbs by altering a small set of process parameters[2]. Despite this, such an approach is still limited in scope to mAbs. A recent study by Vecchiarello et al. showed that by creating a database of HCP retention behavior and by utilizing orthogonal, non-affinity chromatography steps, it is possible to develop a purification process for a given molecule in just 1-2 weeks[9]. One limitation of this approach, however, is that orthogonality was defined with respect to the

HCP profile and molecule of interest, limiting the approach's applicability to specific separation challenges. Here, we build upon this work to define a flexible framework for identifying sets of resins and conditions which can optimally remove orthogonal sets of impurities from a wide range of products.

Historically, orthogonality in process development has been assessed in a largely heuristic manner, wherein resins that operate using different modes of interactions, such as ion exchange (IEX) and hydrophobic interaction chromatography (HIC), are thought to be orthogonal. Although this may sometimes be true, differences in base matrices, ligand chemistries, and ligand densities can impact selectivities and complicate this definition of orthogonality [10]. Further, the advent of multimodal resins in industrial process development has resulted in resins with unique and often unintuitive selectivity trends, whose orthogonality is challenging to characterize[11, 12, 13]. As a result, it is of interest to develop a methodology to quantify the extent of orthogonality between resins to guide the selection of an optimally orthogonal resin set.

In the field of analytical chromatography, many methods have been developed to quantify orthogonality in resin systems with the goal of improving detection for liquid chromatography-mass spectrometry (LC-MS). These methods aim to identify resin systems which provide the highest peak capacity[14]. Geometric-based approaches are often used to quantify orthogonality and efficiency of separation space utilization by plotting peptide retention times for resin pairs as scatter plots [14]. Techniques such as the Geometric Surface Coverage (SCG), Gilar-Stoll Surface Coverage (SCs), and the Convex Hull Surface Coverage (SCCH) segment these scatter plots into bins of equal areas and quantify the extent to which these points are spread through the separation space[15, 16, 17]. Other techniques such as correlation functions and mutual information have also been used in an attempt to quantify the degree of similarity between in the retention behavior or resin pairs [18]. A

more detailed reivew of these techniques and their respective limitations can be found elsewhere[14].

Although these techniques have led to the development of efficient 2D LC techniques (e.g. high- and low- pH RPLC pairing), a similar effort to quantify orthogonality in preparative chromatography is lacking [16, 19]. While analytical techniques can inform our understanding of orthogonality in preparative chromatography, they cannot be easily reapplied because they are highly dependent on the number of molecules considered, they overestimate the separation space (by considering space that exceeds baseline resolution between peaks), and they cannot be easily extended to systems of 3 or more resins. It is therefore necessary to define a methodology to quantify orthogonality within the context of preparative chromatography.

Here, we develop a framework for quantifying the ability of a given resin or resin set to separate a set of proteins. Orthogonality in preparative chromatography can then be understood as the improvement in this separability associated with combining multiple resins together in a set. We then perform a series of gradient screens of model proteins on a variety of ion exchange and multimodal media and use our approach to evaluate orthogonality between resins at different pHs. Our work provides a foundation for quantifying and understanding orthogonality with respect to resin design and downstream process development.

3. Experimental Methods

Materials: Sodium chloride, sodium phosphate monobasic, sodium phosphate dibasic, citric acid, trisodium citrate dihydrate, sodium hydroxide, Tris base, Chromasolv grade acetonitrile (ACN), sodium azide, α-Lactalbumin from bovine milk, α Chymotrypsinogen A (Type II) from bovine pancreas, Concanavalin A from *Canavalia ensiformis*, Lysozyme from chicken egg white, Cytochrome C from equine heart, α-Chymotrypsin (Type II) from bovine

pancreas, Ribonuclease B from bovine pancreas, Conalbumin from chicken egg white, Ubiquitin from bovine erythrocytes, Carbonic anhydrase from bovine erythrocytes, Lactoferrin from bovine milk, β-Lactoglobulin B from bovine milk, and album (rabbit) were purchased from Sigma-Aldrich (St. Louis, MO). hGH was generously donated by Novo Nordisk (Bagsværd, Denmark). Mab was generously donated by MedImmune (Gaithersburg, MD). 96-well 350 μL sample collection plates, 96-well plate mats, Acquity UPLC Protein BEH C4 columns (300 angstrom, 1.7 μm, 2.1 mm x 100 mm), and Acquity UPLC Protein BEH VanGuard Pre-Columns (300 angstrom, 1.7 μm, 2.1 mm x 5 mm) were purchased from Waters Corporation (Milford, MA). Pre-packed OPUS ®200 μL MiniChrom columns (5 mm x 10 mm) were purchased from Repligen (Waltham, MA) and packed with the following resins: Q Sepharose HP, SP Sepharose HP, Capto Adhere, Nuvia cPrime, Capto MMC, Capto MMC ImpRes, HyperCel STAR AX, HyperCel STAR CEX, CMM HyperCel, Eshmuno Q, Eshmuno HCX, BAKERBOND PolyQuat, BAKERBOND PolyABx, and Toyopearl MX-Trp-650M. 350μL, 0.2μm Supor AcroPrep Advance filter plates were purchased from Pall Corporation (Port Washington, NY). Mylar plate sealers and HPLC grade trifluoroacetic acid (TFA) were purchased from Thermo Fischer Scientific (Pittsburgh, PA). 0.2μm nylon centrifugal filters, 30mL Luerlock syringes, 96-well 2mL collection plates, and 0.2μm PES syringe filters were purchased from VWR (Radnor, PA).

Methods: Overview: A set of 15 model proteins were chromatographically screened using linear salt gradients at pH 5.0, 6.0, and 7.0 in order to determine their retention behaviors on a set of 14 multimodal resins. Given the large number of chromatographic screens required in this work, a workflow was developed which enabled the simultaneous screening of multiple proteins in a single chromatography run. Mixtures containing 5 different model proteins were loaded for each chromatographic run and fractions were collected

throughout each gradient. Fractions containing a UV signal greater than baseline at 280nm were subsequently analyzed by efficient UP-RPLC assays in order to determine their compositions. Finally, these compositions were used to deconvolute and construct chromatograms for each individual protein and these data were stored in a continually updated retention database. This workflow represents a novel and highly parallel strategy for screening the retention behavior of large numbers of proteins at the lab scale. The following subsections provide further experimental details of the chromatographic screens and UP-RPLC analysis.

UP-RPLC Assay Development: The set of 15 model proteins shown in **Table 1** were chosen for screening in this work. These proteins were grouped into mixtures as mentioned above such that proteins in a given group could be well-resolved by UP-RPLC. To determine optimal protein groupings, each lyophilized protein was first solubilized to 2mg/mL in buffer containing 20mM sodium phosphate at pH 6.0 containing 0.02% azide as a preservative. Proteins were subsequently filtered using 0.2µm centrifugal filters and individually analyzed by UP-RPLC using a 10-minute linear acetonitrile gradient from 0-70% containing 0.1 percent trifluoroacetic acid. A Matlab script was created to identify the optimal protein sets (**Table 1**) with the greatest average difference in retention times by UP-RPLC. For each of the 3 sets of proteins, rapid UP-RPLC assays were developed by trial-and-error to arrive at efficient assays requiring less than 4 minutes. The UP-RPLC method details for these assays and the resulting analytical chromatograms for these protein mixtures are provided in SI.

Chromatographic Screening of Model Proteins: Protein load mixtures for chromatographic screens were prepared by solubilizing each individual lyophilized protein in Buffer A at the appropriate pH. Upon centrifugal filtration with a 0.22μm nylon filter, a Nanodrop Spectrophotometer was used to measure the protein concentration at 280nm and proteins were diluted us-

Table 1: Table of model proteins considered in this analysis. Isoelectric points (pI) and retention behaviors on HIC in a 1500-0mM ammonium sulfate gradient (40CV) on Capto Phenyl ImpRes at pH 7.0 are shown. HIC retention is shown as a general indicator of protein hydrophobicity. Proteins are grouped as indicated by shading according to 3 groupings in the AKTA and UP-RPLC runs.

Protein	PI	Retention in HIC (% Gradient)
Alpha-Lactalbumin	5.0	85.5
Alpha-Chymotrypsinogen A (Type II)	8.5	66.3
Horse Cytochrome C	10.3	0.0*
hGH	5.1	64.8
mAb A	8.3	84.1
Concanavalin A, Type VI	5.0	82.4
Lysozyme	11.4	44.1
Alpha-Chymotrypsin	9.2	95.9
Ribonuclease B	8.9	37.4
Albumin (Rabbit)	5.8	87.3
Conalbuin	6.7	40.6
Ubiquitin	6.8	37.5
Carbonic Anhydrase	6.4	59.6
Lactoferrin	8.7	65.9
Beta-Lactoglobulin B	5.1	49.7

ing Buffer A to a final concentration of 2 mg/mL (note: for cases where the solubility limit of a protein was less than 2 mg/mL, proteins were prepared at this limit). Proteins belonging to a set were mixed in equal volumetric ratios and were subsequently loaded onto columns packed with the resins listed in **Table 2** to a total protein load of 10mg/mL resin. This load challenge was chosen since it provided reasonable limits of detection while also minimizing competitive binding effects.

Table 2: Resins used in chromatographic screens.

Resin	Class
Capto MMC	Multimodal Cation Exchange
Capto MMC ImpRes	Multimodal Cation Exchange
Nuvia cPrime	Multimodal Cation Exchange
CMM HyperCel	Multimodal Cation Exchange
ToyoPearl	Multimodal Cation Exchange
Eshmuno HCX	Tentacular Cation Exchange
Bakerbond PolyABx	Tentacular Cation Exchange
STAR AX	Salt Tolerant Anion Exchange
Capto Adhere	Multimodal Anion Exhange
Q Sepharose HP	Strong Anion Exchange
Bakerbond PolyQuat	Tentacular Anion Exchange
Eshmuno Q	Tentacular Anion Exchange
SP Sepharose	Strong Cation Exchange
HyperCel STAR CEX	Salt Tolerant Cation Exchange

Screens were performed on an Akta Explorer system running Unicorn 5.0 software and equipped with a P960 sample pump, Frac-950 fraction collector, and 10mm flow cell. 10 column volumes (CVs) of protein mixtures were loaded onto each column followed by 5 CVs of re-equilibration with buffer (0M NaCl). Linear salt gradients were operated at constant pH and extended from 0M (Buffer A) to 1.5M (Buffer B) sodium chloride over 40CVs, followed by 15CVs of Buffer B. A 20mM sodium citrate buffer system was used for gradients operated at pH 5.0 and a 20mM sodium phosphate buffer system was used for gradients at pH 6.0 and 7.0. All buffers contained 0.02 percent sodium azide as a preservative. Following gradient elution, resins were stripped with 10 CVs of 100mM citric acid containing 1M NaCl for resins anion exchange functionality, or 100mM Tris base containing 1M NaCl for resins with cation exchange functionality. Columns were subsequently regenerated using 0.5M sodium hydroxide (NaOH). For all experiments the

flowrate was kept to 1CV/min.

For each chromatography run, the flowthrough, re-equilibration, gradient, and strip were collected in fractions at a resolution of 1CV in a 96-well plate (note: fractions were not collected during regeneration due to denaturing of protein in the presence of NaOH. Fractions containing a UV signal greater than 5mAU at 280nm were analyzed by UP-RPLC with the appropriate method from Table SI.1 and using a 20μL injection volume. Peak integration was performed using the built-in Empower™ 3 integration pool in order to determine the relative quantity of each model protein in a given fraction. These data were input into a Matlab script and were used to construct the peak profiles for each individual protein in a given gradient. To account for differences in the extinction coefficients of proteins, reconstructed chromatograms of individual proteins were normalized by area.

4. Theory

In this work, we aim to develop an approach to quantify a given resin's ability to separate proteins and to determine the extent of orthogonality for sets of resins, independent of protein identities. We begin by making the assumption that a pool of n proteins can be defined, such that every protein that might need to be separated is approximately represented within this set. This leads to the question: What is the probability that a given resin will be able to successfully separate two proteins arbitrarily chosen from our pool of n proteins? This probability is quantified as the separability factor, S.

To calculate this probability, it is necessary to first address the question: what does it mean to "successfully separate" proteins? Here, a "successful separation" is defined based on differences in elution salt concentration, ΔC_s, in a gradient chromatography experiment. For every pair of proteins a and b, a weight, w_{ab}, is calculated to represent the degree of success associated with separating those proteins:

$$w_{a,b} = \begin{cases} 0 & \Delta C_s < r_{low} \\ \frac{\Delta C_s - r_{low}}{r_{high} - r_{low}} & r_{high} \geq \Delta C_s \geq r_{low} \\ 1 & \Delta C_s > r_{high} \end{cases} \quad (1)$$

where r_{low} is a lower bound below which all proteins are taken to be co-eluting and r_{high} is an upper bound above which all proteins are taken to be fully separated. The separability factor is then calculated based on the average value of these weights:

$$S = \frac{1}{\binom{n}{2}} \sum_{a=1}^{n-1} \sum_{b=a+1}^{n} w_{ab} \quad (2)$$

Thus, S represents the ability of a given resin to separate all protein pairs from the pool of n proteins. It is important to note that S is not only a function of resin, but also of mobile phase conditions, particularly pH.

The separability factor, S, is based on a series of assumptions:

- Salt concentration is an appropriate selectivity handle (when other variables such as pH or co-solutes are held constant)
- Chromatography is being carried out under non-competitive conditions
- Protein elution salt concentration is not significantly affected by gradient slope

Here, we make these assumptions in order to arrive at a simple framework for calculating a resin's ability to generally separate proteins with the understanding that this will be dependent on the protein set. For problems that require it, it is possible to derive straightforward extensions to this approach that allow the relaxation of these assumptions.

While it is of interest to assess separability of single resins, most downstream bioprocesses involve two or more non-affinity separation steps in order to achieve the desired purity. It is therefore of interest to not only select or design resins that are individually capable of separating proteins, but also to identify resin sets which, when used together, achieve high separabilities.

To this end, the definition of w_{ab} and S can be expanded to apply to sets of m resins. Previously, each resin's ability to separate proteins was evaluated based on differences in elution salt concentration. For a set of m resins, this can be captured by defining a distance between proteins a and b as:

$$d_{ab} = \max(\Delta C_s^1, \Delta C_s^2 ... \Delta C_s^m) \tag{3}$$

where ΔC_s^m is the difference in elution salt concentration between proteins a and b on resin m. Thus, the distance between two proteins on a set of resins is taken as the maximum distance between those proteins on any individual resin. The weight, w_{ab} is then redefined as:

$$w_{a,b} = \begin{cases} 0 & d_{ab} < r_{low} \\ \frac{d_{ab} - r_{low}}{r_{high} - r_{low}} & r_{high} \geq d_{ab} \geq r_{low} \\ 1 & d_{ab} > r_{high} \end{cases} \tag{4}$$

This newly defined w_{ab} can then be combined with Equation 2 to obtain the separability factor for a resin set containing any number of resins.

While the ultimate goal when designing a multi-step separation process is to successfully remove impurities, it is also desirable to make the process efficient and non-redundant. To this end, it is desirable to not only maximize separability for sets of resins, but also to evaluate their orthogonality, i.e. their ability to separate non-overlapping sets of impurities.

To quantify the degree of orthogonality within a resin set, we define an enhancement factor E as the fractional enhancement in the separability factor

associated with adding an additional resin to the resin set:

$$E_m = \frac{S_m}{\max(S_{m-1} \forall (m-1) \in m)} - 1 \tag{5}$$

Thus, for two resins which are highly orthogonal, the separability factor associated with the resins combined would be higher than either of the individual resins, leading to a high enhancement factor. In contrast, two non-orthogonal resins would not have a significantly improved separability factor when combined, and thus the enhancement factor would be low.

5. Results

5.1. Applying Separability and Enhancement to a Sample System

It is useful to first illustrate how separability and enhancement are calculated by applying our theory to a single, multimodal cation exchange ligand, Nuvia cPrime, operated in a pH 5.0 salt gradient. **Figure 1a** shows the elution salt concentrations of 15 proteins on Nuvia cPrime. As described in the theory section, the differences between the elution salt concentrations of each pair of proteins (ΔC_s) were calculated first, yielding a distribution of distances (**Figure 1b**). This distribution of protein-protein elution distances can be thought of as capturing the resin's ability to generally separate proteins in the protein pool.

To facilitate straightforward comparisons between resins, it is useful to collapse this distance distribution into a single number that captures the resin's ability to separate the pool of proteins. This requires addressing the question posed earlier: what does it mean to "successfully separate" proteins? Here, we created a function (defined in the Theory section) that maps each ΔC_s to a weight, w_{ab}, that varies between 0 and 1. To define r_{high} and r_{low}, it was assumed that each ΔC_s was not a function of gradient slope and that each gradient could be rescaled based on the elution salt concentrations of the least and most strongly retained proteins. Based on these assumptions,

r_{high} was set to $\frac{1}{2}$ of that rescaled gradient, or $\frac{1}{2}(\max(C_s) - \min(C_s))$, and r_{low} was set to $\frac{1}{8}$ of the rescaled gradient, or $\frac{1}{8}(\max(C_s) - \min(C_s))$. While these values of r_{high} and r_{low} were employed for the scaling here, any scaling strategy could be used with our approach. Thus, any function that maps ΔC_s to $w_{a,b} \in [0, 1]$ can be used to calculate a separability score and that different applications may require different mapping functions.

For the case of Nuvia cPrime, **Figure 1b** illustrates that the distribution of ΔC_s is bimodal, with some proteins eluting relatively closely, and some farther apart. Comparison with **Figure 1a** illustrates that this arises from the fact that there are two groups of closely eluting proteins, one eluting in the 300-600 mM range, and the other eluting at higher salt concentraions. Based on these 15 proteins, the separability score for Nuvia cPrime at pH 5.0 was 0.46.

How did this separability change when Nuvia cPrime was combined with another resin? **Figure 2a** illustrates the elution salt concentrations for each of the 15 proteins on Nuvia cPrime at pH 5.0 and Capto MMC (another multimodal cation exchange resin) at pH 5.0. Interestingly, the distances between proteins on each resin differed significantly, and as a result, each resin was capable of separating different pairs of proteins. **Figure 2b** shows the distribution of protein-protein distances, $d_{ab} = \max(\Delta C_s^1, \Delta C_s^2)$, for the resin pair (orange). This distribution is shifted to the right, illustrating that the separation improved because the two resins separated non-overlapping set of protein pairs. This resulted in a pair separability score of 0.76 and a pair enhancement factor of 0.31, defined previously in the Theory section.

5.2. One-Resin Separabilities

Figure 3 shows the individual resin separability scores for each of the resins tested at each pH. The highest scores generally occurred for the resin/pH combinations at which the proteins were most strongly bound. Generally, this corresponded to pH 5.0 for resins with cation exchange functionality and pH

7.0 for resins with anion exchange functionality. In some cases, however, binding conditions were too strong, causing many of the proteins to elute in the strip. For these cases, high separability scores did not trend as expected with binding strength. For example, for the tentacular multimodal cation exchanger, Eshmuno HCX, separability scores increased with increasing pH. At pH 5.0, many proteins eluted in the denaturing strip on Eshmuno HCX (shown in SI Figure 9), resulting in a large cluster of proteins whose ΔC_s values equaled 0. With increasing pH and decreasing binding strength, many of these proteins eluted in the gradient resulting in higher separability scores. Conversely, for some resins, binding strengths were too weak to provide high separability scores. This was best illustrated by some of the resins with anion exchange functionality at pH 5.0 for which all 15 proteins flowed through, resulting in separability scores of 0.

The Eshmuno HCX behavior illustrates an important aspect of the separability scores calculated in this analysis: Resins and conditions with many proteins eluting in the denaturing strip can result in lower separability scores. In addition, this analysis assumes that proteins eluted in the strip can be separated from proteins eluted in the gradient. Practically, if the protein of interest does not elute in the gradient, it must be recovered and not denatured during the strip. For generality, a single regenerative strip was implemented in this work. For specific sets of proteins with known stability windows, a non-denaturing strip can be added to linear gradient screens and used to determine the feasibility of successfully recovering each protein.

Figure 4 shows a histogram of the distribution of separability scores for all individual resins and conditions considered in this study. A peak at $S_m = 0$ was observed for the resins with anion exchange functionality at pH 5.0 (a condition at which all proteins flowed through). The majority of conditions, however, had a non-zero separability factor with a peak centered at approximately 0.40. It is interesting that for the resins, conditions, and

proteins studied here, the maximum individual separability factor is only 0.58. This indicates that a single resin is incapable of successfully separating all 15 proteins from each other and additional resins are required.

5.3. Two-Resin Separabilities and Enhancement

How do these separabilities change when each resin is paired with a second resin? As shown in **Figure 5a**, the distribution of two-resin separability scores (orange) is shifted to the right compared to the distribution of one-resin scores (blue). This illustrates that by pairing resins with one another, it was possible to separate a larger number of protein pairs. 56% of the two-resin separability scores (orange) were found to exceed the largest one-resin separability score of $S_m = 0.58$ (dotted line). This demonstrates that in order to achieve the highest separabilities, it is necessary to strategically pair chromatography resins (as opposed to focusing on individual resins).

Figure 5b shows the distribution of the enhancement factor, E_m for all combinations of resins and pHs. The distribution was bi-modal, where the majority of resin combinations yielded enhancements ranging from 0.2 to 0.5 and a few yielded enhancements extremely close to or equal to 0.0. No resin combinations achieved enhancements close to or equal to 1.0, indicating that no resin combinations separated protein pair sets that were entirely mutually exclusive. This was unsurprising for two reasons. First, to our knowledge, no resin pair in our set has been specifically designed to optimize orthogonality. Second, no separability score can exceed 1.0, regardless of the number of resins involved. Thus, individual resins whose separability scores exceed 0.5 cannot, by definition, be part of a pair whose enhancement factor is 1.0.

It is useful to consider the relationship between the separability score and the enhancement factor (shown in **Figure 5c**). Although we found that a high enhancement factor was associated with a high separability score, the relationship between these two quantities was more complex. A few resin pairs corresponding to high enhancements had relatively low pair separabil-

ity scores (illustrated by scatter in the top left-hand of **Figure 5c**). Since, the enhancement factor was calculated as the percent enhancement in separability over the best performing resin in the pair, resins that individually performed poorly could have high enhancement factors, while resins that perform well individually tended to have lower enhancement factors. Thus, we found that the resin pairs with the highest separability scores did not necessarily have the highest enhancement factors. It is important to remind the reader that our primary goal is to produce the best overall performing resin set. Orthogonality can be thought of as a secondary goal that will likely accompany any high performing resin set.

In addition to the overall trends observed in **Figure 5c**, it is interesting to note that many points appear to fall along lines with slightly different slopes. These lines contain points corresponding to resin pairs that share a common high performing resin. This was a natural consequence of our approach for calculating the enhancement factor, which included the separability score of the better performing resin in the denominator. To illustrate this effect, **Figure 5d** shows all points corresponding to resin pairs that include Capto MMC at pH 5.0 (orange) and all points corresponding to resin pairs that include Baker Bond Poly Abx at pH 5.0 (blue). Capto MMC at pH 5.0 had a high individual separability score (0.58), and as a result, all resin pairs that included Capto MMC at pH 5.0 fell on a line. In contrast, Baker Bond Poly Abx had a low individual separability score (0.25) and, as a result, resin pairs that included Baker Bond Poly Abx at pH 5.0 did not fall on a line. For ligand pairs that fell on a line, the x intercept of this line was equal to the separability of the better performing resin, $\max(S_1, S_2)$, and the slope of this line was equal to $\frac{1}{1-\max(S_1,S_2)}$. In this way, as individual separability scores improve, larger increases in separability are required to achieve equivalent increases in enhancement (illustrated in **Figure 5b**).

5.4. Effect of pH on Enhancement Factor

In this work, we evaluated the retention behavior of proteins using salt gradients and, as a result, explicitly evaluated separability and enhancement factors using salt as the separation handle. Ideally, in order to take into account pH as a possible separation handle, an optimally orthogonal resin set should be designed to contain resins that are orthogonal to all other resins within the set *and* themselves at different pHs. We were interested in examining whether changing the operating pH of a resin could create selectivity changes that were comparable to changing the resin itself.

Figures 6a and **6b** show the distribution of separability scores and enhancement factors, respectively, when all resin pairs are considered (blue) and when resins are only compared to themselves at different pH values (orange). Unsurprisingly, evaluating the same resin at different pH values tended to result in lower separability scores, shown by the shifts to the left on the blue curves. This difference became even larger when comparing the distributions of enhancement factors, indicating a lower degree of orthogonality when resins are compared to themselves at different pH conditions. Shifting pH can change selectivities by altering the charge distribution on proteins as well as the charge state on the resin. In this analysis, many of the resins contained strong ion exchange groups and would not change charge states within the selected pH ranges. We therefore hypothesize that this may contribute to the relatively low enhancement factors observed in this case.

Some resins, however, were found to be orthogonal to themselves only within specific pH windows. For example, **Figure 7a-c** shows the protein retention scatter plots for Capto MMC compared in all combinations at pH 5.0, 6.0, and 7.0. Interestingly, the pH 6.0 and 7.0 conditions were not orthogonal to one another, $E_M = 0.098$. This is can be seen in **Figure 7b** where many of the elution salt concentrations were highly correlated. In contrast, the pH 5.0 condition was more orthogonal when paired with either

the pH 6.0 and 7.0 condition as seen in **Figure 7a** and **c** ($E_M = 0.257$, 0.300 when paired with pH 6.0 and 7.0 respectively), illustrated by the spread of points in separation space. As discussed above, one hypothesis for this phenomenon is that the the weak cation exchange groups on Capto MMC as well as specific amino acids on the proteins (i.e. histidines) are beginning to titrate and opening new windows of selectivity.

5.5. Case Studies

In the previous sections we have developed an approach for evaluating the separability and orthogonality of two-resin systems by measuring a separability score and an enhancement factor. With this approach, we can now ask: which resin/pH pairs are the most orthogonal? Similarly, which resin/pH pairs are the least orthogonal? Previously, orthogonality within process development has been assessed in a heuristic manner, such that resins interacting with proteins through different modes were thought to be orthogonal. How do our measurements of separability and orthogonality compare with these intuitions? To explore this, we performed a more thorough analysis of a series of case studies within our data set.

5.5.1. Strong Cation and Anion Exchangers

Strong cation and anion exchange resins are often thought of as orthogonal and are used together in many processes. It was therefore expected that these resin types would separate non-overlapping sets of proteins and share a relatively large enhancement factor. To explore this, **Figure 8** shows protein retention on Q Sepharose HP at pH 7.0, a strong anion exchanger, and SP Sepharose HP at pH 6.0. As expected, **Figure 8a** shows that different sets of proteins were separated by each resin. In particular, we found that proteins that flowed through on one resin were often separated by the other resin and vice versa. The resulting separability score was 0.64 and the enhancement factor was 0.31. Thus, our approach reflects the commonly held intuition

that these two modes of chromatography are orthogonal.

5.5.2. Strong and Salt Tolerant Ion Exchangers

Salt tolerant ion exchangers are generally described as a type of ion exchanger that strongly retains proteins in the presence of moderate conductivities, as compared to a traditional ion exchanger. This salt tolerance may be achieved by adjusting the ligand or base matrix chemistry to reduce the sensitivity of protein-resin interactions to salt concentration. One might therefore expect that salt tolerant ion exchangers would not be orthogonal to traditional strong ion exchangers, but would separate the same sets of protein pairs (although perhaps at a different salt concentration). On the other hand, since secondary interactions may be contributing to this salt tolerance, one might expect some selectivity differences between salt tolerant and traditional ion exchangers.

To investigate this, **Figure 9** compares protein retention on Q Sepharose HP, a strong anion exchanger, at pH 7.0, and HyperCel STAR AX, a salt tolerant anion exchanger, at pH 7.0. Interestingly, as seen in **Figure 9a**, protein retention times on the two resins were highly correlated, resulting in both resins separating approximately the same sets of proteins. This effect resulted in a relatively low enhancement factor of 0.16 for this resin pair.

Despite this non-orthogonality, the resulting two-resin separability score was 0.61, which was approximately average for a two-resin separability score (see **Figure 5a**). This was because each of the individual separability scores, 0.42 and 0.49 for HyperCel STAR AX and Q Sepharose HP respectively, were among the best in our resin set. Thus, while each of the individual resins efficiently separated protein pairs, they were highly redundant, resulting in an intermediate two-resin separability score and a low enhancement factor.

5.5.3. Salt Tolerant and Multimodal Ion Exchangers

Multimodal ion exchangers are generally defined as a type of ion exchanger that employs at least one additional mode of interaction (often hydrophobic interaction). Multimodal ion exchangers are explicitly described as having unique selectivities that differ from traditional ion exchangers. In fact, previous work in our group has illustrated that some multimodal ion exchange resins exhibit different selectivities on several homologous and non-homologous protein libraries[20, 11, 13, 21, 22, 23]. Multimodal ion exchangers achieve these unique selectivities by leveraging synergistic combinations of ionic, hydrophobic, and hydrogen bonding interactions. Since the specific ligand chemistries and the nature of protein-ligand interactions have not been disclosed for salt tolerant ion exchangers, we were interested in evaluating the orthogonality of multimodal resins to this class of resins.

To explore this question, **Figure 10** compares protein retention on a multimodal cation exchanger, CMM HyperCel at pH 6.0, and a salt tolerant cation exchanger, HyperCel STAR CEX at pH 6.0. Interestingly, **Figure 10a** shows that protein retention on the two resins was less correlated than the results obtained when combining the salt tolerant and strong ion exchangers (**Figure 9**). Further, the results from our analysis indicated that there were several non-overlapping protein pairs that could be separated with this resin combination, resulting in a separability score of 0.67 and an enhancement factor of 0.28. Thus, CMM HyperCel and HyperCel STAR CEX were moderately orthogonal at pH 6.0. While many multimodal resins in our anlaysis were found to be orthogonal to salt tolerant ion exchangers, the degree of orthognality varied with resins as can be seen SI Figures 1-8.

5.5.4. High Separability Resin Pair

Finally, it is interesting to take a closer look at a resin pair that was highlighted by our approach as having a particularly high separability. **Figure 11** shows protein retention on Capto MMC, a multimodal cation exchanger,

at pH 5.0 and Eshmuno Q, a tentacular anion exchanger, at pH 7.0. **Figure 11a** illustrates that protein elution salt concentrations varied widely on the two resins and were highly uncorrelated. This led to proteins being well separated by the resin pair (**Figure 11b**). The resulting separability score was 0.827 and the enhancement factor was 0.374.

As described previously, depending on the conditions, proteins that elute in the strip might be denatured and therefore, if they are the protein of interest, may not be thought of as successfully separated. Since many proteins eluted in the strip on Capto MMC at pH 5, **Figure 11c-d** show the protein retention behavior for Capto MMC ImpRes at pH 5.0 (a lower ligand density) and Eshmuno Q at pH 7.0 for comparison. By reducing the ligand density, more proteins eluted in the gradient. The resulting separability score and enhancement factor remained relatively unchanged (0.81 and 0.40 respectively), now with fewer proteins eluting in the strip.

6. Conclusions

Here, we reported the development of a framework for assessing the separability and orthogonality of multi-dimensional separations for process development. This approach relied on transforming a set of separation distances (in our case, elution salt concentration distances in a salt gradient) into a set of weights based on a simple mapping function. These weights were then averaged to obtain a score that represents the ability of one or more separation steps to separate pairs of proteins. Orthogonality can then be quantified as the fractional improvement of this score upon adding additional separation steps. To explore how this approach can be used in practice, we performed chromatographic gradient screens of 15 proteins on 14 resins. We found that although some resins were individually able to separate proteins, separability was significantly improved by pairing individually high-performing resins that were also orthogonal to one another. We believe that by strategically

designing resins to optimize these parameters, either for specific protein sets or for proteins in general, it is possible to obtain resin sets which, when used together, have a high probability of being able to separate an arbitrary product-impurity pair.

In this work, linear salt gradients were chosen as the method for measuring protein retention times used to calculate separability scores and enhancement factors. This method was selected due to its speed and equipment availability, however, modifications can be easily implemented in order to relax several assumptions if required. Firstly, in multimodal systems with U-shaped log k' vs. salt plots, gradient slope can affect the selectivity between proteins. To account for this, thermodynamic data and column modelling can be used as a means to simulate protein retention with different gradient slopes. This type of modification can be particularly important for cases where high salt concentrations are required or kosmotropic fluid phase modifiers are used. Secondly, although this analysis focused on salt gradients at constant pH, modifications such as pH gradients or the addition of fluid phase modifiers can easily be employed to create selectivities. Since this approach is agnostic to the type of gradient, separability scores and enhancement factors can easily be calculated for these data sets as well.

As described in the Theory section, the separability score and enhancement factors are only accurate if the protein set used can be assumed to be representative of the proteins you wish to separate. In this manuscript, we focused our attention on assessing the separability and orthogonality of resins based on the retention behavior of 15 proteins. While these proteins are reasonably diverse and easily available, our measurements are only accurate if the product-impurity pair in question is sufficiently similar to these proteins. In the future, we therefore plan to apply this approach to two parallel problems: 1) assessing context-specific separability and orthogonality and 2) assessing generalizable separability and orthogonality. Since many

non-affinity separation steps are used as polishing steps in mAb separations, it may be useful to select a large set of mAb variants as the protein pool for this approach. This would lead to the identification of resin sets with optimized separabilities and orthogonalities for the specific challenge of mAb variant separations. In contrast, it may be useful to identify resins which are the most globally separable and orthogonal for any protein class. This would require screening with an extremely large protein pool whose constituents represent all of protein diversity as a whole.

Currently, resins are typically not designed in the context of a set. It is therefore our opinion that it is possible to design optimally separable and orthogonal sets which outperform commercially available resins by the metrics described in this paper. Our group has previously shown that multimodal systems can create unique and unintuitive selectivities that depend on charge, hydrophobicity, and hydrogen bonding which make them strong candidates for this next-generation resin set[12, 11, 20, 24, 25, 13]. Given the relatively complex and synergistic interactions in multimodal systems, the mathematical framework developed here provides a straightforward method for screening new prototypical resin sets with new and exciting orthogonal selectivities.

Supporting Information. Separability and enhancement scores for combinations of multimodal resins and protein elution salt concentrations on Eshmuno HCX.

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Fig. 1: a.) Elution salt concentrations of 15 proteins on Nuvia cPrime in a pH 5.0 salt gradient. b.) Histogram (bars, light blue) and corresponding Gaussian kernel density estimate (line, dark blue) of protein pair elution salt concentration differences for this gradient.

Fig. 2: a.) Scatter plot of elution salt concentrations for 15 proteins on Capto MMC (x-axis) vs. Nuvia cPrime (y-axis) in pH 5.0 salt gradients. b.) Histograms (bars, light blue and light orange) and Gaussian kernel density estimates (lines, dark blue and dark orange) of protein pair elution salt concentration differences for Nuvia cPrime (blue) and the set of Nuvia cPrime + Capto MMC (orange) in pH 5.0 salt gradients.

Fig. 3: Heat map of 1D separability scores for each resin at pH 5, 6, and 7 using the 15 proteins selected in this study.

Fig. 4: Histogram (bars, light blue) and Guassian kernel density estimate (line, dark blue) of 1D separability scores for all 14 resins at pH 5.0, 6.0, and 7.0.

Fig. 5: a.) Histograms (bars, light blue and light orange) and Gaussian kernel density estimates (lines, dark blue and dark orange) of 1D separability scores for individual resins (blue) and 2D separability scores for all possible resin-pH pairs (orange). b.) Histogram (bars, light blue) and Gaussian kernel density estimate (line, dark blue) of enhancement factors for all possible resin-pH pairs. c.) Scatter plot of 2D separability scores vs. enhancement factors for all possible resin-pH pairs. Contours are shaded according to density of points. The x-axis and y-axis show the distribution of separability scores and enhancement factors, respectively. d.) Scatter plot of 2D separability scores vs. enhancement factors for all resin-pH pairs that contain Bakerbond PolyABx in a pH 5 salt gradient (blue) and Capto MMC in a pH 5 salt gradient (orange).

Fig. 6: Histograms (bars, light blue and light orange) and Gaussian kernel density estimates (lines, dark blue and dark orange) of a.) 2D separability scores and b.) enhancement factors for all possible resin-pH pairs (blue) and cases where the resin is only compared to itself at different pH values (orange).

Fig. 7: Scatter plots of protein pair elution salt concentration differences for Capto MMC in different pH combinations: a.) pH 5.0 and pH 6.0, b.) pH 6.0 and pH 7.0, and c.) pH 5.0 and pH 7.0

Fig. 8: a.) Scatter plot of elution salt concentrations for Q Sepharose HP in a pH 7.0 salt gradient (x-axis) and for SP Sepharose HP in a pH 6.0 salt gradient (y-axis). b.) Histogram (bars, light blue) and Gaussian kernel density estimate (line, dark blue) of protein pair elution salt concentration differences for these two resins and conditions with $S_m = 0.64$ and $E_m = 0.31$.

Fig. 9: a.) Scatter plot of elution salt concentrations for Q Sepharose HP in a pH 7.0 salt gradient (x-axis) and for HyperCel STAR AX in a pH 7.0 salt gradient (y-axis). b.) Histogram (bars, light blue) and Gaussian kernel density estimate (line, dark blue) protein pair elution salt concentration differences for these two resins and conditions with $S_m = 0.61$ and $E_m = 0.16$.

Fig. 10: a.) Scatter plot of elution salt concentrations for HyperCel STAR CEX in a pH 6.0 salt gradient (x-axis) and for CMM HyperCel in a pH 6.0 salt gradient (y-axis). b.) Histogram (bars, light blue) and Gaussian kernel density estimate (line, dark blue) of protein pair elution salt concentration differences for these two resins and conditions with $S_m = 0.67$ and $E_m = 0.28$.

Fig. 11: a.) Scatter plot of elution salt concentrations for Eshmuno Q in a pH 7.0 salt gradient (x-axis) and for Capto MMC in a pH 5.0 salt gradient (y-axis). b.) Histogram (bars, light blue) and Gaussian kernel density estimate (line, dark blue) of protein pair elution salt concentration differences for these two resins and conditions with $S_m = 0.83$ and $E_m = 0.37$. c.) Scatter plot of elution salt concentrations for Eshmuno Q in a pH 7.0 salt gradient (x-axis) and for Capto MMC ImpRes in a pH 5.0 salt gradient (y-axis). d.) Histogram (bars, light blue) and Gaussian kernel density estimate (line, dark blue) of protein pair elution salt concentration differences for these two resins and conditions with $S_m = 0.81$ and $E_m = 0.40$.