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# Improving selectivity and performing online on-column fractioning in liquid chromatography for the separation of therapeutic biopharmaceutical products

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ABSTRACT

A novel column-coupling approach is suggested to improve both the selectivity and efficiency of protein separations in liquid chromatography. Protein separations often suffer from limited selectivity or not appropriate resolving power. For a new biopharmaceutical product, the identification of the main and minor variant species is required. For that purpose, often offline collection fractioning is applied which is time consuming and regularly dilute the samples to an unacceptable extent. By serially coupling columns in the order of their increasing retentivity and applying "multi-isocratic" elution mode, indeed any (arbitrary) selectivity can be attained. Moreover, if a protein peak is trapped at the inlet of a later column segment – of a coupled system -, its band will be refocused and elute in unprecedented sharp peak. Furthermore, it becomes possible to perform online on-column fractioning of protein species within a very short analysis time (~ 1 min) and without sample dilution. Two-, three- or multiple column systems can be developed and applied for complex sample separations (such as antibody mixtures). This new methodology can be particularly useful to improve the analysis (and therefore, safety and quality) of therapeutic mAbs and related products and offers benefits compared to offline fractionating. It is also demonstrated in this proof of concept study, that methyl (C1) modified RP phase has a great potential for protein separations despite it is not commercially available in state-of-the-art wide pore superficially porous particle format..

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## 1. Introduction

Protein biopharmaceuticals have emerged as important therapeutic options for the treatment of various diseases (e.g. cancer, cardiovascular diseases, inflammatory and autoimmune disorders, asthma, Alzheimer's disease and migraines). Based on this broad range of applications, pharmaceutical companies are increasing their efforts aimed at the research and product development of innovative biological drugs. Given their obvious benefits in terms of efficacy, monoclonal antibodies (mAbs) are the most successful therapeutic biopharmaceutical products. To increase the potential success rates and improve the quality and safety of biopharmaceutical products as approved drugs, a comprehensive set of analytical and functional techniques must be applied. Among the available analytical strategies, liquid chromatography (LC) is commonly used for the detailed evaluation of new protein-based drugs. However, LC separations often suffers from inadequate resolving power for closely related proteins (large solutes). There is a need to improve the separation power of current LC methods.

Large solutes such as proteins and peptides, show a particular retention behavior in reversed phase liquid chromatography (RPLC). This behavior is often called as "on/off" or "bind and elute" retention mechanism. A consequence of this mechanism is that a minor variation in the mobile phase composition causes an important change in solute retention [1–4]. At a given mobile phase composition, the solute binds at the column inlet (highly retained). Then, a small increase in the eluent strength, will result in a much lower retention. The retention factor drops to practically zero and the molecules will be unleashed from the column inlet and travel through the entire length of the column without further physicochemical interaction. A recent study demonstrated, that for an intact monoclonal antibody (mAb), only a 0.8% change in mobile phase composition shifts the retention by a factor of ten [4]. While, for a common small molecule solute (e.g. aspirin), the same shift in retention was observed for a 33% change of organic solvent content in the mobile phase. It was found that the *S* parameter of the linear solvent strength (LSS) model is a good measure of the on/off

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type retention behavior [4,5]. $S$ is a constant for a given compound at fixed experimental conditions (in practice, it describes how sensitive is the solute retention to mobile phase composition). For small solutes, this $S$ value is typically comprised between 2 and 6, while it gives $S \geq 15$ for a 25 kDa solute, $S \geq 25$ for a 50 kDa solute and $S \geq 100$ for an intact mAb (150 kDa) [5]. In practice, we can estimate that solutes with $S \geq 15$ follow the on/off type behavior.

The chromatographic separation of solutes following the on/off mechanism requires necessarily gradient elution mode. For analytical scale separations, therapeutic proteins are therefore analyzed mostly by running linear gradients, however nonlinear (concave or convex) gradients can also improve the selectivity [6–8]. In addition to those gradients, the so-called one-segment-per-component or one-segment-per-group-of-components approach can also be useful [9]. Here, the idea is to adjust the slope of the gradient after the elution of each individual component of the sample.

Very recently, it has been shown that the possible highest selectivity and resolution for protein separations can be achieved by applying a so-called multi-isocratic elution mode [4]. Such elution mode consists in the combination of (1) binding isocratic segments and (2) eluting steep gradient segments which in theory performs much higher selectivity than any linear or nonlinear gradient does. By utilizing the potential of multi-isocratic elution mode, a uniform peak distribution (equidistant band spacing) can be achieved. Moreover, the elution distance between the peaks can be adjusted arbitrary (in most cases), by setting the length of the isocratic segments. Furthermore, the compounds can be eluted in very sharp peaks, thanks to the band compression caused by the short and steep eluting gradient segments.

To improve the separation power in chromatography, column coupling is a promising approach and has been applied quite early in the history of chromatography [10–15]. There are two ways to combine two or more columns in uni-dimensional separations, namely parallel and serial arrangements. In many cases, the aim of column coupling remains to increase the chromatographic performance by finding the optimal column length [16,17]. Next to kinetic performance, selectivity can also be improved by serially coupling columns of different selectivity. This methodology is often referred to as stationary phase optimized selectivity liquid chromatography (SOSLC) [13,14]. This SOSLC approach has been commercialized under the name of POPLC (as phase optimized liquid chromatography, provided by Bischoff Chromatography), and several studies have reported the possible increase in selectivity resulting in improved separation quality, compared to the use of a single column. Recently, an interesting approach was suggested to benefit from a so-called “peak-sharpening” effect [18,19]. The idea was to couple columns made of the same stationary phase but packed with particles possessing different diameters (and thus different plate heights). When combining such columns in their order of decreasing particle size (increasing efficiency), then the gradient band compression effect can compensate and outperform the competing band broadening caused by dispersive and diffusive processes.

In this work, our purpose was to combine the “multi-isocratic” elution mode with column coupling approach to further improve selectivity. When combining columns with a comparable selectivity, but different retentivity, then selectivity and resolution can be further improved for large solutes compared to the recently studied single column multi-isocratic elution mode. Moreover, if the column segments provide appropriate differences between their retention and are coupled in the order of their increasing retentivity, an online on-column protein fractioning is feasible within a very short time. This approach enables to trap the different protein species on the different column segments. Then the trapped species can be eluted from the individual columns by applying any elution mode (linear-, multi-linear-, nonlinear gradient or multi-isocratic modes).

## 2. Materials and methods

### 2.1. Chemicals and samples

Acetonitrile (AcN) and water were purchased from Fisher Scientific (Reinach, Switzerland). Trifluoroacetic acid (TFA) and dithiothreitol (DTT) were purchased from Sigma-Aldrich (Buchs, Switzerland). Commercial therapeutic monoclonal antibody (mAb) (i.e. rituximab, cetuximab, daratumumab, ramuricumab, natalizumab, infliximab and obinutuzumab) samples were obtained as European Union pharmaceutical-grade drug products from their respective manufacturers.

### 2.2. Chromatographic system, columns and software

Measurements were performed on a Waters Acquity UPLC I-Class system equipped with a binary solvent delivery pump, an autosampler and fluorescence (FL) detector. The system includes a flow through needle (FTN) injection system with 15 µL needle and a 2 µL FL flow-cell. The overall extra-column volume was about 7.5 µL as measured from the injection seat of the autosampler to the detector cell. The dwell volume was measured as $V_d = 0.110$ mL. Data acquisition and instrument control were performed by Empower Pro 3 software (Waters).

Commercial C4, ES-C18 and diphenyl (DP) columns packed with superficially porous 2.7 $\mu$m 1000 Å particles (50 × 2.1 mm and 150 × 2.1 mm formats) were generous gift from Advanced Materials Technology (Wilmington, DE, USA). Prototype C4 and DP columns with 10% and 50% surface coverage (low ligand density) and prototype C1 and C2 columns (100% surface coverage) were provided also by Advanced Materials Technology and were also packed with superficially porous 2.7 $\mu$m 1000 Å particles.

Data processing was performed with Excel and Drylab (4.2) software.

### 2.3. Sample and mobile phase

MAb samples were analyzed at their intact and subunit levels. Intact mAbs were diluted to 1 mg/mL with water and injected without further preparation.

Preparation of mAb subunits (heavy chain and light chain) was performed on the basis of a previously published protocol [20]. Daratumumab’s interchain disulfide bonds were reduced by adding freshly prepared 1 M DTT solution to 1 mg/mL intact daratumumab solution (to have approximately 100 mM DTT concentration in the sample). Reduction was performed at 45 °C for 30 min. After sample preparation, samples were kept at 4 °C.

For all separations, mobile phase A was 0.1% TFA (v/v) in water, while mobile phase B was 0.1% TFA in acetonitrile.

### 2.4. Apparatus and methodology

Sample volume of 1 $\mu$L was injected using linear gradients and various multi-isocratic conditions. Temperature was set to 80 °C (when using the commercial HALO 1000 Å C4, ES-C18 and DP columns and prototype C4 and DP columns) and to 85 °C (for the prototype C1 and C2 phases). For the two-column system (C4 and DP) temperature was set to 80 °C, while for the three-column system (C1, C4 and DP) it was set to 85 °C. Data was acquired using 280 nm excitation and 350 nm emission wavelengths (FL).

The LSS parameters were derived from two linear gradient runs (30–45% B) performed at different gradient steepness (gradient time, tG). On the 150 × 2.1 mm columns, the flow rate was

**Table 1**
Parameters of the linear solvent strength (LSS) models.

| Solute/column | C4 (150 mm, $T = 80$ °C) | | DP (150 mm, $T = 80$ °C) | |
|---|---|---|---|---|
| | log $k_w$ | $S$ | log $k_w$ | $S$ |
| Rituximab | 56.7 | 156.6 | 45.8 | 121.2 |
| Cetuximab | 61.6 | 161.9 | 60.5 | 153.5 |
| Daratumumab LC | 6.0 | 18.8 | 14.7 | 40.3 |
| Daratumumab HC | 10.5 | 27.6 | 17.1 | 42.2 |

| | C1 (50 mm, $T = 85$ °C) | | C4 (50 mm, $T = 85$ °C) | | DP (50 mm, $T = 85$ °C) | |
|---|---|---|---|---|---|---|
| | log $k_w$ | $S$ | log $k_w$ | $S$ | log $k_w$ | $S$ |
| Rituximab | 36.8 | 109.1 | 44.3 | 129.4 | 34.3 | 96.4 |
| Ramuricumab | 35.1 | 101.3 | 57.5 | 163.6 | 46.0 | 126.3 |
| Obinutuzumab | 29.4 | 80.2 | 43.9 | 117.1 | 40.5 | 105.2 |

set to 0.4 mL/min and $tG_1 = 10$ min and $tG_2 = 30$ min were applied. When operating the $50 \times 2.1$ mm columns, the flow rate was set to 0.5 mL/min and $tG_1 = 6$ min and $tG_2 = 18$ min were applied. Then the log $k_w$ and $S$ parameters of the LSS models were obtained using DryLab software. The parameters of LSS models are listed in Table 1.

For the multi-isocratic conditions, the binding and eluting mobile phase compositions were determined from the LSS models, using the recently suggested criteria [4].

The isocratic conditions to perform online on-column protein fractioning on coupled systems are detailed in the corresponding sections.

## 3. Results and discussion

### 3.1. The general concept of on-column online protein fractioning

Due to the on/off retention mechanism of large proteins, if two proteins possess difference between their retention, then it is possible to find an isocratic mobile phase composition which results in the binding of the more retained protein on the column inlet, while the less retained protein just travels through the column without any further interaction (not retained). Then, if we imagine two serially coupled columns with increasing retentivity, it may happen that the less retained protein does not interact with the first (less retentive) column, but adsorbs on the second (more retentive) column. At the end, the more retained protein can be trapped on the less retentive (first) column, while the less retained protein can be trapped on the more retentive (second) column. Fig. 1 shows a schematic view of a two-column system. Here, it is assumed that column A provides lower retention for all proteins compared to column B ($k_{col\,A} < k_{col\,B}$), and that protein 1 is less retained than protein 2 ($k_1 < k_2$). When coupling the two columns in the order of their retentivity (A → B) and setting weak mobile phase strength (operating the columns in isocratic mode), then the retention of both proteins is high enough and they will bind at the inlet of the first column. Increasing the mobile phase strength to reach a sufficient composition will cause the elution of the less retained protein from the first column, while the more retained protein will still remain on the first column. The less retained protein then can travel through the first column and bind on the more retentive second column. In this case, the two proteins are bound at the inlet of the two individual columns. Such situation can be called as on-column fractioning. Then, when further increasing the mobile phase strength, sooner or later the less retained protein will elute from the second column, while the more retained protein will either move on to the second column or will remain bound to the first column. Finally, when setting strong enough mobile phase composition, the more retained protein will also elute from the entire system.

Such setup can be utilized either to improve the resolution between peaks and set any selectivity between proteins species, or to trap the different proteins on the individual columns (on-column fractioning). To fractionate more species, obviously more columns can be coupled (e.g. three columns to fractionate three proteins, or $n$ columns to fractionate (or partially fractionate) $n$ proteins).

### 3.2. Selecting columns for serially coupled systems

The purpose of serial column coupling is generally either to improve the kinetic performance or to change the separation selectivity. In the present study, the idea is however different and the goal is to perform online fractioning or improve the resolution between large protein species, such as mAbs. Therefore, we need columns providing – ideally – comparable selectivity, but significantly different retention. In other words, the relative retention of protein species should be maintained, while their absolute retention needs to be increased on the column segments along the serially coupled system. In the case of silica-based stationary phases, the type of alkylsilane bonded to the surface can influence the retention of proteins and can therefore be used to manipulate the retention, and to a lesser extent, selectivity [21]. It is known, that the relative hydrophobicity of the ligand, surface coverage, ligand density, carbon load, ligand flexibility, and the degree of exposure of the surface silanols all impact solute retention. In addition, the choice of ligand chemistry can influence the recovery and conformational integrity of the protein species [21]. When analyzing proteins, it is always important to consider possible secondary interactions with residual silanols, due to the high number of charges on the protein surface, compared to small solutes. In some cases, the ligand density of column bonded with shorter alkyl chains can be higher than that of longer chains (less steric hindrance), thus the accessible hydrophobic surface area may even be larger for phases modified with short alkyl ligands. In addition, if there are residual unbounded silanols present on the silica surface, they will be more accessible in cases where the stationary phase is composed of short chain ligands or made with lower ligand surface coverage. Therefore, it is not obvious for large solutes how ligand density and chain length will impact the overall retention.

To select the appropriate columns for a coupled system, several stationary phases were screened. First, we have tested various commercial wide-pore 1000 Å phases packed with superficially porous particles (i.e. C4, ES-C18 and DP). In addition, some prototype C4 and DP columns having only 10 and 50% coverage - compared to the commercial products – were also prepared, as well as some additional prototype stationary phases bonded with C1

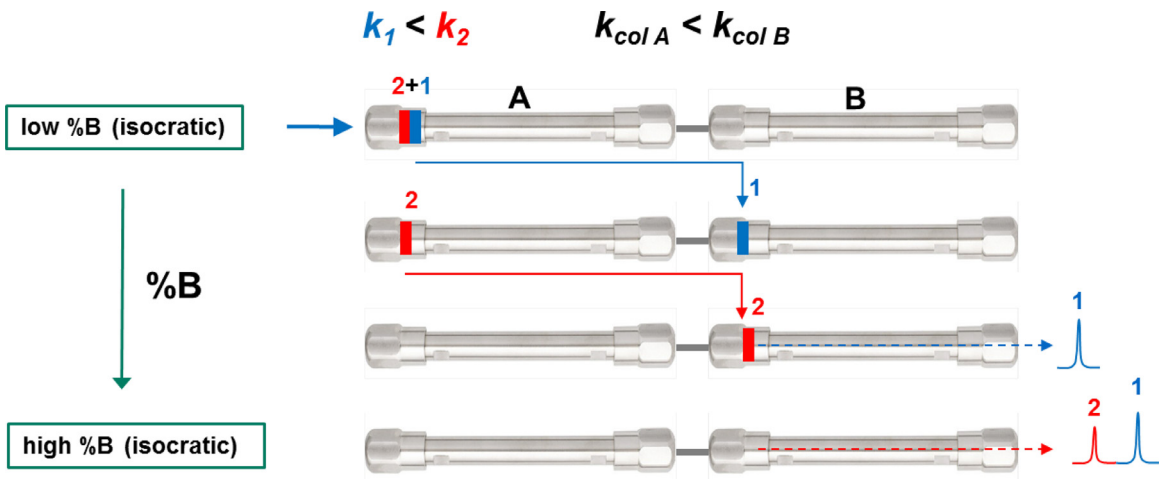



**Fig. 1.** Schematic representation of the elution of two large proteins from a two-column system. Conditions: The retention of protein 1 (blue) is lower than of protein 2 (red). The first column (A) provides lower retention than the second one (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and C2 ligands having normal (nominal) coverage. Then, seven intact mAbs (representing a wide range of both hydrophobicity and isoelectric points [22,23]) were injected using linear gradients (at two different gradient steepness). Then, retention and peak shapes were compared for the nine different stationary phases and seven mAbs.

It was found that the different stationary phases provided different absolute retention, but similar relative retention (selectivity). Among the commercial columns, the DP phase systematically showed the highest retention for all samples (probably due to its ability to form $\pi$-$\pi$ interactions), while the C4 and ES-C18 columns showed significantly lower – and quite similar - retentivity. In some cases, the shorter chain C4 even provided slightly higher retention than the ES-C18. When comparing the efficiency of the C4 and ES-C18 phases, the C4 showed somewhat higher peak capacity and more symmetrical peaks for most mAbs. Therefore, considering the commercial columns (C4, ES-C18 and DP), the C4 and DP have been selected as candidates for a coupled system.

Then, partially covered (i.e. 50% and 10% surface coverage) versions of these two phases were also evaluated. The stationary phases with 50% coverage showed very similar or even slightly higher retention than the commercial phases (100% coverage for both the C4 and DP), but mAbs eluted in somewhat broader peaks on the 50% coverage phases. The slightly higher retention and broader peaks on the partially covered phases might be explained by the more accessible surface silanols, which probably promote additional electrostatic interactions. Then, the less covered phases (10% C4 and phenyl surface coverage) showed slightly lower retention, but broad and asymmetrical peaks. This observation suggests that too much silanols became accessible and broaden the peaks (probably through strong ion-exchange interactions), while on the other hand, the lower retention is probably due to the much lower ligand density and thus lower hydrophobicity. At the end, to create less retentive phases than the commercial ones, we disclaimed the partially covered RP phases, as there is a risk of peak tailing and less controlled retention mechanism (mixed mode).

Another idea was to try very short alkyl chains instead of the commonly used C4–C18 ones. Therefore, C1 and C2 bonded stationary phases were prepared and tested. The C2 phase showed practically the same retentivity as the C4, but the C1 modification seemed to be very promising, since it gave significantly lower retention compared to both the commercial C4 and DP phases, while maintaining high peak capacity. Moreover, in many cases, the C1 phase showed better resolution between mAb variants than the commercial C4 phase.

Finally, we selected the following three columns as potential candidates for serially coupled system, since they possess enough difference in retentivity: prototype C1 (1), commercial C4 (2) and commercial DP (3).

### 3.3. Performing arbitrary resolution by combining multi-isocratic elution mode and column coupling

Based on the preliminary experiments, two intact mAbs (rituximab (1) and cetuximab (2)) have been selected, since they showed sufficiently large differences in retention. Rituximab showed indeed the lowest retention on all columns. Regarding stationary phases, the commercial C4 and DP phases were considered for a coupled system. First, the LSS parameters were determined on the individual columns. For rituximab, we obtained log $k_w = 56.7$, $S = 156.6$ (C4) and log $k_w = 45.8$, $S = 121.2$ (DP), while for cetuximab we found log $k_w = 61.6$, $S = 161.9$ (C4) and log $k_w = 60.5$, $S = 153.5$ (DP). The parameters of LSS models are listed in Table 1. Fig. 2 shows the obtained log $k$–$\varphi$ plots. Purple arrows indicate the isocratic conditions which can be set to perform multi-isocratic elution on a coupled system (by coupling the columns in their order of increasing retentivity: C4 → DP). Setting 33% B results in sufficient retention for both mAbs on the two columns ($k_1 = 9.3 \times 10^4$, $k_2 = 5.2 \times 10^7$ on the C4 column and $k_1 = 6.5 \times 10^5$, $k_2 = 6.6 \times 10^9$ on the DP column). Therefore, such composition can be considered for initial binding step on the two-column system. When setting 38.6% B eluent, rituximab can be eluted from both columns ($k_1 = 2 \times 10^{-4}$ on the C4 and $k_1 = 0.1$ on the DP column), while cetuximab can be eluted only from the C4 column ($k_2 = 0.04$) but will be retained on the DP column ($k_2 = 17$). Then, when setting 42% B, the more retained cetuximab can be released from the DP column ($k_2 = 1 \times 10^{-4}$).

The conditions suggested by the log $k$–$\varphi$ plots were experimentally verified. The C4 and DP columns (150 × 2.1 mm) were coupled using a 1 $\mu$L stainless steel connecting tube (please note that this additional void volume is negligible as the total accessible porous volume of the two columns is about 640 $\mu$L). An isocratic segment of 33% B eluent was set as the initial binding step (Fig. 3). Then at 2 min, a 0.1 min long gradient step was set to reach 38.6% B and this isocratic condition was maintained for different times such as 0.9 min (1 min in total), 1.9 min (2 min in

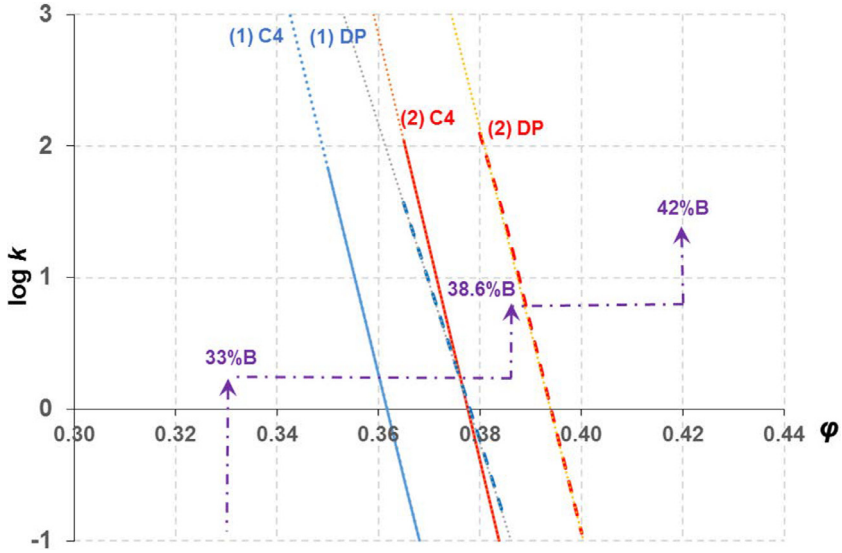



**Fig. 2.** log $k$–$\varphi$ plots obtained for rituximab (blue) and cetuximab (red) on C4 (straight line) and diphenyl (DP, dashed) stationary phases. The purple dashed lines indicate the mobile phase composition (%B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

total) and 3.9 min (4 min in total). This eluent strength allows to elute the first peak (rituximab) from both columns, while it elutes the second mAb (cetuximab) only from the first column, and sufficiently retain this mAb on the second (more retentive) column. On Fig. 3, the purple, blue and green lines indicate the gradient time program observed on the columns and the orange dashed lines indicate the total delay time originating from the column hold up time and system delay time. Therefore, at about 1.6 min, shift can be seen between the set and measured time program. Finally, inserting again a 0.1 min long gradient step to reach 43% B resulted in the elution of cetuximab from the second column. This mobile phase strength was hold until 10 min and at the end, the columns were equilibrated for 4 min before next injection. As shown on Fig. 3, the elution distance between the peaks can be arbitrary set by adjusting the length of the second isocratic segment. As it was recently shown, such multi-isocratic mode works perfectly on a single column [5]. However, this study demonstrates that a two-column system will result in higher resolution compared to a one-column system, since the more retained peak will benefit from band-refocusing at the inlet of the second column and thus will elute in much sharper peak than the first eluting solute which does not bind to the second column (the peak just travels through the column with the mobile phase velocity). Rituximab peak elutes with a peak width of $w_{1/2} = 0.0319$ min (corresponding to a peak variance of $\sigma^2 = 29\ \mu L^2$), while cetuximab peak elutes with $w_{1/2} = 0.0143$ min (corresponding to a peak variance of only $\sigma^2 = 6\ \mu L^2$). Rituximab peak is focused at the inlet of the first column only while disperses along its travel through two column length. While cetuximab is refocused on the inlet of the second column and then travels and disperses only through one column length. (Please note that on the individual columns, when running linear gradients, the two solutes eluted with very similar peak widths.) This way the second column itself does not play in the elution (and thus in the selectivity) of the first peak but is does for the second peak with additional retention and band focusing. The resolution values observed between the two peaks were $Rs = 21.1$ (1 min hold segment), $Rs = 44.7$ (2 min hold segment) and $Rs = 93.7$ (4 min hold segment).

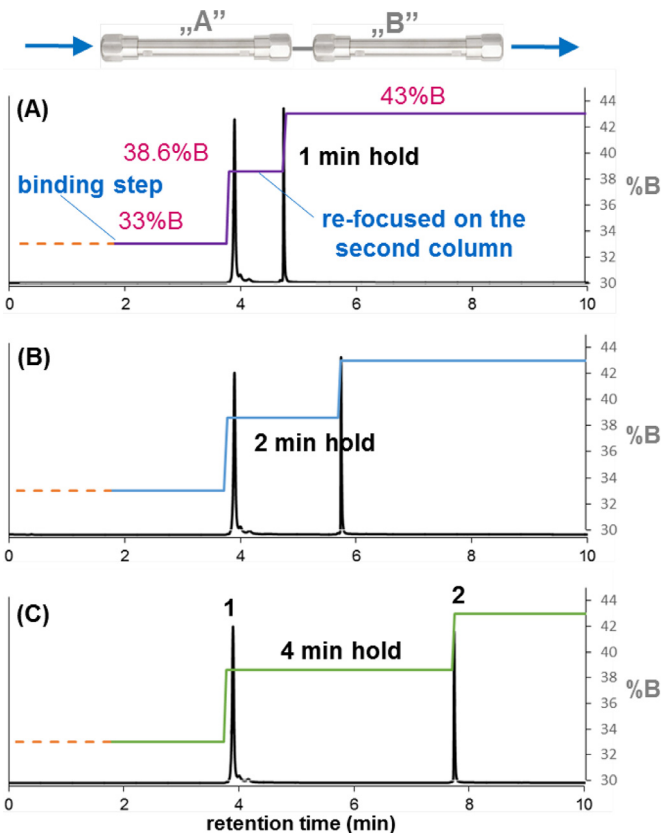



**Fig. 3.** Separation of rituximab (1) and cetuximab (2) on a two-column system (C4 as column "A" and diphenyl as column "B") applying multi-isocratic elution mode. The holding time of the second isocratic step was set as 1 (A), 2 (B) and 4 min (C). Column length: 2 × 150 mm (300 mm), flow rate: 0.4 mL/min, temperature: 80 °C.

### 3.4. On-line, on-column fractioning on a two-column system

If there is enough difference between the retentivity of columns in a two column system, then two proteins (possessing sufficiently different retention) might be fractioned on the two individual columns, applying appropriate isocratic mobile phase composition. To prove the applicability of this concept, the light chain (LC) and heavy chain (HC) fragments of a commercial therapeutic mAb (daratumumab) were attempted to be fractionated on a C4 → DP

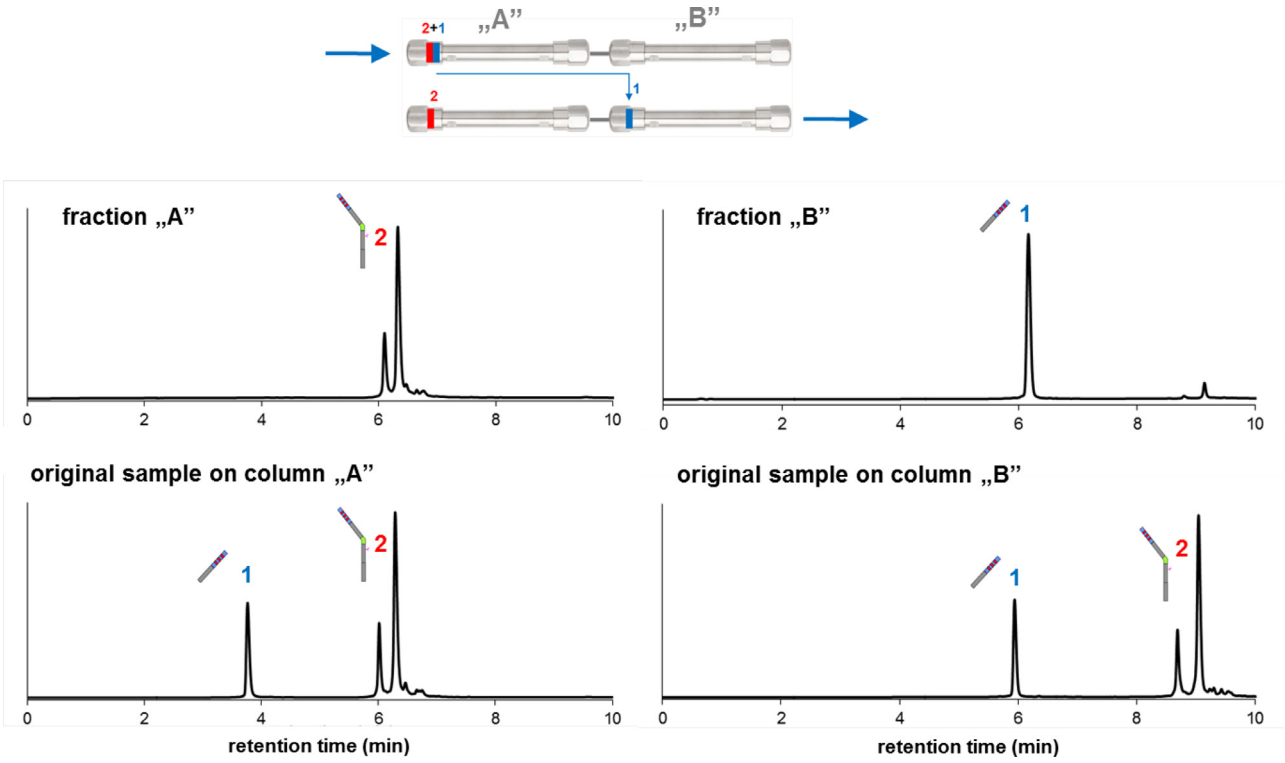



**Fig. 4.** Fractioning the light chain (1) and heavy chain (2) of daratumumab on a two-column system (C4 as column "A" and diphenyl as column "B") applying isocratic conditions (34.5% B for 1.6 min). After that, the fractionated peaks were eluted with a 10 min long generic gradient from the disconnected individual columns, and compared to the original sample including both fragments.

two-column system. The LSS parameters were first measured on the individual columns. For the LC fragment, we found log $k_w = 6.0$, $S = 18.8$ (C4) and log $k_w = 14.7$, $S = 40.3$ (DP), while for the HC fragment we obtained log $k_w = 10.5$, $S = 27.6$ (C4) and log $k_w = 17.1$, $S = 42.2$ (DP). So, both solutes were more retained on the DP column compared to the C4 phase. The parameters of LSS models are listed in Table 1. Constructing the log $k$–$\varphi$ plots enables to quickly find the appropriate mobile phase composition to fractionate the solutes in a coupled system (data not shown). Setting 34.5% results in $k_{LC} = 0.3$ and $k_{HC} = 8.5$ on the first (less retentive C4) column. It means that the HC peak will be retained (trapped), while the LC peak will be eluted from the column. Then, on the second column (more retentive DP), the entering LC fragment will be sufficiently retained ($k_{LC} = 6.9$). To experimentally verify these calculations, the two columns were coupled in series and isocratic mode (34.5% B eluent) was applied on the system. The run time ($t_{run}$) was set to the sum of column dead times, $t_{run} = t_0$ (C4) + $t_0$ (DP). (This run time corresponds to about 1.6 min in case of two 150 × 2.1 mm columns, operating at 0.4 mL/min flow rate.) After the isocratic run, the flow was stopped and the columns were disconnected. Then, a generic 10 min long gradient (30–45% B) was performed on the individual columns (without any additional injection). This generic gradient eluted the fractioned solutes from the individual columns. We also injected the initial sample (containing both LC and HC fragments) and compared the chromatographic profiles. Fig. 4 verifies that the two compounds were perfectly fractionated on the coupled system. On the first column (fraction "A"), we only saw the HC fragment peak, while on the second column (fraction "B"), only the LC fragment peak could be seen.

This example illustrates that two protein species (which follow the on/off retention behavior) can easily be fractionated online, using a two-column serially coupled system. The only criterion is that columns need to possess enough difference in their retentivity and need to be placed in their order of increasing retentivity.

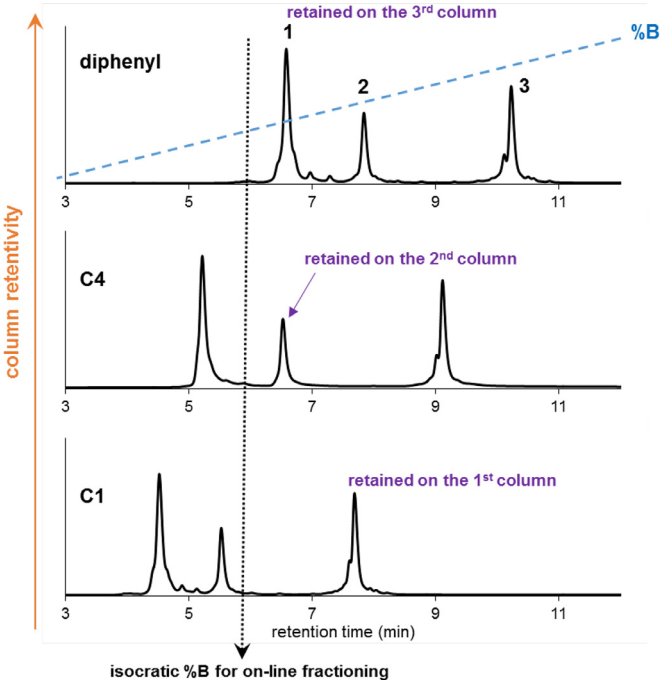



**Fig. 5.** Selecting isocratic composition (%B) to perform on-line fractioning on a three-column coupled system and to trap each compound on different columns. Peaks: rituximab (1), ramuricumab (2) and obinutuzumab (3). Columns: 50 × 2.1 mm C1, C4 and diphenyl, flow rate: 0.5 mL/min, tG = 18 min (30–45% B), temperature: 85 °C.

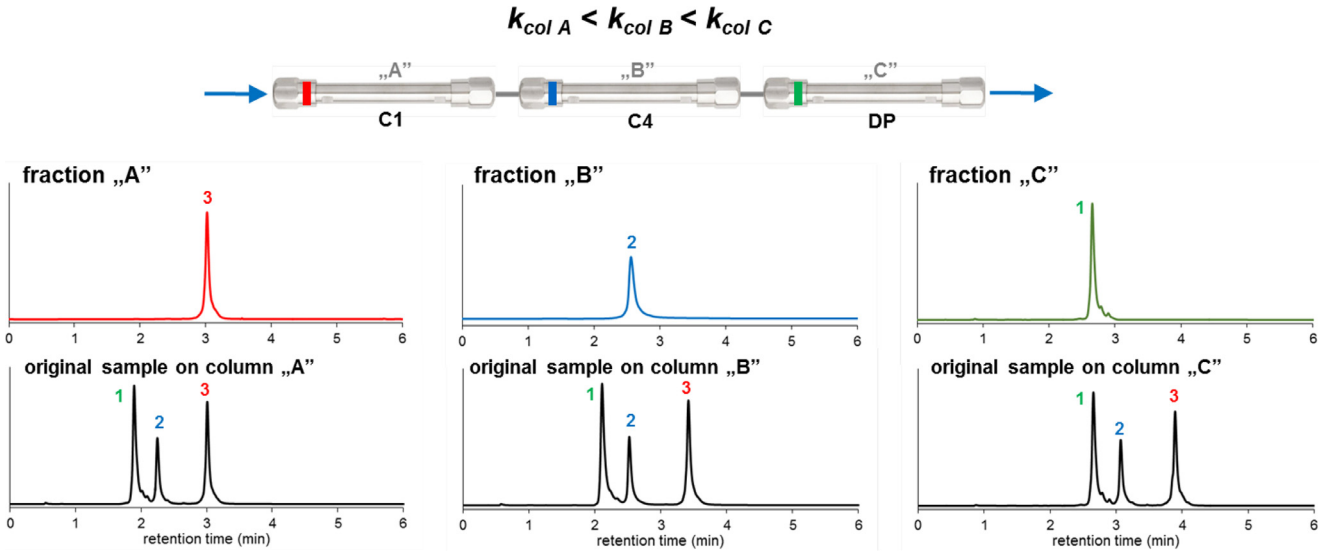



**Fig. 6.** A three-column system to fractionate intact mAbs (rituximab (1), ramucirumab (2) and obinutuzumab (3)). The chromatograms show the elution profile of the mAbs from a C1 (A), a C4 (B) and a diphenyl (DP) (C) columns.

### 3.5. Online, on-column fractioning on a three-column system

It is easy to imagine that three protein species can be fractionated on a three-column system if the following criteria are fulfilled: At a given isocratic composition, the first column retains only the third protein (most retained solute) (1), the second column retains both the second and third proteins (2), the third column retains all the three proteins (3) and the elution order of the compounds is maintained on all columns (4). The condition which fulfill the criteria can be easily found on log $k$–$\varphi$ plots, but also on the basis of overlaid chromatograms measured on the individual columns. Fig. 5 illustrates the selection of appropriate mobile phase composition to fractionate three solutes (intact mAb mixture) in a three-column system. (The parameters of LSS models are listed in Table 1.)

The three columns were then coupled in their order of increasing retentivity (C1 → C4 → DP) and 34.5% B was set as isocratic mobile phase composition. The run time was set to the sum of column dead times, $t_{run} = t_0$ (C1) + $t_0$ (C4) + $t_0$ (DP). (This run time corresponds to about 0.65 min in the case of three 50 × 2.1 mm columns, operating at 0.5 mL/min flow rate.) With these conditions, rituximab and ramucirumab were not retained on the first column (C1) and therefore travel until the second column, while obinituzumab has high enough retention ($k = 53.6$) to be trapped at the inlet of the first column. Then, only two solutes enter the second column (C4). Among them, rituximab was not retained, but ramucirumab was trapped ($k = 12.8$) on the second column segment. Thus, only rituximab will reach the third column (DP) and its retention is high enough ($k = 11.2$) to be parked at the column inlet.

After the isocratic run, the flow was stopped and the columns were disconnected. A generic 6 min long gradient (30 – 45% B) was performed on the three individual columns (without any additional injection). This generic gradient eluted the fractioned intact mAbs from the individual columns. Then, the original mAb mixture was also injected and the chromatographic profiles were compared. Fig. 6 confirms that the three intact mAbs were indeed

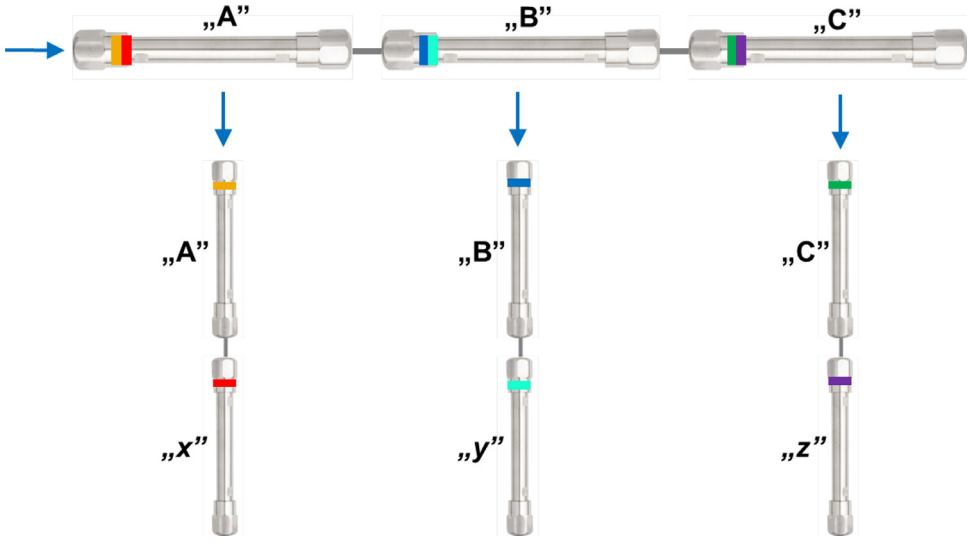



**Fig. 7.** Schematic view of a multiple on-line fractioning system for complex samples.

collected on the individual column segments. On the first column (fraction "A"), only obinutuzumab was eluted, while ramuricumab was eluted from the second column (fraction "B") and rituximab was eluted from the last column (fraction "C").

### 3.6. Multiple on-line fractioning

Obviously, there are situations where the criteria mentioned in the previous section cannot be fulfilled for the following reasons: there is not enough difference between solute retentions (1), not enough difference between column retentivity (2) or more peaks should be fractionated than the number of available column segments (complex sample) (3). Currently some complex mixtures of recombinant therapeutic antibodies combine several mAb products (2–25) with the desired specificities into a single drug product. Such mixtures could require the use of more complex setup. A possible way to handle this issue is to first perform a so-called pre-fractioning on few number of selected columns, and then the required pre-fractions can be further fractionated by coupling the selected column segment to other columns. Fig. 7 illustrates the schematic view of a multiple on-line fractioning system in the case of six mAb species and $3 \times 2$ column segments. In this example, a three-column pre-fractioning (columns A, B and C) was assumed for six compounds. During the pre-fractioning, the solutes were trapped as three pairs on the three segments. If we have additional columns (x, y and z) which show higher selectivity for the critical peak pairs than the pre-fractioning columns, then those can be combined (coupled) with the pre-fractioning column segments and the critical peak pairs can be separated and trapped on individual columns.

## 4. Conclusion

We suggest a column-coupling approach, which can be a powerful tool to improve both the selectivity and efficiency of protein separations (RPLC) compared to a single column separation. When combining this approach with the recently proposed multi-isocratic elution mode, the elution space between peaks can be further improved. In addition, if a solute is trapped at the inlet of a later column segment, its band will be refocused and will elute in unprecedented sharp peak. Furthermore, this novel approach makes possible to perform online on-column fractioning of protein species within a very short analysis time and without sample dilution. Then, the peaks of interest can be eluted with any gradient program from the selected column segment without time limitation.

The methodology is based on the coupling of the columns in the order of their increasing retentivity. The columns however should provide similar selectivity. (Please note the difference compared to SOSLC or POPLC where the selectivity is tuned by the combination of different stationary phases, therefore – in contrast to our approach - in POPLC there is a need for different column selectivities.) In most cases similarity in column selectivity is not a problem, as large therapeutic protein samples almost always show a very similar chromatographic profile (relative retention, selectivity) on various RP stationary phases, except that their absolute retention might be different on the different columns. In other words, in practice, wide-pore RP columns applied for protein separations, show very similar selectivity but different retentivity. Thus, one can select two or three columns which show sufficient difference in retentivity (eg. alkyl and phenyl modified phases).

We also demonstrated here, that methyl (C1) modified RP phase has a great potential for recent protein separations and provides significantly lower retentivity than commercial C4, C18 or phenyl phases commonly used for modern protein separations. Therefore this C1 phase can be especially interesting for column coupling purposes. In addition, as it complements the retentivity scale of currently available state-of-the-art wide pore superficially porous materials (C4, C8, C18 and DP), the C1 phase can be a good candidate in method screening too.

The on-line on-column fractioning approach could also potentially improve the efficacy of preparative scale protein separations.

Please note that it is a proof of concept study and its applicability will be further studied for more difficult and more substantial cases such as the separation of antibody isoforms from each other which is a major separation challenge today. In addition to reversed phase chromatography, ion-exchange chromatography will also be studied to improve the separation of protein charge variants.

## Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## CRediT authorship contribution statement

**Szabolcs Fekete:** Writing - original draft, Methodology. **Harald Ritchie:** Writing - review & editing. **Jason Lawhorn:** Conceptualization. **Jean-Luc Veuthey:** Supervision, Resources, Project administration, Writing - review & editing. **Davy Guillarme:** Supervision, Methodology, Writing - review & editing.

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