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Negative gradient slope methods to improve the separation of closely eluting proteins

Szabolcs Fekete[a,b,*], Amarande Murisier[a,b], Jennifer M. Nguyen[c], Matthew A. Lauber[c], Davy Guillarme[a,b]

[a] School of Pharmaceutical Sciences, University of Geneva, CMU-Rue Michel Servet 1, 1211 Geneva 4, Switzerland
[b] Institute of Pharmaceutical Sciences of Western Switzerland, University of Geneva, CMU-Rue Michel Servet 1, 1211 Geneva 4, Switzerland
[c] Waters Corporation, 34 Maple Street, Milford, MA 01757-3696, United States

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ABSTRACT

In the present work, we describe the fundamental and practical advantages of a new strategy to improve the resolution of very closely eluting peaks within therapeutic protein samples.

This approach involves the use of multiple isocratic steps, together with the addition of a steep negative gradient segment (with a decrease in mobile phase strength) to "park" a slightly more retained peak somewhere along the column (at a given migration distance), while a slightly less retained compound can be eluted.

First, some model calculations were performed to highlight the potential of this innovative approach. For this purpose, the retention parameters ($\log k_0$ and S) for two case studies were considered, namely the analysis of a mixture of two therapeutic mAbs (simple to resolve sample) and separation of a therapeutic mAb from its main variant (challenging to resolve sample). The results confirm that the insertion of a negative segment into a multi-isocratic elution program can be a good tool to improve selectivity between critical peak pairs. However, it is also important to keep in mind that this approach only works with large solutes, which more or less follow an “on-off” type elution behavior.

Two real applications were successfully developed to illustrate the practical advantage of this new approach, including the separation of a therapeutic mAb from its main variant possessing very close elution behavior, and the separation of a carrier protein from an intact mAb as might be encountered in a quantitative bioanalysis assay. These two examples demonstrate that improved selectivity can be achieved for protein RPLC through the inclusion of a negative gradient slope that selectively bifurcates the elution of two or more peaks of interest.

1. Introduction

Liquid chromatographic separations of proteins are performed in gradient elution mode. In general, simple linear gradients are performed since they are easy to generate and control, and consequently those methods can be easily transferred [1]. However, a simple linear gradient is often unable to provide sufficient chromatographic resolution. Therefore, segmented gradients can be applied to improve separation quality [2]. Two-segment (“bi-linear”) gradients are often used to shorten the analysis time when a separation includes a few well-resolved late-eluting peaks. Then, a steeper gradient segment can be set for the late eluting peaks [1]. For more complex samples, multiple gradient segments can be combined to attain suitable separation. In order to facilitate the elution of the peaks, it is common knowledge that gradient slopes should always be positive; however occasionally, one or more isocratic steps can be inserted to obtain the most optimal separation [2].

Besides multi-linear gradients, non-linear gradients might also provide some benefits. Power function based gradients have been successfully applied for therapeutic protein separations for both reversed phase (RPLC) and ion-exchange (IEX) chromatography [3,4]. Another type of non-linear gradient can also be useful when the

* Corresponding author.
E-mail address: szabolcs.fekete@unige.ch (S. Fekete).

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compounds of interest belong to a series of increasingly more retained analytes (e.g. members of homologues series). In this case, a logarithmic shape gradient profile provides the best overall selectivity [5]. Customized non-linear gradients (including both concave and convex segments) were also developed by Kall *et al.* to separate complex peptide mixtures and were successfully applied in shotgun proteomics [6]. Some other complex gradient profiles were also applied based on considering either the elution of only the first and last eluting peaks [7] or the elution of each individual peak [8].

For a preparative scale separation (purification) of peptides and proteins, step or step-wise gradients have been extensively used in flash chromatography and counter-current chromatography [9,10]. The idea behind such step gradients is that only one or a very few number of components have to be separated, while the other sample components are just washed out from the column.

Timperman and co-workers demonstrated that a "saw-tooth" gradient program allows small subsets of proteins to be eluted from the column intermittently by using short gradient steps separated by a negative slope and isocratic holding segments. The isocratic holding periods can be used to perform additional sample processing (e.g. online fractioning for a second dimension analysis) [11]. The saw-tooth gradient was found superior to a common segmented linear gradient/isocratic mode, since the negative steps prevents band broadening that takes place during isocratic elution steps. This saw-tooth gradient was set to achieve complete sample transfer between the first- and second-dimension for protein and peptide identification [12]. Saw-tooth gradients are also applied for polymer separations [13].

Armstrong *et al.* discussed the possibility to run a simple negative linear gradient in RPLC for protein separations [14]. The authors referred to their separation as "non-traditional reverse gradient". Unusual convex log$k$–φ plots with global minima were reported for ribonuclease, insulin and myoglobin, and the authors explained that those observations were not in agreement with previously reported results. Nevertheless, it was pointed out that if there is a minima on a log$k$–φ plot, then retention and elution can be attained either with positive or negative gradients. The authors also explained that such unusual behavior was probably related to solubility-based phenomenon. Despite that the conditions used in the study were not ideal (narrow pores of 60 Å–RPLC phase, 1% TFA as mobile phase additive and ambient temperature), the three proteins were successfully separated with a linear reverse gradient.

Recently, an innovative strategy termed as "multi-isocratic" elution has been shown to provide exponential increase in selectivity between protein variants. It was demonstrated that the combination of multi-isocratic steps and very short, yet steep gradient segments (with steepness close to infinity) at solute elution allows one to set the selectivity as desired while maintaining sharp peaks due to significant band compression effects [15]. This method was successfully applied for the analytical scale separation of intact and subunit digested samples of monoclonal antibodies (mAbs) as well as antibody-drug conjugates (ADCs). Uniform peak distribution (equidistant band spacing) and much higher resolution could be achieved than with common linear, multilinear, or nonlinear gradients. In a following study, this approach was combined with column coupling to further improve separation power. In such a setup, if a protein peak is trapped at the inlet of a later column segment - of a serially coupled system - then its band is refocused and it elutes as an unprecedented sharp peak [16]. Furthermore, it became possible to perform online on-column fractioning of protein species within a very short analysis time (~1 min) and without sample dilution. Similar idea has already been reported to sharpen peaks utilizing post-column refocusing and remobilization on trapping columns [17,18,19].

In the present work, the goal was to further improve the resolution of RPLC separations of very closely eluting peaks of therapeutic proteins. Therefore, the recently developed "multi-isocratic" elution mode was upgraded by adding a steep negative gradient segment between the "eluting" and "non-eluting" isocratic segments. As a result of this negative slope segment, it becomes possible to "park" a slightly more retained peak along the column bed (at a given migration distance) with a condition that still allows the slightly less retained compound to be eluted. Therefore, this approach has the potential to resolve compounds possessing very similar retention properties, which are difficult to separate, even with the multi-isocratic technique. Here, we present some theoretical considerations and illustrate the capabilities of this approach for large solutes. The expected benefit of inserting negative gradient steps (short segments) into a "multi-isocratic" program is also discussed. Two applications were developed to show the practical advantage of this new approach. These two separations were not feasible by applying the multi-isocratic elution mode (let alone linear or multi-linear gradients).

2. Materials and methods

2.1. Equipment and software

Chromatographic experiments were performed on a Waters Acquity UPLC I-Class system equipped with a binary solvent delivery pump, an autosampler, a fluorescence (FL) detector and a flow through needle injection system with 15 µL needle and a 2 µL FL flow-cell. The overall extra-column volume was about 8.5 µL as measured from the injection seat of the auto-sampler to the detector cell. The dwell volume was measured as V_d = 0.09 mL. Data acquisition and instrument control were performed by Empower Pro 2 software (Waters). Calculation and data processing were done by using Drylab (4.2) and Excel (Microsoft) software.

2.2. Chemicals and columns

LC-MS grade acetonitrile (ACN) and LC-MS grade water were purchased from Fisher Scientific (Reinach, Switzerland). ULC/MS grade formic acid (FA) and ULC/MS grade trifluoroacetic acid (TFA) were purchased from Biosolve (Dieuze, France).

Intact mAb Mass Check Standard (murine anticitrinin IgG1) was obtained from Waters. Bovine Serum Albumin (BSA) was purchased from Sigma-Aldrich (Buchs, Switzerland). Therapeutic mAb (eculizumab) was obtained as European Union pharmaceutical-grade drug product from its respective manufacturer.

Prototype columns (15 × 2.1 mm) packed with 3 µm 2000 Å polystyrene divinylbenzene (PS-DVB) particles as well as a commercial BioResolve RP mAb Polyphenyl column (50 × 2.1 mm, 2.7 µm, 450 Å) were provided by Waters (Milford, USA). To prepare the prototype PS-DVB column, a specialized guard column was constructed with a low clearance endnut and a low dispersion coupler to give a standard female inlet/female outlet configuration.

2.3. Sample and mobile phase preparation

Intact eculizumab was diluted to 1 mg/mL with water and injected without further preparation. Waters Intact mAb Mass Check Standard was diluted to 0.025 mg/mL with water and mixed with BSA diluted to 0.25 mg/mL with water.

For the separation of intact eculizumab variants, the mobile phase A was 0.1% TFA (v/v) in water and B was 0.1% TFA (v/v) in ACN.

For the separation of anti-citrinin mAb (Waters Intact mAb Mass Check Standard) and carrier protein (BSA), the mobile phase A was 0.1% FA (v/v) in water and B was 0.1% FA (v/v) in ACN.

2.4. Chromatographic conditions

To improve the selectivity of closely eluting proteins, a linear gradient separation was compared to separations achieved with a multi-isocratic elution technique that either contained or did not contain a negative gradient segment ("negative segmented multi-isocratic elution"). First, the parameters of the linear solvent strength (LSS) model were determined from two linear gradients.

For eculizumab, the flow rate was set at 0.5 mL/min, column temperature was set at 85°C, and 0.5 µL of intact eculizumab sample was injected. For the initial linear gradient experiments, the gradient times were set as $t_{G1} = 4$ and $t_{G2} = 10$ minutes and a 25–50%B gradient was run.

For the separation of the anti-citrinin mAb and carrier protein (BSA), the flow rate was set at 0.4 mL/min and the column temperature was set at 80°C. Sample injection volume was 0.1 µL. For the initial linear gradient experiments, the gradient times were set as $t_{G1} = 4$ and $t_{G2} = 10$ minutes and a 20–50%B gradient was run.

For all measurements, data were acquired at 280 nm excitation and 350 nm emission wavelengths (FL).

The optimized conditions for multi-isocratic and "negative segmented" programs are detailed in the *Results and Discussion* section.

2.5. Calculations

In LC, the LSS model - sometimes called exponential model - is commonly used to describe the relationship between solute retention (k) and mobile phase composition (φ) [20]:

$$\log k = \log k_0 - S\varphi \tag{1}$$

where k is the solute retention factor, φ is the volume fraction of mobile phase "B" (stronger eluent), S is a constant for a given solute (it describes how sensitive is the solute retention to mobile phase composition) and k_0 is the (extrapolated) value of k for $\varphi = 0$ (i.e., the retention factor observed in pure mobile phase "A").

The migration velocity (u) of a solute along the column (measured at a given φ) depends on the interstitial mobile phase velocity (u_0) and retention factor:

$$u = \frac{u_0}{1 + \mathrm{k}} \tag{2}$$

Expressing k from Eq. (1) and substituting to Eq. (2) enables one to describe the relative solute migration speed (u_{rel}) as:

$$u_{rel} = \frac{u}{u_0} = \frac{1}{1 + k_0 10^{(-S\varphi)}} \tag{3}$$

The time spent to reach position z can be expressed as [21]:

$$t(z) = t_0\left[\frac{z}{\mathrm{L}} + \frac{1}{b}\log\left(1 + k_{in} b \frac{z}{L}\right)\right] \tag{4}$$

Where k_{in} is the retention factor at the starting mobile phase composition (inlet retention factor), b is the gradient steepness ($b = S \cdot \Delta\varphi \cdot \frac{t_0}{t_G}$), t_0 is the column dead time, L is the column length and z is the solute position along the column. Then, the time to travel the entire column ($z = L$) corresponds to the retention time (t_r) and can be written as:

$$t_r = t(L) = t_0\left[1 + \frac{1}{b}\log(1 + k_{in} b)\right] \tag{5}$$

Please note that Eqs. (4) and (5) assume linear gradient. For our calculations, the parameters $\log k_0$ and S were derived from experimentally measured retention times data of two preliminary linear gradient experiments–performed with different gradient times (t_G) (corresponding to different gradient steepness) - using DryLab 4 software. (Please note that, deviations from the LSS model might be observed, especially when working in a broad Δ%B range–e.g. $\Delta\varphi > 0.5$, then other non-linear models can be used - e.g. Neue-Kuss or quadratic models - instead of the LSS model [22]. In our practice, for peptides and proteins, less than 5% deviation is observed in reversed phase chromatography. However here in this study, very narrow $\Delta\varphi$ ranges were set (< 0.1) and LSS models were found to be appropriate.)

2.5.1. Studying the evolution of selectivity

Then, from $\log k_0$ and S, the retention factor (k) was estimated for a given mobile phase composition (φ) for any set of %B program (e.g. linear-, multi-isocratic or negative segmented multi-isocratic program). The solute relative velocity and the travelled distance can be calculated for any time point of a given %B program. To illustrate solute migration and study selectivity, plots of (1) B fraction vs t_G, (2) u/u_0 vs z and (3) distance travelled vs t were constructed. Simulated chromatograms were also plotted assuming the common gradient band compression factor; $G = \frac{\sqrt{1+p+p^{2/3}}}{1+p}$, with $p = 2.3b$ [23]. Please note that this factor G is only valid for linear gradient. For our calculations for multi-isocratic separations, we assumed consecutive linear gradient and isocratic segments.

2.5.2. Optimization of multi-isocratic and negative segmented separations

It is known that the retention of large solutes such as therapeutic proteins is very sensitive to the mobile phase composition. A minor change in the mobile phase composition can indeed drastically affect their retention (very high S value in the LSS model). Snyder explained this phenomenon by the fact that large solutes are either fully captured at the column inlet or completely released from the column [20,24,25]. This behavior is today often termed as an "on-off" or "bind-and-elute" mechanism. Very recently, it was indeed shown that the retention of large proteins can only be controlled in a very narrow mobile phase composition range (e.g. with gradients applying only 3.5–5 %ΔB for intact mAbs) [15,16]. Their relative migration speed varies within the $0 < u_{rel} < 1$ range only in this very narrow %B window, otherwise it is either 0 or 1 (corresponding to "on"–fully captured–state or to "off"–released–state). Therefore, the mobile phase composition required to start the migration of a large molecule ($\varphi_{(urel=0.01)}$; when the solute starts traveling with only 1% of the mobile phase velocity, $u/u_0 = 0.01$) can be estimated as:

$$\varphi_{(u_{rel}=0.01)} = -\frac{\log\left[\frac{1}{\frac{0.01}{k_0}} - 1\right]}{S} \tag{6}$$

Similarly, the mobile phase composition to reach the "off" state ($\varphi_{(urel=0.99)}$; unbound state with $u/u_0 = 0.99$) can be written as:

$$\varphi_{(u_{rel}=0.99)} = -\frac{\log\left[\frac{1}{\frac{0.99}{k_0}} - 1\right]}{S} \tag{7}$$

On the other hand, eluting mobile phase composition with very low retention factor ($\varphi_{k<0.1}$) and binding mobile phase composition with high retention factor ($\varphi_{k>100}$) for a multi-isocratic elution separation can be estimated as [15]:

$$\varphi_{k<0.1} > \frac{\log k_0 + 1}{S} \tag{8}$$

$$\varphi_{k>100} < \frac{\log k_0 - 2}{S} \tag{9}$$

The $\varphi_{k<0.1}$ and $\varphi_{k>100}$ can be good starting points for the optimization of a multi-isocratic protein separation. However, in practice, there is sometimes only a minor difference between the model parameters of closely related proteins (e.g. variants of intact mAbs) and thus it is hard to predict whether they can be separated

or not. For such a situation, we found that performing a "screening multi-isocratic gradient" can be very helpful. To realize such, a 5-segmented multi-isocratic condition was set (please note that any number of segments can be set). The mobile phase composition for the initial step (φ_{in}) was set to be retentive enough (eg. $\varphi_{k>100}$), while the composition of the last segment (φ_{last}) was set to be able to elute all compounds (eg. $\varphi_{k<0.1}$). Then, five equidistant segments (2 minute long intervals) were set between the initial and final compositions. The difference ($\Delta\%B_{segment}$) between the mobile phase compositions of the consecutive segments for the case of n isocratic segments can be determined as:

$$\Delta\%B_{segment} = \frac{\varphi_{\text{last}} - \varphi_{\text{in}}}{n-1} \quad (10)$$

After performing the first screening run, one can fine-tune the number of steps and the mobile phase composition of the isocratic steps to improve the separation by performing a so-called "stretched" multi-isocratic run. (Supplementary fig. 1 shows a schematic view of the optimization procedure, including (1) a preliminary linear gradient run, (2) a "screening" multi-isocratic run and (3) a "stretched" multi-isocratic run). As a generic suggestion, for intact mAbs, an effective screening run may consider a 5% B difference between φ_{last} and φ_{in} (which is due to the high S value, typically ranging between 90 and 150 under RPLC conditions).

4. Results and discussion

4.1. Model calculations - potential of inserting a negative gradient step

It was recently shown that a so-called multi-isocratic elution technique could produce uniform peak distribution (equidistant band spacing) for a separation of protein species (assuming they obey an on-off type elution mechanism) [15]. Ideally, the elution distance between peaks can be adjusted arbitrarily by changing the length of the holding isocratic segments. However, this is only feasible if just one of the peaks of interest starts migrating within a given elution gradient–or isocratic hold - segment. In practice, it may happen that not only one, but two or more compounds start to migrate along the column at a given segment (because of a lack of selectivity, their retention behavior and thus model parameters are very similar). Such cases represent the limits of the multi-isocratic approach. In case of co-migration (even if the solutes migration speed is slightly different), the selectivity cannot be increased anymore without boundaries [15]. Accordingly, we were motivated to find a better solution to resolve such closely eluting protein compounds. The idea was to park ("freeze") a migrating - more retained - compound somewhere along the column, while letting a less retained compound complete its elution through the column. For this, we explored the use of a negative gradient segment along with a "holding" isocratic segment immediately after the elution of the less retained compound.

First, some model calculations were performed to highlight the potential of a multi-isocratic separation and the effect of inserting a negative gradient segment into a multi-isocratic elution program. Two sets of compounds were studied, namely a "simple to resolve sample" and a "challenging to resolve sample". For the simple-sample, a mixture of intact rituximab and ramucirumab was considered, since there is enough difference between their retention to separate them either with a linear gradient or multi-isocratic elution technique. For the challenging-sample, intact atezolizumab and its main variant were chosen since this sample already faced the limits of the multi-isocratic elution mode in a former study [15]. (The parameters of retention models used for these calculations were taken from our previous studies [15,16].)

Fig. 1 A-D shows the evolution of solute migration, the travelled distance along a column and the calculated chromatograms for the simple-sample when performing linear gradient and multi-isocratic separations. Based on Fig. 1 B and 1C, it is clear that once the two compounds start their migration (switching to "off" mode), their relative migration speed and acceleration are nearly the same along the entire column. However, peak 1 starts migrating ($\varphi_{(urel=0.01)} = 0.335$) far earlier than peak 2 does ($\varphi_{(urel=0.01)} = 0.366$). With a 10 min long linear gradient (32 to 42 %B), peak 1 starts migrating after a parking time (t_{park}) of 1.5 min, while peak 2 parks at the column inlet until 4.6 min. After their release from the head of the column, they travel through the chromatographic bed within nearly the same amount of time ($t_{trav} = t_r - t_{park}$, gives 2.67 and 2.52 min, respectively). Therefore, the selectivity is mostly determined by the difference of their parking times ($\Delta t_{park} = +3.1$ min) and not by their travelling time ($\Delta t_{trav} = -0.15$ min). Due to the large difference between the parking times, the separation of those peaks is easy to achieve with a linear gradient. Figs. 1 E-H show the case of a multi-isocratic separation. Setting 28%B for the starting isocratic binding segment resulted in very high initial retention for both compounds ($k_1 = 2.03{*}10^7$ and $k_2 = 1.11{*}10^{11}$). Over a 2 min isocratic segment, the two peaks practically do not move from the head of the column. Changing to 36.8%B mobile phase composition resulted in the immediate elution of the less retained peak ($k_1 = 0.07$). Meanwhile, peak 2 remained to be strongly retained ($k_2 = 61.15$), albeit with an indication of some very slow migration ($u_{rel} = 0.016$). Holding this second isocratic segment for 4 min (6 min of total run time) resulted in $z = 0.9$ cm travelled distance for peak 2 (Figs. 1 F and G, red curve). Subsequently setting the mobile phase composition to 45%B resulted in the immediate elution of peak 2 ($k_2 = 1.44{*}10^{-7}$). In conclusion, due to the large difference of retention between the two mAbs (determined by $\log k_0$ values), either linear gradient or multi-isocratic separations are easy to implement. In the end, the latter technique has the advantage to drastically improve selectivity. With the conditions set in this example (36.8%B for the second isocratic segment), the elution distance (selectivity) between the two peaks can be increased up to ~40 min. (With 36.8%B, it takes about 44 min for peak 2 - migrating with $u_{rel} = 0.016$ - to travel the entire column length of 10 cm.)

Figs. 2 A-D illustrate the challenge to separate intact atezolizumab and its main hydrophobic variant by applying a linear gradient. Peak 1 starts migrating at $\varphi_{(urel=0.01)} = 0.335$ (33.5%B), while peak 2 begins travelling at $\varphi_{(urel=0.01)} = 0.338$ (33.8%B). By setting a 10 min long linear gradient (32 to 42 %B), peaks 1 and 2 will start migrating after $t_{park} = 1.50$ and 1.76 min, respectively. Following their release from the column inlet, their travelling times are also nearly the same ($t_{trav} = 2.66$ and 2.58 min, respectively). Since both their parking times ($\Delta t_{park} = +0.26$ min) and travelling times ($\Delta t_{trav} = -0.08$ min) are almost identical, it is hardly possible to afford selectivity through the application of linear gradients. However, by running a multi-isocratic program, the selectivity can be slightly increased (Figs. 2 E-H). By setting 33%B as initial isocratic segment, both compounds were found to be highly retained ($k_1 = 312$ and $k_2 = 590$). After two minutes of holding time and a switch to 34.5% B, both compounds started migrating with $u_{rel} = 0.09$ and 0.05 (Fig. 2 F). At the end of the second segment, peak 1 traveled $z = 2.5$ cm while peak 2 traveled $z = 1.6$ cm (Fig. 2 G). Then, during the third segment (35.3%B hold for 2 min), peak 1 accelerated and left the column, while peak 2 approached $z = 9.1$ cm. Finally, the last segment (35.9%B) quickly eluted peak 2 from the column. It is important to notice that once peak 1 started migrating (switches to "off" mode) so too did peak 2, albeit with a slightly lower velocity. Since the difference between their migration speed is limited (a factor of 1.2–1.6 difference can be real-

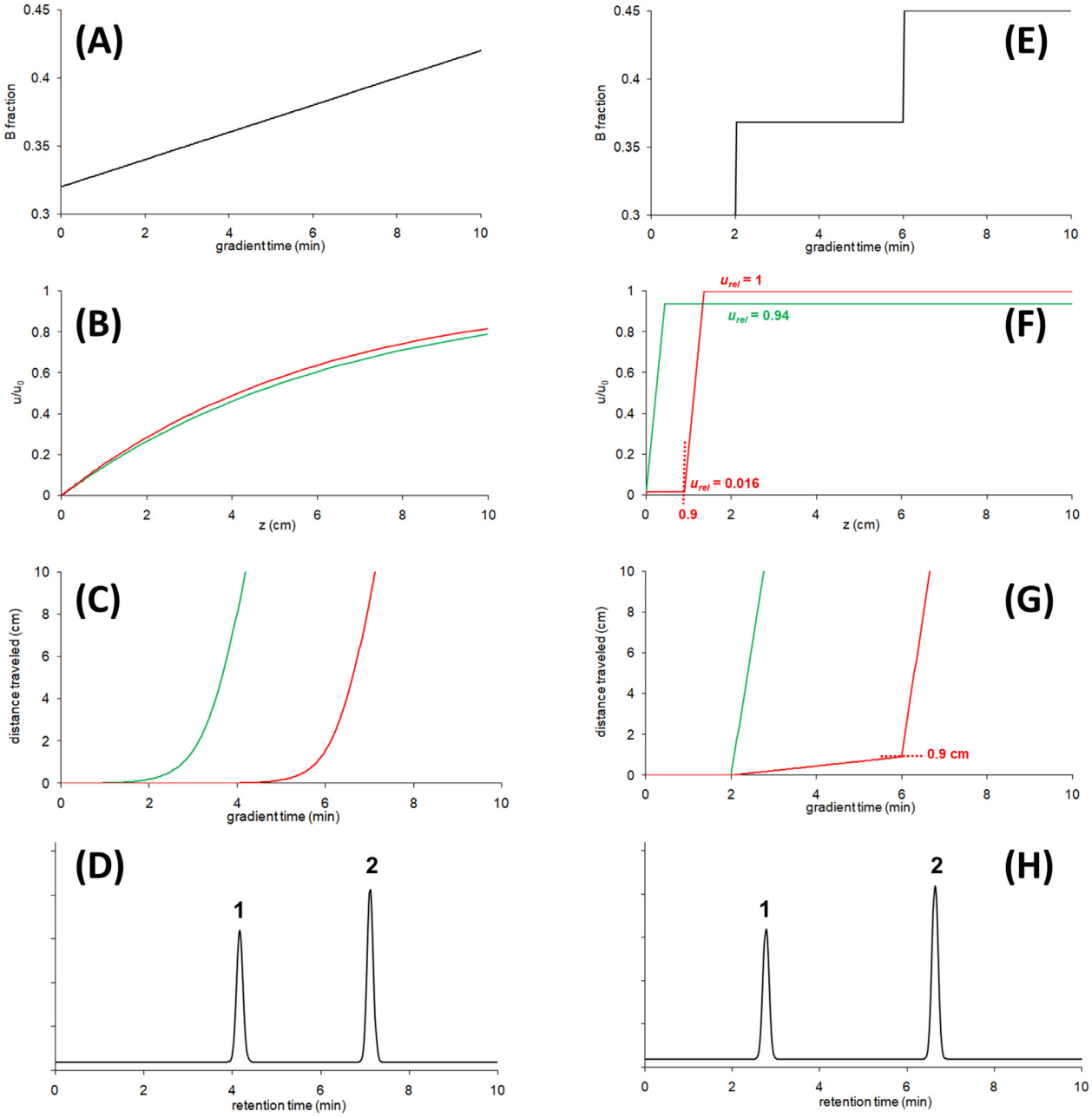

Fig. 1. Evolution of selectivity ("simple-sample") with linear gradient 32–42 %B in 10 min (A,B,C,D), and with multi-isocratic (E,F,G,H) elution. The %B program for the multi-isocratic run was 28%B (0–2 min), 36.8%B (2.01–6 min) and 45%B (6.01–10 min). F = 0.3 mL/min, 100 × 2.1 mm column, $\varepsilon = 0.62$, rituximab peak (1): $S = 96.4$, log $k_0 = 36.3$, ramucirumab peak (2): $S = 105.2$, log $k_0 = 40.5$.

ized), it was not possible to find conditions that simultaneously yielded high velocity for one compound ("off" mode) and low velocity for the other (close to "on" mode). In contrast, for the case of the simple-sample, a factor of 58 was obtained between the migration speeds of the two solutes during the second segment of the multi-isocratic program, as shown on Fig. 1 F.

The challenging-sample can be used to illustrate the limitations of the multi-isocratic elution technique and the beneficial effects of inserting a negative gradient segment. Fig. 2 G shows an example wherein a short, negative and steep gradient segment was inserted just as peak 1 left the column ($t_G = 5.5$ min, grey dashed line in Fig. 2 G). With this, the migration of the more retained compound was stopped at the position to which it had traveled up until that point in time($z = 7.2$ cm, the crossing point of the grey dashed line and red curve on Fig. 2 G). Fig. 3 illustrates the separation for the case when at 5.5 min, the mobile phase composition was set back from 35.3 to 33%B and held until 8 min (to give a 2.5 min holding (parking) time). As suggested by Figs. 3 B and 3 C, peak 2 did not move to any appreciable extent during this negative step. Upon setting a stronger mobile phase composition (e.g. 38%B) peak 2 was made to immediately elute ($k = 5.2^{*}10^{-3}$). Based on these model calculations, it is proposed that a negative segment (decrease of mobile phase strength) can be inserted into a multi-isocratic elution program to improve selectivity between critical peak pairs found in large biopharmaceutical drug products.

To the best of our knowledge, this is the first time that such a combination of positive and negative gradients (and isocratic holding) segments are combined and applied.

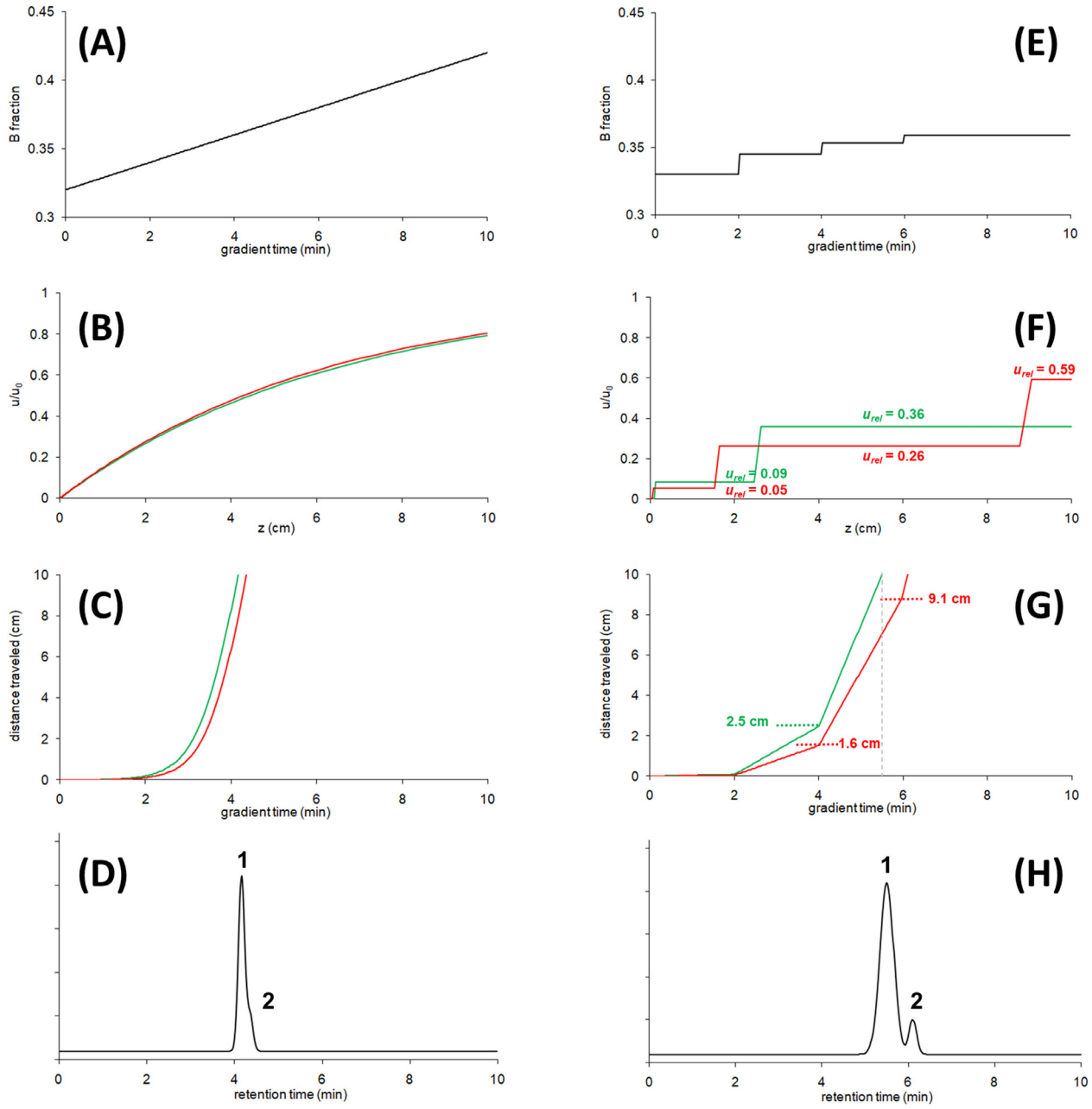

Fig. 2. Evolution of selectivity ("challenging-sample") with linear gradient 32–42 %B in 10 min (A,B,C,D), and with multi-isocratic (E,F,G,H) elution. The %B program for the multi-isocratic run was 33%B (0–2 min), 34.5%B (2.01–4 min), 35.3%B (4.01–6 min) and 35.9%B (6.01–10 min). F = 0.3 mL/min, 100 × 2.1 mm column, $\varepsilon = 0.62$, main peak (1, atezolizumab): $S = 97.53$, log $k_0 = 34.68$, minor peak (2): $S = 101.2$, log $k_0 = 36.17$.

4.2. Application to the separation of intact mAb variants

One of the most challenging tasks in the field of therapeutic protein analysis is the RPLC separation of protein variants at the intact level. Hence, we tried our new approach for such challenging sample. Eculizumab (humanized therapeutic mAb product) has been selected as an example, since it contains hydrophobic variants. We have already made several attempts to separate the two main variants of this mAb by linear gradient separations but have always failed. Our new method development approach (see description in Section 2.5.2.) has been applied to define the optimal conditions for a multi-isocratic or negative step inserted multi-isocratic separation. LSS parameters were derived from two initial linear gradients and then a screening multi-isocratic run was performed to estimate the binding (parking) and eluting compositions (Supplementary Figure 2). It was found that 33.8%B mobile phase provided sufficiently high retention for both compounds to be parked at the column inlet ($k_1 = 856$, $k_2 = 1062$). Then, four different %B compositions were employed as eluting compositions (37.3, 36.8, 36.3 and 35.8 %B). Whatever the composition, the two peaks co-migrated with only a minor difference between their velocities (Supplementary Figure 3, left panel). The lower the %B - during the elution step/hold - the higher the selectivity was, but sensitivity decreased drastically due to band broadening of the macromolecules during isocratic migration with $k \geq 1$. Baseline separation was therefore not feasible with a multi-isocratic elution mechanism. A mobile phase composition of 36.3%B was selected as the first eluting segment of the program - as it showed a good

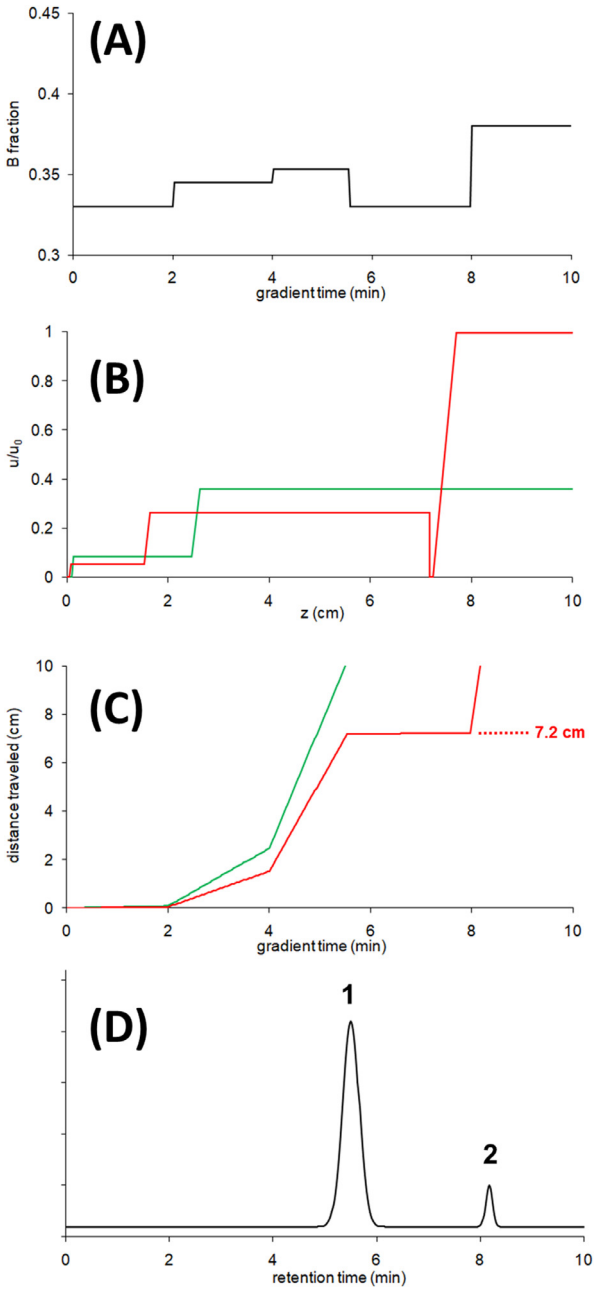

Fig. 3. Evolution of selectivity ("difficult-sample") with multi-isocratic elution, when inserting a negative ("parking") gradient step. Conditions and samples as described in Fig. 2, except the %B program was: 33%B (0–2 min), 34.5%B (2.01–4 min), 35.3%B (4.01–5.5 min), 33%B (5.51–8 min) and 38%B (8.01–10 min).

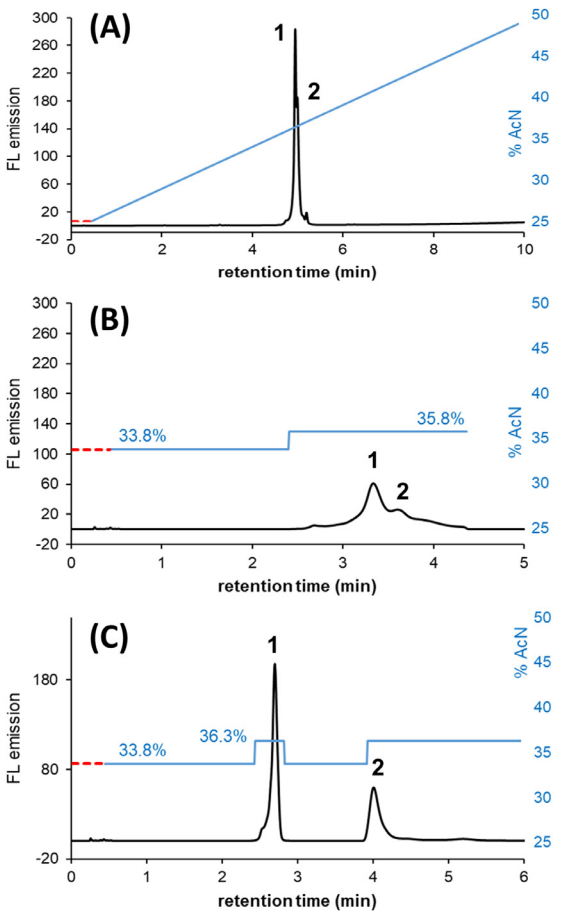

Fig. 4. Separation of intact eculizumab variants with a linear gradient (A), multi-isocratic elution mode technique (B) and multi-isocratic mode including negative gradient step technique (C). F = 0.5 mL/min, column: BioResolve RP 50 × 2.1 mm, mobile phase A: water + 0.1% TFA, mobile phase B: acetonitrile + 0.1% TFA, temperature = 85 ^{0}C, main peak (1, eculizumab): S = 129.46, log k_0 = 46.69, minor peak (2): S = 124.42, log k_0 = 45.08. The %B program for the negative step inserted multi-isocratic run (panel C) was 33.8%B (0–2 min), 36.3%B (2.01–2.4 min), 33.8%B (2.4–3.5 min) and 36.3%B (3.51–6 min). Red dashed lines correspond to the sum of column dead time and gradient delay time. The blue curves (%ACN) were corrected for the total (system + column) delay time.

compromise between selectivity and sensitivity–and then negative gradient and holding segments were added with an attempt to improve the separation. After the eluting segment, a 0.01 min long steep negative ramp - from 36.3%B to 33.8%B - was added to stop the migration of the second peak and this composition (33.8%B) was held until 4 min. Then at 4.01 min, a positive step was added to reset to 36.3%B and resume elution of the more retained peak. The purpose was to elute the entire peak of the less retained compound during the first eluting step while parking the more retained compound at a given migration distance (negative step), and then finally to elute the parked compound by returning to the elution condition (last positive step). To realize this, the length of the first eluting isocratic step (36.3%B) has to be optimized. Various holding times were tried ranging from 0.25 to 0.5 min (Supplementary Figure 3, middle panel). In the case of holding times that were too short, peak 1 was not completely eluted, while in case of a too long holding time, a fraction of peak 2 eluted together with peak 1. At the end, 0.4 min was found to be the optimal holding time since the entire peak of the first compound was eluted without allowing through a fraction of the second compound. For one last step of optimization, the selectivity between peaks 1 and 2 was changed by adjusting the length of the negative isocratic parking segment (Supplementary Figure 3, right panel). Ultimately, adding a negative gradient and holding step into a multi-isocratic program enabled us to achieve arbitrary selectivity between critical peak pairs, which was not possible with a linear gradient or multi-isocratic elution technique. Fig. 4 shows the comparison of experimentally measured chromatograms obtained by performing an optimized linear gradient (A), multi-isocratic elution (B) and negative step inserted multi-isocratic elution mode (C) separation. It is worth mentioning that the shape of peak 1 is more fronted with the optimized negative step gradient compared to the linear gradient elution. The reason is probably that the pre-peak variant (minor peak eluting in the front part of the main peak 1) is better separated from peak 1 (Fig. 4 A *vs* C) since both peaks elute isocratically with very small k values. What is important to say is that

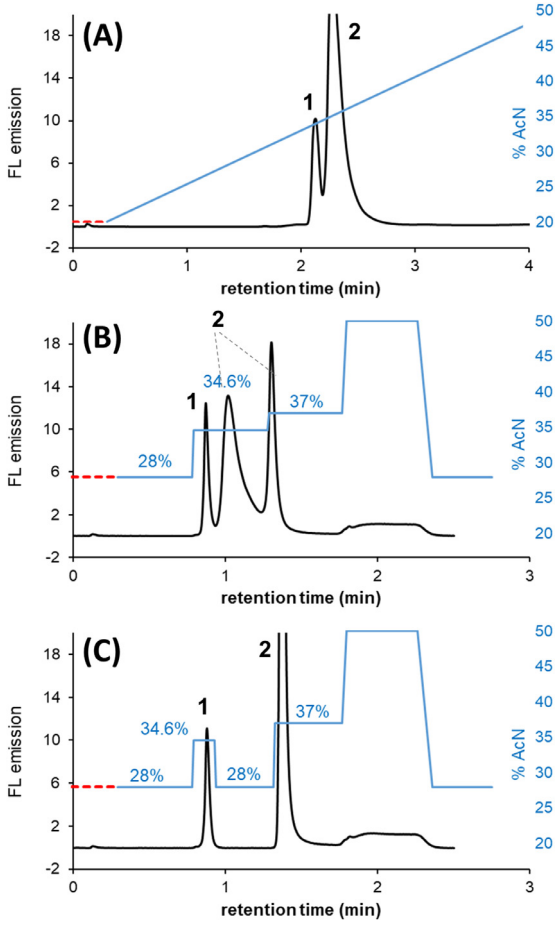

Fig. 5. Separation of anti-citrinin mAb and carrier protein (BSA) with a linear gradient (A), multi-isocratic elution mode technique (B) and with multi-isocratic mode including negative gradient step technique (C). F = 0.4 mL/min, column: PS-DVB, 15 × 2.1 mm, mobile phase A: water + 0.1% FA, mobile phase B: acetonitrile + 0.1% FA, temperature = 80 ^{0}C, peak 1 (mAb): S = 54.48, log k_0 = 18.87, peak 2 (BSA): S = 50.87, log k_0 = 18.29. The %B program for the negative step inserted multi-isocratic run (panel C) was 28%B (0–0.5 min), 34.6%B (0.51–0.65 min), 28%B (0.66–1.05 min), 37%B (1.06–1.5 min) and 50%B (1.51–2 min). Red dashed lines correspond to the sum of column dead time and gradient delay time. The blue curves (%ACN) were corrected for the total (system + column) delay time.

no signal loss is observed, the entire quantity of solute 1 elutes in peak 1–it is supported by Supplementary Figure 3.

4.3. Application to the separation of an intact mAb from a carrier protein

RPLC analysis of mAbs often suffers from the loss of recovery and, in addition, solutes may undergo some non-desired on-column aggregation or degradation [26]. To prevent intact mAbs from self-aggregation and non-specific binding, albumin (like bovine serum albumin, BSA) can be added into the sample as a so-called carrier protein. The carrier protein helps to improve protein recovery and can be especially useful when very low concentrations of proteins need to be analyzed [27,28]. However, it may happen that the separation of the mAb of interest and the carrier protein is challenging.

In the example reported in Figs. 5 A and 5 B, we could not achieve appropriate resolution between an anti-citrinin mAb and BSA peak, neither with linear gradient nor with multi-isocratic elution separation techniques. We again found that once the slightly less retained mAb starts its migration, BSA also begins to migrate. Despite the large difference between the molecular structures of the two proteins, they possess very similar retention model parameters (anti-citrinin mAb: S = 54.48, logk_0 = 18.87, BSA: S = 50.87, logk_0= 18.29) (as measured for the utilized PS-DVB stationary phase and selected mobile phase). The only chance to separate these compounds was to add a negative segment immediately after the elution of the mAb peak in order to stop the migration of the BSA, and thereby improve overall selectivity. The same optimization procedure was applied as in Section 4.2. For the initial binding step, mobile phase composition was set to 28%B (k_1 = 4.1*10^3, k_2 = 1.1*10^4) and held for 0.5 min. Switching to 34.6%B eluted both the mAb peak (k_1 ~ 0.9and BSA (k_1 ~ 3.9) with noticeably broadened peak shape. Adding another positive step (37%B) at 1 min resulted in the prompt elution of the remaining portion of BSA in a sharp (compressed) peak. This interesting behavior is portrayed in Fig. 5 B, where BSA was split into two peaks; the first fraction experienced isocratic elution, while the second fraction eluted by a very steep gradient segment (24%B/min). Finally, going back to 28%B after holding the elution segment (34.6%B) until 0.15 min (0.65 min in the elution program) made it possible to completely elute the mAb peak and to park the entirety of BSA. At the 1.05 minute mark, the mobile phase was then changed to 37%B to yield immediate elution of BSA in a single sharp peak (Fig. 5 C).

4.4. Robustness of the measurements

Since very minor changes need to be set in the %B program when running multi-isocratic and negative step inserted multi-isocratic separations, it is essential to consider the repeatability of the technique. To this end, five consecutive replicates of the eculizumab sample were injected and the same replicates were re-injected again on the next day. The relative standard deviations (RSD) of the retention times obtained for the two peaks over 2 days were lower than 0.1%. Consequently, the results suggest that the accuracy and precision of current modern UHPLC instrumentation–at least in terms of mobile phase delivery - are sufficient to perform these negative step inserted multi-isocratic separations.

In many cases, protein samples need only be separated with a change in organic modifier content of no greater than 5–10%. As a result, it might be preferred to prepare mobile phases A and B as premixed solvents (e.g. A: 70% aqueous + 30% organic solvent, and B: 50% aqueous + 50% organic solvent). The multi-isocratic separations (including those with negative slope segments) can be performed with broader absolute ranges for pump operation. This contributes to improve the repeatability of the measurements.

5. Conclusion

. Separation of therapeutic proteins by RPLC is most commonly performed by using linear gradients. However, in many cases, common linear gradients do not offer sufficient selectivity and resolving power. For this reason, a recently developed multi-isocratic elution mode should be considered to enhance the separation of challenging samples. Even still, unsatisfactory levels of resolution might be encountered in protein RPLC separations. In this study, we explored the use of a negative gradient segment along with a “holding” isocratic segment to beneficially affect critical pairs of peaks within a protein sample. With the insertion of a negative gradient, the migration of the more retained compound was stopped (“parked”) somewhere along the column, while a less retained compound was successfully eluted. Note that this approach only works for large solutes, which approach an “on-off” type elution behavior. For the separation of challenging protein samples, we suggest the combination of a so-called (1) binding isocratic segment with (2) eluting short steep gradients and holding segments along with (3) “parking” segments consisting of short steep

negative gradients and holding steps. Please note that true "on-off" mechanism does not exist, large solutes just approach this behavior. In our practice, we saw that solutes possessing molecular weights of MW > 20 - 25 kDa are close enough to a retention behavior which can benefit a lot from the multi-isocratic and the negative gradient slope methods.

The theoretical benefits of the negative segmented multi-isocratic elution mode have been demonstrated in comparison with common linear and optimized multi-isocratic separations. Two real applications have also been developed and we have proven their utility and the significance of this new separation mode.

We have also reported a fast and efficient optimization procedure to develop multi-isocratic and negative segmented multi-isocratic separations. The proposed procedure includes (1) two initial linear gradients, followed by a (2) "screening" multi-isocratic run and one (or few) (3) "stretched" multi-isocratic runs. In the end, the length of the eluting and parking isocratic segments need to be empirically determined. The total time of method development only takes a few hours.

This negative segmented multi-isocratic elution mode can potentially be applied to improve the separation of notoriously heterogeneous biopharmaceutical samples (e.g. intact mAb variants, Fc-fusion proteins, bispecific-mAb, antibody mixtures, or ADC species). Moreover, a uniform peak distribution (equidistant band spacing) can be achieved if so desired.

Declaration of Competing Interest

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, Investigation. **Amarande Murisier:** Conceptualization, Writing - original draft. **Jennifer M. Nguyen:** Writing - review & editing. **Matthew A. Lauber:** Resources, Writing - review & editing. **Davy Guillarme:** Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461743.

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