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Communication

Boosting the Liquid Chromatography Separation of Complex Bispecific Antibody Products by Using the Multi-Isocratic Elution Mode

Amarande Murisier [1,2], Valentina D'Atri [1,2], Vincent Larraillet [3], Sebastian Pirner [3] and Davy Guillarme [1,2,*]

[1] School of Pharmaceutical Sciences, University of Geneva, CMU-Rue Michel Servet 1, 1206 Geneva, Switzerland
[2] Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO), University of Geneva, CMU-Rue Michel Servet 1, 1206 Geneva, Switzerland
[3] Roche Diagnostics GmbH, Nonnenwald 2, 82377 Penzberg, Germany
* Correspondence: davy.guillarme@unige.ch; Tel.: +41-22-379-34-63

Abstract: In reversed-phase liquid chromatography (RPLC), the selectivity between major species and minor variants of protein biopharmaceutical products is always limited. Unfortunately, the stationary phase chemistry, type of mobile phase (organic modifier and salts) and temperature only have a very limited impact on selectivity. Therefore, instead of using a linear elution gradient, we evaluated a recently developed strategy, named the multi-isocratic elution mode, to improve the chromatographic resolution. In this contribution, a generic workflow involving the use of an Excel spreadsheet is provided for the rapid and successful development of multi-isocratic elution methods, without the need to use HPLC modeling software. This simple strategy was then successfully applied to very complex biopharmaceutical products; these included one reduced mAb-cytokine fusion protein and a mAb-domain-fusion (C-terminal) protein sample, containing numerous minor variants that were poorly separated from the major species. The addition of several isocratic steps during the chromatographic run provides a clear added value in terms of chromatographic selectivity for several variants, simplifying characterization of the sample with advanced MS tools. In addition to these advantages, some of the limitations of the multi-isocratic elution mode were also highlighted; these included the need to use a highly precise pumping device (preferably, a binary pumping system) and the need to prepare highly accurate mobile phases.

Keywords: multi-isocratic elution conditions; bispecific antibodies; reversed-phase liquid chromatography; better selectivity; method robustness

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1. Introduction

Bispecific antibodies (bsAbs) have become an increasingly relevant and important class of protein biopharmaceuticals [1], making up about 160 products in clinical studies [2]. With the benefit of combining several antigen recognition sites in a single unique protein format, bsAbs can be applied in innovative therapeutic applications; however, this also confers an extreme structural complexity. Indeed, the ability to recognize multiple antigen sites requires complex engineering of the structure in terms of format (fragment-based, symmetric and asymmetric) and valency (number of binding sites, generally 1 + 1, 1 + 2 or 2 + 2) that can be mixed in more than 100 different combinations of antigen-binding moieties and (homo/hetero) dimerization modules [2–4]. This makes bsAbs one of the most complex formats to characterize in the field of biopharmaceutical proteins; thus, it is one of the most suitable samples to be analyzed to test new analytical strategies.

Very recently, innovative analytical approaches have been proposed in the field of liquid chromatography to improve the selectivity between closely eluting protein species [5–8]. In this context, the so-called 'multi-isocratic elution mode' has pushed protein subunits separations achievable in reversed-phase liquid chromatography (RPLC) to the next level [5].

This elution mode is based on the principle that large solutes (proteins in this case) analyzed in RPLC follow a particular retention behavior (generally referred to as on/off mechanism), for which minor changes of mobile phase composition have a "butterfly effect" on solute retention. Indeed, the *S* value—describing how sensitive the solute retention is relative to mobile phase composition in the linear solvent strength (LSS) model—is particularly high for large solutes (i.e., more than 100 for intact proteins); this means that at a given composition, the retention factor is so high that the solute is infinitely retained in the chromatographic column. However, with a small increase in the eluent strength, the retention factor quickly drops and the solute elutes. By exploiting this principle, our group recently demonstrated that the combination of multi-isocratic steps and very steep gradient segments (at solute elution) enables arbitrary set selectivity [5].

The multi-isocratic strategy has been successfully applied to the analysis of monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs) at both intact and subunits levels [5,9]. Compared to previously published work, the novelty of this contribution can be summarized as follows: i) we provided a complete workflow to rapidly and successfully develop the multi-isocratic elution mode of proteins, without the need for HPLC modeling software; ii) we demonstrated the applicability of this approach to very complex biopharmaceuticals samples containing numerous minor variants; and iii) we have also highlighted some of the limitations of this approach, which were not previously reported in the literature (pumping precision, mobile phase preparation, inter-day variability/robustness and so forth).

2. Materials and Methods

2.1. Chemicals and Reagents

Type 1 water was provided by a Milli-Q purification system from Millipore (Bedford, MA, USA). Acetonitrile (ACN) was purchased from Fisher Scientific (Reinach, Switzerland). MS-grade difluoroacetic acid (DFA) (IonHance DFA) was obtained from Waters (Milford, MA, USA). DL-Dithiothreitol (DTT) (for molecular biology, ≥98% HPLC); guanidine hydrochloride (GuaHCl for molecular biology, ≥99%); TRIZMA base (≥99.8%) and hydrochloric acid (HCl) solution (1.0 N, BioReagent) were obtained from Sigma-Aldrich (Buchs, Switzerland). Bispecific (bsAb) mAb-related complex samples, including mAb-cytokine-fusion protein (∼165 kDa) and mAb-domain-fusion (C-terminal) protein (∼200 kDa) samples, were provided by Roche (Penzberg, Germany).

2.2. Apparatus and Methodology

Experiments were performed on a Waters Acquity UPLC I-Class system, equipped with a binary solvent delivery pump, an autosampler and a fluorescence (FL) detector (2 µL FL flow cell). The system includes a flow through needle (FTN) injection system with a 15 µL needle. FL-chromatograms were acquired at λ_{ex} = 280 nm and λ_{em} = 350 nm. The BioResolve Polyphenyl column (2.7 µm, 100 mm × 2.1 mm, 450 Å) from Waters was used. The column temperature was set at 70 °C. Mobile phase A was 0.05% (*v*/*v*) DFA in water and mobile phase B was 0.05% (*v*/*v*) DFA in ACN. Linear gradients were realized from 10 to 90% of mobile phase B, at a flow rate of 0.3 mL/min. Optimized linear gradients (step 3), optimized linear gradients with a premixed mobile phase (step 4) and optimized multilinear gradients (step 5) were automatically calculated through an Excel spreadsheet (Microsoft) and reported in Tables S1–S6. Data acquisition and instrument control were performed by Empower Pro 3 software (Waters).

2.3. Sample Preparation

Denaturing the buffer for reduction (400 mM Tris, 8 M GuaHCl, pH 8.0) was prepared by dissolving 4.85 g Tris base and 76.42 g guanidinium hydrochloride in 100 mL of water. The pH was adjusted to 8 by using a solution of 25% HCl. DTT was added in the denaturing buffer to obtain a final concentration of 40 mM. The DTT solution was freshly prepared before use and added to a 2 mg/mL solution of bsAb in water (1:1) to obtain a final

concentration of bsAb of 1 mg/mL. The DTT antibody solution was then incubated for 30 min at 37 °C. The prepared samples were analyzed within 2–3 days if stored at 4 °C.

The mAb-Cytokine fusion protein and mAb-domain fusion (C-terminal) protein samples were stressed over 6 and 12 weeks at 40 °C, respectively (forced degradation study).

3. Results and Discussion

3.1. Workflow to Develop an Optimal Multi-Isocratic Method

First, we wanted to justify why the method was called the multi-isocratic elution mode rather than the multi-step gradient. In a multi-step gradient, the analyzed compounds are eluted during parts of the gradient having different slopes or possibly during isocratic parts. In the multi-isocratic elution mode, the principle is rather different. Indeed, gradient profiles include parts of the gradient with a very strong slope (change in composition was performed in only 0.02 min) followed by relatively long isocratic parts (equal to 1.5 min); this allows sufficient selectivity between the successive peaks. This means that the isocratic sections are 75-fold more important in time than the gradient sections. In other words, isocratic steps constitute more than 95% of the total gradient time; hence, the importance we wish to give to the isocratic steps by calling this method the "multi-isocratic elution mode" to differentiate it from a more classical "multi-step gradient".

To develop an optimized multi-isocratic method in RPLC, without the need for modelling software (Drylab, Chromsword, etc.), preliminary experiments following the five steps listed below are required; these are also summarized in Figure 1:

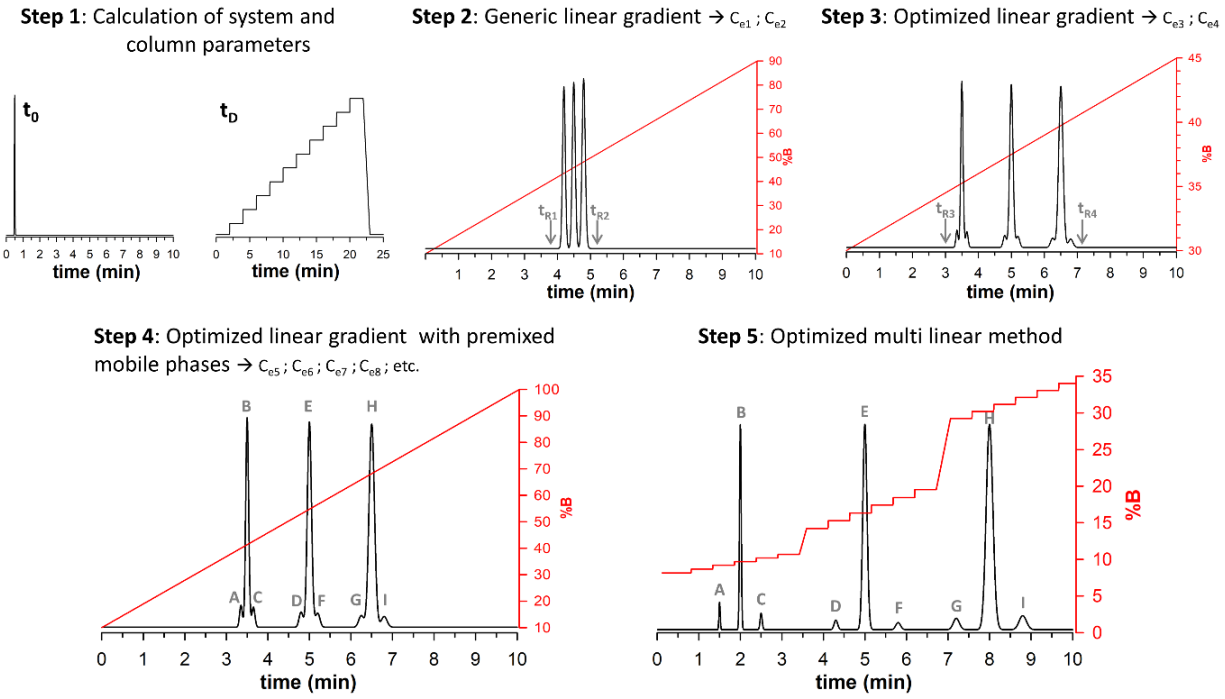

Figure 1. Workflow to develop an optimized multi-isocratic method in RPLC for the analysis of protein biopharmaceuticals. Step 1 consists of measuring the system and column parameters. Step 2 consists of analysis with a generic linear gradient using common gradient conditions. Step 3 is performed with an optimized linear gradient. Step 4 involves the application of the optimized linear gradient using premixed mobile phases. Based on Step 4, the elution composition of peaks of interest can be found. Step 5 consists of finding the optimal multi-isocratic method based on the different values found at Step 4.

The first step is straightforward and consists of measuring the system and column parameters, namely system dwell time (t_D) and column dead time (t_0). For this purpose, some generic procedures already described elsewhere can be followed [10,11].

The second step is also quite simple and consists in performing a first generic linear gradient experiment. For successful operation, water + 0.1% trifluoroacetic acid (TFA), formic acid (FA) or DFA should be used as mobile phase A; while mobile phase B is composed of ACN + 0.1% of the same additive. Then, a generic gradient program from 10 to 90% B is performed at a column temperature of 60–90 °C, with a gradient time of 10 min. These conditions are in line with what is commonly used for the analysis of protein biopharmaceuticals [12]. Based on this initial experiment, the retention times at the start of the first peak and the end of the last peak are obtained. These two retention times values (t_{r1} and t_{r2}) are then transformed into elution compositions (C_{e1} and C_{e2}) using the following equation:

$$C_e = C_i + \frac{C_f - C_i}{t_g} \times (t_r - t_0 - t_D) \quad (1)$$

where C_i and C_f represent the initial and final composition of the initial gradient, respectively, and t_g is the gradient time.

In a third step, an optimized linear gradient is performed using the elution compositions (C_{e1} and C_{e2}) calculated with equation 1 as the initial and final compositions; while the gradient time remains constant (10 min). Again, from this gradient, the retention times corresponding to the start of the first peak and the end of the last peak (t_{r3} and t_{r4}, respectively) are transformed into elution compositions (C_{e3} and C_{e4}) using Equation (1).

The fourth step consists of performing an experiment with the optimized linear gradient using C_{e3} and C_{e4} as the initial and final compositions; however, using premixed mobile phases rather than a purely aqueous or organic mobile phase to have the most precise mobile phase delivery. Indeed, it is important to keep in mind that very small variations of mobile phase composition are required in the multi-isocratic elution method (see step 5). This will improve the repeatability and robustness of the final method. This step is important since modifications of the compound elution can take place between non-premixed and premixed mobile phases; this is because the pump does not work always identically in a narrow vs. large composition range. To provide a sufficient level of flexibility when preparing the premixed mobile phases, the initial elution composition of the optimized linear gradient (C_{e3}) has to be decreased by 5%; while the final elution composition found at step 3 (C_{e4}) should be increased by 5%. With this premixed mobile phase, the final and initial compositions (C_{i2} and C_{f2}) of the optimized linear gradient from step 3 can be recalculated using the following equations:

$$C_{i3} = \frac{5 \times 100}{C_{f2} - C_{i2}} \quad (2)$$

$$C_{f3} = 100 - \frac{5 \times 100}{C_{f2} - C_{i2}} \quad (3)$$

Once the gradient is performed, it may contain several zones of interest corresponding to different regions containing peaks that require resolution improvement. For each zone of interest, it is required to estimate the retention times at the beginning and end of the zone that can both be transformed into elution compositions using equation 1. For example, if there are three zones of interest, the user will need to calculate C_{e5}, C_{e6} and C_{e7} as start compositions of each zone; and C_{e8}, C_{e9} and C_{e10} as end compositions of each zone.

The last step of the process consists of finding the optimal multi-isocratic method based on the different values found at step 4, namely C_{e5}, C_{e6}, C_{e7}, C_{e8}, C_{e9} and C_{e10}. However, it is also important to consider some additional parameters in each zone, namely the number of isocratic steps that will be required to improve the selectivity in each zone (generally comprised between 3 and 6); the duration of each step (usually comprised between 0.5 and 1.5 min); and the time required to move from one step to the next one (usually 0.01 min). Finally, it is also important to define the time between each zone (usually 1 min). From all of these values, the gradient profile can then be constructed either in an automatic way using an Excel spreadsheet or manually.

As highlighted in the next sections, the gradient profile complexity increases with the number of zones of interest and the number of isocratic steps in each zone.

3.2. Application to mAb-Cytokine Fusion Protein Sample

In the first instance, the multi-isocratic workflow described in Section 3.1 was applied to a complex mAb-cytokine fusion protein sample [13] of about 165 kDa that was reduced and temperature-stressed using the procedures described in the experimental section (the corresponding gradient conditions are summarized in Tables S1–S3). For this complex molecule, the chemical reduction with DTT should generate three different sub-units corresponding to two identical light chains (LC_A) of about 24 kDa; one heavy chain (HC_H) having a size of 50 kDa; and one heavy chain linked to a cytokine (HC_K), corresponding to a size of 66 kDa. As expected, three main peaks were observed in the RPLC chromatograms obtained using the optimized linear gradient (Figure 2A; gradient conditions provided in Table S2) and the optimized multi-isocratic gradient (Figure 2B; gradient conditions provided in Table S3). The elution order of the different sub-units was LC_A, followed by HC_H and HC_K.

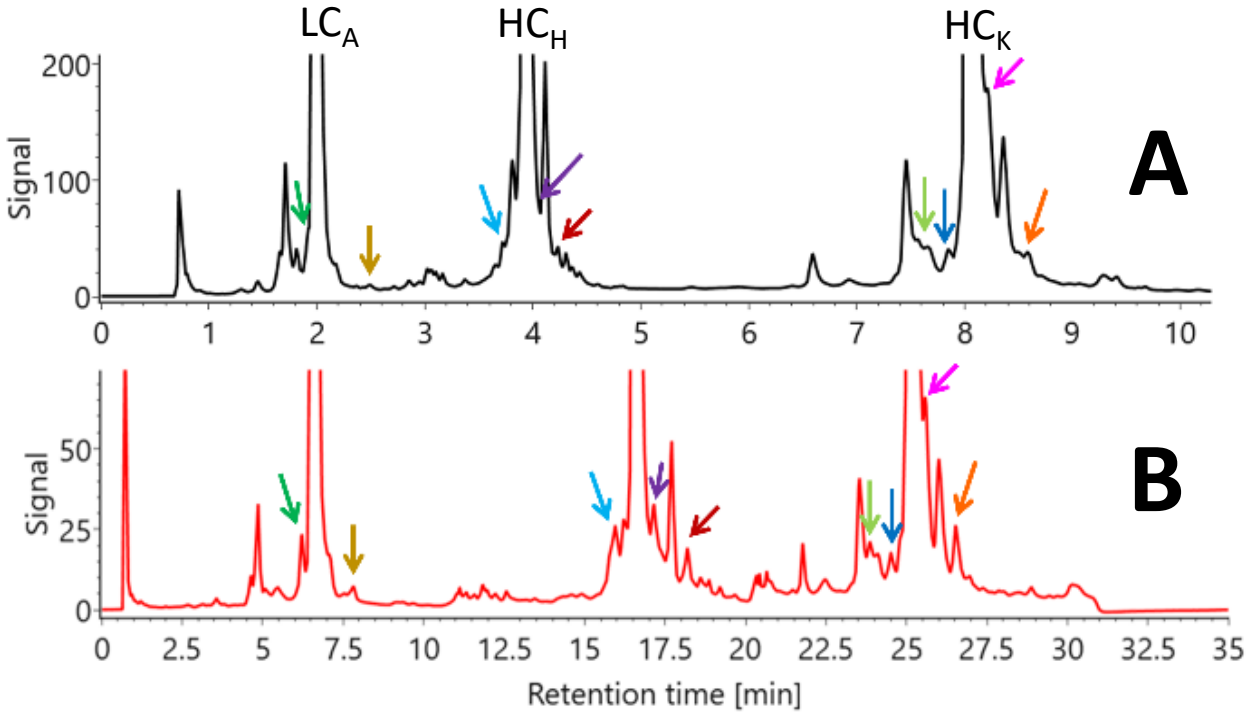

Figure 2. Analysis of the mAb-cytokine fusion protein by RPLC. The chromatograms were obtained by applying: (**A**) the optimized linear (black) vs. (**B**) the optimized multi-isocratic elution (red) gradients.

The chromatogram shown in Figure 2A corresponds to that obtained at step 4 of the workflow described in Section 3.1, using premixed mobile phases, as well as optimized initial and final mobile phase conditions. Combining 0.05% DFA as a mobile phase additive [14], a temperature of 70 °C [15] and a modern stationary phase [16], very good resolving power and sharp peaks can be obtained under RPLC conditions. However, the forced degraded sample [17] also contains numerous minor species that were only partially separated from the three main sub-units. The resolving power of the method proved too limited to obtain sufficient separation for all these variants.

In this context, a multi-isocratic method was developed to further improve the selectivity for the minor variants. In this example, three zones of interest (each corresponding to the elution of a sub-unit species) were considered. These three zones cover the ranges

1.50 to 2.20 min; 3.50 to 4.20 min; and 6.95 to 8.60 min in the chromatogram shown in Figure 2A. In each of these zones, six isocratic steps of 1.5 min were added to further improve the selectivity and overall separation quality. The mobile phase composition range over a given zone was divided by 5 and this number (rounded) was used to create six steps of equally incrementing composition. As an example, in the first zone, the initial and final retention times of 1.5 and 2.2 min were transformed into mobile phase compositions of 16% and 25.9% using equation 1. Then, the six isocratic steps were performed at 16, 18, 20, 21.9, 23.9 and 25.9%. The change between each mobile phase composition was performed in only 0.01 min. The same strategy was applied to the two other zones and the final gradient conditions are provided in Table S3 of the Supplementary Material.

As shown in Figure 2B, the separation can be improved when applying the multi-isocratic vs. linear gradient approach. This is illustrated with the different arrows highlighting the much better separation observed with the multi-isocratic method; this leads to an easier deconvolution of the signals with MS and better characterization of the sample. It is important to note that sensitivity remains comparable between the two chromatograms; in addition, no additional peak broadening was noticed with the multi-isocratic method, despite the addition of several isocratic steps. This observation is clearly due to the on–off retention mechanism observed for proteins and the strong compressibility factor under gradient conditions. It is, however, important to notice that the analysis time became longer when adding numerous isocratic steps during the run. Therefore, to have a fair comparison and obtain comparable retention times between the multi-isocratic and linear gradient approach, we also performed a 30 min long linear gradient using the mAb-cytokine fusion protein sample stressed for 2 weeks at 40 °C. As shown in Figure S1, selectivity was not improved when extending the analysis time for the optimized linear gradient; moreover, the overall separation was evidently not better than the one corresponding to the multi-isocratic elution mode. In conclusion, the addition of several isocratic steps during the run provides a clear added value in terms of chromatographic selectivity.

3.3. Application to mAb-Domain-Fusion (C-Terminal) Protein Sample

The same strategy was also applied to another complex biopharmaceutical product; namely a bispecific antibody [2,18] consisting in a mAb-domain fusion at the C terminal, having a size of about 200 kDa (the corresponding gradient conditions are summarized in Tables S4–S6). For this molecule, the chemical reduction with DTT generated four different sub-units, corresponding to two identical light chains (LC_A) of about 24 kDa; another light chain (LC_B) of 22 kDa; one heavy chain (HC_H), corresponding to a size of 50 kDa; and one heavy chain linked with domain fusion (HC_K), having a size of 76 kDa.

With this bispecific antibody, the elution order of the different sub-units is expected to be LC_B, followed by LC_A, HC_H and HC_K. However, a significant hydrophobicity difference was noticed between the two light chains and the two heavy chains; therefore, two groups of peaks were observed under RPLC conditions (see Figure 3A). Only one major species was observed for each of the LC_B, LC_A and HC_H chains; however, several major variants were observed for the HC_K chain, generating at least three main species eluted towards the end of the gradient. It is important to note that the analyzed sample was stressed for 12 weeks at 40 °C, attributing to the heterogeneity of the species observed.

The chromatogram reported in Figure 3A corresponds to the one obtained at the step 4 of the workflow described in Section 3.1; using premixed mobile phases (mobile phases A and B were composed of 26 and 42% ACN, respectively), as well as optimized initial and final mobile phase conditions (linear gradient from 33 to 100% B in 10 min; full gradient conditions are provided in Table S5). Several minor species were observed near the six major peaks and all the major peaks were not fully resolved.

Since the peaks can be grouped into two distinct zones in the chromatogram shown in Figure 3A, a multi-isocratic method was developed; this expanded the elution over 3.55 to 5.20 min and 5.20 to 7.40 min. The chromatogram shown in Figure 3B corresponds to the optimized multi-isocratic method obtained by using six isocratic steps over the two

zones of elution, using the procedure explained in Section 3.1 and already applied to the mAb-cytokine fusion sample. The final gradient conditions were provided in Table S6 of the Supplementary Material.

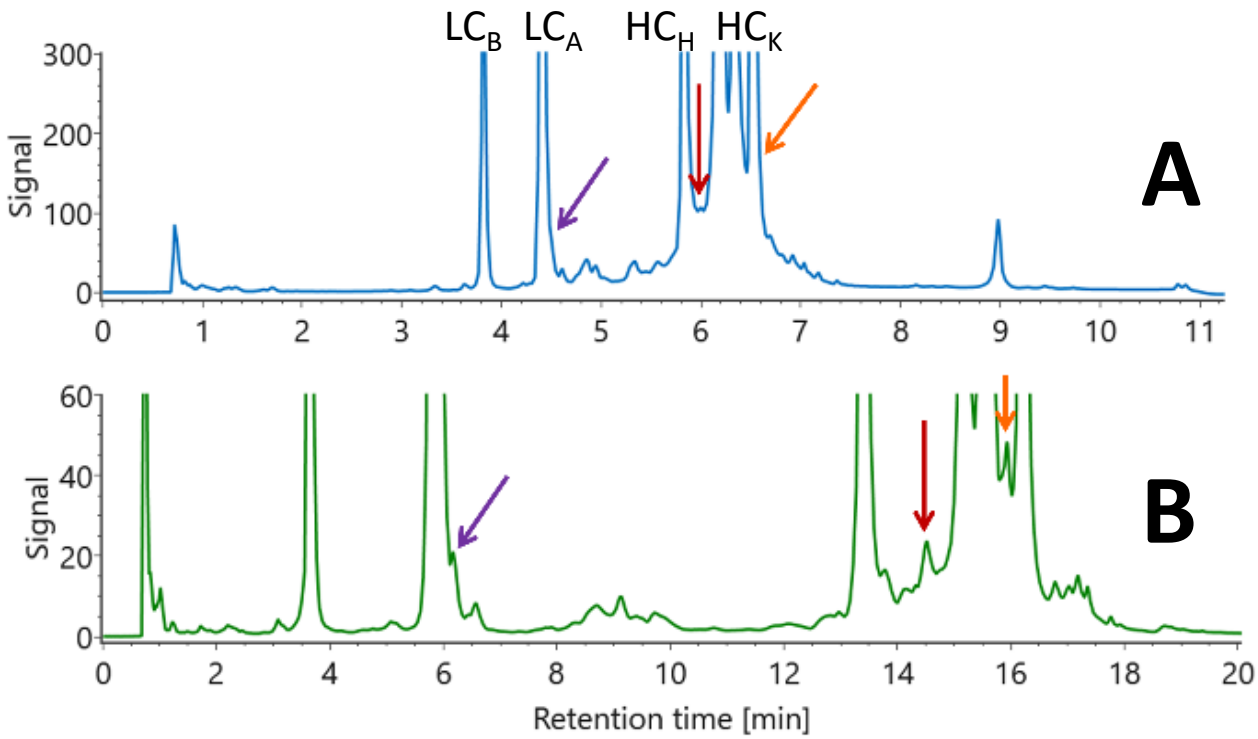

Figure 3. Analysis of the mAb-domain-fusion (C-terminal) protein. The chromatograms were obtained by applying: (**A**) the optimized linear (blue) vs. (**B**) the optimized multi-isocratic elution (green) gradients.

As previously observed, the separation of the hydrophobicity variants was improved using the multi-isocratic vs. linear gradient; as highlighted with the different arrows reported on Figure 3. However, it is important to note that the gradient becomes quite complex when adding several isocratic steps in different zones of elution (see Tables S3 and S6 of the Supplementary Material). Even if the mobile phases were premixed to improve pump delivery precision, method robustness may be compromised due to the highly precise mobile phase compositions that are required for the gradient. Experimentally, we observed that retention times remain stable (RSD values were lower than 1% for all the peaks when performing three replicate analysis) in multi-isocratic experiments during one single day of experiments, when using the same mobile phase. Such RSD values are in line with what is commonly obtained under UHPLC conditions and correspond exclusively to the variability of the pumping system. However, when performing experiments over several days (with the instrument being switched off at the end of each day and a new mobile phase prepared daily), the variability of the pumping system is superimposed with the precision of the mobile phase preparation. Therefore, the chromatographic profile can vary, both in terms of selectivity and retention times. When performing the same experiment over three consecutive days, inter-day variability on retention times for the different peaks observed on the chromatogram of the reduced mAb-domain-fusion (C-terminal) protein sample was comprised between 2 and 4%. Here, the non-negligible variation of retention times was mostly attributed to the preparation of the mobile phase. Indeed, premixed mobile phases are quite difficult to prepare precisely. In our opinion, the premixed mobile phases should ideally be prepared by gravimetric rather than volumetric method to further decrease RSD values on retention times.

This means that such a complex multi-isocratic method is not recommended for routine use such as quality control environments; however, when applied in R&D laboratories, it offers significant benefits in order to gain as much information as possible on unknown samples.

4. Conclusions

Applying the generic procedure described in this contribution, consisting of only five steps, a multi-isocratic elution method can be rapidly and successfully developed without the need for HPLC modeling software. This strategy is well-suited to protein biopharmaceuticals, as they offer an on–off retention mechanism and strong compressibility factor under gradient conditions.

A linear gradient (reference method) and a multi-isocratic elution mode (innovative method) were systematically compared for the analysis of two complex stressed biopharmaceutical samples; namely one mAb-cytokine fusion and a bispecific antibody sample. These two samples were analyzed at the subunit level, after chemical reduction with DTT.

As expected, the addition of several isocratic steps during the run provides a clear added value in terms of chromatographic selectivity; this leads to an improved characterization of the sample. It is also important to note that sensitivity and peak width remain comparable between the linear gradient and multi-isocratic elution methods; this is due to the large S values of proteins and strong compressibility factors in the gradient mode. However, the gradient profile becomes quite complex when using the multi-isocratic method. Despite the mobile phase premixing to improve pump precision, the method robustness was found insufficient over several days of experiments. Therefore, such a methodology should be only considered in R&D environments to enhance sample characterization and knowledge; however, it should not be considered in QC laboratories.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9090243/s1. Table S1: Optimized linear gradient (Step 3) for the analysis of a mAb-cytokine fusion protein sample; Table S2: Optimized linear gradient with premixed mobile phase (Step 4) for the analysis of a mAb-cytokine fusion protein sample. Mobile phase A consisted of 25% ACN + 0.05%DFA and mobile phase B of 55% ACN + 0.05% DFA; Table S3: Multi-step gradient profile (Step 5) for the analysis of a mAb-cytokine fusion protein sample; Table S4: Optimized linear gradient (Step 3) for the analysis of a mAb-domain-fusion (C-terminal) protein sample; Table S5: Optimized linear gradient with premixed mobile phase (Step 4) for the analysis of a mAb-domain-fusion (C-terminal) protein sample. Mobile phase A consisted of 26% ACN + 0.05%DFA and mobile phase B of 42% ACN + 0.05% DFA; Table S6: Multi-step gradient profile (Step 5) for the analysis of a mAb-domain-fusion (C-terminal) protein sample; Figure S1: Separation of a complex reduced mAb-cytokine fusion protein stressed for 2 weeks at 40 °C, using the optimized linear gradient (step 3, 10 minutes run), the multi-isocratic elution mode (step 4, 30 minutes run) and a long linear gradient similar to step 3, but with gradient time of 30 minutes. In this figure, the focus was put on the elution of the third zone in the chromatogram, corresponding to the elution of HCK.

Author Contributions: Conceptualization, A.M., V.D., V.L., S.P. and D.G.; methodology, A.M., V.D., V.L., S.P. and D.G.; validation, S.P. and V.L.; formal analysis, A.M., V.D., V.L., S.P. and D.G.; investigation, A.M., V.D. and D.G.; resources, D.G., V.L. and S.P.; writing—original draft preparation, A.M., V.D. and D.G.; writing—review and editing, A.M., V.D., V.L., S.P. and D.G.; supervision, D.G.; project administration, D.G.; funding acquisition, D.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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