Journal of Pharmaceutical and Biomedical Analysis 203 (2021) 114162

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

# Superheated reversed phase chromatography with ultrashort columns for the analysis of therapeutic proteins

Balázs Bobály*, Alex Keresztfalvi, Thomas Gräber, Maria Anna Schwarz

*Solvias AG, BU Biopharmaceuticals, Römerpark 2, 4303 Kaiseraugst, Switzerland*

ARTICLE INFO

*Article history:*
Received 16 April 2021
Received in revised form 18 May 2021
Accepted 20 May 2021
Available online 24 May 2021

*Keywords:*
Superheated reversed phase chromatography
Non-covalent protein complexes
Ultrashort column
On-column degradation
Bispecific

ABSTRACT

Mild or elevated temperatures are routinely used for the analysis of therapeutic proteins by reversed phase liquid chromatography. Generic conditions can be used for the analysis of monoclonal antibodies, and may be adapted for species derived thereof, for instance their immuno-conjugates. Beyond platform monoclonal antibodies, many novel, non-covalent protein complexes are also frequently pursued as protein therapeutics. These complexes, in reverse phased chromatography, may require extremely harsh, superheated conditions to dissociate and elute as interpretable profiles. In order to minimize on-column degradation under superheated conditions, the analysis time has to be reduced as much as possible. Using ultrashort columns and fast gradients is a promising approach in achieving informative separations within a minute, or even faster. Here the applicability of this approach, which supports maintaining levels of degradation products close to the intrinsic sample composition without further on-column degradation is demonstrated. NISTmAb as conventional IgG, a bispecific homodimer and a bispecific homotetramer were used for demonstrating differences in the elution characteristics and the necessity of using the proposed approach. The analysis of the bispecific homodimer was discussed in detail as a case study.



## 1. Introduction

Advances in the discovery and development of protein therapeutics have led to unprecedented increase in the number of new biologics approvals by the EMA and FDA in the past few years. The number of approvals approximately doubled between 2013–2019, compared to between 2007–2013 [1]. Antibodies represented 44% of the new biologics approvals by the FDA and/or EMA between 2014 and 2018, and were the largest category among 155 biopharmaceutical approvals [2]. Besides monoclonal antibodies, novel protein constructs addressing a variety of therapeutic targets attract significant attention. These emerging classes of novel protein modalities set new challenges for their development, including analytics [3–5]. Conventional conditions and platform analytical methods working well for the majority of antibodies [6–10] may not be directly applicable to fusion proteins, antibody fragments, immuno-conjugates and non-antibody proteins. These materials introduce additional levels of molecular complexity and/or behave substantially differently in the analytical system [11,12]. Thus, adaptation of generic approaches or development of product specific methods may be inevitable for these novel modalities.

In this study, we present a potential new application by adapting generic, elevated temperature reversed phase liquid chromatography (RPLC) conditions for the analysis of stable, non-covalent therapeutic protein complexes. The idea of using elevated (e.g. up to 90 °C), or even superheated temperature conditions (also called high temperature chromatography – HTLC; above the atmospheric boiling point of the mobile phase, e.g. up to 120 °C) has been considered, and confirmed to be beneficial for the fast and efficient analysis of proteins decades ago by the pioneering work of Horvath and co-workers [13,14]. These temperature conditions, in combination with further settings (e.g. 4.6 mm ID × 10–30 mm length ultrashort columns packed with pellicular particles, and high flow rates of 5 mL/min) enabled the separation of model proteins in seconds [14], due to drastically improved mass transfer. The use of ultrashort columns for sub-minute analysis of therapeutic proteins has recently been further described in detail by Fekete et al. [20,21]. Reduced analysis time is not just the outcome, but also a need, when using such harsh conditions. High temperatures and strongly acidic mobile phases (0.1 % TFA, pH ~ 2) inherently lead to sample degradation during analysis. Such on-column sample degradation can then confound interpretation of results of the quality (e.g. purity) of a sample. Therefore, there is a continuous search

* Corresponding author.
*E-mail address:* Balazs.Bobaly@solvias.com (B. Bobály).

https://doi.org/10.1016/j.jpba.2021.114162
0731-7085/

for improving separation conditions while also limiting chromatography related artefacts in RPLC. Thanks to innovations in modern column technologies [15,16], efforts made on selecting appropriate mobile phase additives [17,18], and fragmentation techniques reducing molecular size and leading to more favorable chromatographic properties, it is possible to analyze therapeutic proteins even at mild temperatures (e.g. 60–70 °C) while maintaining sufficient resolving power and limiting risk of on-column sample degradation [19].

In the particular cases presented here, however, superheated conditions are necessary for obtaining interpretable elution profiles. The elution of the proteins in their functional form (dimeric or tetrameric complexes) is not feasible with mild or elevated temperatures. Dissociation of the complex occurs during the analysis, and monomers leaching from the bound protein result in transformational profiles. Using superheated conditions, complete dissociation of these complexes occurs, which enables the elution of well-defined monomer peaks. In order to limit on-column degradation, the analysis time was reduced to ~ 1 min with the application of an ultrashort (guard) column. Combining superheated conditions with ultrashort columns in reversed phase chromatography, the bispecific homodimer and the bispecific homotetramer species are all well resolved with minimal on-column sample degradation.

## 2. Materials and methods

### 2.1. Equipment and software

Analyses were performed on an Agilent 1290 Infinity II UHPLC, equipped with a binary pump, autosampler with flow through needle (FTN) injector, thermostated column compartment, and diode array detector (DAD), followed by an Agilent 1260 Infinity II fluorescence (FL) detector. Analyses up to 110 °C were performed using the G7116B column compartment with ultra-low dispersion preheater, and without any custom modification. The column outlet was directly connected to the detector inlet without post-column temperature control. DAD signals were acquired at 220 and 280 nm (10 Hz sampling rate), excitation and emission wavelengths for the FL detection were set to 280 nm and 350 nm, respectively (10 Hz sampling rate). The modules were connected with the shortest possible 0.075 mm ID stainless steel or PEEK capillaries. The nominal volume of DAD and FL flow-cells are 1 and 8 μL, respectively. The system volume from the injector to the DAD cell was 21 μL. For injections with reduced injection volumes (4 μL for the 100 mm column and 0.3 μL for the 10 mm column) made onto the ultrashort columns, only FL detection was used, since DAD intensity (or S/N) was insufficient. Thus, despite of known peak broadening effect originating from the application of the 8 μL cell, this channel was used for the evaluation. The gradient delay volume of the system was measured to be 190 μL. System control, data acquisition and evaluation were performed using Chromeleon 6.8 CDS (Thermo Fisher Scientific, Waltham, MA, USA). For the optimization of the separations, DryLab 4 software (Molnar Institute, Berlin, Germany) was used.

MS analyses were performed on a Thermo Scientific Q-Exactive plus Orbitrap mass spectrometer, coupled with the UHPLC system. Heated positive ESI mode was used. The capillary temperature and probe heater were set to 275 °C. Sheath gas and auxiliary gas flow were set to 35 and 10 units, respectively. Full scans were acquired between *m/z* 650–6000 (resolution: 17500, S-lens: 100, SID: 80 eV, 10 microscans). The MS data were evaluated with PMI Intact v3.5 (Protein Metrics, Cupertino, CA, USA). Graphics were prepared from exported raw data using Excel (Microsoft).

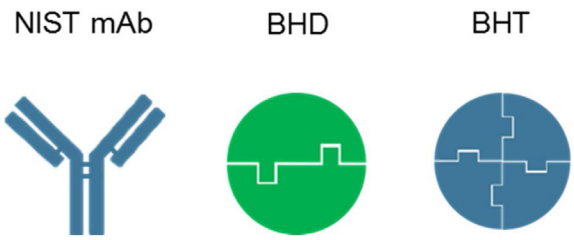


**Fig. 1.** Schematic respresentation of the proteins used in the study. BHD: bispecific homodimer, BHT: bispecific homotetramer.

### 2.2. Chemicals and columns

Mobile phases were prepared using gradient grade acetonitrile, purified water, obtained from a Merck Millipore purification system (Merck, Darmstadt, Germany), trifluoroacetic acid (TFA) and methanesulfonic acid. (MSA) Hydrogen peroxide and sodium hydroxide was used for the preparation of forced degradation samples. All chemicals were purchased from Merck (Buchs, Switzerland).

The MabPac RP columns (100 × 3.0 mm 4 μm, and 10 × 3.0 mm 4 μm with Acclaim cartridge holder # 069580) used in the study have been purchased from Thermo Fisher Scientific (Reinach, Switzerland) and were selected based on their expected operating stability at superheated temperatures. The estimated column volumes based on solvent disturbance, and corrected for the system volume were 513 μL for the 100 mm, and 54 μL for the 10 mm column with the cartridge holder.

### 2.3. Samples

NIST Monoclonal Antibody Reference Material 8671 was purchased from Merck (Buchs, Switzerland). A bispecific homodimer (BHD), consisting of 2 non-covalently bound ~ 50 kDa protein chains forming a homodimer; and a bispecific homotetramer (BHT), formed from monomers of ~ 60 kDa were used in this study. The schematic of the protein used in the study is shown in Fig. 1. BHD and BHT are related to customer projects of Solvias AG, and were used within this study with the agreement of their proprietors. Intact samples were diluted to 1 mg/mL with water and injected without any further preparation. Forced degradation BHD samples were prepared as follows. Oxidized BHD: 1 % $H_2O_2$ was added to BHD and incubated at 37 °C for 1 h. pH-stressed BHD: the pH of the sample was set to 9 with 0.1 M sodium hydroxide and the sample was incubated at 45 °C for 24 h. Stored (stability) BHD: the sample was stored at 25 °C for 6 months.

## 3. Results and discussion

### 3.1. The need for superheated conditions when analyzing BHD and BHT in RPLC

The majority of conventional IgGs, like the NIST Monoclonal Antibody Reference Material do not require exceptional conditions and can be analyzed with generic settings [7,8,19]. However, these generic conditions may need to be optimized, in order to achieve the desired separation of the specific protein species [22,23]. As shown in Fig. 2, a temperature screening was performed with two different mobile phase additives, and generic scouting gradient conditions for NISTmAb, as well as BHD and BHT. Mobile phase A was water with 0.1 % TFA or MSA, mobile phase B was acetonitrile with 0.1 % TFA or MSA. A scouting gradient of 10–100 %B in 10 min was used with a flow of 0.6 mL/min. 4 μL was injected onto the 100 mm long analytical column. The proteins eluted were detected

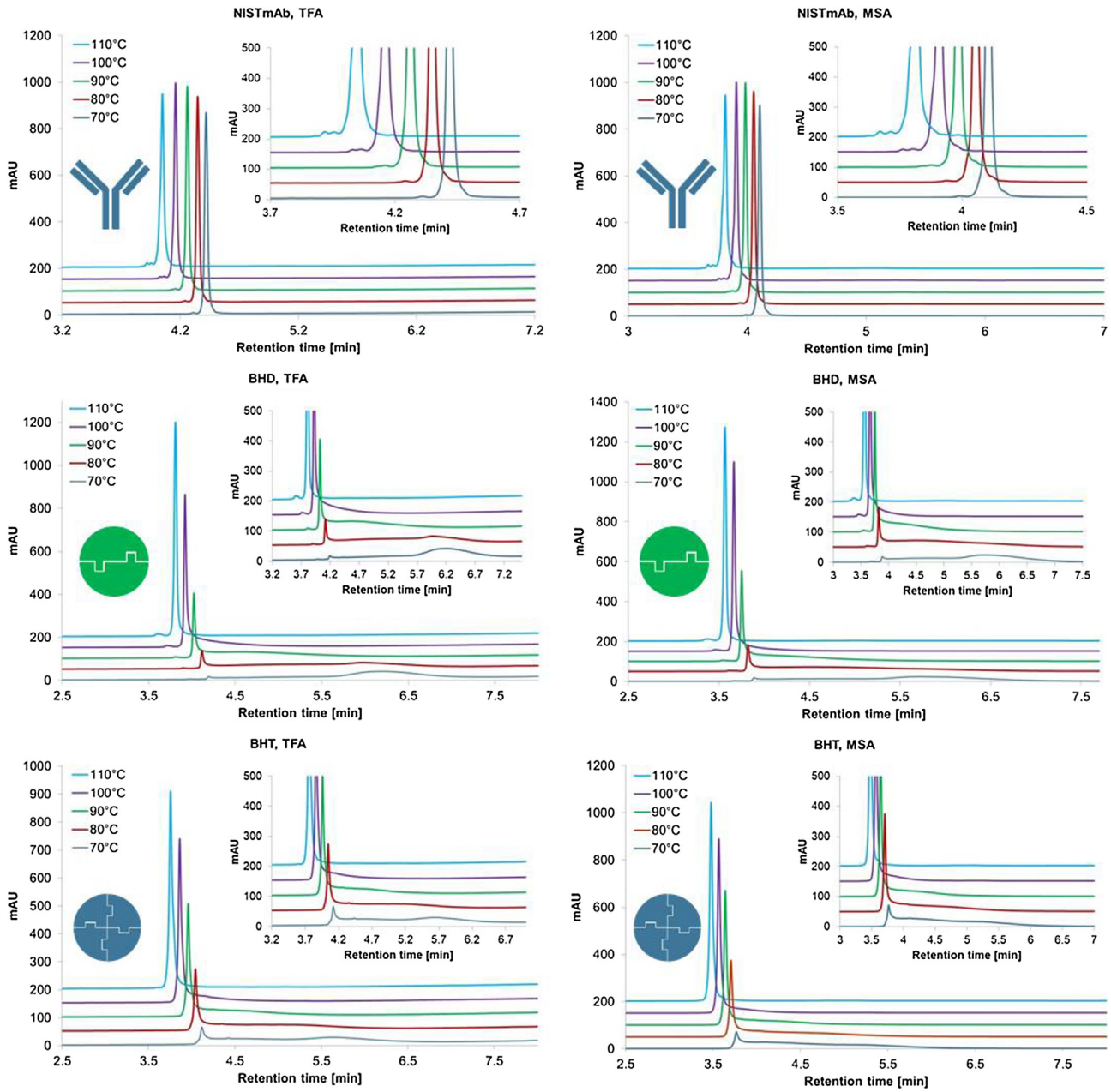


**Fig. 2.** Chromatograms obtained with temperature screening generic scouting RPLC gradients on a 100 mm column. Left panels: 0.1 % TFA, right panels: 0.1 % MSA in mobile phase. BHD: bispecific homodimer, BHT: bispecific homotetramer.

by their absorbance 214 nm (10 Hz). When analyzing BHD and BHT with generic RPLC conditions, profiles similar to on-column interconversion [24] have been observed (Figs. 2 and 3). These are substantially different form the simply tailing profiles observed if sub-optimal conditions are used and adsorption issues deteriorate peak shape [19,25–27]. The profiles are significantly affected by the column temperature, developing from "batman"-like [24] peaks to a well-defined monomeric peak when approaching superheated conditions. Besides TFA, MSA was also investigated as mobile phase additive. Based on the results published by McCalley and Guillarme [17], this additive can be a potential replacement for TFA and may enable the use of reduced temperatures compared to TFA, while maintaining similar separation properties. The importance of reducing column temperature will be discussed in Section 3.2. For BHD and BHT, no significant benefits of using MSA in the elution profiles have been observed. This is not contrary to [17], since there peak broadening at lower column temperatures can be explained by adsorption. Here, it appears that peak broadening at elevated temperatures is related to uncontrolled dissociation during the analysis (see discussion later in this section), while in case of regular IgGs, it is a sign of non-specific adsorption to the stationary phase. RPLC-ESI-MS analysis was performed with the TFA containing mobile phase (MSA is not MS compatible) at 70 °C, confirming the elution of the monomer both for BHD and BHT in the entire elution profile (Fig. 3). Only minor dimeric species were observed in the transitional profile. All this led to the following assumption: upon injection and focusing at the column inlet, the complexes - at least partially - maintain their associated form. During analysis at mild (e.g. 70 °C) or elevated temperature (e.g. up to 90 °C), the monomer is partially being eluted at a certain eluent strength, and is continuously dissociating from the bound fraction (presumably still from the complexes which cannot be eluted) when increas-

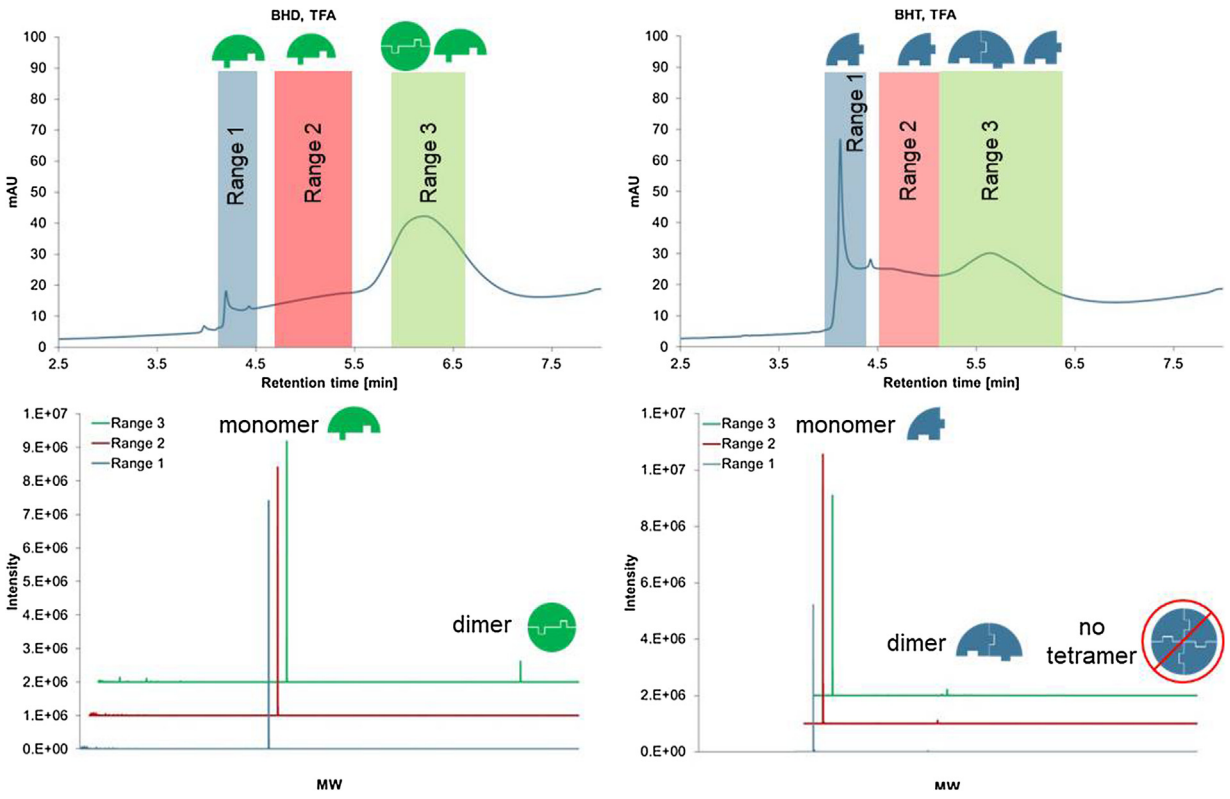


**Fig. 3.** Elution profiles of BHD and BHT at 70 °C (top, also shown in Fig. 2) with the deconvoluted mass spectra (bottom) obtained for the retention time ranges in marked in the chromatograms. Deconvoluted mass spectra were shifted for better visibility.

ing the mobile phase organic concentration. The complexes cannot be eluted, as they are strongly adsorbed at mild temperature, or being dissociated by heating. Applying elevated and superheated temperature conditions facilitates the dissociation, therefore the development of a sharp monomer peak can be observed. As a shown in Fig. 2, superheated conditions are required for the analysis of the BHD and BHT constructs. The effect of superheating on the sample consistency and column selection is discussed in detail in the next section.

### 3.2. On-column sample degradation and intrinsic sample composition

Harsh conditions with temperatures above 100 °C and strongly acidic mobile phase inherently lead to on-column degradation (e.g. hydrolysis of the protein chain) of the samples injected, resulting in artefact peaks not related to the real sample composition. Thus, it is particularly important to consider the contribution of on-column degradation artefacts to the overall profile obtained. Injecting the sample and parking the band with initial focusing isocratic conditions before elution helps in estimating how residence time (retention time) of the protein contributes to the on-column formation of impurities. Plotting relative area (A%) of species of interest as the function of main peak (MP) residence time shows how artefact degradants are formed, and how the peaks vanish which they originate from. The slopes of the fitted functions help in estimating degradation/formation rates at a given temperature based on relative peak areas (e.g. ± A%/min), while their intercepts can be used for extrapolating to their hypothetical intrinsic sample composition without on-column degradation (analysis time is 0). For the following sections, results obtained with BHD will be shown as a case study. Following the experiments described in Section 3.1, gradient adjustment was performed, in order to better separate species indicating on-column degradation. DryLab software was used for this purpose. Note, the goal of the optimization was to improve the separation of pre- and post-peaks from the main peak, while maintaining similar elution profiles for both additives. Based on input experiments at 100 °C and 110 °C with gradients of 25–40 %B times in 8 and 16 min, profiles with comparable retention times and resolution were optimized for both additives. For MSA additive, the following adjusted gradient was used for estimating on-column degradation: parking time + 27.0–29.7 %B in 0.5 min followed by a 29.7–32.5 %B in 4.5 min segment. For TFA additive, a slightly different gradient was used since this additive is more retentive: parking time + 29.0–31.4 %B in 0.5 min, followed 31.4–34.5 %B in 4.5 min segment. The applicability of these optimized gradients were then reinvestigated at temperatures of 110−107−104 and 101 °C. After evaluating the profiles between 101–110 °C, it was concluded that temperature can be decreased to 108 °C for TFA and to 105 °C for MSA without significant peak broadening or area decrease of the main peak (data not shown). In further experiments, 108 °C was used for TFA and 105 °C for MSA. BHD was injected and parked for 0–2–4–6 and 8 min, before eluting with an adjusted gradient (Fig. 4). Relative area of pre-peaks, main peak and post-peaks of BHD were plotted against the residence time of the main peak and a linear fit was evaluated for estimating the real sample composition without on-column degradation. From the slopes of the pre- and main peaks one can conclude that the main peak is directly degraded and converted into pre-peaks with a rate of 3.7–3.8 A%/min. The intrinsic sample composition was estimated between 90–94 % for the main peak (intercept of the fit), 5–8 % for the pre-peaks and 1–2 % for the post-peaks.

### 3.3. Controlling on-column degradation

As described in Section 3.2, as a result of fast degradation at superheated conditions, analysis time is a crucial parameter affecting the resulting sample composition. With the optimized

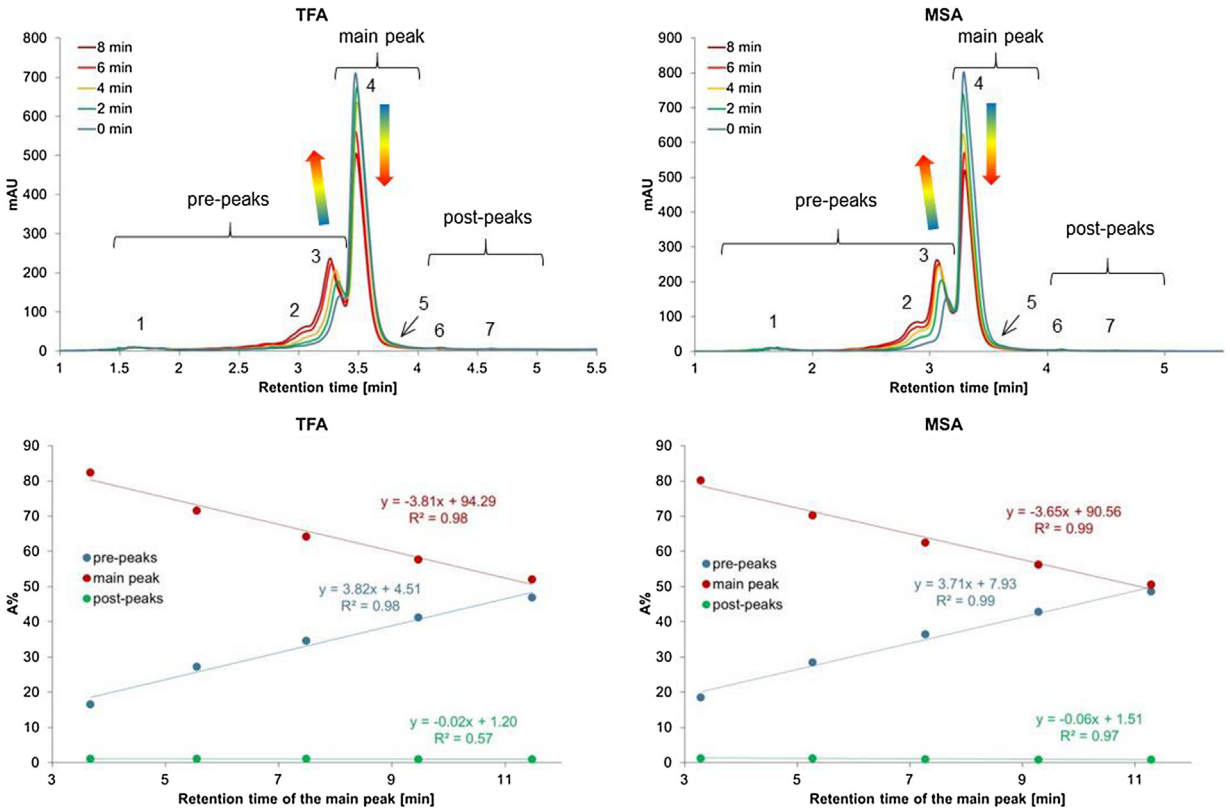


**Fig. 4.** On-column degradation of BHD at superheated conditions, visualized by overlaid, retention time normalized profiles. Sample parking was performed on the 100 mm column by using an initial isocratic segment before starting gradient elution. Parking time (duration of the initial isocratic segments) is shown in the top panels. Left panels: 0.1 % TFA, right panels: 0.1 % MSA in mobile phase. Bottom panels: evaluation of relative area (A%) of $\sum$pre-peaks, main peak and $\sum$post-peaks as function of the main peak retention time (residence in the superheated system). Slope of the fit: estimated degradation rate [ΔA%/min], intercept: estimated intrinsic sample composition. BHD species are annotated for comparison of the profiles only.

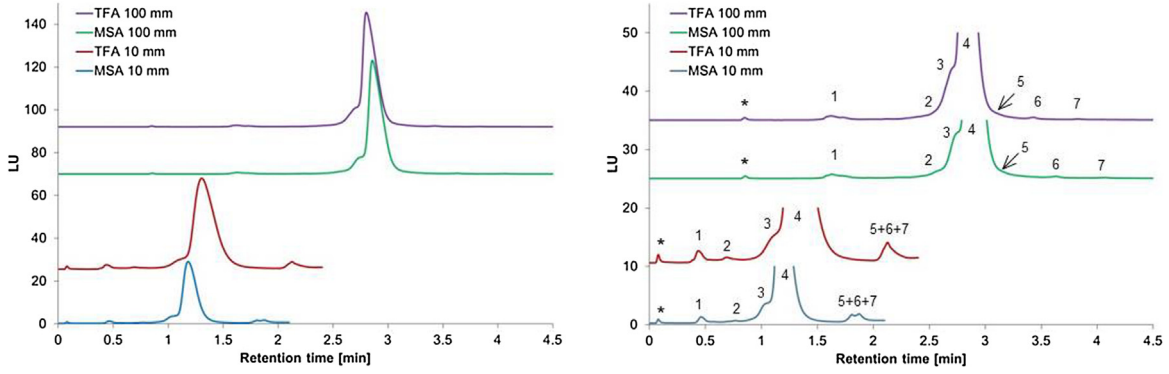


**Fig. 5.** Software assisted optimized conditions for the separation of BHD species on the 3 × 10 mm and 3 × 100 mm columns, using 0.1 % TFA and 0.1 % MSA in the mobile phase. Left panel: expanded view, right panel: zoomed view with peaks annotated for comparison of the profiles only, * denotes dead time.

conditions on the 100 mm column, it was possible to reduce the analysis time to around 3–4 min (Fig. 4). As a result of the generic "on-off" retention mechanism of proteins, very short column can be used, since large proteins are mainly retained at the first few millimeters of the column [20,21]. This part of the column, where the sample components are focused and then eluted, is responsible for the separation. Once the protein is eluted, the interaction with the stationary phase is negligible and the remaining part of the column plays only a minor role in the separation. Thus, applying ultrashort columns, the analysis time can drastically be decreased, even allowing sub-minute separations. Limited column volumetric load, sensitivity to extra-column system volumes, and currently not commercially available optimized columns with such dimensions have been summarized as limitations of this approach [21].

Considering the above observations, and following optimization on the analytical column, the same procedure was executed, but now using a 10 mm guard column packed with the same material. As the previously optimized profile with MSA showed a slightly better separation for pre- and main peaks compared to TFA, using this additive was continued for the optimization on the guard column as well. Input experiments for optimization were executed as follows: gradients of 29–33.5 %B for TFA and 27–33 %B for MSA were run in 2 and 4 min, at 108 °C for TFA and 105 °C for MSA with a flow of 1 mL/min. Injection volume of 0.3 μL was used, taking into consideration the limited loading capacity of the short column. The optimized

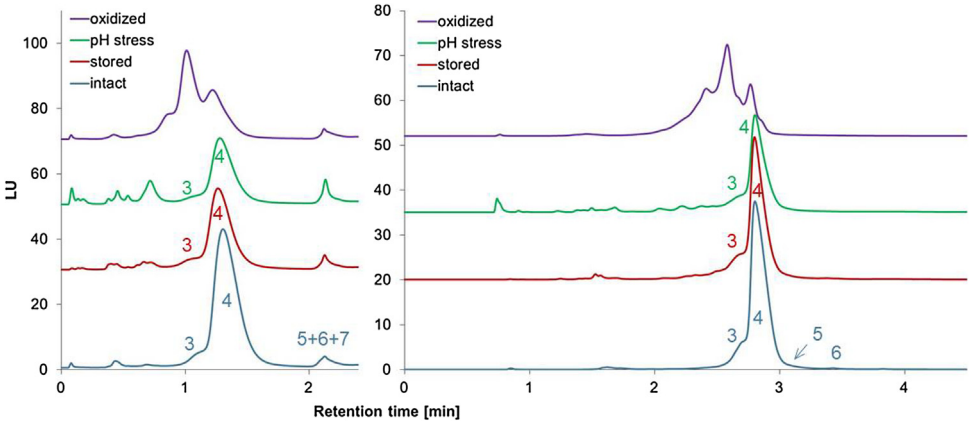


**Fig. 6.** Analysis of stressed BHD samples using the 3 × 10 mm (left) and 3 × 100 mm columns (right) with the TFA containing eluent. Other conditions as in Figs. 2 and 3 (except detection, here fluorescence detection was used for both columns). See sample preparation in section 2.4. BHD species are annotated for comparison of the profiles only, * denotes dead time.

**Table 1**
Comparison of BHD relative peak areas obtained on ultrashort and conventional analytical columns using superheated conditions. Note, pre-MP (MP: main peak) levels directly related to on-column degradation (Fig. 4) are significantly lower on the ultrashort column, due to reduced analysis time. Expected A% values are deduced from intercepts in Fig. 4, bottom panels.

| | Measured relative area (A%) | | | Expected relative area (A%) without on-column degradation | | |
|---|---|---|---|---|---|---|
| Column length, mobile phase additive | ∑pre-MP | MP | ∑post-MP | ∑pre-MP | MP | ∑post-MP |
| 10 mm, 0.1 % TFA | 9.4 | 87.4 | 3.2 | 4.5 | 94.3 | 1.2 |
| 100 mm, 0.1 % TFA | 13.3 | 85.5 | 1.2 | | | |
| 10 mm, 0.1 % MSA | 8.1 | 88.9 | 3.0 | 7.9 | 90.6 | 1.5 |
| 100 mm, 0.1 % MSA | 12.4 | 86.6 | 1.0 | | | |

multi-segmented gradient on the 10 mm column for MSA additive was 25.0 %B for 0.1 min, 25.0–28.6 %B in 0.1 min, 28.6–29.0 %B in 1.3 min and finally 29.0–50.0 %B in 0.5 min. For the TFA additive, a different segmented gradient was used for obtaining similar separation profiles: 27.5 %B for 0.1 min, 27.5–30.1 %B in 0.1 min, 30.1 %B for 1.6 min and finally 30.1–50.0 %B in 0.5 min. The optimized methods on the ultrashort column enabled further decreasing the analysis time below 1.5 min, as shown in Fig. 5. Note the resolution between the main pre-peak and main peak is lower than in Fig. 4. This is most likely related to the band broadening effect of the fluorescence cell volume. For the 0.3 μL injections the DAD intensity was insufficient, therefore the column outlet was directly connected to the FL detector, and this traces have been compared on both columns. Relative peak areas obtained on the 10 mm and 100 mm columns were evaluated. As expected, A% of pre-peaks further approached hypothetical intrinsic levels and are in-line with the estimations made in Section 3.2 (Table 1). The pre-peak levels are now decreased from 12–13 A% to 8–9 A%, only slightly above the 5–8 % expected for 0 analysis time. Similar observations can be made for the main peak. For estimating the significance of the decrease in ∑pre-peak relative area, repeated injections (n = 6) of the intact sample were evaluated using the 10 mm column. Relative standard deviations of A% were < 5% for the ∑pre-peaks, < 0.3 % for the main peak, and < 7% for the ∑post-peaks, respectively. Note, separation of the post-peaks was improved by changing the gradient shape, and post-peaks are therefore better split from the main peak compared to the separation on the 100 mm column. This, and the fact that post peak levels remain practically unaffected by on-column degradation should be taken into consideration, when evaluating main peak A% levels (~ 2% post-peak were integrated with the main peak in 3.2, thus adding this 2% to main peak in Table 1 results around 89–91 A% for the main peak).

### 3.4. Capabilities of the short gradient

In order to demonstrate the capabilities of the very short gradient, stressed samples were injected and their profiles were compared using the optimized conditions on 10 and 100 mm columns. Note, that the conditions were optimized for separating the main peak and its pre- and post-peaks, and not for separating the individual species appearing upon stress. Fig. 6 shows overlaid chromatograms of stressed and intact samples analyzed on the 10 mm and 100 mm columns. The profiles of intact, oxidized, pH stressed and ambient stored samples remained comparable on the 10 mm column (except post-peaks, see explanation in Section 3.3) and maintained the stability indicating capability of the method. These results showed that indeed, it is feasible to perform very fast analyses on ultrashort columns, and so limit on-column degradation under superheated conditions, while maintaining the capabilities of the separation. According to our experience, the divinylbenzene polymer stationary phase is very stable under extreme chemical and thermal conditions. The routine applicability of the current 10 mm guard column is rather limited by the hardware design, not fully fitting for this purpose. A properly designed ultrashort column hardware may allow routine analysis not only under superheated conditions, but also in high-throughput analyses [28] and comprehensive 2D LC separations, where the second dimension separation is optimally in the 1–2-min range or less [29,30].

## 4. Conclusion

In this study, we investigated the applicability of superheated conditions for the analysis of novel therapeutic protein constructs, which form stable complexes in solution. The dimeric/tetrameric

form of the complexes could not be eluted at mild or elevated temperatures, and transformation-like profiles were observed, most probably corresponding to the dissociation of the bound complex during analysis. These profiles are difficult to interpret. At superheated conditions, however, well- defined monomer peaks could be attained. The major limitation of applying superheated conditions is the fast on-column degradation of the samples. Software assisted method development was used to identify conditions forultrashort columns that maintained peak resolution but minimized on-column sample degradation. The optimized profile on a 10 mm column remained comparable to that one obtained on a regular 100 mm column. With around 1 min elution time for the main peak, it was possible to minimize the on-column degradation to acceptable levels, well approaching estimated intrinsic sample composition without on-column degradation. Moreover, the 10 mm column does not limit the method's stability indicating capability. Note also, all experiments in this study have been performed using commercially available instrumentation, columns and consumables, and without excessive system optimization. A system configuration that enables operation at 100−110 °C column temperature while using low volume FL cell would clearly be beneficial for such applications. It is expected, that decreasing extra-column volumes and optimizing column dimensions and hardware could further improve the separation profiles and allow the routine application of ultrashort columns [21].

## Author contributions

B. Bobaly: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision.

A. Keresztfalvi: Investigation, Writing - Review & Editing.

T. Gräber: Investigation, Writing - Review & Editing.

M. A. Schwarz: Review & Editing, Project administration, Funding acquisition.

## 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.

## Acknowledgement

The authors are grateful to Cristina Montealegre-Dondarza, Clifford Barrow, Markus Markl and the proprietors of the molecules used in this study for the valuable discussions and support in the preparation of the manuscript.

## References

[1] H. Kaplon, J.M. Reichert, Antibodies to watch in 2021, mAbs 13 (2021), 1860476.
[2] G. Walsh, Biopharmaceutical benchmarks 2018, Nat. Biotechnol. 36 (2018) 1136–1145.
[3] U. Brinkmann, R.E. Kontermann, The making of bispecific antiboides, mAbs 9 (2017) 182–212.
[4] R. Vazquez-Lombardi, T.G. Phan, C. Zimmermann, D. Lowe, L. Jermutus, D. Christ, Challenges and opportunities for non-antibody scaffold drugs, Drug Discov. Today 20 (2015) 1271–1283.
[5] N. Joubert, A. Beck, C. Dumontet, C. Denevault-Sabourin, Antibody–drug conjugates: the last decade, Pharmaceuticals 13 (2020) 245.
[6] A. Goyon, V. D'Atri, B. Bobaly, E. Wagner-Rousset, A. Beck, S. Fekete, D. Guillarme, Protocols for the analytical characterization of therapeutic monoclonal antibodies. I - Non-denaturing chromatographic techniques, J. Chrom. B 1058 (2017) 73–84.
[7] B. Bobaly, A. Goyon, V. D'Atri, O. Colas, A. Beck, S. Fekete, D. Guillarme, Protocols for the analytical characterization of therapeutic monoclonal antibodies. II – Enzymatic and chemical sample preparation, J. Chrom. B 1060 (2017) 325–335.
[8] V. D'Atri, A. Goyon, B. Bobaly, A. Beck, S. Fekete, D. Guillarme, Protocols for the analytical characterization of therapeutic monoclonal antibodies. III – Denaturing chromatographic techniques hyphenated to mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1096 (2018) 95–106.
[9] L. Verscheure, M. Oosterlynck, A. Cerdobbel, P. Sandra, F. Lynen, K. Sandra, Middle-up charcatreization of monclonal antibodies by online reduction liquid chromatography-mass spectrometry, J. Chrom. A 1637 (2021), 461808.
[10] V. D'Atri, A. Goyon, B. Bobaly, A. Beck, S. Fekete, D. Guillarme, Protocols for the analytical characterization of therapeutic monoclonal antibodies. III – Denaturing chromatographic techniques hyphenated to mass spectrometry, J. Chrom. B 1096 (2018) 95–106.
[11] T. Graf, K. Heinrich, I. Grunert, H. Wegele, M. Haberger, P. Bulau, M. Leiss, Recent advances in LC–MS based characterization of protein-based bio-therapeutics – mastering analytical challenges posed by the increasing format complexity, J. Pharm. Biomed. Anal. 186 (2020), 113251.
[12] S. Fekete, D. Guillarme, P. Sandra, K. Sandra, Chromatographic, electrophoretic, and mass spectrometric methods for the analytical characterization of protein biopharmaceuticals, Anal. Chem. 88 (2016) 480–507.
[13] F.D. Antia, Cs. Horvath, High performance liquid chromatography at elevated temperatures: examination of conditions for the rapid separation of large molecules, J. Chrom 435 (1988) 1–15.
[14] H. Chen, Cs. Horvath, High-speed high-performance liquid chromatography of peptides and proteins, J. Chrom. A 705 (1995) 3–20.
[15] B. Bobaly, M. Lauber, A. Beck, D. Guillarme, S. Fekete, Utility of a high coverage phenyl-bonding and wide-pore superficially porous particle for the analysis of monoclonal antibodies and related products, J. Chrom A 1549 (2018) 63–76.
[16] S. Fekete, A. Murisier, A. Beck, J. Lawhorn, H. Ritchie, B. Boyes, D. Guillarme, New wide-pore superficially porous stationary phases with low hydrophobicity applied for the analysis of monoclonal antibodies, J. Chrom. A 1642 (2021), 462050.
[17] D.V. McCalley, D. Guillarme, Evaluation of additives on reversed-phase chromatography of monoclonal antibodies using a 1000 Å stationary phase, J. Chrom A 1610 (2020), 460562.
[18] H. Lardeux, B.L. Duivelshof, O. Colas, A. Beck, D.V. McCalley, D. Guillarme, V. D'Atri, Alternative mobile phase additives for the characterization of protein biopharmaceuticals in liquid chromatography - mass spectrometry, Anal. Chim. Acta 1156 (2021), 338347.
[19] B. Bobaly, V. D'Atri, M. Lauber, A. Beck, D. Guillarme, S. Fekete, Characterizing various monoclonal antibodies with milder reversed phase chromatography conditions, J. Chrom. B 1096 (2018) 1–10.
[20] S. Fekete, B. Bobaly, J.M. Nguyen, A. Beck, J.-L. Veuthey, K. Wyndham, M.A. Lauber, D. Guillarme, Use of ultrashort columns for therapeutic protein separations. Part 1: theoretical considerations and proof of concept, Anal. Chem. 93 (2021) 1277–1284.
[21] S. Fekete, A. Murisier, J.M. Nguyen, M.J. Bolton, J. Belanger, A. Beck, J.-L. Veuthey, K. Wyndham, M.A. Lauber, D. Guillarme, Use of ultrashort columns for therapeutic protein separations. Part 2: designing the optimal column dimension for reversed-phase liquid chromatography, Anal. Chem. 93 (2021) 1285–1293.
[22] S. Fekete, S. Rudaz, J. Fekete, D. Guillarme, Analysis of recombinant monoclonal antibodies by RPLC: toward a generic method development approach, J. Pharm. Biomed. Anal. 70 (2012) 158–168.
[23] S. Fekete, I. Molnar, D. Guillarme, Separation of antibody drug conjugate species by RPLC: a generic method development approach, J. Pharm. Biomed. Anal. 137 (2017) 60–69.
[24] A. Sepsey, D.R. Nemeth, G. Nemeth, A. Felinger, Rate constant determination of interconverting enantiomers by chiral chromatography using a stochastic model, J. Chrom. A 1564 (2018) 155–162.
[25] B. Bobály, A. Beck, J. Fekete, D. Guillarme, S. Fekete, Systematic evaluation of mobile phase additives for the LC–MS characterization of therapeutic proteins, Talanta 136 (2015) 60–67.
[26] S. Fekete, S. Rudaz, J.-L. Veuthey, D. Guillarme, Impact of mobile phase temperature on recovery and stability of monoclonal antibodies using recent reversed-phase stationary phases, J. Sep. Sci. 34 (2012) 3113–3123.
[27] S. Fekete, A. Beck, E. Wagner, K. Vuignier, D. Guillarme, Adsorption and recovery issues of recombinant monoclonal antibodies in reversed-phase liquid chromatography, J. Sep. Sci. 38 (2015) 1–8.
[28] J.M. Nguyen, X. Liu, M. DeLoffi, A. Murisier, S. Fekete, D. Guillarme, M.A. Lauber, Aptamer-based immunoaffinity LC-MS using an ultra-short column for rapid attomole level quantitation of intact mAbs, Chromatogr. B Analyt. Technol. Biomed. Life Sci 1173 (2021) 122694.
[29] B.W.J. Pirok, D.R. Stoll, P.J. Schoenmakers, Recent developments in two-dimensional liquid chromatography: fundamental improvements for practical applications, Anal. Chem. 91 (2019) 240–263.
[30] S. Jaag, M. Shirokikh, M. Lämmerhoffer, Charge variant analysis of protein-based biopharmaceuticals using two-dimensional liquid chromatography hyphenated to mass spectrometry, J. Chrom. A 1636 (2021), 461786.