Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 24–32

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Analysis of recombinant monoclonal antibodies in hydrophilic interaction chromatography: A generic method development approach

Balázs Bobály[a], Valentina D'Atri[a], Alain Beck[b], Davy Guillarme[a], Szabolcs Fekete[a,*]

[a] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Centre Médical Universitaire (CMU), Rue Michel-Servet 1, 1206 Geneva, Switzerland

[b] Institut de Recherche Pierre Fabre, Centre d'Immunologie, 5 Avenue Napoléon III, BP 60497, 74160 Saint-Julien-en-Genevois, France[1]

ARTICLE INFO

Article history:
Received 12 May 2017
Received in revised form 7 June 2017
Accepted 11 June 2017
Available online 15 June 2017

Keywords:
HILIC
Method development
Monoclonal antibody
Recovery
Drylab

ABSTRACT

Hydrophilic interaction liquid chromatography (HILIC) is a well-established technique for the separation and analysis of small polar compounds. A recently introduced widepore stationary phase expanded HILIC applications to larger molecules, such as therapeutic proteins. In this paper, we present some generic HILIC conditions adapted for a wide range of FDA and EMA approved recombinant monoclonal antibody (mAb) species and for an antibody-drug conjugate (ADC). Seven approved mAbs possessing various isoelectric point (pI) and hydrophobicity as well as a cysteine conjugated ADC were used in this study. Samples were digested by IdeS enzyme and digests were further fragmented by chemical reduction. The resulting fragments were separated by HILIC. The main benefit of HILIC was the separation of polar variants (glycovariants) in a reasonable analysis time at the protein level, which is not feasible with other chromatographic modes. Three samples were selected and chromatographic conditions were further optimized to maximize resolution. A commercial software was used to build up retention models. Experimental and predicted chromatograms showed good agreement and the average error of retention time prediction was less than 2%. Recovery of various species and sample stability under the applied conditions were also discussed.

1. Introduction

Hydrophilic interaction chromatography (HILIC) is an important and popular complementary separation method to reversed phase liquid chromatography (RPLC). It can be utilized in optimizing information regarding the constituents of a polar mixture, especially of small molecules [1–5].

Thanks to recent developments in column technology, widepore HILIC phases are now commercially available and enables the separation of peptides, protein fragments and intact proteins with high efficiency [6,7]. Several highly-relevant samples such as insulins, interferon alpha-2b and monoclonal antibody (mAb) have been recently analysed in HILIC [6]. It was shown that a mobile phase composition between 80 and 65% acetonitrile in presence of 0.1% trifluoroacetic acid (TFA) provided optimal conditions to retain proteins and obtain appropriate peak shapes. The selectivity of these HILIC separations have proven to be highly orthogonal to RPLC and some hydrophilic protein-variants (mAb glycoforms) were better resolved in HILIC than in RPLC.

HILIC has already been applied in the past to the field of biopharmaceuticals for released glycan profiling and glycopeptide separations [8,9]. Wide-pore HILIC phases now offer new possibilities in glycan analysis at intact or middle-up levels of analysis [7,10]. This approach also allows the qualitative comparison of the glycosylation profiles between originator and biosimilar products. By evaluating the chromatographic profile of the HILIC middle-up analysis, it was possible to quickly assess the most abundant glycosylation moieties and to highlight the differences between an originator and a biosimilar [7].

HILIC offers several additional benefits for biopharmaceutical characterization, as inherent compatibility with MS (better sensitivity in many cases compared to RPLC [3,11]), the use of moderate mobile phase temperature for several proteins that are poorly recovered in RPLC, and the possibility to couple several columns in series to improve resolving power (peak capacity), thanks to comparatively low mobile phase viscosity [6].

* Corresponding author.
E-mail address: szabolcs.fekete@unige.ch (S. Fekete).
[1] www.cipf.com.

http://dx.doi.org/10.1016/j.jpba.2017.06.016
0731-7085/

In spite of the potential benefits of HILIC, chromatographers often fear this method because the retention mechanism is more complex than in RPLC and very sophisticated retention models are often required for method development. Trial and error method development approach is still usually performed for HILIC separations. HILIC retention can be considered as a mixed-mode mechanism, combining hydrophilic partitioning, adsorption through hydrogen bonds and various types of possible electrostatic and ionic interactions [12] which may be attractive as well as repulsive [13]. Therefore HILIC retention models do not follow a perfect linear relationship in most cases [14]. Polynomial, empirical and mixed retention models are often applied for HILIC method development [12,14–16]. Such models regularly require several initial experimental runs, which make this approach time-consuming. Recently some quantitative structure-retention relationship (QSRR) based approaches were also reported for HILIC method development and retention prediction [3,17–19]. In these studies, retention modeling was restricted to small molecules.

The aim of this study was to propose a generic method development approach for the HILIC separation of mAb subunits and their hydrophilic variants. The impact of gradient steepness and mobile phase temperature on retention as well as on recovery were investigated in detail. A generic approach based on linear relationships and four initial experimental runs was suggested to provide good accuracy and a fast procedure in the practically useful range of the method variables. The impact of sample solvent on mAb subunits' peak shape was also discussed.

2. Experimental

2.1. Chemicals and samples

Acetonitrile and water were purchased from Fisher Scientific (Reinach, Switzerland). Trifluoroacetic acid (TFA), dithiothreitol (DTT) and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Sigma-Aldrich (Buchs, Switzerland). IdeS (FabRICATOR®) was purchased from Genovis AB (Lund, Sweden). FDA and EMA approved monoclonal antibody (cetuximab, rituximab, panitumumab, ipilimumab, denosumab) and antibody-drug conjugate (brentuximab vedotin) samples were kindly provided by the Center of Immunology Pierre Fabre (Saint-Julien-en-Genevois, France). NISTmAb was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.2. Sample preparation

The main advantage of HILIC separation is the possible characterization of polar mAb species, such as glycovariants, which represent one of the most important polar modifications of biopharmaceutical products [20]. Due to their poor molecular diffusion properties, the separation of polar variants at intact protein level is hardly feasible. To separate glycovariants, mAbs can therefore be fragmented into smaller subunits [21]. Except in few cases, glycosylation takes place only on the fragment crystallizable (Fc) region. Glycosylated fragments can be cleaved from the antigen binding region ($F(ab')_2$) with the IdeS enzyme [22]. IdeS cleaves under interchain disulfides of the hinge region resulting in Fc/2 (or single chain Fc, sFC) and $F(ab')_2$ fragments. The molecular size of the resulting $F(ab')_2$ fragment can be further decreased to get light chain (LC) and Fd subunits, by chemical reduction. After this combined sample preparation of IdeS digestion and reduction, the sample contains glycosylated Fc/2, LC and −in certain cases glycosylated- Fd fragments. The resulting pool of these ~25 kDa fragments is favourable for chromatographic and mass spectrometric characterization [22].

5000 U lyophilized IdeS enzyme was reconstituted in 75 μL water (67 U/μL). 2 μL of this IdeS solution was added to 100 μL mAb or ADC solution, which was previously diluted from the commercial formulation in 10 mM pH 7.5 TRIS buffer to get 1 mg/mL protein concentration. Samples were incubated and mixed at 45 °C for 30 min. After digestion, samples were reduced by adding DTT. 1 M DTT solution was freshly prepared prior to sample preparation. 10 μL 1 M DTT was then added to the previously digested sample to have approximately 100 mM DTT concentration in the sample solution. Reduction was performed in the same thermomixer at 45 °C for 30 min. Finally, samples were diluted in acetonitrile containing 0.1% TFA to have 60% organic solvent in the sample solution. No precipitation was observed and samples were stable for at least 2 days at 4 °C.

2.3. Chromatographic system

Samples were analyzed using a Waters Acquity UPLC I-Class® system equipped with a binary solvent delivery pump, an autosampler (possessing flow-through needle (FTN) injection port with a 15 μL needle) and FL detector. The dwell volume was experimentally measured as 110 μL. Data acquisition, data handling and instrument control were performed by Empower 2. The Acquity UPLC Glycoprotein BEH Amide chromatographic column (150 mm × 2.1 mm, 1.7 μm, 300 Å) was purchased from Waters (Milford, MA, USA).

Mobile phase A was 0.1% TFA in water, mobile phase B was 0.1% TFA in acetonitrile. A volume of 0.5 μL was injected using a generic linear gradient from 75% to 60% B in 15 min. Gradients were further optimized in some cases. Flow rate was set to 0.45 mL/min, mobile phase temperature was 80 °C, unless otherwise mentioned. Purge solvent was 85% and wash solvent was 50% acetonitrile in water. Data were acquired using 280 nm excitation and 360 nm emission wavelengths with 5 Hz sampling rate and 0.4 s time constant. Data were processed using Excel and Drylab (4.2) software.

3. Results and discussion

3.1. Development of a general HILIC gradient

The solvent generally used as the injection solvent is an aqueous buffer, which may result in solvent mismatch and distorted peaks when injecting into highly organic mobile phase in HILIC [23,24]. This is due to the high solvent strength of water in HILIC conditions which may decrease the peak focusing effect at the column inlet. In our previous studies, it was shown that a fast and steep gradient ramp at the beginning of the chromatographic run can compensate for solvent incompatibility [6,7]. The initial highly organic mobile phase composition supported the appropriate adsorption of the sample components at the column inlet, which were then eluted in regular, symmetrical peaks. The absence of this initial gradient ramp resulted in seriously fronting peaks of proteins when injecting from aqueous solvents.

The present study was aimed to propose a general HILIC method development approach for the characterization of monoclonal antibodies and related proteins. Previous studies showed the power of quality by design (QbD) based method development approach [25–31]. This methodology is based on the prediction of retention times based on initial experimental runs as input data for retention models. Retention modeling software can handle only linear gradients as input data. As being a multilinear segmented gradient, the previously used initial gradient rump is not suitable for chromatographic retention prediction. To address this problem, samples were diluted in acetonitrile containing 0.1% TFA. TFA was supposed to have stabilizing effect on protein species

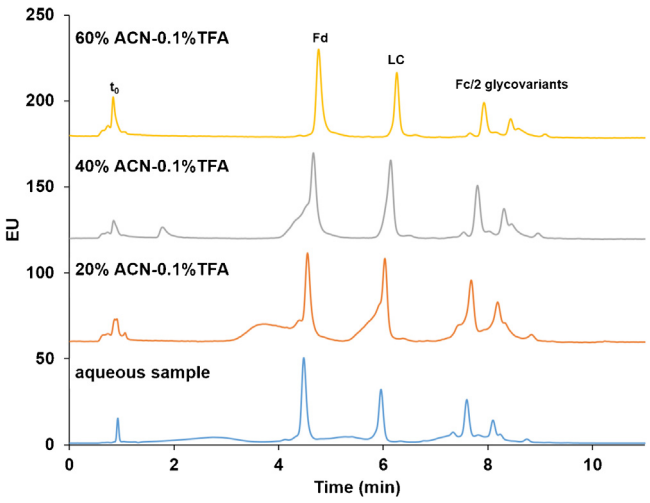

Fig. 1. HILIC chromatograms of IdeS digested and reduced trastuzumab when injecting from different sample solvents and using linear gradient without initial fast gradient ramp. Gradient: 74–68%B in 15 min. A: H2O + 0.1% TFA, B ACN + 0.1% TFA. Other chromatographic conditions are detailed in Section 2.3.

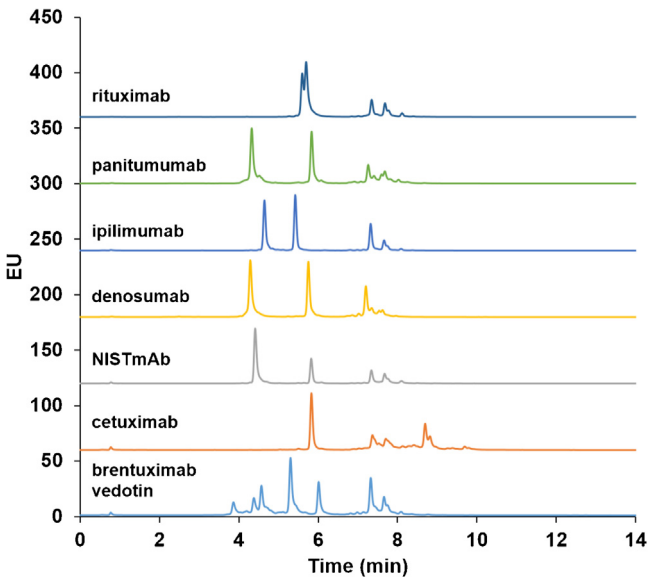

Fig. 2. General HILIC chromatograms of IdeS digested and reduced mAb and ADC samples. Gradient: 75–60%B in 15 min. A: H_2O + 0.1% TFA, B ACN + 0.1% TFA. T: 80 °C, flow: 0.45 mL/min. Column: Waters Acquity UPLC Glycoprotein BEH Amide (300 Å, 1.7 μm, 2.1 mm × 150 mm).

in highly organic solutions and is considered as a good solubilizing agent at low concentrations (e.g. ~0.1%). Then simple linear gradient from 74% to 68% B in 10 min was applied and various trastuzumab sample solutions were injected containing 0, 20, 40 and 60% organic solvent. In this chromatographic setup, samples containing less than 60% acetonitrile showed fronting peaks indicating insufficient focusing of the sample zone at the column inlet. Fig. 1 shows that acceptable peak shapes were observed with solvent containing 60% acetonitrile. It is worth noting that addition of acetonitrile may potentially effect the conformation and so the retention behaviour of the solute being analyzed. In this case this effect seemed to be negligible, since the chromatograms obtained with 60% organic solvent composition was highly comparable with the reference chromatograms using the initial gradient ramp and aqueous sample solvent [6]. Flourescence emission spectra of peaks eluting close to column dead time were compared to protein peaks and confirmed as not being of protein origin. Based on these preliminary results, digested and reduced samples were diluted in 60% acetonitrile containing 0.1% TFA. Preliminary experiments showed that a 75-60%B linear gradient provides acceptable retention for many mAbs. For this reason, a simple linear gradient was run from 75% to 60% B in 15 min to study the applicability of generic HILIC conditions. Various additional mAb samples were selected according to their diverse physicochemical properties. Rituximab (pI of ~9.1) and panitumumab (pI of ~6.7) are intended to represent basic and acidic mAbs, respectively [26]. Ipilimumab is hydrophobic, while denosumab can be considered as a relatively hydrophilic mAb [32]. NISTmAb is currently the only universal standard reference material (SRM) for monoclonal antibody therapeutics. NISTmAb is a widely available, well characterized and well documented humanized IgG1κ monoclonal antibody, which makes it a promising supplement to in-house developed product-specific reference standards [33–37]. It is expected to greatly facilitate qualitative and quantitative drug substance/product characterisation [38]. Thus, the inclusion of NISTmAb into upcoming studies may further improve the comparability and so the quality of the published data. Cetuximab is glycosylated in both the Fc and Fd region, resulting in complex elution profile for the glycovariants [7,10]. Brentuximab vedotin is currently the only marketed antibody-drug conjugate using cysteine conjugation chemistry. Fig. 2 shows general HILIC chromatograms of reduced and digested samples after sample dilution. As expected, all the fragments were eluted within an acceptable elution window with reasonable peak shape, regardless of their acidic/basic properties or hydrophobicity.

Peaks were eluted in the following order: Fd fragment first, followed by the LC and finally by the Fc/2 glycovariants, except for cetuximab and brentuximab vedotin that showed a different elution order, which annotation is described in Section 3.2 and represented in Fig. 3B.

3.2. Recovery of mAb species in HILIC conditions

The prerequisite of an unbiased separation relies on obtaining an acceptable recovery for all the species since it can influence the evaluation of the chromatograms. Higher temperature generally enhances mass transfer for proteins, leading to higher separation performance. In addition, temperature is a common parameter in chromatographic method development since selectivity and resolution can be tuned by changing this variable [25,27,29,31,39]. For the analysis of proteins under RPLC conditions, temperature affects solute adsorption on the stationary phase. Previous studies showed that intact mAbs, as well as mAb subunits, show poor recovery in RPLC conditions when working at moderate temperature (e.g. ≤80 °C) [31,40,41], thus demonstrating the need to work at 80–90 °C to avoid adsorption issues and reach acceptable recovery (e.g. above 90% of the injected protein amount). Conversely, it was also reported that in HILIC, lower temperature (e.g. 50–60 °C) might result in appropriate recovery [6]. Before starting method development and considering temperature as a variable, its effect on recovery was thus investigated. Recovery of digested and reduced NISTmAb, cetuximab, and brentuximab vedotin subunits were monitored and relative recoveries of the main peaks were calculated (peak areas of the subunits were normalized to their largest peak areas obtained at different temperatures [40]). Fig. 3A shows the relative recovery (recovery was calculated from peak areas normalized to the highest observed peak area [40]) of the mAb subunits between 40 and 90 °C. Peak areas were measured in duplicates and their deviation remained always below 1–2%. Dotted black line represents 90% relative recovery.

Recovery of NISTmAb subunits (Fig. 3A, first panel) was higher than 90% above 70 °C, and remains still higher than 70% at 40 °C. Similarly, cetuximab showed an acceptable recovery above 70 °C

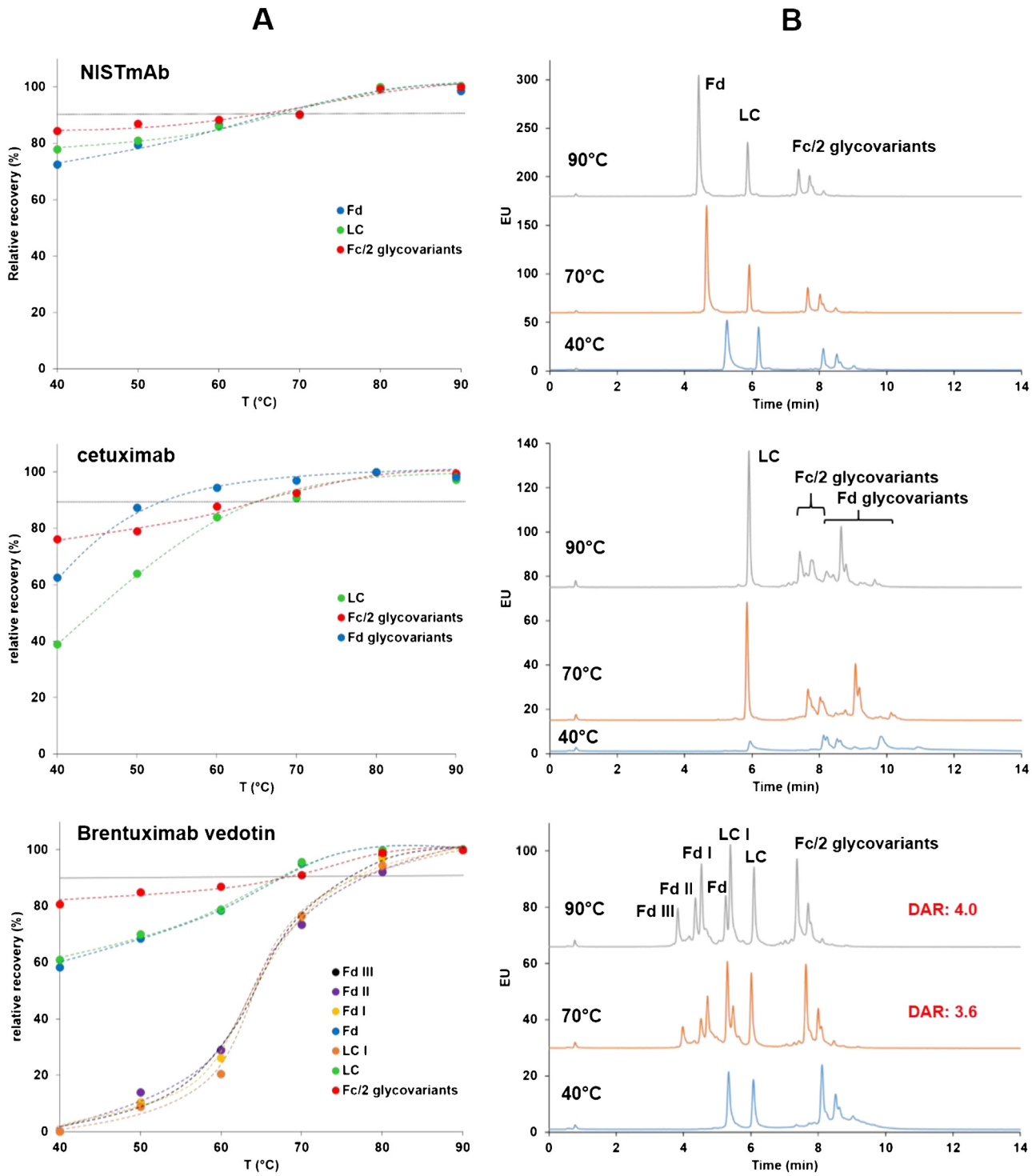

Fig. 3. (A) Relative recovery of NISTmAb, cetuximab and brentuximab vedotin subunits as a function of mobile phase temperature. (B) Corresponding chromatograms at 40 °C, 70 °C and 90 °C. Gradient: 75–60%B in 15 min. A: H_2O + 0.1% TFA, B: ACN + 0.1% TFA. Flow: 0.45 mL/min. Column: Waters Acquity UPLC Glycoprotein BEH Amide (300 Å, 1.7 μm, 2.1 mm × 150 mm).

(Fig. 3A, second panel), but a lower recovery of the LC and the Fd glycovariants to 39% and 62% was observed at 40 °C. Brentuximab vedotin has several highly hydrophobic loaded fragments due to cytotoxic drugs attached to them (LC I, Fd I, Fd II and Fd III fragments, with I, II and III corresponding to the number of bound payloads). The proper recovery of these loaded subunits is important for the estimation of the average drug-to-antibody ratio (DAR), which is a critical quality attribute (CQA) of the antibody drug conjugates [31,42–44]. Average DAR can be calculated based on the formula reported by Wagner-Rousset et al. [45] and it is generally close to 4.0 [46,47]. Fig. 3A (third panel) shows that at least 80 °C is required to achieve 90% recovery of the loaded subunits, whereas some of the subunits were completely adsorbed at 40 °C. Interestingly, the adsorption behaviour of the three different categories of subunits (glycosylated, loaded and naked) can clearly be differentiated, with the most critical group represented by the loaded species, and the

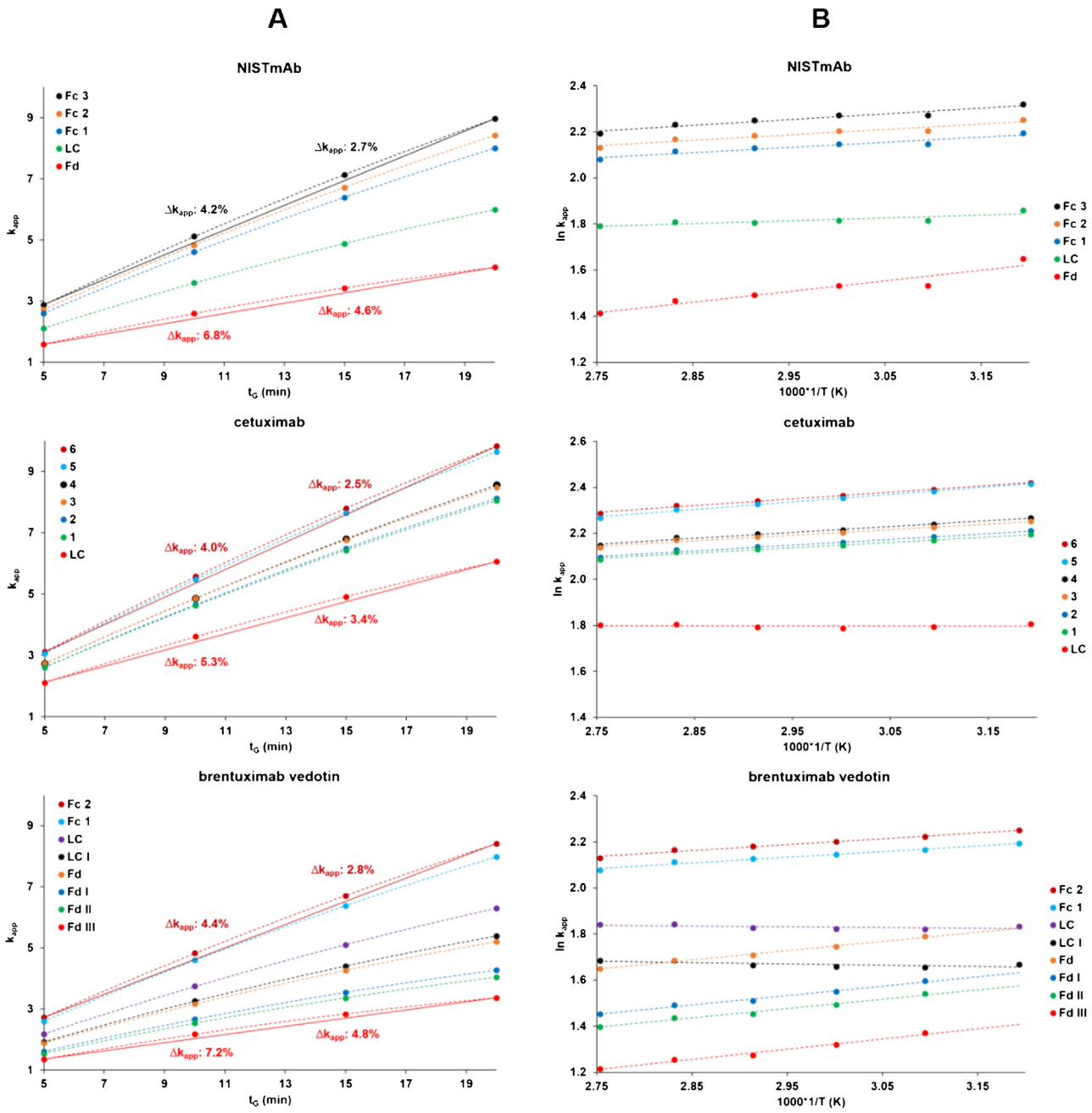

Fig. 4. (A) Retention as a function of gradient time, (B) Van't Hoff plots of NISTmAb, cetuximab and brentuximab vedotin subunits. Gradient: 75-60%B. A: H2O + 0.1% TFA, B: ACN + 0.1% TFA, flow: 0.45 mL/min. Column: Waters Acquity UPLC Glycoprotein BEH Amide (300 Å, 1.7 μm, 2.1 mm × 150 mm). Other chromatographic conditions are detailed in Section 3.3.

most hydrophilic glycosylated subunits showing the highest recovery in all cases. Average DAR calculation was not possible at 40 °C, whereas it resulted in 3.6 at 70 °C and 4.0 at 90 °C, meaning that evaluation of mild temperature chromatograms might be heavily biased by incomplete recovery of loaded species. Fig. 3B shows the chromatograms of NISTmAb, cetuximab and brentuximab vedotin subunits obtained at 40, 70 and 90 °C. Peaks were annotated based on previous studies [7,31]. Finally, recovery may not be sufficient at lower temperature and this behaviour also depends on the selected protein. For the selected mAbs and ADCs species, a minimal temperature of 70–80 °C is recommended for HILIC operation, which should provide acceptable recovery for most mAbs. In special cases – such as brentuximab vedotin – highly hydrophobic loaded species may suffer from strong adsorption and necessitates to work at high temperature (80–90 °C). In addition, retention times and chromatographic profiles may slightly vary in HILIC during first injections when using brand new columns. Saturation of active sites by serial injections of concentrated protein samples might be necessary prior to run the sequence on a new column. This can be monitored by the stabilization of retention times and elution profiles. According to our experience, carefully equilibrated and properly saturated HILIC columns provide comparable retention time repeatability, as usually observed in reversed phase conditions.

3.3. *Retention properties of mAb subunits in HILIC*

Digested and reduced samples of NISTmAb, cetuximab and brentuximab vedotin were eluted in HILIC conditions using lin-

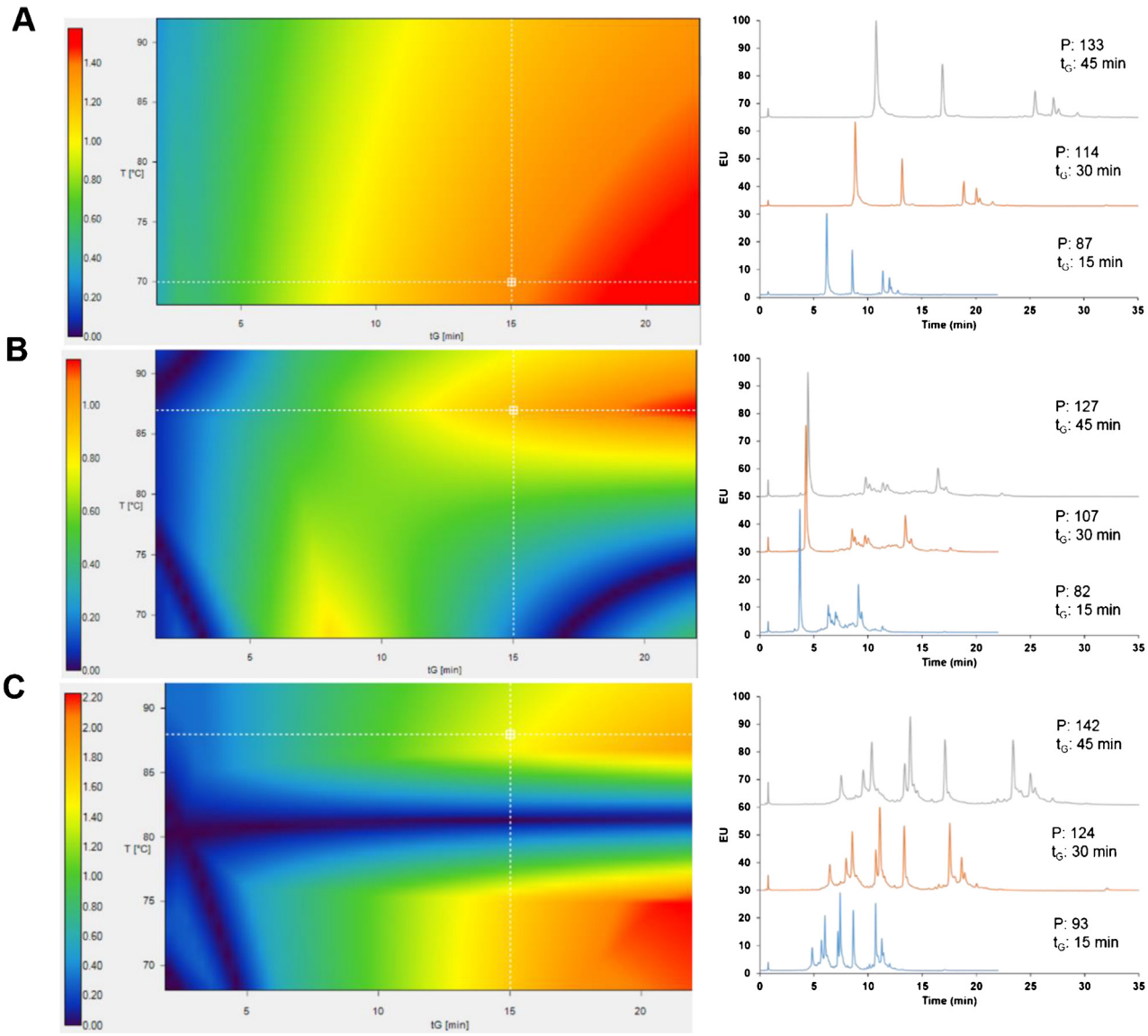

Fig. 5. Resolution maps and peak capacities at different gradient times of (A) NISTmAb, (B) cetuximab and (C) brentuximab vedotin using optimized gradient conditions described in Section 3.4.

ear gradients. The effect of gradient steepness and temperature on retention and selectivity was studied. Results of these preliminary studies were used to perform a QbD based method development, as detailed in Section 3.4.

First, the effect of gradient steepness on retention and selectivity was evaluated. A generic linear gradient starting from 75% to 60%B was applied; flow rate was set to 0.45 mL/min and temperature was set to 90 °C. It was assumed that using a relatively limited, practically relevant design space, a linear or nearly linear correlation between the gradient time and retention can be observed (linear solvent strength model like behaviour for $k_{app}(t_G)$). Four gradient times of 5, 10, 15 and 20 min were set to study the retention (t_G/t_0 = 7.0, 14.1, 21.1 and 28.2, respectively). Fig. 4A shows the apparent retention factors (k_{app}) plotted against gradient time (t_G). The peaks selected for constructing the plots are shown in Fig. S1 and comprise the Fd and LC fragments plus three abundant Fc/2 glycovariants for NISTmAb; the LC fragment and six intense glycovariants peaks for cetuximab; and conjugated and unconjugated subunits as well as the two most abundant Fc/2 glycovariants for brentuximab vedotin. Fig. 4A shows the impact of gradient time (steepness) on retention, which can be approached by a quadratic function in all cases. Deviations from linear fit are shown on the diagrams in percentages. Deviation seemed to decrease with increased retention. Selectivity slightly increased in some cases (up to 9.6% for NISTmAb, 7.5% for cetuximab and 6.3% for brentuximab vedotin peak pairs, when changing gradient time from 5 to 20 min), but elution order remained the same with longer gradient times. Selectivity change of the selected peaks are detailed in supporting information.

The effect of mobile phase temperature on retention was evaluated. The same chromatographic conditions were used, as previously described in this section. Gradient time was fixed at 20 min and temperature varied from 40 to 90 °C with 10 °C increments. The constructed Van't Hoff plots are shown in Fig. 4B. Linear fits properly approached the experimental points. In the case of NISTmAb and cetuximab, retention order of the peaks did not change with temperature. Taking into account the restricted range of applicable temperature between 70 and 90 °C – which is mandatory to reach appropriate recovery – this factor may be used for fine tuning of NISTmAb and cetuximab subunits resolution. Concerning the digested and reduced brentuximab vedotin, the presence of glycosylated, loaded and naked species, having very different

elution properties, turns the temperature into a more interesting chromatographic variable, as respected to the two other tested samples. This ADC sample should be injected at least at 80 °C to get 90% recovery for all peaks. On the other hand, a coelution of Fd and LC I peaks occurs at 80 °C due to the selectivity change of this two distinct peaks between 70 and 90 °C. This temperature range was used only to explore retention properties. With respect to recovery, practical temperature range was restricted to 80–90 °C. For this particular sample, temperature is not only useful to tune the selectivity, it can even change the elution order. It has been previously discussed in Section 3.2 that adsorption properties (recovery) of similar solutes can be categorized according to their structure (e.g. LCs, Fc/2 glycovariants, conjugated fragments). Similar observation was done when studying the impact of temperature on retention properties. Slopes of the Van't Hoff plots ($\ln(k_{app})$*1000/T) at 95% confidence level are 0.425 ± 0.025 (n = 5) for Fd fragments, 0.245 ± 0.008 (n = 9) for Fc/2 glycovariants and 0.007 ± 0.126 (n = 4) for LCs. The mean slope for the two considered glycosylated cetuximab Fd peaks was 0.309. Fragments possessing various nature (e.g. conformational-dependent response to temperature changes, hydrophobicity, etc.) may interact differently with the stationary phase, which might lead to the observed retention behaviour. Slopes of Van't Hoff plots indicate the contribution of enthalpy to the retention process in the following order: Fd fragments > Fd glycosylated fragments > Fc fragments > LCs. Whereas contribution of entropy can be ranked by the intercept of the Van't Hoff plots: Fc and Fd glycosylated fragments > LCs > Fd fragments at 90 °C.

3.4. Method development in 2D experimental domain

Based on the results of the preliminary experiments, a two dimensional experimental design ($t_G \times T$) is suggested. A linear model was applied for the gradient time (quadratic gradient time model was not available) in the limited – practically relevant – range. The impact of both parameters was studied at two levels. Temperature was set to 70 °C and 90 °C, while gradient time varied between 10 and 20 min. Gradient window was 75–60% B. Other chromatographic conditions remained unchanged. Peaks possessing area ratio higher than 1% have been included in the model. This simple model requires only 4 experimental runs/sample, and just 1 working day for performing the initial runs, optimization and verification of 3 samples. The calculated resolution maps are shown in Fig. 5. First, the gradient window was adjusted with the help of the modeling software. Starting mobile phase composition from 75%B was modified only for cetuximab, since the later eluting LC fragment enabled the setting of the initial mobile phase composition to 72%B without occurring peak distortion. For NISTmAb and brentuximab vedotin, starting mobile phase composition from 73%B and 74%B were initially calculated to eliminate the empty chromatographic window at the beginning of the run and expand the gradient span. However, below 75%B, peak fronting was observed for these two samples, presumably due to inappropriate focusing at the column inlet. For this reason, starting mobile phase composition for NISTmAb and brentuximab vedotin samples was kept at 75%B and only the final mobile phase composition was optimized. Based on the calculated resolution maps, gradient times of 15 min seemed to be a good compromise between resolution and analysis time in all cases. Optimized gradient conditions from the red-orange areas on Fig. 5 (these parts of the design plane show the best available resolution for the closest eluting peak pair) were determined as follows: gradient of 75–67%B and T = 70 °C for NISTmAb with a resolution of the closest eluting peak pair (Rs_{crit}) of 1.36; 72–66%B and T = 87 °C for cetuximab with Rs_{crit} = 0.94; and finally, 75–67%B and T = 88 °C for brentuximab vedotin with Rs_{crit} = 1.19. The resolution map of brentuximab vedotin showed a promising region below 75 °C, but recovery of loaded species was poor in this temperature range. This map is horizontally bisected by a low resolution zone, which corresponds to the coelution of Fd and LC I at around 80 °C. Predicted chromatograms at the working points (optimized conditions) were validated experimentally. Predicted and experimental retention times showed good agreement with an average deviation of retention times equal to 0.78% for NISTmAb, 1.57% for cetuximab and 2.17% for brentuximab vedotin species. Based on model approximation errors (linear retention fit instead of quadratic, see Fig. 4) and on previous studies [14,48], these values are clearly acceptable for HILIC retention prediction. As shown by the resolution maps, resolution can subsequently be improved through the increase of gradient time without significant changes in selectivity. Additionally, this observation was in line with retention behaviours observed in preliminary experiments (see Fig. 4A). Therefore, gradient times were increased from 15 to 30 and 45 min using the optimized chromatographic conditions and peak capacities were compared. Peak capacity is a good indicator of the chromatographic resolving power under gradient elution conditions. It represents the number of symmetrical peaks which can be theoretically separated in the applied gradient window with unique resolution. Peak capacity was calculated as reported in previous studies [49–51] and chromatograms were compared in Fig. 5. Increasing gradient time from 15 to 30 min enhanced peak capacity by around 30%. A further increase of gradient time to 45 min resulted in an additional 20% increase in peak capacity. Indeed, resolutions of consecutive peaks were notably enhanced, while selectivity was kept nearly the same. It is worth mentioning here that in spite of using long gradient times and high temperatures with strongly acidic mobile phase, no sample degradation was observed on the chromatograms. Conversely, significant degradation was already observed under reversed phase conditions [40]. The absence of this phenomenon might be explained by the fact that the samples were different between the previous study and this one, and above all by the strong differences in mobile phase composition (highly aqueous in RPLC vs. highly organic in HILIC), which may influence protein stability. Indeed, water is generally known as more reactive media than acetonitrile.

4. Conclusion

HILIC separates protein variants based on their differences in hydrophilic properties, which may co-elute under RPLC conditions. This feature can beneficially be utilized for the rapid evaluation of the glycosylation profile of mAbs and ADCs. Moreover, glycosylation profile and average DAR of ADCs can be evaluated from a single chromatogram. These HILIC benefits are clearly demonstrated in this study. A generic linear gradient was developed for mAb and ADC subunits using sample dilution to avoid solvent mismatch commonly observed in HILIC. Samples were digested by IdeS enzyme and then reduced by DTT to get LC, Fc/2, and Fd fragments for subsequent HILIC separation. The proposed HILIC conditions were verified by analyzing a wide range of mAbs including acidic, basic, hydrophilic and hydrophobic ones, as well as a cysteine conjugated ADC and a reference standard mAb. First, recovery and retention behaviour of the protein species were evaluated. Recovery of mAb fragments was poor below 60–70 °C, while for loaded ADC fragments at least 80 °C is required to reach 90% relative recovery. In a limited range of conditions, retention can be approached by linear functions which allowed a simple retention modeling. To our knowledge this is the first study presenting a quality by design (QbD) based generic method development approach for HILIC characterization of antibodies and conjugates. An experimental design using two factors (temperature and gradient time) at two levels was proposed. A commercial chromatographic modeling software was

used to predict optimum conditions for the separation of protein subunits based on only 4 initial experimental runs/sample. Using this relatively simple experimental design, method optimization for three different samples took only one working day. Predicted and experimental chromatograms were in good agreement and the average error in retention time prediction was less than 2%. Resolution of the peaks was further enhanced by extending gradient time. No sample degradation was observed at higher temperatures up to 80–90 °C and longer gradients up to 45 min. Based on these results HILIC is a promising chromatographic approach for therapeutic protein characterization. New widepore HILIC materials with different morphologies (e.g. core-shell) are expected to appear, which may open new ways in the analysis of biopharmaceuticals.

Acknowledgements

Davy Guillarme wishes to thank the Swiss National Science Foundation for support through a fellowship to Szabolcs Fekete (31003A_159494). Jean-Luc Veuthey from the University of Geneva is acknowledged for his support and discussions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at **http://dx.doi.org/10.1016/j.jpba.2017.06.016**.

References

[1] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, J. Chromatogr. A 499 (1990) 177–196.
[2] A. Periat, I.S. Krull, D. Guillarme, Applications of hydrophilic interaction chromatography to amino acids, peptides and proteins, J. Sep. Sci. 38 (2015) 357–367.
[3] M. Taraji, P.R. Haddad, R.I.J. Amos, M. Talebi, R. Szucs, J.W. Dolan, C.A. Pohl, Rapid method development in hydrophilic interaction liquid chromatography for pharmaceutical analysis using a combination of quantitative structure-retention relationships and design of experiments, Anal. Chem. 89 (3) (2017) 1870–1878.
[4] A. Periat, J. Boccard, J.L. Veuthey, S. Rudaz, D. Guillarme, Systematic comparison of sensitivity between hydrophilic interaction liquid chromatography and reversed phase liquid chromatography coupled with mass spectrometry, J. Chromatogr. A 1312 (2013) 49–57.
[5] A. Kumar, J.C. Heaton, D.V. McCalley, Practical investigation of the factors that affect the selectivity in hydrophilic interaction chromatography, J. Chromatogr. A 1276 (2013) 33–46.
[6] A. Periat, S. Fekete, A. Cusumano, J.-L. Veuthey, A. Beck, M. Lauber, D. Guillarme, Potential of hydrophilic interaction chromatography for the analytical characterization of protein biopharmaceuticals, J. Chromatogr. A 1448 (2016) 81–92.
[7] V. D'Atri, S. Fekete, A. Beck, M. Lauber, D. Guillarme, Hydrophilic interaction chromatography hyphenated with mass spectrometry: a powerful analytical tool for the comparison of originator and biosimilar therapeutic monoclonal antibodies at the middle-up level of analysis, Anal. Chem. 89 (2017) 2086–2092.
[8] M. Mancera-Arteu, E. Gimenez, J. Barbosa, V. Sanz-Nebot, Identification and characterization of isomeric N-glycans of human alfa-acid-glycoprotein by stable isotope labelling and ZIC-HILIC-MS in combination with exoglycosidase digestion, Anal. Chim. Acta 940 (2016) 92–103.
[9] J. Ahn, J. Bones, Y.Q. Yu, P.M. Rudd, M. Gilar, Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent, J. Chromatogr. A 878 (2010) 403–408.
[10] M.A. Lauber, S.M. Koza, Mapping IgG Subunit Glycoforms Using HILIC and a Wide-pore Amide Stationary Phase, 2015, Waters application note 720005385EN.
[11] A. Periat, I. Kohler, A. Bugey, S. Bieri, F. Versace, C. Staub, D. Guillarme, Hydrophilic interaction chromatography versus reversed phase liquid chromatography coupled to mass spectrometry: effect of electrospray ionization source geometry on sensitivity, J. Chromatogr. A 1356 (2014) 211–220.
[12] G. Greco, S. Grosse, T. Letzel, Study of the retention behavior in zwitterionic hydrophilic interaction chromatography of isomeric hydroxy- and aminobenzoic acids, J. Chromatogr. A 1235 (2012) 60–67.
[13] A.J. Alpert, Electrostatic repulsion hydrophilic interaction chromatography for isocratic separation of charged solutes and selective isolation of phosphopeptides, Anal. Chem. 80 (2008) 62–76.
[14] E. Tyteca, A. Périat, S. Rudaz, G. Desmet, D. Guillarme, Retention modeling and method development in hydrophilic interaction chromatography, J. Chromatogr. A 1337 (2014) 116–127.
[15] U.D. Neue, H.J. Kuss, Improved reversed-phase gradient retention modeling, J. Chromatogr. A 1217 (2010) 3794–3803.
[16] G. Jin, Z. Guo, F. Zhang, X. Xue, Y. Jin, X. Liang, Study on the retention equation in hydrophilic interaction liquid chromatography, Talanta 76 (2008) 522–527.
[17] M. Taraji, P.R. Haddad, R.I.J. Amos, M. Talebi, R. Szucs, J.W. Dolan, C.A. Pohl, Prediction of retention in hydrophilic interaction liquid chromatography using solute molecular descriptors based on chemical structures, J. Chromatogr. A 1486 (2017) 59–67.
[18] S.L. Maux, A.B. Nongonierma, R.J. FitzGerald, Improved short peptide identification using HILIC–MS/MS: Retention time prediction model based on the impact of amino acid position in the peptide sequence, Food Chem. 173 (2015) 847–854.
[19] E. Tyteca, M. Talebi, R. Amos, S.H. Park, M. Taraji, Y. Wen, R. Szucs, C.A. Pohl, J.W. Dolan, P.R. Haddad, Towards a chromatographic similarity index to establish localized quantitative structure-retention models for retention prediction: use of retention factor ratio, J. Chromatogr. A 1486 (2017) 50–58.
[20] D. Reusch, M.L. Tejada, Fc glycans of therapeutic antibodies as critical quality attributes, Glycobiology 25 (2015) 1325–1334.
[21] E. Largy, F. Cantais, G. Van Vyncht, A. Beck, A. Delobel, Orthogonal LC/MS methods for the comprehensive characterization oftherapeutic glycoproteins, from released glycans to intact protein level, J. Chromatogr. A 1498 (2017) 128–146.
[22] J. Sjörgen, F. Olsson, A. Beck, Rapid and improved characterization of therapeutic antibodies and antibody related products using IdeS digestion and subunit analysis, Analyst 141 (2016) 3114–3125.
[23] J.C. Heaton, D.V. McCalley, Some factors that can lead to poor peak shape in hydrophilic interaction chromatography, and possibilities for their remediation, J. Chromatog. A 1427 (2016) 37–44.
[24] F. Gritti, J. Sehajpal, J. Fairchild, Using the fundamentals of adsorption to understand peak distortion due to strong solvent effect in hydrophilic interaction chromatography, J. Chromatogr. A 1479 (2017) 95–106.
[25] 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.
[26] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part I: Salt gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 33–44.
[27] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, Part II: pH gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 282–289.
[28] M. Rodriguez-Aller, D. Guillarme, A. Beck, S. Fekete, Practical method development for the separation of monoclonal antibodies and antibody-drug-conjugate species in hydrophobic interaction chromatography, part 1: optimization of the mobile phase, J. Pharm. Biomed. Anal. 118 (2016) 393–403.
[29] A. Cusumano, D. Guillarme, A. Beck, S. Fekete, Practical method development for the separation of monoclonal antibodies and antibody-drug-conjugate species in hydrophobic interaction chromatography, part 2: optimization of the phase system, J. Pharm. Biomed. Anal. 121 (2016) 161–173.
[30] B. Bobály, G.M. Randazzo, S. Rudaz, D. Guillarme, S. Fekete, Optimization of non-linear gradient in hydrophobic interaction chromatography for the analytical characterization of antibody-drug conjugates, J. Chromatogr. A 1481 (2017) 82–91.
[31] S. Fekete, I. Molnár, D. Guillarme, Separation of antibody drug conjugate species by RPLC: a generic method development approach, J. Pharm. Biomed. Anal. 137 (2017) 60–69.
[32] S. Fekete, J.-L. Veuthey, A. Beck, D. Guillarme, Hydrophobic interaction chromatography for the characterization of monoclonal antibodies and related products, J. Pharm. Biomed. Anal. 130 (2016) 3–18.
[33] T. Formolo, M. Ly, M. Levy, L. Kilpatrick, S. Lute, K. Phinney, L. Marzilli, K. Brorson, M. Boyne, D. Davis, J. Schiel, Determination of the NISTmAb primary structure, in: J.E. Schiel, D.L. Davis, O.V. Borisov (Eds.), State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study, American Chemical Society, Washington, DC, 2015, pp. 1–62.
[34] J.M. Prien, H. Stöckmann, S. Albrecht, S.M. Martin, M. Varatta, M. Furtado, S. Hosselet, M. Wang, T. Formolo, P.M. Rudd, J.E. Schiel, Orthogonal technologies for NISTmAb N-glycan structure elucidation and quantitation, in: J.E. Schiel, D.L. Davis, O.V. Borisov (Eds.), State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization, Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study, American Chemical Society, Washington, DC, 2015, pp. 185–235.
[35] D. Saro, A. Baker, R. Hepler, S. Spencer, R. Bruce, S. LaBrenz, M. Chiu, D. Davis, S.E. Lang, Developability assessment of a proposed NIST monoclonal antibody, in: J.E. Schiel, D.L. Davis, O.V. Borisov (Eds.), State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization, Volume 2. Biopharmaceutical Characterization: The NISTmAb Case Study, American Chemical Society, Washington, DC, 2015, pp. 329–375.
[36] I.D.G. Campuzano, C. Larriba, D. Bagal, P.D. Schnier, Ion mobility and mass spectrometry measurements of the humanized IgGk NIST monoclonal

antibody, in: J.E. Schiel, D.L. Davis, O.V. Borisov (Eds.), State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization, Volume 3. Defining the Next Generation of Analytical and Biophysical Techniques, American Chemical Society, Washington, DC, 2015, pp. 75–112.
[37] Y.J. Kil, M. Bern, K. Crowell, D. Kletter, N. Bern, W. Tang, E. Carlson, C. Becker, Towards a comprehensive bioinformatic analysis of the NIST reference mAb, in: J.E. Schiel, D.L. Davis, O.V. Borisov (Eds.), State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization, Volume 3. Defining the Next Generation of Analytical and Biophysical Techniques, American Chemical Society, Washington, DC, 2015, pp. 395–414.
[38] J.E. Schiel, A. Mire-Sluis, D. Davis, Monoclonal antibody therapeutics: the need for biopharmaceutical reference materials, in: J.E. Schiel, D.L. Davis, O.V. Borisov (Eds.), State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization, Volume 1. Monoclonal Antibody Therapeutics: Structure, Function, and Regulatory Space, American Chemical Society, Washington, DC, 2015, pp. 1–34.
[39] S. Fekete, J.-L. Veuthey, D. Guillarme, New trends in reversed-phase liquid chromatographic separations of therapeutic peptides and proteins: theory and applications, J. Pharm. Biomed. Anal. 69 (2012) 9–27.
[40] 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. 35 (2012) 3113–3123.
[41] T.M. Dillon, P.V. Bondarenko, D.S. Rehder, G.D. Pipes, G.R. Kleemann, M.S. Ricci, Optimization of a reversed-phase high-performance liquid chromatography/mass spectrometry method for characterizing recombinant antibody heterogeneity and stability, J. Chromatogr. A 1120 (2006) 112–120.
[42] T. Chen, Y. Chen, C. Stella, C.D. Medley, J.A. Gruenhagen, K. Zhang, Antibody-drug conjugate characterization by chromatographic and electrophoretic methods, J. Chromatogr. B 1032 (2016) 39–50.
[43] A. Beck, G. Terral, F. Debaene, E. Wagner-Rousset, J. Marcoux, M.-C. Janin-Bussat, O. Colas, A.V. Dorsselaer, S. Cianférani, Cutting-edge mass spectrometry methods for the multi-level structural characterization of antibody-drug conjugates, Expert Rev. Proteomic 13 (2016) 157–183.
[44] R.Y.-C. Huang, G. Chen, Characterization of antibody-drug conjugates by mass spectrometry: advances and future trends, Drug Discov. Today 21 (2016) 850–855.
[45] E. Wagner-Rousset, M.-C. Janin-Bussat, O. Colas, M. Excoffier, D. Ayoub, J.-F. Haeuw, I. Rilatt, M. Perez, N. Corvaïa, A. Beck, Antibody-drug conjugate model fast characterization by LC-MS following IdeS proteolytic digestion, MAbs 6 (2014) 173–184.
[46] F. Debaene, A. Bœuf, E. Wagner-Rousset, O. Colas, D. Ayoub, N. Corvaïa, A. Van Dorsselaer, A. Beck, S. Cianférani, Innovative native MS methodologies for antibody drug conjugate characterization: high resolution native MS and IM-MS for average DAR and DAR distribution assessment, Anal. Chem. 86 (2014) 10674–10683.
[47] B. Bobály, A. Beck, J.-L. Veuthey, D. Guillarme, S. Fekete, Impact of organic modifier and temperature on protein denaturation in hydrophobic interaction chromatography, J. Pharm. Biomed. Anal. 131 (2016) 124–132.
[48] M.R. Euerby, J. Hulse, P. Petersson, A. Vazhentsev, K. Kassam, Retention modelling in hydrophilic interaction chromatography, Anal. Bioanal. Chem. 407 (2015) 9135–9152.
[49] S. Fekete, R. Berky, J. Fekete, J.-L. Veuthey, D. Guillarme, Evaluation of a new wide pore core–shell material (Aeris™ WIDEPORE) and comparison with other existing stationary phases for the analysis of intact proteins, J. Chromatogr. A 1236 (2012) 177–188.
[50] S. Fekete, M.W. Dong, T. Zhang, D. Guillarme, High resolution reversed phase analysis of recombinant monoclonal antibodies by ultra-high pressure liquid chromatography column coupling, J. Pharm. Biomed. Anal. 83 (2013) 273–278.
[51] B. Bobály, D. Guillarme, S. Fekete, Evaluation of new superficially porous particles with carbon core and nanodiamond–polymer shell for proteins characterization, J. Pharm. Biomed. Anal. 104 (2015) 130–136.