Journal of Chromatography A 1713 (2024) 464498

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Improved sample introduction approach in hydrophilic interaction liquid chromatography to avoid breakthrough of proteins

Raquel Pérez-Robles [a,b,c], Szabolcs Fekete [d], Róbert Kormány [e], Natalia Navas [a,b], Davy Guillarme [f,g,*]

[a] Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA), Granada, Spain
[b] Department of Analytical Chemistry, Science Faculty, University of Granada, Granada, Spain
[c] Fundación para la Investigación Biosanitaria de Andalucía Oriental-Alejandro Otero, Granada, Spain
[d] Waters Corporation, Geneva, Switzerland
[e] Egis Pharmaceuticals Plc., Keresztúri út 30-38, 1106, Budapest, Hungary
[f] School of Pharmaceutical Sciences, University of Geneva, CMU - Rue Michel Servet 1, 1211 Geneva 4, Switzerland
[g] Institute of Pharmaceutical Sciences of Western Switzerland, University of Geneva, CMU - Rue Michel Servet 1, 1211 Geneva 4, Switzerland

ARTICLE INFO

Keywords:
Hydrophilic interaction liquid chromatography
Breakthrough
Solvent mismatch
Therapeutic proteins

ABSTRACT

When therapeutic proteins are analysed under hydrophilic interaction liquid chromatography (HILIC) conditions, there is an inherent mismatch between the sample diluent (proteins must be solubilised in aqueous media) and the mobile phase, which is mostly composed of aprotic solvent (acetonitrile). This difference in eluent strength between sample diluent and mobile phase is responsible for severe analyte breakthrough and peak distortion. As demonstrated with therapeutic proteins of different sizes (insulin of 6 kDa, anakinra of 17 kDa and rituximab subunits of 25 and 50 kDa), only very small volumes of 0.1–0.2 μL can be injected without breakthrough effects, when performing rapid analysis on short HILIC columns of 20–50 mm, leading to poor sensitivity. In order to avoid the undesired effect of the strong sample diluent, a special injection program should be preferred. This consists in the addition and automatic injection of a defined volume of weak solvent (acetonitrile) along with the sample to increase retention factors during sample loading. Various injection programs were tested, including the addition of a pre-injection or post-injection or both (bracketed injection) of acetonitrile plugs. Several weak to strong injection solvent ratios of 1:1, 1:2, 1:4 and 1:10 were tested. Our work proves that the addition of a pre-plug solvent with a weak vs. strong injection solvent ratio of 1:10 is a valuable strategy to inject relatively large volumes of proteins in HILIC, regardless of column dimensions, thus maximising sensitivity. No peak deformation or breakthrough was observed under these conditions. However, it is important to note that peak broadening (40 % larger peaks) was observed when the injection program increased the injection solvent ratio from 1:1 to 1:10. Finally, this strategy was applied to a wide range of therapeutic mAb products with different physico-chemical properties. In all cases, relatively large volumes can be successfully injected onto small volume HILIC columns using a purely aqueous sample diluent, as long as an appropriate (weak) solvent pre-injection is applied.

1. Introduction

Hydrophilic interaction liquid chromatography (HILIC) is a powerful solution for the separation of polar and/or charged compounds. In HILIC, analytes are interacting with a hydrophilic stationary phase, such as bare silica particles, or bounded amine, hydroxy, amide or zwitterionic particles, by applying a polar mobile phase, composed of water and acetonitrile, and elution is achieved by a water gradient [1]. It is believed that the hydrophilic stationary phase enriches water from the mobile phase and thus generates a thin aqueous layer surrounding the stationary phase. This allows for hydrophilic analytes to partition between the hydrophilic layer and the mobile phase mostly composed of acetonitrile. Elution is then achieved by increasing the amount of water in the mobile phase. However, the retention mechanism of HILIC is

* Corresponding author at: Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO), School of Pharmaceutical Sciences, University of Geneva, CMU - Rue Michel Servet 1, 1211 Geneva 4, Switzerland.
E-mail address: davy.guillarme@unige.ch (D. Guillarme).

https://doi.org/10.1016/j.chroma.2023.464498
Received 4 October 2023; Received in revised form 7 November 2023; Accepted 8 November 2023
Available online 10 November 2023

likely a superposition of hydrophilic partitioning, electrostatic interactions and hydrogen bonding to the stationary phase [2].

HILIC has been successfully applied in many areas for the analysis of proteins [3,4], small drugs [5], released glycans of therapeutic proteins [6], sugars and polar metabolites in metabolomic studies [7]. HILIC separation is often referred as being orthogonal to reversed phase (RP) separation, making it suitable for multidimensional separation of complex samples [8,9].

HILIC separations also have some drawbacks such as longer equilibration times, and limited kinetic efficiency when compared with RP [10]. Similarly to what can be observed in other chromatographic modes, sample injection in HILIC may also causes serious problems due to the higher eluent strength of the injection solvent vs. mobile phase (solvent mismatch between the mobile phase composition and the injection solvent), as the sample is inadequately focused at the column inlet [11]. A fine adjustment of the injection diluent, could minimize this mismatch (i.e. increasing the percentage of acetonitrile in the sample diluent). However, this adjustment is not always possible due to the nature of the sample, i.e. for peptide or protein analysis; the use of diluents with a high proportion of acetonitrile is counterproductive as denaturation phenomena, insolubility, induction of post-translational modifications, or even insolubility (precipitation) may occur.

The differences in elution strength between the sample solvent and the mobile phase usually give rise to undesirable effects on the chromatographic separation, ranging from slight broadening to severe peak deformation or even splitting and analyte breakthrough. Breakthrough is a phenomenon where part of the sample migrates through the column with very little interaction with the stationary phase and hence elutes very close to the column dead time, while the other part of the peak elutes at the normal retention time, resulting in the presence of the expected retained peak. However, it is common for the retained peak to exhibit a wide range of deformations during breakthrough, and multiple retained peaks to appear for a given injected analyte [12,13]. The shape of the retained peaks depends on (i) the solute retention in the injection solvent, (ii) the solute retention in the mobile phase (iii) the gradient elution conditions (iv) the column temperature, (v) the injected sample concentration and (vi) the injection volume [8].

Several strategies have been proposed in the literature to overcome mobile phase incompatibility in HILIC. One valuable approach is to introduce a rapid, high acetonitrile initial gradient at the beginning of the chromatographic process, but this approach is only attractive with low injection volumes. Alternatively, an on-line solid phase extraction (SPE) step can be incorporated. Proteins are first loaded onto a short RP cartridge under aqueous conditions. After loading, the SPE cartridge and HILIC column are placed in-line and the highly organic mobile phase at the beginning of the HILIC gradient elutes the mAb sample from the SPE cartridge onto the HILIC column where separation occurs [14]. However, this approach remains complex to apply routinely. Recently, the implementation of the Performance Optimizing Injection Sequence (POISe) technique has been investigated to avoid the solvent mismatch problems which cause breakthrough and extra band broadening effects [15] (exacerbated by the use of ultra-short columns) generated within the injection system on HILIC [11,16]. The POISe technique involves injecting a defined volume of weak solvent (usually similar to the initial mobile phase composition) along with the sample in order to increase retention factors during sample loading and to eliminate the incompatibilities between the sample solvent and mobile phase. This technique is effective in gradient elution mode and can also reduce pre-column band broadening effects in isocratic elution mode [17].

In this work, the effect POISe-like injection has been evaluated on peak shape for the analysis of proteins and monoclonal antibodies (mAbs) subunits (sizes comprised between 5 and 50 kDa). To this end, a systematic study of the injection mode and loading capacity was carried out using programmed sequenced injection. Various injection programs were tested, including the addition of a pre-injection or post-injection or both (bracketed injection) of acetonitrile plugs. Various weak to strong injection solvent ratios were tested. The influence of column length (volume) and flow rate was also investigated. Finally, this strategy was applied as a generic approach to a wide range of therapeutic mAbs.

2. Materials and methods

2.1. Chemicals, reagents and sample preparation

Acetonitrile (ACN) and water were purchased from Fisher Scientific (Reinach, Switzerland). Trifluoroacetic acid (TFA), formic acid (FA), DL-dithiothreitol (DTT) were purchased from Sigma-Aldrich (Buchs, Switzerland). Rituximab (10 mg/mL), cemiplimab (50 mg/mL), ramucirumab (10 mg/mL), bevazizumab (25 mg/mL), casirivimab (120 mg/mL), daratumumab (20 mg/mL), ipilimumab (5 mg/mL), nivolumab (10 mg/mL) and anakinra (150 mg/mL), were purchased from their respective manufacturers as EU pharmaceutical grade products. Insulin, USP specification quality grade was supplied by Sigma-Aldrich (Buchs, Switzerland).

Light (LC) and heavy (HC) chains were obtained by the addition of 10 μL DTT (1 M) to 50 μg of the mAb (10 mg/mL), and water to obtain a final volume of 50 μL. The mixture was incubated for 30 min at 45 °C.

The mAb subunits and anakinra samples were off line diluted up to 0.5 mg/mL in 100 % of pure water meanwhile the insulin standard was diluted in water +0.1 % FA to enhance the solubility of the protein in the solvent.

2.2. Instrumentation, columns and chromatographic conditions

A Waters ACQUITY™ UPLC™ I-Class System equipped with a binary solvent delivery pump, autosampler (FTN), thermostated column compartment, and photodiode array UV detector was used for this study. A low dispersion volume flow-cell was used (0.5 μL) for the UV detection. An extended loop tubing was coupled to the injection system, allowing up to 50 μL to be injected into the system. The UV detections were carried out at $\lambda = 214$ and $\lambda = 280$ nm at sampling rate of 20 Hz and filter time constant of 0.005 s. The extra-column volume of the system was measured as $V_{EC} = 4.6$ μL, while the gradient delay volume was $V_d = 100$ μL. Data acquisition and instrument control were carried out using Empower™ Pro 3 software (Waters). Several prototypes and commercial columns were provided by Waters (Milford, USA), including very short prototype 300 Å Amide 1.7 μm, 2.1 × 20 mm; and commercial Glycoprotein BEH™ 300 Å Amide 1.7 μm; 2.1 × 50 and 2.1 × 150 mm columns.

The mobile phases were composed of water +0.1 % TFA (mobile phase A) and ACN + 0.1 % TFA (mobile phase B). The retention of therapeutic proteins and the column length (L) were considered for the linear gradients. The gradient time (t_G) was adjusted proportionally with the column volume, i.e. from 72 to 64 % of B for HC and LC, from 78 to 64 % of B for anakinra and from 85 to 71 % of B for insulin, over 10 min ($L = 50$ mm) and 30 min ($L = 150$ mm); and from 74 to 66 % of B for HC and LC, from 80 to 66 % for anakinra and from 85 to 71 % of B for insulin over 4 min for $L = 20$ mm column. The column temperature was set at 60 °C. Two flow rates (0.3 mL/min and 0.6 mL/min) were tested during the study of the conditions recorded in Table 1.

A multiple injection mode was used in this work. The ACQUITY FTN autosampler allowed a multi-step (sequenced) injection mode to be configured using the Auto-Additions option in Empower. This mode allows to automate and program the pre-, post- and bracketed injections. A time delay between sample and solvent aspiration and injection ``stroke'' can also be considered (distance a sample is moved within the sample loop. This movement is μL of air introduced in the sample loop). In this work, no delay time was used. ACN was always used as solvent plugs for pre-, post- and bracketed injections.

Table 1
Pre-column dilution using POISe injection.

Column	Column length L (mm)	Column volume (mL)	Sample volume (μL)	Pre-plug volume of ACN (μL)
Prototype, BEH, amide, 300 Å, Waters.	20	0.06	0.1	0.1; 0.2; 0.4; 1
			0.5	0.5; 1; 2; 5
			1	1; 2; 4; 10
			2	2; 4; 8; 20
ACQUITY UPLC GlycoProtein BEH, amide, 300 Å, Waters	50	0.13	0.5	0.5; 1; 2; 5
			1	1; 2.; 4; 10
			2	2; 4; 8; 20
			5	5; 10; 20; 45
ACQUITY UPLC GlycoProtein BEH, amide, 300 Å, Waters	150	0.36	1	1; 2; 4; 10
			2	2; 4; 8; 20
			5	5; 10; 20; 45
			10	10; 20; 40

2.3. Pre-plug, post-plug and bracketed injection

The influence of the pre- plug, post- plug and combined pre/post-plugs of ACN was systematically studied. In the present work, 3 μL of anakinra were injected on the 2.1 × 50 mm column. Among the tested solutions, the influence of ACN dilution (seven times the sample volume) was studied using different injection modes: (i) pre-plug (21 μL of ACN), (ii) post-plug (21 μL of ACN) and (iii) bracketed injection (21 μL of ACN before the sample and 21 μL of ACN after the sample aspiration).

2.4. Pre-plug solvent injection

An experiment design was planned and carried out considering the sample volume (0.5–10 μL) and solvent (pre)plug volumes as variable. The sample injection volume was adjusted according to the column volume (i.e. for $L = 20$ mm column; 0.1, 0.5, 1 and 2 μL, which corresponds to V_i/V_0 between 0.2 and 4.6 %; for $L = 50$ mm column, 0.5, 1, 2 and 5 μL, which corresponds to V_i/V_0 between 0.4 and 4.6 %; for $L =$ 150 mm column, 1, 2, 5 and 10 μL, which corresponds to V_i/V_0 between 0.3 and 3.1 %). A number of ACN pre-plug volumes were tested in proportion to the sample volume (one, two, four and ten times the sample volume). The column volume was measured experimentally from the t_0 determination ($V_0 = t_0 \times$ flow rate). t_0 was determined experimentally from the measured solvent breakthrough time, considering the extra-column volume and injection delay into consideration. The injection programs used in these experiments are detailed in Table 1.

2.5. Generic application of the sequenced injection approach: mAbs subunits separation

Several mAbs (i.e. rituximab, cemiplimab, ramucirumab, bevazizumab, casirivimab, daratumumab, ipilimumab, nivolumab) subunits with different physico-chemical properties in terms of isoelectric points (pI) and hydrophobicity were separated using two chromatographic columns of different lengths: Waters prototype column 300 Å Amide 1.7 μm, 2.1 × 20 mm and Glycoprotein BEH 300 Å Amide 1.7 μm; 2.1 × 50 mm Column. 2 μL and 1 μL of the reduced mAbs samples followed by 20 μL and 10 μL injection of ACN pre-plug were analyzed on the $L = 50$ mm and $L = 20$ mm columns, respectively (weak vs. strong solvent ratio of 1:10).

3. Results and discussion

3.1. General considerations

In HILIC conditions, the sample diluent (injection solvent) is often much stronger than the mobile phase. In most cases, the samples are actually dissolved in water (or buffered aqueous solution), while the mobile phase contains high proportion of ACN. This inherent solvent incompatibility (mismatch) often results in analyte breakthrough effects. Such a phenomenon occurs when the injected volume is relatively large or when the retention of the solute in the sample diluent is very different from that in the initial mobile phase. As a result, a fraction of the solute molecules elutes with the injection solvent peak. The other fraction of the solute leaves the injection solvent, resulting in a retained peak with a distorted shape [12,18]. In addition to the retained peak, a breakthrough peak is thus eluted near the column dead time (or even before the dead time, due to exclusion or repulsion effects).

Since the breakthrough effect is related to the injection volume, the term "critical injection volume" (V_{crit}) is often used to express the maximum injectable volume that will not result in breakthrough [12, 19]. This critical volume directly depends on the column volume (V_{col}) and the retention factor (k) of a solute experienced in the injection solvent. Therefore, V_{crit} can be estimated from retention model parameters for a given column volume and for any diluent composition. The parameters of the linear solvent strength (LSS) model was used here to estimate retention factor (k), as the calculation of breakthrough volume requires the knowledge of k value at a given diluent composition. In the present work, retention model parameters were experimentally measured for insulin, anakinra and the subunit fragments of rituximab (light chain (LC) and heavy chain (HC)). The model parameters were then used to estimate and plot V_{crit} as a function of diluent composition. To compare the breakthrough phenomenon between large and small solutes, retention parameters for small molecules (rhodamine and homovanillic acid) were taken from the literature to estimate V_{crit} [20, 21]. Finally, V_{crit} was normalized to the column volume (to have values independent of column dimensions) and plotted against the ACN volume fraction (ϕ_{ACN}) of the sample diluent. Such plot shows the injectable volume (to avoid breakthrough) as a function of sample diluent composition. The higher the vertical position of the curve for a given diluent composition, the greater the volume that can be injected without breakthrough. If the y-value is zero, breakthrough is practically unavoidable. Calculations were performed in Excel™ Software (Microsoft) and Statistica™ Software (Tibco Software Inc.). Fig. 1 shows the corresponding plot, highlighting the fact that large molecules result in a more abrupt transition between the absence of breakthrough and the onset of breakthrough (very steep ascending curves). For example, when anakinra is diluted in $\phi_{ACN} < 0.64$, breakthrough is inevitable, no matter how small the volume injected. If $\phi_{ACN} = 0.65$, $V_{crit} = 0.01 \times V_{col}$, and if the ACN fraction is increased to $\phi_{ACN} = 0.69$ then $V_{crit} = 0.1 \times V_{col}$ (the injection volume can be up to 10 % of the column volume). In contrast, the breakthrough of small molecules peaks is less sensitive to changes in diluent composition. For homovanillic acid, when $\phi_{ACN} = 0.40$, 0.50, 0.60 and 0.70, the expected critical injection volumes are 1, 3, 8 and 20 % of V_{col}, respectively. Fig. 1 also suggests that for the selected proteins, $\phi_{ACN} \sim 0.65$–0.70 (as sample diluent) would result in no breakthrough and would allow the injection of relatively large volumes, but these proteins have limited solubility in such solvents. However, please note, that the absence of breakthrough does not necessarily result in a perfect peak shape. There are several sources of additional band broadening such as mass overload, volumetric overload and very often in HILIC the unwanted secondary interactions.

3.2. Impact of injection volume in HILIC for proteins

To assess the effect of injection solvent in HILIC, a first series of experiments was carried out by injecting different volumes of anakinra (a therapeutic protein of approximately 17 kDa) onto a 50 × 2.1 mm, 1.7 μm column. The tested injection volumes ranged from 0.5 to 5 μL (corresponding to about 0.4–4 % of the column volume, V_{col}). The commercial formulation of anakinra is aqueous and was further diluted with water to 0.5 mg/mL prior to analysis, which is clearly not an ideal

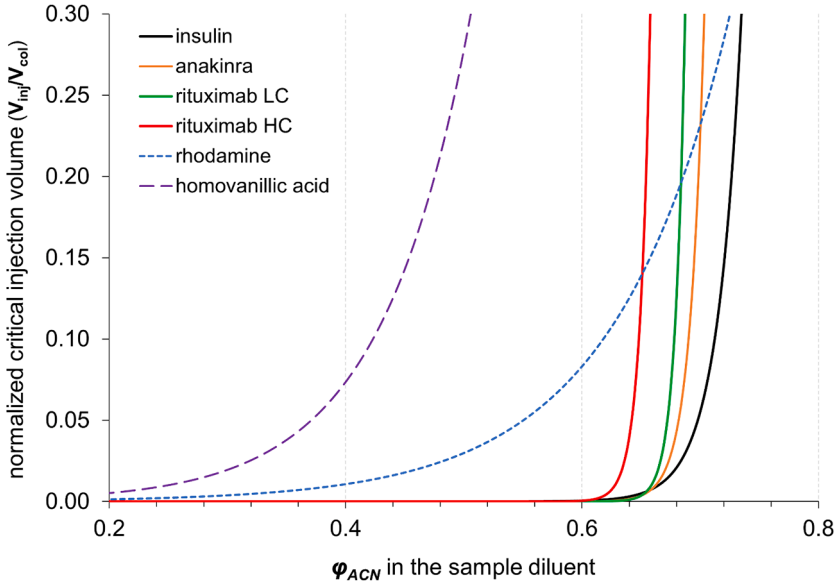

Fig. 1. Estimated critical injection volume (normalized) as a function of sample diluent composition (volume fraction of acetonitrile, ϕ_{ACN}).

sample diluent. Fig. 2 shows the results obtained for the analysis of anakinra. For injection volumes comprised between 2 and 5 μL (corresponding to approximately 1.6–4.0 % of V_{col}), a significant breakthrough phenomenon was observed, with an intense peak eluted in the column dead time, at approximately 0.45 min. This breakthrough peak has an area 10-times higher than the retained peak, when injecting a volume of 5 μL, while the value drops to 7-fold for an injection volume of 2 μL. This total breakthrough phenomenon was already described under HILIC conditions, but only with positively charged compounds such as peptides and weak bases. In the present work, a strong total breakthrough was also observed with proteins (positively charged molecule under acidic conditions) for injected volumes comprised between 2 and 5 μL. When decreasing the injection volume to 1 μL and 0.5 μL, the ratio of peak area for unretained vs. retained peak was reduced to 3.6 and 1.3-fold, respectively. However, even if the unretained peak had a lower intensity, an additional peak was observed (relatively broad eluted at 4.25 min when injecting 0.5 μL and very broad peak eluting at 3.2 min for an injection volume of 1 μL). These peaks are typical of breakthrough phenomenon, which corresponds to an intermediate scenario between the total breakthrough (only an unretained peak is observed) and no breakthrough (only a retained peak is observed). In this example, the reduction of peak area corresponding to total breakthrough at lower injection volume always results in the appearance of an additional peak eluting closer (partially retained) to the peak of interest (double or split peak). For injection volume of 0.5 and 1 μL, the breakthrough peak has an area corresponding to 1.6 and 0.4-fold of the anakinra peak area,

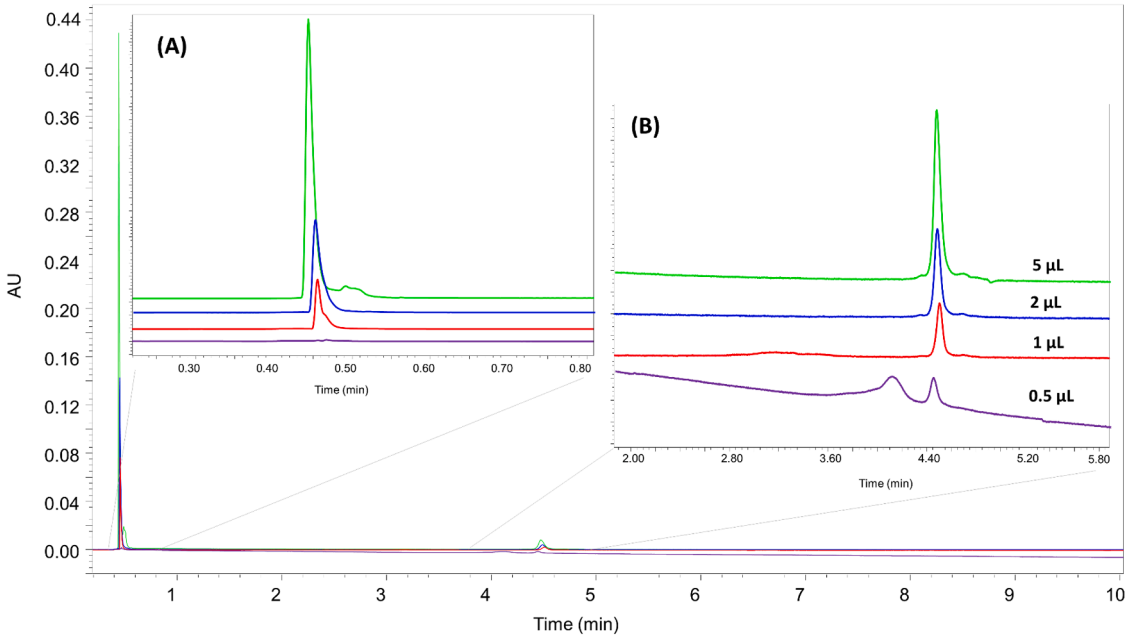

Fig. 2. Overlaid chromatograms in HILIC mode of 0.5–5 μL anakinra solution (0.5 mg/mL in water) obtained using $L = 50$ mm (1.7 μm, 2.1 × 50 mm) column. Enlarged view of the shifted chromatograms from (A) 0.2 to 0.8 min (breakthrough region) and (B) from 2.0 min to 5.8 min (sample elution region). Injected volume: 0.5 μL, purple trace; 1 μL, red trace; 2 μL blue trace; and 5 μL green trace. Generic conditions: flow rate 0.3 mL/min; gradient 78 to 64 % B in 10 min; temperature 60 °C; UV detection 280 nm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respectively. When cumulating all the peaks, the loss of observed peak area was equal to about 5-fold for the injection volumes of 0.5 and 1 μL.

In all cases, the anakinra peak eluted at 4.5 min was fairly symmetrical and had a suitable peak width at half height equal to 0.049, 0.054, 0.056 and 0.058 min for the 0.5, 1, 2 and 5 μL injection volumes, respectively. So, the peak broadening associated with the increase of injection volume was always reasonable. On the other hand, the peak area obtained for the different injection volumes was equal to 4300, 10,800, 17,000 and 34,000 for the 0.5, 1, 2 and 5 μL injection volumes, respectively. In theory, it should be directly proportional to the injection volume, which was almost the case here. However, if breakthrough would not exist, sensitivity would be 5-times higher at 0.5 and 1 μL, 7-fold higher at 2 μL and 10-times larger at 5 μL injection volume. Breakthrough phenomenon is therefore particularly critical for sensitivity, even if symmetrical/narrow peaks can be obtained.

As shown in Fig. 2, a volume of 0.5 μL is still too large and leads to breakthrough and a peak splitting. A volume of 0.1–0.2 μL would certainly be ideal in terms of peak shape for this HILIC analysis. However, using such a very low volume would lead to extremely limited sensitivity and is therefore an unacceptable solution.

3.3. Evaluation of various injection programmes for proteins in HILIC

A few years ago, an interesting approach was proposed to minimise the effect of the injection conditions on chromatographic peak broadening when using very efficient and small volume columns in RPLC and operating them in isocratic elution mode. This approach, which consists in performing a pre-column dilution, was named as Performance Optimizing Injection Sequence (POISe), and involves automatic injection of a defined volume of weak solvent with the sample to increase retention factors during sample loading [17]. This method has recently been revisited by McCalley et al. for the analysis of small molecules under HILIC conditions [11]. In the present work, this POISe approach was reconsidered to avoid the problems encountered in Fig. 2. Fig. 3 shows the analysis of anakinra using four different injection programmes, and a constant injection volume of 3 μL on a 50 mm column length. In our experiments, ACN solvent plugs were co-loaded onto the column together with the sample plug in different configurations. Among the tested configurations, the addition of a pre-plug or post-plug or both (bracketed injection) of ACN was evaluated and corresponding results were compared with the classical (non-POISe) injection. As expected, the classical injection of 3 μL (light blue trace) provided a significant breakthrough peak, corresponding to 7.5-fold the retained peak, leading to non-negligible loss in sensitivity on the retained peak, but no peak deformation and a good peak width at 50 % equal to 0.058 min were observed. In previous RPLC work, the optimal ratio of weak to strong injection solvent was 4:1 [17]. However, as the impact of injection solvent when analyzing proteins in HILIC was found to be significant, a ratio of 7:1 was applied in Fig. 3 (this ratio will be further explored in Fig. 4). When adding a post-plug injection of ACN equal to 7-fold the injected volume (green trace), the breakthrough peak was slightly reduced (the ratio of unretained vs. retained peak now corresponds to 3-fold) and the retained peak offer a better sensitivity (peak area was increased by 2.3-fold). This result can be explained by the increased focusing effect, as the ACN proportion in the injection plug is increased. When adding a pre-plug of ACN (dark blue trace), significant benefits were noticed, with no peak deformation, and no breakthrough peaks. In this case, the sensitivity was strongly enhanced compared to the classical injection (peak area was increased by 9-fold, for the same injection volume of 3 μL). However, we also noticed a peak broadening when adding the pre-plug of ACN. Indeed, peak width at 50 % was equal to 0.099 min, corresponding to 70 % increase in comparison with the regular injection of 3 μL. In addition, we also observed a gradual shift of the peak apex towards lower retention times and a triangular shape towards the front. These phenomenon can be attributed either to an increase in band broadening during the special injection programme, or to concentration overloading effects, as injection volume of 3 μL corresponds to about 3 % of the column volume, which is quite large under HILIC conditions. Besides the addition of a pre-plug, the interest to simultaneously add a post-plug (red trace) was found to be very limited. Indeed, the peak area and peak width remain comparable to the case where only the pre-plug was added.

In the end, it is clear that the addition of a pre-plug solvent is a valuable strategy to inject relatively high volume when analyzing proteins under HILIC conditions and maximize sensitivity.

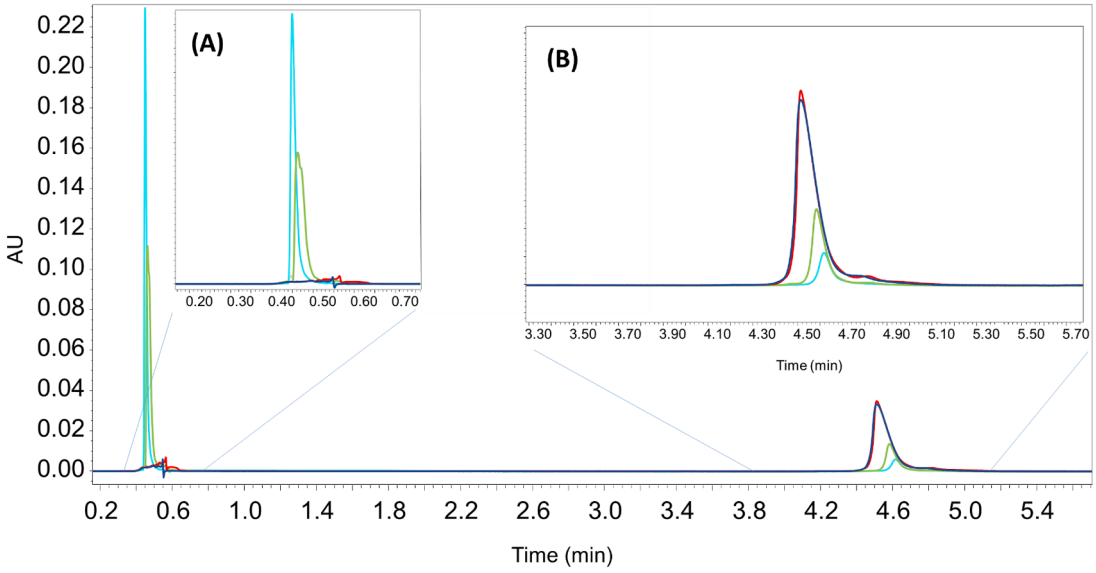

Fig. 3. Overlaid HILIC chromatograms injecting 3 μL of anakinra sample (0.5 mg/mL diluted in water) using pre-plug (pre-CD) and post-plug sample column dilution (post-CD) with ACN, i.e. 1) 21 μL of pre-plug ACN + 3 μL of sample, dark blue trace; 2) 21 μL of pre-plug ACN + 3 μL of sample + 21 μL of post-plug ACN, red trace; 3) 3 μL of sample + 21 μL of post-plug ACN, green trace; and 4) 3 μL of sample (no column dilution), light blue trace. Enlarged view of the chromatograms from (A) 0.2 to 0.7 min (breakthrough region) and (B) from 3.3 min to 5.7 min (sample elution region). Generic conditions: $L = 50$ mm (1.7 μm, 2.1 × 50 mm) column; flow rate 0.3 mL/min; gradient 78 to 76 % B in 10 min; temperature 60 °C; UV detection, 280 nm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

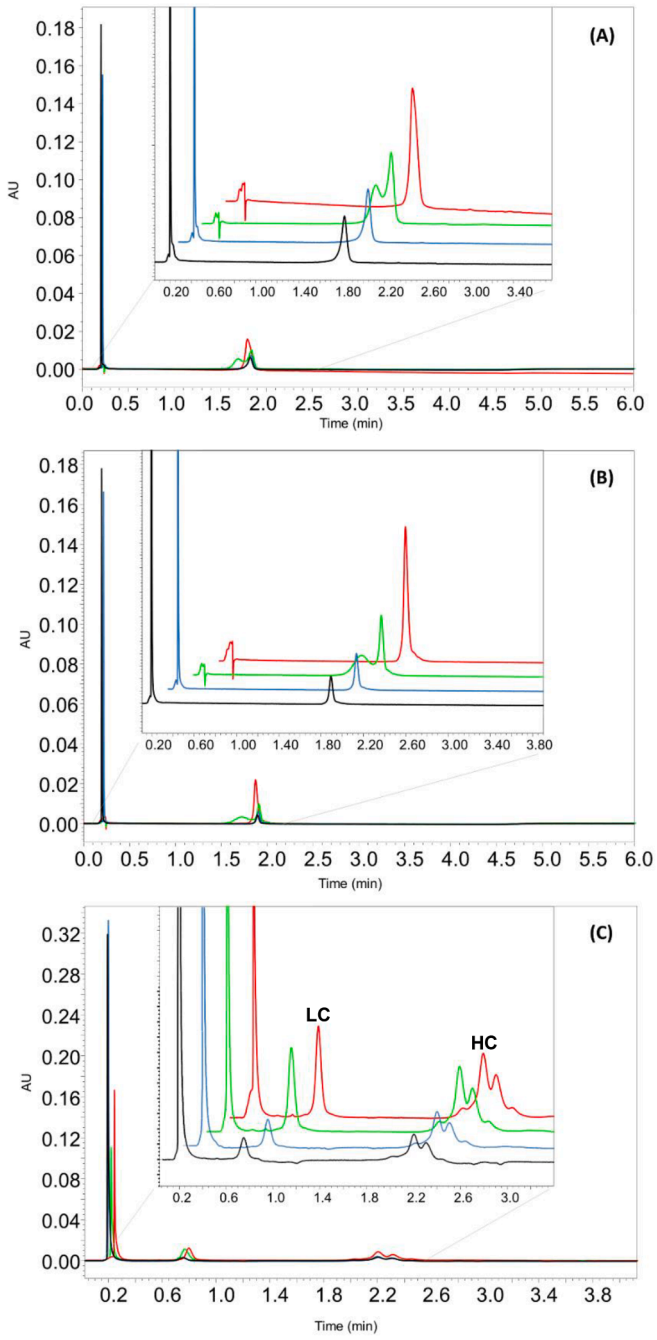

Fig. 4. Overlaid HILIC chromatograms of (A) insulin (0.5 mg/ mL), (B) anakinra (0.5 mg/ mL) and (C) reduced rituximab (0.5 mg/ mL), injecting 1–10 μL of ACN in pre-plug column dilution mode (pre-CD) (1 μL, black trace; 2 μL, blue trace; 4, green trace; and 10 μL, red trace) followed by 1 μL of sample. Generic conditions: $L = 20$ mm (1.7 μm, 2.1 × 20 mm) column; flow rate 0.3 mL/min; gradient 85 to 71 % B for insulin, 80 to 66 % B for anakinra, 74 to 66 % B for reduced rituximab in 4 min; temperature 60 °C; UV detection, 280 nm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Impact of pre-plug volume of an injection sequence for proteins in HILIC

The following experiments were performed on a shorter HILIC column of only 20 mm, with the same chemistry and particle size as the one used in the previous experiments. Three different samples were considered, namely insulin (therapeutic protein of about 6 kDa), anakinra and a reduced rituximab sample of which we analysed the light chain (LC) and heavy chain (HC) sub-units of 25 and 50 kDa, respectively. The injected volume was fixed at 1 μL and different ACN pre-plug volumes of 1, 2, 4 and 10 μL were tested, corresponding to a ratio of weak vs. strong injection solvent equal to 1:1, 1:2, 1:4 and 1:10. The gradient conditions were optimised for each protein sample to achieve reasonable retention for all species. As shown in Fig. 4(A), three different scenarios were observed for insulin, depending on the pre-plug volume of ACN, with total breakthrough, classical breakthrough or adequate elution of the peaks with no breakthrough. With a weak vs. strong injection solvent ratio of 1:1 or 1:2, a total breakthrough phenomenon was observed, with a relatively large peak eluted at the column dead volume (corresponding to 2.4 and 1.8-fold the peak of interest for injection solvent ratios of 1:1 or 1:2, respectively), and a symmetrical peak of limited intensity retained on the column. When the ratio was increased to 1:4, a classical breakthrough was observed, with no more peaks eluted at the column dead volume, but the appearance of two peaks eluted at 1.68 and 1.82 min. In this case, the two peaks have a comparable intensity. Finally, the best separation was obtained when the ratio was increased to 1:10, corresponding to a pre-plug of 10 μL for an injection volume of 1 μL. Under these conditions, the separation was perfectly acceptable, and the sensitivity was significantly improved compared to the other three conditions (peak area was increased by about 3-fold, compared to the cases where the injection solvent ratios were equal to 1:1 or 1:2). These results confirm the interest of applying sequenced injection by loading an ACN pre-plug. However, it is important to mention that peak width at half-height was increased by 40 % when increasing the injection solvent ratio from 1:1 to 1:10. As the injection volume was very low in this case (1 μL only), this behaviour cannot be attributed to column overloading, and it is clear that the special injection sequence employed could contribute to peak broadening, when a significant injection solvent ratio is employed.

Exactly the same behaviour was observed for anakinra (Fig. 4(B)), but the total breakthrough peak observed for a weak vs. strong injection solvent ratio of 1:1 or 1:2 was even higher (breakthrough peak corresponds to 6 and 4-fold the peak of interest, respectively). In addition, the peak width at half-height was increased by only 20 % when increasing the injection solvent ratio from 1:1 to 1:10. This means that more benefits of the injection sequence were observed with anakinra than with insulin, and again the 1:10 ratio was found to give the best performance (symmetrical peaks and high sensitivity).

For the reduced rituximab sample (Fig. 4(C)), the interpretation of the breakthrough was more difficult because dithiothreitol (DTT) was used to reduce rituximab and the excess DTT eluted at the column dead time. It is therefore difficult to interpret whether or not there is a complete breakthrough. However, the peak intensity at column dead time decreases by a factor 2, when pre-plug with a ratio of 1:10 is applied, and the peak areas of the LC and HC peaks also increased significantly, by about 4-fold. The occurrence of a total breakthrough phenomenon can be confirmed as the same reduced rituximab sample was injected to obtain the four chromatograms shown in Fig. 4(C), and only the weak vs. strong injection solvent ratio changed. On the other hand, no partial breakthrough was observed in Fig. 4(C). For the rituximab sample, it appears that the peak widths at half height remains constant for the HC peak, whatever the weak vs. strong injection solvent ratio. This behaviour can be attributed to the fact that HC has a larger size compared to insulin and anakinra. Indeed, it seems that the impact of sequenced injection on peak widths decreases with solute size, probably due to the "on-off" like retention mechanism and a stronger band focusing effect at the column inlet with larger proteins. For the LC peak, the peak widths were decreased by about 20 % at 1:10 vs. 1:1 injection solvent ratio. This result is quite different from the ones previously obtained. It could be attributed to the fact that LC peak is eluting much earlier ($t_r = 0.75$ min) than insulin, anakinra and HC of rituximab (t_r comprised between 1.8 and 2.2 min) and is therefore differently affected by the injection conditions. Based on these observations, it

appears that the 1:10 ratio, corresponding to a 10 μL pre-plug, was again the most appropriate solution to utilize the full performance (suitable peak shapes and high sensitivity) of the very short HILIC column.

We were interested in further investigating the effect of injection conditions (different combinations of sample volumes and ACN plug ratios) and mobile phase flow rate on breakthrough phenomenon. Therefore, various sample volumes were set (0.5, 1.0, 2.0 and 5 μL) and pre-plug volumes were adjusted to 1, 2, 4 and 10-times the sample plug volume. Two flow rates were tested (F = 0.3 and 0.6 mL/min). Fig. 5 shows a representative contour plot (anakinra at F = 0.3 mL/min) showing the representation of the peak distortion including overall breakthrough (in %) as a function of the pre-plug solvent (ACN) to sample plug volume ratio and also the sample volume. As expected, the lower the sample volume, and the larger the solvent plug volume, the lower the breakthrough is (dark green areas on Fig. 5). At a sample volume of 1 μL, a 10-fold solvent pre-plug injection appears to be safe enough to avoid breakthrough. If the sample volume is reduced to 0.5, 0.2 and 0.1 μL, then the ratio of solvent pre-plug volume to sample volume can be set to lower values such as 9, 8 and 2, respectively, to limit breakthrough. In other words, a smaller injection volume requires a relatively smaller solvent pre-plug volume. Interestingly, as the sample volume is increased beyond 1 μL, the required ACN pre-plug volume ratio does not necessarily increase, suggesting a sort of convergence towards a 10:1 ratio even at 2 μL sample volume. The same trends were observed for all protein samples and at both flow rates (data not shown).

3.5. Applicability of sequenced injection to various mAb products in HILIC

Finally, to demonstrate the potential and broad applicability of the weak solvent pre-plug injection strategy, we selected several mAbs with different physico-chemical properties in terms of isoelectric points (pI) [22] and hydrophobicity [23]. These different mAb samples were reduced and then their sub-units were analysed on either the 50 or 20 mm HILIC columns. The gradient times were also adjusted according to the column length, 10 min and 4 min on the 50 and 20 mm columns, respectively. For the 50 mm column, the injection volume was set to 2 μL and a 20 μL injection pre-plug was included in the injection sequence. For the shorter column, the injection volume was reduced to 1 μL and the pre-plug volume to 10 μL to maintain the same weak vs. strong solvent ratio of 1:10. The corresponding chromatograms are shown in Fig. 6. Irrespective of the mAb sample, it is clear that most of the peaks observed for LC and HC fragments were symmetrical. In all cases, several variants can be separated around the HC peak. These additional species probably correspond to glycoforms that can be resolved under HILIC conditions [24,25]. On the other hand, some other hydrophilic variants can also be resolved in the time window corresponding to the LC peak. This was for example the case for nivolumab or cemiplimab samples. When comparing the 20 and 50 mm column length, the average peak widths at half-height for the LC peaks were equal to 0.067 and 0.078 min on the 20 and 50 mm long column, respectively. However, since the gradient time was longer on the 50 mm column, peak capacity was equal to 60 and 129 on the 20 and 50 mm long column, respectively. So, this is primarily the gradient time that determines the peak width and not the column length when analyzing proteins, which agrees with the on-off like behaviour [26]. Independently on the mAb hydrophobicity and pI, there is no breakthrough for all these separations, and a suitable sensitivity was obtained in all cases with a reasonable injection volume, even on the very short HILIC columns. Here again, these results confirm the possibility to inject relatively large volumes onto small volume HILIC columns using a purely aqueous sample diluent, only if an appropriate (weak) solvent pre-plug injection is applied.

4. Conclusion

This work is dedicated to the analysis of therapeutic proteins using short HILIC columns. One of the main problems when injecting proteins on short HILIC columns is related to the mismatch between the sample diluent (proteins are formulated in aqueous media) and the mobile phase (mostly composed of acetonitrile). This solvent mismatch is indeed detrimental to the separation and is responsible for peak deformation and breakthrough phenomena. To evaluate this problem and propose possible solutions, therapeutic proteins of different sizes (insulin of 6 kDa, anakinra of 17 kDa and rituximab subunits of 25 and 50 kDa) were selected and short HILIC columns of 20–50 mm were used.

Due to the on-off retention mechanism of proteins under HILIC

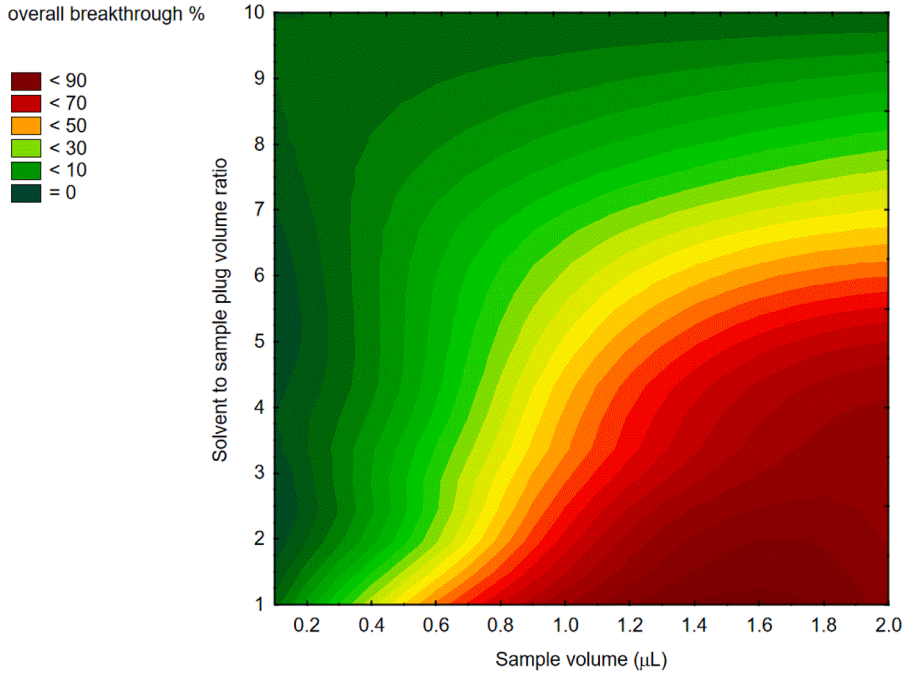

Fig. 5. Contour plot of overall breakthrough (expressed in %) as function of pre-plug solvent (ACN) to sample plug volume ratio (vertical axis) and of sample volume (horizontal axis). Sample: anakinra, F = 0.3 mL/min, 20 × 2.1 mm column.

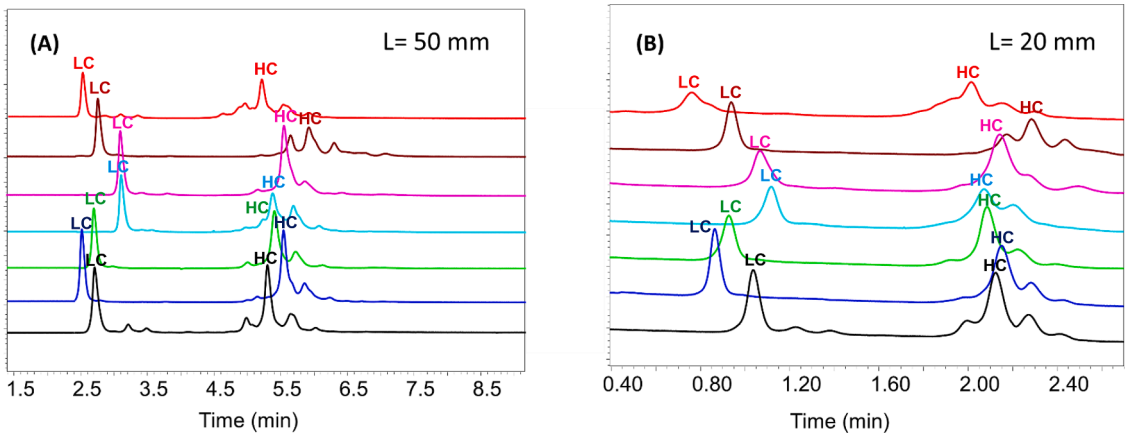

Fig. 6. Representative chromatograms in HILIC mode of several mAbs (0.5 mg/mL) reduced with DTT: Nivolumab, black trace; Ipilimumab, dark blue trace; Daratumumab, green trace; Casirivimab, light blue trace; Bevacizumab, pink trace; Ramucirumab, brown trace; and Cemiplimab, red trace, using different column lengths, (A) $L = 50$ mm and (B) $L = 20$ mm. Generic conditions: 20 μL pre-plug column dilution (pre-CD) with ACN followed by 2 μL of sample in $L = 50$ mm column and 10 μL pre-CD with ACN followed by 1 μL of sample in $L = 20$ mm column; flow rate 0.3 mL/min; gradient 72 to 74 % B in 10 min ($L = 50$ mm) and gradient 74 to 66 % B in 4 min ($L = 20$ mm); temperature 60 °C; UV detection 280 nm.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conditions, we demonstrated that large molecules lead to an abrupt transition between the absence of breakthrough and the onset of breakthrough, which was not necessarily the case for small molecules. For the selected proteins, $\phi_{ACN} \sim 0.65$–0.70 (as sample diluent) would result in no breakthrough and would allow the injection of relatively large volumes. However, proteins are not soluble in such solvents and this solution was unsuitable.

Next, we tried to better understand the effect of injection volume in HILIC of proteins. It appears that a volume of 0.5 μL on a 50 × 2.1 mm column was still too large, leading to breakthrough and peak splitting. A volume of 0.1–0.2 μL would certainly be ideal for this type of analysis. However, using such a very small volume would result in very low sensitivity and is again not an acceptable solution.

The best solution to avoid the solvent mismatch problem is to modify the injection program. Automatic injection of a defined volume of weak solvent with the sample was tested to increase retention factors during sample loading. In these experiments, ACN solvent plugs were loaded on the column together with the sample plug in different configurations. The addition of either a pre-plug, a post-plug or both (bracketed injection) of ACN was evaluated and the results compared to classical injection. Irrespective of the protein selected, the addition of a pre-plug solvent is a valuable strategy for injecting relatively large volumes when analysing proteins in HILIC and for maximising sensitivity. Various weak to strong solvent ratios of 1:1, 1:2, 1:4 and 1:10 were then tested and the best compromise was always achieved with the 1:10 ratio. This means that for a sample volume of 1 μL, a pre-injection of 10 μL ACN is required to avoid peak deformation and breakthrough. However, it is also important to note that the peak width at half height increased by 40 % when the injection solvent ratio was increased from 1:1 to 1:10 due to the injection sequence.

Finally, the strategy (pre-injection of ACN at a ratio of 1:10) was successfully applied to the HILIC separation of different mAb sub-units using 20 and 50 mm long columns. These results confirm the possibility of injecting relatively large volumes onto small volume HILIC columns using a purely aqueous sample diluent, provided that a suitable (weak) solvent pre-plug injection is used.

CRediT authorship contribution statement

Raquel Pérez-Robles: Investigation, Visualization, Formal analysis, Writing – original draft. **Szabolcs Fekete:** Conceptualization, Writing – review & editing. **Róbert Kormány:** Writing – review & editing. **Natalia Navas:** Funding acquisition, Writing – review & editing. **Davy Guillarme:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Waters, ACQUITY, UPLC, Empower, and BEH are trademarks of Waters Technologies Corporation. Excel is a trademark of Microsoft Corporation. Statistica is a trademark of Cloud Software Group, Inc.

Data availability

Data will be made available on request.

Acknowledgements

Raquel Pérez-Robles currently holds a postdoctoral position granted by the Junta de Andalucía, Spain (ref: DOC_01694).

References

[1] B. Buszewski, S. Noga, Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique, Anal. Bioanal. Chem. 402 (2012) 231–247, https://doi.org/10.1007/s00216-011-5308-5.
[2] P. Hemström, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29 (2006) 1784–1821, https://doi.org/10.1002/JSSC.200600199.
[3] B. Bobály, V. D'Atri, A. Beck, D. Guillarme, S. Fekete, Analysis of recombinant monoclonal antibodies in hydrophilic interaction chromatography: a generic method development approach, J. Pharm. Biomed. Anal. 145 (2017) 24–32, https://doi.org/10.1016/J.JPBA.2017.06.016.
[4] 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. Chromatogr. B 1058 (2017) 73–84, https://doi.org/10.1016/J.JCHROMB.2017.05.010.
[5] M.R. Gama, C.B.G. Bottoli, Hydrophilic interaction liquid chromatography of small molecules, Encycl. Anal. Chem. (2018) 1–23, https://doi.org/10.1002/9780470027318.A9385.
[6] S. Carillo, R.P. Erez-Robles, C. Jakes, M. Ribeiro Da Silva, S. Mill An Martín, A. Farrell, N. Navas, J. Bones, Comparing different domains of analysis for the characterisation of N-glycans on monoclonal antibodies, J. Pharm. Anal. 10 (2019) 23–34, https://doi.org/10.1016/j.jpha.2019.11.008.
[7] G. Paglia, G. Astarita, A high-throughput HILIC-MS-based metabolomic assay for the analysis of polar metabolites, Methods Mol. Biol. 2396 (2022) 137–159, https://doi.org/10.1007/978-1-0716-1822-6_11.
[8] S. Chapel, F. Rouvière, S. Heinisch, Comparison of existing strategies for keeping symmetrical peaks in on-line hydrophilic interaction liquid chromatography ×

reversed-phase liquid chromatography despite solvent strength mismatch, J. Chromatogr. A 1642 (2021), 462001, https://doi.org/10.1016/J.CHROMA.2021.462001.
[9] P.J. Boersema, S. Mohammed, A.J.R. Heck, Hydrophilic interaction liquid chromatography (HILIC) in proteomics, Anal. Bioanal. Chem. 391 (2008) 151–159, https://doi.org/10.1007/S00216-008-1865-7.
[10] D.V. McCalley, Managing the column equilibration time in hydrophilic interaction chromatography, J. Chromatogr. A 1612 (2020), 460655, https://doi.org/10.1016/J.CHROMA.2019.460655.
[11] M.R. Taylor, J. Kawakami, D.V. Mccalley, Managing sample introduction problems in hydrophilic interaction liquid chromatography, J. Chromatogr. A 1700 (2023), 464006, https://doi.org/10.1016/j.chroma.2023.464006.
[12] H.C. Van De Ven, J. Purmova, G. Groeneveld, T.S. Bos, A.F.G. Gargano, S. Van Der Wal, Y. Mengerink, P.J. Schoenmakers, Living with breakthrough: two-dimensional liquid-chromatography separations of a water-soluble synthetically grafted biopolymer, Separations 7 (2020) 41, https://doi.org/10.3390/separations7030041.
[13] S. Chapel, F. Rouvière, V. Peppermans, G. Desmet, S. Heinisch, A comprehensive study on the phenomenon of total breakthrough in liquid chromatography, J. Chromatogr. A 1653 (2021), 462399, https://doi.org/10.1016/J.CHROMA.2021.462399.
[14] I. Vandenheede, K. Sandra, E. Dumont, V. D'Atri, D. Guillarme, P. Sandra, Hydrophilic interaction chromatography for the characterization of therapeutic monoclonal antibodies at protein, peptide, and glycan levels, LCGC Europe 30 (2017) 424–434.
[15] K. Vanderlinden, K. Broeckhoven, Y. Vanderheyden, G. Desmet, Effect of pre- and post-column band broadening on the performance of high-speed chromatography columns under isocratic and gradient conditions, J. Chromatogr. A 1442 (2016) 73–82, https://doi.org/10.1016/J.CHROMA.2016.03.016.
[16] J.A. Anspach, A.C. Sanchez, T. Farkas, Injection sequence for optimizing performance in UHPLC separations, Phenomenex, http://phx.phenomenex.com/lib/po89900911_L_2.pdf, consulted on 25th of September 2023.
[17] A.C. Sanchez, J.A. Anspach, T. Farkas, Performance optimizing injection sequence for minimizing injection band broadening contributions in high efficiency liquid chromatographic separations, J. Chromatogr. A 1228 (2012) 338–348, https://doi.org/10.1016/J.CHROMA.2012.01.038.
[18] S. Chapel, F. Rouvière, S. Heinisch, Pushing the limits of resolving power and analysis time in on-line comprehensive hydrophilic interaction × reversed phase liquid chromatography for the analysis of complex peptide samples, J. Chromatogr. A 1615 (2020), 460753, https://doi.org/10.1016/J.CHROMA.2019.460753.
[19] S. Chapel, S. Heinisch, Strategies to circumvent the solvent strength mismatch problem in online comprehensive two-dimensional liquid chromatography, J. Sep. Sci. 45 (2022) 7–26, https://doi.org/10.1002/JSSC.202100534.
[20] J.J. Baeza-Baeza, M.C. García-Alvarez-Coque, Extension of the linear solvent strength retention model including a parameter that describes the elution strength changes in liquid chromatography, J. Chromatogr. A 1615 (2020), 460757, https://doi.org/10.1016/J.CHROMA.2019.460757.
[21] B.W.J. Pirok, S.R.A. Molenaar, R.E. van Outersterp, P.J. Schoenmakers, Applicability of retention modelling in hydrophilic-interaction liquid chromatography for algorithmic optimization programs with gradient-scanning techniques, J. Chromatogr. A 1530 (2017) 104–111, https://doi.org/10.1016/J.CHROMA.2017.11.017.
[22] A. Goyon, M. Excoffier, M.C. Janin-Bussat, B. Bobaly, S. Fekete, D. Guillarme, A. Beck, Determination of isoelectric points and relative charge variants of 23 therapeutic monoclonal antibodies, J. Chromatogr. B 1065–1066 (2017) 119–128, https://doi.org/10.1016/J.JCHROMB.2017.09.033.
[23] A. Goyon, V. D'Atri, O. Colas, S. Fekete, A. Beck, D. Guillarme, Characterization of 30 therapeutic antibodies and related products by size exclusion chromatography: feasibility assessment for future mass spectrometry hyphenation, J. Chromatogr. B 1065–1066 (2017) 35–43, https://doi.org/10.1016/J.JCHROMB.2017.09.027.
[24] 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, https://doi.org/10.1021/acs.analchem.6b04726.
[25] 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, https://doi.org/10.1016/J.CHROMA.2016.04.056.
[26] S. Fekete, D. Guillarme, Ultra-short columns for the chromatographic analysis of large molecules, J. Chromatogr. A 1706 (2023), 464285, https://doi.org/10.1016/J.CHROMA.2023.464285.