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Computer assisted liquid chromatographic method development for the separation of therapeutic proteins

Eva Tyteca,[a] Jean-Luc Veuthey,[b] Gert Desmet,[a] Davy Guillarme[b] and Szabolcs Fekete*[b]

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This review summarizes the use of computer assisted liquid chromatographic method development for the analytical characterization of protein biopharmaceuticals. Several modes of chromatography including reversed-phase liquid chromatography (RPLC), ion exchange chromatography (IEX), hydrophobic interaction chromatography (HIC) and some perspectives are discussed. For all these chromatographic modes, the most important variables for tuning retention and selectivity are exposed. Then, the retention models that were applied in the literature in RPLC, IEX and HIC are described and critically discussed. Finally, some representative examples of separation of therapeutic proteins and mAbs are shown, to illustrate the possibilities offered by the retention modeling approach. At the end, the reliability of the models was excellent, whatever the chromatographic mode, and the retention time prediction errors were systematically below 2%. In addition, a significant amount of time can be saved during method development and robustness testing.

1. Introduction

While most traditional pharmaceuticals represent chemically synthesized small-molecular weight molecules, the number of biopharmaceuticals that enter the market today is rapidly increasing.[1] Particularly, recombinant monoclonal antibodies (mAbs) are delivering interesting results in the treatment of autoimmune, cardiovascular and infectious diseases, cancer and inflammation.[2] Benefits for the patients of these large biomolecules include high efficacy, specificity, wide therapeutic range and limited side effects.[3] Moreover, generic biologics or "bio-similars" are entering the market, as the patents of the oldest approved biopharmaceuticals have expired. A thorough drug characterisation is required by regulatory bodies such as FDA (Food and Drug Administration) and EMA (European Medicines Agency), because the variability in the production of these biopharmaceuticals is typically greater than for conventional pharmaceuticals. To fully describe these complex samples, a variety of orthogonal methods is usually required. Spectrophotometry, electrophoresis, chromatography and mass spectrometry represent the most relevant techniques.[1]

Different chromatographic modes, possessing different separation mechanisms, have been applied for the analysis of protein samples. Reversed-phase liquid chromatography (RPLC) separates proteins based on their hydrophobicity, while size exclusion chromatography (SEC) and ion-exchange chromatography (IEX) protein separations are based on their molecular size and charge, respectively. Hydrophobic interaction chromatography (HIC), based on the protein salting-out principle, is the technique of interest to determine the relative hydrophobicity of mAbs and to separate different populations of antibody-drug conjugates (ADC) that differ in the number of drugs per antibody,[4] and is often also used for protein purification. Hydrophilic interaction chromatography (HILIC) is particularly interesting for glycoprofiling, at the peptide and protein level.[5]

Another challenging analytical project is the analysis of the proteome or "proteomics". While the genome is constant, the proteome is dynamic in nature as it is defined by both the genome, the environment and the cell history. The analytical requirements include high sensitivity, high resolution and high throughput. Hence, liquid chromatography is recognized as an indispensable tool in proteomics research.[6] Prior to protein identification *via* mass spectrometry (MS), the proteins can either first be separated and then fragmented in the MS or first digested and then separated. These proteomics approaches are respectively called "top-down" and "bottom-up", respectively. As a compromise between these two

[a]*Vrije Universiteit Brussel, Department of Chemical Engineering, Pleinlaan 2, B-1050 Brussels, Belgium*
[b]*School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland. E-mail: szabolcs.fekete@unige.ch; Fax: +41 22 379 68 08; Tel: +41 22 37 963 34*

approaches, "middle-down" proteomics is applied to proteins that have been cleaved into a few large fragments (larger than tryptic peptides but smaller than intact proteins).

Protein purification is often a bottleneck during protein generation for large molecule drug discovery. The process need to be optimized to perform high throughput purification. Similar concepts and methodology can be applied as for analytical scale chromatography.

The aim of this review is to highlight the possibilities of automated method development in liquid chromatography for large biomolecules, such as therapeutic proteins. Retention models applied in RP, IEX and HIC are reviewed and some practically useful approaches (designs) are suggested to save time spent on method development and understand retention behaviors.

2. Computer assisted method development

Method development (MD) in chromatography is the search for the optimal chromatographic operating conditions (type of mobile and stationary phase, temperature, gradient steepness, pH, ionic strength, *etc.*) resulting in the required separation of a mixture into its constituents. Because of the high probability for peak overlap and the sensitive dependency of the retention time on the employed chromatographic parameters, the MD process is often tedious and time-consuming (up to several weeks of work).[7] MD requires knowledge and expertise from the analyst and still involves a lot of trial and error processes. Computer-assisted MD has the potential to speed up the MD process significantly, if adequate retention models exist. The gain in analysis time can be particular interesting for peptides and proteins, which respond more strongly to changes in solvent strength than small molecules and hence often require very shallow and long gradients (on–off retention mechanism). The development of such long gradients without retention modeling is therefore iterative and time-consuming.[8]

MD typically involves a scoping (screening, scouting) and an optimization phase. During the optimization phase, very accurate retention modeling is required to find the optimal separation conditions (errors as low as 1–2% in retention time). On the other hand, during the scoping phase, including *e.g.* the choice of the chromatographic technique and column stationary phase, prediction errors up to 10% could be tolerated. Quantitative Structure Retention Relationships (QSRR), which could potentially replace the initial exploratory experiments by prediction solely based on the structure of the molecule, are of interest to speed up this scoping phase. However, much lower prediction errors can be achieved using analytical, empirical models established through fitting of a limited number of experimental retention data. Different software packages have been commercialised, employing empirical models (DryLab, Osiris) or a combination of *ab initio* and empirical models (ChromSword, ACD/Chromgenius).[9–12] ChromSwordAuto provides a fully automated MD tool, initiating 30 to 40 injections overnight. The analyst can then in the morning select the best chromatograms for future work.[9] Today, the most successful and widespread software package is Drylab. Other optimization strategies reported in literature were developed in house, using Matlab software or Microsoft Excel.[13–15] Besides speeding up the MD process, the systematic experimentation using these software packages also allow to meet the Quality by Design (QbD) practices, required in industrial laboratories by providing a tool to improve the robustness of a chromatographic method.[16] At last, computer-assisted MD reduces the solvent consumption by limiting the required number of experiments. Hence, it can be considered as a green strategy in liquid chromatography.[17]

Note that for proteomic applications, involving a high number of compounds, peak capacity optimization can be considered as an alternative to modeling the resolution between each peak pair in the chromatogram.[18]

3. Method development for reversed phase applications

In 1995, Horváth *et al.* pointed out the potential of HPLC as a quasi real-time monitoring tool for biotechnological processes.[19] Improvements in column technology led to a dramatic increase in the speed of analysis for these biological macromolecules with much smaller diffusivities compared to small molecules. Fast separations of peptides and proteins can be achieved thanks to the use of short columns packed with small particles, stationary phases with low mass transfer and kinetic resistance and elevated temperatures, combined with steep gradients and high mobile phase velocities.[19] As an illustration, a separation of five proteins in 20 s was reported in 1987 using a short column (30 × 4.6 mm) packed with a micropellicular sorbent (*n*-octyl bounded 2 μm silica particles). The separation was carried out at high flow rate (4 mL min^{-1}) and elevated temperature (80 °C), using a linear gradient from 15 to 95% ACN (0.1% TFA) in 48 s.[20] Recently, the potential of polymer monolithic capillary columns for ultra-fast gradient separations of large biomolecules has been shown by Vaast *et al.*[21] These high-speed separations could be applied in high-throughput screenings, such as biomarker-validation studies or therapeutic drug monitoring.

In 1995, Karger and co-workers reported on the strong influence of mobile phase and temperature effects on the gradient elution RP chromatography of proteins. They found significantly lower retention on more hydrophilic stationary phases (*e.g.* cyano or non-endcapped butyl) compared to *n*-alkyl bonded phases using acetonitrile while in the case of 1-propanol, no retention difference was observed. Column temperature, salt type and concentration induced dramatic changes in peak shapes for certain proteins.[22]

Different retention mechanisms for proteins in RPLC have been proposed: (1) an on/off mechanism retaining the macromolecules at the column inlet until at some point in the gradient they are desorbed and then move through the column

without any further interaction (2) precipitation–redissolution, *i.e.*, separation based on solubility instead of interaction with the stationary phase (3) multipoint attachment to the surface of the stationary phase.[23] While these mechanisms are fundamentally different from those observed for small molecules, the gradient separation of macromolecules can still be predicted from the "small molecule" Linear Solvent Strength (LSS) theory.[24] Indeed, the solutes are also eluted in their order of increasing hydrophobicity.[25,26] Moreover, the retention is strongly dependent on small changes in the fraction of organic modifier. Gradient elution mode, whereby the fraction of organic modifier is continuously increased, is therefore preferred over isocratic elution.[4] Jandera reported the validity of the LSS-model for various types of columns, packed with fully porous, superficially porous or non-porous particles, silica-based and organic-polymer monolithic columns.[27] However, conformational changes in proteins at variable chromatographic conditions could be very complex and may result in deviations from the LSS-behavior. Improved predictions can thus be obtained using a quadratic retention model.[4]

Vaast *et al.* reported accurate retention time predictions of high-speed gradient separations of intact proteins in capillary liquid chromatography using the non-linear Neue–Kuss model (<2% error). On the other hand, conventional gradient slopes could be predicted using the simple LSS-model.[28] Using standard bore columns, the LSS-model was again able to describe the retention behavior of intact proteins. This result was attributed to the strong effect of organic modifier on retention (*cf.* the large *S*-values of proteins), and the corresponding small elution window that is attained.[29]

Besides the mobile phase composition, the column temperature is also known to affect the retention behavior of proteins. Depending on the extent of denaturation (unfolding) of the biomolecules, they will more or less strongly interact with the stationary phase. This temperature-induced unfolding is protein dependent. Hence, the temperature can also be considered as an optimization parameter during method development.[4] However, as thermal degradation of proteins is possible at high temperatures, a compromise between residence time (gradient time) and temperature should be found.[30]

3.1 "Gradient steepness – temperature" model in RP mode

After selection of an appropriate stationary phase and mobile phase, the selectivity can be optimized using a multi-factorial (3^2) optimization including gradient steepness and temperature. For a 150 × 2.1 mm column, initial gradient experiments from 30 to 40% B (ACN + 0.1% TFA) in 4, 8 and 12 min are proposed at temperatures of 70, 80 and 90 °C.[30] (Mobile phase "A" is typically 0.1% TFA in water). Importing the experimental retention times, peak widths and asymmetries into a modelling software (*e.g.* DryLab), a quadratic two-dimensional model and resolution map can be fitted. The critical resolution ($R_{s,crit}$) is then plotted against the two optimization parameters to find out the optimal conditions. Fig. 1 shows a two dimensional resolution map based on the proposed experiments. On the resolution map, a colour-code is often applied that allows to easily visualize the optimal conditions. An average retention time prediction error of 1.0% was found for this approach. The average R_s prediction error, which also includes peak width and peak symmetry errors, was 16.1%. The proposed generic MD approach was applied to the analysis of large mAb fragments, obtained *via* limited proteolytic digestion (*e.g.* by adding papain, pepsin or IdeS) and/or reduction (by adding dithiotreitol).[30] These strategies require only limited sample preparation and therefore offer a high-throughput alternative to the conventional peptide mapping involving a complete proteolytic digestion of mAbs to identify mAbs chemical modifications. As shown by Chloupek *et al.*, accurate retention modeling, together with peak matching, can still be used for the separation optimization of such complex small peptide mixtures.[31–34]

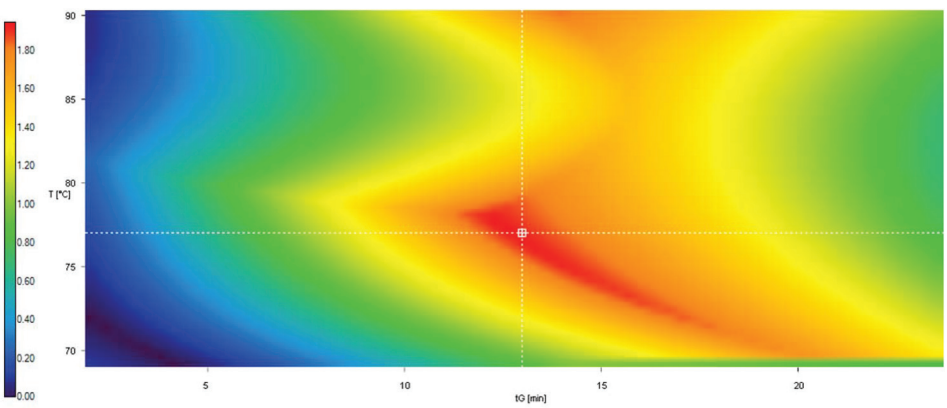

Fig. 1 Two dimensional resolution map as response function for the optimization of the separation of reduced mAb sample in RP mode. The retention model is based on nine initial gradients performed at three gradient steepness and three temperatures (generated with DryLab4 software). The red colour corresponds to the highest resolution, while blue area indicates co-elution. Data taken from the authors' laboratory.

3.2 "Gradient steepness – temperature – ternary composition" model in RP mode

A three-dimensional model including gradient steepness, temperature as well as ternary composition can also be considered for the computer-assisted optimization of protein separations in RPLC. Besides acetonitrile, an alcohol (protic), such as methanol or isopropanol, can be used for ternary elution of both small molecules as well as intact proteins.[35,36] The three-level multi-factorial optimization now consists of 27 experiments (3^3) when considering a full quadratic model for each of the parameters.

In another study, a fractional design consists of 2 × 2 × 3 experiments have been applied. The conventional, small-molecule linear relationship was used to model the gradient steepness and temperature, while the ternary composition was modeled using a quadratic relationship. For each of the three experimental sets (ternary composition) two-dimensional resolution maps (gradient time against temperature) are generated. These are then used to create a three-dimensional resolution cube to visualize the combined influence of the optimization parameters.[36] Fig. 2 represents a resolution cube based on three method variables, obtained for a reduced mAb sample (heavy- and light chain fragments). The part of the "design-space" where $R_s > 1$ is shown in red.

3.3 Possible issues in RP mode

Inherent problems associated with the analysis of proteins by RPLC include the numerous different conformations, post-translational modifications or multiple isoforms resulting in broadened band widths and shifted retention times.[4] Moreover, the lower diffusion coefficients of large molecules compared to small molecules result in lower column efficiencies and broader peaks. Also, the slow equilibrium between different conformations, present at high concentrations of organic solvents, can dramatically increase the band widths.[27] Similar retention behavior can typically be expected for biopharmaceutical samples, due to a small difference in physicochemical properties. As a result, high efficiency columns, such as the ones packed with wide-pore sub-2 μm porous particles (PP), superficially porous particles (SPP) or wide-pore monolithic columns, are required to enhance the resolution of separations with unsatisfactory selectivity.[4]

When working under UHPLC conditions (pressure >400 bar), pressure and temperature strongly influence the retention of large molecules. The increase in retention with pressure (at 800–1000 bar) is related to a change in molar volume and the solvation layer of an alkyl-bonded phase. The effect of longitudinal temperature gradients due to frictional heating (at high linear velocities) was found to be less pronounced for large biomolecules compared to small molecules.[37] Temperature related retention effects were found to be more complex than the conventional van't Hoff relationship. Concave plots (ranging from 25 to 75 °C) with a maximum retention factor were reported, indicating changes in secondary structure/conformation of the proteins at a certain pressure and temperature. Moreover, this conformational change was found to be protein-dependent.

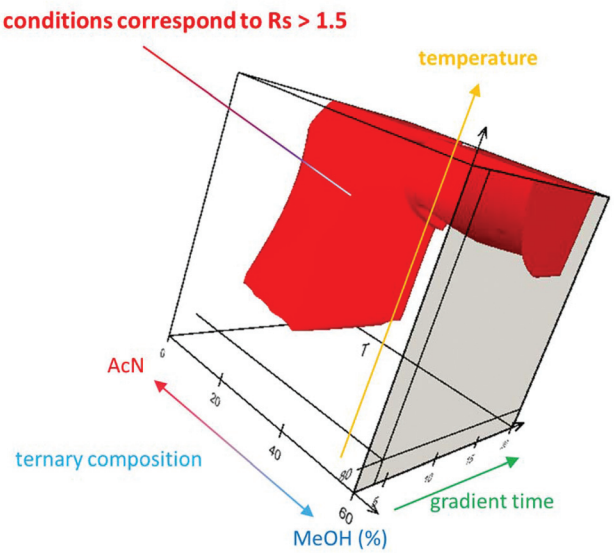

Fig. 2 Three dimensional resolution cube as response function for the optimization of the separation of reduced mAb sample in RP mode. The retention model is based on twelve initial experiments performed at two gradient steepness, two temperatures and three ternary mobile phase compositions (generated with DryLab4 software). The red colour corresponds to the conditions where the resolution of any critical peak pair was higher than 1.5. Data taken from the authors' laboratory.

4. Method development in ion-exchange chromatography

IEX is a historical and non-denaturing chromatographic mode widely used for the characterization and separation of charge variants of therapeutic proteins. It is considered as a reference technique for the qualitative and quantitative evaluation of charge heterogeneity of therapeutic proteins.[38] The history and current applications of IEX were recently reviewed.[39] Among the different IEX modes, cation-exchange chromatography (CEX) is the most widely used for protein characterization, due to the fact that most therapeutic proteins (mAbs) possess high isoelectric point (pI).[40] CEX is considered as the gold standard for charge variants analysis, but method parameters, such as column type, mobile phase pH, and salt concentration gradient, often need to be optimized.[41] In addition to cation-exchange, anion-exchange chromatography (AEX) was also applied and found suitable for the separation of oxidized variants of intact mAbs.[42]

In classical IEX, a linear salt-gradient is applied for the elution and therefore, a constant mobile phase pH is maintained. Several retention models have been reported for IEX based on the molecular structures.[43] The retention models can be classified as stoichiometric and non-stoichiometric ones. Stoichiometric models describe the multi-faceted binding of the protein molecules to the stationary phase as an exchange

of mobile phase protein and bound counter-ions.[44] This stoichiometric displacement model (SDM) predicts that the retention of a protein is related to counter-ion concentration. This model was extended to describe protein retention under linear gradient elution conditions (LGE model), as well as under non-linear protein adsorption conditions (steric mass action (SMA) model) in both isocratic and gradient elution modes.[45–47] Another extension of the model for the ion-exchange adsorption which accounts for charge regulation was recently developed.[48,49]

Various non-stoichiometric models have also been proposed.[50–53] Quantitative structure–property relationship (QSPR) models have been derived for protein retention modeling in IEX by means of different numerical approaches that attempt to correlate retention to functions of descriptors derived from the three-dimensional structure of the proteins.[54–56] More recently, theories used in colloid and surface chemistry to describe electrostatic and other interactions have also been applied to describe retention properties of proteins in IEX.[57–61]

4.1 "Gradient steepness – pH" model in salt gradient mode

In most cases, the charge heterogeneity and the variants distribution of therapeutic proteins are not known (or only after their thorough characterization), thus retention modelling based on molecular structure is hardly feasible. Therefore, it is more practical to perform some scouting chromatographic runs to have an idea about the species that have to be separated and about their retention behavior, instead of performing time-consuming calculations based on protein structures and possible interactions (using descriptors). It has been recently shown that the LSS model can be applied for mAbs under common salt gradient conditions.[62] Then – from practical point of view – the fastest procedure is based on a limited number of scouting runs followed by building the retention model and then finding the optimal conditions. The two most important variables for tuning selectivity in salt gradient mode were found to be (1) gradient steepness and (2) mobile phase pH. In salt gradient mode, the impact of mobile phase temperature on selectivity was found to be minor. Due to the LSS behavior, the impact of gradient steepness can be studied only at two levels, which simplifies the number of required experiments. However, retention not always depends linearly on mobile phase pH, especially when working at pH close to the protein's pI. Therefore, accurate modelling of the change of retention with mobile phase pH requires at least three experiments (within a limited pH range). When combining these two method variables, an experimental design based on 6 experiments (2 gradient steepness × 3 pH) enables to model retention, resolution and determine the optimal conditions.[62] Such a procedure can be applied routinely and the time spent for method development can be shortened. The relative error in retention time prediction was reported to be lower than 1% when using DryLab software for such a design, making this approach highly accurate for intact mAb and mAb fragments separations.

4.2 "Gradient steepness – temperature" model in pH gradient mode

Ion-exchange chromato-focusing represents a useful alternative to salt-gradient elution, in particular for separating protein isoforms with minor differences in their pI. Chromato-focusing is performed on an ion-exchange column employing a pH gradient.[63–67] Highly linear, controllable, and wide-range pH gradients (from pH 5.6 to 10.2) can be performed by using specific mobile phase buffers.[67–70]

The number of publications dealing with the retention modeling of linear pH gradient elution in IEX is rather limited.[71] To describe the elution behavior of proteins in linear pH gradient IEX, a pH dependence parameter has to be incorporated into the ion-exchange model. In pH-gradient mode, the protein's net charge is modified during the pH gradient, due to protonation–deprotonation of the functional groups. In CEX, the protein is expected to elute at a pH equal or close to its pI. The applied pH range therefore clearly determines the proteins that can possibly be eluted. The effect of pH gradient steepness (gradient time) on the retention of large proteins (intact mAbs and their variants) was recently studied and showed an LSS-like behaviour.[72] Mobile phase temperature was also an important method variable to optimize pH gradients (probably through the temperature dependence of pH and pI). Because the retention models were always linear, only four initial experiments (2 gradients steepness × 2 temperatures) were required to model the retention behaviour in CEX pH-gradient.[72] The predicted retention times of mAb sub-units were in good agreement with the experimental ones. The average retention time relative errors was systematically under 1.0%, which can be considered as excellent. The highest individual deviation was ~1.5%.[72]

4.3 Applications of computer modelling in IEX

Computer assisted method development (retention modelling) was successfully applied for the separation of Fab (fragment antigen binding) and Fc (fragment crystallisable region) variants of cetuximab in both salt- and pH gradient modes.[62,72] In salt gradient mode, 10 and 30 min gradients were performed on a 100 mm long standard bore column at pH = 5.6, 6.0 and 6.4 to build up the models (DryLab). In the pH gradient mode, the experiments were performed with 10 and 30 min gradients at mobile phase temperatures of 25 and 55 °C. Finally, similar separation quality was achieved in the two modes and the analysis times were comparable. These examples suggest that the two modes can routinely be applied for the optimization of mAb fragments separation.

Another important application of IEX is the possibility to perform a generic multi-product intact mAb separation and to elute as many mAbs as possible under one given condition (for screening purposes and to evaluate mAbs' pI). DryLab software was used to develop multiproduct mAb methods in both salt- and pH gradient modes.[62,72] Again, the two modes of elution gave comparable results in terms of analysis time and separation power.

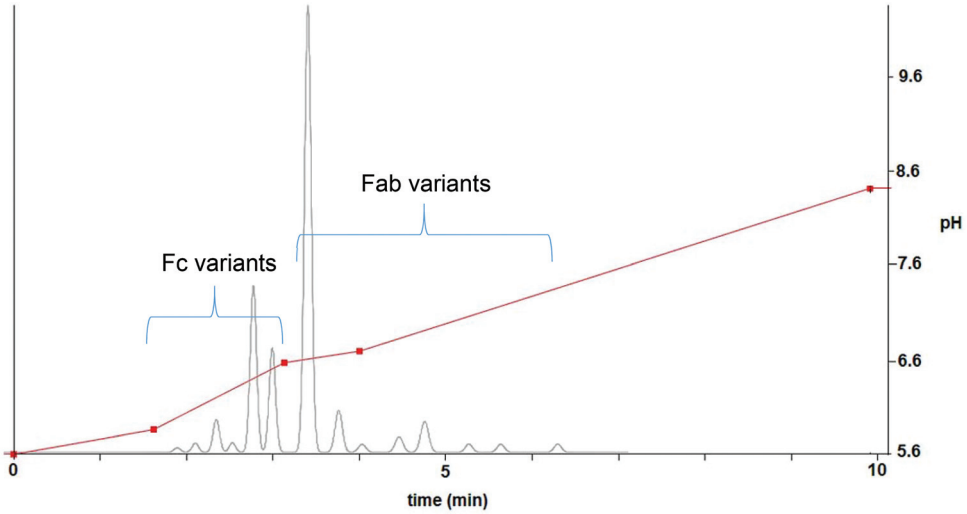

Fig. 3 Modelling of a multi-linear pH gradient for complex digested mAb sample in CEX. The retention model is based on two initial gradients performed at different gradient steepness (linear pH gradient between pH = 5.6 and 10.2) and generated with DryLab4 software. Data taken from the authors' laboratory.

There are also some additional benefits of using modelling software, such as the possibility to simulate the impact of various method parameters, including column dimension, flow rate or system gradient delay volume, on the separation. Based on LSS retention behaviour, multi-linear gradients can be calculated too, which often improves the selectivity and reduce analysis time. Fig. 3 shows a calculated multi-linear gradient separation for a complex papain digested mAb sample analysed in pH gradient mode.

5. Method development in hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is also a historical technique used for the analytical purification and characterization of proteins. HIC is able to separate protein species based on their hydrophobicity, under non-denaturing conditions (*i.e.* physiological pH conditions, ambient mobile phase temperature and no organic solvents). Currently, HIC is mostly applied for the characterization of mAbs, ADCs and bispecific antibodies (bsAbs).[73] One of its most important applications is the separation of different populations of ADC molecules that differ in their number of drugs per antibody, which are often known as DAR (drug-to-antibody ratio) species.[74,75]

In HIC, the separation of proteins is based on a negative salt gradient performed on a slightly hydrophobic stationary phase, using physiological mobile phase pH conditions. The retention mechanism in HIC is often misunderstood and none of the proposed theories has received general acceptance.[73] Different interpretations and approaches, including hydrophobic interaction, solvophobic theory, salting-out effect, dehydration of proteins or structural rearrangement of proteins are often confused. All the different interpretations of retention in HIC have recently been reviewed.[73]

The salting-out effect is based on electrolyte-nonelectrolyte interactions (corresponding to mobile phase–protein interaction in HIC), in which the protein becomes less soluble at high salt concentrations.[76] In aqueous solutions, proteins fold and the hydrophobic amino acids usually form some protected hydrophobic areas, while hydrophilic amino acids form hydrogen bonds with the surrounding water. If the hydrophilic surface of the protein is large enough, then the protein can be dissolved in water. When adding salts, water molecules will solvate predominantly salt ions. Therefore, the number of water molecules available to interact with the hydrophilic part (charged) of the protein will decrease. Under these conditions, the protein–protein intermolecular interactions become stronger due to the decreased amount of surrounding water molecules. At the end, the protein molecules can associate by forming hydrophobic interactions with each other. This mechanism seems to be strongly affected by the temperature.[77–81] It was shown that hydrophobic interactions are entropy-driven at low temperatures, but enthalpy-driven at elevated temperatures.[82] Such model experiments provided the basis of a more detailed understanding of the influence of temperature on hydrophobic interactions and salting out effect.

Horváth *et al.* developed the basis for describing retention mechanisms in HIC employing the framework of the solvophobic theory.[83] A simplified mass balance equation was introduced and led to an expression of the different free energy contributions to the overall retention process. It was shown that the structural forces of H-bond interlinked water molecules represent an energetically low state of the water structure. In contrast, in the neighborhood of the stationary phase

alkyl-chains, the water (or aqueous solvent) is under an energetically "excited" state as it has no contact with neighboring water molecules.[83] One way to return to an energetically low state is through an enforced association among the alkyl-chains, and another way is to combine the alkyl-ligand and hydrophobic solute to form an association complex. The disappearing contact surface area multiplied by the surface tension represents the released energy upon association of the hydrophobic ligand with the analyte molecule, suggesting that the retention is an exothermic process. It was also found that the dominant term in the free energy equation was the so called "cavity term", which expresses the large energy required to separate neighboring water molecules to form a cavity in the aqueous mobile phase around the alkyl chains.[84]

By accounting for the effect of salt concentration on the mobile phase surface tension, the magnitude of solute retention has been expressed in terms of the molar salt concentration in HIC.[85,86] The theory predicts that for sufficiently high salt concentrations – where the retention is governed predominantly by hydrophobic interactions – the retention increases with both the molar salt concentration (in the mobile phase) and the size of the solute (protein) – or its hydrophobic moiety.

5.1 Optimization of the phase system in HIC

Retention in HIC mostly depends on the ligand type, ligand chain length and ligand density. It has been shown that the salt type can have different effects on the retention, depending on the hydrophobicity of the protein to be separated, and emphasized the importance of a well selected phase system (the combination of stationary phase, salt type and salt concentration).[87] The influence of salts on hydrophobic interactions follows the lyotropic (Hoffmeister) series for the precipitation of proteins from aqueous solutions.[88] In practice, sodium or ammonium sulfate effectively promote stationary phase–protein interactions and have a stabilizing effect on protein structure. Hence, the most commonly used salts are ammonium sulfate, sodium sulfate as well as sodium chloride and ammonium acetate. It has also been demonstrated that salt nature affects differently the retention on different stationary phases. It can both increase and decrease the retention of hydrophobic and hydrophilic proteins, respectively.[89,90] The above results also imply that the effect of a salt cannot be predicted in advance but should always be experimentally tested as an early step of method development.[91]

Besides the salt type, the other parameter for varying HIC retention is the salt concentration. Depending on the lyotropic strength of the various salts, different concentrations are required to maintain the same salting-out effect. Stronger salts are efficient in 1–1.5 M concentration, but weaker salts require higher concentration (3–5 M) to maintain the same retention. On the other hand, peak widths also vary with salt concentration, since it impacts the concentration gradient (steepness) and therefore the gradient band focusing effect. A systematic study showed the possibility to combine different salts (binary and ternary salt systems) to modify selectivity and retention in HIC.[91] In this phase system optimization concept, the experimental design was based on gradient experiments performed on three different columns and with three different buffers (salts). A very recent study demonstrated that for ADC DAR species separations, similar selectivity can be achieved with any type of salts, provided that its lyotropic strength is corrected on a given stationary phase.[4,72] In this study, equivalent molarity of the different salt systems were also determined for ADC separations on various columns.

Hydrophobicity indexes were also derived for various mAbs and ADC species on several stationary phases using various salt systems.[72] Hydrophobicity indexes can be derived from the LSS retention model parameters on the basis of two scouting gradients performed at different gradient steepness. These indexes can be determined on various columns with different salts and then, the optimal combination of salt and stationary phase can be obtained and the elution window be set. For mAbs, the hydrophobicity indexes varied significantly within the different phase systems, but the elution order remained identical in each condition. Hydrophobicity indexes were found to be dependent on the stationary phase for the same salt system. This suggests that mAb retention (and therefore selectivity) strongly depends on the stationary phase. The selectivity of mAb separations performed on one given column can also be tuned by changing the salt type.

5.2 "Gradient steepness – temperature" model in HIC

For mAbs, it was shown that the most important method variables were the gradient steepness and mobile phase temperature.[72] LSS and van't Hoff type behavior have been found, therefore a two dimensional retention model can be built on the basis of four initial runs (2 gradient steepness × 3 pH). Please note that temperature cannot be increased above 40–45 °C since at higher temperature, proteins can undergo conformational changes and denaturation that should be avoided in HIC. Based on the four initial runs, the average relative error of retention time prediction was around 1% for various mAbs inside the applied design space. After the phase system optimization this "gradient steepness – temperature model" can be performed on the best phase system to speed up the method development procedure. With a commonly used HIC column dimension of 100 × 4.6 mm, a 10 and a 30 min linear gradient provide accurate retention model.

5.3 "Gradient steepness – organic modifier" model in HIC

The addition of organic modifier to the mobile phase can improve the recovery of the most hydrophobic species (*e.g.* high DARs of ADCs) and can also impact their retention.[4,72] Therefore, organic modifier content can also be a useful method variable when very hydrophobic proteins have to be analyzed in HIC. Both protic and aprotic solvents can be used (*e.g.* isopropanol, acetonitrile) since they can affect the salting out process in different ways through their different solvation. Recently, a linear HIC retention model has been derived for organic modifier content, therefore the effect of organic modifier can be easily studied using only two levels

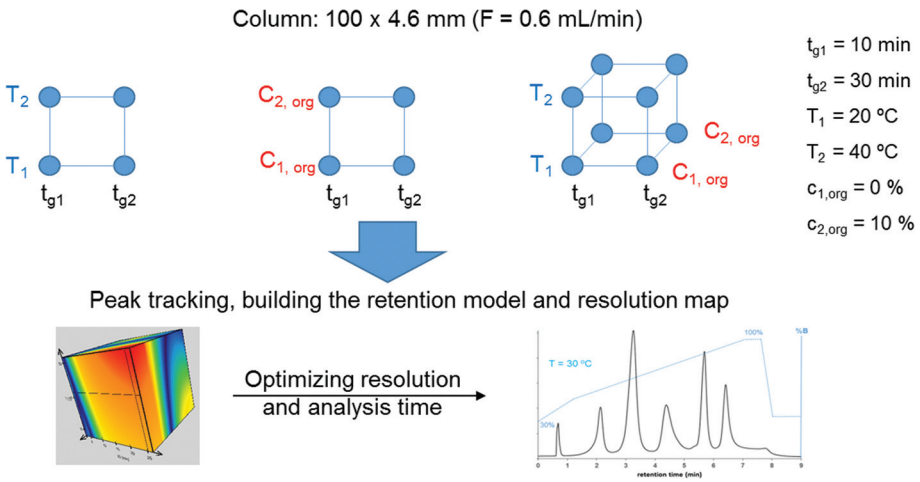

Fig. 4 Summary of possible HIC optimization processes using the most popular column dimension (100 × 4.6 mm). Both two and three dimensional models can be developed.

(*e.g.* 5 and 15%).[4] In this case, it is useful to combine the effect of organic modifier with gradient steepness to build up a thorough retention model. This approach again requires four initial experiments performed at two gradient steepness and at two organic modifier contents.

5.4 Applications of computer modelling in HIC

Custom made DryLab models (DryLab 2010) have been successfully applied for the separation of intact mAbs and ADC species for both "gradient steepness – temperature" and "gradient steepness – organic modifier" modes.[4,72] It seemed that for ADC species, a multi-linear gradient (steeper at the beginning and flatter at the end) gives better selectivity than linear gradients, probably due to the fact that ADC species consist in a homologous series of linkers and cytotoxic drugs.

A three-dimensional model was also proposed recently with gradient steepness (1), temperature (2) and organic modifier (3) as variables, however experimental results have not been reported yet.[73] This model would require 8 initial experiments (2 gradient steepness × 2 organic modifier content × 2 temperature).

Fig. 4 summarizes the possible retention models and required experiments for the optimization of HIC separations.

6. Robustness testing

A fundamental quality criterion in High Performance Liquid Chromatographic (HPLC) is robustness.[92] Guidelines define the robustness of an analytical procedure as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". providing "... an indication of its reliability during normal usage".[93] Historically, robustness testing was usually carried out as the final step of a method development process, during the validation stage, which often led to unexpected observations.[92,94] However, since a method considered as non-robust should be adapted/redeveloped and revalidated, this could lead to a substantial increase of development time and costs. Therefore, robustness should be evaluated earlier in the lifetime of a method, *i.e.* at the method development stage or at the beginning of the validation procedure.[95–97] The robustness testing is often based on either one factor at time (OFAT) approach or complex experimental design.

A new feature of commercial modelling software (such as DryLab 4) has been shown to perform an in-depth "modelled" robustness testing.[98] From the design space, as defined in a resolution map or cube, it is possible to get robustness information for the measured parameters, including gradient time (t_g), mobile phase temperature (T, °C) mobile phase pH or ternary composition. In addition, based on the models included in the software, the retention time of any compound can be calculated for the influence of additional parameters such as flow rate or start- and end-% B of the gradient. Consequently, the impact of changes in any of these parameters on the resolution can be assessed using simulated two levels (2^4, 2^5, 2^6) or three levels (3^4, 3^5, 3^6) type factorial designs (including 16 to 729 simulated experiments, depending on the number of factors and their levels). No additional experiments are necessary for performing the simulated robustness calculation.[98,99] The possible deviations from the nominal values have simply to be defined and then the software makes the calculations for all the conditions. At the end, a 'frequency distribution graph' showing how often (N) a certain critical resolution occurs under any combination of possible parameters is provided. This graph clearly shows the failure rate, *i.e.* number of experiments that could fall outside the required critical resolution. On the other hand, 'regression coefficients' can also be obtained to show the effect of each parameter, related to the selected deviation from the nominal value, for the critical resolution.

Fig. 5 shows an example of a simulated robustness test based on gradient steepness (1) – temperature (2) – ternary

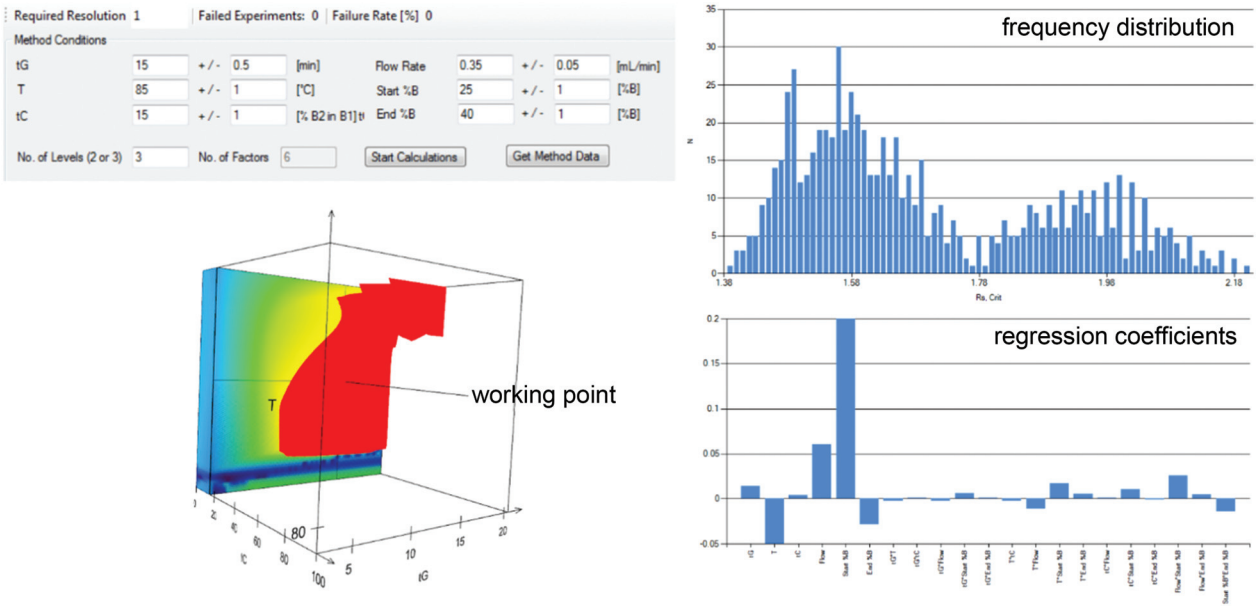

Fig. 5 Example of simulated robustness testing for partially digested and reduced mAb. The retention and resolution calculations were based on gradient steepness (1) – temperature (2) – ternary mobile phase composition (3) 3D model and generated with DryLab 4 software. On the resolution map, the red space indicates the conditions where R_s = 1 criteria is fulfilled. Data taken from the authors' laboratory.

mobile phase composition (3) 3D retention (resolution) model for a partially digested and reduced mAb. For the initial runs, the gradient steepness was studied at two levels (4 and 12 min gradient time at 0.35 mL min^{-1} on a 150 × 2.1 mm column), temperature at two levels (80 and 90 °C) and ternary composition at three levels (100% acetonitrile, 50–50% acetonitrile–methanol and 100% methanol). Then, the working point was selected at 15 min gradient time, 85 °C mobile phase temperature and 85–15% acetonitrile–methanol composition in mobile phase B. A 3D resolution map was created with R_s = 1 as resolution criteria (see Fig. 5). Next, the effect of the three model variables and other three parameters (flow rate, initial mobile phase and final mobile phase composition) on the critical resolution was calculated at three levels, simulating 729 experimental conditions around the selected working point. The low, medium and high values of the variables are shown in Fig. 5. Finally, frequency distribution and regression coefficients were determined. It was shown that initial mobile phase composition (start% B) had the highest impact on the resolution and failure rate was 0% (with R_s = 1 as criterion) suggesting that the method is robust around the working point. It is worthy to note that the calculation and simulation of 729 conditions required less than 1 minute.

7. Perspectives: possibilities of HILIC

7.1 New wide-pore HILIC stationary phases for intact and fragmented mAbs

HILIC is recognized as a powerful technique for peptide analysis and peptide mapping (proteomics). Until recently it has however found only limited application in the analysis of proteins. Successful applications are membrane proteins, histones and glycosylated isoforms of intact proteins.[100] Nevertheless, with the introduction of a new sub-2 μm stationary phase consisting of wide-pore amide-bonded hybrid silica, therapeutic proteins could be analysed using HILIC.[101] The large pore size (300 Å) does not restrict diffusion of the large biomolecules into the pores. Moreover, the amide ligand provides high HILIC retention, which enables the use of high water content mobile phases. While the employed HILIC and RPLC stationary phases (Acquity UPLC Glycoprotein BEH Amide 300 Å 1.7 μm and Peptide BEH C18, respectively) showed comparable kinetic performance, the two techniques were found to be orthogonal for the analytical characterisation of therapeutic proteins.[101] The elution order was different but not opposite as retention in RPLC and HILIC are determined by the hydrophobic and the hydrophilic amino acid residues, respectively. It was found that optimized aqueous injections can be achieved by decreasing the injected volume to 0.1–0.2% of the column volume and adding a fast initial ramp starting form a high ACN condition. Moreover, reasonable temperatures (40 °C) could be employed without significant intensity loss (appropriate protein recovery), in contrast to RPLC analysis. The HILIC analysis of the mAb trastuzumab (~150 kDa) could separate the glycovariants, which was not possible by RPLC or IEX.[101] In conclusion, HILIC can offer some "hydrophilic selectivity" while RPLC can deliver "hydrophobic selectivity" for mAb fragment analysis.

Other manufacturers also provide wide-pore HILIC stationary phases (polyHYDROXYETHYL A is available with pore sizes up to 1500 Å, ZORBAX RRHD HILIC 300 Å 1.8 μm). The

separation of native ApoA-I and recombinant ApoM (~20 kDa) on the polyHYDROXYETHYL A 300 Å has been reported by Tetaz *et al.*[102]

7.2 Possible retention models and optimization strategies

A multi-factorial optimization strategy including gradient steepness and temperature, could also be envisaged for the analysis of intact and fragmented mAbs in HILIC. The complex, mixed-mode retention mechanism in HILIC typically results in non-linear retention relationships for small molecules.[103,104] Hence, for large biomolecules, a quadratic model can again be expected to perform better than the conventional LSS-model. Future studies will answer the question whether these quadratic models will be sufficient to model the retention of biomolecules in HILIC with adequate accuracy to find the optimal separation conditions. Particularly, the requirement of a fast initial ramp to ensure optimal injection conditions, and resulting in a two-step gradient profile, should be incorporated in the method development strategy. Drylab offers the possibility to explore multi-segment gradients in a trial-and-error fashion, starting from the best linear gradient, then inserting a number of node points and "drag and drop" each node. The corresponding simulated chromatogram can be evaluated until a satisfactory separation is obtained.[105] However, other multi-segment gradient optimization strategies have been reported in the literature,[106–108] which could in the future be incorporated in the multi-factorial optimization.

8. Conclusion

Computer assisted liquid chromatographic method development is a well established strategy to save time and money, when developing method for small molecules. However, such an approach is less common when dealing with the analytical characterization of protein biopharmaceuticals. The goal of this review was therefore to highlight the benefits and limitations of automated method development for large biomolecules, such as therapeutic proteins and mAbs. As shown in this paper, retention modeling can be successfully performed in several chromatographic modes, including RPLC, IEX and HIC. In all these modes, the prediction of retention time and resolution was highly accurate (as example, the retention time prediction errors were systematically below 2% for protein biopharmaceuticals), providing that the adequate model was used and suitable variables were selected. Similarly to what happen with small molecules, the time spent for method development and robustness evaluation can be significantly shortened with biopharmaceuticals, thanks to automated method development tool. Last but not least, method transfer between different column geometries or instruments having different dwell volumes becomes straightforward with computer assisted liquid chromatographic method development tool.

By taking into account all these positive features, such softwares will certainly be more and more widely used in a close future in academia but also in the pharmaceutical industry to rapidly develop HPLC methods for the analytical characterization of biopharmaceuticals.

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