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SHORT COMMUNICATION

# Enhancing the Quality of Separation in One-Dimensional Peptide Mapping Using Mathematical Transformation

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**Abstract** In this study, some practical examples are presented that show the quality of separations using very efficient columns packed with the latest generation of core shell sub-3 μm and fully porous sub-2 μm particles in one-dimensional peptide separations. This paper shows an approach for the analysis of proteins, such as high-resolution separations, and a data transformation process to improve peak recognition and analysis. Applying power functions on raw chromatographic data can be a neat tool in the field of biosimilar analysis, especially in comparability studies regarding the quality (primary structure) of proteins. Based on the results presented here, it can be stated that the use of power functions is beneficial for the comparison of chromatograms when peak areas are considered but has no effect when using peak heights. In this study, the new Acquity CSH columns (C18 and phenyl-hexyl) and the core–shell type wide pore Ascentis Express Peptide ES C18 material were applied with great success in peptide mapping. Finally, using phenyl-hexyl stationary phase in peptide separation seems to be a good alternative to the generally applied C18 or C4 phases.

**Keywords** Liquid chromatography · Peptide map · Acquity CSH column · Core–shell particles · Enhanced resolution · Biosimilar

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## Introduction

In pharmaceutical industry, the quality of the different pharmaceuticals has to be controlled by validated analytical methods. Pharmacopeial organizations (ICH, FDA, USP, JP, PhEUR) worked out several guidelines for the analytical method validation. The fulfillment of the predefined requirements (accuracy, precision, limit of detection, limit of quantitation, etc.) is in most cases an achievable task when the targets of the corresponding analysis are small pharmaceuticals. These substances are often well characterized (the exact structure, physical and chemical properties are known) and with few exceptions, the standards are also available in a high-purity form, so one can obtain an authentic reference material and perform quantitation.

On the contrary, there is no obvious regulation when protein-derived high molecular weight components are considered. Reversed-phase (RP), ion-exchange (IEX) and size exclusion (SEC) chromatographic separations (HPLC) are widespread in biopharmaceutical analysis. These high molecular weight compounds may have numerous possible conformations, posttranslational modifications, multiple isoforms (microheterogeneity), which can cause broadened peak shape and shifted retention in the chromatograms. Another reason for broadened peaks is the very low molecular diffusion coefficient ($D_M$) of these compounds due to their large size. Finally, an additional serious problem in biopharmaceutical analysis is the lack of high-purity reliable reference materials [1]. Since proper standards are often not available, it is impossible to carry out quantitation and method validation according to the existing guidelines for the low molecular weight substances. Currently, there is no specific guideline for the method validation of the complex biomedicals analysis. The

guidelines of International Conference on Harmonisation (ICH) such as Q2(R1) was originally developed for low molecular weight products and cannot really be applied for biotechnology-based pharmaceuticals. On the other hand, Q6B does not describe true validation guidelines [2, 3]. However, manufacturers have to demonstrate that the biotechnological production has good batch-to-batch reproducibility and the product has suitable stability.

ICH Q5E is concerned with the comparison of the biotechnological and biological products [4]. The guide suggests considering more than one analytical procedure to evaluate the same quality attribute. Since there are many unique properties of a biotechnology-based product (molecular weight, size, isoelectric point, structure, purity, charge state, charge microheterogeneity, stability, aggregates, secondary and tertiary structure and so forth) manufacturing of biomedicals requires greater number of batches (>250) and quality tests (>2,000) than in the case of small pharmaceuticals (batches <10, and tests <100) [5].

Peptide mapping is a basic technique regarding the primary structure of proteins (single-point mutation). RP-HPLC plays a key role among other techniques because of the high reproducibility of the retention times of the different peptide fragments which arise as a result of the peptide digestion by an enzyme. The co-elution of critical peaks is often a problem in peptide mapping [6]. To solve this issue, several very efficient columns packed with core shell particles or very fine fully porous particles such as sub-2 μm packing have been introduced for commercial use [7].

Computer simulation can also be helpful in speeding up the method development and finding the parameters that provide the optimal separation. Indeed, developing a chromatographic separation necessitates a few basic runs, whose data serve as a basis for the computer program (for example DryLab) in constructing the resolution map. A critical step in understanding the data sets is the peak tracking (finding the peaks in the different chromatograms but representing the same component). In peptide mapping, it can be very difficult and time consuming because of the large number of fragments arising from the digestion of the protein so the use of computer simulations can be very difficult in peptide mapping [8].

Resolution and signal-to-noise ratio can be enhanced by means of application of power functions to the chromatographic data. Guiochon et al [9]. used such methodology on diastereomer separations and two-dimensional HPLC separations. Applying power functions can be a neat tool in the field of biosimilar developments, especially in comparability studies. In this study, some practical examples show how to enhance the quality of the separation obtained using very efficient columns packed with core shell sub-3 μm and fully porous sub-2 μm particles in one-dimensional peptide separations.

## Experimental

### Chemicals, Columns

Acetonitrile (gradient grade) and trifluoroacetic acid (TFA) (Uvasol) were purchased from Merck (Darmstadt, Germany). For measurements water was prepared freshly using Milli-Q® equipment (Milli-Q gradient A10 by Millipore). TRIS (ultrapure grade; 99.9%) and trypsin (for protein sequencing) were obtained from Sigma-Aldrich (Steinheim, Germany).

Model samples used in this study were tryptic digests of experimental model proteins from the authors' laboratory (trypsin was used as enzyme in TRIS buffer solution, the samples were thermostated at 37 °C during the digestion process). For peptide separation on the Ascentis Express Peptide ES C18 and Waters CSH C18 columns, digests of a polypeptide mixture were used to illustrate the chromatogram transformation and different batches were compared in terms of statistical evaluation. In case of the column packed with Waters CSH phenyl-hexyl particles, the peptide map of a moderate-sized protein is presented.

The new Waters Acquity CSH columns (phenyl-hexyl and C18) packed with 1.7 μm particles (100 mm × 3 mm) were generous gifts from Waters Ltd., Budapest. Ascentis Express Peptide ES C18 column (Sigma-Aldrich) with a particle size of 2.7 μm (100 mm × 3 mm) was purchased from Sigma–Aldrich Ltd., Budapest. All of the columns used in this study were brand new; no other experiments were performed on them.

### Equipment, Software

All measurements were performed using a Waters Acquity system equipped with binary solvent delivery pump, an autosampler and a photo diode array detector (Waters Ltd., Budapest, Hungary). The UPLC system had a 20-μL injection loop and a 500-nL flow cell (path length = 10 mm). A polyether ether ketone (PEEK) tube (15 cm × 0.1 mm) is located between the column outlet and the detector. The measured dwell volume is 130 μL. Data acquisition with a 20-Hz data-sampling rate and instrument control were performed by Empower 2 Software (Waters).

Calculation and data transferring to obtain the transformed chromatograms was achieved with OriginPro 8 (OriginLab Corporation, USA). Curve fittings and confidence interval calculations were performed using Statistica 9.0 (StatSoft Inc., USA). Solvent and gradient optimization

was performed using Dry Lab 2000 Plus chromatography optimization software (Molnar-Institute Berlin, Germany).

## Apparatus and Methodology

### *Optimization of the Chromatographic Conditions*

The mobile phase was prepared by mixing appropriate amount of HPLC gradient grade acetonitrile, Milli-Q water and TFA. The mixtures were degassed by sonication for 5 min. The weak mobile phase (A) consisted of water/TFA 100/0.1 (*v/v*), while the strong mobile phase (B) consisted of acetonitrile/water/TFA 90/10/0.1 (*v/v*). Four input experiments (gradient time–column temperature model) were initially performed to optimize the separations on the different columns (100 × 3 mm). Linear gradients with 10 and 30 min (0.75 mL $min^{-1}$) at 30 and 60 °C column temperature were performed. DryLab software was used to predict the optimal solvent ratio and gradient program, which would give the best selectivity and resolution. Since the protein and polypeptide digest contained numerous peaks and because the correct peak tracking was not feasible, only the 10 most intense peaks were chosen to evaluate an appropriate separation.

### *Transformation of Chromatographic Data*

The raw data were extracted from Empower Pro into ASCII-format. These files were then imported into OriginPro 8 mathematical data-handling software. The absorbance data were shifted to have no negative absorbance values and preserve the absolute differences. After this procedure, the chromatographic data were transformed using power functions according to the following equation:

$$f(x)^n = (ae^{-(x-b)^2/2c^2})^n \quad (1)$$

The equation represents the ideal situation when the chromatographic peaks can be described by the Gaussian distribution. $a$ represents the height, $b$ the location of the centre and $c$—standard deviation—the width of the peak. During the analysis of the data power ($n$ in Eq. 1) of 2, 4 and 8 were used. Nineteen peaks with the most intense signal from the original and the transformed chromatograms were integrated and the peak heights were also measured. The previously described data were normalized to the highest peak and expressed as relative peak height or relative peak area percentages. This procedure can also be applied for all of the peaks in the chromatogram but due to the limitations of the OriginPro 8 software and the complexity of the data-handling procedure, only 19 peaks were used for demonstrating how this novel method of comparing peptide maps works.

### *Statistical Evaluation, Comparison of the Peptide Maps*

The data described in the previous section were imported into Statistica software and sorted according to the needs of the analysis. The results of three repetitions of each sample from different batches were used (three digestions per sample). The relative peak area or peak height percentages of the first sample were plotted against the other. Linear regression was applied (Eqs. 2–4) and confidence intervals ($\alpha = 0.95$) were also calculated and plotted (Eq. 5). Theoretically, when the peptide map of biopharmaceuticals are similar, all the data points should be aligned along the line described by the equation $y = x$.

$$Y(x) = \alpha + \beta(x - \bar{x}) \quad (2)$$

$$\hat{\alpha} = a = \frac{\sum y_i}{n} = \bar{y} \quad (3)$$

$$\hat{\beta} = b = \frac{\sum y_i \times (x_i - \bar{x})}{\sum (x_i - \bar{x})^2} \quad (4)$$

$$P(\hat{Y} - s_{\hat{Y}} \times t_{0.025} < Y < \hat{Y} + s_{\hat{Y}} \times t_{0.025}) = 0.95 \quad (5)$$

Equation 2 describes the relationship between the $Y(x)$ as dependent and $x$ as independent variables in linear regression. The Eqs. 3–4 show how the parameters of the Eq. 2. $(\alpha;\beta)$ can be estimated $(\hat{\alpha};\hat{\beta})$ using the experimental data. The confidence intervals of the regression line can be calculated according to Eq. 5, where $\hat{Y}$ is the estimated value of $Y$, $s_{\hat{Y}}$ is the standard deviation of $\hat{Y}$, $t_{0.025}$ is the Student parameter for the two-sided 0.95 confidence level.

## Results and Discussion

Several types of column packing are available for achieving very fast or very efficient chromatographic separations. In the recent development of particle technology for liquid chromatography, the use of core–shell particles has received considerable attention [10–16]. The particle size is also a beneficial factor as it improves the rate of mass transfer and reduces the eddy dispersion effect, resulting in smaller plate height and higher optimum linear velocity as the particle size is reduced [17, 18]. In this study, the new 1.7 µm Waters CSH (charged surface hybrid) packing and the recently introduced wide pore 160 Å Peptide-ES core–shell type packing (Sigma-Aldrich) were applied in order to enhance the efficiency in peptide separation compared to conventional HPLC peptide maps. Three examples were presented to demonstrate the high efficiency of these new generation stationary phases and to show the benefits of applying power functions on chromatographic data.

Peptide Separation on Wide Pore Core–Shell Particles (C18 phase)

This example presents a very efficient gradient separation of a protein digest using 2.7 μm Ascentis Express Peptide ES C18 100 mm × 3 mm column. Initially, four input experiments (gradient time–column temperature model) were performed to optimize the separation. The optimal conditions were found as: linear gradient program from 0 to 12%B min (in 20 min) then 30%B at 24 min, followed by a 4 min reequilibrating. The mobile phase flow rate was set to 0.75 mL $min^{-1}$. The column was thermostated at 30 °C and 10 μL of the sample solution was injected. The chromatograms were recorded at 280 nm. The samples contained several peptides (approximately 0.7–2 kDa fragments). The baseline separation of all peaks was not achieved. Figure 1 shows the original and transformed chromatograms. It can obviously be seen on the original peptide map that in spite of the high separation power of this column, allowing very high peak capacity ($P_c = 176$ in 24 min, peak widths were measured at peak half-height) there were lots of critical peak pairs with insufficient resolution and the evaluation of peak area was also difficult and inaccurate. Due to the transformation with power functions using different powers, the chromatogram was simplified and the integration much easier to carry out.

Peptide Separation, on CSH C18 1.7 μm Totally Porous Phase

In this section, the applicability of the recently introduced CSH C18 phase is presented. Our results confirmed that this stationary phase can be very useful in peptide separations because of its very high efficiency and good selectivity. In this example, the separation of the peptide mixture used in the previous section was demonstrated on CSH C18 phase. Because of the different selectivity of CSH C18 phase compared to other common C18 phases, the chromatographic conditions were adapted. The optimal conditions were found as: linear gradient program from 0 to 4%B min

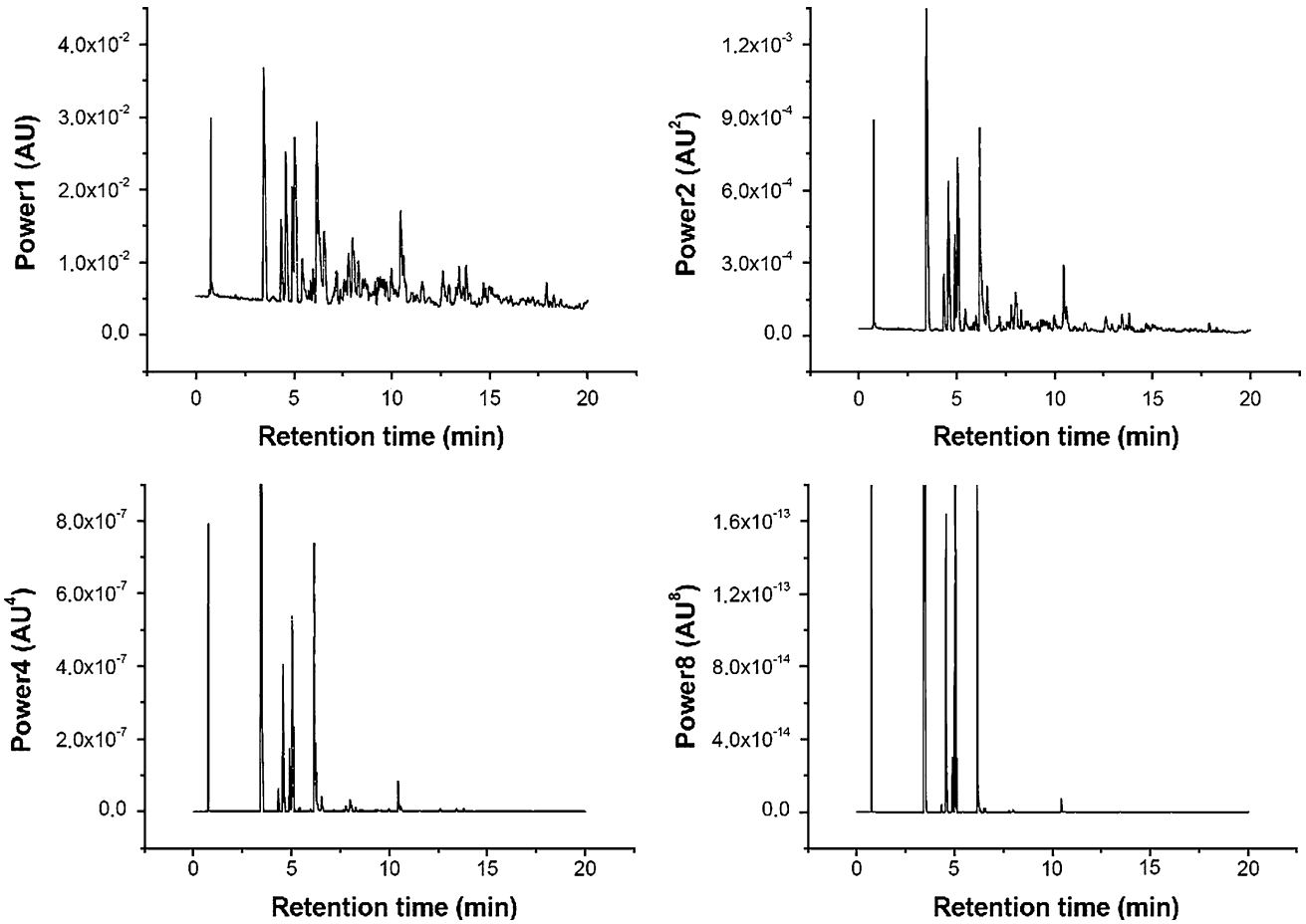



**Fig. 1** Peptide separation on wide pore core–shell particles (C18 phase). Power 1 indicates the original chromatogram acquired on the UPLC system. Chromatograms labeled with powers 2, 4, and 8 represents the transformed chromatograms with the power function using the powers of 2, 4, and 8, respectively. The UV absorbance is indicated in absorbance units (AU) and the retention time in minutes (min)

(in 20 min), then 10%B at 30 min and 30%B at 35 min (4 min reequilibrating time). It is important to notice that the retention on this CSH C18 phase was significantly lower compared to the Ascentis Express Peptide ES-C18 phase. The mobile phase flow rate was set to 0.75 mL $min^{-1}$, column was thermostated at 30 °C and 10 µL of the sample solution was injected. The chromatograms were recorded at 280 nm. Figure 2 shows the original and transformed chromatograms. It can be seen that in spite of the moderate pore size (130 Å) of this packing, very efficient peptide separations were achieved. A peak capacity of $P_c = 160$ was obtained (10 cm long column, 30 min gradient span, measured at peak half-height). With the application of power function on the raw data, the same tendency was observed as in case of Ascentis Express Peptide ES C18 column. The resolution was improved when the highest power was used during the transformation process. Figure 2 highlights the effect of power functions on the resolution. The resolution was increased from $Rs = 0.96$ to $Rs = 2.06$ between peak 2 and peak 3, and from $Rs = 1.79$ to $Rs = 3.95$ between peaks 3 and 4 when the data were transformed with a power 8 function.

## Fast Peptide Mapping Using CSH Phenyl-hexyl Stationary Phase

The most popular and widespread stationary phase chemistries in peptide separation and peptide mapping are the C18 or sometimes the C4 phases. The new CSH phenyl-hexyl phase was studied as an alternative possibility in reversed phase peptide separation. The following fast gradient program was used: linear gradient from 0 to 70%B within 7 min, followed by an isocratic step at 70%B for 8 min and a 4 min reequilibrating time. The mobile phase flow rate was set as 0.85 mL $min^{-1}$, column was thermostated at 60 °C and 10 µL of the sample solution was injected. The detection wavelength was set to 280 nm. Figure 3 shows the results of the peptide mapping

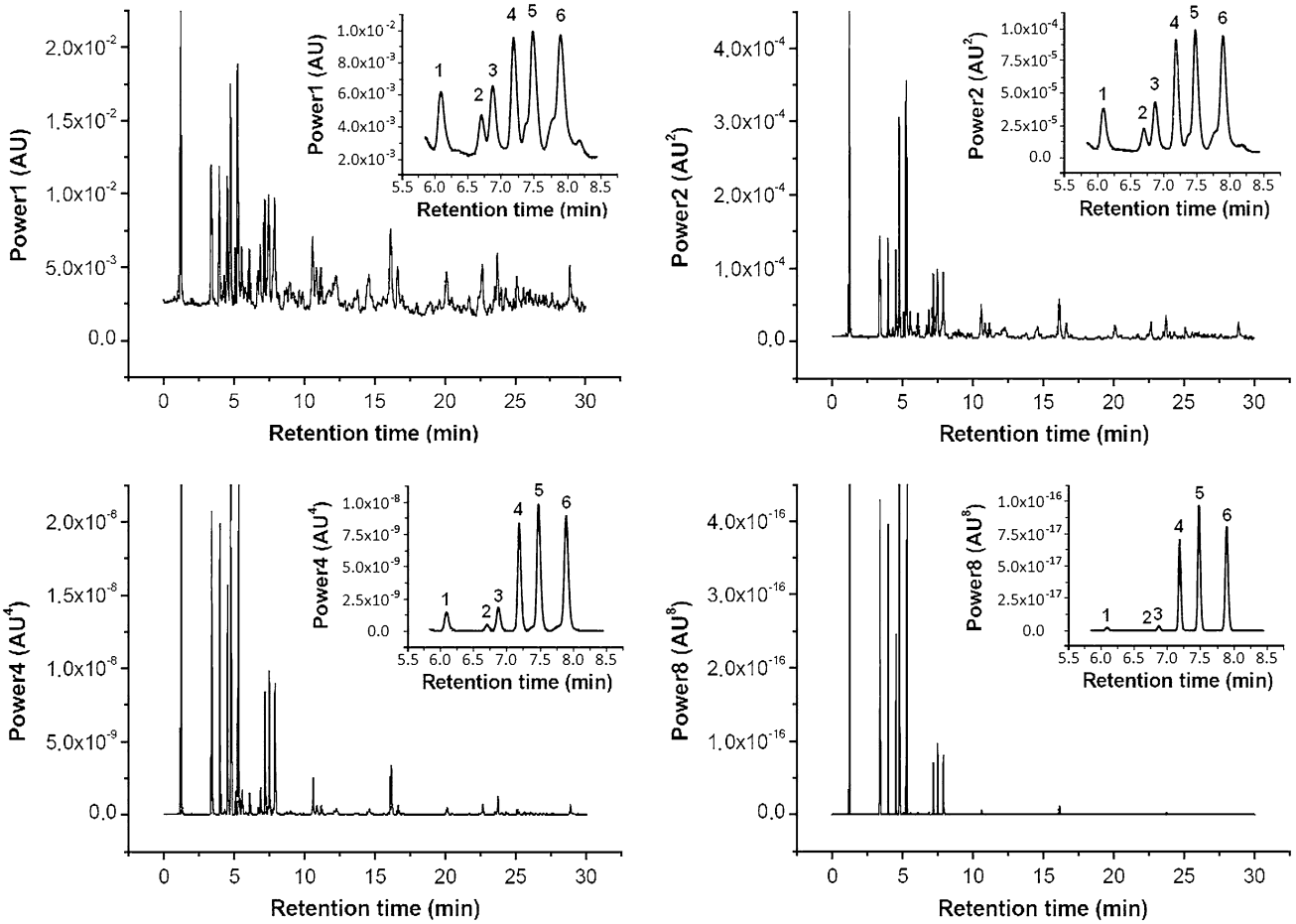



**Fig. 2** Peptide separation on the new CSH C18 1.7 µm totally porous phase. Power 1 indicates the original chromatogram acquired on the UPLC system. Chromatograms labeled with powers 2, 4, and 8 represents the transformed chromatograms with the power function using the powers of 2, 4, and 8, respectively. The effect of the transformation with different powers on the resolution can be seen on the zoomed chromatograms. The UV absorbance is indicated in absorbance units (AU) and the retention time in minutes (min)

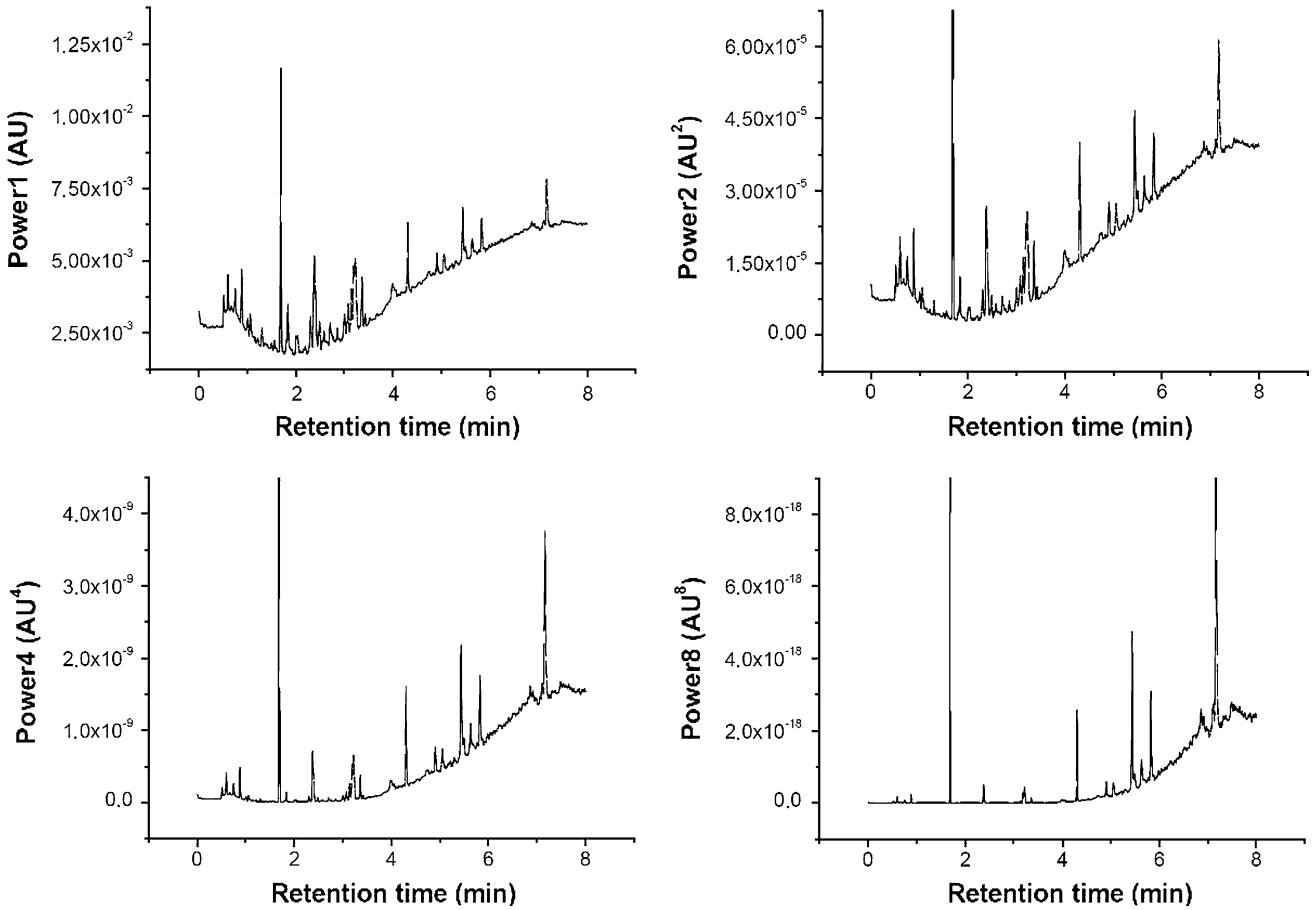



**Fig. 3** Peptide separation on the new CSH phenyl-hexyl packing. Power 1 indicates the original chromatogram acquired on the UPLC system. Chromatograms labeled with powers 2, 4, and 8 represents the transformed chromatograms with the power function using the powers of 2, 4, and 8, respectively. The UV absorbance is indicated in absorbance units (AU) and the retention time in minutes (min)

obtained with the CSH phenyl-hexyl phase. The use of this column for peptide mapping was also a good choice as shown on the original chromatogram (power 1) where several fragments were separated with very high efficiency. A peak capacity of $P_c = 151$ was achieved within 8 min. Nevertheless, there are several critical peak pairs with poor resolution. The use of the transformation process is in this case also a good tool for improving the resolution.

## Statistical Evaluation of the Similarity of Peptide Maps

There is no clear cut definition of similarity. When comparing peptide maps, the similarity can be related either to the retention times of fragments (quality of the protein) or to the peak area ratios of fragments. In this section, a simple statistical method is presented for the correct comparison of the peptide maps of different batches.

The method described in this paper, which includes transformation of chromatographic data to facilitate the comparison of the peptide maps, can be used to simply decide whether two different biopharmaceuticals are similar or not. By means of this procedure only information about the similarity of the primary structure can be extracted. The main goal of this method was that simple graphical representations were evaluated as useful tools in making a decision regarding the similarity. However, it is necessary to mention that more sophisticated, complex methods such as cluster analysis, factor analysis or principal component analysis are often used to compare chromatographic data [19, 20].

Ideally, two different batches of a biopharmaceutical product give exactly the same peptide map (same peak areas and heights) and when they would be carried through this data-handling procedure, a regression line with an intercept of 0 and a slope of 1 would arise independently of using peak heights or areas. Nevertheless, errors can occur during sample preparation, acquisition, processing and due to this fact the results always present uncertainty. The goodness-of-fit in the linear regression is characterized by the $r^2$ value. The closer it is to 1, the better the regression line fits to the data points.

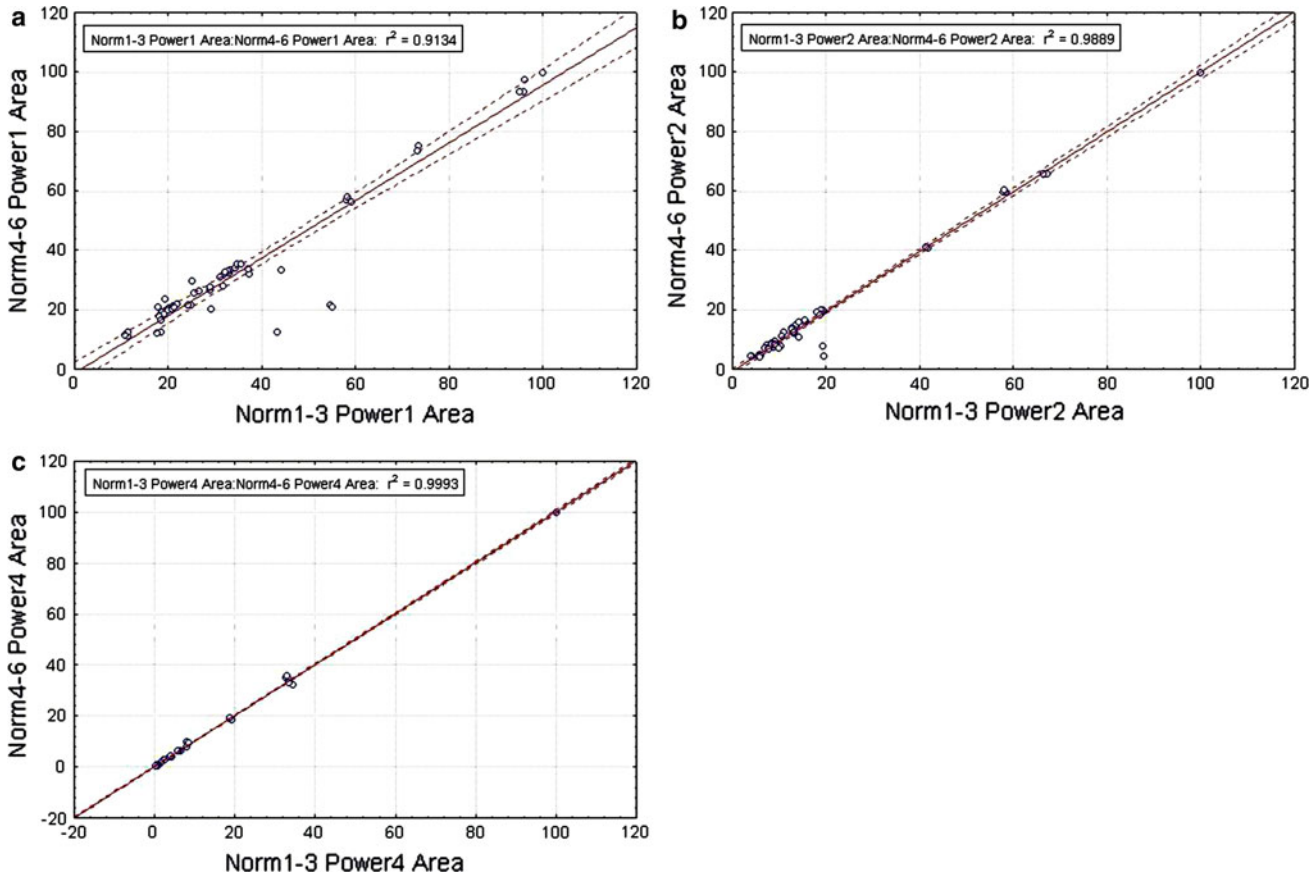



**Fig. 4** Statistical evaluation of the similarity in peptide mapping on wide pore core–shell particles (C18 phase) using peak areas obtained from the original and transformed chromatograms. Two different samples from different batches were used with three repetitions each (three digestions per batch). *Norm1–3* indicates the three repetitions of the first, *Norm4–6* those of the second batch. **a** The original data set labeled as power 1, **b** data set from the transformed choromatogram with the power of 2, **c** data set from the transformed choromatogram with the power of 4

When peak areas are used for the comparison, the goodness-of-fit was improved when the highest power was used. The $r^2$ was found as 0.9134, 0.9889 and 0.9993 with the power of 1 (original data), 2 and 4, respectively (19 peaks were considered in each case with three replicates). The reason for this phenomenon is that in peptide mapping, the chromatograms contain very high number of peaks among which there are in most of the cases critical peak pairs with poor resolution that hinders the correct evaluation of the peak area. This tendency is presented in Fig. 4.

In case of using peak heights for the comparison, no such tendency was observed. Applying the same data set as formerly for the comparison using peak areas; $r^2$ was found as 0.9985, 0.997 and 0.9965 with the power of 1 (original data), 2 and 4, respectively (19 peaks were considered in each case with tree replicates). Based on these results, it is clear that the use of power functions was beneficial when peak areas were used but has no effect with peak heights. In addition, the evaluation of the peak area was easier in the case of the chromatograms transformed with higher powers since the resolution was improved.

In this methodology of comparison, the similarity of the corresponding biopharmaceuticals was indicated by the good fitting of regression line to the data. When there were larger differences between peptide maps than expected due the random errors, it can obviously be seen on the graphic results. Finally, the value of $r^2$ also indicates the rate of similarity.

Unfortunately there are some drawbacks of this transformation process. Power transformation can filter the lower abundant species as the power transform increases while this transformation tends to favor large peaks. The loss of data may hinder the total fingerprint identification. Therefore, using this approach can result in false matches in some cases especially when a transformation with power 8 is applied. Applying a power of 2 or 4 seems to be a good compromise between improving the resolution and losing data. A systematic further study will be conducted to clarify the uncertainty of this process. Moreover the suitability of the statistical evaluation will be investigated; the effect of different numbers of repetition and consecutive samples will be compared.

## Conclusion

The resolution in peptide map chromatograms was significantly increased using power functions on the raw chromatographic data. Applying power functions could be a neat tool in the field of biosimilar developments, especially in comparability studies if one-dimensional peptide mapping is used. When power 2 or power 4 functions were applied, the resolutions were usually acceptable. Another advantage of applying power transformations is that when peak areas were used for the comparison of peptide maps, the goodness of linear regression fit can be improved. Therefore, peak areas can also be used for comparing the maps instead of peak heights. Though without any transformation, using peak heights is more accurate than using peak areas to evaluate the chromatograms.

Using phenyl-hexyl stationary phase in peptide separation seems to be a good alternative for the generally applied C18 or C4 phases, which is also shown by the achieved peak capacity values. Nevertheless, we must emphasize that peak capacity values originating from chromatographic runs with different gradient programs are hardly comparable.

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