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Exploring better column selectivity choices in ultra-high performance liquid chromatography using Quality by Design principles

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ABSTRACT

An older method for amlodipine was reworked with the goal to reduce the analysis time of 60 min below 6 min. To select the best column for short and robust analysis, 9 different UHPLC column chemistries were investigated using 3-dimensional resolution spaces based on 12 experiments using modelling software. The main variables used were gradient time (tG), temperature (*T*) and the pH of eluent A. The best critical resolution was calculated and located in a 3-dimensional space in an automated fashion and the corresponding best experiments were carried out. The work ($9 \times 12 = 108$ runs) was finished with an UHPLC instrument in less than 24 h. The comparison between predictions and real experiments showed an excellent correlation with differences typically less than 0.04 min (<3 s) in average, although the set points were located at quite different conditions on gradient times, pH's and temperatures for the individual columns. All columns could perform the required baseline separation at their individual best working points with satisfactory results.

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1. Introduction

An older method for amlodipin [1] was reworked with the goal to reduce the analysis time of 60 min below 6 min, as regulatory agencies request increasingly the application of Quality by Design (QbD) principles in liquid chromatography method development [2]. Investigating how the best possible separation could be achieved, solid science should be applied and a Design Space should be established [3]. Trying to achieve these goals, Erxleben et al. used computerized design of modelling UHPLC, which plays an increasingly important role in establishing robust conditions [4]. Modelling allows to screen experiments in seconds, test different variants of working points for QbD and establish their stability for routine applications faster than in the past [5–10].

According to the requirements of a "control strategy", methods should be regularly checked how they perform and how they fulfil acceptance criteria. Another request is the "continual improvement" of the method, if possible [5]. This includes the application of better columns with improved performance, shorter column lengths with reduced diameter, which can also better be used with the mass spectrometer.

In a recent work Euerby and his group have shown, that modelling using gradient chromatography with rapid column formats generates excellent results, i.e. Retention modelling in ternary solvent-solvent gradient elution reversed-phase chromatography using 30 mm columns [9]. In a later paper they controlled the precision of DryLab® predictions and measured with a mix of 22 pharmaceutical compounds at different positions inside of a ternary Cube (Fig. 1A and B). Euerby et al. came up with precise results, showing >99.9% accuracy in average of retention times in 5 different positions inside the cube (Fig. 1A), if compared with predicted values [10]. Their high precision results motivated us to test the reliability of the accuracy of predicted retention times also with a larger number of differently selective columns in the present work.

2. Column selectivity database

Searching for suitable columns, including equivalent stationary phases, there are a number of excellent papers about column characterization procedures, developed by different research groups under the leadership of Snyder and Dolan, Tanaka, Euerby and Petersson [11–13]. To evaluate columns with differing selectivities, we selected a number of UHPLC columns. According to the Snyder–Dolan hydrophobicity subtraction database [11], we found for the Fs-values of selectivities the following set of data (Table 1).

The Fs-values <3 mean excellent similarity of selectivity between the compared columns, between $3 < Fs < 5$ the selectivity comparison is moderate, between $5 < Fs < 10$ there is a questionable but still fair comparability of selectivity. Our observations,

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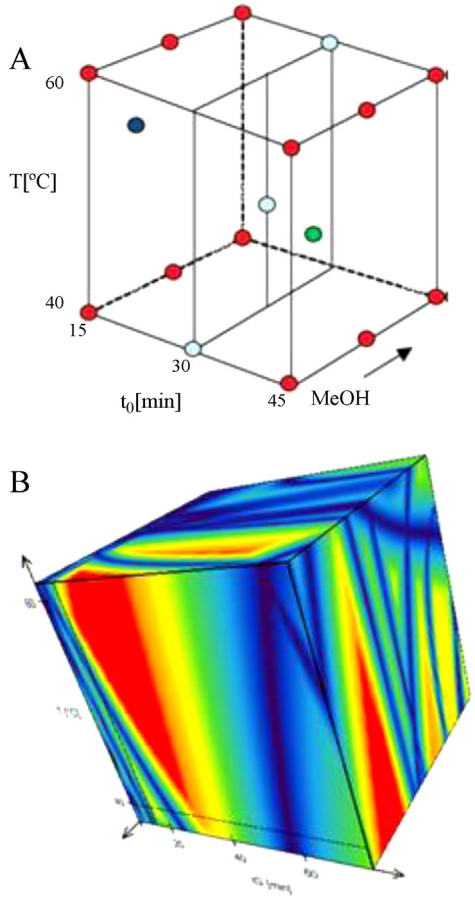


Fig. 1. (A, top): Experimental design for the 3D retention model and its control by Melvin Euerby and coworkers. Red circles represent the twelve input experiments for the 3D model, the light blue circles the validation experiments and the dark blue (tG = 22 min, T = 55 °C and methanol in eluent B) and green (tG = 45 min, T = 50 °C and methanol:acetonitrile (80:20, v/v in eluent B) circles optimum conditions for comparison. Retention times were predicted with 99.9% accuracy as shown in the original publication (from Chromatography Today with permission) [15]. (B, bottom): The investigated 3D-Cube of Mel Euerby [15] showing robust conditions in methanol (in red) as the organic eluent.

which we will report in this paper, are in good agreement with the predicted Fs-values, as we will show it later in this paper.

The database is comparing column selectivities in an isocratic system at 35 °C in (acetonitrile:water) (50:50 (V:V)) and pH 2.8 in eluent A. Peaks should be eluted in isocratic conditions between $1 < k < 10$. In our cases this range was between 1 and 120, so we had to use gradient elution. Therefore we were interested in an extended comparison of the columns in a gradient elution system.

Table 1
Column selectivity comparison according to the Snyder–Dolan hydrophobicity subtraction database (ColumnMatch).

Column	Fs (ColumnMatch)
XBridge (BEH) C18	0.0
HSS C18	1.6
XSelect CSH C18	3.9
Kinetex XB C18	4.1
HSS T3	5.5
YMC Triart C18	5.8
HSS C18 SB	21.7

HSS PFP and HSS CN columns are not yet included in the database.

In 2000 Dolan and Snyder published a paper on the comparison of column selectivity's using 2-dimensional resolution maps with 4 experiments for each column and two samples. They found after establishing the best working point, that all columns were separating the given mixture with the best possible results for every column [14].

We extended this work from the tG-*T*-model to a 3-dimensional model as described in [3,4,7,10] and combined the investigation of individual column characteristics and elution conditions together to find the best separation at the highest critical resolution, giving maximum separation robustness and short analysis time.

3. Experimental

3.1. Chemicals

Eluents: The mobile phase was a mixture of acetonitrile and 5 mM ammonium dihydrogen phosphate buffer. Acetonitrile (gradient grade), ammonium dihydrogen phosphate, phosphoric acid and standard reference buffers (pH 2.00, 4.01 and 7.00) (Merck, Darmstadt, Germany). For measurements water was prepared freshly using ELGA Purelab UHQ water (ELGA, Lane End, UK). The buffer was filtered before use on regenerated cellulose filter membrane, 0.2-μm pore size (Sartorius, Goettingen, Germany).

The sample containing 10 μg/ml Amlodipine and its Ph.Eur. impurities (A, B, D, E, F, G, and H). There were purchased from European Directorate for the Quality of Medicines & HealthCare (EDQM). Sample solvent was (acetonitrile:water) (30:70(V:V)).

3.2. Equipment, software

UPLC™ was performed using a Waters Acquity system equipped with binary solvent delivery pump, an auto sampler, a photo diode array detector and Empower software (Waters, Milford, USA). Detection was done at 230 nm.

The UPLC™ system had 5 μl injection loop and 500 nl flow cell. The dwell volume of the system was measured to be 0.125 ml.

MP 225 pH-metre was purchased from Mettler-Toledo (Greifensee, Switzerland).

Method development and method modelling was performed using DryLab® 2010 v.3.9 optimization software, consisting of the DryLab® Core-module, the PeakMatch® and the 3D-Resolution Space feature called the Cube (Molnár-Institute, Berlin, Germany).

3.3. Preliminary experiments

First investigations with the above selected stationary phases gave a variety of different selectivities, which are demonstrated in Fig. 2. They are showing separation variabilities in column selectivities, based on alternative column chemistries of the different types of columns used. As we can see, only the Acquity CSH C18 column gave a reasonable baseline separation for all components. All other columns show non-robustness, i.e., double peak formation under the selected conditions. In this situation we wanted to understand influences of elution conditions, temperature and pH in more detail and started to carry out a systematic study to evaluate the Design Space for the compounds of interest.

3.4. Design of experiments

Experimental design for simultaneous optimization of gradient time (tG), temperature (T) and pH requires 12 experiments, as illustrated in Fig. 3 [3]. Two linear gradients with a factor 3 different gradient times, 3 and 9 min, from 30 to 90%B, were carried out at two different column temperatures, at 15 and 45 °C. The

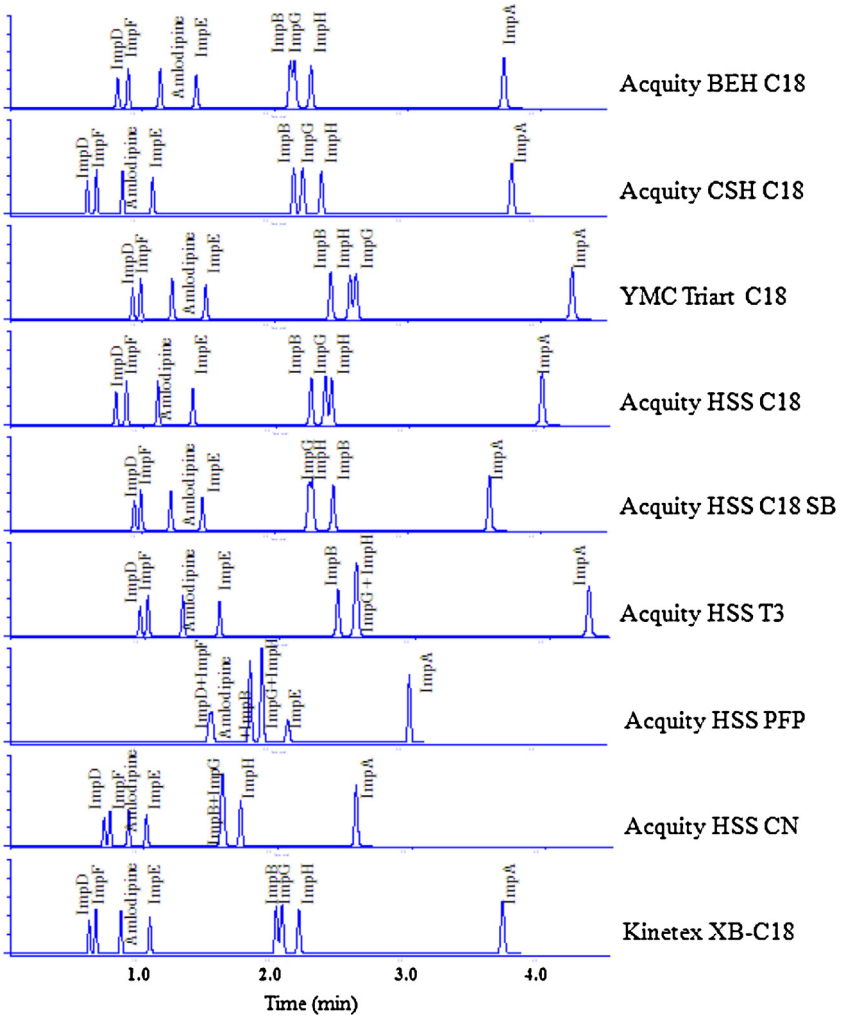


Fig. 2. Column selectivity comparison. Chromatographic parameters under the conditions tG = 6 min (from 30%B to 90%B), T = 30 °C, pH = 2.5, for other conditions see Section 3.

mobile phase A consisted of 5 mM ammonium dihydrogen phosphate buffer with 3 different pH values, 2.0, 2.5 and 3.0. Mobile phase B was acetonitrile, because its low viscosity and favourable UV cut-off. The flow-rate was at 0.5 ml/min. The injection volume was 1 μl.

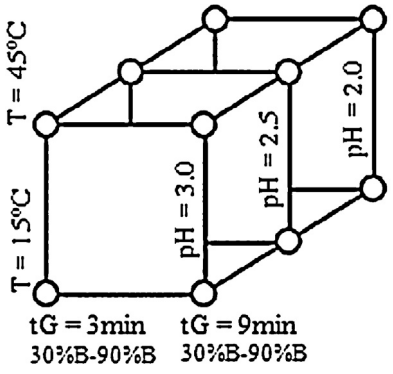


Fig. 3. Design of experiments (DoE) for the simultaneous optimization of gradient time (tG), temperature (T) and pH of the eluent A. The basic element here are 3 measured tG-T-sheets with 3 × 4 experiments. Further 97 tG-T-Sheets are additionally calculated and form in this way a continuum of > a million virtual experiments. Each data point in this Design Space represents a chromatogram. The chromatograms can be seen visually and the separations can be judged whether they are meaningful or not. Circles represent the twelve input experiments for the 3D model.

3.5. Columns

To measure selectivity differences we used 9 different type of modern 5-cm long, narrow bore (2.1 mm I.D.) columns with sub-2-μm particles. If we want to use an equivalent column it is not enough to know in which USP group the stationary phase belongs, i.e., to the USP-class L1 (octadecylsilane chemically bonded to porous silica or ceramic particles-1.5–10 μm diameter), L10 (nitrile groups chemically bonded to porous silica particles-3–10 μm diameter) or L43 (pentafluorophenyl groups chemically bonded to silica particles-5–10 μm diameter).

The modern sorbents have also low metallic ion concentration (<10 ppm) and a uniform particle size distribution. The different companies prepare their sorbents in different ways, which has an influence on the retention of the sample components.

In the measurements we used three different types of 5-cm narrow bore sub-2-μm columns:

A:	Hybrid particles:	Acquity BEH C18, Acquity CSH C18 and YMC Triart C18;
B:	Fully porous particles:	Acquity HSS C18, Acquity HSS C18 SB, Acquity HSS T3, Acquity HSS PFP and Acquity HSS CN;
C:	Core shell particle:	Kinetex XB-C18.

The Acquity columns were purchased from Waters (Milford, USA). Kinetex XB-C18 column were purchased from Phenomenex (Torrance, USA).

Table 2
Properties of the used columns.

Column	Length (mm)	I.D. (mm)	Particle size (μm)	Silica type	Pore size (Å)	Surface area (m^2/g)	Surface coverage ($\mu mol/m^2$)	Porous shell (μm)
Acquity BEH C18	50	2.1	1.7	Hybrid	130	185	3.0	–
Acquity CSH C18	50	2.1	1.7	Hybrid	130	185	2.3	–
YMC Triart C18	50	2.0	1.9	Hybrid	110	370	1.5	–
Acquity HSS C18	50	2.1	1.8	Fully porous	100	230	3.2	–
Acquity HSS C18 SB	50	2.1	1.8	Fully porous	100	230	1.8	–
Acquity HSS T3	50	2.1	1.8	Fully porous	100	230	1.7	–
Acquity HSS PFP	50	2.1	1.8	Fully porous	100	230	3.2	–
Acquity HSS CN	50	2.1	1.8	Fully porous	100	230	2.0	–
Kinetex XB-C18	50	2.1	1.7	Core shell	100	200	1.8	0.23

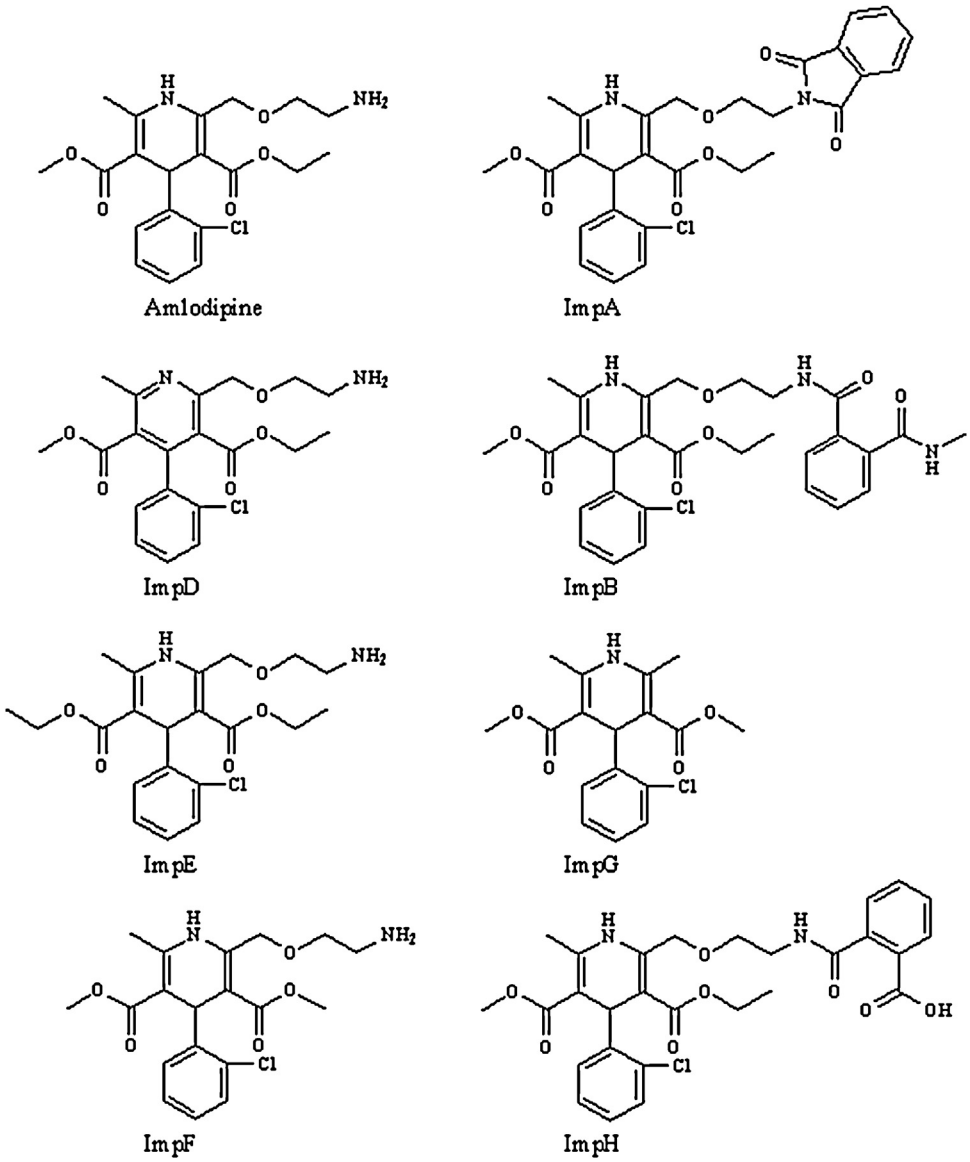


Fig. 4. Structure of amlodipine and its impurities. Amlodipine, ImpD, ImpE and ImpF contain free amino groups. ImpH contains free carboxylic group. There is a movement of ImpH with increasing pH to shorter retention times, which is of strong influence on the elution order. The basic groups have at the low pH rather limited influence.

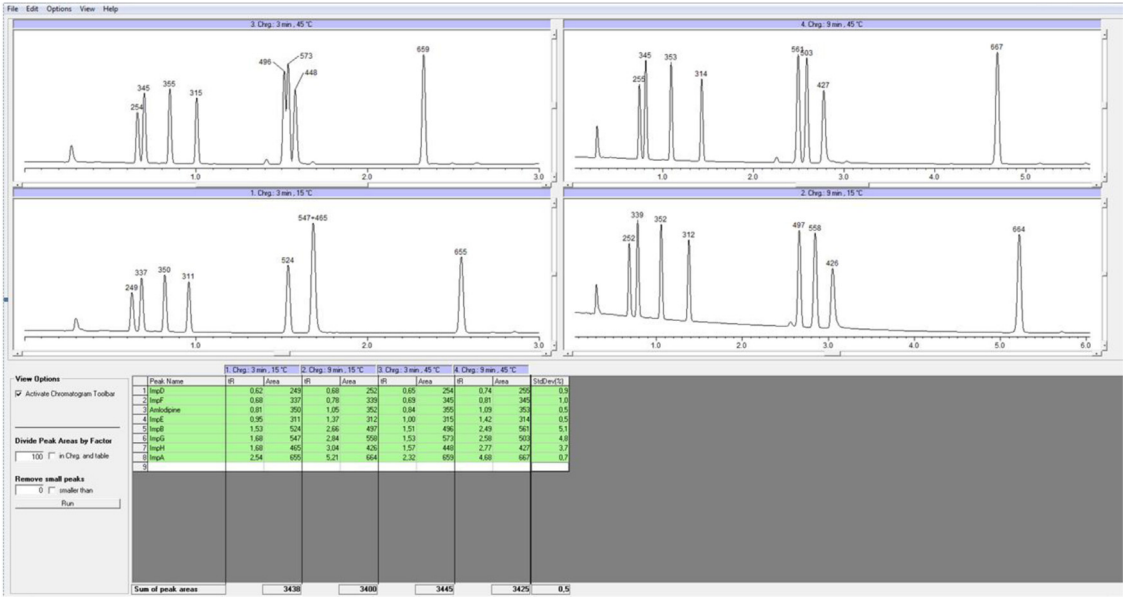


Fig. 5. Peak tracking data table including the original chromatograms. As we can see at the bottom right corner, the sums of the peak areas in each experiment are fairly similar (Std. Dev. only 0.5%). The figure is from the PeakMatch-software. It is relevant as it shows the differences between the experiments of the 4 basic runs of the tG-*T*-model in a very compact way and helps to understand, why the tG-*T*-model is necessary in a method development protocol according to QbD-principles. It serves as a document for the basic runs, which are organic part of the whole method development process. It allows judging the quality of the basic runs in a simple visual way.

YMC Triart C18 column was purchased from YMC (Kyoto, Japan).

The properties of the used columns are summarized in Table 2.

4. Results and discussion

4.1. Peak tracking

With this mixture of compounds the DryLab® approach can be used, which is based on the measurement of the retention behaviour of organic compounds in Reversed Phase (RP) HPLC. Csaba Horváth published the observation first, that almost all compounds can be eluted with a proper gradient between 0 and 100%B (B: acetonitrile or methanol or their mixtures). Only if the carbon number exceeds 50 (f.e. in case of triglycerides) we need 20%THF added to the organic eluent. They named their results the "Solvophobic Theory" [17]. Later Snyder et al. applied the gradient elution as a dominant principle in the DryLab® software with extended modelling of HPLC-retention. The measured retention times are highly accurate and can be used for precise prediction of chromatographic behaviour for almost every compounds in life science [18].

Using the simple DoE, shown in Fig. 3, all experiments are carried out with the same gradient range, but with gradient times differing by a factor 3 and at two temperatures (with a difference of ca. 30 °C) and at three pH values, differing 0.5–0.6 pH-units, one receives typically 12 chromatograms with quite different selectivities.

Structures of amlodipine and its impurities are illustrated in Fig. 4. There are several basic and acidic groups in the structures, which suggest to investigate the influence of the pH, the gradient time and the temperature.

The identification of peaks was carried out by their UV spectra and their peak areas using the corresponding features in DryLab®-s peak tracking modul, called PeakMatch®. Here the alignment of the peaks using peak areas and retention times of one compound are required to be placed in a horizontal line, to be able to calculate and graphically show chromatograms in the Design Space (see Fig. 5).

Fig. 5 shows the differences between the experiments of the 4 basic runs of the tG-*T*-model in a very compact way and helps to understand, why the tG-*T*-DoE is necessary. Furthermore it serves in the method development record as a document for the basic runs, which become part of the whole model. It allows judging the quality of the basic runs in a simple visual way.

To be able to model peak positions, each peak is identified with 12 sets of retention times and 12 peak area values. With this data

Table 3
Predicted DryLab parameters at the "working point" (highest critical resolution in the Cube) and difference of predicted vs. experimental retention times.

Columns	pH	Column temperature (°C)	Gradient time (min)	Rate (%B/min)	Average of retention time	
					Difference[a]	%Error[b]
Acquity BEH C18	2.1	13.5	8.1	7.41	0.008	0.25
Acquity CSH C18	3.0	13.5	9.8	6.13	0.017	0.88
YMC Triart C18	3.0	13.5	7.4	8.08	0.011	0.57
Acquity HSS C18	2.1	24.0	9.8	6.13	−0.038	−1.95
Acquity HSS C18 SB	2.0	30.0	9.8	6.13	−0.014	−0.37
Acquity HSS T3	2.0	31.5	9.6	6.28	−0.023	−0.97
Acquity HSS PFP	2.0	19.5	9.8	6.13	−0.005	−0.28
Acquity HSS CN	3.0	13.5	7.9	7.61	0.000	−0.15
Kinetex XB-C18	2.2	13.5	9.9	6.13	0.013	0.81

[a] Difference (min): predicted retention time − experimental retention time.
[b] %Error: [(predicted retention time − experimental retention time)/experimental retention time] × 100.

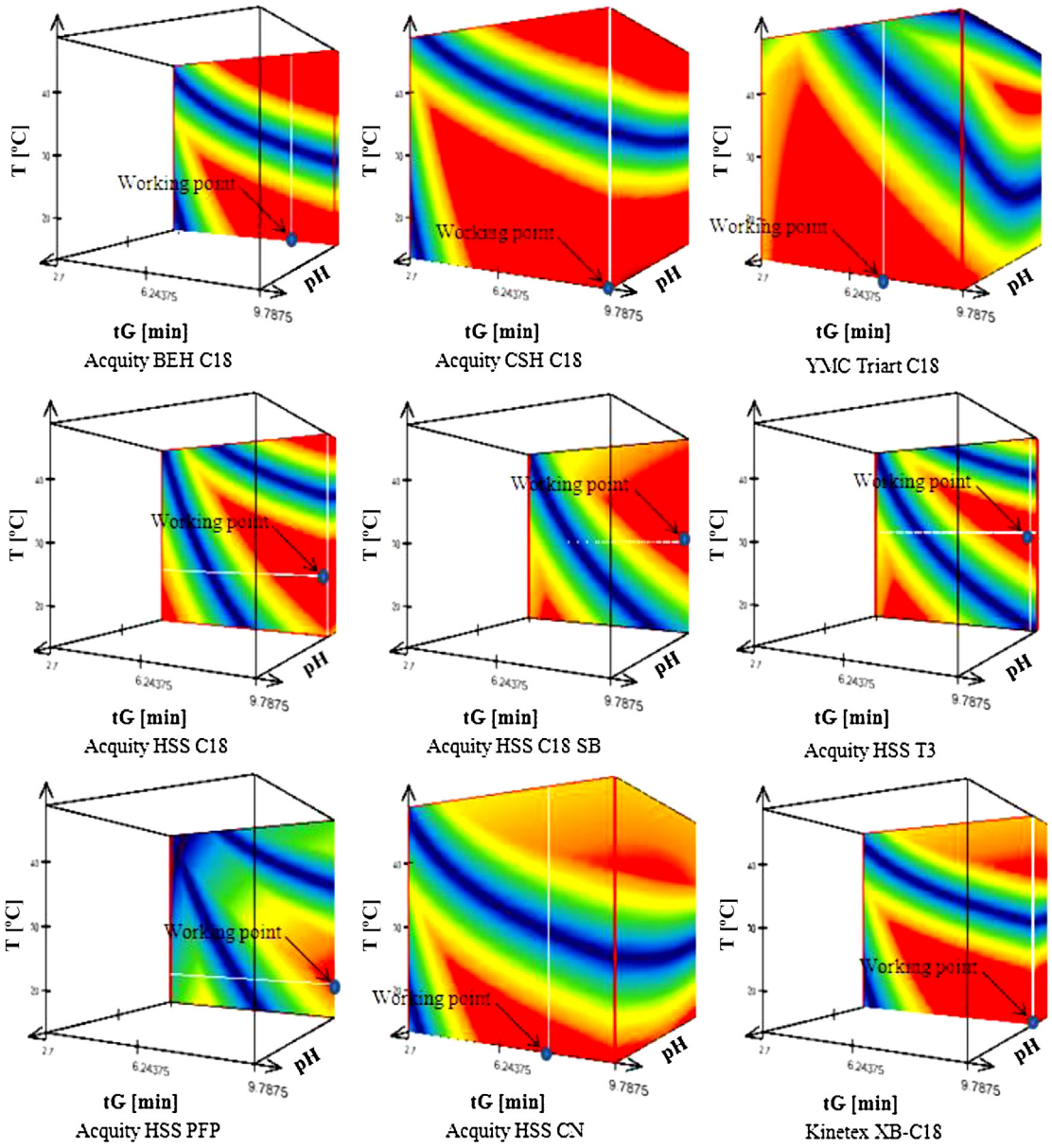


Fig. 6. Three-dimensional tG-*T*-pH models (cubes) on sub-2-μm silica particle showing the best separations at the "Working points". Note that although the working points are all at different positions, but we have in each cases a sufficient baseline separation. Red colours mean "baseline separation" ($R_{s,crit} > 1.5$), blue colours indicate coelution ($R_{s,crit} > 0$) of some peak pairs (experimental conditions see Section 3).

any peak position can be calculated inside the resulting Cube [3] (Fig. 6).

The Design Space (shown in red colour in Fig. 6) allows alteration of the position of the "working (or set) point" without the need for a new validation, allowing a high flexibility in the HPLC/UHPLC laboratory. After the 12 experiments according to the experimental design in Fig. 3 were carried out, chromatograms were exported into PeakMatch® as AIA-files and the 12 chromatograms were aligned with each of the 9 different columns. Consequently all nine tG-*T*-pH-Cubes were calculated using the 3D-cube calculation module (Fig. 6).

After the cubes were all calculated, the points of the highest critical resolution, the so-called "Working points" were established automatically, as shown for each Cube. The highest critical resolution is the location for the best "equal band spacing", and exhibits the best working point for robust routine work.

The predicted retention times were in good agreement with the experimental ones; the errors in retention times were less than 0.04 min in average for each column type. The best predicted separation parameters were established for each column (see Table 3). Baseline separation was established and proven for all components and for all columns (Fig. 7), however the retention times of the components were different on the different columns.

The alteration of pH between 2 and 3 and the effect of changes in %B had small effects, the retention times not changed remarkably, as long the change in temperature had significant effects on the retention and critical resolution of the components. Red colour means "baseline separation" and blue colour means coelution in Cubes (see Fig. 6) so if we are approaching the blue colour, the critical resolution is decreasing and if we cross over a blue coloured band, the resolution increases while the retention sequence is inverted.

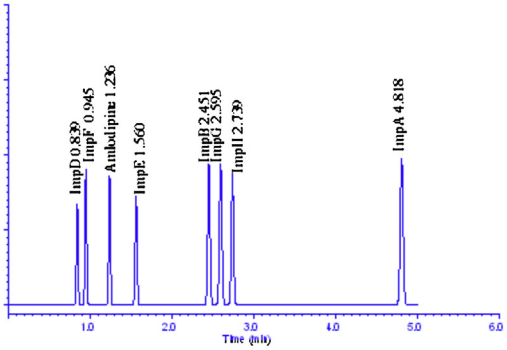


Fig. 7.1.a Predicted chromatogram on Acquity BEH C18 column, Rs,crit = 2.73 (ImpD-ImpF)

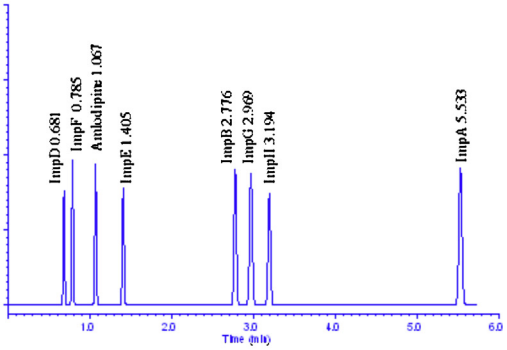


Fig. 7.2.a Predicted chromatogram on Acquity CSH C18 column, Rs,crit = 3.06 (ImpD-ImpF)

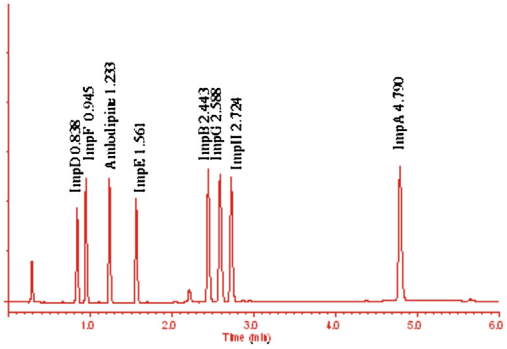


Fig. 7.1.b Experimental chromatogram on Acquity BEH C18 column, Rs,crit = 2.92 (ImpD-ImpF)

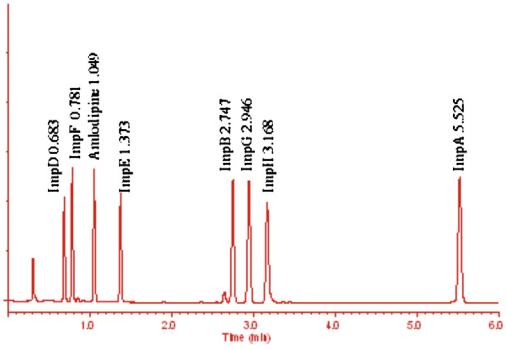


Fig. 7.2.b Experimental chromatogram on Acquity CSH C18 column, Rs,crit = 2.91 (ImpD-ImpF)

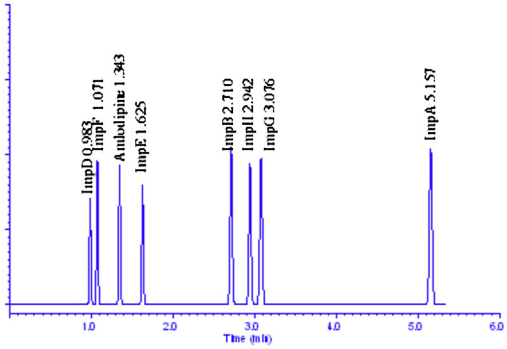


Fig. 7.3.a Predicted chromatogram on YMC Triart C18 column, Rs,crit = 2.46 (ImpD-ImpF)

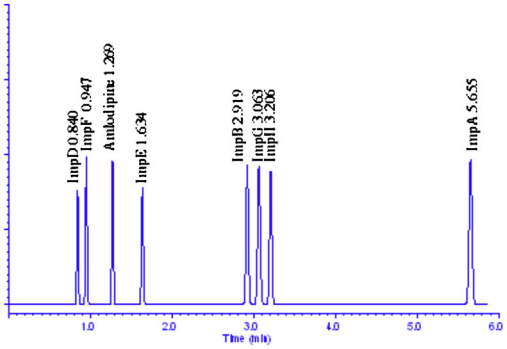


Fig. 7.4.a Predicted chromatogram on Acquity HSS C18 column, Rs,crit = 2.45 (ImpB-ImpG)

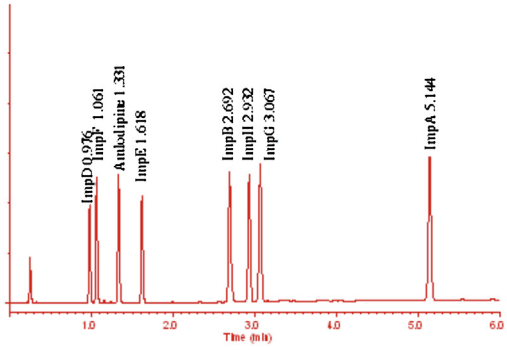


Fig. 7.3.b Experimental chromatogram on YMC Triart C18 column, Rs,crit = 2.36 (ImpD-ImpF)

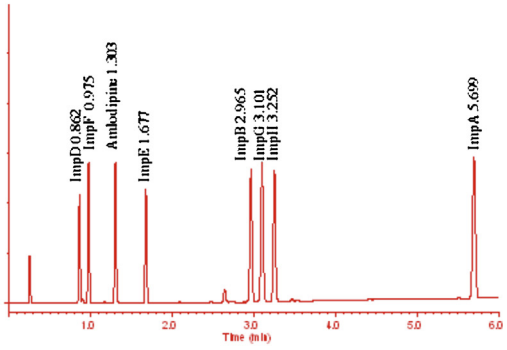


Fig. 7.4.b Experimental chromatogram on Acquity HSS C18 column, Rs,crit = 2.63 (ImpB-ImpG)

Fig. 7. Predicted (blue) and experimental (red) chromatograms (experimental conditions see Section 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

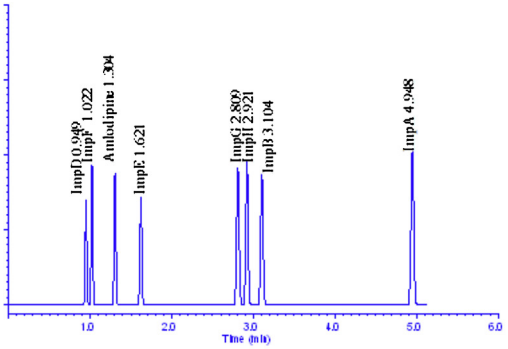


Fig. 7.5.a Predicted chromatogram on Acquity HSS C18 SB column, Rs,crit = 2.01 (ImpD-ImpF)

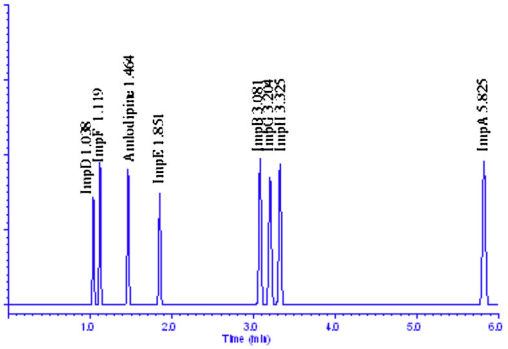


Fig. 7.6.a Predicted chromatogram on Acquity HSS T3 column, Rs,crit = 2.17 (ImpB-ImpG)

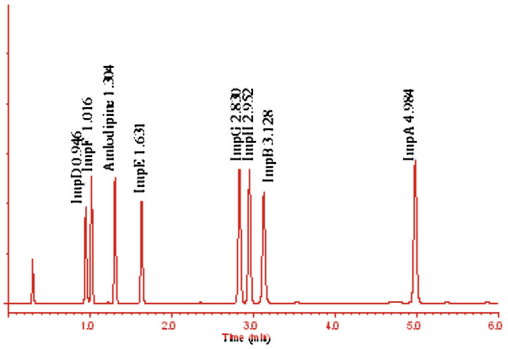


Fig. 7.5.b Experimental chromatogram on Acquity HSS C18 SB column, Rs,crit = 1.91 (ImpD-ImpF)

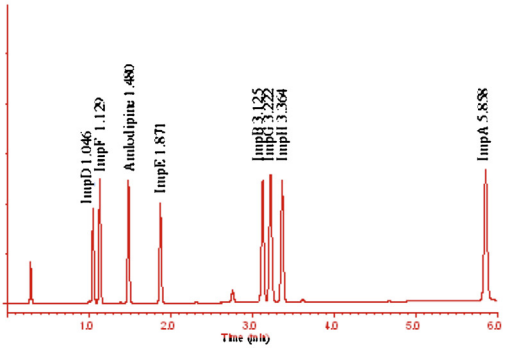


Fig. 7.6.b Experimental chromatogram on Acquity HSS T3 column, Rs,crit = 1.73 (ImpB-ImpG)

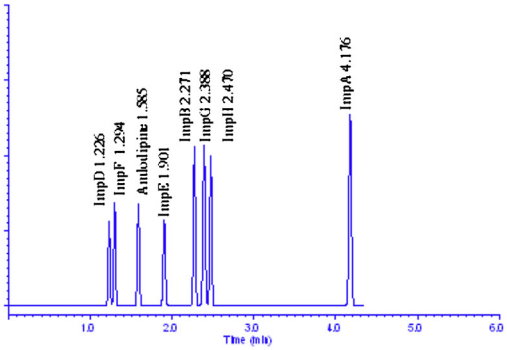


Fig. 7.7.a Predicted chromatogram on Acquity HSS PFP column, Rs,crit = 1.58 (ImpD-ImpF)

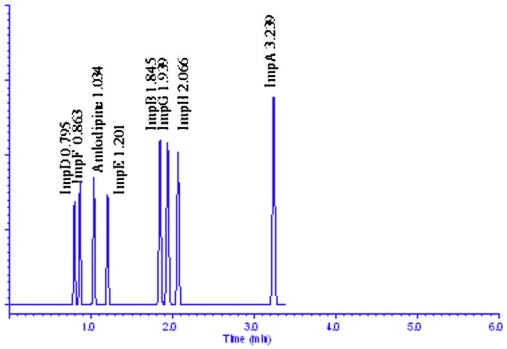


Fig. 7.8.a Predicted chromatogram on Acquity HSS CN column, Rs,crit = 1.95 (ImpD-ImpF)

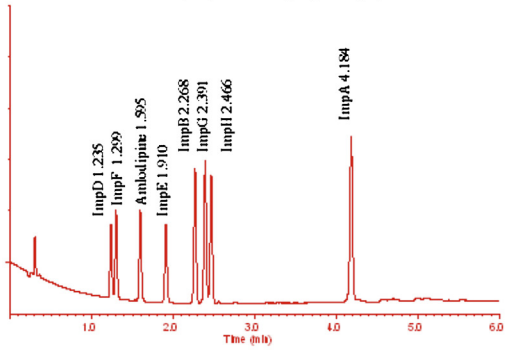


Fig. 7.7.b Experimental chromatogram on Acquity HSS PFP column, Rs,crit = 1.51 (ImpD-ImpF)

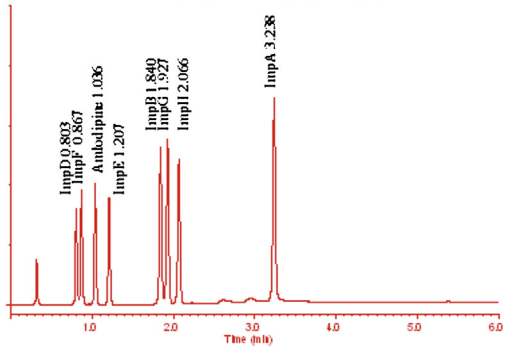


Fig. 7.8.b Experimental chromatogram on Acquity HSS CN column, Rs,crit = 1.73 (ImpD-ImpF)

Fig. 7. (*continued*).

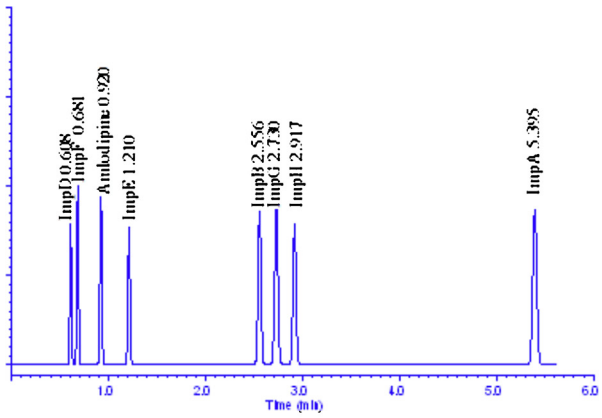


Fig. 7.9.a Predicted chromatogram on Kinetex XB-C18 column, $R_{s,crit} = 2.18$ (ImpD-ImpF)

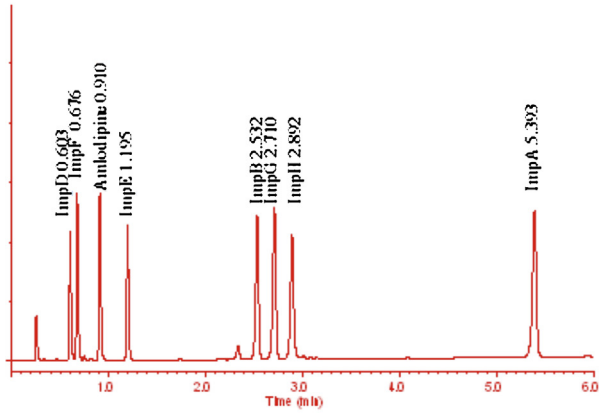


Fig. 7.9.b Experimental chromatogram on Kinetex XB-C18 column, $R_{s,crit} = 2.33$ (ImpD-ImpF)

Fig. 7. (*continued*).

Resolution in model chromatograms were always compared with real experiments and adjusted if needed in the model, comparing measured and modelled peak widths and the calculated plate number, until resolution of critical bands was identical in both model and experiment.

The time spent for this set of experiments and developing the methods including 2 gradient time × 2 temperature × 3 pH = 12 experiments on all of the 9 columns, was less than ca. 24 h (3 workdays). The best results are shown below. The results are in accordance with QbD principles and allow a more flexible way to deal with method variabilities [15,16,20].

4.2. Precision of modelled retention values

Concerning precision, one of us [19] reviewed in 2002 a number of articles, to show what the user can expect on precision with a planned design of separations. The precision is better than 99.8% in tR and it is depending of course on the quality of the input data. Gradient elution for complex mixtures is working today with very high precision, due to the high accuracies in pump flow delivery of the aqueous mobile phase. The amount of water is responsible to retard the sample in the first place.

Snyder and Dolan's column database ("ColumnMatch"), which is part of DryLab 2010 and also included in the USP-Website, contains over 500 different columns [11]. It is interesting to see, that the predictions of column equivalency are in good agreement with our data for the 9 columns, which we used in our investigations (Table 1 and Fig. 2).

The 3D models of DryLab® are predicting furthermore different chromatograms in the DoE under a great variety of conditions with high precision. Among the 3 measured factors, DryLab® is able to predict more than 6 additional factors by calculation: column length- and ID, flow rate, dwell volume, $\%B_{start}$ and $\%B_{end}$, etc. If steps in the gradient are included, their positions are further additional factors due to rounding effects. In their recent work Mel Euerby and his group measured with a mix of 22 pharmaceutical compounds, what would happen, if acetonitrile would be replaced by methanol – in times of "acetonitrile shortage" – and which precision of predictions can be achieved with DryLab® at different positions inside of the cube (Fig. 1 A and B). Mel Euerby and Gesa Schad came up with their excellent and highly precise results, showing in 5 different Cube-positions inside the Cube, that the precision was better than 99.9% accurate between the average of predicted and of experimental retention times (Fig. 1A) [10]. The above results in the present work on accuracy are further extending the work of the Euerby group by demonstrating the excellent reliability of the predicted retention times, shown in Figs. 7 and 8 for each of the 9 different columns (Table 3) (Fig. 8).

The prerequisite for the impressive precision values with the Waters UPLC™ is of course, that the instrument works precisely, which is demonstrated also in this paper. Similar excellent prediction values were obtained with the Shimadzu UHPLC system [4]. With such systems the reduction of 160 min to 3 min analysis time was recently achieved, showing new ways of rejuvenating older methods [21].

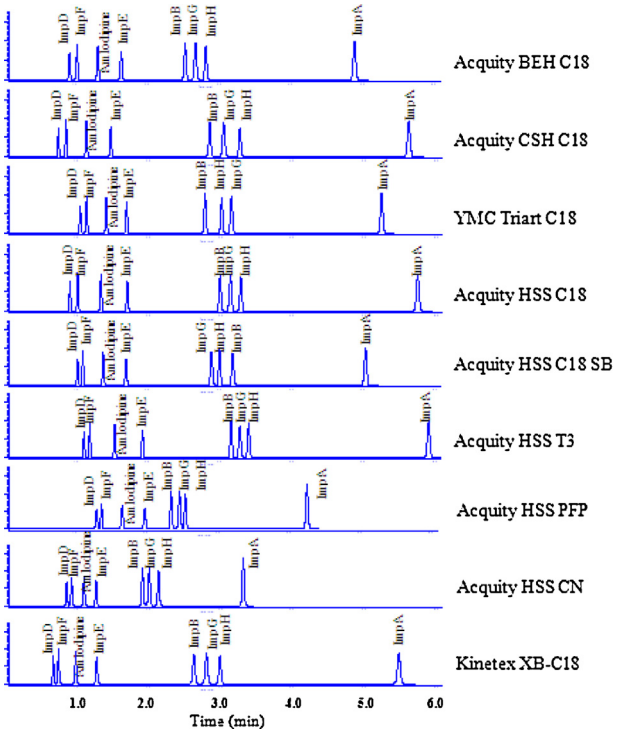


Fig. 8. The best separation on each column (experimental conditions see Section 3). Comparison of the 9 different columns after evaluating the best separation working point for each column. The selectivities are comparable and all peaks are baseline separated. The best robustness is provided with the CSH C18 column.

5. Conclusions

In the method development for ultra-high performance liquid chromatography separations, according to Quality by Design principles, it could be shown, that if eluent properties are carefully considered with a wide variety of column chemistries, the solution for the best separation can easily be found for almost every column using retention modelling. Furthermore the reduction of the analysis time could be achieved from 60 min to less than 6 min. Although there are in terms of robustness observable differences between the individual columns, the total results are showing a much easier handling of the separation as such. This is a great advantage in the rapid development of the best possible separation in industrial units, helping to develop new drugs faster for many diseases, which could not be treated before.

The novelty of the work is in the scientifically reliable way of comparing columns in a highly variable multifactorial Design Space, which has to reflect not only column chemistries, but also the influence of gradient time, pH, ternary eluent composition, flow rate, starting and final % organic eluent composition at the same time. This is a new scientific approach, which enables us to gain insight of the fundamentals of multifactorial variabilities of UHPLC methods. Besides of the scientific importance of precise predictions of chromatograms, the paper is also important for the application of reliable science at reduced costs in the pharmaceutical industry, helping to update older pharmacopoeia methods.

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