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Reliability of simulated robustness testing in fast liquid chromatography, using state-of-the-art column technology, instrumentation and modelling software

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ABSTRACT

The goal of this study was to evaluate the accuracy of simulated robustness testing using commercial modelling software (DryLab) and state-of-the-art stationary phases. For this purpose, a mixture of amlodipine and its seven related impurities was analyzed on short narrow bore columns (50 × 2.1 mm, packed with sub-2 μm particles) providing short analysis times. The performance of commercial modelling software for robustness testing was systematically compared to experimental measurements and DoE based predictions. We have demonstrated that the reliability of predictions was good, since the predicted retention times and resolutions were in good agreement with the experimental ones at the edges of the design space. In average, the retention time relative errors were <1.0%, while the predicted critical resolution errors were comprised between 6.9 and 17.2%. Because the simulated robustness testing requires significantly less experimental work than the DoE based predictions, we think that robustness could now be investigated in the early stage of method development.

Moreover, the column interchangeability, which is also an important part of robustness testing, was investigated considering five different C8 and C18 columns packed with sub-2 μm particles. Again, thanks to modelling software, we proved that the separation was feasible on all columns within the same analysis time (less than 4 min), by proper adjustments of variables.

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1. Introduction

A fundamental criteria of quality in a High Performance Liquid Chromatographic (HPLC) separations, is robustness [1]. 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" [2]. 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 [1,3]. 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 is verified earlier in the lifetime of a method, i.e. at the method development stage or at the beginning of the validation procedure [4–6].

Generally two approaches are used to evaluate robustness according to the ICH definition in pharmaceutical analytical practice. Either a one-factor-at-a-time (OFAT) procedure or an experimental design (DoE) procedure could be applied. The OFAT procedure varies the levels of a given factor, while keeping the other factors at nominal levels, to evaluate the effect of the former factor on the method response(s) [4]. The results obtained after varying one factor, are then compared to that of the experiment with all factors at nominal levels. This univariate approach is sometimes performed when a factor is varied in a relatively wide range to understand the peak movements. In the past, this approach was frequently used for method development and screening purposes. But for other reasons, this OFAT approach is not recommended for robustness testing. The most important one is that when the factors are examined in given intervals, the effects are estimated for a smaller domain around the nominal levels with the OFAT compared to the experimental design approach. When applying a DoE, the effect of a given factor is calculated at several level combinations of the other factors, while with the OFAT approach this is only at one level. Thus, in DoE, a reported factor effect is an average value for the whole domain, and it represents more globally what happening around the nominal situation. Moreover, the univariate approach requires more experiments and time, especially when the number of examined factors becomes larger, and secondly, the

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importance of factor interactions cannot be taken into account [4]. In pharmaceutical industrial practice, the DoE approach is clearly preferred. Plackett-Burman, full factorial, nested factorial, fractionated factorial and asymmetrical factorial experimental designs are often carried out [7–10]. These types of robustness testing typically allow the investigation of 3–15 factors (variables) based on 8–16 experiments. Beside continuous quantitative factors e.g. gradient program, mobile phase composition, pH, temperature or flow rate, the effect of qualitative factors such as column or instrument (laboratory) could also be studied and included in robustness testing.

The development of a method cannot only be based on quality but has also to be based on assurance of quality, taking into account the variability of the quality [11]. Evaluating the robustness of a method is equivalent to find its design space (DS), defined as "The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality" [12–14]. Therefore, it is obviously preferred to perform the robustness testing during the method development. For that purpose, state-of-the-art chromatographic modelling softwares offer a very efficient and straightforward way of robustness testing incorporated in method development, based on modelling the retention properties. Computer modelling programs can be employed to improve the analysis throughput as well as maximize information about method specificity during method development. One of the most successful and widespread modelling programs (DryLab) optimizes the DS mainly by measuring and visualizing the effects of the mobile phase composition: gradient time and shape, pH, ionic strength, ternary eluent, additive concentrations and temperature [15–18]. For this purpose, the program suggests a relatively well-defined number of experiments on a particular stationary phase; furthermore it can predict the separation inside the DS based on changes in mobile phase composition, mode of elution (either isocratic or gradient), temperature, pH or column parameters such as column length, internal diameter, particle size and flow-rate [19].

During the robustness testing in pharmaceutical industry, among the several method variables, the column itself is always of great interest. A method validation report has to suggest an alternative column that is able to perform nearly the same quality of separation as the one using the "primary column". Finding the alternative column (column interchangeability) is often difficult. Generally, the method is developed using one given column and then an alternative column is considered at the validation procedure under the optimized conditions. In many cases, the alternative column has not the same working point (optimal conditions in a robust zone) as the primary column. Therefore, this "trial and error" approach at the end of method development often fails. However, it is worth mentioning that the alternative column is also probably able of separating the analytes, but under different analytical conditions. Column databases could be helpful for selecting an appropriate column but generic stationary phase tests (e.g. Tanaka test, hydrophobic subtraction model [20,21]) are not always able to predict certain column similarity for special separations.

In this study, the simulated robustness testing, included within commercial modeling software, was systematically studied and compared to experimental measurements and DoE based predictions. The reliability of this "early stage" simulated robustness approach was critically evaluated for real-life separations applying short narrow bore columns (50 × 2.1 mm) and fast separations. Moreover, as a continuation of a previous study, the column interchangeability was further studied applying five different C8 and C18 sub-2 μm packings. By varying properly the variables, the separation was feasible on all columns within the same timescale (less than 4 min). This work demonstrates the accuracy of simulated robustness testing and shows that nearly the same quality of separation can be achieved on different stationary phases.

2. Experimental

2.1. Chemicals, columns

The mobile phase used in this work was a mixture of acetonitrile and 10 mM citrate buffer. Acetonitrile (gradient grade), citric acid, sodium hydroxide, standard reference buffers (pH 2.00, 4.01 and 7.00) were purchased from Merck (Darmstadt, Germany). For the 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, Göttingen, Germany).

The test samples contained 10 μg/ml Amlodipine and its European Pharmacopoeia (Ph. Eur.) impurities (A, B, D, E, F, G, H). Real life samples were prepared from amlodipine API (1 mg/ml) and spiked with all the impurities at 0.1% level. Amlodipine and its impurities were purchased from European Directorate for the Quality of Medicines & HealthCare (EDQM, Strasbourg, France). Sample solvent was acetonitrile:water 30:70 (v/v).

The Acquity columns (50 × 2.1 mm, 1.7 μm BEH C18, BEH C8, HSS C18) were purchased from Waters (Milford, USA), Hypersil columns (50 × 2.1 mm, 1.9 μm GOLD C18, GOLD C8) were purchased from Thermo Scientific (Waltham, USA).

2.2. Equipment and softwares

UHPLC experiments were performed on a Waters Acquity UPLC system (Milford, USA) equipped with binary solvent delivery pump, autosampler, photodiode array detector and Empower software. This UHPLC system had 5 μl injection loop and 500 nl flow cell. The dwell volume of the system was measured as 125 μl.

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

Modelling was carried out using DryLab v.4.0 and the quantitative robustness evaluation of generated models was performed in the latest DryLab Robustness Module v.1.0. (Molnár-Institute, Berlin, Germany). StatSoft Statistica v.11 was used for the evaluation of robustness testing (StatSoft Inc., Tulsa, OK, USA).

2.3. Apparatus and methodology

2.3.1. Considerations and initial runs for setting up the model

The selected example describes a fast and efficient method development for the determination of impurities and degradation products of a long-acting calcium channel blocker dihydropyridine (DHP) class active pharmaceutical ingredient (amlodipine), utilizing the separation power of sub-2 μm packed columns. Due to the basic character of the solutes, the mobile phase pH should play an important role in tuning the selectivity; therefore this factor was considered as a variable of robustness testing and method development. When dealing with low molecular weight analytes, the most common strategy in method development consists in selecting suitable stationary phase chemistry, organic modifier nature and mobile phase pH [11]. In a second instance, the gradient program and mobile phase temperature are optimized as complementary parameters, for fine tuning the optimum after selection of the correct combination of stationary phase, organic modifier and mobile phase pH.

Snyder, Dolan and co-workers recommended initial basic runs for multifactorial experimental designs already in the 1990s [22]. A general methodology is to simultaneously model the effect of temperature and gradient steepness on selectivity with a given RP column [23,24]. Thanks to the recent developments in chromatographic modelling softwares, it is now possible to model the effect of three variables simultaneously on a given separation and calculate the effect of additional factors like flowrate, column length,

internal diameter, particle size, initial- and final mobile phase composition, gradient-step-points or dwell volume [1,17,18,25]. In our case, gradient steepness, temperature and mobile phase pH were selected as model variables to create a cube resolution map, which shows the critical resolution of the peaks to be separated against the three factors. Probably, these selected variables have the most significant effect on the selectivity and resolution for this type of analytes.

In RPLC, the effect of temperature on retention factor (k) can generally be expressed by the van't Hoff equation:

$$\log\ k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \log\ \beta \quad (1)$$

where ΔH the enthalpy change associated with the transfer of the solute between phases, ΔS the corresponding entropy change, R the molar gas constant, T the absolute temperature and β the column phase ratio. When the logarithm of retention factor ($\log(k)$) is plotted against $1/T$, the enthalpy is given by the slope of the curve. In most cases, these van't Hoff plots follow a linear relationship. However, quadratic dependence of $\log(k)$ versus $1/T$ over a wide range of temperature by using silica-based as well as non silica-based stationary phases was also observed by several authors [26]. Melander et al. [27] as well as Castells et al. [28] have developed complex relationships to describe the retention of a partially ionized solute with a unique acid-base equilibrium, according to the assumption that the retention factor is considered as the weighted mean of the retention factors of the individual forms. Based on previous screening measurements and due to the restricted range of investigated temperature, a linear relationship described well the impact of temperature on retention change [18]. Therefore, temperature was studied at two levels (T_1 = 20 °C, T_2 = 50 °C), when creating the three dimensional model.

In RPLC, the linear solvent strength (LSS) model is the widely accepted theory which describes the analyte retention as a function of the volume fraction (ϕ) of the B solvent. For gradient elution mode, the following general equation can be written:

$$\log\ k^* = \log\ k_w - S\phi^* \quad (2)$$

where k^* is the apparent value of k during gradient elution (corresponds the band has reached the column mid-point), k_w is the value of k in pure water, S is a constant for a given compound (slope of the curve) and ϕ^* is the corresponding value of ϕ. It is practical to show the dependence of k^* on the gradient time (t_g) and for this purpose, the following equation was derived [29,30]:

$$k^* = \frac{t_g}{1.15 t_0 \Delta\phi S} \quad (3)$$

where t_0 is the column dead time. For practical reasons, modeling software such as DryLab generally deal with transformed variables of k or k^* to log (k) or log (k^*) to build a mathematical model. On the basis of Eq. 2 and 3, $\log(k^*)$ should follow a linear model when plotted against the logarithm of gradient time (which is related to the gradient steepness) in case of "regular" samples. Since the validity of this linear model was accepted in this study, the effect of gradient steepness (gradient time) was investigated at two levels and was set to t_{g1} = 2 min and t_{g2} = 6 min at a flow rate of 0.7 ml/min. The amount of B solvent was varied between 30 and 90% through the gradient program. Previous works demonstrated the suitability of these very short gradients on 50 × 2.1 mm columns [18,25].

When separating ionizable compounds, pH related changes in retention occur for pH values within ± 1.5 units of the pKa value [24]. Outside this range, the compound is considered as mostly ionized or non-ionized, and its retention is not significantly altered with pH. In a relatively small pH range - within the ±1.5 units of the pK_a value -, the dependence of retention on the mobile phase pH can be described with a quadratic model:

$$y = b_0 + b_1 x_1 + b_{11} {x_1}^2 \quad (4)$$

where y is the response (retention time or its transformation), x_1 is a model variable e.g. pH while b_0, b_1, b_{11} are the model coefficients. Then, the effect of mobile phase pH had to be investigated at least for 3 levels. Since the pK_a values of the investigated compounds possess a wide pK_a values range, and because some of the compounds have various ionisable functional groups, it was better to cover a relatively wide pH range (i.e. from pH 2.8 to 6.4). Obviously, in this broad pH range, the investigation of this variable at three levels probably will not give an accurate model for all the analytes. Thus, the investigated pH range was cut to equidistant periods by 0.6 pH increments and its effect on resolution was studied at 7 levels (i.e. 2.8, 3.4, 4.0, 4.6, 5.2, 5.8 and 6.4).

In the proposed final model, two variables (temperature and gradient steepness) were set at two levels while the third factor (pH) was set at 7 levels. This full factorial experimental design required 28 experiments (2 × 2 × 7). To achieve good accuracy, three independent models were built up (based on the 28 experiments) with DryLab, in which the pH was studied at only three levels. For the first model, the pH was studied between 2.8 and 4.0. In the second model, it was investigated between 4.0 and 5.2, while in the third model it was set between 5.2 and 6.4. Fig. 1 shows the design of experiments for the simultaneous optimization of gradient time (t_g), temperature (T) and pH of the eluent A. Circles represent the input experiments for the 3-D models.

2.3.2. Column interchangeability

Searching for alternative columns, while keeping the quality of a given separation is always one of the key purposes of robustness testing. Several papers on stationary phase characterization procedures, developed by Snyder, Dolan, Tanaka, Euerby and Petersson are available and could be helpful for users, in finding a similar column during the method validation [20,21,31,32].

In this study, the Snyder–Dolan hydrophobicity subtraction database was used to evaluate the selectivity of five stationary phases [21]. This model takes into account the hydrophobicity (H), hydrogen bond basicity (B), ionic interactions at two pH (C(2.8) and C(7.0)), hydrogen bond acidity (A) and steric selectivity (S). Table 1 shows the corresponding H, S, A, B and C values for the BEH C18, BEH C8, HSS C18, Hypersil Gold C8 and C18 phases. This table also reports the degree of selectivities similarity by means of the similarity factor (Fs). The Fs < 3 means excellent similarity of selectivity between the compared columns; between 3 < Fs < 5, the selectivity similarity is moderate, and between 5 < Fs < 10, there is a questionable but still fair comparability of selectivity. The selectivity was compared to the BEH C18 phase (as a reference) and it appears that the Fs values range between 5.8 and 10.8 for the other columns, meaning that differences in selectivity should be expected. For this column interchangeability study, columns of somewhat different selectivities were deliberately chosen. Indeed, our purpose was to demonstrate that it could be possible to find conditions on all the five columns, performing comparable selectivity and analysis time.

Several years ago, Snyder and Dolan showed the comparison of column selectivities using 2-dimensional resolution maps based on 4 experiments for each column and proved that all columns were able to separate a given mixture of analytes [33]. In the present study, this approach was extended to a 3-dimensional model (t_g, T, pH, requires 12 experiments for one 3-D model). It 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 shortest analysis time [25,34].

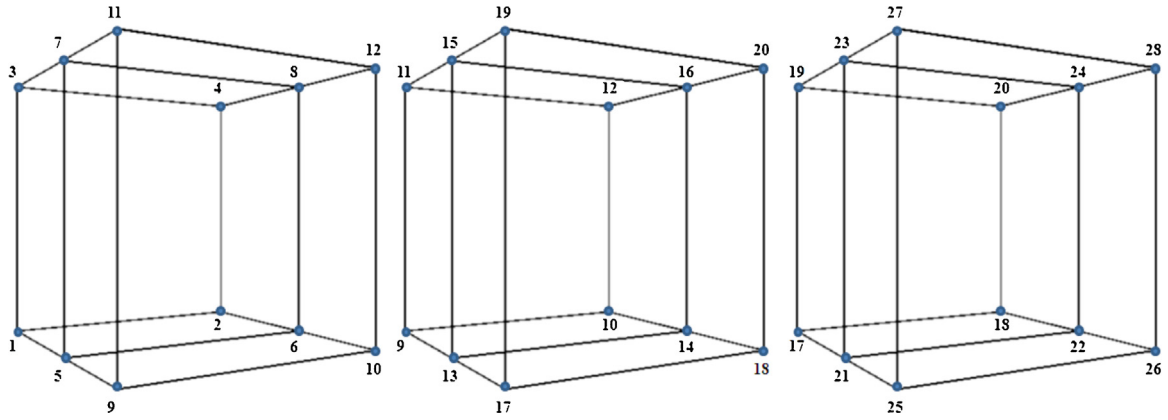


Fig. 1. Design of Experiments (DoE) for the simultaneous optimization of gradient time (t_g), temperature (T) and pH of the eluent A. Circles represent the input experiments for the 3-D models. The short gradient time t_{g1} = 2 min (30%B→90%B) was at the points 1, 5, 9, 13, 17, 21, 25 and 3, 7, 11, 15, 19, 23, 27, while the long gradient time t_{g2} = 6 min (30%B→90%B) was at the points 2, 6,10, 14, 18, 22, 26 and 4, 8, 12, 16, 20, 24, 28. The low temperature T_1 = 20 °C experiments were: 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22, 25, 26, while the high temperature runs at T_2 = 50 °C were at 3, 4, 7, 8, 11, 12 15, 16, 19, 20, 23, 24, 27, 28. Using the seven pH values, three pH ranges were determined: pH 2.8 (1, 2, 3 and 4)–3.4 (5, 6, 7 and 8,)–4.0 (9, 10, 11 and 12) was called low pH range, pH 4.0 (9, 10, 11 and 12)–4.6 (13, 14, 15 and 16)–5.2 (17, 18, 19 and 20) was called middle pH range and pH 5.2 (17, 18, 19 and 20)–5.8 (21, 22, 23 and 24)–6.4 (25, 26, 27 and 28) was called high pH range.

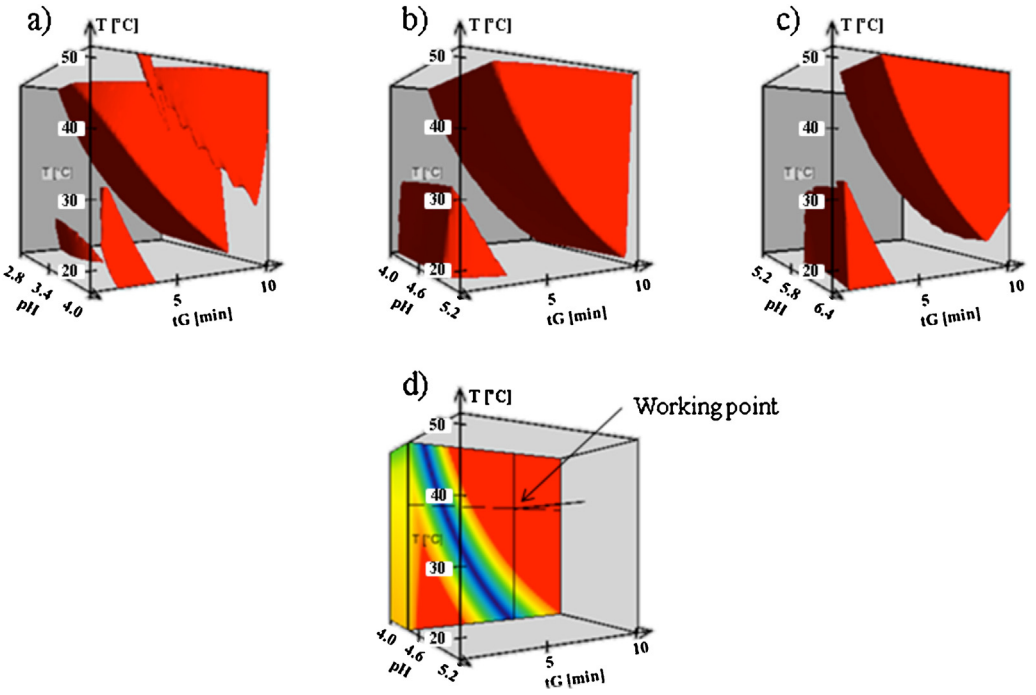


Fig. 2. Design spaces for the 3 models (low, middle and high pH). The red zones indicate the design space where the critical resolution was higher than 1.5. (a), (b) and (c) cubes correspond to the three models built up for low, middle and high pH range, respectively. (d) illustrates the working point in the middle pH range model.

For comparing the column selectivity and evaluating their interchangeability, the following model was built up for the five columns. Similarly to the model described in section 2.3.1, the gradient time was set at two levels (t_{g1} = 2 min and t_{g2} = 6 min at a flow rate of 0.7 ml/min), the temperature was also set at two levels (T_1 = 20 °C, T_2 = 50 °C) while mobile phase pH was set at three levels (pH_1 4.0, pH_2 4.6 and pH_3 5.2), corresponding to the middle pH range (see in Section 3.1). Then, cubic resolution maps

Table 1
Comparison of the applied columns based on hydrophobicity substraction database.

Fs	Column	*H*	*S*	*A*	*B*	*C* (2.8)	*C* (7.0)
0.0	Acquity BEH C18	1.007	0.028	−0.097	0.009	0.178	0.138
5.8	Hypersil GOLD C18	0.881	0.002	−0.017	0.036	0.162	0.479
7.8	Acquity BEH C8	0.805	0.018	−0.296	0.018	0.129	0.063
8.2	Hypersil GOLD C8	0.825	0.016	−0.157	0.030	0.093	0.215
10.8	Acquity HSS C18	1.022	0.039	−0.136	−0.020	0.059	−0.009

H: hydrophobicity; *S*: steric selectivity; *A*: hydrogen bond acidity and *B*: hydrogen bond basicity; *C*(2.8) and *C*(7.0) ionic interactions at two pH.

were created based on 12 experiments for all the five columns and attempts were made to perform the analysis within 4 min on all columns, while ensuring similar selectivity and appropriate resolution.

2.3.3. Robustness testing

A new feature of commercial modelling software DryLab 4 is the possibility to perform an in-depth "modelled" robustness testing. From the design space, as defined in a resolution map or cube, it is possible to get robustness information for the measured parameters, including t_g, T and pH. 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 6 parameters on the resolution can be assessed using a simulated 2^6 or 3^6 type factorial design. No additional experiments were necessary for performing the simulated robustness calculation [35]. The possible deviations from the nominal values have simply to be defined and then the software makes the calculations for 64 or 729 conditions. At the end, the software provides a 'frequency distribution graph' showing how often (N) a certain critical resolution occurs under any combination of possible parameters. 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.

This simulated robustness test was performed around the selected working point (t_g = 7 min, T = 40 °C, pH 4.4, flow rate: 0.7 ml/min, start %B: 30, end %B: 90). Several experiments were carried out at different settings and the calculated resolutions were compared to experimentally measured values.

In addition, a 2^3 full factorial DoE (including center point) was also performed and used for predicting resolution under different conditions. The 2^3 full factorial DoE is probably the most popular one in routine pharmaceutical analysis, and this was the reason why this type of DoE was selected. The levels of variables were set as: t_{g1} = 6.8 min, t_{g2} = 7.2 min, T_1 = 38 °C, T_2 = 42 °C, pH_1 4.2, pH_2 4.6 and the center point: t_{gc} = 7 min, T_c = 40 °C, pH_c 4.4. The results of this "conventional" robustness test were compared to the simulated test and predicted resolution values were also evaluated.

According to the best of our knowledge, the reliability of the simulated robustness testing has not yet been reported in LC. The aim of this study was to compare the result of the simulated robustness testing to "conventional" robustness testing, and to evaluate its accuracy by the comparison of experimental and predicted values.

3. Results and discussion

3.1. Determining the optimal working point

First, the working point was selected using the BEH C18 column. Fig. 2 shows the obtained resolution cubes. Fig. 2a corresponds to a pH range of 2.8–4.0 while Fig. 2b and c correspond to pH between 4.0–5.2 and 5.2–6.4, respectively. The red zones fulfil a criterion of Rs,crit > 1.5. Since the largest robust space was observed on Fig. 2b, the middle pH model, corresponding to 4.0 ≤ pH ≤ 5.2, was chosen for further experiments and calculations. A working point at t_g = 7 min, T = 40 °C and at pH 4.4 was selected as it provides a robust and high throughput analysis (the last peak was eluted at 3.5 min). Fig. 2d illustrates that the method (critical resolution) was not sensitive to changes in t_g, T°, and pH around the working point in a relatively large space. In a previous work, a 3-D model was build for the separation of the same compounds, but the effect of pH was studied only between 2 ≤ pH ≤ 3 using 5 mM ammonium dihydrogen phosphate buffer [25]. Thanks to the new extended model, the quality of the separation between amlodipine and related impurities was further improved. Indeed, somewhat better overall selectivity and more robust separation could be achieved when working at a pH of 4.4 compared to 2 ≤ pH ≤ 3.

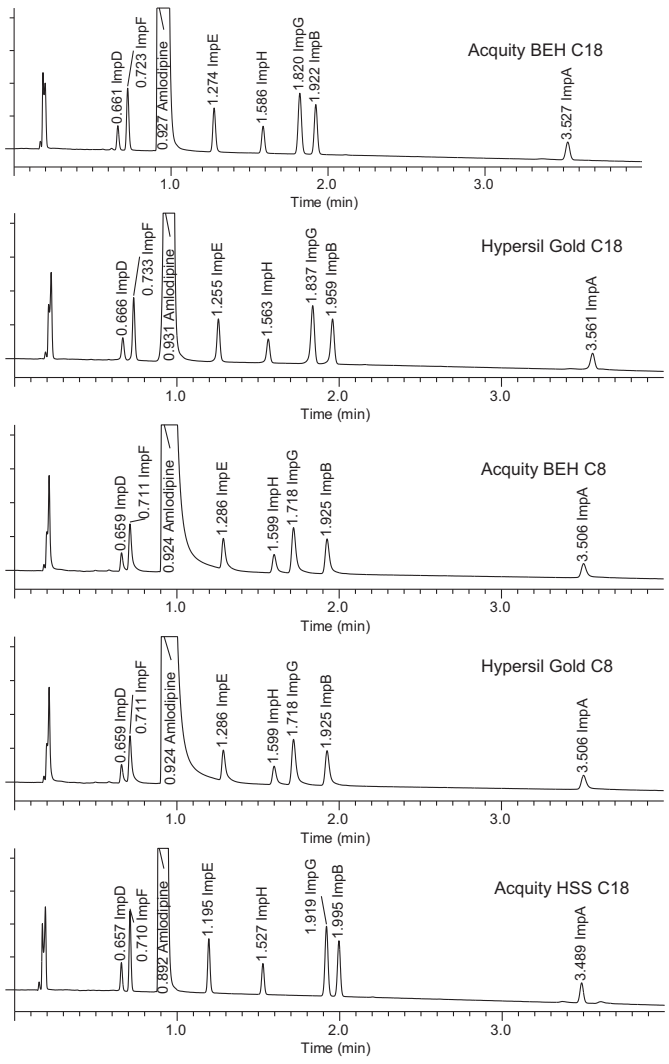


Fig. 3. Column interchangeability: fast separation of Amlodipine related impurities on 50 × 2.1 mm columns packed with sub-2 μm particles.

3.2. Impact of stationary phase

Based on our experience, it appears that the best method development strategy consists in performing initial runs and building up the 3 dimensional models using different columns at the early stage of method development. In this way, the working points,

Table 2
Experimental conditions for the different columns that provide similar selectivity and analysis time.

Column	t_g (min)	Start B%	End B%	T (°C)	pH
Acquity BEH C18	7.0	30.0	90.0	40	4.4
Hypersil GOLD	8.0	29.5	90.5	40	4.6
Acquity BEH C8	9.5	30.0	90.0	40	4.4
Hypersil GOLD C8	9.0	30.0	92.0	40	4.6
Acquity HSS C18	6.3	29.0	90.0	50	4.6

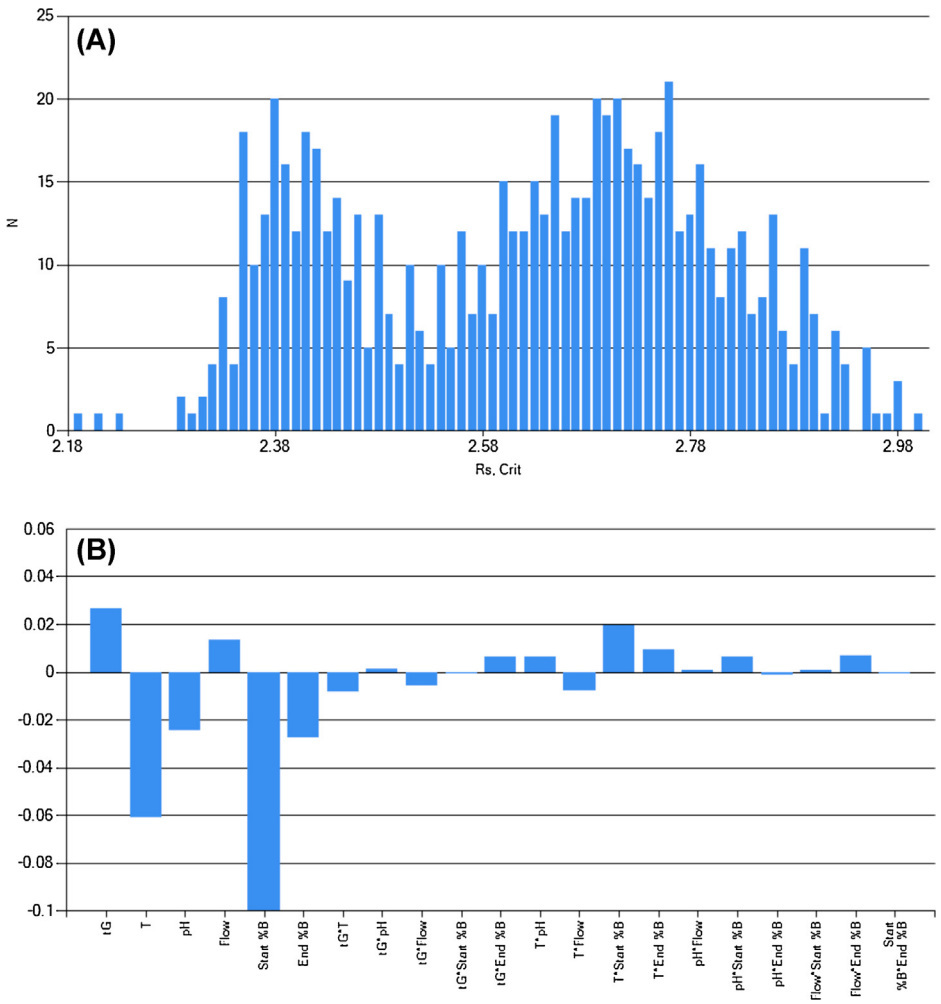


Fig. 4. Frequency of resolution values of most critical peak pairs (A) and the relative effects of the chromatographic parameters on the critical resolution (B).

robust zones, analysis time, selectivity and critical resolution can be compared for any mobile phase, temperature and gradient conditions. In contrast, when the column interchangeability is a part of robustness testing, the alternative column is only tested under the conditions that were optimized using the primary column. In many cases, the robustness testing fails at this stage since slight differences in selectivity and efficiency could manifest in inappropriate resolution.

A previous work illustrated that the baseline separation of amlodipine impurities was feasible on nine different 50 × 2.1 mm columns packed with sub-2 μm fully porous or sub-3 μm core-shell particles [25]. In that work, the authors compared the selectivity and achievable analysis time when selecting the condition that ensures the highest possible resolution. Obviously, each column had different working points and in some cases the analysis time was significantly different and important differences in selectivity (or even changes in elution order) were also noticed.

In this current study, our purpose was to perform very similar separations on the different columns. The middle pH range model and cubes were created for all columns. The conditions that provide similar selectivity, elution order and analysis time–compared to the reference BEH C18 column - were selected. Please note that these conditions were not identical with the working points of the different columns (higher critical resolution may reach, but more differences were observed in selectivity and retention when operating the columns at their individual working points). Fig. 3 presents comparative chromatograms of spiked amlodipine samples and Table 2 summarizes the corresponding experimental conditions. As shown in Fig. 3, all the five columns performed baseline resolution within the same analysis time (3.5 min). In agreement with the hydrophobic subtraction model (see data in Table 1), the most diverse conditions had to be set for the HSS C18 column. The Fs value of the HSS C18 column was the highest among all the columns, compared to the BEH C18 material. As reported in Table 2, the temperature had to be elevated to 50 °C instead of 40 °C.

When the conditions optimized for the BEH C18 reference column were applied to the four other columns, significantly different selectivities were observed. This proves that columns are not directly interchangeable but by adjusting analytical conditions, it is possible to achieve very similar separations. In conclusion, the evaluation of column interchangeability should be a part of early stage method development and not of the method validation.

3.3. Modelled robustness testing

A modelled robustness testing was performed thanks to commercial modelling software. Beside the three model variables (t_g, T°, pH), the flow rate, as well as initial and final compositions of the mobile phase represent the investigated factors in the built up model. The effect of these six factors was calculated, at three levels. The modelled deviations from the nominal values were the following: The gradient time was set to 6.8, 7.0 and 7.2 min, temperature

Table 3
Experimental conditions around the working point when performing the robustness test.

Parameter	Nominal value	Robust test 1		Robust test 2		Robust test 3		Robust test 4	
		Deviation	Set value	Deviation	Set value	Deviation	Set value	Deviation	Set value
t_g (min)	7.000	−0.005	0.695	−0.010	0.690	−0.015	0.685	−0.020	0.680
T (°C)	40.0	+0.5	40.5	+1.0	41.0	+1.5	41.5	+2.0	42.0
pH	4.40	+0.05	4.45	+0.10	4.50	+0.15	4.55	+0.20	4.60
Flow (ml/min)	0.700	−0.005	0.695	−0.010	0.680	−0.015	0.685	−0.020	0.680
Start %B	30.0	+0.5	30.5	+1.0	31.0	+1.5	31.5	+2.0	32.0
End %B	90.0	+0.5	90.5	+1.0	91.0	+1.5	91.5	+2.0	92.0

was set to 38, 40 and 42 °C, mobile phase pH was set to 4.2, 4.4 and 4.6, flow rate was set to 0.68, 0.70 and 0.72 ml/min, initial mobile phase composition was set to 28, 30 and 32% B and its final composition was set to 88, 90 and 92% B. Then, the 729 experiments were simulated in less than 1 min. A criterion of Rs,crit > 1.5 was considered. Fig. 4A shows the results of the experiments expressed in frequency as a function of critical Rs. As can be seen, the most frequent resolution was Rs,crit = 2.76 (21 conditions provided this Rs value), while the lowest predicted resolution was Rs,crit = 2.18. Therefore, the method can be considered as robust, since the failure rate was 0% in the studied design space. Another feature of the modelling software employed in this study is the calculation of individual and interaction parameter effects. Fig. 4B describes the importance of each parameter, related to the selected deviation from the nominal value, for the critical resolution. This figure shows that the "start %B" (initial mobile phase composition) has the most significant influence on the critical resolution (i.e., a negative change in "start %B" would increase the critical resolution), followed by T°, t_g, end %B and pH as the most important parameters. Some interactions between the factors i.e. T^{*} Start %B also have an impact on critical resolution.

As a next step, the reliability of this simulated robustness test was evaluated. Experimental verifications of calculated chromatograms were performed. The values of the six factors were systematically changed around the working point. Experiments under four different conditions (corresponding to the edges of the evaluated robust space) were carried out. Table 3 summarizes the experimental conditions while Table 4 compares the predicted and experimentally observed retention times within the design space. The predicted retention times were in good agreement with the experimental ones since the average retention time relative errors were <1.0% (the individual values scattered between −3.57 and 2.27%, see Table 4), which can be considered as an excellent prediction with such ballistic gradient. The accuracy of critical resolution prediction was also assessed. As illustrated in Table 4, the predicted critical resolution errors were between 6.9 and 17.2%. Obviously, the errors on resolution values contain both the retention time error and also the uncertainty of peak width prediction. This explains why the errors on resolution were systematically higher than the one observed on retention time. Even though there are slight differences, the separation is still sufficient as all critical resolutions were larger than 1.5. In our opinion, the prediction provided by modelling software can be considered as reliable and suggest that the accuracy of the simulated robustness test is indeed acceptable.

Robustness testing was also performed using experimental design and the final results were compared to the simulated one. The 2^3 full factorial DoE is probably the most popular one in routine pharmaceutical analysis and this is the reason why this type of DoE was selected in this study (due to time constraints, 3^6 DoE is never performed in analytical laboratories). The t_g, T° and pH levels were set at the same values as in the simulated robustness testing. Fig. 5 shows the corresponding Pareto chart, and the obtained results were in good agreement with the predicted frequency plot from the modelling software. Among these three factors, the temperature had the most significant effect on the resolution (negative effect) followed by pH (also negative effect) and gradient time (positive effect). Finally, Table 5 shows several conditions (where the three parameters were randomly varied) and the calculated values of expected critical resolutions. The differences in terms of predicted critical resolution between the DoE and modelling software approach values ranged between −0.77 and +0.38%. These results also confirm the reliability of the simulated robustness testing.

Please note that in the simulated robustness test, there are only 3 "true" variables, namely the pH, t_g and T° even if it is named as 3^6 DoE. Only the effects of these three variables were experimentally measured for the simulated robustness testing. The effect of initial-, final mobile phase composition and flow rate were calculated, based on built in models. Our purpose was to experimentally determine the effect of the same factors for our 2^3 DoE. The aim was to compare the predictions of the common 2^3 DoE and the DryLAb's "apparent" 3^6 DoE based predictions for given conditions.

Table 4
Comparison of predicted and experimentally observed retention times within the design space.

	Working point			Robust test 1			Robust test 2			Robust test 3			Robust test 4		
	Pred.	Exp.	Error%	Pred.	Exp.	Error%	Pred.	Exp.	Error%	Pred.	Exp.	Error%	Pred.	Exp.	Error%
Retention times															
Imp D	0.67	0.67	0.00	0.63	0.63	0.00	0.59	0.60	−1.67	0.56	0.57	−1.75	0.54	0.56	−3.57
Imp F	0.73	0.73	0.00	0.69	0.69	0.00	0.65	0.65	0.00	0.62	0.63	−1.59	0.59	0.61	−3.28
Amlodipine	0.98	0.98	0.00	0.94	0.92	2.17	0.9	0.88	2.27	0.85	0.85	0.00	0.81	0.82	−1.22
Imp E	1.27	1.27	0.00	1.22	1.21	0.83	1.18	1.16	1.72	1.13	1.11	1.80	1.08	1.07	0.93
Imp H	1.57	1.58	−0.63	1.50	1.5	0.00	1.42	1.40	1.43	1.35	1.36	−0.74	1.36	1.37	−0.73
Imp G	1.81	1.82	−0.55	1.77	1.76	0.57	1.73	1.71	1.17	1.69	1.69	0.00	1.61	1.61	0.00
Imp B	1.92	1.92	0.00	1.87	1.86	0.54	1.83	1.81	1.10	1.78	1.78	0.00	1.71	1.7	0.59
Imp A	5.5	5.53	−0.54	3.46	3.45	0.29	3.41	3.39	0.59	3.37	3.36	0.30	3.23	3.24	−0.31
Average			−0.22			0.55			0.83			−0.25			−0.95
Critical resolution															
	2.64	2.47	6.88	2.56	2.27	12.78	2.48	2.14	15.89	2.40	2.06	16.50	2.32	1.98	17.17

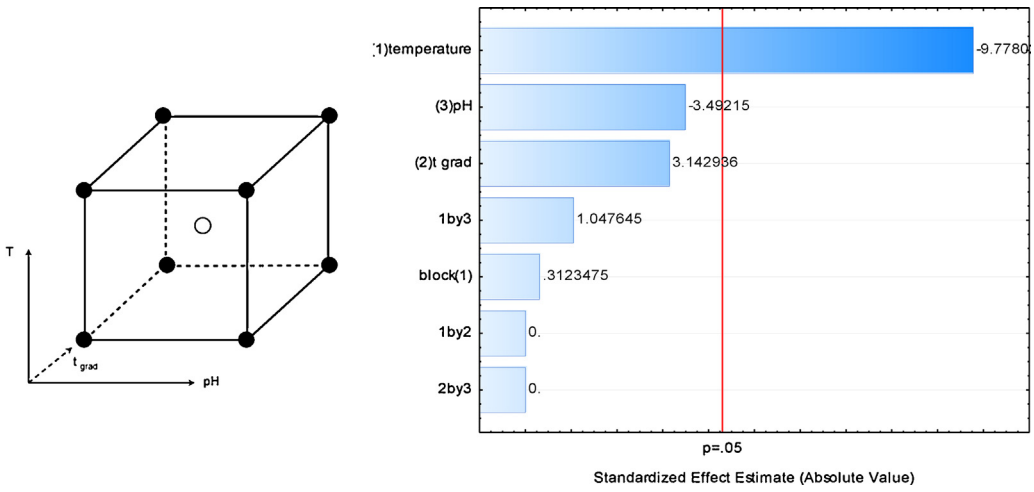


Fig. 5. Schematic view (on the left) and Pareto chart of the 2^3 DoE (on the right). The effect of the three parameters and their interactions are plotted in absolute values. The red line indicates the significant effects.

Table 5
Conditions with randomly varied parameters and the comparison of expected critical resolutions based on DryLab and Statistical predictions.

T (°C)	t_g (min)	pH	Rs (statistically predicted)	Rs (DryLab predicted)	Difference (%)
39	6.9	4.3	2.70	2.69	0.37
41	7.1	4.5	2.62	2.61	0.38
39	7.1	4.2	2.74	2.74	0.00
41	6.9	4.6	2.59	2.59	0.00
37	6.7	4.1	2.79	2.80	−0.36
43	7.3	4.7	2.57	2.59	−0.77

4. Conclusion

Because robustness testing could be a long and tedious task in liquid chromatography, the possibility to employ commercial modelling software was evaluated to expedite this task. In the present study, state-of-the-art stationary phases (short narrow bore columns of 50 × 2.1 mm packed with sub-2 μm particles) were exclusively employed for the separation of amlodipine and related impurities.

In a first instance, the possibilities offered by modelling software for performing robustness testing were systematically compared to experimental measurements and DoE based predictions. The reliability of predicted retention times and resolutions were compared for the working point and at the four edges of the design space. It appears that the retention time relative errors were on the maximum equal to 4% for the eight different compounds (lower than 1% for the average relative error) and the four tested conditions (corresponding to the edges of the design space). On the other hand, the predicted critical resolution errors were comprised between 6.9 and 17.2%. Based on these observations, it appears that the prediction reliability was satisfactory and then, robustness could be investigated in the early stage of method development, without generating an unacceptable amount of work for the analyst.

Another important part of robustness testing is the column interchangeability. This was also evaluated for the mixture of amlodipine and related impurities. For this purpose, five different C8 and C18 columns packed with sub-2 μm particles were tested. Using the same modelling software, we proved that this separation was feasible on all columns within the same analysis time (less than 4 min), by proper adjustments of variables (pH, temperature, gradient conditions).

This confirms that modelling software is an important tool for chromatographists, to expedite method development but also robustness testing.

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