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Simultaneous optimization of mobile phase composition and pH using retention modeling and experimental design

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ABSTRACT

Chromatographic methods are progressing continuously. Increasing sample complexity and safety expectations lead to higher regulatory demands, hence challenges in liquid chromatography analysis are rising, even today, when faster and faster chromatographic systems are extensively employed and become widely accessible for successful method development.

The goal of this study was to investigate the impact of mobile phase influences as important factors of selectivity tuning in method development. This would mitigate mobile phase-related robustness issues throughout the method's lifecycle.

To discover and understand these effects, a new module of chromatographic modeling software DryLab (ver. 4.3.4. beta) was introduced and a special experimental design (DoE) was tested, allowing the simultaneous optimization of solvent-dependent parameters, such as gradient time (t_G), ternary eluent composition (t_C) and pH, requiring 18 input experiments ($2 \times 3 \times 3 = 18$).

Additionally, the model creation, using a UPLC system and a narrow bore column (50×2.1 mm), the entire experimental work could be finished in 2–3 hours. To demonstrate the applicability of this new design, amlodipine and its related pharmacopoeia impurities (A–H) were subjected to be used in a case study. Predicted vs. Experimental (or Verification) runs showed excellent agreement, average retention time deviations were typically less than 1 s. Modelled robustness testing was also performed, elucidating all important mobile phase and instrument parameters that could influence a method's lifetime performance. Furthermore, as the in silico robustness testing is the least time consuming part of the method development process, it can be used extensively to evaluate robustness even at the very early part in stage 1 of the Method Life Cycle (MLC).

1. Introduction

High pressure liquid chromatography (HPLC and UHPLC) method development assisted by software modeling shows an increased tendency of regulatory expectations to be getting to a higher level in the quality of submissions. Regulatory agencies have too much trouble with unreliable, underperforming methods, which were developed by trial and error, as the many "Out of Specification" (OoS) cases cost a great amount of time, hinder the development of new drugs and more importantly a chance for fast help for the patients [1,2].

On the one hand, considering the recent advances in medicine, such as the headway of therapeutic proteins, multi-API drug products, the overall complexity of drug analysis has risen, bringing a whole set of new challenges for the analysts working in this field. Therefore, systematic developments of HPLC methods, according to Quality by Design principles, are requested today. On the other side, chemical understanding, combined with modern computer ("in silico") science and quality-oriented methodology can rationalize these difficulties and help achieving the primary goal, among others, to speed up analytical processes and reduce time to market. Since Arieh Warshel, Michael Levitt, and Martin Karplus received in 2013 the Nobel-Prize for computer modeling, this new tool became accepted in the scientific community.

The history of separation modeling stretches back to the late 1980ś, when Lloyd Snyder and his workgroup established the first

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One-Factor-At-the-Time (OFAT) retention modeling for isocratic [3] and gradient [4] methods. This allowed the experiment-based optimization of influential chromatographic parameters on selectivity, such as isocratic organic percentage (%B), gradient time- (t_G) and -profile (start[B%], end[B%], steps), temperature (T), pH and buffer concentration ($c_{buff.}$). Soon, a two-dimensional (2D) optimization followed, which is now considered to be a messenger of a new, systematic concept, known as the Analytical Quality by Design (AQbD) movement [5].

The next milestone was the introduction of a three-dimensional (3D) model, called the "Cube" [6], whereby relying on 12 input experiments, simultaneous optimization of 3 factors, gradient time (t_G), temperature (T) and pH or ternary composition (t_C) or buffer concentration ($c_{buff.}$) or additive concentration ($c_{additive}$) could be achieved [7].

Having another advantage of virtual modeling, not just experimentally measured, but also other relevant chromatographic parameters, like flow-rate (F), column length [L], and inner diameter [I.D.], particle size [d_p]), instrument dwell-volume (V_d), and extra-column volume (V_{ec}) can be modeled by calculation, allowing a deeper understanding, broader flexibility and space for prospective changes.

DryLab modeling is now extensively used by research facilities and by pharmaceutical companies to reduce method development time [8], create fast-, robust- [9], or update old pharmacopeia methods [10,11] and transfer them between different columns and instruments [9], in various applications of reversed phase, ion-pair- [12], ion-exchange [13], hydrophobic interaction chromatography (HIC) [14] and HILIC [15], both for the analysis of small and large molecules, showing the broad spectra of possibilities in software aided HPLC modeling.

While the scientific and regulatory recognition of Quality by Design, that is being conducive to enhance overall pharmaceutical quality, has already been advocated for drug analysis, the analogous systematic, science-based methodology for robustness is still missing in the relevant guidelines.

ICH identifies 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"* [16]. The necessity of controlling all relevant analytical variables, including solvent-, instrument-, and column-dependent differences are also highlighted, but the question on how to do that, applying QbD-principles, remains open.

Similarly, there is no further instruction in the United States Pharmacopeia (USP <1225>, even if the technical implementation of other analytical terms regarding performance characteristic, are well detailed.

Although method qualification (validation and system suitability test) might indicate if a method is not robust, working with a failing method, only limited, and without a systematic knowledge, untargeted possibilities are available for subsequent corrective actions in compliance with USP <621>. However, if this does not succeed, partial or full re-validation becomes inevitable.

In earlier works, model-based systematic robustness evaluation of 6 chromatographic parameters, such as gradient time, temperature, flow rate, start-, final organic percentage, and either pH or ternary composition or additive concentration was performed and experimentally verified. [7,17,18]. Additionally, using the full-potential of method modeling, the effects of column batch-to-batch variations [19] and typical instrument differences, occurring at method transfer [7] were also explored.

At the same time, there were some cases, when both pH-control and the use of ternary eluent composition were required to achieve the desired selectivity [20,21].

In such cases, the mobile phase can affect the quality of the separation in many ways. On one hand, the pH change may alter the ionization degree of molecules with dissociable functional groups. On the other hand, change of ternary composition may influence the selectivity through mobile phase strength or by shifting from an aprotic to a protic solvent, the selectivity changes through H-bonded solvent-solute complex formations, and through a reduced solvation (better "silanol-masking") of the alkyl-chains. All of these effects can have a strong influence on the chromatographic selectivity. Previous works revealed that modeling software can be used to study retention properties in the process of method optimization [9,22,23] and besides of gradient time (t_G) and temperature either pH or ternary eluent concentration (t_C) gave interesting selectivity changes for better separations. However t_G, pH and t_C were never combined before in a Cube. Therefore a special resolution cube was developed, incorporating both nonlinear factors pH and t_C at the same time using 18 input runs.

As described in previous works, for gradient time two level measurement [2], while for pH and t_C three levels are necessary [24–26], resulting in 18 ($2 \times 3 \times 3$) experiments needed to be performed for a t_G-pH-t_C-Cube. With this combination a new view might be found on how to implement an early phase robustness calculation, with a special highlight on mobile phase influences on the separation.

2. Experimental

2.1. Chemicals

The mobile phases used in this work were a mixture of acetonitrile (MeCN, Eluent B1), methanol (MeOH, Eluent B2) and water (Eluent A) buffered with 10 mM sodium formate and ammonium acetate. Acetonitrile, methanol (gradient grade), formic acid, sodium hydroxide, ammonium acetate and standard reference buffers (pH 2.00, 4.01 and 7.00) were purchased from Merck (Darmstadt, Germany). Water was prepared freshly, using ELGA Purelab UHQ water (ELGA, Lane End, UK).

Sample was prepared from amlodipine API (0.5 mg/mL) and spiked with its impurities at 1% level. The structures of the compounds are shown in Fig. 1. Amlodipine and its impurities were obtained from Egis (Egis Pharmaceuticals Plc., Budapest, Hungary). Sample solvent was acetonitrile/water = 30/70 (v/v).

2.2. Equipment and software

UHPLC experiments were performed on a Waters Acquity UPLC system (Milford, USA) equipped with binary solvent delivery pump, autosampler, photodiode array detector and Empower 3 software. This UPLC system had a loop sample injector and 500 nL flow cell. The dwell volume of the system was measured as 0.1 mL. The Hypersil GOLD C18 column (50×2.1 mm, 1.9 μm) was chosen as stationary phase because of its relatively high surface coverage with endcapping [18]. The column was purchased from Thermo Scientific (Waltham, USA).

UHPLC method development and modeling were performed by using DryLab4, v.4.3.4 (beta) modeling software (Molnár-Institute, Berlin, Germany). The buffers were made freshly and the pH was measured using MP 225 pH-meter, which was purchased from Mettler-Toledo (Greifensee, Switzerland).

2.3. Preliminary experiments

Amlodipine and its Ph. Eur impurities exhibit a wide variety of different chromatographic properties; therefore they were selected for the experiments as model compounds. Based on former experiences, amlodipine and its impurities were found to be relatively lipophilic, hence the starting mobile phase composition was set to 40% eluent B. However, the ImpA was found to be highly lipophilic, therefore high organic content, i.e., over 90% B was required at

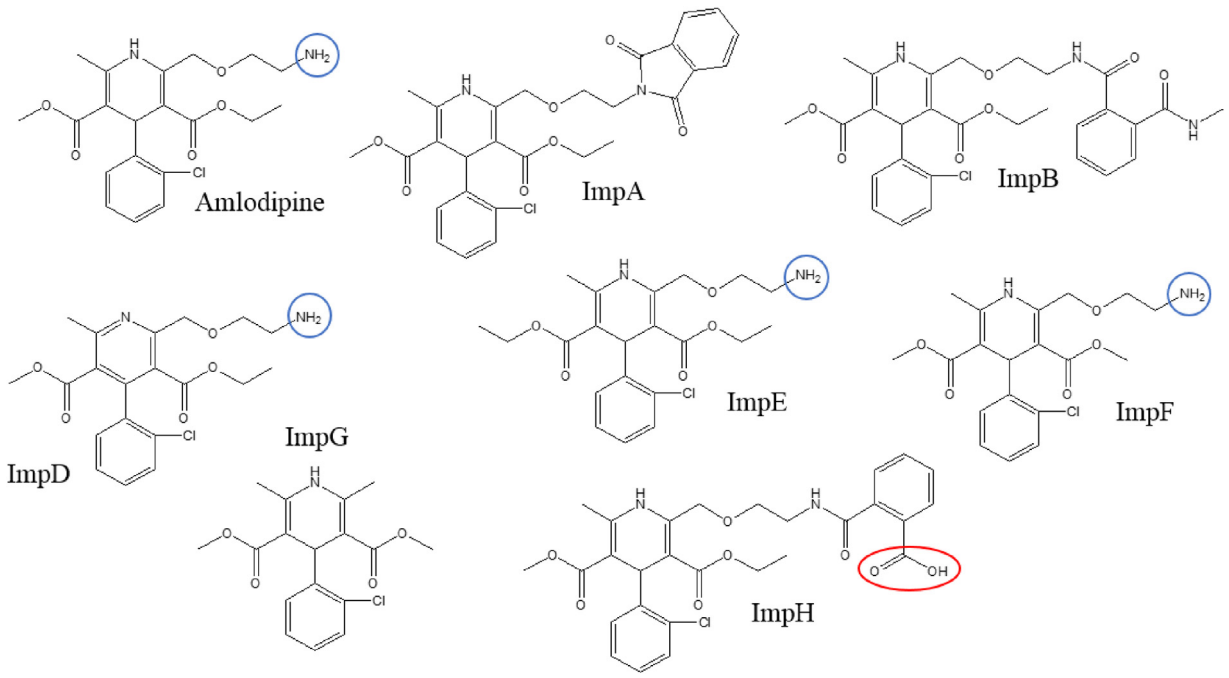

Fig. 1. Molecular structures of the compounds used in this work; amlodipine and impurities A, B, D, E, F, G, H.

the end of the gradient to elute this substance. Furthermore, there was a structural similarity between amlodipine, ImpD, ImpE and ImpF as all of them contained a basic primary amino group (pK_a > 10), meaning that all these substances were assumed to be ionized under common reversed phase (RP) conditions. On the contrary, due to the carboxylic group attached to an aromatic structure (pK_a ~ 4), ImpH had acidic character, so far, depending on the applied RP conditions, it could be either fully ionized or neutral [22,23,27].

The aim of the new experimental design was to create a model, where the baseline separation can be attained for each peak pair and nonetheless, the effect of the methodical change of the aqueous phase (Eluent A) with the pH and the organic (Eluent B) component of the mobile phase can be studied for further selectivity influences. Furthermore, using the full potential of "in silico" modeling, our goal was to implement an early-stage robustness calculation, which can deliver a clear answer about mobile phase influences, with the main focus on the expected long-term method performance.

2.4. Design of experiments

Experimental design for simultaneous optimization of gradient time (t_G), pH, and ternary composition (t_C) required 18 experiments, as illustrated in Fig. 2. Two linear gradients with a factor 3 difference in gradient times, 3 and 9 min, in a range from 40 to 90%B were used. The mobile phase A consisted of 10 mM sodium formate buffer with 3 different pH values, 3.2, 3.8 and 4.4, respectively. The mobile phase B consisted of acetonitrile, methanol and a mixture of both with a ratio of 50/50 (v/v). The flow rate was set to 0.5 mL / min, the temperature was set to 30 °C. The detection was performed with DAD-detector, chromatograms were extracted at 237 nm. The injected volume was 1 μL.

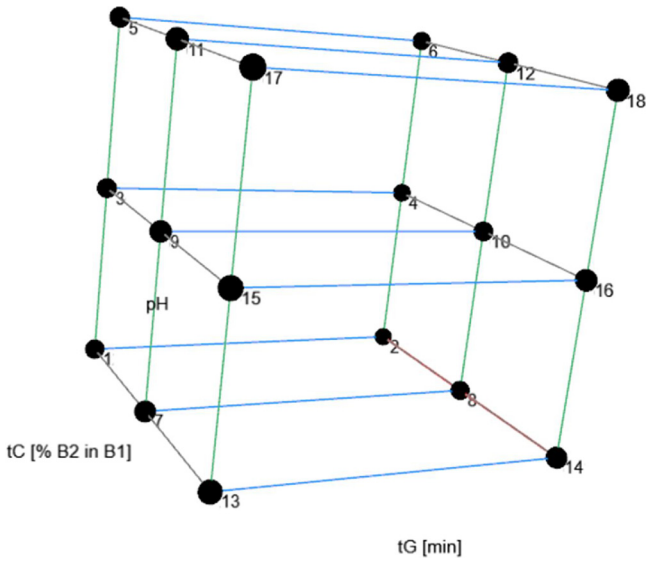

Fig. 2. Design of Experiments (DoE), used to obtain the 3D-models. The experiments 1, 3, 5, 7, 9, 11, 13, 15 and 17 were carried out with a steep gradient (i.e. t_G = 3 min), 2, 4, 6, 8, 10, 12, 14, 16 and 18 with a flat gradient (i.e. t_G = 9 min). The pH of eluent A was 3.2 with experiments 1, 2, 7, 8, 13 and 14; it was 3.8 with experiments 3, 4, 9, 10, 15 and 16, and it was 4.4 with experiments 5, 6, 11, 12, 17 and 18. The ternary composition (t_C) of eluent B (the ratio of MeCN vs. MeOH) was 100% MeOH with experiments 13, 14, 15, 16, 17 and 18, it was MeCN/MeOH = 50/50 (v/v) with experiments 7, 8, 9, 10, 11 and 12, and it was 100% MeCN with experiments 1, 2, 3, 4, 5 and 6.

3. Results and discussion

The importance of ternary composition can be seen in Fig. 3. The baseline separation could not be achieved in this pH range neither with 100% MeOH nor at 100% MeCN with the Hypersil GOLD C18 column. The introduction of a variable composition of organic phases was therefore essentially needed.

The target was the selection of a set point, where baseline separation can be achieved for all peak pairs and the analysis time is short as well. Keeping these expectations in mind and analyzing Fig. 4, the middle robust area of the upper "Method Operable Design Region" (MODR) seemed to be a rational choice, therefore the setpoint was selected at t_G = 4.0 min, pH = 4.2 and t_C = 60% (B2

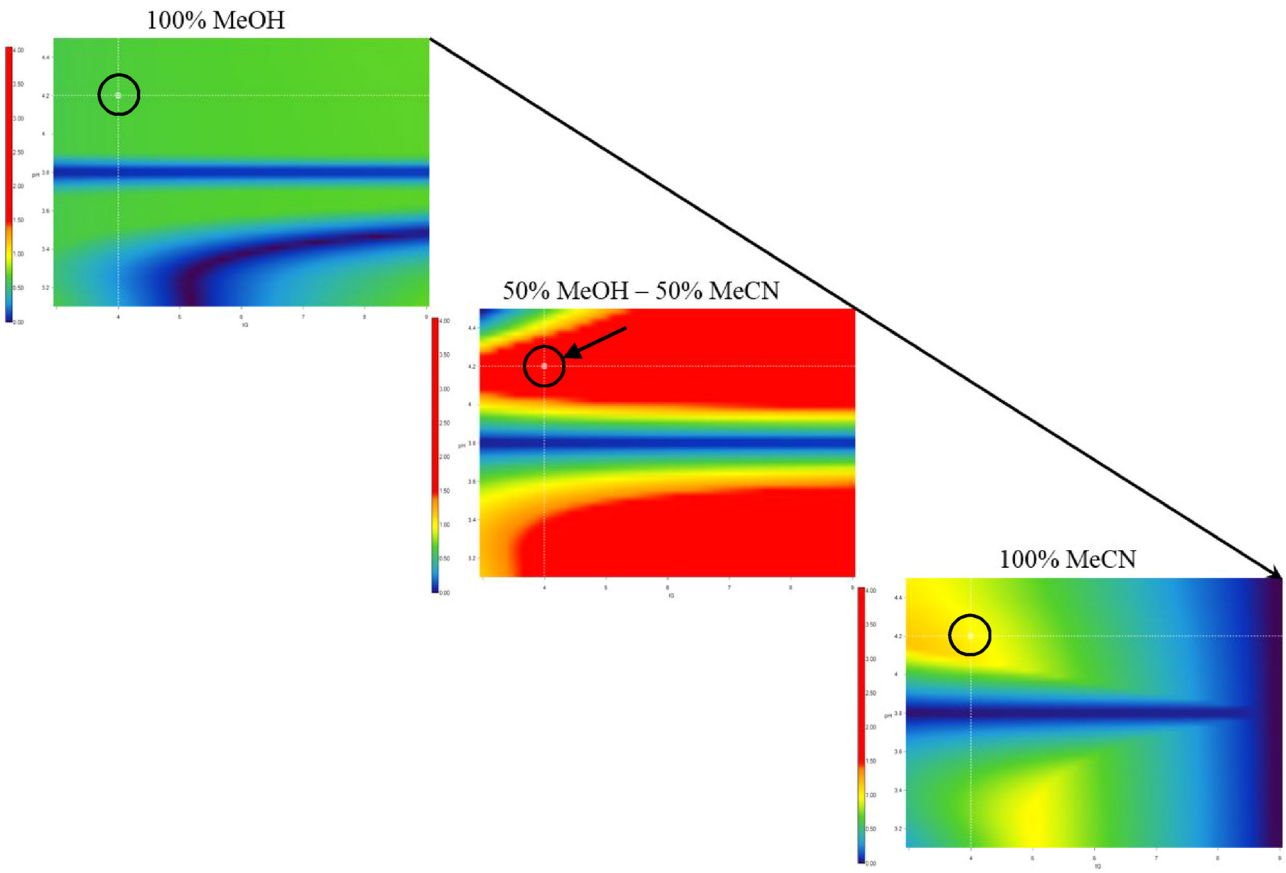

Fig. 3. Changes in critical resolution $R_{s,crit}$ along the t_C-axis shows, that regardless all the possible combination of t_G and pH, adequate separation is not achievable neither with pure MeOH, nor with MeCN. However, it is achievable with the application of both, t_c = 50%, see at the arrow. For the set points (encircled), the other conditions remained unchanged pH = 4.2, t_G = 4.0 min.

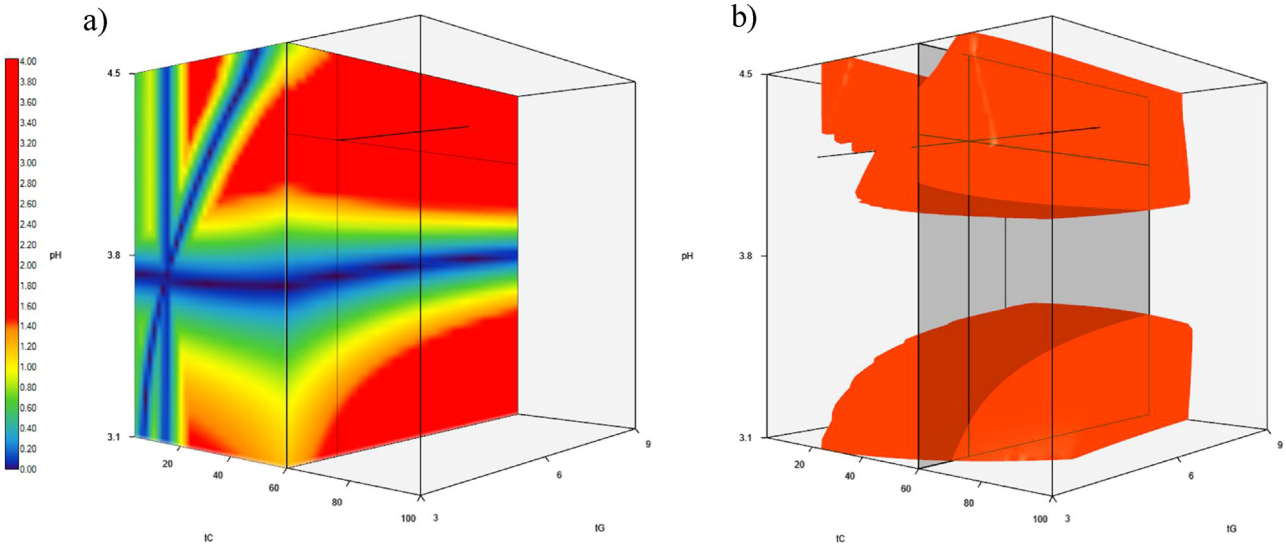

Fig. 4. Three-dimensional resolution map based on t_G-pH-t_C model. a) shows the influence of t_G, pH, and t_C on the critical resolution. t_C is referred to as $\%B_2$ in B_1 (B_1 is MeCN, B_2 is MeOH). Red color indicates critical resolution values higher than 1.5 ($R_{s,crit} \geq 1.5$, baseline separation), while blue indicates co-elution ($R_{s,crit} = 0$), and b) shows only the robust zones where $R_{s,crit} \geq 1.5$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

in B1) (B1:MeCN/B2:MeOH = 40/60 (v/v)). Verification run of the model at this point showed high correlation with the predicted chromatogram as seen on Fig. 5. There is no significant difference between the modelled and the experimental chromatogram.

3.1. Robustness

Direct fulfilment of the initial analytical expectations with the Analytical Target Profile (ATP), such as the baseline separation of the critical peak pair, minimum analysis time, etc., is self-evidently

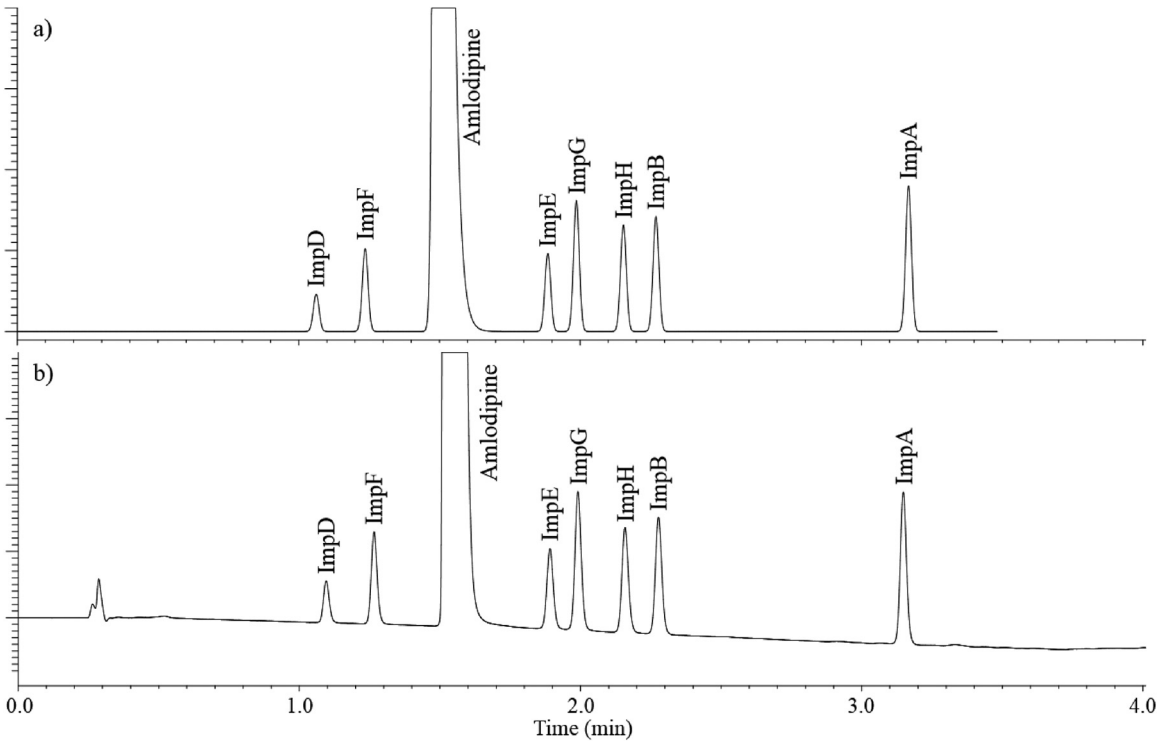

Fig. 5. Simulated (a) and experimental (b) chromatograms at the set point (t_G = 4.0 min, pH = 4.2 and t_C = 60%).

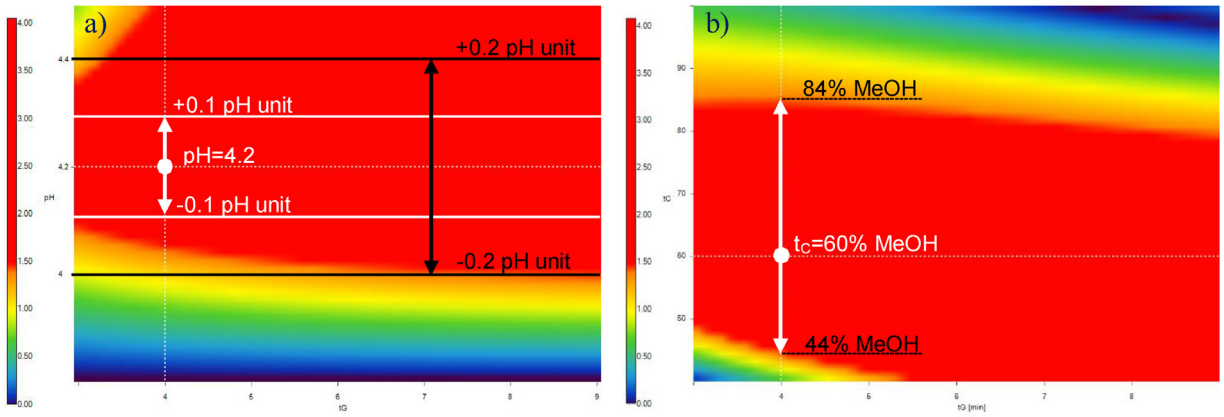

Fig. 6. The effect of varying pH under the defined tolerance limits around the set point (t_G = 4 min, pH = 4.2 ± 0.1 and pH = 4.2 ± 0.2). As a conclusion, strict control of pH (4.2 ± 0.1 units) is essential to avoid OoS-results. Fig. 7b shows the ruggedness of the set point against the change of ternary composition. (Tolerance limit for robustness calculation was specified as t_C = 60 ± 1 and t_C = 60 ± 2%).

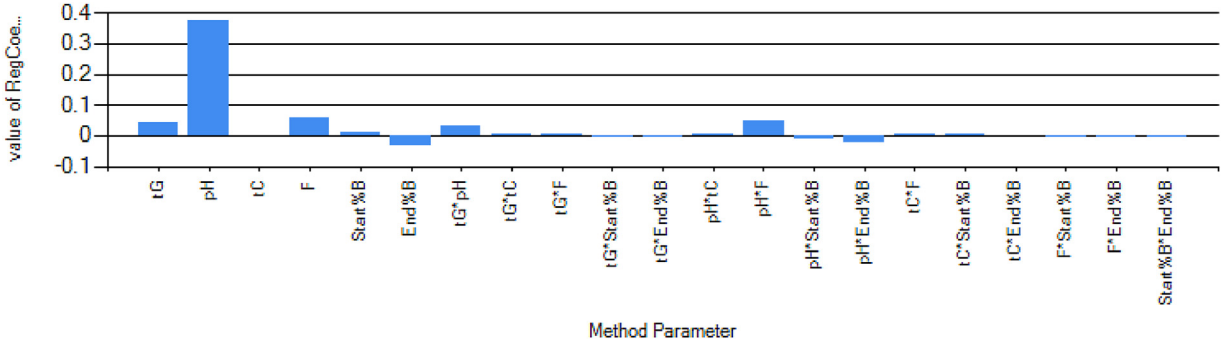

Fig. 7. Regression coefficients of the robustness calculation show pH as the most influential factor on the separation in the set point (pH = 4.2 ± 0.2, t_C = 60 ± 2%).

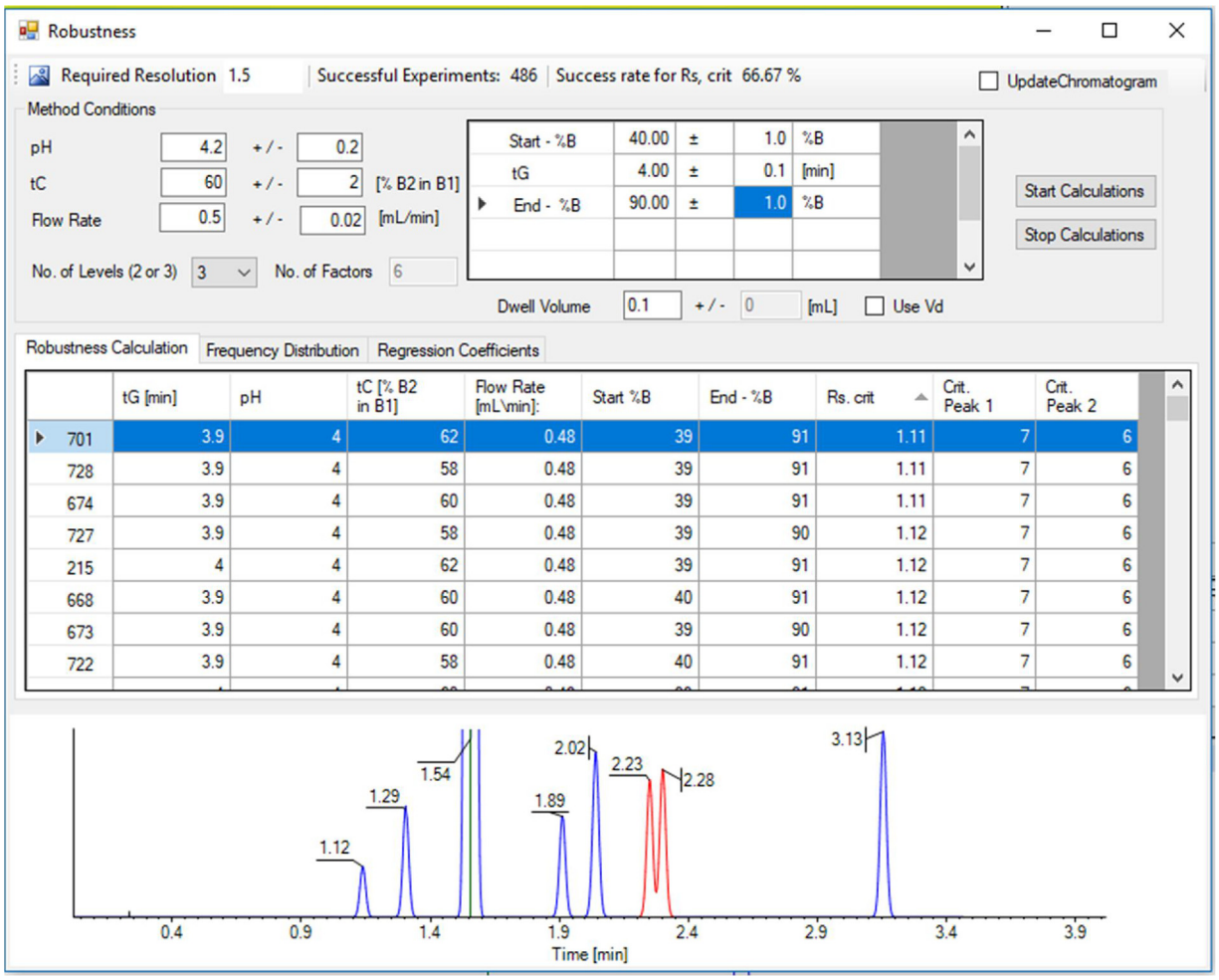

Fig. 8. Calculation of 729 robustness experiments ($3^6 = 729$) with a gradient from 40 →90%B in $t_G = 4$ min as shown at the top part of the table. The set tolerances are for pH ± 0.2, for t_C ±2%, for F ± 0.02 mL/min, for Start[%B] ±1, for End[%B] ±1 and for t_G ± 0.1 min. Virtual results were sorted by increasing resolution values. The lower chromatogram shows the first worst-case combination from the list (out of 729 virtual experiments), as selected in the table. Seemingly, baseline separation is not attained under these conditions ($R_s = 1.11$).

important, however the method must be capable of producing adequate results, not only for short term and in a limited environment but also for a long time period and in a wide, often hardly predictable, complex analytical environment, e.g. method transfer across locations and instruments. These aspects are one of the main topics of recent discussions considering the lifecycle management (LCM) of analytical methods [28].

In fact, during a daily application of a method, possible alterations of chromatographic parameters and consequently, slight fluctuations of method performance can be expected. On one part these alterations are caused by normal variations of instrument properties, such as solvent delivery, solvent mixing, and temperature-controlling that all can be characterized with so called instrument specification limits (ranges). On the other side, and as discussed earlier, it is caused by some less evident variations, like batch-to-batch differences of columns, of which effects might be less substantial, but requires a deep understanding and a systematic plan [19,22].

Robustness, i.e., the ability of a method, to resist against these possible alterations is well highlighted but less detailed in the relevant guidelines. As mentioned, while there is a plain description on how to characterize method performance in terms of accuracy, specificity, linearity, precision, detection limit, qualification limit in ICH Q2 (R1) [16] and USP <1225> [29], it is less obvious how to evaluate a method's robustness in practice. Nevertheless, the ICHś Q2 (R1) clearly formulates that *"The evaluation of robustness should be considered during the development phase. . ."* (Stage 1). At the same time consequences of non-systematic method development comes to the fore very often only at a later phase, namely at the performance qualification (Stage 2) or even later, at Stage 3 [30]. This brings to the relevance of an early-stage robustness evaluation.

In previous works, commercial software's robustness calculation was utilized in many ways for method optimization [9,17,18]. In this study however, we focused on the issues of method robustness, considering the aspects of the mobile phase influences.

3.2. Investigating single solvent effects

At first, tolerance limits, with a special focus on solvent changes, i.e., possible alterations of method conditions were needed to be thoroughly considered. To be able to further specify these limits, we took the UPS's <621> [31] guideline, but from a different perspective. As described, for a validated method ±0.2 pH-unit subsequent adjustment, while for minor solvent component 30 rel. %, and according to FDA's corresponding guide (ORA Lab.5.4.5. attachment A) ±2 abs.% adjustments are permitted, without a compelling need of a revalidation. In this regard, we investigated the solvent effect in a theoretical case of a validated environment, specified with these tolerance limits, calculating with ±0.2 pH-unit and ±2% of ternary composition difference. The rest of the possible alterations

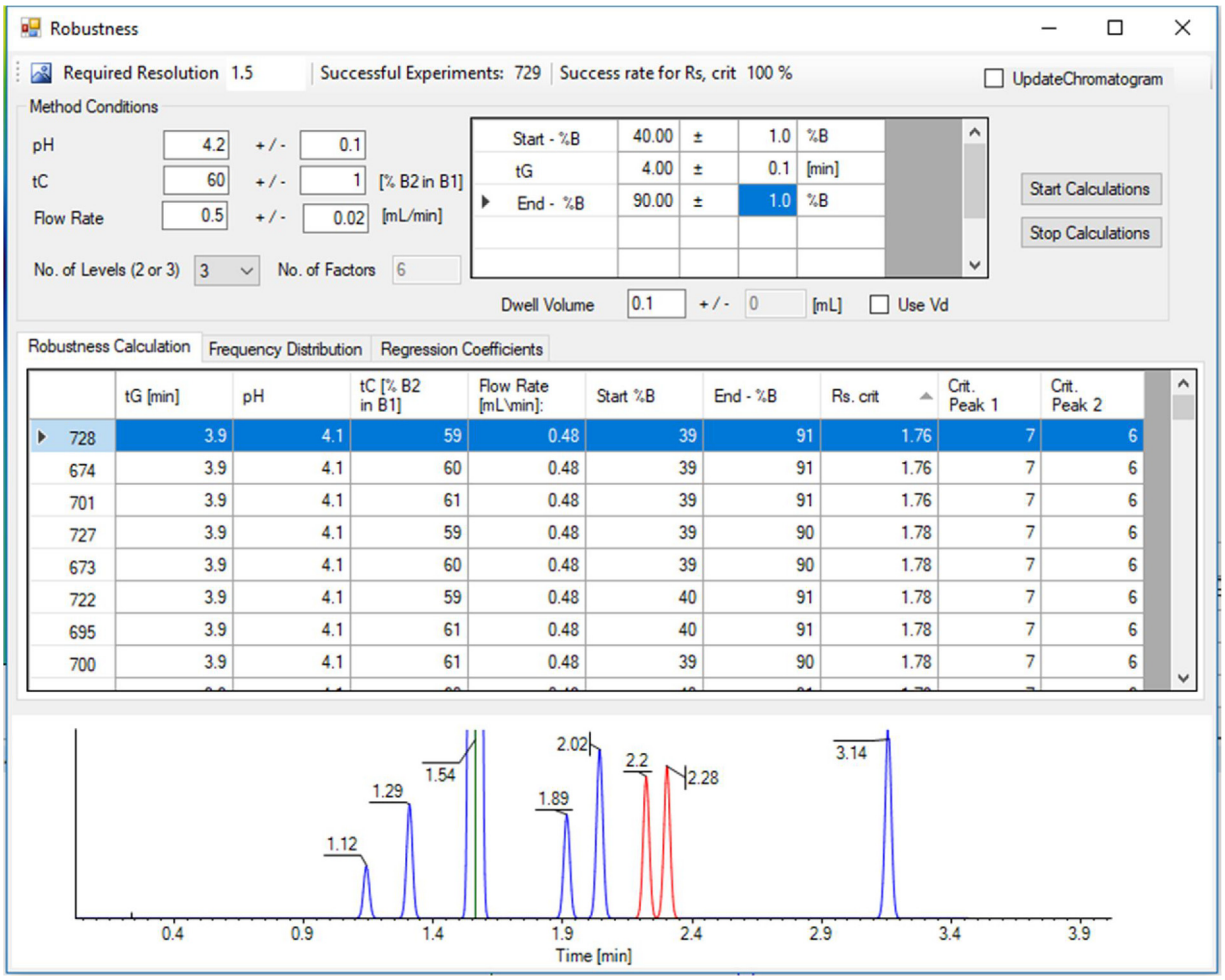

Fig. 9. Robustness calculation based on reduced tolerance limits for the pH from 4.20 ± 0.2 to 4.2 ± 0.1 and at the same time the accuracy in t_C was improved to 60 ± 2 to 60 ± 1 (%B2 in B1). This increased the success rate from 66% to 100%. The lower chromatogram shows the first worst-case combination from the list (out of 729 virtual experiments), as selected in the table. Even for the worst case baseline separation is guaranteed (R_s = 1.71).

for the robustness calculation (t_G, flow-rate, start[B%], end[B%]) were determined taking the typical instrument specification limits from the manufacturer's handbook.

First, to investigate the single effect of the pH on the separation, a section of the 3 dimensional models (t_G-pH layer at 60% MeOH content) was prepared and shown (Fig. 6a). This elucidates that if the pH decreases to a lower extreme of the specification limit (pH = 4.0), the method starts to produce OoS-results. However, if a more precise control of the pH is established (±0.1 pH-unit), the method produces satisfactory ($R_s \geq 1.5$) results.

Investigating the influence of a single change in the organic composition around the set point (Fig. 6b) shows that the method's performance is relatively indifferent on these changes. Presuming other chromatographic parameters remain constant, even with 16% less or 24% more methanol content would provide reasonable results.

3.3. Software-based robustness calculation

ICH Q2 (R1) implies that not only solvent, but also instrument effects can have an impact on the method's robustness. Additionally the guideline (USP <621>) says that *"multiple adjustments can have a cumulative effect on the performance of the system and are to be considered carefully before implementation"*. Software-based robustness calculation has the advantage that not only all single solvent and instrument effects, but also all conceivable combinations of them can be, in a model-mediated way, calculated.

For these purposes DryLab robustness module was used. The set point was at pH = 4.2, t_C = 60%, F = 0.5 mL/min; Start[%B] = 40%, End[%B] = 90 and t_G = 4.0 min.

Fig. 7 shows that in case of taking all six parameters into account at three levels (+, 0, -), regardless if pH and ternary composition is on a broader (±0.2, t_C ±2%) or narrower range (pH ± 0.1, t_C ±1%) specified, pH had the highest influence on the resolution, therefore this parameter must be controlled with a special care. Smaller, although considerable impact had the gradient time, flow-rate, and the pH-flowrate cross-effect on the separation. As expected, ternary composition had under none of these specification limits a special influence.

To quantify and to compare the results with the two different solvent specification limits, those virtual runs of the calculation which fulfilled the predefined requirements ($R_s \geq 1.5$) were listed and counted, resulting in a success rate percentage. By defining the solvent specification limits on a broader range (pH ± 0.2, t_C = ±2%), only 66% (486 experiments out of 729) provided greater than or equal to 1.5 resolution (Fig. 8). This would be an insufficient value in quality control.

However, if the tolerance limits were more rigorously (pH ± 0.1, t_C ±1%) defined, the success rate improved to 100%. This indicated the importance of a proper pH-adjustment. In this regard, with a modern pH-meter a more accurate pH setting can be maintained,

thus the limit can be reduced to ±0.1 or even less. This means, that using this set point, the performance of the method can be easily guaranteed, if other instrument-dependent parameters do not exceed their outlined specifications.

On the other side, in some other cases the ternary composition and its decisive influence were reported, showing the importance of both solvent-dependent parameters [32,33]. Similarily to the pH, the error of mixing two organic solvents can be lowered to less than ±1%, if the solvents are either premixed with precise graduated cylinders or by employing two ("organic") pumps for ternary blending [18]. In this way, high succces rate and long-time reliablity of the method can be achieved (Fig. 9).

4. Conclusion

The study shows the dependence of the selectivity on mobile phase composition from gradient time (t_G), ternary eluent composition (t_C) and pH. The resolution cube with 18 runs was well suited for selectivity change testing and for studying the mobile phase effects on the critical resolution.

The novelty of the work was providing a scientific way to visualize these dependencies in a highly variable multifactorial design space. In addition, compiling all possible combinations of solvent- (t_C, pH), and instrument-dependent (t_G, flow rate, start[B%], end[B%]) factors, specified with their tolerance limits, modeling allowed an integrated early-stage multi-parameter robustness characterization.

This, in the light of scarcity of relevant regulation guidelines, can effectively support the analyst to design methods with long-term separation quality, already at the development phase.

In the proposed case study, three-dimensional (t_G-t_C-pH) solvent optimization for amlodipine and its Ph. Eur impurities was carried out, showing that proper separation is neither in pure MeCN nor in pure MeOH feasible. However, by using 60/40 MeOH/MeCN (v/v) as organic and setting pH to 4.2, baseline separation even at low gradient time (4 min) could be easily achieved. In addition, subsequent robustness calculation elucidated all influential chromatographic parameters, showing the necessity of accurate pH-adjustment as of ±0.1 unit.

For the last, this real life-related case study was a perfect example on how to implement an early stage robustness, with a particular interest in solvent effects on selectivity. This we believe, can contribute to a better control strategy of mobile phase-, and from instrument dependent chromatographic parameters. This can safeguard methods, regarding expected performance across all stages of the life-cycle.

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