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Analytical quality by design-compliant retention modeling for exploring column interchangeabilities in separating ezetimibe and its related substances

Elek Ferencz[a,b], Arnold Zöldhegyi[c], Éva-Katalin Kelemen[b], Mona Obreja[b], Melinda Urkon[a], Emese Sipos[a], Gergő Tóth[d], Imre Molnár[c], Zoltán-István Szabó[a,e,*]

[a] George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Târgu Mureș, Romania
[b] Gedeon Richter Romania S.A., Târgu Mureș, Romania
[c] Molnár-Institute for Applied Chromatography, Berlin, Germany
[d] Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary
[e] Sz-imfidum Ltd., RO-525401 Lunga, Romania

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ABSTRACT

There are several potential advantages of using experimental design-based retention modeling for chromatographic method development. Most importantly, through the model-delivered systematic understanding (Design Spaces), users can benefit from increased method consistency, flexibility and robustness that can efficiently be achieved at lesser amount of development time. As a result, modeling tools have always been great supplementary assets and welcomed by both the pharmaceutical industry and the regulatory authorities. Most recently published chapters of ICH however – Q2(R2) and Q14 (both currently drafts) – evidence a further paradigm shift, specifying the elements of model-based development strategies in the so-called "enhanced approach".

The main aim of this study was to investigate the impact of stationary phase chemistries on chromatographic method performance in the application example of ezetimibe and its related substances. A commercial modeling software package (DryLab®) was used to outline three-dimensional experimental design frameworks and acquire model Design Spaces (DSs) of 9 tested columns. This was done by performing 12 input calibration experiments per column, systematically changing critical method parameters (CMPs) as variables such as the gradient time (tG), temperature (T) and the ternary composition (tC) of the mobile phase. The constructed models allowed studying retention behaviors of selected analytes within each separation systems.

In the first part of our work, we performed single optimizations for all nine stationary phases with substantially different surface modifications based on their highest achievable critical resolution values. For these optimum points *in silico* robustness testing was performed, clearly showing a change of CMPs, depending on the column, and specified optimum setpoint.

In the second part of our work, we simultaneously compared the three-dimensional virtual separation models to identify all method parameter combinations that could provide at least baseline separation ($R_{s,\ crit.} > 1.50$). These overlapping areas between the models described a common method operational design region (MODR) where columns were considered completely interchangeable – in terms of their baseline resolving capability – regardless of their exact physicochemical properties. A final optimized, column-independent working point within the common MODR was selected for verification. Indeed, experimental chromatograms showed excellent agreement with the model; all columns in the common condition were able to yield critical resolution values higher than 2.0, only their retentivity (elution window of peaks) was found different in some cases.

Our results underline that a profound understanding of the separation process is of utmost importance andthat in some cases, adequate selectivity is achievable on various stationary phases.

* Corresponding author at: Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Sciences and Technology of Targu Mures, Gh. Marinescu 38, RO-540139 Târgu Mureș, Romania, Sz-imfidum Ltd, RO-525401, Lunga no. 504, Romania.
E-mail address: zoltan.szabo@umfst.ro (Z.-I. Szabó).

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1. Introduction

Ezetimibe is an azetidinone derivative, chemically described as (3*R*,4*S*)−1-(4-fluorophenyl)−3-[(3*S*)−3-(4-*fluorophenyl*)−3-*hydroxypropyl*]−4-(4-hydroxyphenyl)azetidin-2-one and acts as a cholesterol absorption inhibitor by physically interacting with cholesterol transporters at the brush border of the small intestine, decreasing the level of cholesterol in the bloodstream. Most notably, ezetimibe was the first agent of a novel class of selective cholesterol uptake inhibitors, which has been widely used since then in both oral monotherapy and in combination with statins to reduce the risk of harmful cardiovascular events [1–3].

Up to this date, several synthetic pathways have been described in the literature for the synthesis of ezetimibe [4,5] implying the possibility of forming various process-related impurities (starting materials, by-products or intermediates) in the final product. Other sources of organic impurities result from the ongoing degradation of the active pharmaceutical ingredient (API) during manufacturing and/or storage that might affect the efficacy and safety of the final drug product. Detection and quantification of impurities which may be present in the API and/or pharmaceutical product are strictly regulated by the authorities [6,7]. Stability indicating analytical procedures with high selectivity and sensitivity are therefore crucial in effective management of impurity profiling to support pharmaceutical development, but also to ensure routine quality control during manufacturing. Among other analytical methodologies, reverse-phase high performance liquid chromatography (RP-HPLC) has become one of the most popular techniques in impurity profiling [8].

Given the high number of synthetic routes and the multitude of possible impurities, there are several RP-HPLC methods for ezetimibe and its achiral impurities described in the literature, which also show a great diversity in their specifications both in their stationary- and mobile-phase conditions [9]. In this sense, the recent review by Rocha et al. provides an excellent overview of analytical methodologies developed by different groups, reflecting the huge confusion of chromatographic method parameters caused by an unsystematic, trial-and-error-based development approach. The significant differences appear due to the applied stationary phases with various chemistries such as C8, C18, pentafluorophenyl (PFP) or phenyl-hexyl types, and due to the different elution modes, including both isocratic and gradient elution with diverse profiles. There are also differences in the mobile phases applied, such as the organic modifier employed (ACN or MeOH or mixtures of these solvents in different proportions and in some cases, a low amount of tetrahydrofuran is also added), the aqueous part of the mobile phase (ultrapure water, diluted aqueous phosphoric or perchloric acid, phosphate- or acetate-based buffer systems with various pH values) [9]. There are also numerous methods that do not appear in this review, such as the method, developed by Desai et al., which could quantify six achiral related substances of ezetimibe in the presence of simvastatin and its impurities [10]. Another method described by Luo et al. was suitable for the simultaneous quantification of eleven related substances, and the effect of the stationary phase chemistry on the separation process was investigated by comparing two different columns [11].

Considering the high number of widely different analytical methods one can use, it can be difficult to justify the actual suitability of one method over another. In addition, these methods were often developed using the traditional “one-factor at a time” (OFAT) approach, which frequently lacks the model-derived systematic understanding between all system components. In contrast to the OFAT approach, experimental design-based, multivariate methodologies enable the simultaneous variation of all investigated CMPs and tracking their effect upon selected method performance indicators. As a result, deeper method understanding can be obtained with significantly fewer experimental runs while mutual interactions between variables can also be detected [12].

Drylab 4 is a commercial software suite that follows this modeling design concept by effectively integrating Design-of-Experiments (DoE) along with chromatographic fundamentals, such as the solvophobic theory and Linear Solvent Strength Model (LSSM) to model and visualize complex chromatographic interdependencies present in HPLC separation systems [13–17]. These virtual models are highly predictive and flexibly suited to be employed for extensive *in silico* studies, such as gradient optimization, robustness quantification – to identify the CMPs as sources of variability – and to facilitate method transfers. The validity of this modeling approach has extensively been described by many authors [13,14,16,18,19] and also getting a spotlight in the recently published ICH guidelines that commit to create a common platform along with well-defined terminologies for analytical quality by design (AQbD). In this sense, having observed the obvious benefits for manufacturing processes, industry practitioners have already adapted QbD-elements with success to design analytical methods “with the end in mind”[20]. Among others, a general aspect of AQbD is to include tolerance limits of the parameters involved along with other systematic elements such as a Design of Experiments (DoE) creating each DS. This greatly facilitates risk-, and knowledge-based decision making, which in the long-term can not only minimize but effectively prevent out-of-specification (OoS) investigations [21–25]. Regulatory intentions to support this by incorporating pharmaceutical product lifecycle elements and establish post-approval changes on a risk-, and knowledge base are clearly represented in the ICH Q12 guideline [26]. Other, current draft quality guidelines – Q14 and Q2(R2) – describe technical enablersd advantages using the “enhanced approach” in the analytical development [27,28]. By gaining understanding of the relationships between analytical variables and measured responses, the DS can be established, which enables easier validation and flexible movements within the parameter ranges. In other words, when working within this multidimensional MODR changes to the workpoint do not require additional regulatory notification. Thus, following such AQbD approaches, reduces the need of regulatory oversight, builds trust and leads to a more effective communication between applicant and regulator [27,29,30].

The other relevant chapter on Lifecycle Management, USP ⟨1220⟩> also points to this direction, by fostering a well-structured holistic way of analytical procedure development. It also exemplifies modeling approaches – mechanistic and empirical – also emphasizing that either may be appropriate depending on the intended use of the analytical procedure and the desired model accuracy [31].

Using such DS-modeling methodology, an alternative to the European Pharmcopoeia method for the impurity analysis of albendazole was developed and described in our earlier study [32]. Prior to that Kormány et al. in their work had already leveraged the advantages of 3D model DS to find optimum separation conditions of amlodipine and seven impurities, described in the European Pharmacopoeia (Ph. Eur.) on nine different C18-type columns. Initially, by fixing method conditions to a generic approach, only one column could offer baseline resolution. Using 3D-models however, it was then clearly shown that all columns could provide excellent baseline separations, but differences arose in their optimum setpoint conditions and their robust separation capability [33]. This methodology was extended to successfully separating multi-API (amlodipine and bisoprolol) samples along with their specified impurities on 24 out of 25 state-of-the-art Ultra-High-Performance Liquid Chromatography (UHPLC) columns [34]. Similar 3D methodology was published by Rácz et al., also visualizing column MODRs to discover batch–to–batch differences of commercial bridged ethylene-hybrid (BEH) columns [35].

In another work, sub-2-μm column entities differing in their residual silanol activity were subjected to 3D modeling. Great differences were observed and intelligent software algorithms – Design Space Comparison (DSC) module – were introduced allowing 2-, and 3D DSs to be simultaneously aligned and cross-sections of overlapping baseline-separating areas manifesting a common MODR were visualized. This could help identify interchangeable regions across various separation systems and alleviate the burden around replacement HPLC-column selection [36]. More recently, the same group published a new impurity profiling method for Terazosin that was developed with this approach and published as part of the official European Pharmacopeia monograph. Remarkably, with the aid of model DSs, overlapping MODRs were found and equivalent setpoints on competitive pentafluoro phases – two batches of a primary and a replacement column could be specified [14].

In the present work, Dryab® was used with the focus on building 3D separation models of ezetimibe and its related achiral impurities on nine RP columns. Based on only twelve input experiments per column, we investigated the impact of all chromatographically relevant method parameters – such as gradient time, column temperature, ternary composition of the mobile phase and other instrument factors – on the efficiency of the separation process. The acquired multivariate DSs provided in-depth characterization of each separation systems with certain tolerances of relevant method parameters, as fostered by the AQbD methodology. Furthermore, using the DSs as comparison tools, we identified both dissimilar and interchangeable areas in their MODRs. All stationary phases were first evaluated individually to determine their optimum working points for each column and finally, a common setpoint was also established and experimentally verified on all nine columns to prove the interchangeability of these stationary phases.

2. Experimental

2.1. Chemicals and samples

2.1.1. Chemicals

Gradient grade methanol (MeOH), acetonitrile (ACN) and the chromatographic grade phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). The aqueous part of the mobile phase during this study was water with 0.1% phosphoric acid. The ultrapure water was freshly prepared each day by a MilliPore MilliQ Integral 10 (Merck Millipore, USA) equipment. Ezetimibe and its impurities (ezetimibe diol, desfluoro ezetimibe, meta-fluoroaniline analog, ezetimibe ketone, ezetimibe THP (tetrahydropyran) compound, benzylated ezetimibe and ezetimibe TBDMS (tert–butyldimethylsilyl) ketone) were from LGC Standards (Teddington, London, United Kingdom). The chemical structures of the analytes are shown in Fig. 1. Their IUPAC names, chemical formulas, molecular weights, and the calculated physical-chemical properties (log*P* and pK_a) of the analytes are summarized in **Supplementary Table 1.**

2.1.2. Sample solutions

Taking into consideration the poor water solubility of ezetimibe and its related substances (ezetimibe is practically insoluble in water, ~4.4 mg/L [37]) , the solvent used for sample preparation was a mixture of gradient grade ACN and purified water in the proportion of 80:20 (V/V %) and all samples were filtered using Whatman Puradisc PTFE syringe driven filter units (Merck, Darmstadt, Germany) with 0.45 µm pore size, to eliminate the potential insoluble residues.

The test sample used for peak tracking and software-aided retention modeling was a mixture with the following composition: 1000 µg/mL ezetimibe spiked with all impurities, in the following concentrations: ezetimibe diol 8 µg/mL, desfluoro ezetimibe 4 µg/mL, meta-fluoroaniline analog 8 µg/mL, ezetimibe ketone 2 µg/mL, ezetimibe THP compound 4 µg/mL, benzylated ezetimibe 4 µg/mL and ezetimibe TBDMS ketone 6 µg/mL.

2.2. Equipment and software

The analytical balance (MT XPE 205) was from Mettler-Toledo (Mettler-Toledo, Greifensee, Switzerland), and the ultrasonic bath (Elmasonic P180 H) used in the process of preparing the sample solutions was ordered from Elma Schmidbauer (Singen, Germany).

The chemical structure of the molecules and the physical-chemical parameter estimation of the compounds were realized using the MarvinSketch software (ChemAxon, Budapest, Hungary).

Chromatographic experiments were performed on two Agilent Infinity chromatographic systems (Santa Clara, California, USA). The first chromatograph was an Agilent Infinity 1260 system equipped with a quaternary solvent delivery pump (G1311B), autosampler (G1367E), autosampler thermostat (G1330B), column thermostat (G1316A) and photodiode array detector (G1315C). The second chromatograph was a similar one, an Agilent Infinity 1260 system equipped with quaternary solvent delivery pump (G1311A), autosampler (G1367E), autosampler thermostat (G1330B), column thermostat (G1316A) and a high dynamic range (HDR) photodiode array detector (diode array detector 1 with long (60 mm, 4µL) flow cell: G4212B and diode array detector 2 with short (3.7 mm, 0.9 µL) flow cell: G4212B). The dwell volume of the systems was measured as 1000 µL. All chromatographic data were acquired and processed by OpenLAB (EZChromEdition, Ver. A.04.09) software (Agilent, Santa Clara, California, USA). After integration, the chromatograms were exported to AIA/ANDI-format (*.cdf) and directly imported to the modeling software for peak tracking and model processing. The experimental design and interpretation of the acquired data, including subsequent method optimization, *in silico* robustness testing and DSC was carried out with DryLab®4 modeling software package. (Molnár-Institute, Berlin, Germany).

Using Drylab®, 3D modeling methodology was employed based on twelve input chromatographic runs according to the suggested 3D (gradient time-temperature-ternary organic composition) DoE plan. With the help of the 3D model DSs, we focused on comprehensive understanding of each HPLC separation systems to find their optimum performance and to allocate points of equivalency, which in turn, allowed us to define a general method specification for multiple columns.

2.3. Selection of HPLC column chemistries

During the preliminary screening study, nine different reversed-phase columns with similar dimensions were tested for better comparability: Inertsil ODS-3 (150 × 4.6 mm, 3 µm, GL-Sciences, Japan), Ascentis Express RP-amide (150 × 4.6 mm, 2.7 µm, Sigma-Aldrich, USA), Pursuit XRs Diphenyl (150 × 4.6 mm, 5 µm, Agilent, USA), Synergy Hydro RP and Synergy Polar RP (150 × 4.6 mm, 4 µm, 80 Å, Phenomenex, USA), Luna Phenyl Hexyl, Luna PFP (2) and Kinetex Biphenyl (150 × 4.6 mm, 5 µm, 100 Å, Phenomenex, USA) Gemini C6 Phenyl (150 × 4.6 mm, 5 µm, 110 Å, Phenomenex, USA), respectively. The tested columns are commonly applied in today's reverse-phase HPLC-separations. Among them, some of the stationary phase chemistries were selected based on existing methods for the separation of ezetimibe and its related substances, for instance, the Inertsil ODS column [10], the Luna Phenyl-Hexyl column [11]. The PFP (pentafluorophenyl)-type column is described in the ezetimibe active substance monograph of the United States Pharmacopeia [38]. Other columns were chosen with the rationale of covering a wider selectivity-range during the preliminary scouting process.

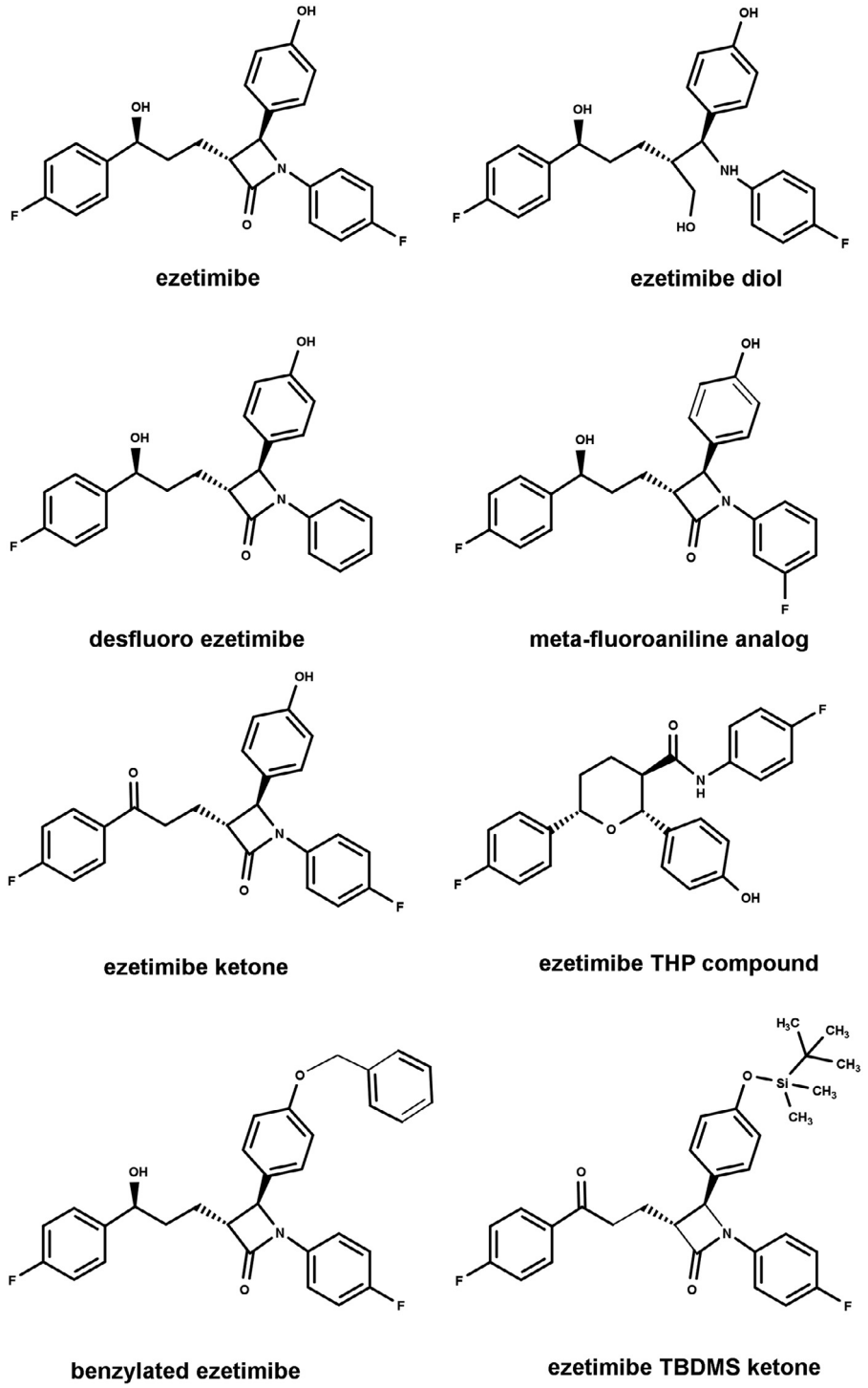

Fig. 1. Chemical structures of ezetimibe and its related substances.

2.4. Preliminary experiments – one dimensional model

The main aim of performing the preliminary experiments was to identify the most influential method parameters for a comprehensive systematic method modeling work.

First, we selected a simple one-dimension of ternary composition model to investigate the chromatographic behavior of the analytes with three corner runs on the arbitrarily chosen reference Inertsil ODS-3 column. A general gradient was applied with 60 min runtime and the gradient ranging from 10 to 95% organic modifier, at fixed 30 °C. The three organic compositions were 100% ACN (tC_1), an equivalent mixture of 50:50 MeOH-ACN (tC_2) and 100% MeOH (tC_3), while the aqueous mobile phase was purified water with 0.1% phosphoric acid. Also, a similar experimental design was performed using purified water without the acidic modifier, to evaluate its effect on separation. The very same amount of test analyte mixture (see in Section 2.1.2) was injected in all abovementioned conditions and the results were evaluated based on the number of separated peaks, the observed peak shapes and retention window of the eluting analytes.

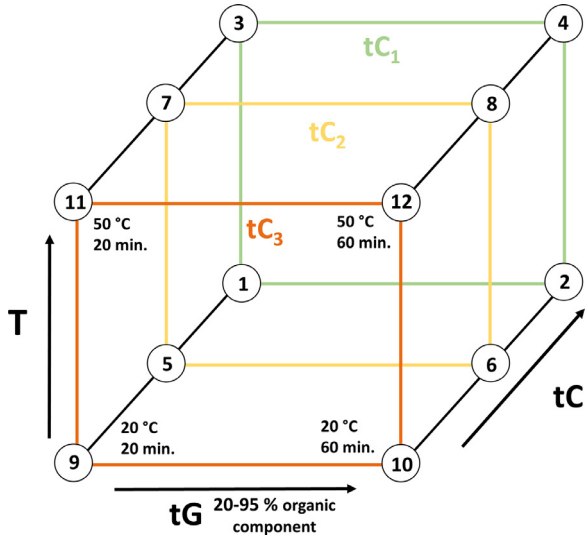

Fig. 2. Schematic view on the DoE of a *tG-T-tC* three-dimensional DryLab® model presented in this study.

2.5. Method optimization – three dimensional models

3D modeling parameters and ranges were selected based on the preliminary runs (one-dimensional tC model), physical-chemical properties of the analytes (**Supplementary Table 1**), previously published similar studies [14,39], and also taking into consideration the basic recommendations of the DryLab® software.

For the sake of objective comparison, the same three-dimensional (3D) gradient time (tG), temperature (T) and ternary composition (tC) experimental framework (*tG-T-tC*) was accomplished using all nine selected columns. Experiments in relation to the temperature and gradient time were performed on two levels, whereas the difference between the short and long gradient time was at a factor of three (tG_1= 20 min and tG_2 = 60 min) and in the case of temperature 30 °C (T_1= 20 °C and T_2= 50 °C). The ternary composition (tC) of the mobile phase was investigated at three levels using different compositions of ACN (Eluent B1) and MeOH (Eluent B2) (level 1: 100% ACN, level 2: 30% MeOH in ACN (30% B2 + 70% B1) and level 3: 60% MeOH in ACN (60% B2 + 40% B1), with a gradient range from 20 to 95% organic component. The aqueous part of the mobile phase (Eluent A) was purified water with 0.1% phosphoric acid. In the case of columns with particle diameter of 4 or 5 µm the flow rate was set to 1.5 ml/min, however using columns with lower particle size the flow rate reduced to 1 ml/min to avoid overpressures in the chromatographic system. In all the experiments mentioned above, the injection volume was 10 µL, and chromatograms were processed at 247 nm. It should also be mentioned that by employing state-of-the-art UHPLC technology, model development time can drastically be reduced.

Thus, construction of the models outlined 12 corner experiments – 2 factors (tG and T) at 2 levels and 1 factor (tC) at 3 levels (2^2 x 3^1= 12). This experimental design is shown in Fig. 2. The different colors of the single layers illustrate the three different ternary composition levels, and the numbers represent each individual method conditions, as they were displayed in the DryLab® software.

3. Results and discussion

3.1. Preliminary experiments – onedimensional tC model

The first step of the study was the selection of the ideal experimental design framework. DryLab® can simultaneously handle up to three experimental variables at a time, either using a *tG-T-tC* or the *tG-T*-pH model. From the practical point of view, the only difference between the two designs is that in the first case the organic modifier composition (tC) of the mobile phase (eluent B) is investigated at three levels, while in the second the pH of the aqueous part of the mobile phase (eluent A) is in focus. In our case, the similar pK_a values of the analytes (**Supplementary Table 1**) and preliminary runs indicated that the pH of the mobile phase would not be a CMP, and that the *tG-T-tC* experimental design is adequate for further studies. However, during the preliminary screening study it was observed that although the addition of 0.1% phosphoric acid did not have a significant effect on the retention times of the analytes, it substantially improved peak symmetry especially for the main, API peak. Therefore, in further studies, 0.1% phosphoric acid solution was used instead of purified water, as eluent A.

During these studies, it was also observed that the selectivity of the method was lower using a high proportion of MeOH. **Supplementary Figure 1** shows the virtual chromatograms of the model with different proportion of MeOH in the mobile phase and it can be observed that the MeOH content has a high impact on retention time, especially for the THP compound (high MeOH content of the mobile phase, resulting in an overlap of the THP compound with the successively eluting meta-fluoroaniline analog, ezetimibe and desfluoro ezetimibe peaks).

Based on these results, ACN was selected as the first organic modifier (B1: 0% MeOH) and 30% MeOH, 60% MeOH (B2, B3 respectively) was added to ACN as second and third levels of the subsequent *tG-T-tC* designs. Also, considering the high lipophilicity of the analytes, the starting organic content of the mobile phase (start%B) was increased to 20 %. Furthermore, benzylated ezetimibe and the ezetimibe TBDMS ketone could be characterized with an even higher lipophilicity which required organic content as high as 95 % at the end of the linear gradient (end%B) to be eluted from the columns.

3.2. Method optimization using three-dimensional tG-T-tC models

Following the software recommended *tG-T-tC* DoE scheme, the same twelve corner runs were performed on all nine HPLC columns of similar dimensions, but with substantially different chemistry. The obtained chromatograms provided the input data for the DryLab ® software to model the three-dimensional resolution maps, which are the visual representations of the critical method attributes (critical resolutions in this case) as a function of the selected method parameters. To prove the validity of these virtual separation models, numerous setpoints – with the highest predicted resolution of critical peak pairs – were selected and verified, by comparing the model-predicted and experimentally acquired chromatograms. Correlations between the predicted and experimental retention times were later investigated by plotting the model vs. experimental retention times and calculating their relative difference (average of retention time errors%). Results were also used for a linear regression analysis to determine goodness-of-fit, i.e., the R^2-values.

Interestingly, the elution order of the analytes was always the same, regardless of the applied stationary phase. In all cases the desfluoro ezetimibe impurity eluted directly before the main peak of ezetimibe and the meta-fluoroaniline analog immediately after it, potentially interfering with any moderate tailing of the main peak. Therefore, two critical peak pairs were identified as the desfluoro ezetimibe-ezetimibe ($R_{s,\ crit.\ 1}$), and the ezetimibe and the meta-fluoroaniline analog ($R_{s,\ crit.\ 2}$) respectively, and these resolution values were used as method performance indicators throughout the study.

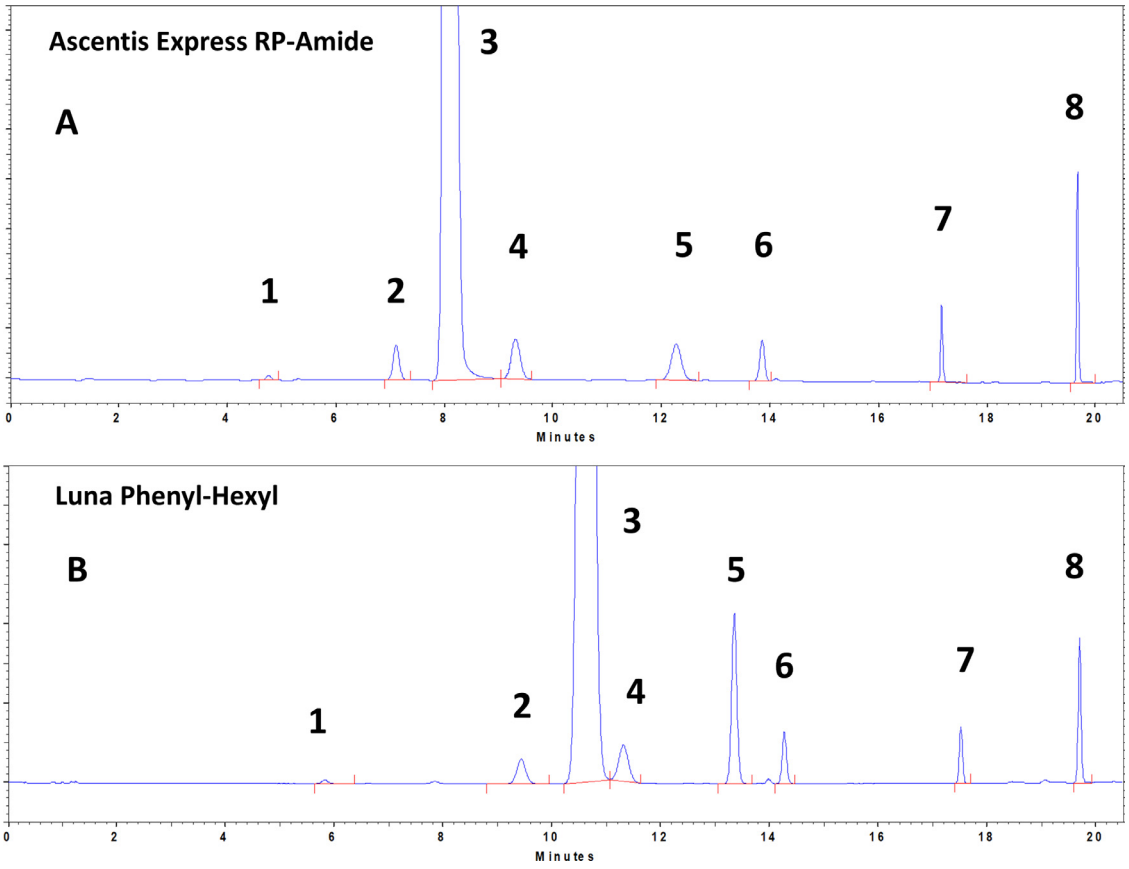

Fig. 3. Verification chromatograms of columns at their optimum setpoint producing the highest critical resolution (**A:** Ascentis Express RP Amide column, $R_{s,\,crit.\,2} = 4.73$) and the lowest critical resolutions (**B:** Luna Phenyl-Hexyl, $R_{s,\,crit.\,2} = 2.06$) among the tested 9 stationary phases (**1**: ezetimibe diol, **2**: desfluoro ezetimibe, **3**: ezetimibe, **4**: meta-fluoroaniline analog, **5**: ezetimibe ketone, **6**: ezetimibe THP compound, **7**: benzylated ezetimibe and **8**: ezetimibe TBDMS ketone).

Experimental results for all tested columns are summarized in Table 1, showing the experimentally tested setpoints, along with correlation between the experimental and predicted retention times (averages of retention time errors,%), and the optimal method conditions yielding the highest critical resolutions. After the model optimization process, it was found that the optimum chromatographic conditions for the tested columns are remarkably similar, with typical operating temperature ranges between 20 and 40 °C, with either neat ACN or low amounts of MeOH (2–15%) as organic solvent. The software-optimized segmented gradients also showed similarity in terms of their profiles with two steps, the first part being an isocratic or a very slight gradient slope within 8–12 min, and the second part with a steep rise within approximately 10 min. Generally, the total analysis time was around 20 min, apart from the Pursuit XRs Diphenyl column which was only able to provide baseline separation of all analytes in ~40 min. Fig. 3 represents two examples from the optimum setpoints. The highest critical resolution was achieved in the case of the Ascentis Express RP Amide column ($R_{s,\,crit.\,2} = 4.73$, Fig. 3A) and the lowest critical resolution value was obtained using the Luna Phenyl-Hexyl column ($R_{s\,crit.\,2} = 2.06$, Fig. 3B).

3.3. In silico *robustness studies*

In case of the optimum setpoints (summarized for all columns in Table 1) the effect of the chromatographic and instrument parameters on the separation process was also evaluated following a multivariate approach using the *in silico* robustness module of DryLab®. Impacts of potential changes around the specified setpoint – eight or nine different method parameters depending on the number of steps in the segmented gradient profile – were assessed at three levels (+/0/-). This defined a full-factorial virtual test with the selected parameters and levels tested described in Table 2. The total number of virtual chromatograms predicted by the software from nine parameters at three levels was 3^9=19.683, for each setpoint of 9 columns. If no MeOH was present in the system, (0% tC indicates a binary system), the number of factors was reduced by one, i.e. 3^8=6.561 virtual chromatograms were calculated.

The impact of the individual method parameters and their cross-effects on $R_{s,\,crit.}$, the predicted chromatograms in all possible conditions and the histogram of the distribution of $R_{s,\,crit.}$ values are summarized in Table 3. In all the possible cases the $R_{s,\,crit.} \geq 1.5$, indicating 100% success rate. We identified the $R_{s,\,crit.}$ values for the best-case and worst-case scenarios and the first three method parameters (as CMPs) with the highest impact on the separation process.

Results from the robustness analysis showed that in most of the cases the starting organic composition of the gradient (start% B), the length of the first gradient step (GP.1 Time) and the flow rate (F) are responsible for most changes in the separation performance, therefore identified as CMPs. Furthermore, the dwell volume was found to be another CMP that relates to the chromatographic system, and its effect is highly influential in the case of method transfer between instruments with different pump systems and solvent mixing mechanisms.

Another aim of the robustness study was to use the model robustness knowledge for setting up individual maximum tolerance limits for each CMP for the establishment of a meaningful control strategy and to help clear definitions of system suitability (SST) specifications. A similar approach was carried out in the case of

Table 1
Summary overview of modeling accuracies of the constructed retention models and specifications of individual optimum run conditions found with the model on the tested stationary phases.

Column	Number of tested setpoints	Average of retention time errors (%) for all tested setpoints	Chromatographic conditions for the setpoint with the highest critical resolutions					Highest critical resolutions (experimental/predicted)	
			Organic composition	Temperature °C	Flow ml/min	Gradient program		Critical resolution1	Critical resolution2
1. Inertsil ODS-3 150×4.6 mm, 3 µm	4	3.33	15% MeOH in ACN	25	1.0	Time (minutes) 0 10 20	Organic (%) 50 55 98	3.62/3.84	3.43/3.72
2. Luna Phenyl Hexyl 150×4.6 mm, 5 µm, 100 Å	11	2.67	2% MeOH in ACN	25	1.5	Time (minutes) 0 10 20	Organic (%) 40 45 98	3.71/3.89	2.06/2.08
3. Gemini C6 Phenyl 150×4.6 mm, 5 µm, 110 Å	6	2.33	2% MeOH in ACN	35	1.5	Time (minutes) 0 10 20	Organic (%) 40 43 98	3.48/3.47	2.33/2.34
4. Luna PFP (2) 150×4.6 mm, 5 µm, 100 Å	8	1.78	10% MeOH in ACN	25	1.5	Time (minutes) 0 12 20	Organic (%) 40 43 98	6.22/6.27	2.19/2.28
5. Kinetex Biphenyl 150×4.6 mm, 5 µm, 100 Å	7	1.65	100% ACN	20	1.5	Time (minutes) 0 10 20	Organic (%) 35 40 98	3.69/3.62	2.16/2.07
6. Ascentis Express Rp-Amide 150×4.6 mm, 2.7 µm, 100 Å	7	4.54	7% MeOH in ACN	10	1.0	Time (minutes) 0 10 18	Organic (%) 48 48 98	4.59/4.82	4.73/4.76
7. Synergi Hydro RP 150×4.6 mm, 4 µm, 80 Å	11	1.19	10% MeOH in ACN	40	1.5	Time (minutes) 0 12 20	Organic (%) 40 45 98	4.20/4.32	3.90/3.79
8. Synergi Polar RP 150×4.6 mm, 4 µm, 80 Å	5	2.05	100% ACN	33	1.5	Time (minutes) 0 12 24	Organic (%) 38 41 98	3.65/3.61	2.22/2.17
9. Pursuit XRs Diphenyl 150×4.6 mm, 5 µm	7	2.52	10% MeOH in ACN	30	1.5	Time (minutes) 0 19 40	Organic (%) 40 40 98	2.22/2.10	2.36/2.24

Retention time error (%) = (Predicted-Experimental)/Experimental×100; **Critical resolution1**: resolution between desfluoro ezetimibe and ezetimibe; **Critical resolution2** resolution between ezetimibe and meta-fluoroaniline analog.

Table 2
Selected parameters and levels for the *in silico* robustness testing.

Parameter	Abbreviation	Levels
Flow rate	F	1.0 ± 0.1 ml/min or 1.5 ± 0.1 ml/min depending on the selected column
Dwell volume	Dwell vol. (Vd)	1.4 ± 0.1 ml
Column temperature	T	± 2 °C
Ternary composition	tC	± 2% MeOH, only if the organic mobile phase was a real ternary (MeOH-ACN) mixture
Starting organic composition of the mobile phase	start% B	± 2% organic mobile phase
Amount of organic of the mobile phase at the first step of the gradient	step 1% B	± 2% organic mobile phase
Final organic composition of the mobile phase at the gradient end	end% B	± 2% organic mobile phase
First step's gradient time	step 1 time (GP.1 Time)	± 1 min
Total gradient time	tG	± 1 min

Table 3
Results obtained during the *in silico* robustness testing. Reduced numbers of virtual experiments indicate combinations of% B-changes that would result in negative gradients in the virtual calculations. By definition of chromatography, these possibilities are automatically not taken into account by the software.

Stationary phase	Number of virtual experiments	Critical resolution for best-case scenario	Critical resolution for worst-case scenario	Success rate	Single parameters or parameter combinations with the highest impact		
					1	2	3
Inertsil ODS-3	3^9=19 683	4.27	3.17	100%	Start %B	F	Vd
Luna Phenyl Hexyl	3^9=19 683	2.42	1.74	100%	Start% B	Vd	GP.1 Time
Gemini C6 Phenyl	$3^7 \times (3^2-1)$ = 17 496	2.63	1.91	100%	Start% B	Vd	GP.1 Time
Luna PFP (2)	$3^7 \times (3^2-1)$ = 17 496	2.50	1.61	100%	Start% B	Vd	F
Kinetex Biphenyl	3^8 = 6 561	2.31	1.58	100%	Start% B	Vd	F
Ascentis Express Rp-Amide	$3^7 \times (3^2-3)$ = 13 122	4.96	3.30	100%	Start% B	Vd	F
Synergi Hydro RP	3^9 = 19 683	4.21	2.75	100%	Start% B	Vd	F
Synergi Polar RP	$3^6 \times (3^2-1)$ = 5 832	2.26	1.70	100%	Start% B	Vd	F
Pursuit XRs Diphenyl	$3^8 \times 2$ = 13 122	2.22	1.88	100%	tC	Start% B	Vd

our previous study regarding chromatographic method development for the determination of albendazole and its related substances [32].

3.4. Design space comparison and column interchangeability

A common industry practice to find replacement columns is analogous to the screening study however, with a different focus. Expected column selectivities are often estimated with the help of various vendor brochures, column guides (USP's L-Classification) and column databases using specific test procedures, like the Snyder-Dolan Hydrophobic Subtraction Model also known as the PQRI-database [40], the Tanaka-test [41], Engelhardt-Jungheim test [42]. Experimental verification of column equivalencies, however, can be still a tedious task, accompanied by time-, and resource-intensive trial-and-error work.

Some of the practical limits of these databases have already been reported, highlighting the shortcomings of these test procedures that are solely based on a few constant sets of isocratic experiments and a handful of test compounds. Conversely, these tests alone cannot reliably predict column equivalencies for impurity profiling or assays, which are the most common pharmaceutical applications [36]. At the same time, existing databases might not always immediately include data for the newest, state-of-the-art stationary phases and the acquired column data can also be dependent on the measurement test lab [43].

To overcome these limitations, in our work the systematic knowledge delivered by the three-dimensional DSs was used to better understand the various contribution of relevant system components – particularly differences among the studied stationary phases – to the separation. In this sense, new, intelligent software algorithms were introduced to find common MODRs where all 9 different columns with respect to their separation performance could easily be interchanged. It was found that despite their inherent chemical differences, each of the studied columns was able to provide with at least a critical resolution of 2.0 or higher when operated at their model-optimum. Similar results – i.e., stationary phase chemistry is not always CMP – were reported by Kormány et al., in the earlier referenced papers [33,34].

On the contrary, in our case, the initial method goal was to identify similar or equivalent setpoints – design regions – and use this to define a common method that works on multiple columns. To facilitate the common workpoint selection, a gradient optimization was undertaken. We found that using ACN as organic solvent with the application of a general segmented gradient (shallow gradient from 38 to 45%, then a steep ramp from 45–98%B) all peaks, especially the highlighted critical peaks around ezetimibe could be better resolved. For the columns packed with smaller particles (Ascentis RP-amide and Inertsil ODS) a virtual transfer was also performed by setting the flow-rates within the *in silico* models to 1.50 mL/min. This yielded significantly improved separation results and because of the lower viscosity of ACN, the operating backpressure still remained well below the specified maximum column limits (<400 bar). (**Supplementary Figure 2**)

Next, the complete 3D separation models (*tG-T-tC*) were recalculated, displayed, and were used to reveal the red, interchangeable MODR-areas, as common spaces. The overlapping separation areas in Fig. 4 clearly show that a common setpoint at tG=20 min, T = 30 °C, tC=0% (ACN) would baseline separate all tested peaks with the very same peak elution order and similar selectivities on eight out of nine columns: Ascentis RP-amide, Synergi Hydro RP, Luna PFP 2, Gemini C6 Phenyl, Synergi Polar RP, Inertsil ODS, Luna Phenyl-Hexyl and Kinetex Biphenyl. For these columns, under the specified setpoint, the critical peak-pair was always ezetim-

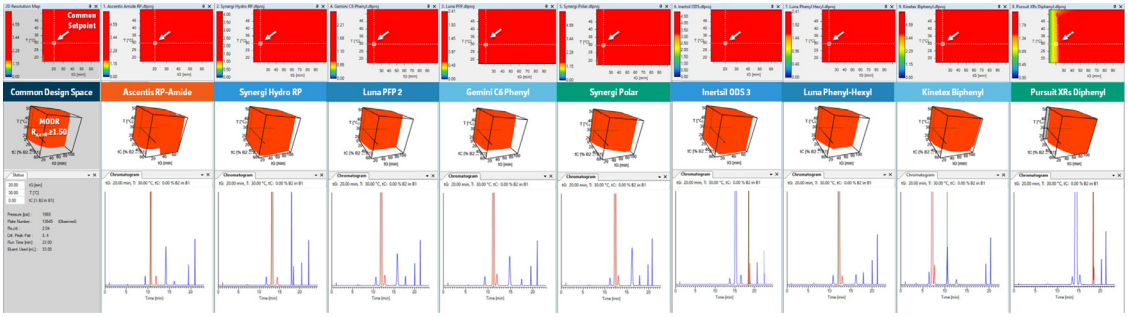

Fig. 4. Comparing the MODRs of all tested columns. Red areas inside the three-dimensional DS correspond to method conditions where baseline separation for all peaks could be achieved. The selected common setpoint is indicated with a white arrow, while the predicted, virtual chromatograms are displayed below.

ibe and the next eluting meta-fluoroaniline analog peak. Although the diphenyl-phase (Pursuit XRs Diphenyl) was also able to resolve the peaks around the API, due to the formation of another critical peak-pair – the overlap between the THP compound and the ezetimibe ketone ($R_{s,\ crit.}$=1.31) – this column under these run conditions would not be interchangeable with the other tested coumns, nor well-suited for a routine analysis.

The best overall results in terms of resolving the API and all other impurity peaks was achieved using the Ascentis RP-Amide phase. However Fig. 4 shows that all other phases (except the Pursuit XRs Diphenyl) could also be conveniently used under the specified setpoint or any other setpoints within the displayed MODR. In this regard, working at 100% ACN would offer potential advantages of not only grantinga larger range of column equivalent points (red areas) but also the lowest backpressure which can be important for small particle packed columns operated at 1.50 mL/min flowrate.

Furthermore, to formulate transparent SST criteria, replacement columns were selected by considering not only the column provided $R_{s,\ crit}$ values, but also similarities in their peak elution window to further facilitate easy peak identification in the routine QC testing, on the basis of peak relative retention times. Taking all of this into account, the RP-Amide phase was chosen as primary column and five other (Synergi Hydro, Luna PFP 2, Gemini C6 Phenyl, Synergi Polar and Luna Phenyl-Hexyl) as potential replacement columns.

As expected, Inertsil ODS column packed with fully-porous 3 µm particles displayed by far the highest overall retentivity of compounds among the studied columns, therefore an amendment in the gradient – higher%Bs for the gradient steps – would be recommended here. The situation is similar for the diphenyl column (Pursuit XRs Diphenyl); high retentivity for all peaks and low selectivity between the THP compound and the ezetimibe ketone peak pair was observed. For the biphenyl phase (Kinetex Biphenyl), higher selectivity changes and somewhat lower overall retention of peaks were observed. It should be noted here that this column has a 5 µm core-shell packing thus, this column was assumed to have the smallest surface area (hydrophobic surface) among the tested stationary phases, which could explain the lower retentivity.

For this reason, it was concluded that the diphenyl and biphenyl phases should be operated under method conditions closer to their optimum, where the two might also be perfectly interchangeable. Therefore, these substantially different stationary phases (Insertsil ODS, Kinetex Biphenyl and Pursuit XRs Diphenyl) should not be considered as replacement columns at uniformly set method conditions.

As a last step, model verification experiments were performed. Based on the obtained results summarized in Table 4, the common setpoint was working well and good correlations were found between the predicted and experimental retention times (low average retention time error). Furthermore, there was a good agreement between the predicted and experimental critical resolution ($R_{s,\ crit.\ 1}$ and $R_{s\ crit.\ 2}$) as well. However, it is notable that on those stationary phases (RP-amide, biphenyl, diphenyl) where there is a higher possibility of secondary interactions (hydrogen bonding, π-

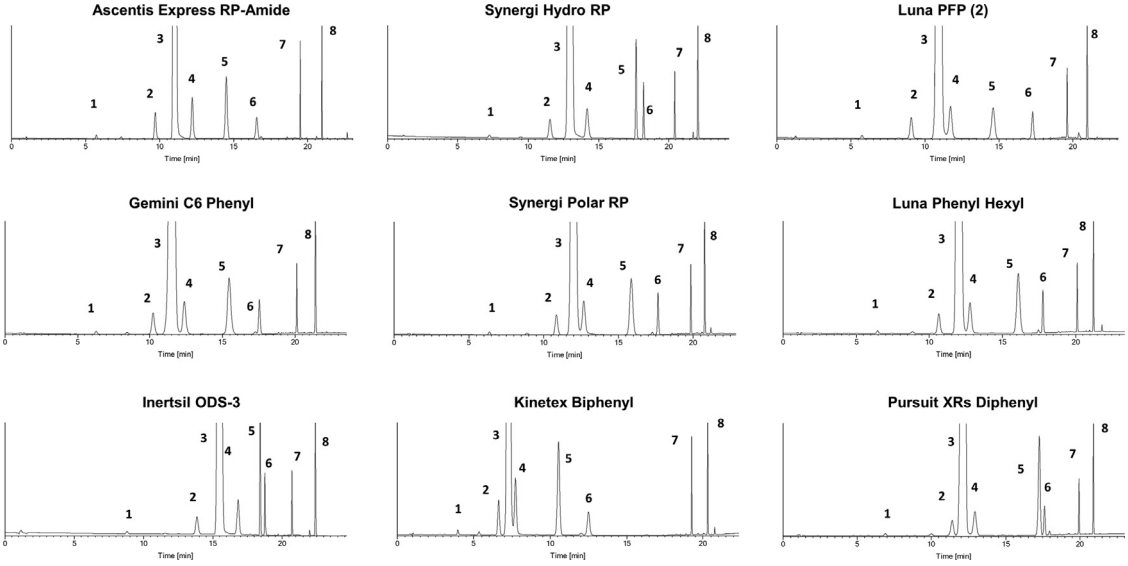

Fig. 5. Experimental chromatograms of the common setpoint selected from the overlapping area of the nine MODRs (**1**: ezetimibe diol, **2**: desfluoro ezetimibe, **3**: ezetimibe, **4**: meta-fluoroaniline analog, **5**: ezetimibe ketone, **6**: ezetimibe THP compound, **7**: benziyated ezetimibe and **8**: ezetimibe TBDMS ketone).

Table 4
The obtained results for the setpoint selected from the common area of all nine DSs (common setpoint).

Column	Average of retention time errors (%)	Chromatographic conditions for the common setpoint		Critical resolutions (experimental/precited)	
				Critical resolution1	Critical resolution2
Inertsil ODS-3 150×4.6 mm. 3 µm	0.87	**Organic mobile phase**: 100% acetonitrile		4.75/4.70	4.22/3.85
Luna Phenyl Hexyl 150×4.6 mm. 5 µm. 100 A	1.17	**Column temperature**: 30 °C		4.02/4.16	2.18/2.11
Gemini C6 Phenyl 150×4.6 mm. 5 µm. 110 A	2.13	**Flowrate**: 1.5 ml/minute		3.61/3.73	2.41/2.32
Luna PFP (2) 150×4.6 mm. 5 µm. 100 A	2.12	**Gradient program**:		5.77/5.74	2.23/2.16
Kinetex Biphenyl 150×4.6 mm. 5 µm. 100 A	2.48	t ime (minutes)	organic (%)	3.02/3.12	2.01/1.91
		0	38		
		15	45		
		20	98		
Ascentis Express Rp-Amide 150×4.6 mm. 2.7 µm. 100 A	2.65			5.99/5.89	5.33/5.18
Synergi Hydro RP 150×4.6 mm. 4 µm. 80 A	1.16			4.42/4.41	3.64/3.54
Synergi Polar RP 150×4.6 mm. 4 µm. 80 A	1.71			3.48/3.55	2.06/2.04
Pursuit XRs Diphenyl 150×4.6 mm. 5 µm	2.80			2.11/2.07	2.26/2.16

Retention time error (%) = (Predicted-Experimental)/Experimental*100; **Critical resolution1**: resolution between desfluoro ezetimibe and ezetimibe; **Critical resolution2** resolution between ezetimibe and meta-fluoroaniline analog.

π interactions, steric effects etc.), retention time predictions were found generally somewhat lower, which could be explained by a possible deviation from LSSM.

At this point, it is also important to highlight the extensive knowledge derived from the model. As mentioned earlier, in the case of the columns with lower particle size (Ascentis RP-Amide and Inertsil ODS) the DoEs were carried out at a flowrate of 1 ml/min, while model verification was performed after a virtual transfer at 1.5 ml/min. Nevertheless, excellent correlation was observed between the virtual (extrapolated) and experimental data, in these cases too.

Fig. 5 represents the experimental chromatograms of the common working point for all nine stationary phases. Based on these chromatograms and on the retention window of the different columns it can be concluded that 6 of 9 tested stationary phases provided very similar outcomes (resolutions and retention times) and these columns could be used interchangeably in routine QC analysis, as equivalents. Moreover, the selected chromatographic conditions of the common setpoint (see Table 4) can be used to conveniently set up run conditions, with less influence from the column chemistry side. Although the other tested columns (Inertsil ODS, Pursuit XRs Diphenyl, Kinetex Biphenyl) were also able to deliver proper separation, they could not be considered as interchangeable at the common setpoint, mostly because their peak elution windows were found to be suboptimal. Certainly, these columns would also be able to offer a much better performance, if they are applied at their respective optimal conditions (see Table 1).

To conclude, these results underline that the column type is not always a CMP (as opposed to a popular belief) and thus, proper understanding of the complete separation process and the underlying effects of CMPs are of significant importance. In this sense, enhanced DoE-modeling tools can effectively support analytical developers to better scrutinize their separation systems and find case-specific optimal separation solutions, instead of an unmethodical testing of a larger set of available stationary phases.

4. Conclusion

In our work, it was shown that the experimental design approach and method modeling tools (like DryLab®) are particularly useful tools in chromatographic method development, providing essential information to profoundly understand the complex separation processes, in accordance with existing and advocated AQbD-principles. Following a systematic development methodology, in this work, the separation of ezetimibe and its studied impurities could easily be achieved on multiple columns. Not only did all stationary phases yield critical resolution of higher than 2.0 at optimum working conditions, but benefits of a multivariate *in silico* robustness testing were also underlined. Robustness results led to clear identification of the CMPs, their allowed tolerance limits and thus, aided the selection of meaningful SST criteria for the routine QC lab testing. With the substantial support of the modeling software's DSC tool, column-independent setpoints (overlapping MODRs) could also be identified, revealing that in our case, most of the stationary phases could generally be considered interchangeable, despite their inherent differences in their chemistries. A common setpoint was found, and later experimentally verified, at which 6 out of 9 tested chromatographic columns could be perfectly interchanged, producing almost identical chromatographic results. This proved that despite the popular belief, column chemistry is not always a CMP in the chromatographic method development, which also implies that model-derived understanding of underlying separation systems is paramount importance.

The main advantage of this approach, its ability to easily identify replacement-, or equivalent columns already in the early phase of method development. Also, it can facilitate the definition of a column-independent method specification, that in turn, can mitigate the possible risks of method related out-of-specification results in QCtest laboratories.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Elek Ferencz: Investigation, Validation, Software, Writing – original draft. **Arnold Zöldhegyi:** Software, Visualization, Writing – original draft. **Éva-Katalin Kelemen:** Resources, Project administration. **Mona Obreja:** Supervision, Resources. **Melinda Urkon:** Formal analysis, Visualization. **Emese Sipos:** Supervision, Writing – review & editing. **Gergő Tóth:** Methodology, Writing – review & editing. **Imre Molnár:** Methodology, Writing – review & editing. **Zoltán-István Szabó:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463494.

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