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Revisiting column selectivity choices in ultra-high performance liquid chromatography–Using multidimensional analytical Design Spaces to identify column equivalency

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ABSTRACT

Current guides and column selection system (CSS) platforms can provide some helpful insights with regard to the selection of alternative phases. Their practical reliability however, can also turn out to be questionable, especially considering the lack of detailed specifics, such as a clear definition of points of equivalence—appropriate running conditions under which the given analytical mixture can be satisfactorily resolved on various stationary phases. In this context, the use of multivariate modeling tools can be highly beneficial. These tools, when applied systematically, are ideal for uniquely characterizing complex LC-separation systems, a fact supported by numerous peer-reviewed papers.

Revisiting our earlier work [1] and the applied systematic workflow [2], we used a Design Space modeling software (DryLab), with the main focus on building and comparing 3-dimensional separation models of amlodipine and its related impurities to identify shared method conditions under which columns are conveniently interchangeable. Our study comprised 5, C18-modified ultra-high performance liquid chromatography (UHPLC) columns in total, in some cases with surprising results. We identified several equivalences between the Design Spaces (DSs) of markedly different columns. Conversely, there were cases where, despite the predicted similarities in column data, the modeled DSs demonstrated clear differences between the selected stationary phases.

1. Introduction

Selectivity is the main measure of how well the actual chromatographic setup is able to distinguish—in time and space—two adjacent peaks from each other. As a result, selectivity is often regarded as the most critical term in Purnell's fundamental resolution formula [3,4].

Knowing and controlling selectivities is key in HPLC separations. In the beginning of the development process, exploring stationary phases with distinct ("orthogonal") selectivities can facilitate the detection and control of unknown impurities in a pharmaceutical sample. However, during method validation, it is essential to find backup or replacement columns with identical or similar selectivities to avoid delay of drug release, caused by interruptions of primary column's market availability or batch-to-batch irreproducibilities. Consequently, one of the method-specific requirements set by regulatory bodies is the robustness testing on columns from different batches, as well as on other competitive columns that are expected to provide similar separation quality [5]. Finding the desired selectivity to separate the actual sample constituents—while fulfilling the primary analytical goals—however, often implies a tedious development process, with the outcome of lengthy methods, inconsistent robustness performance and thus, uneconomic use of resources [6–9].

In partitioning LC-techniques such as reversed-phase (RP), ion-exchange chromatography (IEX), hydrophobic (HIC) and hydrophilic chromatography (HILIC), the separation is established on the basis of different sample affinity to the packing material. There are nearly a thousand different LC-columns available on the market only for reversed phase separations today, some with significant differences in selectivity as well as manufacturing standards. However, it is important to highlight modern columns differ only marginally from their kinetic standpoint but more widely in their engineered selectivity. Undisputedly, the variability of provided selectivities might be beneficial to the analyst, for

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the cases where compounds to be separated are closely related to the test substance. This might compel analytical laboratories to stock a variety of columns, some with almost identical physicochemical characteristics. On the other hand, the lack of clear comprehensive characterization of column provided selectivity impedes the definition of a generic "multi-purpose" column or any vendor-independent general specification of methods [10].

This said, evaluation of column selectivities has always been an important focus of both scientific and industrial interest. In the early days of chromatography, trial-and-error column selection based on empirical knowledge and chemical intuition was preferred. The problem recognized—as far back as 1978—United States Pharmacopeia (USP) addressed this issue with the "L-classification" guide of column packings, that 45 years after, still serves as the main regulatory directive for replacing columns with «ideally» equivalent selectivity. This orders stationary phases into several groups, aligning some of the most relevant physicochemical, but at the same time, overlooking other important properties. For example, their L1-group specifies *"Octadecylsilane chemically bonded to porous or non-porous silica or ceramic microparticles, 1.5–10 μm in diameter"*. This includes both irregular and spherical particles, Type A and B silica variants, not differentiating between fully-porous, pellicular particles, pore sizes, and any substantial C18 surface modifications, such as stable bonded and end-capped materials. As a result, within the group, carbon load, accessible hydrophobic surface area, and residual silanol-activity can be significantly different, thus variations in separation selectivities are likely to occur. What's more, today in the L1-group there are some '"exotic" C18-phases with heterogeneous ligands listed as well, with added perfluorophenyl-, ether- and amide-groups on the C18 base ligands. Inherent issues and failures in method transfers, robustness, and subsequent troubleshooting have highlighted the need to extend the USP's approach to a more sophisticated column classification. These works aimed to simplify the end users' choice of RPLC columns and led to the emergence of new comprehensive column characterization and selection schemes [11–13].

With the advent of modern column technology and the popularity of pre-packed columns, various testing methods for characterizing column selectivity were introduced. These methods, based on objective numerical criteria, facilitated the ranking of columns. These are called 'Column Selection Systems', following specific test procedures, like the Engelhardt-Jungheim-test that focused on surface coverage and hydrophobic properties of columns. Soon after, this was followed by the development of more complex characterization methods like the Tanaka-test and Hydrophobic Subtraction Model (HSM) that are still popular today [10,14]. An excellent, comprehensive summary of these testing routines was published a few years ago [15].

For instance, the Tanaka-test method uses retention factors (k) of test compounds to measure specific column properties. Surface coverage ($k_{\text{pentylbenzene}}$) and the selectivity contribution coming from hydrophobic "methylene" groups ($\alpha_{CH2}=k_{\text{pentylbenzene}}/k_{\text{butylbenzene}}$), shape selectivity ($\alpha_{T/oT}=k_{\text{triphenylene}}/k_{\text{o-terphenyl}}$) and selectivity as the results of residual silanol activity described by their hydrogen-bonding capacity ($\alpha_{C/Ph}=k_{\text{caffeine}}/k_{\text{phenol}}$) and ion-exchange properties ($\alpha_{Ba/Ph}=k_{\text{benzylamine}}/k_{\text{phenol}}$ measured at pH 2.7 and 7.6) was assessed on the basis of 4 isocratic experiments. This selectivity hexagon can be used to display the specific properties of columns, including ligand density, hydrophobicity, steric selectivity, ion-exchange capability, and hydrogen bonding capacity [16].

Adopting a similar approach, Lloyd R. Snyder used 16 model compounds to assess their elution properties under standardized conditions, normalized to the retention of ethyl-benzene. The resulting six isocratic experiments at a fixed eluent and temperature after subtracting the largest hydrophobicity term allowed them to describe other important column interaction terms, such as steric effects, hydrogen-bond acidity, hydrogen-bond basicity, and ion-exchange contributions. Placing these attributes in a five-dimensional space, specified with a Euclidean distance between them, similarity factors (F_s) between columns could be calculated. The resulting HSM-database yielded quantitative results for comparing column selectivities, which was included in the modeling software used, and also later adopted by USP (PQRI-database) [11,17]. An interesting application of leveraging column-provided selectivities to resolve complex sample matrices is also seen in Phase Optimized Liquid Chromatography (POPLC), that aimed to couple columns in series in pursue of achieving high-selectivity separations [18].

While column selection systems (CSS) often provide reasonable preliminary information on comparable columns, they rely on data from a limited number of model compounds and under a preset of isocratic conditions and constant temperatures. However, when deviating from these ideal conditions, such as in real-life analyses with different samples and gradient conditions, the practical utility and reliability of these systems is likely to become less certain [19–21].

In contrast, a general aspect of Quality by Design (QbD) is to include tolerance limits of relevant parameters changes established within a multidimensional Design Space (DS). This is being achieved by setting up meaningful Design of Experiments (DoE) to acquire multidimensional DSs. Over the past decades, DSs have become key to the pharmaceutical product lifecycle by establishing flexible ways for managing post-approval changes (PACMPs)—on a risk-, and knowledge basis—as clearly described in ICH Q12 guideline [22]. Although QbD was initially intended for manufacturing purposes, innovators have successfully adopted vital QbD elements for analytical development support as well [13,23]. In response, ICH recently published two new, guidelines on Analytical Quality by Design (AQbD) that have been recently finalized: Q14 describe the technical enablers and list several key advantages of using "enhanced approaches" in the analytical development, while Q2 (R2) shows how a simplified validation can be performed following such a structured approach. In this context, the main advantage of acquiring a multidimensional DS is that it provides a profound understanding of the relationships between relevant analytical variables and measured responses. Within this space, Method Operable Design Region (MODR) can be outlined, which is a smaller subset of parameter ranges where the analytical procedure performance criteria (Analytical Target Profile, ATP) are continuously met and the quality of the measured results assured. In other words, working within the multidimensional MODR, changes to the workpoint do not require additional regulatory notification. Thus, by following AQbD approaches, out-of-specification (OoS) issues can be avoided, and post-approval changes can be more easily managed with reduced regulatory oversight. AQbD builds trust and leads to a more effective communication between the applicant and regulatory [24,25].

Nevertheless, correctly identifying and including chromatographically relevant parameters to construct meaningful DSs remains crucial. As stated in the USP ⟨621⟩ document: *"Multiple adjustments can have a cumulative effect on the performance of the system and are to be properly evaluated by the users"* [26]. Integrating basic chromatographic knowledge, derived from fundamental theories rather than relying on 'black-box' analysis, can further aid in proper risk identification, assessment, and subsequent risk management. USP ⟨1220⟩ gives a more detailed insight on how risk should be evaluated and later managed across the three lifecycle stages of the analytical procedure. "Assessment is driven by prior knowledge and scientific expertise, but some factors with unknown influence may need to be considered higher risk until further knowledge is available". In a more specific modeling context, it also states *"when available, mechanistic models can be used to understand the effect of procedure parameters on performance. Use of mechanistic models can reduce experimental work and provide a reliable estimate of the behavior of the analytes of interest"* [27].

In summary, the new guidelines can greatly alleviate the burden around the traditional "one-off" routine of method development and remove the barrier to accommodate changes over time—which has also been one of the main obstacles for new technology investment. Aligned with the principle of continuous improvement, post-approval changes are permissible without additional regulatory notification if the

associated risk is deemed low. This pushes forward the constant review of methods in order to rationalize analytical processes, while continuously meeting the ATP.

The development of methods with scientific support of computerized tools to build *in-silico* knowledge of complex chromatographic systems was introduced by Laub and Purnell. As early as in the late 1970ies, they developed simple algorithms to plot "window diagrams" that visualized relevant parameter effects both in GC and LC [28,29]. A few years later, with the new era of personal computers, more sophisticated computational solutions started to emerge. Lloyd Snyder and his co-workers launched their first, commercially available, mechanistic HPLC modeling platform, DryLab in 1986. The team made their first systematic 2-D gradient time-temperature (t_G-T) modeling maps in 1997 to display separation changes on monomeric and polymeric C18 columns. Interestingly, the acquired monochromic resolution maps showed not only great differences but also some overlapping regions, where the two columns could provide with similar (or virtually equivalent) separation performance [30]. In follow-up research, they revealed some remarkable similarities between different brands of C18 and also polar embedded C8-phases. However, in accordance with USP-grouping, significant differences were only found when the two chemistries (C8 and C18) were directly compared to each other. In another study, they used t_G-T maps once again to suggest replacement column options and described generic method conditions that could work across a number of different stationary phases. Furthermore, they employed 2-D resolution models with success to safeguard failing methods due to column batch-to-batch inconsistencies [31,32].

Building on this methodological development, Kormány employed innovative 3-D resolution models, known as 'cubes,' to optimize the separation conditions of amlodipine and seven impurities described in the European Pharmacopoeia, using eight different L1-class columns. First, by choosing a single linear gradient method, only one column could give baseline resolution. Using 3-D models as a basic tool to identify optimum separation conditions, it was clearly shown that all columns could provide excellent baseline separations, however, with differences in robustness of the separation capability. This applied methodology was later extended to successfully separate another combination drug (amlodipine and bisoprolol) samples along with European Pharmacopeia specified impurities on 24 out of 25 state-of-the-art Ultra-High Performance Liquid Chromatography (UHPLC) columns. A similar 3-D modeling study was published by Rácz et al. to visualize column MODRs and discover batch-to-batch differences of commercial, bridged ethylene-hybrid (BEH) columns. In another work, competitive sub-2 μm column entities differing in their residual silanol activity were subjected to 3-D modeling. Great differences were found and intelligent software algorithms—Design Space Comparison (DSC) module—were introduced allowing 2-, and 3-D DSs to be simultaneously aligned and shared regions of baseline-separating areas manifesting an intercolumn MODR to be visualized. This visualized graphics could greatly help to identify interchangeable regions across various separation systems and alleviate the burden around replacement HPLC-column selection. 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. The knowledge provided by the comprehensive characterization and comparison of DSs allowed Ferencz et al. to identify shared, intercolumn MODR of 8 stationary phases that possessed substantially different chemical properties. Based on that, they were able to establish similar separation results of ezetimibe and several of its impurities, using a single, general method specification. Similar workflow was utilized by Duivelshof to separate COVID-19-related therapeutic monoclonal antibodies as well. By comparing two elution modes of ion-exchange chromatography, superior performance was found for a pH-elution mode over a traditional NaCl gradient method, at any given combination of gradient time and temperature [8,9,12,33–38]. These features made chromatography-based modeling approach attractive for industry and academia users in both small-, and large molecule applications [7,8,39].

These examples also highlight the need for powerful modeling approaches that can simultaneously align and model the effects of all system components, including the stationary phase, mobile phase, column, and the actual sample.

In this retrospective study, we revisited the question of column provided selectivities from the Design Space perspective. Motivated by recently developed «Design Space Comparison» (DSC) option of a commercial software package (DryLab), we analyzed several, previously acquired Design Spaces [1] of various USP L1-group C18-stationary phases to find answers on how a robust way for the identification of replacement column options and conditions can easily be achieved. For an objective comparison, we also conducted other traditional CSS approaches, including the Tanaka test and Snyder-Dolan HSM evaluation. As a matter of fact, it should also be noted that previous to this study, a complete method redesign on cetirizine impurities with a simplified generic workflow was also showcased and successfully tested. This work described a fast, 4-run based 2-D (t_G-T) DSC of columns, making it ideal for early-phase testing column equivalences or using it for screening purposes [2].

2. Materials and methods

2.1. Chemicals

Chromatographic analyses were conducted using premixed eluents: The aqueous mobile phase (eluent A) consisted of 5 mM phosphate buffer (pH 2.0, 2.5, and 3.0) and acetonitrile (ACN) in a volumetric ratio of 30:70. Eluent B was 10:90 aqueous buffer:ACN (V/V).

For measurements water was prepared freshly using ELGA Purelab UHQ water (ELGA, Lane End, UK). The buffer was filtered before use through a regenerated cellulose filter membrane with a 0.2 μm pore size (Sartorius, Goettingen, Germany). The sample consisted of 10 μg/mL amlodipine and its European Pharmacopoeia-specified impurities (A, B, D, E, F, G and H). Reference standards were purchased from European Directorate for the Quality of Medicines & HealthCare (EDQM). Sample solvent was ACN:H_2O 30:70 (V/V).

2.2. Equipment, software

Design Space modeling experiments were conducted using a Waters Acquity Classic UPLC system equipped with a binary solvent delivery pump, an autosampler, and a photodiode array detector. The chromatographic system had a 5 μL injection loop and 500 nL flow cell, system dwell volume measured as 125 μL. Instrument control and data acquisition were managed using the Empower CDS (Waters, Milford, USA), chromatographic processing done at 230 nm.

DS-models were built using DryLab 4 (Ver. 4.5) modeling software package (Molnár-Institute, Berlin, Germany), including direct comparison of the multidimensional DSs with the help of the Design Space Comparison Module. The pH adjustments were performed using an MP 225 pH-meter, purchased from Mettler-Toledo (Greifensee, Switzerland).

2.3. Stationary phase selection

To measure and the selectivity differences of stationary phases, we selected five reversed-phase columns, all with an identical short, narrow-bore format (50 × 2.1 mm): Acquity™ HSS C18, Acquity™ BEH C18, Acquity™ HSS C18 SB, Zorbax SB-C18, Hypersil Gold™ C18. Although possessing some differences in their physicochemical properties, these stationary phases are listed in the L1-group of USP and therefore, virtually interchangeable.

3. Theory and calculations

3.1. Column selectivity testing procedures

3.1.1. Snyder–Dolan test

Results from the Snyder–Dolan test were obtained using the ColumnMatch module of DryLab. This module offers access to a database containing information on more than 500 stationary phases built on the account of the HSM. The calculated similarity factors (F_s) gave quantitative estimates of expected column selectivity, both column similarities and dissimilarities. Stationary phases are considered equivalent if their F_s-values are less than 3. If their F_s lies between 3 and 5 their equivalence is somewhat questionable and if >5 they are considered to be dissimilar in terms of their selectivities [11,17].

3.1.2. Tanaka-test

Similar to the HSM, the Tanaka-test outlines predefined test mixtures and experimental conditions to assess stationary phase properties. The first two of the isocratic experiments were performed at 80:20 MeOH: H_2O (V/V) and 30 °C to determine surface coverage ($k_{pentylbenzene}$), selectivity contribution coming from hydrophobic "methylene" groups ($\alpha_{CH2}=k_{pentylbenzene}/k_{butylbenzene}$) and shape selectivity ($\alpha_{T/oT}=k_{triphenylene}/k_{o\text{-}terphenyl}$). The selectivity resulting from the hydrogen-bonding capacity of residual silanols was determined at 70:30 MeOH: H_2O (V/V) and 30 °C ($\alpha_{C/pH}=k_{caffeine}/k_{phenol}$). Ion-exchange properties of the residual silanol groups were estimated with two additional isocratic experiments performed at two pH values 2.7 and 7.6 under 70:30 MeOH:20 mM phosphate buffer (V/V) isocratic conditions and 30 °C ($\alpha_{Ba/pH}=k_{benzylamine}/k_{phenol}$). After the experiments performed, the 6 obtained terms were plotted and displayed as a selectivity hexagon (radar-map) [16].

3.1.3. Design space modeling and comparison

The workflow of this study involved four consecutive steps, as illustrated in Fig. 1. First, the tested stationary phases were selected. Next, with modeling support, unique DSs were built on each. In order to map all the underlying interactions—similarities and differences of column-provided separation results—well-structured DoE according to the modeling software recommendations were carried out. Finally, the resulting DSs were used as the foundation for an objective comparison.

A crucial component of any DS model is the selection of relevant modeling parameters and ranges. As an early step in the systematic workflow, a single preliminary experiment per each stationary phase was performed, at the run condition $t_G = 6$ min (30→90 %B), $T = 30$ °C, pH = 2.5. This step ensured suitable elution windows and a chromatography that meets basic expectations of gradient elution ($= 1<k^*<20$) for the runs that would later serve as the modeling input.

The second step was to select a meaningful 3-dimensional DoE for outlining the modeling framework. Considering established chromatographic best practices, highly influential method parameters such as gradient steepness (t_G) and temperature (T) were selected, as varying these parameters is likely to have a significant effect on selectivity changes in reversed-phase chromatography [6,40]. Furthermore, it was known that the active pharmaceutical ingredient (amlodipine) and several of its impurities (Imp D, E, F, H) have ionizable functional groups, as well as the silica backbone, might have some residual silanol activity (pK_a for "Type B" high purity is estimated to be ~6–7) [33,41,42]. Instead of untapping pH as a very influential selectivity tuning parameter, we aimed to maintain maximum robustness by establishing pH-control conditions. This was achieved with 5 mM phosphate buffer, commonly used in analytical laboratories. Although the phosphate buffer offers a relatively broad acidic spectrum of pH with good buffering capability, keeping the long-term chemical stability of the column also in mind, we ruled out the lowest pH-end and only considered the practical range of pH=2.0–3.0.

In the next step, we selected the multidimensional t_G-T-pH mode of the software with an experimental set consisting of 2 linear gradients with a factor 3 difference in gradient times, two temperatures with a difference of ~20–30 °C and three pH-inputs with 0.5–0.6 steps each. In our case, t_G were 3 and 9 min (30→90 %B), T were 15 and 45 °C and pH were 2.0, 2.5 and 3.0. The mobile phase B was acetonitrile (ACN). The flow rate was set to 0.50 mL/min, the injected volume to 1.0 μL.

The 12 input experiments were conducted on all five tested columns, following the optimal implementation order as previously reported by Rácz and colleagues [34]. In total, the whole set of experiments required approx. 5 × 120 min (~10 h) experimental work, including necessary run-to-run equilibration methods prior to each analysis. In this regard, another convenient automated experimentation option using direct connectivity to chromatographic data system (CDS) was introduced by Duivelshof and his colleagues [35]. This interfaced solution allowed one-click implementation of the required injections, for instance acquisition of runs on multiple columns and samples, with the addition of pre-calculated equilibration times and a scientifically established order of runs. The runs could then be processed in the chromatography data system (CDS) and directly imported into the modeling software for subsequent peak matching. In consequence, a specific model could easily be created for each phase system to be later compared using the modeling software's Design Space Comparison Module.

4. Results and discussion

4.1. Traditional comparison of the selected HPLC—Columns

The five C18 stationary phases selected for further modeling study represented a relative broad spectrum—in terms of their exact physicochemical characteristics—within USP's L1-group. The HSS C18 is a well-covered octadecyl-silica phase with additional end-capping with trimethylsilyl(TMS)-chloride. This phase, expectedly, has the highest carbon content and lowest residual silanol activity. In contrast, the HSS C18 SB "selectivity for bases" is another C18-phase where the residual silanol groups were left intact. As a result, among the tested columns this was expected to have the largest residual silanol activity. BEH C18 is another type of a well-covered stationary phase but here the backbone consists of silica and crosslinked ethylene hybrid. This reinforced structure makes this phase suitable for high-pH (up to pH = 10–11) operations. The Zorbax "Stable Bonded" C18 features diisobutyl-groups

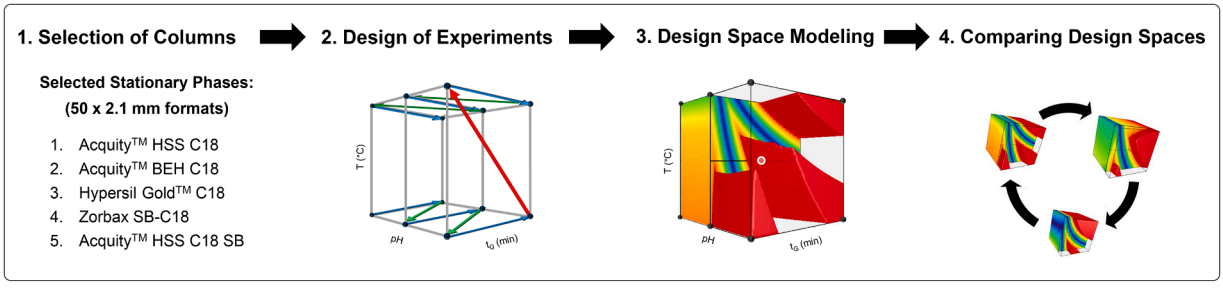

Fig. 1. Applied systematic development scheme for building up and comparing individual Design Space Models of the selected stationary phases.

(*reader should note 'SB' abbreviation has two different meaning*) to sterically shield the acid-labile siloxane-bonds. According to the manufacturer, this column can routinely be operated as low as pH=1 conditions. Finally, the selected Hypersil column represents a proprietary C18 phase with ultrapure silica backbone. Clearly, based solely on the column-specific data, these C18 phases are not expected to be interchangeable 'out-of-the-box'.

The available physicochemical properties of each stationary phase are detailed in Table 1.

To create an objective reference, two CSS procedures were employed. HSM-results were directly retrieved from ColumnMatch database of DryLab, while the Tanaka test injections were performed on the actual chromatographic system. Indeed, the test results in Table 2 support the assumed dissimilarity between these phases: F_s-values of the HSM-model ranged from 11.5 to 85.5 (BEH C18 and HSS C18 SB, respectively) referenced to the HSS C18 column (Fs = 0). This means all columns could be considered much different, the high F_s-values even indicating orthogonal selectivities.

More interesting was to visualize all the selectivity-terms of the Tanaka-test in the conventional radar-plot that could give a deeper insight into how the selected stationary phases differ from each other.

As shown in Fig. 2, the selectivity maps of the HSS C18 and HSS C18 SB columns largely deviated, as well as for shielded Zorbax SB-C18. Interestingly, the selectivity hexagon of the BEH C18 and Gold C18 (both endcapped) were found not so different. Here, the third endcapped stationary phase, the HSS C18 column, also gave a similar plot, albeit with less methylene-group selectivity.

To summarize, traditional methods of comparing columns—such as by means of manufacturers' data and various column testing protocols (HSM, Tanaka test, etc.)—assume fixed selectivities of columns. Unfortunately, this limits their applicability for practical applications, as the presented impurity analysis of amlodipine shows. Here, as generally in LC, strong and complex interactions between stationary phase, sample, and mobile phase impact the analytical procedure's outcome and must be properly addressed. In addition, mobile phase parameters can be varied in certain ranges to further fine-tune or discover new chromatographic selectivities. At this point, it is easy to understand why traditional comparison methods are limited in their ability to predict column selectivity changes. Here, tools capable of measuring and assessing the LC-inherent, systemic interactions, are required.

4.2. Systematic characterization of columns

Unlike CSSs procedures, chromatography-based DS modeling equally aligns the system components to accurately describe the underlying interactions within each of the selected separation system unit. The acquired multidimensional DSs are immanently suited to simply describe complex chromatographic interdependencies, while minimizing the experimental work.

The modeled DSs illustrate how the separation responds to simultaneous changes in gradient time (t_G), temperature (T), and pH. The color coding within the DSs explains the dynamics of the separation: blue curves indicate coelutions of critical peak pairs, green-yellow areas peak overlaps, red areas baseline separation. Although DSs in Fig. 3. might appear to be similar at first glance, depending on combination of selected phase and method conditions there are different critical peak pairs forming the coleutions—usually those are between the three impurity peaks (Imp B-G-H) in various elution orders. The lower MODR of columns elucidates only the baseline separating areas with $R_{s,crit.} \geq 1.50$, showing columns could deliver sufficient separation with different workable combination of method parameters. Certainly, DSs can also be employed to determine optimum working points with maximized resolution outcomes [1] and for adjusting experimental conditions in order to minimize differences between the separation results [32]. Furthermore, the displayed DSs confirmed that the change of pH in range of 2–3 has no significant effect on the selectivity, i.e., pH-control conditions were successfully established. However, as reported elsewhere, the impact of pH on selectivity of amlodipine peaks becomes increasingly pronounced at pH greater than 3, particularly affecting the separation of several acidic impurities [42].

Essentially, Design Space models function as custom-built feasibility maps, highlighting all scenarios where a single separation system—a combination of method parameters on a chosen stationary phase—is capable of resolving a specific analytical challenge. By comparing and overlaying individual MODRs, a shared, intercolumn MODR can be identified, within which all the columns can achieve at least baseline separation. This space can then be utilized, for example, to establish general working conditions that are applicable across various stationary phases [38]. The presence of such a shared MODR area also implies that some stationary phases may not only achieve baseline separation results but might also offer comparable or even equivalent selectivities.

In this context, Fig. 4 provides a detailed illustration of the significant impact of method parameters on chromatographic selectivity. By setting a constant gradient time (t_G = 12 min) and pH (pH = 2.5) and varying temperatures between 20 and 40 °C, the chromatograms exhibit considerable changes. At the lower temperature setting (t_G = 12 min, T = 20 °C, pH = 2.5), several columns achieve baseline separation ($R_{s,crit.} \geq 1.50$), except for the BEH C18 and Hypersil Gold C18, which struggle to separate two key impurities (Imp B and G). Notably, under these conditions, the overall selectivity among HSS C18, Zorbax SB-C18, and HSS C18 SB is strikingly similar. However, increasing the temperature to 40 °C causes the peaks to shift at varying rates, substantially altering the chromatograms. The peaks most affected by this temperature shift are those forming various coelutions (Imp B-G-H). In the context of the visualized DSs, this indicates a reversal in the order of peak elution when moving into temperature conditions on either side of the blue curve (as shown in Fig. 3). If the chosen temperature point remains within the cooler-colored region of the DS, the reversal in elution order may not be complete, leading to peak overlap or coelution. Consequently, the temperature change adversely affects both HSS C18 and Zorbax SB-C18, resulting in peak overlaps for Imp B and G. In contrast, BEH C18 and Gold C18 adapt well to the temperature increase, successfully separating the active pharmaceutical ingredient (API) from all its impurities with similar selectivities. The only exception is the HSS C18 SB phase, which consistently maintains baseline separation across both temperature levels. In conclusion, regardless of the HSM's F_s-values, the columns exhibit either baseline separation or poor separation. This unpredictability in actual peak selectivities underscores the necessity of using acquired DS models for accurate identification.

Table 1
General physicochemical properties of the selected stationary phases according to manufacturers' specification.

	1. Acquity HSS C18	2. Acquity BEH C18	3. Zorbax SB-C18	4. Hypersil Gold C18	5. Acquity HSS C18-SB
Column format (cm)	5 × 0.21 (1.8 µm)	5 × 0.21 (1.7 µm)	5 × 0.21 (1.8 µm)	5 × 0.21 (1.9 µm)	5 × 0.21 (1.8 µm)
Surface modification	Octadecyl-silica (endcapped)	Octadecyl-silica (endcapped)	Octadecyl-silica	Octadecyl-silica (endcapped)	Octadecyl-silica
Base material	Silica	Ethylene-hybrid	Silica	Silica	Silica
Packing morphology	Fully-porous				
USP-Category	L1				
Pore size (Å)	100	130	80	175	100
Surface coverage (µmol/m²)	3.2	3.0	N/A	N/A	1.8

Table 2
Two CSS results: Tanaka-test and similarity factors (F_s) according to the Snyder-Dolan Hydrophobic Subtraction Model (HSM) were retrieved from DryLab ColumnMatch module. *Because of the lack of BEH C18 column in the HSM-database, results were taken from its HPLC counterpart (XBridge C18). The Tanaka column selectivity test assesses stationary phase surface coverage ($k_{pentylbenzene}$), selectivity contribution coming from hydrophobic "methylene" groups ($\alpha_{CH2}=k_{pentylbenzene}/k_{butylbenzene}$), shape selectivity ($\alpha_{T/oT}=k_{triphenylene}/k_{o\text{-}terphenyl}$) and selectivity as the results of residual silanol activity described by their hydrogen-bonding capacity ($\alpha_{C/Ph}=k_{caffeine}/k_{phenol}$) and ion-exchange properties ($\alpha_{Ba/Ph}=k_{benzylamine}/k_{phenol}$ measured at pH 2.7 and 7.6).

Tanaka-test results (selectivity terms)		1. Acquity HSS C18	2. Acquity BEH C18	3. Zorbax SB-C18	4. Hypersil Gold C18	5. Acquity HSS C18-SB
Retention factor for pentylbenzene	$\mathbf{k_{Pb}}$	8.69	5.87	4.86	6.74	4.20
Hydrophobicity or hydrophobic selectivity	$\mathbf{\alpha_{CH2} = k_{Pb}/k_{Bb}}$	1.52	1.48	1.42	1.52	1.38
Shape selectivity	$\mathbf{\alpha_{T/oT} = k_T/k_{oT}}$	1.57	1.47	1.32	1.29	2.09
Hydrogen bonding capacity	$\mathbf{\alpha_{C/Ph} = k_C/k_{Ph}}$	0.40	0.41	0.47	0.78	2.43
Acidic ion-exchange capacity	$\mathbf{\alpha_{Ba/Ph} = k_{Ba}/k_{Ph}}$	0.12	0.14	0.18	0.11	0.20
Total ion-exchange capacity	$\mathbf{\alpha_{Ba/Ph} = k_{Ba}/k_{Ph}}$	0.36	0.27	0.57	1.92	2.40
HSM-Similarity factor (F_s)*		Reference	11.5	29.8	60.9	85.5

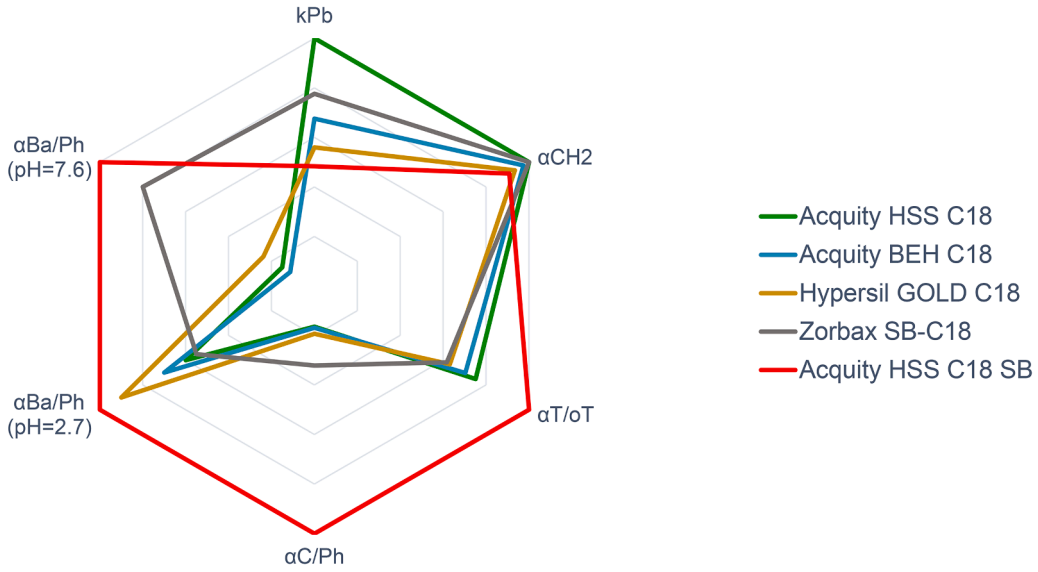

Fig. 2. The displayed Tanaka radar plots of the selected stationary phases. As seen, these plots align well with the general column specifications (Table 1): HSS C18 (green) represents a well-covered stationary phase with less silanol-attributed selectivity, while the less covered HSS C18 SB (red) features a more expressed selectivity contribution from the silanol-groups. Among the studied phases, the Zorbax SB-C18 (grey) can be considered as a balance between hydrophobic- and silanol-based selectivity, while the endcapped BEH C18 (blue) Hypersil Gold C18 (gold) shares a similar general profile (both are designed for high-pH operations).

4.3. Identification of replacement columns and run conditions

To validate this concept, DSs of the two most distinct stationary phases were directly compared, as shown in Fig. 5. The modeling results demonstrate that appropriate run conditions can be identified for each stationary phase. However, within the range of conditions modeled, the columns yield differing separation outcomes. For example, at longer run times, the HSS C18 column exhibits a shift in elution order from Imp B-G-H to G-B-H at higher temperatures. In this scenario, intermediate temperatures result in unsatisfactory resolution ($R_{s,crit.}<1.50$). Conversely, under similar run conditions, the HSS C18 SB maintains the B-G-H elution order while consistently achieving baseline separation of the API and seven impurities. Despite hypothetical differences, the findings indicate that the two columns can be effectively interchangeable—without altering the elution order—when operated at lower temperatures ($T = 20$ °C) and longer gradient times ($t_G = 12$ min), across a broad pH range (2.0–3.0). Identifying this narrower subset within the DSs is highly beneficial, as the significant similarity in performance greatly reduces the effort needed to establish phase equivalence.

To the best of our knowledge, this is the first reported instance of two L1-type columns, characterized by markedly different carbon loads and residual silanol activities, being identified as equivalent. This suggests that 3-D DSs are effective tools for identifying equivalences between any given DSs: if such equivalences exist, they will be visualized as an 'intercolumn MODR'. The case study presented here illustrates an extreme scenario among the tested stationary phases. Future studies could explore similarities between more closely related phases (such as HSS C18 vs. BEH C18) or between competitive phases and different batches of the same columns [12,32,34].

4.4. Finding replacement options of C18- and C8-phases

To broaden the scope of this study and further challenge the proposed approach, we revisited the intriguing question of whether it's possible to find replacements without substantial adjustments in method conditions between L1 and other groups. For this purpose, we selected two brands of C18 and C8 (L7-group) stationary phases. We calculated their F_s-values and retrieved chromatograms from the DS models at predetermined working points ($t_G = 12$ min, $T = 40$ °C, pH = 2.5).

The F_s-values from the HSM shown in Fig. 6 indicated a significant selectivity difference between the shielded Zorbax SB-C18 and the traditional Hypersil Gold C18 phases ($F_s = 33.4$), and a smaller difference between the reference and the shielded Zorbax SB-C8 ($F_s = 9.8$). However, the chromatographic results painted a different picture: the similarity between the two octadecyl phases appeared more pronounced than that with the shielded octyl phase. Adding to the unpredictable nature of actual selectivity, at the selected conditions, the two

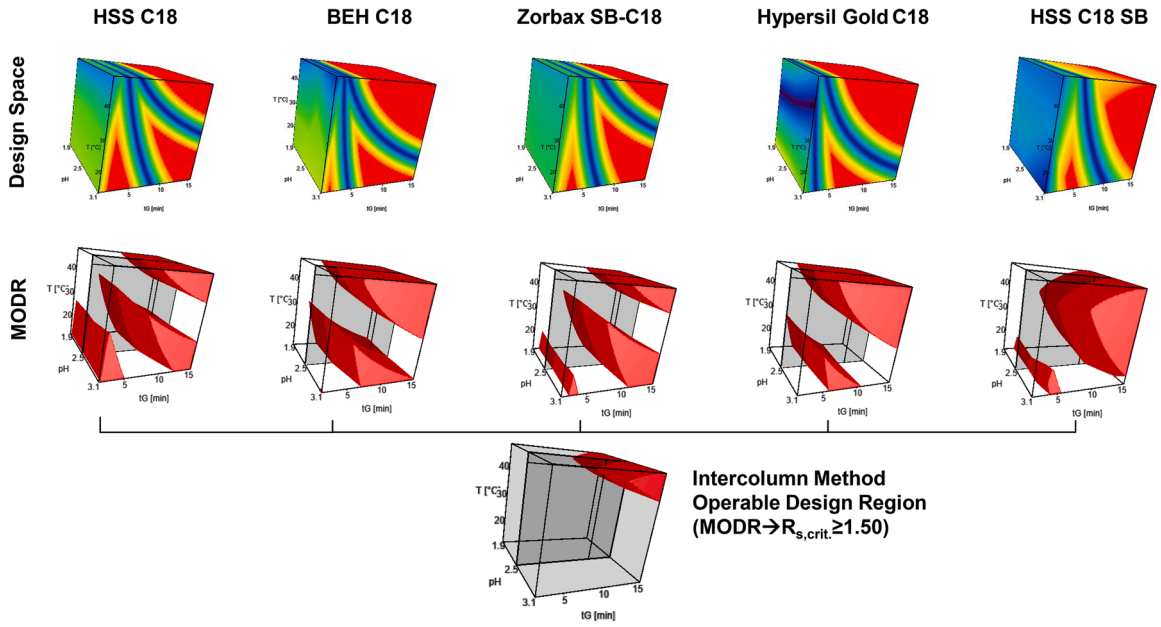

Fig. 3. Model-identification of Design Spaces and red-colored MODRs areas of the selected stationary phases. Based on the displayed MODRs, comprehensive study with regard to replacement column options and suitable method operation conditions (Fig. 4) can easily be performed. The lower, intercolumn MODR shows where baseline separation with each of the studied stationary phases can be realized.

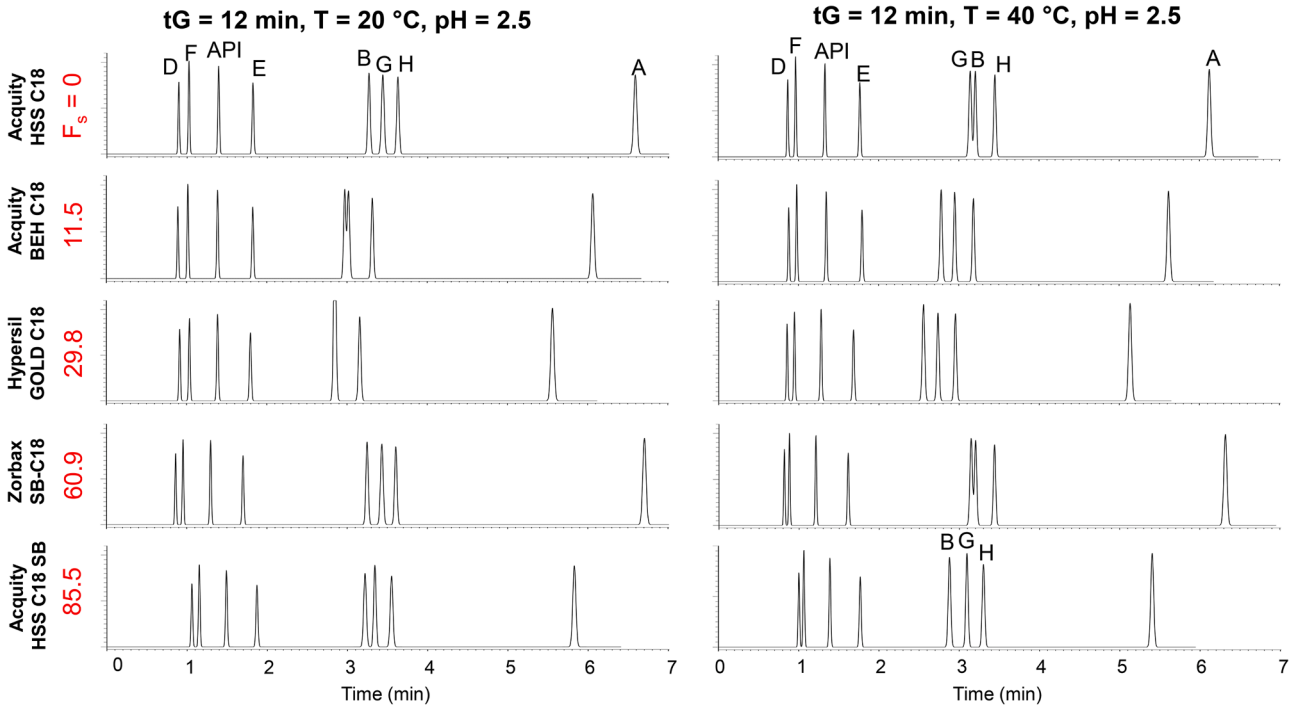

Fig. 4. Comparison of two selected working points at different temperatures (20 and 40 °C).

unshielded phases (Hypersil C18 and C8) exhibited nearly identical selectivities, despite their large F_s-value difference (Fs = 16). Practically, they could be considered as potential replacement options for each other. This contrasts with the current USP guidelines, which do not allow for column equivalence specifications between different groups — here, the C18 phases represent the L1 group, and C8 phases the L7 group.

In the previously referenced study, Snyder et al. had identified a shared baseline separating area (intercolumn MODR) between a larger set of C18 columns with comparable selectivities and a single C8 column, albeit with altered selectivity [31]. It was assumed that the selectivity difference between a C8 and C18 phase is likely due to a variation in shape-selectivity caused by differing alkyl-ligand lengths. However, in our study, considerable selectivity changes were already observed between the Zorbax SB-C18 and Hypersil Gold C18 columns. Given that the Tanaka test yielded reasonably similar selectivity contributions ($\alpha_{T/oT}$) for both, shape-selectivity differences are less likely for these phases. Thus, conducting additional Tanaka tests on the C8 phases was deemed unnecessary, as the DS models already provided deeper insights into the separation capabilities of the columns.

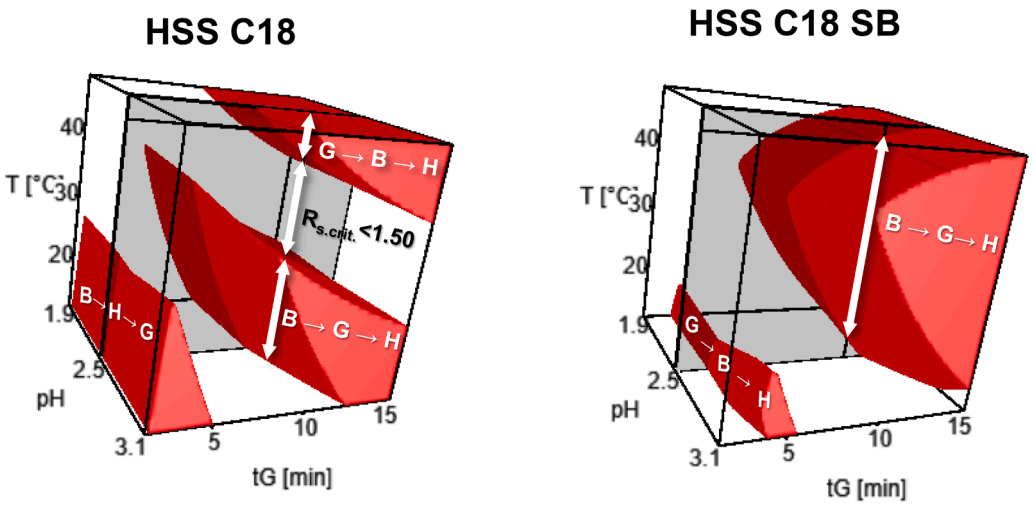

Fig. 5. Acquired MODRs of HSS C18 and HSS C18 SB with the displayed elution order changes of the critical peak pairs (Imp G-B-H). According to general physicochemical characteristics and also the HSM and Tanaka-test results these were identified as the two, most distinct stationary phase.

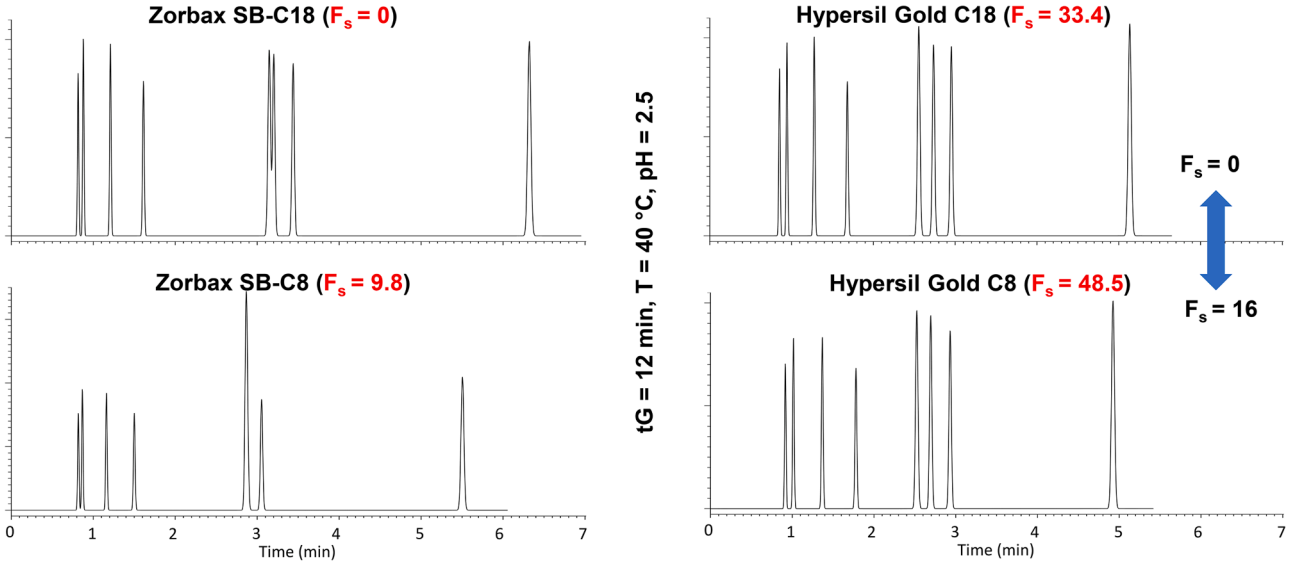

Fig. 6. Model chromatograms of the DS models for the two C8 and C18 stationary phases at a selected working point condition (t_G = 12 min, T = 40 °C, pH = 2.5).

Overall, the results of this study suggest that a proper evaluation of column-provided selectivities can only be achieved with the assistance of AQbD-based modeling solutions. A significant advantage of using multivariate DSs is their capability to offer customized descriptions of complex chromatographic changes within each studied separation system.

5. Conclusions

The challenge of finding alternative stationary phases with equal or comparable chromatographic selectivity is a common issue in pharmaceutical laboratories. The objective of this study was to identify replacement options for various C18-phases and determine suitable method conditions. Initially, we gathered manufacturers' specifications and conducted standard column tests, such as the Snyder-Dolan Hydrophobic Subtraction Model (HSM) and the Tanaka test, to estimate column selectivities, albeit with limited success. These conventional column tests are well suited to quantitatively describe selectivity terms as the result of individual interactions. In real analytical mixtures however, compounds can intensively interact with the stationary phase ligands in various ways (mixed mechanism always occurs) which ultimately can rapidly change in response to the applied method conditions as well.

Bearing this in mind, we developed a straightforward, AQbD-compliant modeling workflow to acquire and compare multidimensional Design Space (DS) models using an actual pharmaceutical sample. This approach enabled us to objectively quantify and compare the chromatographic separations achievable on USP L1-type C18-phases. Although the tested stationary phases displayed a wide range of physicochemical properties within the L1 group, as also evidenced by traditional methods, the DS models uncovered surprising equivalences between columns. For example, the 3-dimensional DSs allowed us to find a shared Method Operable Design Region (MODR) with established baseline separation and identical elution order for two distinctly different C18 phases (HSS C18 and HSS C18 SB).

To validate the efficacy of our modeling approach further, we also aligned two seemingly non-interchangeable C18 and C8 phases. The analysis revealed several equivalences between these markedly different columns. However, there were also instances where, despite the predicted similarities in column data, the model Design Spaces and

chromatograms highlighted distinct differences between the selected stationary phases (C8 vs. C8). This underscores the limitations of current column testing practices and affirms that identifying alternative phases and suitable conditions is feasible only through AQbD-based modeling methodologies.

CRediT authorship contribution statement

Arnold Zöldhegyi: Writing – original draft, Methodology, Investigation. **Krisztián Horváth:** Writing – review & editing. **Róbert Kormány:** Writing – original draft, Methodology, Investigation, Conceptualization.

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.

Data availability

Data will be made available on request.

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