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Updating the European Pharmacopoeia impurity profiling method for cetirizine and suggesting alternative column, using design space comparison

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ABSTRACT

The goal of the present study was to develop a generic workflow to evaluate the chromatographic resolution in a design space and find replacement column for the new method. To attain this objective, a limited number of initial experiments have been performed, and a modeling tool was employed to study and compare design spaces obtained with different columns. By overlaying the different individual resolution maps (design spaces), it is possible to quickly identify a robust zone where the different columns meet a given resolution criterion. This new feature of the modeling tool is very useful for finding alternative columns for a given separation, rather than the usual column tests. It was also demonstrated that two different columns can be used as equivalents (replacement columns), providing sufficient resolution at the same working point and with a high degree of robustness.

1. Introduction

In this work, a difficult to reproduce method for cetirizine dihydrochloride was revised. The method in European Pharmacopoeia (Ph.Eur.) uses a mobile phase with a high (93 %) acetonitrile content and a silica gel stationary phase, so it is a hydrophilic interaction liquid chromatgraphy (HILIC) method that only works on traditional irregular silica gel with high metal ion content [1], more modern regular stationary phases with low metal ion content are not suitable for the analysis. The structure of the molecules to be separated does not justify the use of the HILIC method, a much more reproducible reversed phase (RP) method can be used instead.

Hundreds of octadecyl silane (C18) liquid chromatographic (LC) stationary phases are commercially available today. On one hand, this can make the method development easier since chromatographers can select the most suitable stationary phase for a given separation. On the other hand, it can be a difficult and time-consuming task to find an appropriate replacement (alternative) column, which provides a very similar separation compared to the original column. Today, it is indeed required to suggest an alternative column in pharmaceutical analytical laboratories, and to prove its equivalency during the method validation process. In fact, the pharmaceutical regulatory guidelines mention that method robustness must be checked on columns from different batches and also on other manufacturer's column providing similar separation quality [2].

The column interchangeability in the U.S. Pharmacopeia Convention is quite straightforward. The LC columns are classified in 'L' groups according to their chemical modification [3]. As an example, columns with octadecyl silane (ODS, C18) groups chemically bonded belong to the L1 group, which is defined as: „*octadecyl silane chemically bonded to porous silica or ceramic particles - 1.5–10 μm in diameter* ". This definition is rather inaccurate, since it contains all the phases with irregular silica particles, high metal ion content as well as the widely used hybrid silica. It does not distinguish between different particle morphologies (e.g. fully porous or superficially porous).

To compare or characterize RP columns, various tests have been developed and proposed in the past [4–10]. Databases are also available based on those tests. The limitation of such tests is that they provide information only on a limited number of test compounds, measured under „one constant set"of particular conditions. Those tests cannot predict the applicability of columns for impurity or degradation profiling, assays or other separations, which are the most common applications in pharmaceutical analysis. In previous studies, a so-called simulated robustness testing and other more common design of experiments (DoE) based approaches have been applied to select alternative LC columns for a given separation [11,12].

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It has also been demonstrated that the most efficient way to find alternative columns is to perform retention modeling or DoE at the early stage of method development on several columns and then – if required –, the selectivity can be further adjusted by the slight modification of other method variables (e.g. gradient steepness, mobile phase temperature, mobile phase pH.). With that approach, it was feasible to perform appropriate separation on several columns however it might happen that different columns require for different working points (experimental conditions) to attain the highest resolution [12]. Commercial software is also available today to help in finding a replacement column. The recently introduced module of DryLab software package (column comparison module) enables to overlay the resolution maps obtained on different columns and can easily point out the conditions where all columns fulfil a pre-defined resolution criterion [13]. Such an approach is used in this current study to identify alternative columns for cetirizine impurity profiling.

2. Experimental

2.1. Chemicals

Eluents: The mobile phase was a mixture of purified water and methanol (MeOH). Water contained 0.05 v/v % cc. perchloric acid solution ($HClO_4$-solution), which provided a slightly acidic eluent (pH of 0.05 % $HClO_4$ in water is about 2.3). Under these conditions, the residual silanol groups of a silica based stationary phase are in ion suppressed form, therefore no (or very limited) electrostatic interaction is expected with basic solutes. MeOH (gradient grade) and $HClO_4$-solution (analytical grade) were purchased from Merck (Darmstadt, Germany). Water was purified, using ELGA Purelab UHQ water (ELGA, Lane End, UK). The model sample contained 1 mg/mL cetirizine and its Ph. Eur. impurities (see Fig. 1) at limit level. They were purchased from EDQM and Egis Pharmaceuticals Plc. chemical standard store. The sample solvent was acetonitrile/water = 90/10 v/v %, all components can be dissolved in this solvent mixture. Methanol would also be a good solvent, but it forms a methyl ester with cetirizine.

2.2. Equipment, software

The instrument used in this study was an Acquity UPLC system with a binary delivery pump. The gradient delay volume (V_D) was measured as 0.1 mL, the system contained 500 nL photo-diode-array (PDA) flow cell (Waters Corporation, Milford, USA).

The Kinetex (KNX) columns (100 × 3 mm, 2.6 μm) were purchased from Phenomenex (Torrance, USA).

All chromatographic data were acquired and processed by Empower3 software (Waters Corporation, Milford, USA). UHPLC method development and modeling was performed by using DryLab4, v.4.3.1 optimization software (Molnár-Institute, Berlin, Germany).

2.3. Preliminary experiments

With the data collected from previous experiments, it has been seen that the solutes cover a broad range of lipophilicity therefore linear gradients were run from 30 % to 90 % organic content, methanol (MeOH), acetonitrile (ACN), and a mixture of two (MeOH/ACN = 50/50 v/v %) to sufficiently retain polar compounds and to elute the lipophilic solutes on KNX EVO C18 stationary phase. It is clear from the preliminary experiments that the pure MeOH mobile phase B is the most suitable for the analysis (Fig. 2.).

In the experiments aimed at finding the optimal pH of the eluent, pH= 2.3 was chosen. At a lower value (around 2.0) there is no baseline separation, and at a higher value (greater than 2.5) the retention of ImpD increases greatly.

After optimizing the aqueous and organic parts of the mobile phase, it is enough to examine only two parameters (gradient steepness/time and temperature) – to further improve the separation -, which can be quickly performed by a simultaneous two variable optimization (i.e. two-dimensional DryLab model).

The KNX EVO C18 column was selected as reference column and our goal was to find the most appropriate and similar replacement column.

2.4. Design of experiments (DoE), method development

Development of the cetirizine liquid chromatographic method was carried out following the general methodology, that consists of simultaneously modeling the effect of several method variables (typically mobile phase temperature (T) and gradient steepness (t_G)) on selectivity (or resolution) for a given column [14–17].

A liquid chromatographic method often needs to be transferred to

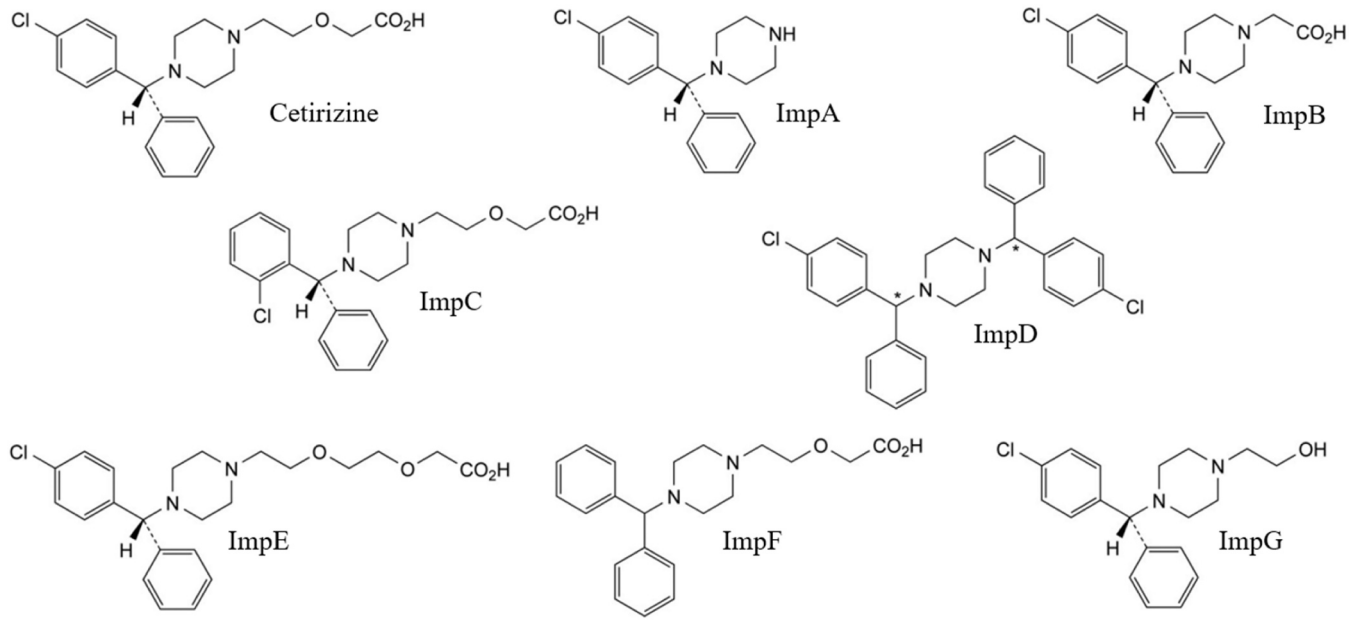

Fig. 1. Cetirizine and its Ph. Eur. impurities [1].

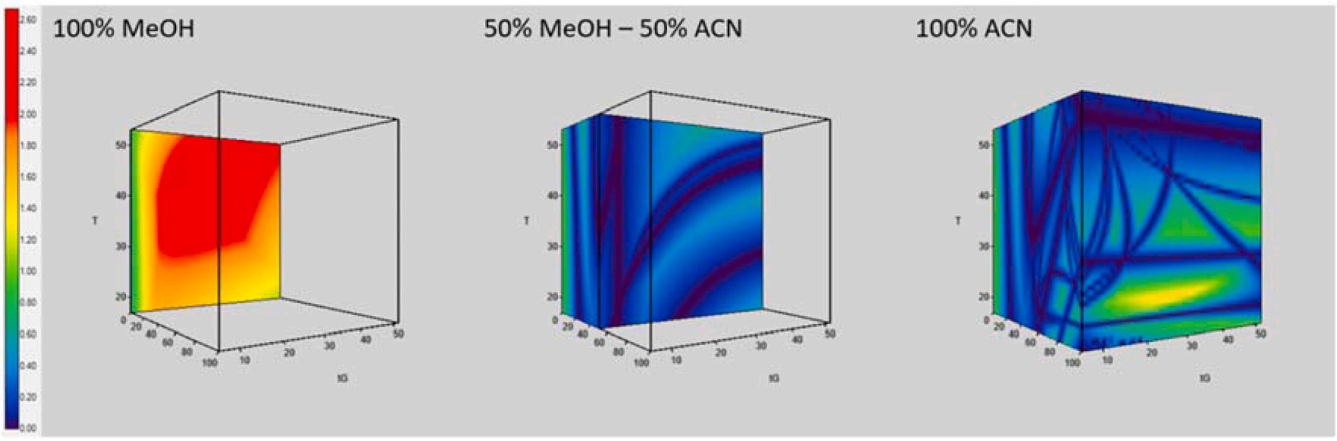

Fig. 2. 3D-critical resolution cube on KNX EVO C18, 100 % MeOH, MeOH/ACN = 50/50 v/v % and 100 % ACN as mobile phase B. Red colour corresponds to conditions where $R_{s,crit} > 1.0$ (resolution of the critical peak pair) while blue colour indicates coelution ($R_{s,crit} = 0$) of the closest ("critical") peaks.

other laboratories or to other chromatographic systems. Therefore, it is important to take the gradient delay time/volume into account. For gradient separations, a good practice is to insert a relatively short (e.g. $t_{iso} = 1$ min) isocratic segment at the very beginning of the separation before the gradient starts. Then, when transferring between different systems, by adjusting the length of the initial isocratic segment, the differences between gradient delay volumes can easily be corrected. In our method, a 1 min long initial isocratic hold is applied. At 0.5 mL/min flow rate, it corresponds to 0.5 mL delay volume.

When running this method on a system possessing $V_D = 0.1$ mL then its delay time corresponds to $t_D = 0.2$ min. Therefore, the isocratic hold time should be set as $t_{iso} = 1–0.2 = 0.8$ min (t_{iso} is the length of the initial isocratic segment). When running the method on systems with $V_D = 0.4$ mL or $V_D = 0.5$ mL then accordingly, the initial isocratic hold time should be decreased to $t_{iso} = 0.2$ min and $t_{iso} = 0$ min, respectively.

For the experimental design, two variables (t_G and T) were set at two levels ($t_{G1} = 10$ min, $t_{G2} = 30$ min and $T_1 = 20$ °C and $T_2 = 50$ °C). This experimental design required for 4 initial experiments (2 ×2) on a given column. Detection wavelength was set at 230 nm, flow rate was 0.5 mL/min and injection volume was 1.0 μL.

The optimal working point can be achieved at $t_G = 20$ min (30–90 % B), T = 30 °C. These conditions correspond to a gradient steepness of 3 %B/min (Fig. 3.).

Under these conditions, the most retained solute elutes below 15 min. Therefore, there is no need to run the gradient until 90 %B. By maintaining the gradient steepness, the analysis can be stopped at 15 min which corresponds to 75 %B eluent. Table 1. contains the final method conditions while Fig. 4. shows the experimental verification of the working point by comparing the predicted and the experimentally measured chromatograms to separate all the possible 8 compounds. Very good agreement was observed between the predicted and measured chromatograms.

3. Results and discussion

3.1. Column comparison

Our goal was to introduce a strategy where - beside method optimization - a substitution (alternative) column can be offered as part of the robustness testing [18].

In addition to the KNX EVO 18 column, four more KNX C18 modified columns with different selectivity (C18, XB-C18, Polar C18 and PS C18) and a pentafluorophenyl KNX column (F5) were selected.

The columns different selectivity performances were first compared

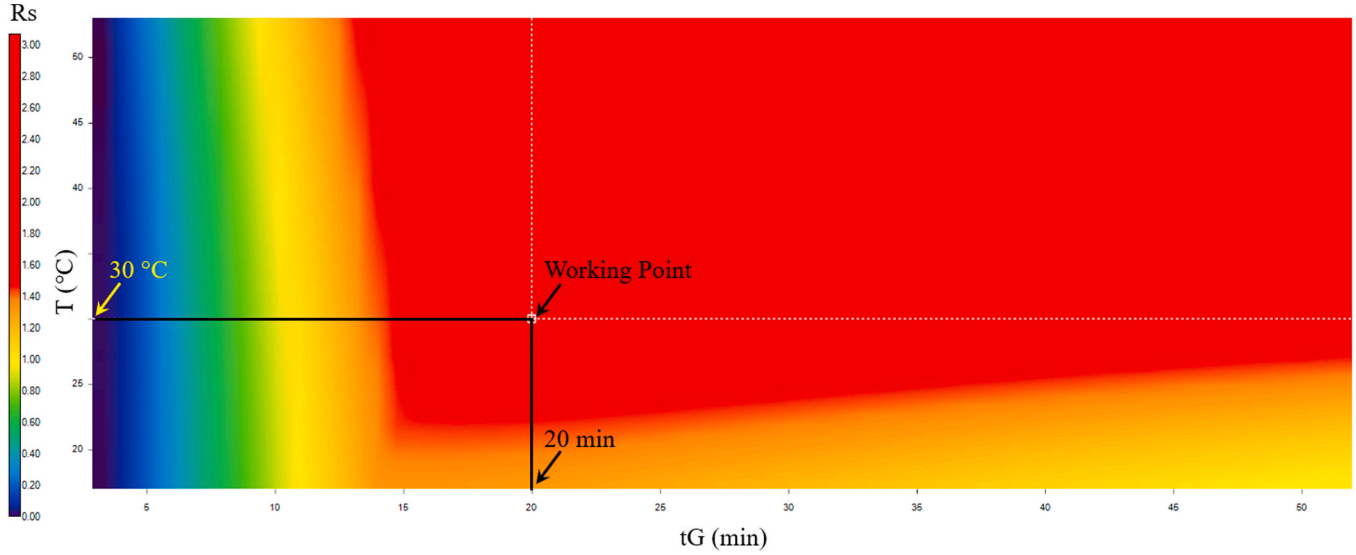

Fig. 3. 2D-critical resolution map with working point on KNX EVO C18. Red colour corresponds to conditions where $R_{s,crit} > 1.0$ (resolution of the critical peak pair) while blue colour indicates coelution ($R_{s,crit} = 0$) of the closest ("critical") peaks.

Table 1
Final UHPLC method conditions.

Instrument	UHPLC system with t_D < 1 min at 0.5 mL/min (V_D < 0.5 mL)		
Column	Kinetex EVO C18, 2.6 m, 100 × 3.0 mm		
Sample solvent	Acetonitrile / Water = 90:10 (v/v %)		
Sample concentration	2.0 mg/mL		
Mobile phase			
"Eluent A"	0.05 % Perchloric acid solution Perchloric acid Preparation: dissolve 0.5 mL cc. Perchloric acid (70 %) in 1000 mL purified water		
"Eluent B"	Methanol		
Method	Gradient		
Gradient table	t [min]	Eluent A [%]	Eluent B [%]
	0	70	30
	1-t_D	70	30
	15 + (1-t_D)	25	75
	t_D: dwell time (min)		
Equilibration time	2 min (start eluent composition)		
Flow rate	0.5 mL/min		
Column temperature	30 °C		
Sample temperature	20 °C		
Detection	230 nm		
Injected volume	1.0 μL		

with the Tanaka test [19]. Different selectivities can be measured with the Tanaka test compounds on the different stationary phases (Table 2. and Fig. 5.), KNX PS C18 and KNX Polar C18 stationary phases show similar selectivity. From these results it would be difficult to say whether we can find a suitable replacement column for the KNX EVO C18.

Besides the KNX EVO C18 column, the same 4 initial runs (see 2.4.) were also performed on various KNX (C18, XB-C18, Polar C18, PS C18 and F5) columns. Retention model and resolution map were built for each column. Preparing these two-dimensional models does not take more time than performing the Tanaka test, but the information content is much more representative for a given separation. The comparison of the stationary phases can be determined most simply based on the color coding (Fig. 6. A) and D)). Red colour corresponds to conditions where $R_{s,crit} > 1.5$ (resolution of the critical peak pair) while blue colour indicates coelution ($R_{s,crit} = 0$) of the closest ("critical") peaks. The separation of cetirizine and its impurities was the most optimal on the KNX EVO C18 stationary phase, of the investigated stationary phases only a similar baseline separation was achieved on the KNX PS C18, therefore it may be suitable as a replacement column. It is interesting that according to the Tanaka test, KNX PS C18 and KNX Polar C18, which are very similar to each other, show a completely different selectivity in this case (Fig. 6. D) and E)). Pentafluorophenyl stationary phase selectivity is different than C18 phases, but baseline separation is not achieved (Fig. 6. F)).

In the modeling software, it is possible to compare the separations by overlaying the resolution maps obtained with different columns (Fig. 7). It is particularly clear in the Fig. 7. A) that there is no condition where baseline separation can be achieved for all KNX C18 stationary phases. Can be clearly seen in the Fig. 7. B), if only KNX EVO C18 and PS C18 are compared, there will be a section where a robust measurement point can be selected.

The advantage of this approach is the mapping of the retention behaviour of the compounds of interest in an entire two-dimensional design space, instead of some selected conditions (as suggested by earlier column tests). By using 100 × 3 mm columns, the entire design space can be mapped on the basis of only 3–4 h experimental work. The approach does not necessarily require UHPLC systems, conventional HPLC may be applied.

A replacement column can easily be proposed at the early stage of method development. The results obtained on five C18 and one pentafluorophenyl modified stationary phases suggest that resolution modelling is practically much more informative for real-life separations than common column tests. During our investigations, we found that two stationary phases cannot be interchangeable for all samples and under all conditions.

3.2. Method validation

The developed method was validated according to the ICH guidelines [2] and the EDQM Techniqual Guide for the Elaboration of Monographs (part III. Analytical Validation) [20] for the following performance characteristics: selectivity, specificity, linearity, precision (system-, method- and intermediate precision), accuracy, sample stability and robustness.

The following solutions and samples were prepared and used for the validation procedure:

Blank solution and sample solvent was acetonitrile/water = 90/10 (v/v %).

Test solution was ~2 mg/mL cetirizine dihydrochloride working standard dissolved and diluted in sample solvent.

To evaluate method selectivity, the following sample was prepared: Imp A, Imp B, Imp C, Imp D, Imp E, Imp F, Imp G, and cetirizine dihydrochloride working standard were dissolved and diluted in sample solvent to obtain a final concentration of ~10.0 μg/mL.

A sample including the impurities in limit concentration (0.15 % or ~1.5 μg/mL) and the cetirizine dihydrochloride in nominal concentration was also prepared.

3.2.1. Selectivity, specificity

Selectivity and specificity of the method was tested by injecting blank solution and "selectivity solution". No interference was detected at the retention time of cetirizine and its impurities. All peaks were separated. The method can be considered selective for the determination of impurities in cetirizine dihydrochloride.

3.2.2. Limit of Detection (LD), Limit of Quantification (LQ)

Detection and quantification limits are calculated by diluting method. A stock solution was prepared, containing the specified impurities at limit level. This stock solution was diluted until peak heights of the impurities met the signal to noise ratio requirements of LD and LQ. The highest LQ level was 0.0217 % (for impurity F), this value is still less than 0.05 %, that is the disregard limit generally applied in pharmacopoeia methods. All requirements were fulfilled for all peaks. In the same manner, the highest LD value obtained was 0.0065 % (for impurity F).

3.2.3. Linearity

The linearity of the method was tested by measuring the area of cetirizine peak at the following levels: LQ (0.03 %, 0.6 μg/mL), 25 % (0.5 mg/mL), 50 % (1.0 mg/mL), 75 % (1.5 mg/mL), 100 % (2.0 mg/mL) and 120 % (2.4 mg/mL). The linearity of impurities was tested at the following levels: LQ (0.03 %, 0.6 μg/mL), 0.10 % (2.0 μg/mL), 0.15 % (3.0 μg/mL), 0.20 % (4.0 μg/mL), 0.25 % (5.0 μg/mL) and 0.30 % (6.0 μg/mL). The correction factors (CF) for impurities of cetirizine dihydrochloride were calculated from the slopes of the individual calibration curves. The results of correction factor obtained are summarized in Table 3. All the results fulfilled the criteria.

3.2.4. Precision

3.2.4.1. System precision. Replicate injections (n = 6) of the limit solution were carried out to determine the statistical error parameters (standard deviation (SD), relative standard deviation (RSD) and confidence interval) of the applied method. The calculated statistical parameters have fulfilled the requirements of system precision. See the results in Table 4.

3.2.4.2. Method precision. Six independent limit spiked test solutions were analyzed. One injection was carried out from each solution. The calculated statistical parameters have fulfilled the requirements of

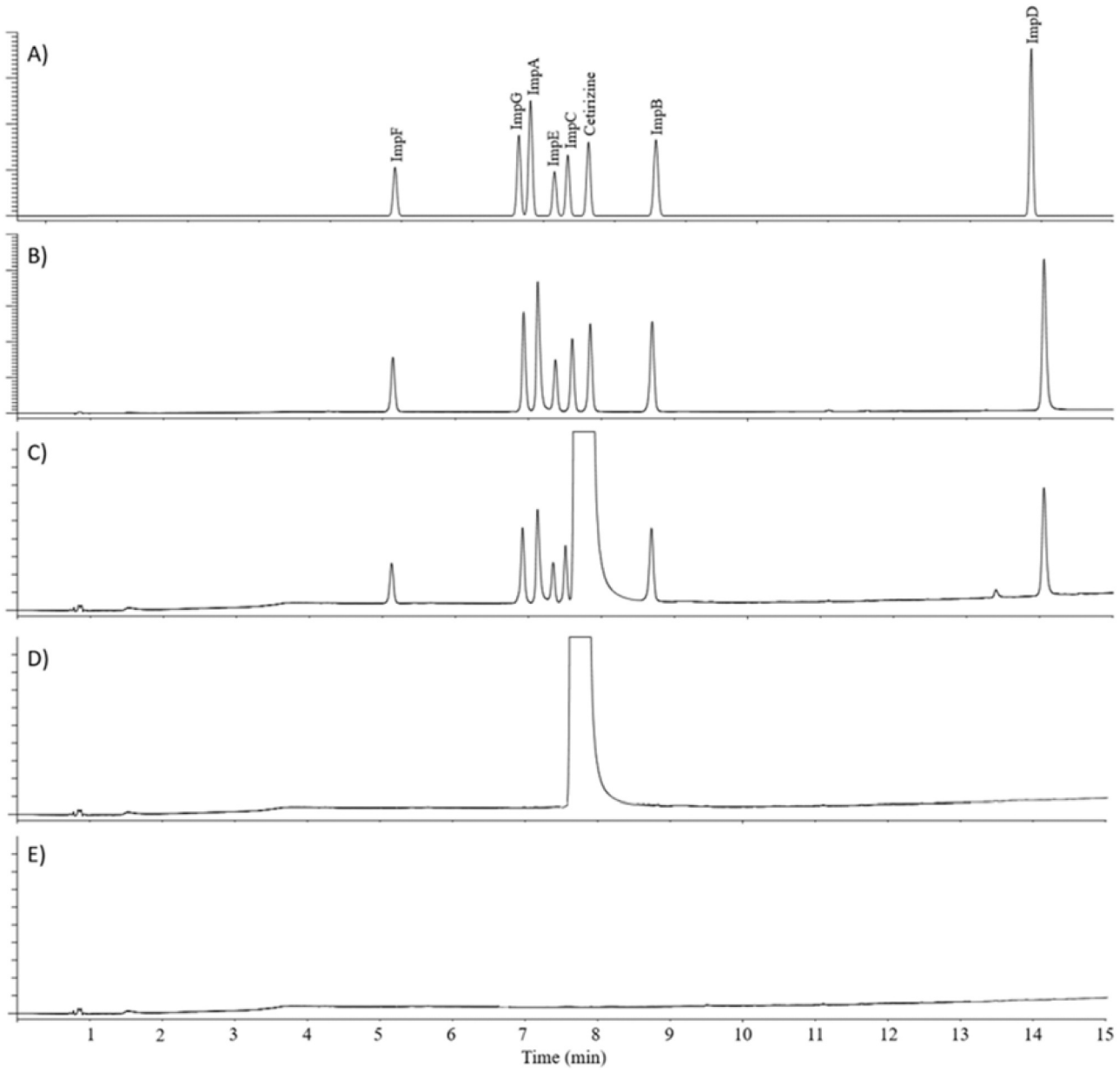

Fig. 4. Predicted chromatogram at 1 % specification level (A), experimental chromatogram at 1 % specification level (B), Experimental spiked chromatogram at 0.15 % impurities level (C), 2 mg/mL sample chromatogram (D) and blank (solvent) chromatogram (E).

Table 2
Tanaka test results on different selectivity KNX columns, where k_{PB} - hydrophobicity, α_{CH2} - hydrophobic selectivity, $\alpha_{T/oT}$ - shape selectivity, $\alpha_{C/Ph}$ - hydrogen bonding capacity, $\alpha_{Ba/Ph\ (pH=2.7)}$ - acidic ion-exchange capacity and $\alpha_{Ba/Ph\ (pH=7.6)}$ - total ion-exchange capacity.

	EVO C18	C18	XB-C18	PS C18	Polar C18	F5
k_{PB}	3.47	4.40	4.50	2.79	2.83	1.68
α_{CH2}	1.47	1.49	1.50	1.47	1.47	1.28
$\alpha_{T/oT}$	1.12	1.33	1.20	1.18	1.20	2.23
$\alpha_{C/Ph}$	0.42	0.50	0.56	0.68	0.67	1.00
$\alpha_{Ba/Ph\ (pH=2.7)}$	0.05	0.11	0.09	0.05	0.09	0.19
$\alpha_{Ba/Ph\ (pH=7.6)}$	0.26	0.32	1.45	0.56	0.50	1.19

method precision. See the results in Table 4.

3.2.4.3. Intermediate precision. The study of intermediate precision was carried out performing the analysis on two different LC systems (Acquity

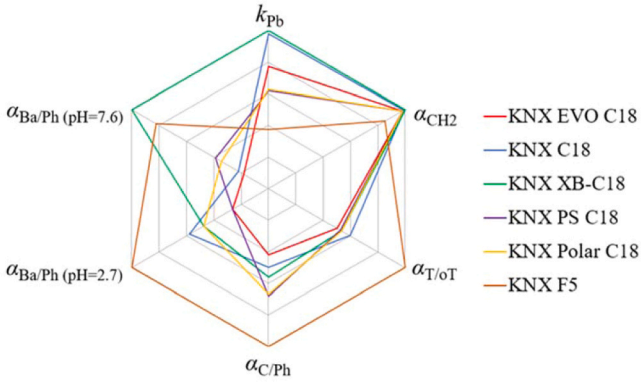

Fig. 5. Tanaka test results for KNX stationary phases with different selectivities represented as hexagons (see Table 2. for symbols).

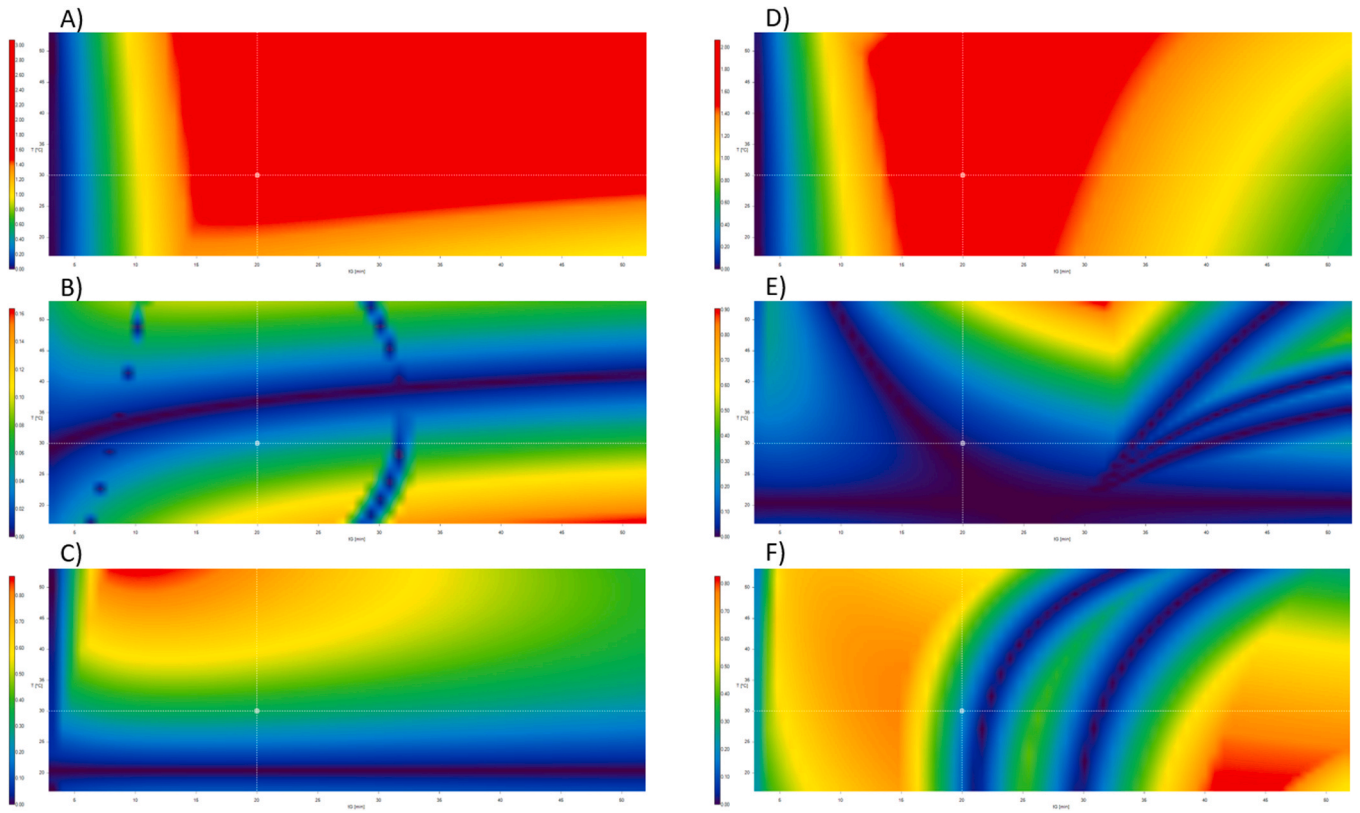

Fig. 6. 2D-critical resolution maps, t_G-T models on KNX columns showing the Working point (t_G = 20 min, T = 30 °C). For EVO C18 (A) and PS C18 (D) red colors mean "baseline separation" (Rs,crit > 1.5), blue colors indicate coelution (Rs,crit > 0) of some peak pairs. In the other cases, the color coding is different because the separation was not successful, C18 (B) Rs,max = 0.16, XB-C18 (C) Rs,max = 0.80, Polar C18 (E) Rs,max = 0.90, F5 (F) Rs,max = 0.80.

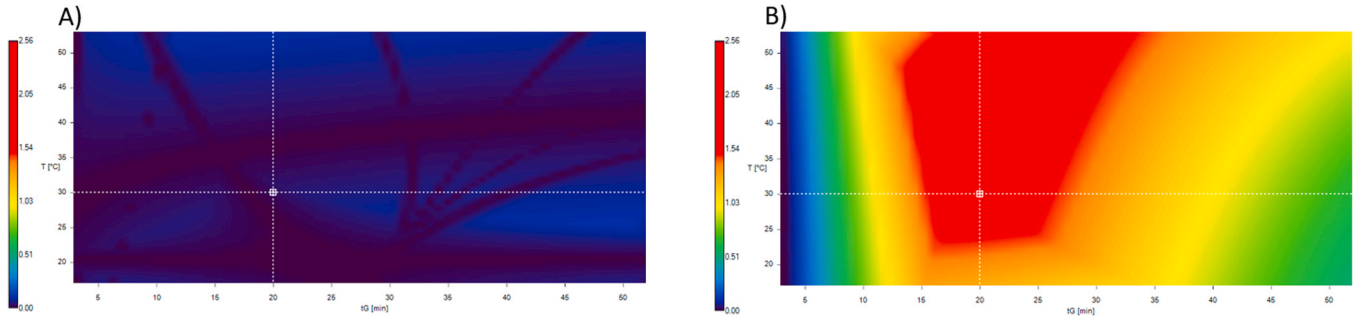

Fig. 7. Design Space Comparison model of KNX all of C18 (A) and KNX EVO C18 and PS C18 (B).

Table 3
Correction factors for impurities of Cetirizine dihydrochloride.

Component	CF
Imp A	0.59
Imp B	1.00
Imp C	1.78
Imp D	0.51
Imp E	1.00
Imp F	1.67
Imp G	1.00

UPLC and Acquity UPLC H-Class), using two different columns and by two different analysts. The measured impurity contents of cetirizine dihydrochloride "limit spiked test solutions" were compared. The absolut difference of any impurities were NMT 0.05 %. The requirements of intermediate precision were fulfilled.

3.2.5. *Accuracy (Recovery)*

Cetirizine dihydrochloride sample was spiked with different quantities of impurities. The measurements were accomplished at three different levels and with triplicate measurements. The three levels were set as limit, 50 % of the limit and 0.03 % of the limit and measured levels were determined in recovery percentage. All the recovery data ranged between 77.80 % and 114.73 %, thus the method was found to be accurate.

3.2.6. *Stability of sample solutions*

The "test solution", the "limit solution" and the "limit spiked test solution" were analyzed over a period of 72 h. The solutions were stored in the autosampler (at 10 °C, protected from light) during that period. The obtained results showed that the solutions were stable for a period of 72 h and all the recovery data ranged between 96.6 % and 108.6 %, which fulfilled the requirements.

Table 4
Comparison of validation criteria and results.

Parameter	Measurement	Requirement	Result	
Selectivity, specificity	-	no interference peak at the retention time of Cetirizine or its impurities	no interference peak at the retention time of Cetirizine or its impurities	Fulfilled
Limit of quantification (LQ)	signal-to-noise ratio	LQ level NMT 0.03 %	0.03 %	Fulfilled
Linearity	Correlation coefficient	NLT 0.990	0.9993–0.9999	Fulfilled
System precision	RSD (%)	NMT 5.0 %	0.23–1.86 %	Fulfilled
Method precision	RSD (%)	NMT 5.0 %	0.27–1.47 %	Fulfilled
Intermediate precision	Difference (%)	NMT ± 0.05%	0.0031–0.027 %	Fulfilled
Accuracy	Recovery (%)	75.0 %–125.0 %	77.80–114.73 %	Fulfilled
Stability of sample solutions	Recovery (%)	85.0 %–115.0 %	96.56–108.56 %	Fulfilled
Robustness	$R_{s,crit}$ between Imp G and Imp A	NLT 1.5	>1.55	Fulfilled

3.2.7. *Robustness*

For robustness test DryLab was used. Software-based robustness calculation has the advantage that not only all single solvent and instrument effects, but also all conceivable combinations of them can be calculated in a model-mediated way [12]. Only 12 measurements were required for the robustness test experiments. It is possible to perform a modeled robustness testing thanks to modeling software. Beside the three main model variables (tG, T and pH), the flow rate, as well as initial and final compositions of the mobile phase represents the investigated variables in a built up model. The effect of these six variables can be calculated at three levels, corresponding to 3^6 = 729 variants in selectivity.

During the virtual robustness study, the impact of the three model variables (t_G, T, pH), and three additional calculated variables (flow rate, initial- and final mobile phase compositions) was studied around the working point (t_G = 20 min – 30–90 %B, T = 30 °C, pH = 2.3) (Fig. 8.).

The effect of these six variables (in this manner they can be considered as factors of a virtual experimental design) was evaluated at three levels (−1, 0, +1). The modeled deviations from the nominal values were the following: gradient time was set to 19.9, 20.0 and 20.1 min, temperature was set to 29, 30 and 31 °C, pH of mobile phase was set to 2.28, 2.30 and 2.32, flow rate was set to 0.49, 0.50 and 0.51 mL/min, initial mobile phase composition was set to 29 %, 30 % and 31 %B and its final composition was set to 89 %, 90 % and 91 %B.

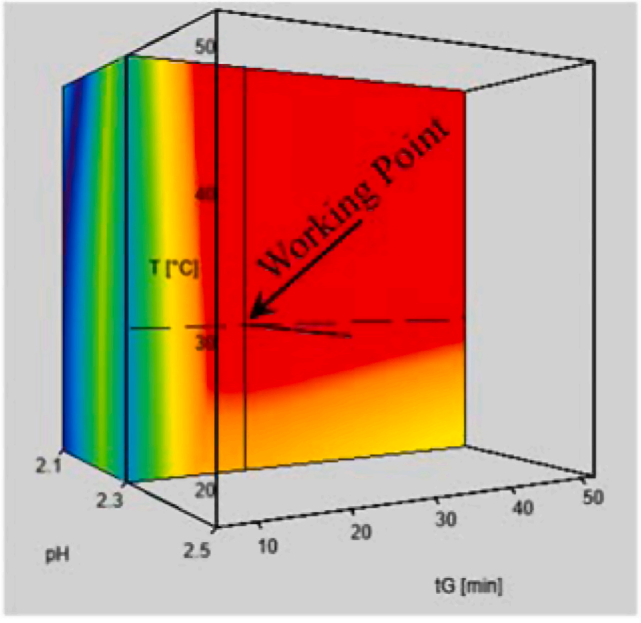

Fig. 8. 3D-critical resolution cube, t_G-T-pH model on KNX EVO C18 column showing the Working point (t_G = 20 min, T = 30 °C, pH = 2.30).

Then, the 729 experiments (3^6) were performed in silico (Fig. 9. A)).

A criterion of $R_{s,crit} > 1.5$ was considered. As shown is Fig. 9. B), the lowest predicted resolution was Rs = 1.55 between peak 2 and 3 (Imp G and Imp A) which is still acceptable (Rs > 1.5). Therefore, the method can be considered as robust, since the success rate to perform $R_{s,crit} > 1.5$ separation was 100 % in the studied range of method variables, pH and temperature have the greatest effect on resolution (Fig. 9. C)).

3.2.8. *Validation results*

Table 4. summarizes the results of method validation criteria for selectivity, limit of quantification, linearity, system precision, method precision, intermediate precision, accuracy, stability and robustness during the validation process. All the results fulfilled the limits of method validation criteria for each parameter.

4. Conclusion

The aim of this work was to actualize the method described for cetirizine related substances in the European Pharmacopoeia monograph. A workflow was proposed for the first time to compare the resolution of an impurity profiling method in a two-dimensional design space and to find possible replacement columns for the method. Our strategy was based on the use of state-of-the-art chromatographic modeling software, allowing to compare the parts of design spaces obtained with different columns, where a pre-defined critical resolution is achieved. A section of robust spaces can then easily be found by overlaying the two-dimensional resolution maps. Despite the fact, that the selected stationary phases showed obvious differences in column tests, two of them were found to provide baseline separation in the same design space. At the end, two of the six columns shared the same working point and resulted in robust separations. Therefore, these two columns can be interchanged. During method development, it is recommended with such a method to look for a suitable interchangeable stationary phase.

Finally, a single robust method was developed and validated in accordance with the requirements of the pharmaceutical authorities and proposed to update the old pharmacopoeia method.

CRediT authorship contribution statement

Róbert Kormány: Conceptualization, Methodology, Investigation, Writing – original draft. **Barnabás Soós:** Investigation, method validation. **Krisztián Horváth:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial

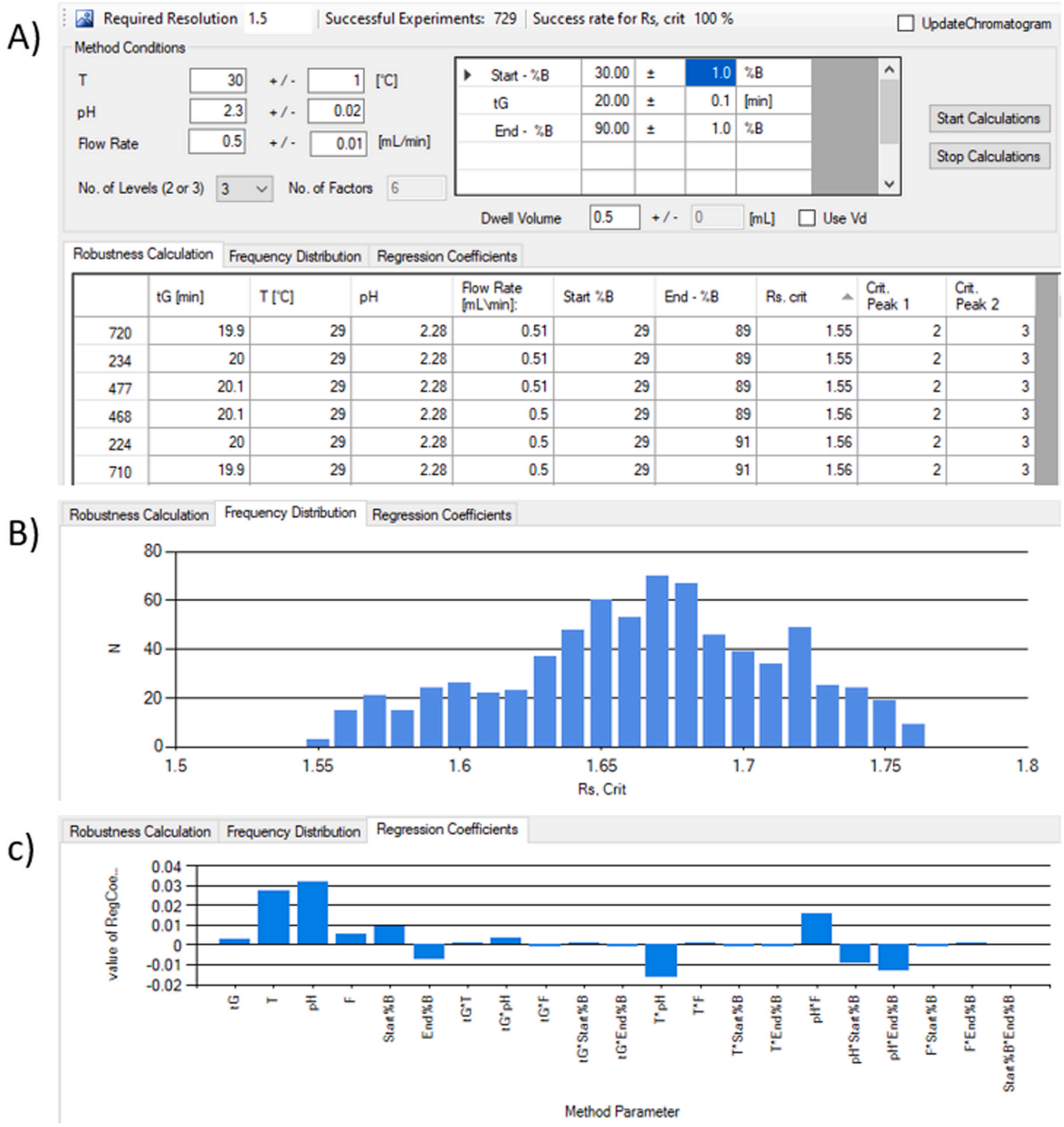

Fig. 9. Deviations (levels) of method variables considered for the virtual robustness study and the calculated results (Rs, crit and critical peak pairs) for the 6 worst separations among the 729 virtual experiments (A), frequency distribution (B) and regression coefficients (C).

interests or personal relationships that could have appeared to influence the work reported in this paper.

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