



Automated UHPLC separation of 10 pharmaceutical compounds using software-modeling

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ABSTRACT

Human mistakes are still one of the main reasons of underlying regulatory affairs that in a compliance with FDA's Data Integrity and Analytical Quality by Design (AQbD) must be eliminated. To develop smooth, fast and robust methods that are free of human failures, a state-of-the-art automation was presented. For the scope of this study, a commercial software (DryLab) and a model mixture of 10 drugs were subjected to testing. Following AQbD-principles, the best available working point was selected and conformational experimental runs, i.e. the six worst cases of the conducted robustness calculation, were performed. Simulated results were found to be in excellent agreement with the experimental ones, proving the usefulness and effectiveness of an automated, software-assisted analytical method development.

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1. Introduction

As defined in Current Good Manufacture Practice (CGMP), "Out of Specification" (OoS) describes the situation, when test results fall outside of the predefined specifications of acceptance criteria. More than 10% of the worldwide pharmaceutical production which is now close to ca. 990 billion US\$, are withheld each year due to OoS-issues [1]. This results in a loss of ca. 99 billion US\$ each year. Regulatory revisions and subsequent post-approval-changes are often long and cumbersome – if they succeed at all. If they don't, they result in unexpected high losses for pharmaceutical companies.

OoS-reasons can be traced back to failure of manufacturing processes (batch-to-batch production) and laboratory processes (Quality Control, Data Integrity) or both. Good manufacturing is obviously essential, but well-performing thoroughly documented supportive analysis, which is free of human mistakes, is just as mandatory.

Since the Thalidomide disaster in 1957, bulk analytical techniques are less common, the separation and identification of all components in a drug being strictly enforced. Among the various analytical techniques, Reversed-Phase High-Performance Liquid

Chromatography (RP-HPLC) constitutes the most popular and successful method for separating mixtures of drug products and related compounds. Although a Quality by Design (QbD) proposed, knowledge-oriented perspective is widely implemented in pharmaceutical production, it is only at the beginning to be applied in HPLC method development. Thus, despite the recent technological advances in HPLC, unsatisfactory analytical results caused by inconsequently developed, trial and error derived, non-robust methods lead to otherwise avoidable debates with the agencies. Subsequent revalidation processes, so called "post-approval changes", always bring with them increased costs, a waste of time and energy, and in the worst case a rejection of commercial batches.

The first in-silico retention modeling initiative, based on solid science, was pioneered by Snyder and his research team in 1986. They gave birth to the "DryLab" software, being at first a "one factor at the time" (OFAT) method development approach for isocratic (DryLab I) [2], and later for gradient installations (DryLab G) [3].

In isocratic HPLC, the concentration of the organic eluent content (%B), in gradient HPLC, the gradient time (tG), the pH, the mixing ratio of two organic eluents ("ternary composition") (B2:B1) and also instrument parameters, such as temperature, dwell volume, column dimensions, flow-rate, etc., all do affect chromatographic selectivity and thus the critical resolution. It is immanent that all these parameters must be thoroughly modeled, based on flawlessly executed experiments, the models predictive

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precision to be subsequently verified per according confirmation runs.

The first modeling software, soon to encompass the optimization of 2 measured parameters to be experimentally verified in several papers of 2-D-modeling [4]. A further step forward was the creation of the 3-dimensional (3-D) resolution map, the “cube” [5], and very recently a state-of-the-art robustness-module [5–7]. The cube can work with 3 experimentally measured parameters (gradient time: tG, temperature: T, pH or ternary composition: tC or additive concentration: aC, for ion-pairing reagent-, or buffer-concentration) and calculate 8 other parameters (dwell-volume: V_d , extra-column volume: V_{ec} , flow-rate: F, startB%, endB%, column length: L, column diameter: I.D. and particle size: d_p), to deliver transparently, why methods might fail, mostly due to peak movements. The robustness calculation can handle 3-level variations for each of the above factors, resulting in $36 = 729$ virtual experiments. Flow-rate and dwell volume tolerances can also be additionally modeled [8].

The creation of this kind of models is most efficient, due to the simple measurement of peak positions and their movement caused by alterations of the most important parameters such as tG, T, pH, tC or aC. With data generated out of only 12 input experiments, once the peak-table is matched, DryLab® 4 can create multi-dimensional resolution models, which are able to predict reliably critical resolution and retention times for millions of chromatograms [9]. Among those, the best robust separation conditions can be easily selected within seconds.

The consistency of this concept was proven by many authors [10–12]. In a recent work, Terazosin's old Ph.Eur. method was remarkably modernized [13]. In another case of the Ph.Eur. method for Ebastine, the analysis time could be reduced from 160 to less than 4 min, at improved selectivity [14]. The work with method development for therapeutic proteins was also supported by DryLab, especially was method development time-reduction reported by Kochling et al. [15,16].

In other cases, the optimized method was effectively transferred between devices of different instrument manufacturers, and between different column geometries. In recent studies, HPLC-method development of monoclonal antibodies (mAb) and antibody-drug conjugates (ADC) were notably facilitated [17–21]. Adding another weight to the balance of multifactorial method development, “Altering the working point in the design space is not considered to be a change. . .”, i.e., in case of moving within the design space (the red region of the cube), there is no revalidation needed, leading to an unparalleled flexibility in routine work [22].

However, manual data handling can still introduce errors into the modeling processes. Laboratory workers, who enter this field in a growing number for the first time, are often not aware of the impacts of their errors. Relevant GMP instruction clarifies that “where human error is suspected or identified as the cause, this should be justified having taken care to ensure that process, procedural or system-based errors or problems have not been overlooked, if present.” [23].

Automation routines can reduce human error, increase productivity and reduce per unit costs. Therefore, in this project, the goal was to develop an automation feature in order to reduce the time to create the necessary experiments, along with the time required to manually enter the run parameters. To achieve this goal, we created a model mixture of blockbuster drug components to be able to follow a large number of experiments and test the influence of variabilities needed to program the automation algorithms in robustness studies and to control the experimental outcome of the individual separation conditions. This would eliminate possible errors on setting the conditions for each single run. The result should be the automated conduction of 12 numbered runs, ready to

be imported into DryLab® 4 and consequently adding to a proficient pharmaceutical Quality Control.

Furthermore, our aim was to find an adequate method which can deliver the fulfilment of the Analytical Target Profile (ATP), in our case baseline resolution for the critical peak pair, even if the variables are altered between their tolerance limits.

2. Experimental

2.1. Chemicals and samples

Gradient grade acetonitrile, water, methanol and ethanol were purchased from Th. Geyer, Berlin, Germany. For the aqueous eluent A, 10 mM formic acid solution (pH 2.9) was prepared freshly using concentrated formic acid (385 μ L 98% HCOOH/1000 mL water) (Fluka, Switzerland). Relevant active pharmaceutical ingredients (API's) were extracted from commercially available drug products using different extraction solvents. Nitrofurantoin (NFT), acetylsalicylic acid (ASA), diltiazem (DIL), dexamethasone (DEX), ketoprofen (KETO), ezetimibe (EZE), atorvastatin (ATV), were extracted in 50:50 methanol-water, diclofenac (DICLO) in 90:10 methanol-water, bromazepam (BRZ) and spironolactone (SPIRO) in pure ethanol. Final theoretical concentrations for each of the samples were ca. 1 mg/mL. Sample solutions were first centrifuged at 3000 rpm for 10 min (Hetovac, VR1), then filtered through a 0.45 μ m filter (Spartan 13/20). Final mixtures were diluted with (MeOH:Water) (50:50)(V:V) and injected consecutively.

2.2. Chromatographic system

For the experiments a Nexera X2 UPLC system (Shimadzu Europe, Duisburg, Germany) equipped with three-pump high-pressure delivery system (dwell volume, $V_d = 0.48$ mL, extra-column-volume, $V_{ec} = 0.004$ mL) cooled autosampler (4 °C), active-heated column oven, DAD-detector (190–800 nm) was taken into service. To change the organic eluent composition (%B), we created a program and used two pumps: B1 and B2, and created with different ratios of B1 (acetonitrile) (=Shimadzu-“Pump B”) and B2 (methanol) (=Shimadzu-“Pump C”). The chromatographic column was a fully porous Merck Purospher Star RP-18 end-capped (50 $\times$ 2.1 mm, 3 μ m) (Order no.1.50651) (Merck Darmstadt, Germany). Flow-rate: see below. Injection volume was 1.0 μ L. Chromatograms were monitored at 240 nm.

Instrumental setup and data collection was performed with Shimadzu Nexera X2 and LabSolutions (Version 5.89). Data processing and automation protocol was achieved with DryLab® 4, version 4.3.2. (Molnár-Institute, Berlin, Germany).

2.3. Chromatographic conditions for the creation of the Cube

12 experiments were carried out, according to Fig. 3.

tG1: 5 min, tG2: 15 min, Gradient range: 10- >95%B; eluent B: B1 + B2, (B1: AN, B2: MeOH); tC1: AN; tC2: B1:B2, 50:50 (V:V); tC3: MeOH
T1: 30 °C, T2: 60 °C
F = 0.6 mL/min.

2.4. Chromatographic conditions at the selected working point

Conditions at the selected optimum working point (see Fig. 6):
tG = 13 min, Gradient range: 10- >95%B; eluent B: B1 + B2, (B1: AN, B2: MeOH), tC = 36:64, AN:MeOH (V:V)
T = 47 °C
F = 0.6 mL/min.

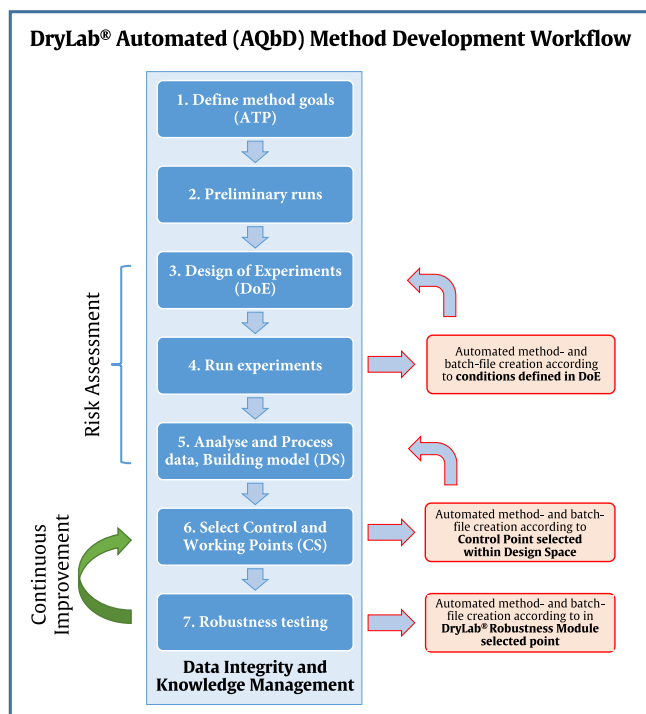


Fig. 1. Scheme of an AQbD method and its automation, developed with DryLab® 4.

2.5. Model-based method development workflow

A well-performing analytical method is considered to be the bottleneck of a chemical analysis, the chemical analysis being one of the most critical parameters in Research & Development-, and the final manufacturing process.

Consequently, an appropriate analytical method is considered not just to be the key factor of a modern analysis, but due to its essential role in Quality Control, the key factor in manufacturing Quality by Design, too. This stresses the importance of a systematic method development, even for analytical methods. Applying a software-based method modeling for the whole stage of analytical method development not only satisfies these requirements but also gives the analyst a deeper understanding of the chromatography and what lies beyond. **There are various commercial software products available on the market, among of which DryLab is a renowned package for analytical method development [24].**

The novelty of this research is that the Design of Experiments (DoE), the creation of the design space verification runs, the establishment of a working point and consequent robustness studies at different points were carried out in an automated way, eliminating human mistakes, reducing time-consuming, fussy analogue work (Fig. 1).

3. Results and discussion

3.1. Determination ATP

Every analytical method development starts with a predefined objective, the Analytical Target Profile (ATP). In most cases the ATP will be to achieve good separation, so called “Baseline Separation” with a critical resolution $R_{s,crit} \geq 1.5$. Other goals might be to minimize analysis time, ruggedness, transferability, economically and possibly environmentally improved methods [15,25].

Main objective of this research was to establish a robust baseline separation ($R_{s,crit} \geq 1.5$) and a reduced analysis time. The mixture that was designed for this research constituted of 10 pharmaceu-

tical compounds, a wide variety of drugs relevant and extensively used today (Fig. 2).

3.2. Preliminary studies

The following screening step (No.2) comprises a rough selection of possible stationary phases, included some preliminary experiments and the selection of main influential parameters for subsequent modeling.

Since the majority of active pharmaceutical ingredients (API's), as well the 10 compounds of this study were considered to be hydrophobic molecules with small polar parts, reversed-phase chromatography was decided for.

As it was experimentally proven in Horváth et. al's “solvophobic theory” [26], under reversed phase conditions the high surface tension of water forces lipophilic compounds out of its structure and lengthens the retention time on C18 columns. With the increase of organic amount in the mobile phase however, due to the reduced surface tension, the excluding force will be less, resulting in a decrease of retention time.

The application of isocratic method development was refused for multiple reasons. One of the main drawbacks of this technique is that technically no or very limited selectivity-range is at the chromatographer's disposal. Here, selectivity-tuning can be realized only through a slight change in mobile-phase composition or a complete replacement of the stationary phase. Another issue with isocratic methods is that for compounds with different polarity, the optimal chromatographic retention factor-(k)-range ($1 < k < 10$) cannot be fulfilled. In addition, with an increase in retention time, a proportional peak-broadening is occurring.

Consequently, apart from very specific applications like the separation of stereoisomers, isocratic elution as a technique that can be considered obsolete in comparison to modern gradient elution [27].

Since there are approximately 1000 RP-columns on the market, proper selection of the stationary phase will often cause a headache to the chromatographer. However, Kormány et al. proved that robust separation of bisoprolol, amlodipine and their impurities can easily be achieved on a wide variety of RP-columns prepared with different ligands or distinct surface coverage after a model-facilitated selectivity-tuning and working point selection [28,29]. This way, for amlodipine and their impurities, analysis time of an old Ph.Eur. method could be reduced from 60 min to less than 6 min [9].

For the above-mentioned reasons, a fully porous Merck Purospher Star RP-18 (50×2.1 mm, $3 \mu\text{m}$, endcapped) was chosen for resolution modeling and method development. In order to avoid pre-eluting ($t_0 + t_D$) and post-eluting ($t_0 + t_D + t_{grad}$) peaks, a few experimental runs were implemented and initial and final organic compositions of the linear-gradient were fixed for the model-runs.

3.3. Design of Experiments (DoE)

A model is as good as the input (runs) it is based on. The selected experimental design is shown in Fig. 3, which allows for a maximum collection of knowledge relying only on a few, in this study 12, real experiments.

Gradient time and temperature were measured at two levels, whereas the difference between short and long run was at a factor of three ($tG_2/tG_1 = 3$) and for temperature 30°C (30 and 60°C). These ranges were determined following software recommendations, however as it was proven by Rácz et al., model's accuracy changes only insignificantly, even at reasonably reduced or extended ranges [30].

For compounds with hydrophobic properties, along with gradient time (tG) and temperature (T), eluent strength is playing the

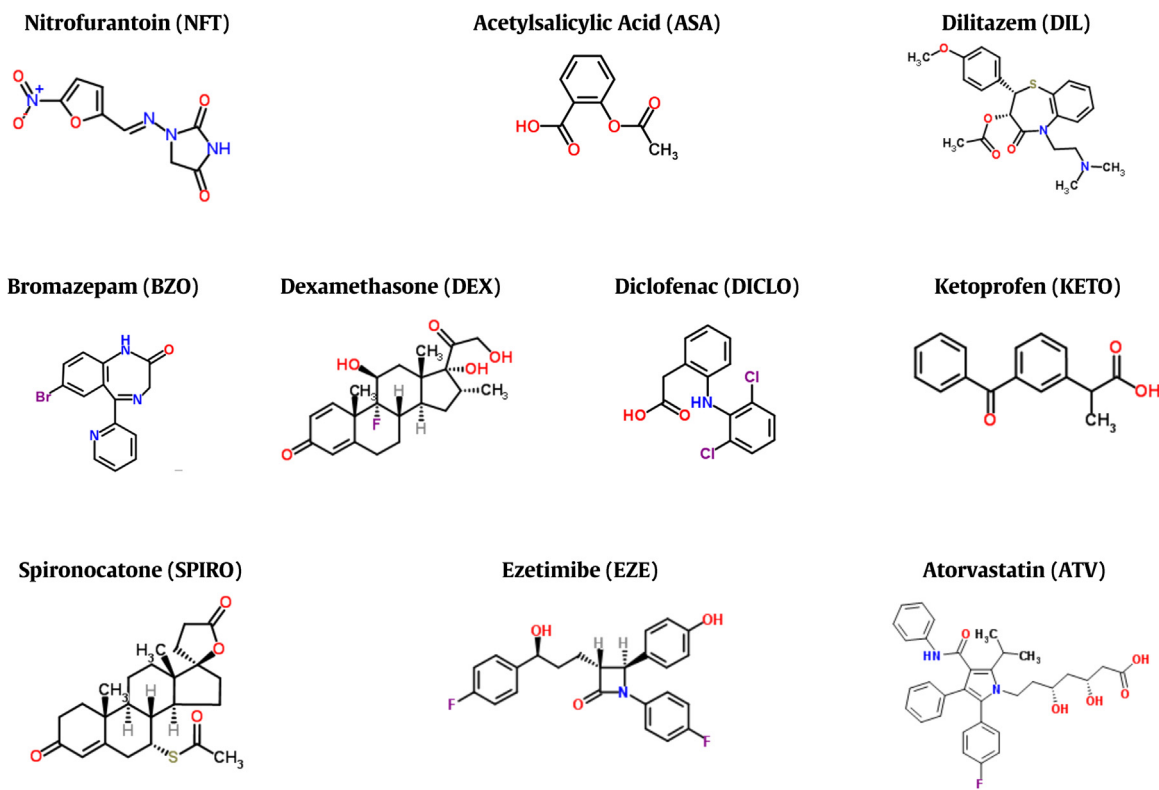


Fig. 2. Molecular structure of the investigated 10 compounds of this study.

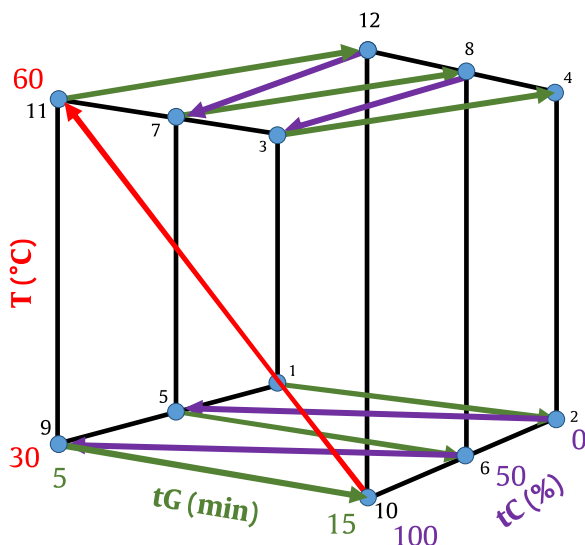


Fig. 3. Automated experimental design (Design of Experiments, DoE) and conditions of tG-T-tC model (12 experimental runs) and best order of execution. Order of runs marked with arrows from 1 to 12. Green arrows represent changes in gradient time (tG), red in temperature (T) and purple in ternary composition (tC). Fine printed numbers indicate DryLab import numbering of the runs for Fig. 4(d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dominant part determining selectivity. Therefore, ternary composition (tC) was selected as the third modeling parameter, while in order to establish uniform ionization of the free silanol-groups in the column and acidic-like compounds of the injected sample (acetylsalicylic acid and diclofenac, atorvastatin, ketoprofen), pH-level was kept constant with 10 mM formic acid (pH ~ 2.9).

As described in previous studies, thermal-, and solvent-equilibria of the chromatographic system play a significant role in reproducibility, therefore precise order of input runs is critical. Starting at low temperature (30 °C) with one organic eluent (B2:0%), at first low, then high values of the gradient time (5 and 15 min, respectively), then proceeding to the next organic composition (B2:50%) and to the third (B2:100%) and repeat the same procedure at elevated temperature (60 °C) afterwards, offers the best systematic design (Fig. 3) [31].

Design of Experiment variables, column-, instrument-, and gradient data were all imported into the data entry page (Fig. 4) and with only one click a batch-file was immediately created.

The relevance of this single but critical step is that it cancels out any risk of incorrect placing of the experimental runs. It may seem very elemental, but recent regulatory implications have shown the existence of such banal issues even for experienced users. Consequences from regulatory side are often serious: deleted, interrupted runs, injections without proper explanation lead to violation against data integrity, therefore subsequent regulatory inspection, withheld and in worst case, complete rejection of commercial batches (s. FDA violation page [32]).

To make the method successful “right first time”, the automation window, predefined on the data entry page contains all the values for the upcoming DoE-experiments (Figs. 3 and 4). From this point, the batch table can be created easily and carried out afterwards with a chromatographic data system, like Labsolutions, Chromeleon or Empower (Fig. 5).

Completed runs can be imported in .cdf-format or, for Labsolutions, in .lcd-format. Time-requirements for one run can be calculated the following way:

$$\text{method time} = t_{\text{grad.}} + t_{\text{eq.}}(V_0, F) + t_D(F) + t_0(V_0, F) + t_{\text{hold}}$$

Equilibrium time (t_{eq}) and dead-time (t_0) are mostly dependent on the net-volume of the column and on the flow rate, while dwell

(a) Mode Selection

Gradient / Temperature / tC (12 runs)	tG 1 [min]	tG 2 [min]	T 1 [°C]	T 2 [°C]	tC 1 [% B2 in B1]	tC 2 [% B2 in B1]	tC 3 [% B2 in B1]
T	5.00	15.00	30.00	60.00	0.00	50.00	100.00

(b) Column Data

Name: Merck Purosper Star RP-18
 Length [cm]: 5
 Inner Diameter [cm]: 0.21
 Particle Size [μm]: 3
 Flow Rate [mL/min]: 0.6
 Injection Volume [μL]: 1

(c) Instrument Data

Name: Shimadzu Nexera X2
 Dwell Volume [mL]: 0.48
 Extracolumn Volume [mL]: 0.004
 Time Constant [s]: 0.1
 Wavelength [nm]: 240
 Selected DataSystem: Lab Solution

(d) AIA - Files

- Run 1: tG1: 5 [min], T1: 30 [°C], tC1: 0 [% B2 in B1] 1_5.0 min_30.0 [°C]_tC0.0R1(PDA-Ch1).CDF
- Run 2: tG2: 15 [min], T1: 30 [°C], tC1: 0 [% B2 in B1] 2_15.0 min_30.0 [°C]_tC0.0R1(PDA-Ch1).CDF
- Run 3: tG1: 5 [min], T2: 60 [°C], tC1: 0 [% B2 in B1] 3_5.0 min_60.0 [°C]_tC0.0R1(PDA-Ch1).CDF
- Run 4: tG2: 15 [min], T2: 60 [°C], tC1: 0 [% B2 in B1] 4_15.0 min_60.0 [°C]_tC0.0R1(PDA-Ch1).CDF
- Run 5: tG1: 5 [min], T1: 30 [°C], tC2: 50 [% B2 in B1] 5_5.0 min_30.0 [°C]_tC50.0R1(PDA-Ch1).CDF
- Run 6: tG2: 15 [min], T1: 30 [°C], tC2: 50 [% B2 in B1] 6_15.0 min_30.0 [°C]_tC50.0R1(PDA-Ch1).CDF
- Run 7: tG1: 5 [min], T2: 60 [°C], tC2: 50 [% B2 in B1] 7_5.0 min_60.0 [°C]_tC50.0R1(PDA-Ch1).CDF
- Run 8: tG2: 15 [min], T2: 60 [°C], tC2: 50 [% B2 in B1] 8_15.0 min_60.0 [°C]_tC50.0R1(PDA-Ch1).CDF
- Run 9: tG1: 5 [min], T1: 30 [°C], tC3: 100 [% B2 in B1] 9_5.0 min_30.0 [°C]_tC100.0R1(PDA-Ch1).CDF
- Run 10: tG2: 15 [min], T1: 30 [°C], tC3: 100 [% B2 in B1] 10_15.0 min_30.0 [°C]_tC100.0R1(PDA-Ch1).CDF
- Run 11: tG1: 5 [min], T2: 60 [°C], tC3: 100 [% B2 in B1] 11_5.0 min_60.0 [°C]_tC100.0R1(PDA-Ch1).CDF
- Run 12: tG2: 15 [min], T2: 60 [°C], tC3: 100 [% B2 in B1] 12_15.0 min_60.0 [°C]_tC100.0R1(PDA-Ch1).CDF

(e) Eluent Data

Gradient: 10 → 95 [%B]
 Eluent A: 1: 100 % Water (H2O)
 pH: 7
 Eluent B1: 1: 100 % Acetonitrile (ACN)
 Eluent B2: 1: 100 % Methanol (MeOH)

Fig. 4. Data Entry page. On the top left is the Design of Experiments selected (a), followed by the column (b), instrument (c) and eluent data (e). Further down are the 12 basic experiments (d), imported in AIA-Format.

Automation conditions

Import runs

Automation Options

Machine Data

Instrument ID: 1 pH Buffer System: No pH Mixing
 No. Pumps: 3 Detector Type: PDA
 Wavelength [nm]: 240 Eluent Comp.: Ternary

Method Options

Basic Lsol Method: 10 API mix.lcm
 Save Data to: Desktop
 Files Name Template: use *.lcm

Eluent B	Eluent C	Vial No.	Tray No.	UV/VIS 1	UV/VIS 2	% B2
ACN	MeOH	1	1	240	0	

Equil. Time before injection [min]: 2.0

Return to Start-%B [min]: 0.1 End-%B Hold: 0.0

Run #	tG [min]	T [°C]	tC	Replicates	File Name
Run 1	5	30	0	1	
Run 2	15	30	0	1	
Run 3	5	30	50	1	
Run 4	15	30	50	1	
Run 5	5	30	100	1	
Run 6	15	30	100	1	

Gradient Program	Time	%B
Gradient Start	0	10
Gradient End	5 / 15	95
End-%B Hold	5 / 15	95
Return to Start-%B	5.1 / 15.1	10
End of Run	5.1 / 15.1	10

Start Runs Close

Fig. 5. Automation conditions, incl. instrument data, method options and run sequence and the conditions of the gradient elution process, incl. re-equilibration.

time (t_D) depends on instrument characteristic and on the flow rate. Applied gradient time (5 and 15 min), i.e. gradient-steepness, was selected after running preliminary experiments (see 2.3.). As during the preliminary runs, there were no signs of post-eluting “sticky” peaks, the application of hold-time (t_{hold}) was disregarded.

As it shown in Fig. 3, model experiments include all possible combinations of 2 temperatures and 3 organic compositions for two gradient times. Considering additional re-equilibration time and the extra time needed due to gradient delay, the complete batch took 152 min, ca. 2.5 h.

$$\text{total run time} = \sum_{i=1}^{12} \text{method time}_i = 2 * 3 * (5 \text{ min} + 15 \text{ min}) + 12 * (2 \text{ min} + 0.48 \text{ min} + 0.19 \text{ min}) = 152 \text{ min}$$

3.4. Design space verification

Correct matching of corresponding peaks under different conditions is often the Achilles-heel of model creation. **Peak tracking in DryLab** bases on the assumption that while the same sample amount was injected all the time, after each run, single and total peak areas remain closely similar. As was presented by Kormány et al., in most of the cases this offers a transparent way on following peak-movements [9], in some cases however, especially for close-eluting and for compounds present in similar amount, peak-tracking becomes difficult and less obvious. If the model turns out to show low predictive validity, troubleshooting has to be performed on previous steps (back steps in Fig. 1).

Evaluation of chromatograms at different wavelengths or using particular mass-data may help remediating these issues [13], but if no simultaneous or specific detection is available for the chromatographer, then there is no other way around than choosing

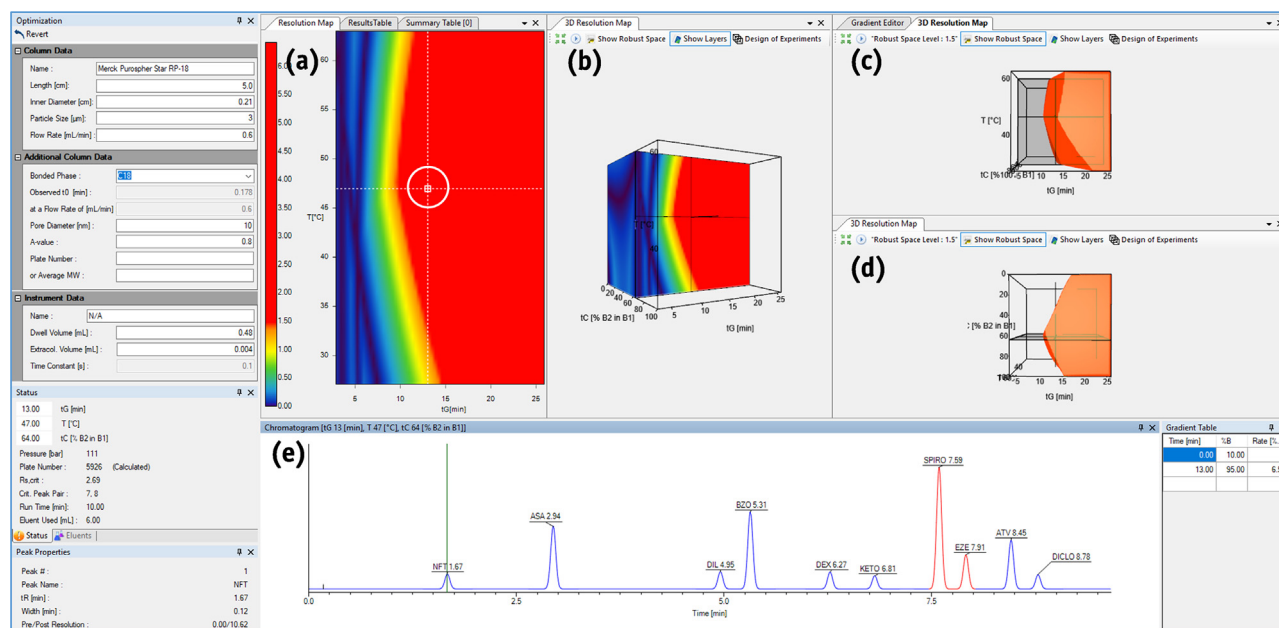


Fig. 6. The screenshot shows the same cube from different perspectives (b, c and d): (b) shows the design space, i.e. the influence of the 3 factors tG, T and tC at the position of the cursor. The corresponding chromatogram (e) shows the critical peaks in red. At the top (c) and (d) show the “Method Operable Design Region” (MODR) from different perspectives (tG-T-tC and tG-tC-T respectively), in which the separation is expected to have baseline resolution for the critical band pair (peaks 7 and 8) with $R_{s,crit} > 1.5$. As can be seen in (c) and (d), large parts of the cube remain empty, pointing to areas yielding critical resolutions below 1.5. Here, experiments are a waste of time and point to the (cost-) inefficiency coming with chromatographic work that is based on Trial and Error. (A) shows the cross-section of the cube as a tG-T-sheet (blue colors indicating coelution, yellow overlapping of peaks). The white cursor indicates the selected point at tC: 64% (MeOH in ACN) (V:V), T: 47[°C] and tG: 13 [min]. The panel on the left shows the parameters at cursor position such as tG, T, tC, column pressure, critical resolution and the critical peak pair along with analysis time, eluent consumption per run and the plate number. On the lower right, the user interface displays gradient data (startB%, endB%, and steepness). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

selected control points (or intended working-points) within the model and comparing with experimental results.

Fortunately, similarly to DoE-runs, in DryLab these runs can be carried out in an automated way. The experimental results showed an excellent match with the modeled ones, average difference was only about 0.026 min (≈ 1.6 s) and exceeded not more than 0.08 min (≈ 4.8 s) for linear gradient (Table 1) and also remained negligibly low for subsequently changed (multi-stepped) gradient profiles ($\Delta t_{ret,avg} = 0.027$ min; $\Delta t_{ret,max} = 0.090$ min). Furthermore, the middle point of the cube showed an excellent match too (see Table 1).

Focusing on the least-resolved peak-pair ($R_{s,crit}$) of any chromatogram, the software visualizes the design space (also called “the cube”) as a 3D resolution map, where all possible outcomes of experimentally measured factor combinations (tG-T-tC) are displayed [5,33]. Hence, blue lines mark complete coelution, yellow areas peak-overlaps and red areas the fortunate interplay of variables where for the critical-peak pair, at least baseline separation can be achieved ($R_{s,crit} \geq 1.5$). The irregular bodies, formed by these red points are also referred to as Method Operable Design Region (MODR) or Critical Resolution Space (CRS) (Fig. 6). Moving within this space presumes that the previously defined ATP-criteria are met.

As it was asserted in 3.1., the main aim of this study was to select an adequate method, which can deliver the fulfillment of the analytical target profile, in our case baseline resolution, even if the variables are varied within their tolerance limits.

The greatest advantage of this kind of modeling is, that within the design space the operator knows exactly the effect of the variables on the chromatography and this can be utilized for achieving the best available working point that delivers direct fulfillment of the goals outlined in the ATP, for the mixture, at a given column and chromatographic system.

Following the above mentioned, in the left-middle region of the cube, at 13 min, 47 °C and 64% of methanol content minimum

analysis time, an adequate and reasonable resolution (2.69) can be achieved (Fig. 6(a) and (e)). Picking this point as working point seemed to be not just an ideal choice because it offered a short, simple method, but heading deeper into the CRS, the expected exposure to a possible alteration of chromatographic parameters, and as a consequence failure of ATP-aims became significantly less likely compared to another working point, for instance at shorter gradient times, i.e. closer to the yellow margin area, so called the Edge of Failure (EoF). On the other side, the cube showed clearly that in general gradient time played a key role in selectivity changes while ternary composition and temperature had less, but still considerable significance (Fig. 6(a)–(d)).

3.5. Robustness

In a routine application however, due to changed chromatographic conditions, deviations and possible fluctuations of the method performance can be expected. This exposure is highly dependent on the selected working point and instrument specifications (tolerance limits) and can be characterized in a robustness study [6,34].

Fortunately, this can be done within the software, evaluating all relevant chromatographic factors influence at three levels (+1, 0, –1), resulting 3^6 theoretical experiments. Main advantage of this evaluation is that according to the instrument’s specification limits, single-, and cross-effects of the factors and their potential influence on the separation performance can be discovered and if necessary, needed steps and preventive actions (e.g. redefinition of working points, replacing instrument parts) can be taken (Fig. 7).

The selected working point under the defined tolerance limits showed perfect robustness (100%), in which gradient time and flow-rate represented the highest influence, starting-, and end B%, temperature had only a limited influence. This indicated that pump condition is relevant and needs to be handled with special care.

Table 1

Singe peak retention time, average and maximum retention time deviations, expressed in minutes between modeled and real chromatographic runs. Design middle point is at tG = 10 [min], T = 45 [°C] tC: 50%.

	tG T							
tC Comp	6 min 35 °C 80%	6 min 50 °C 18%	10 min 28 °C 100%	10 min 45 °C 50%	10 min 45 °C 62%	14 min 35 °C 25%	15 min 50 °C 40%	6.5 min 35 °C 50%
NFT	0.003	0.022	0.002	0.053	0.016	0.022	0.009	0.029
ASA	0.013	0.026	0.005	0.060	0.012	0.038	0.017	0.045
BZO	0.072	0.031	0.026	0.016	0.074	0.005	0.018	0.080
DIL	0.053	0.011	0.045	0.041	0.072	0.037	0.002	0.006
DEX	0.056	0.032	0.021	0.018	0.074	0.045	0.015	0.023
KETO	0.015	0.012	0.021	0.022	0.045	0.001	0.001	0.022
SPIRO	0.018	0.000	0.019	0.007	0.040	0.017	0.006	0.021
EZE	0.024	0.004	0.011	0.050	0.066	0.012	0.015	0.005
ATV	0.015	0.008	0.036	0.019	0.027	0.026	0.013	0.008
DICLO	0.007	0.028	0.014	0.020	0.028	0.072	0.048	0.020
AVG dev.	0.026 [min]							
MAX dev.	0.080 [min]							

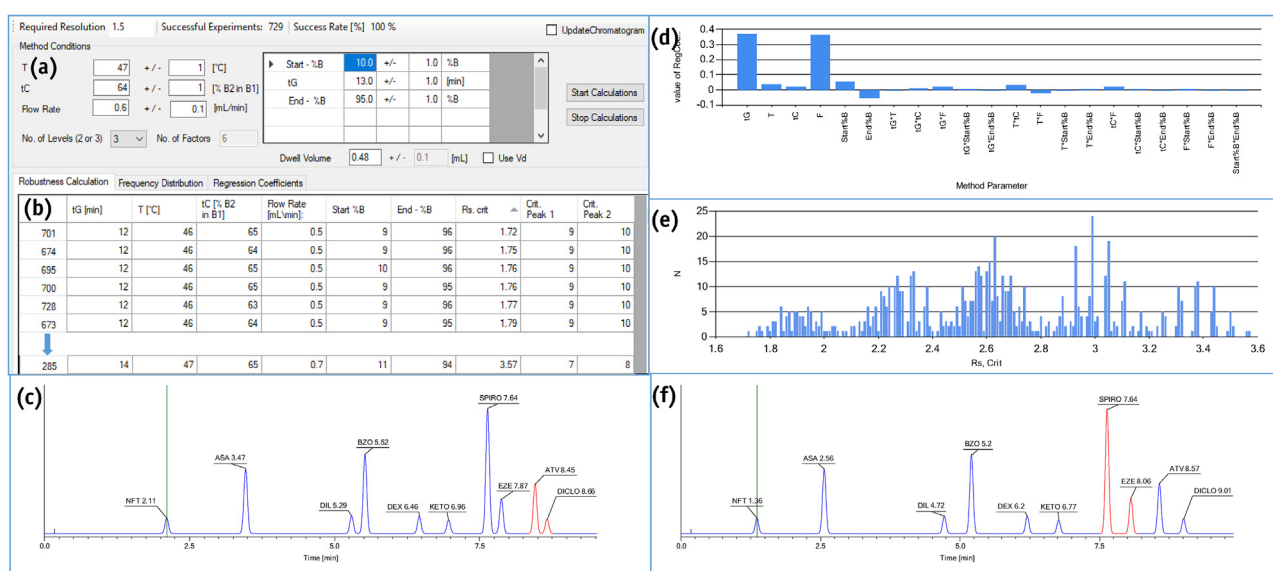


Fig. 7. Evaluation of method robustness at the selected work point (a): 6 variables (T, tC, flow-rate, start%B, tG and end%B) were varied between their individual tolerance limits in $3^6 = 729$ experiments. (b) shows their table, here sorted according to critical resolution, the six worst experiments with the lowest $R_{s,crit}$ at the top. Each experiment is backed up with a chromatogram and quantified in a distribution of $R_{s,crit}$ (e). The chromatograms of the worst (701) and best case (285) are displayed (c and f). Critical peak-pairs are marked in red. The plot of the regression coefficients (d) shows, which parameters and cross effects are the most influential regarding critical resolution.

These specification limits were chosen rather conservatively to withstand the worst case. For the flow rate $\pm 1\%$, for the gradient mixing $\pm 0.5\%$ and for the column thermostat ± 0.1 °C is specified by the instrument producer, nonetheless this can deviate depending on the operating range (e.g. at very low flow-rate gradient formulation) and the time of operation [35].

In addition to the above mentioned, model-based calculation of robustness has the exceptional advantage to reliably simulate all the possible outcomes of any changes in influential conditions, supported by corresponding chromatograms. For instance, in Fig. 7(c) and (f) a changed critical peak pair can be observed.

Out of the 729 experiments modeled in DryLab's robustness module, the worst cases are those that are most likely to fail if performed under varying, in-vivo lab conditions. Automation was executed to confirm those experimental runs too. Similarly to Kormányi et al. previous analogous work [7], the automated results showed an excellent agreement with in simulation predicted values (Table 2 and Fig. 8).

3.6. Continuous improvement

Another advantage of model-based chromatographic separation is that numerous other virtual experiments can be calculated

within no time that enables to select other possible working points or to investigate the effect of a changed gradient profile, modified chromatographic conditions or to evaluate a method's transferability between an UHPLC and HPLC instruments [16,36]. The possibilities are almost unlimited, materializing a Continuous Improvement Strategy based on solid science.

3.7. Knowledge management

As a general documentation to be used in regulatory and other contexts, a Knowledge Management Document (KMD) was created. This report, in a compliance with QbD, incorporates each steps of a consistent method development, including a step-by-step justification of method choices (Fig. 9). This comprehensible and harmonized documentation ensures a high standard for knowledge transfer between laboratories, helps to facilitate regulatory submissions (e.g. post-approval changes) and should provide answers in cases of general liability issues [8].

4. Summary

Creating well-performing, robust methods is the main focus of analytical work. However, from a practical perspective, reducing

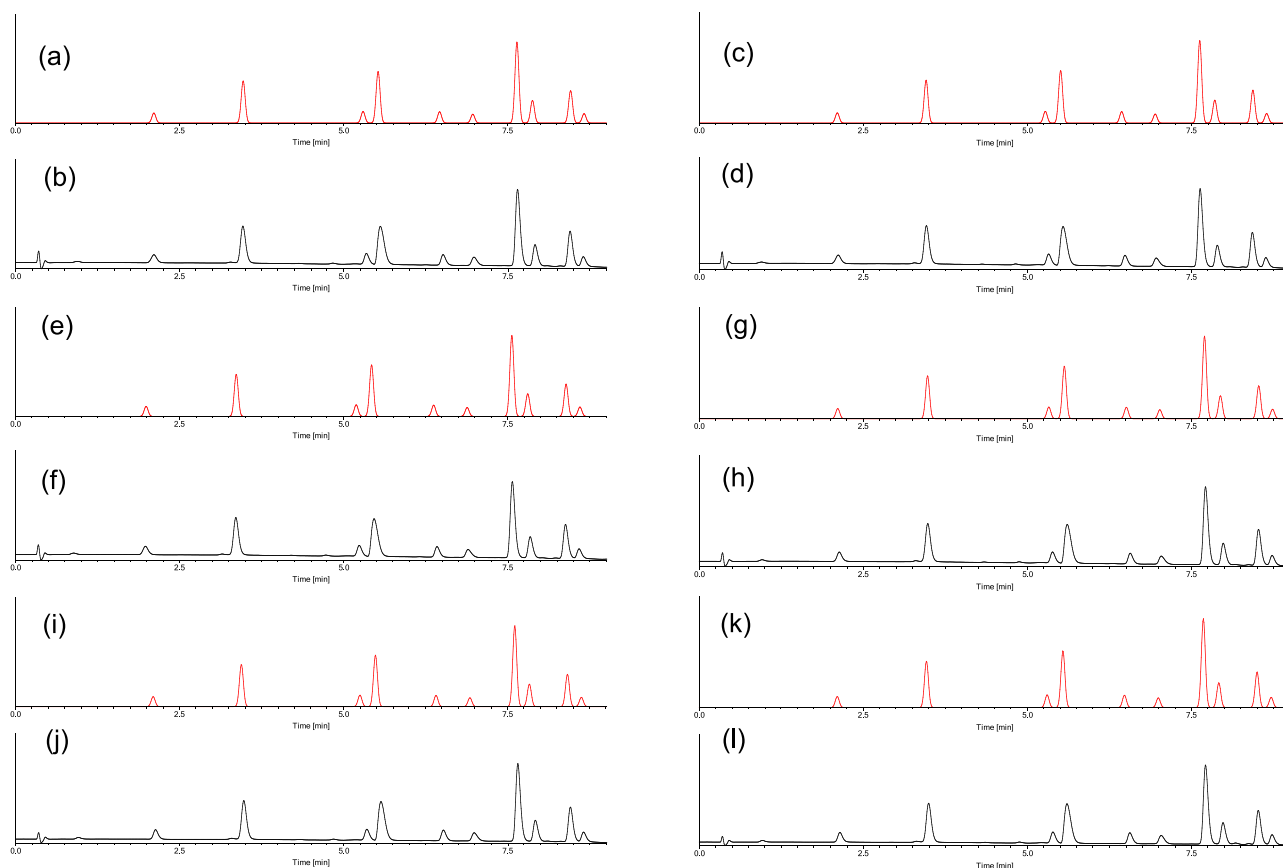


Fig. 8. Comparison of worst case experiments for robustness evaluation between DryLab models and in-vivo experiments: run 701: modelled (a) and experimental chromatogram (b), run 674: model (c) and experiment (d), run 695: model (e) and experiment (f), run 700: model (g) and experiment (h), run 728: model (i) and experiment (g), run 673: model (k) and experiment (l), respectively (for quantified comparison see Table 2).

DryLab Knowledge Management

Project Information
Explain your Method

Project Information

Project Name: Automated UHPLC separation of 10 pharmaceutical compounds Responsible: A. Zöldhegyi, I. Molnár, L. Fekretidnova

Project Description: Automated separation of 10 comp. Analytical Target Profile (ATP): Robust separation for all API's

Automated UHPLC separation of a test mixture, containing 10 pharmaceutically relevant compounds. Establishing robust baseline separation ($R_s=1.5$ or higher) for each of the API peaks in minimum analysis time and possibly at mild conditions.

Risk Management (RM): RM to fill Method Control Strategy (MCS): MCS to fill

Selection Critical Separation Parameters were based on prior knowledge and experience with similar projects: gradient time (tG), temperature (T) and ternary composition (B2 in B1) of the eluent B were judged to be the most potential chromatographic factors. The assurance of analytical performance and quality based on a three level control establishment. On one hand, created model-input runs (Comparison module), on the other hand selected point-experimental runs (Run experiment module) were tested out and compared. The deviation of peak retention times exceeded not more than 0.1 min. For the last, a System Suitability Test was carried out, where the resolution criteria for the critical peak pair (peak 7 and 8) met in the ATP defined ($R_s>1.5$) requirements.

Justification of the Initial Conditions: Gradient / Temperature / IC (12 runs) Justification of the Set Point: Standard Justification

After implementation of preliminary experiments (screening) on the selected column (Merck Purospher Star RP-18 50x2.1 mm, 3 μ m), the effect of most influential factors were evaluated in a systematic way. Gradient time was studied at two levels (5 and 15 min), temperature at 2 levels (30 and 60 °C) and ternary composition (MeOH in ACN) at 3 levels (0,50,100%, respectively). pH-control was maintained with the application of 10 mM formic acid (eluent A).

Selection of the working point was carried out within the pronounced Eluent Design Space by visual inspection, considering ATP-defined goals, high critical resolution (R_s , crit), good robustness and fast separation of the method. Robustness evaluation of the selected point was subsequently examined in Robustness simulation.

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Fig. 9. Final compendium of the project according to in Fig. 1 defined steps. On the next page of the document method's robustness, experimental comparison and the DoE-fashion were also recorded. Final document was exported in pdf-format.

Table 2

The 3-digit numbers are the run-numbers of the modeled 729 virtual experiments. Retention time deviations between modelled and real chromatographic runs for the six worst cases in robustness calculations, expressed in minutes (for specified worst case run conditions the reader is redirected to Fig. 7(b)).

Comp.	701	674	695	700	728	673
NFT	0.002	0.015	0.010	0.023	0.019	0.041
ASA	0.003	0.006	0.005	0.003	0.010	0.032
BZO	0.054	0.053	0.049	0.056	0.050	0.088
DIL	0.037	0.040	0.041	0.044	0.042	0.062
DEX	0.056	0.053	0.054	0.059	0.050	0.082
KETO	0.021	0.019	0.017	0.022	0.015	0.044
SPIRO	0.014	0.011	0.011	0.015	0.008	0.032
EZE	0.043	0.043	0.041	0.044	0.042	0.065
ATV	0.004	0.004	0.001	0.003	0.006	0.019
DICLO	0.007	0.008	0.009	0.008	0.009	0.014
AVG dev.	0.029 [min]					
MAX dev.	0.088 [min]					

development time by eliminating possible human mistakes should receive more attention. Automatedly created methods can remediate issues and help to reduce the failure rate, achieving better transparency, unviolated data integrity, and a higher throughput.

As it was demonstrated in this study, automated execution can be utilized for reliable and accurate chromatographic model creation, verification and working point robustness simulation for a real-life sample. Modeled results were subsequently underpinned per experimental confirmation using an automation tool and were found to be in excellent agreement. These possibilities will allow a step forward to the upcoming tendency of in silico analytical method development.

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