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Aspects of the "Design Space" in high pressure liquid chromatography method development

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ABSTRACT

The present paper describes a multifactorial optimization of 4 critical HPLC method parameters, i.e. gradient time (t_G), temperature (T), pH and ternary composition (B_1:B_2) based on 36 experiments. The effect of these experimental variables on critical resolution and selectivity was carried out in such a way as to systematically vary all four factors simultaneously. The basic element is a gradient time–temperature (t_G–T) plane, which is repeated at three different pH's of the eluent A and at three different ternary compositions of eluent B between methanol and acetonitrile. The so-defined volume enables the investigation of the critical resolution for a part of the Design Space of a given sample. Further improvement of the analysis time, with conservation of the previously optimized selectivity, was possible by reducing the gradient time and increasing the flow rate. Multidimensional robust regions were successfully defined and graphically depicted.

1. Introduction

In 1989 a concentrated effort was made by Snyder and Glajch to ease the HPLC method development process with the aid of computer supported technologies [1] including one- (one-factor-at-a-time OFAT) and two-dimensional modelling. Since then, retention mapping coupled with experimental design strategies have continued to be used as a powerful method development and optimization technique. Indeed, HPLC method development software such as DryLab® [1–3], as well as other software from ACD [4] and Merck's ChromSword [5], though tackling the method development task in different ways, still today use similar resolution maps to clearly identify optimal conditions. They are continually looking for ways to easily and practically optimize many factors simultaneously and change critical resolution seamlessly.

HPLC method robustness and Quality by Design (QbD) [6] are playing an important role in the global economy, where pharmaceutical and chemical products are distributed worldwide and the method transfer process has been running smoothly for the same product in different countries and in different laboratories. Regulatory authorities (FDA, ICH, etc.) nowadays are promoting and requesting the application of QbD principles to ease the exchange of complex information about chromatographic selectivities and critical resolution values to support better method control, including method transfer. The result is a better understanding of how a method should be communicated and adjusted to ensure a given requested performance.

Furthermore, the ICH Q8 (R2) [7] made a clear movement towards more flexibility to support the development of new products in industrial environments. The appearance of terms such as Quality by Design (QbD) and Design Space (DS) are an indication of this growing trend [8] requiring also a high level of understanding of the basic roles of HPLC.

The present paper combines a more advanced resolution and selectivity mapping technology of the four above-mentioned factors with changes in column dimensions, gradient shape and flow rate, with the new mind set of the regulatory authorities to build in a more scientific and multifactorial approach to HPLC method development to reduce costs caused by trial and error.

The shortcomings of an OFAT approach or of a 2D model are presented in Fig. 1 where an edge of the cube presents an OFAT method and a plane inside is presenting a 2D model. In Fig. 1 we can see three planes, each of them corresponding to a 2D resolution map. The total space as such however, is experimentally not fully explored showing large volumes of unknown space. To experimentally fill out this space, we designed a new approach, where the corners of the cube are measured and each point in the volume is calculated and corresponds to a highly precise chromatogram.

A further aspect was to try to reduce costs by searching for robust methods with less acetonitrile and more methanol in eluent B.

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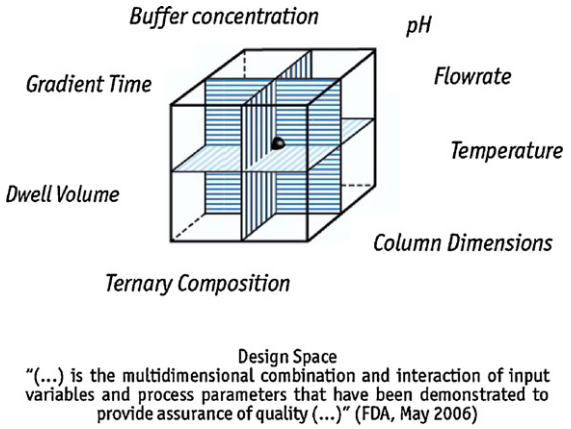

Fig. 1. Presentation of the Design Space for three factors described by 2D planes. The working point is in the middle of the cube and represents a result of the best critical resolution. Three different resolution maps are shown here as individual parts of the whole Design Space.

2. Experimental

2.1. Eluents

Methanol and acetonitrile (gradient grade) and HPLC–water were purchased from Merck (Darmstadt, Germany). Eluent A was prepared by combining varying volumes of aqueous buffers of differing pH (A1 and A2): A1 was a solution 0.1 M phosphoric acid and A2 was a solution 0.1 M monobasic sodium monophosphate. For pH 2.4 we mixed 31% A1 and 68% A2 (V:V), for pH 3.0 10% A1 and 89% A2 (V:V) and for pH 3.6 3% A1 and 96% A2 (V:V).

Eluent B was varied between acetonitrile (B1) and methanol (B2). Gradient elution between 0% and 100% B was used at a flow rate of 0.8 mL/min unless indicated otherwise. In this work gradient times of 30 min and 90 min, temperatures of 30 °C and 60 °C and pH values of 2.4, 3.0 and 3.6 were selected, largely in compliance with recommendations from Snyder et al. [9]. Ternary eluents were acetonitrile (100%) methanol (100%) and a mix of (acetonitrile:methanol) (50:50) (V:V).

2.2. Sample

Model substances and reference materials used were DL-mandelic acid, phthalic acid, *m*-hydrophenylacetic acid, isovanillic acid, paracetamol, *p*-hydroxyphenylacetic acid, benzyl alcohol, vanillic acid and 4-hydroxy-3-methoxy benzyl alcohol incl. some impurities and degradation products were purchased from Merck (Darmstadt, Germany). For systematic studies with three-dimensional resolution models we needed a stable sample mix which would be available over a longer period of time. More practical examples from our work are published in the book of Ermer and Miller [10].

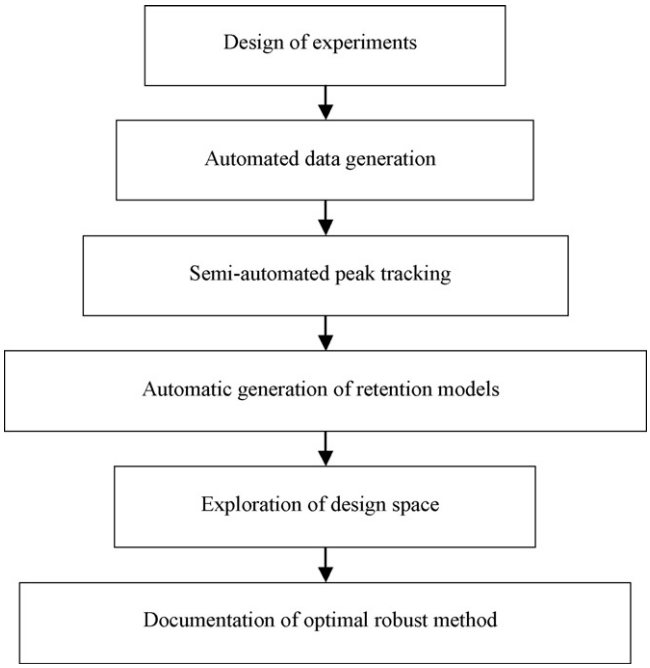

Fig. 3. Work flow scheme followed during the present study.

2.3. Equipment

HPLC separations were performed on a Shimadzu LC-2010C with integrated 4-liquid gradient system, high-speed and cooled autosampler, temperature controlled column compartment and Shimadzu UV–VIS detector (Shimadzu Europe, Duisburg, Germany). UV detection was performed at 254 nm. The dwell volume was 1.06 mL. ACE C18 columns (150 mm × 4.6 mm, 3 μm) were provided by HiChrom (Reading, United Kingdom) and HALO C18 columns by MacMod Inc., (Chadds Ford, PA 19317, USA).

2.4. Software

HPLC separations were generated using the automation option of DryLab®2010, which includes PeakMatch® v. 3.60 and DryLab® v. 3.95 (Molnár-Institute, Berlin, Germany) coupled with Shimadzu's LCsolution integration software. Peaks were identified and aligned based on peak areas using user friendly tools, such as peak turnover and peak splitting functions of the software, reducing the usual problems of common misalignments between peaks. Modelling was performed in DryLab®2010 and predictions were compared with the original experiments to control the validity of the

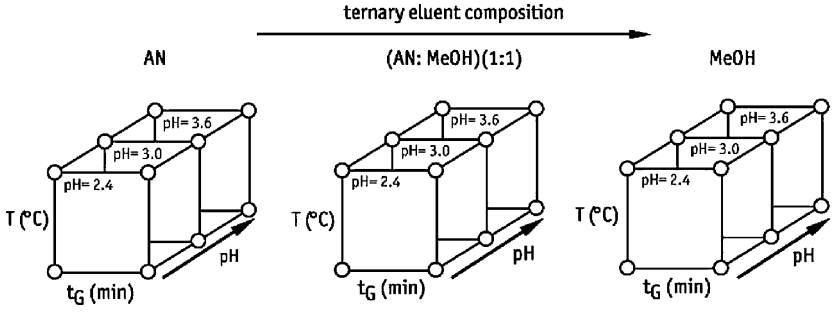

Fig. 2. Experimental design for a four-dimensional HPLC method optimization.

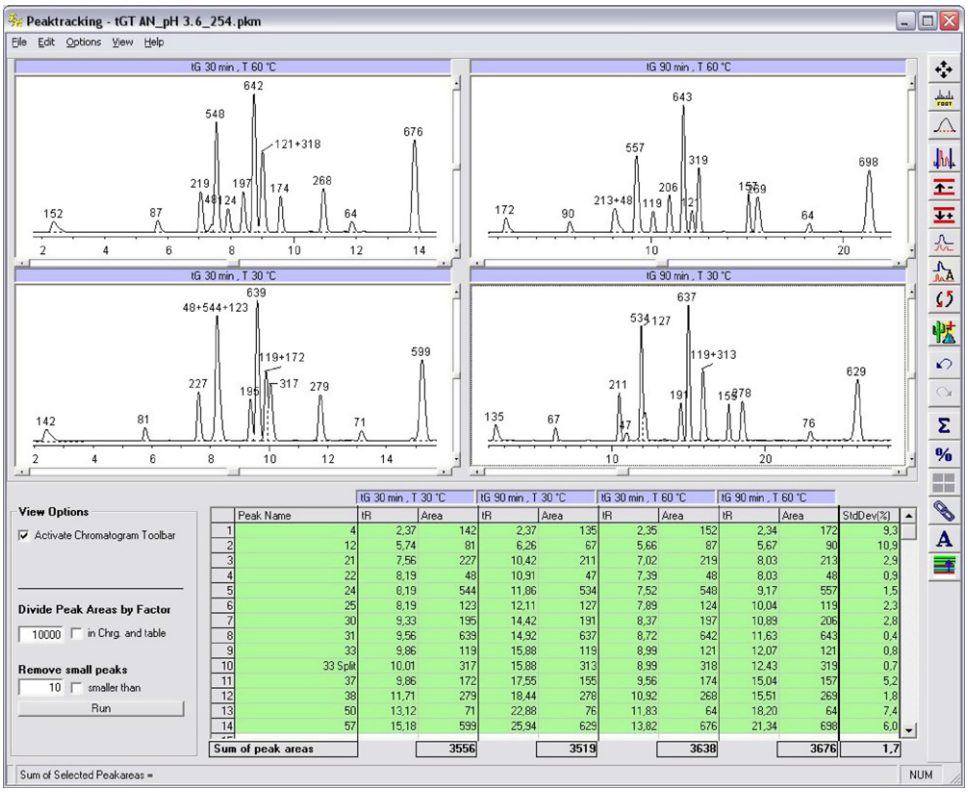

Fig. 4. Four different runs constituting a gradient time–temperature (t_G–T) model, showing different selectivities and the corresponding matched peak table. t_G: 30 and 90 min, T: 30 °C and 60 °C, pH 3.6, 100% acetonitrile, other conditions as described in Section 2.

modelling process. Generation of 3D resolution models was carried out with a new proprietary algorithm in DryLab®2010.

2.5. Experiments for modelling

Initial input data were acquired under the following conditions: gradient times (t_G) of 30 and 90 min, temperatures of 30 °C and 60 °C, eluent A was 100 mM phosphate buffer and had pH values of 2.4, 3.0 and 3.6.

Ternary eluent compositions were of (a) 100% AN, (b) AN:MeOH (50:50) (V:V), and (c) 100% MeOH and were combined to produce 36 experiments as shown in Fig. 2. Input data, 4 × 9 experimental runs, were performed overnight automatically with the Shimadzu LC-2010C controlled from PeakMatch®. After the runs were finished, they were exported automatically to PeakMatch for the peak tracking process. Finally the data were transferred to DryLab®. The plate number was adjusted in various computer simulations of separation to the real column performance.

3. Results and discussion

We know one-dimensional and two-dimensional resolution spaces quite well. The goal in this work was to add further parameter to three-dimensional resolution spaces and increase in this

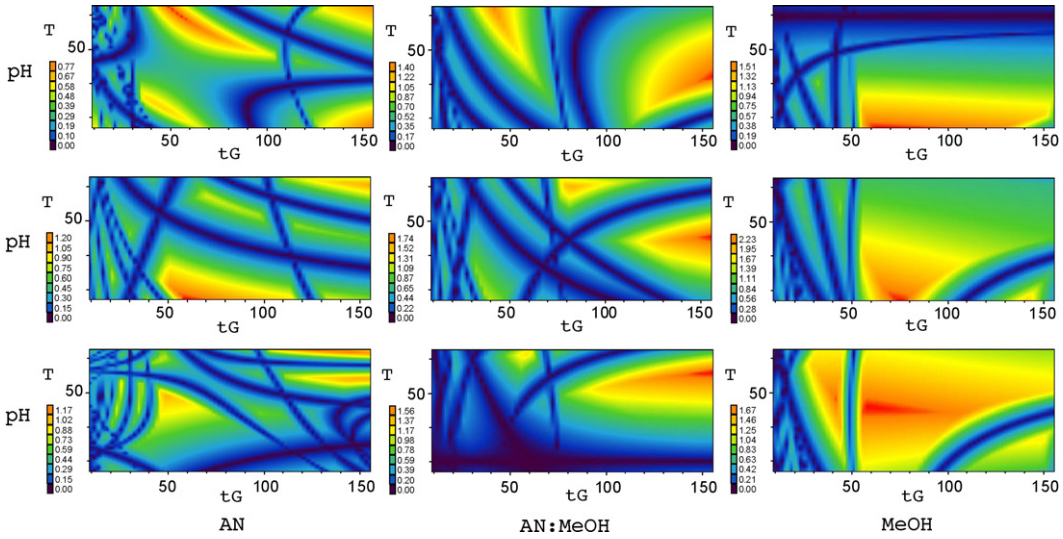

Fig. 5. Two-dimensional resolution maps of gradient time, t_G, [min] against the column temperature T [°C] for each of the nine experimental sets.

way the choices for establishing robust HPLC regions which will give more flexibility in routine work.

3.1. Work scheme

The work flow followed in this project can be divided into six steps, which can be found schematically in Fig. 3 and are described in detail.

3.1.1. Design of experiments

This initial step consisted of the selection of critical parameters which were to be optimized and the establishment of the range over which they were to be varied.

3.1.1.1. Description of the sample. The selection of the critical parameters was done independently of the nature of the sample. Therefore, it is believed that all so-called "regular" samples could be treated in the same manner as described here. Special samples, such as of pharmaceutical origin, but also organic ions, isomers, enantiomers, biological samples, peptides and proteins and other macromolecules such as oligonucleotides are also thought to be successfully separated following these steps, though slight adjustments are likely to be necessary.

3.1.1.2. Definition of goals. Defining separation goals before method development commences both prevents unnecessary experimentation and enables building in the required flexibility from the outset. Depending on the particular requirements of a separation, these goals can vary to have more or less constraints, though typically maximum separation of all or selected peaks and minimum run times are key. As previously stated, the goal of this optimization was the maximum separation of all compounds with a minimum on run time, all within a known robust working region.

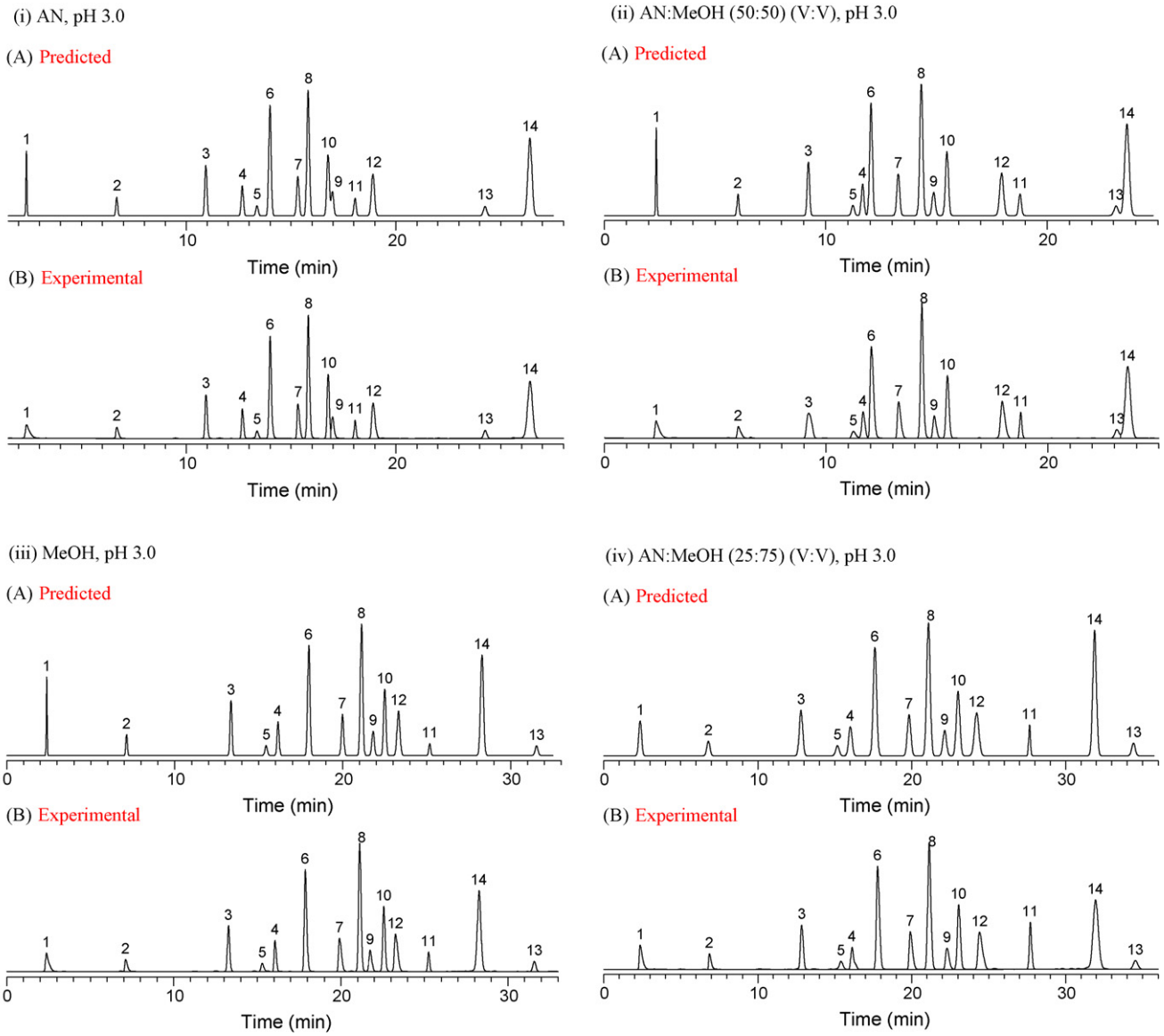

Fig. 6. Predicted (A) and experimental (B) chromatograms for optimal conditions of temperature, gradient time, ternary eluent composition and pH. Column: ACE C18 (150 mm × 4.6 mm), 3.0 μm, mobile phase "A": 100 mM phosphate buffer pH 3.0, mobile phase "B": acetonitrile (i), acetonitrile:methanol (50:50) (V:V) (ii), methanol (iii), acetonitrile:methanol (25:75) (V:V) (iv), gradient elution: 0–100% B, t_G and T at: (i) 57 min, 29 °C, (ii) 153 min, 45 °C, (iii) 74 min, 27 °C, (iv) 120 min, 42 °C, analytes: (1) impurity, (2) impurity, (3) paracetamol, (4) 4-hydroxy-3-methoxy benzyl alcohol, (5) DL-mandelic acid, (6) phthalic acid, (7) *p*-hydroxyphenylacetic acid, (8) vanillic acid, (9) *m*-hydrophenylacetic acid, (10) isovanillic acid, (11) 4-hydroxy-3-methoxy benzyl alcohol degradation product, (12) impurity, (13) benzyl alcohol degradation, (14) benzyl alcohol.

3.1.1.3. Definition of critical parameters. The four parameters gradient time, temperature, pH and ternary eluent composition were chosen to be optimized first due to their known strong influential effect on selectivity. Flow rate, gradient slope, gradient shape, column parameters, dwell- and extra column volume can be varied within the software to investigate selectivity changes, which is a well known feature of DryLab®, but they are not treated here. In order to keep the number of experiments within a practical range, buffer type and concentration (ionic strength) were kept constant and an assessment of further additives was not carried out.

Column switching is a good way to change chromatographic selectivity. However even after choosing the "best" column, selectivity is changing in gradient elution strongly with temperature, gradient form and shape, pH, ternary eluent, flow rate, column length and diameter, just to mention the most important influences. We intended to study primarily the above-mentioned factors on an excellent C18 column to be able to control the method better according to the principles of Quality by Design (QbD). All experiments took place therefore on the same ACE C18 column (150 mm × 4.6 mm, 3 μm), selected on the basis of good peak shape and maximum number of obtained peaks, determined by preliminary basic scouting runs. Additional work on HALO columns showed also excellent performance [11].

Two gradient times, two temperatures, three pH values and three ternary eluent compositions were necessary in order to reliably create retention models of these factors with DryLab®. Gradient times should differ in a factor 3, temperatures in 30 °C, pH in 0.6 pH units and ternary eluent composition was varied between MeOH and AN. These ranges are large enough to induce significant band movement to discover hidden peaks and small enough to enable relatively easy peak tracking.

3.1.1.4. Replacement of acetonitrile by methanol. Due to the actual shortage and high price of acetonitrile we tried to find eluent compositions to reduce the amount of acetonitrile in the mobile phase. The ternary eluent was chosen so as to encompass the whole range from 100% acetonitrile to 100% methanol. This way the replacement of acetonitrile by methanol could be assessed to a large degree.

Table 1
Highest critical resolution for all nine experimental sets (combinations of pH and ternary eluent composition). Other experimental conditions are omitted for clarity.

Ternary eluent	pH	$R_{s,crit}$ (max.)
AN	2.4	1.25
AN	3.0	1.31
AN	3.6	0.80
AN:MeOH (1:1)	2.4	1.63
AN:MeOH (1:1)	3.0	1.85
AN:MeOH (1:1)	3.6	1.51
MeOH	2.4	1.77
MeOH	3.0	2.44
MeOH	3.6	1.63

3.1.2. Automated data generation

Once the experiments were designed, chromatograms were acquired by means of automated data generation. This was executed within the software PeakMatch® coupled with Shimadzu's LCsolution software.

Chromatographic conditions, equipment, column and eluent data are first keyed into the input data interface. Then, upon "starting runs" the relevant experimental data are loaded into a batch file and read by the chromatographic system. Once the batch has been completed, chromatograms are automatically integrated and imported ready for peak tracking.

3.1.3. Peak tracking

Upon changing eluent properties and the temperature, chromatographic selectivity (the separation) also changes. Therefore prior to importing data into DryLab® for modelling, peak tracking had to be carried out. Peak tracking refers to the matching of bands for the same compound between runs where conditions have been changed. In a t_G–T-model four experiments have to be aligned in a way, that all the data (tR's and peak areas) of one substance are in one horizontal line. The peak identification procedure was carried out on the basis of peak areas, which are fairly constant in the four runs of a t_G–T-model. The prerequisite for this process is the injection of the same sample mix and of identical sample volume. In this case peak areas represent sample mass (concentration × volume) and are at constant flow rate constant in each individual run, fairly independent of other experimental conditions.

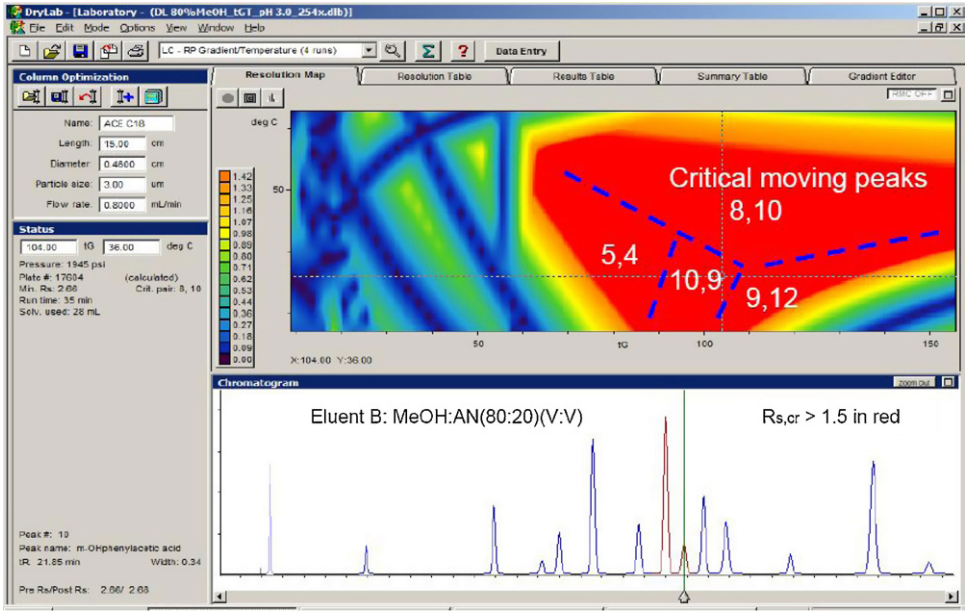

Fig. 7. Critical moving peaks. Resolution map showing a t_G–T-model for pH 3.0 and 80% methanol. Represented is a robust region $R_{s,crit} > 1.5$, in which four critical moving peaks are present. In order to build in flexibility and a long life into a method, critical moving peaks should be identified and documented in the validation process.

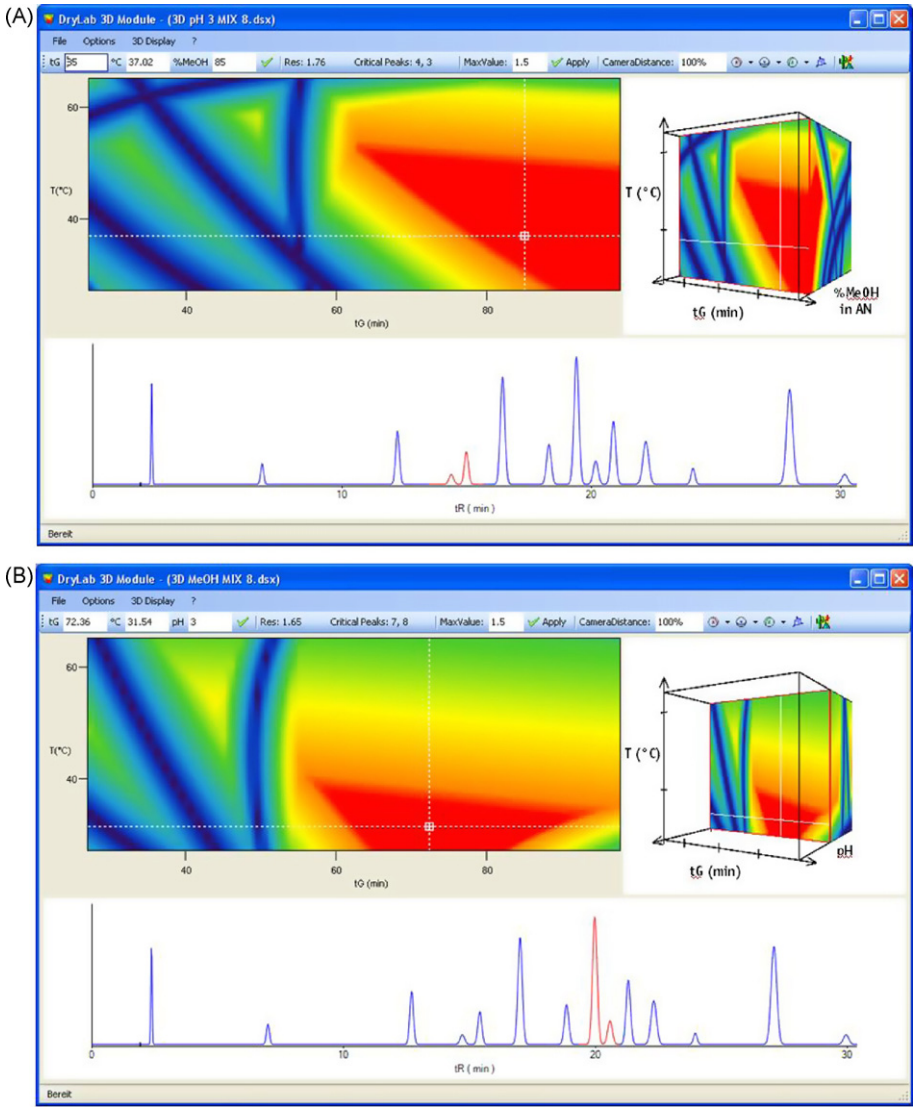

Fig. 8. 3D resolution spaces. (A) Gradient time–temperature–ternary eluent composition and (B) gradient time–temperature–pH models for the same sample as described in Fig. 6.

As mentioned above peak tracking was accomplished in a semi-automated fashion within the programme PeakMatch®. First general complexity, as shown in Fig. 4 was reduced by dividing large peak area numbers to memorable ones, relevant chromatogram areas were zoomed into and irrelevant bands below 0.1% were deleted. Then an automatic operation function was used to match peaks followed by manual adjustments. The result of this step is a fully matched peak table.

3.1.4. Automatic generation of the retention model

Once a matched peak table was obtained, all data were automatically transferred into DryLab®, where the resolution map was generated and optimal conditions could be ascertained by a special comparison of the original experiments with their modelled versions.

In this study nine t_G–T-resolution maps were produced, corresponding to three pH values and three ternary eluent compositions (Fig. 5).

3.1.5. Exploration of the Design Space for t_G, T, pH and ternary composition

Two-dimensional resolution maps were used to create three-dimensional resolution spaces, in which the combined influence of four parameters could be simultaneously assessed, visualized and optimized. This was carried out first for t_G–T–pH and then for t_G–T–ternary eluent compositions. By resetting the resolution options shown in the resolution maps, robust regions ($R_{s,crit} > 1.5$) could be isolated and studied and it was found that these robust regions were continuous voluminous shapes. With this, optimal working conditions could be ascertained visually within the described Design Space.

3.2. Input resolution maps

To briefly recapitulate, it is recommended to set the starting point for any RP-HPLC method optimization to the evaluation of influences exhibited in t_G–T-models [3]. From four basic experiments, t_G–T-models were created in the form of resolution maps, in which ca. 4000 highly precise virtual chromatograms were represented each. These so-called t_G–T-models were taken as a basic element of the models and subjected systematically to three different pH values and three different ternary eluent compositions resulting in nine resolution maps containing predicted chromatograms for ca. 36,000 experiments (Fig. 5). The best one could be selected after a few minutes.

Table 2
Retention time comparison between predicted and experimental data for pH 3.0 using eluent B: (acetonitrile:methanol) (25:75) (V:V), at $T = 42\,^{\circ}C$, $t_G = 120$ min, gradient range 0–100% B, $R_{s,crit} = 2.67$.

Peak no.	Retention time (min)			
	Experimental	Predicted	Difference[a]	% error[b]
1	2.36	2.36	0.00	0.00
2	6.86	6.79	0.06	0.96
3	12.85	12.81	0.04	0.34
4	15.40	15.17	0.23	1.51
5	16.12	16.02	0.11	0.67
6	17.78	17.61	0.17	0.98
7	19.91	19.83	0.08	0.38
8	21.13	21.09	0.04	0.21
9	22.29	22.14	0.15	0.65
10	23.05	23.01	0.04	0.18
11	24.41	24.22	0.19	0.77
12	27.70	27.66	0.05	0.17
13	31.94	31.88	0.06	0.19
14	34.52	34.40	0.12	0.35

[a] Difference = experimental − predicted retention time.
[b] % error = [(experimental − predicted)/predicted] × 100.

In Table 1 the highest critical resolution for all nine experimental sets are shown. As can be seen, critical resolution was highest for pH 3.0, lowest for 3.6 and intermediate for pH 2.4 independently of the ternary eluent. Additionally, the replacement of acetonitrile for methanol results in an increase in critical resolution at each pH value. The best separation out of these initial nine models was found to be for pH 3.0 using 85% methanol, but in pure MeOH as eluent B, there is still a better resolution, than in acetonitrile.

Experiments were run at the optimal conditions of temperature and gradient time for all three ternary eluent compositions at pH 3.0. A comparison of predicted and experimental chromatograms is presented in Fig. 6. An excellent correlation between experimental and modelled data was found. One exception is peak 1 which shows a larger peak width and some tailing. This effect is a result of the dilution of the sample in the injection loop.

It was observed that the critical peak pair was not constant between the chromatograms, indicating the presence of several "critical moving peaks" (=strongly moving peak) in the mixture. This observation prompted an examination on the critical peak pairs within each individual resolution map, and it was found that each robust region was made up of sub regions corresponding to different critical peak pairs, as shown in Fig. 7.

The importance of this finding is, that the fixation of the method to only one single critical band pair is not sufficient and is not practical, as small changes in experimental conditions could change the critical peak pair, having the consequence, that a new validation has to be carried out. It is more reasonable to define the Design Space for several of critical peak pairs above $R_{s,crit} > 1.5$ (or another value) in the method description as shown in Fig. 7.

3.3. Experiments to explore parts of the HPLC Design Space

The posterior evaluation of the 36 input runs after carrying out 9 peak tracking processes led to 6 different 3D resolution data sets, which enabled the creation of intermediate resolution maps to be plotted. The nine 2D resolution maps were used for the calculation of additional t_G–T-planes, resulting in six 3D-cubes obtained by using a proprietary algorithm. Two of these cubes are shown in Fig. 8.

3D resolution spaces represent the simultaneous influence of three parameters on selectivity and critical resolution. The region for method robustness can be visualized as a geometrical body within the resolution space, in which critical resolution does not fall below base-line separation ($R_{s,crit} > 1.5$). Fig. 8(A) shows a

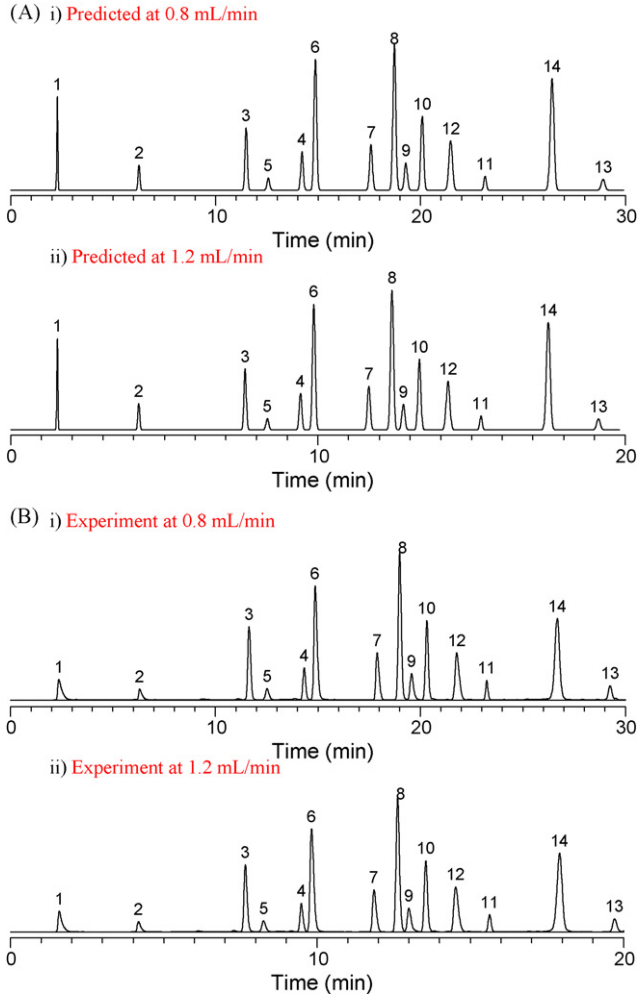

Fig. 9. Reduction of run time. (A) Predicted and (B) experimental chromatograms at flow rate (i) 0.8 mL/min and (ii) 1.2 mL/min.

t_G–T–ternary resolution space at a fixed pH value of 3.00. In this special cube robustness could be defined as: t_G: 85 ± 3 [min], T: 37 ± 3 [°C], ternary eluent composition: 85 ± 3 [% MeOH in AN] at pH 3.0. The robustness space can be moved also to other points within the same cube by changing the coordinates of the middle point of the robust sphere. However, the size of the tolerances may change to smaller values.

There were more than one robust region for a given mixture and different robust spheres can be defined within different cubes for different conditions. Another possibility would be to work for economic reasons at eluent B: 100% MeOH and change the pH having the best conditions at pH 3.0 as shown in Fig. 8(B). In some regards this working point is more favourable than the previously described one as the ternary eluent composition error is reduced to zero. At this working point the pH tolerance can be assessed and a new robust sphere defined with an $R_{s,crit} > 1.20$ at t_G: 76 ± 1 [min], T: 32 ± 1 [°C] and pH: 3.00 ± 0.1 pH unit.

Another possible working point is that of highest overall critical resolution. A comparison of predicted and experimental retention times for this working point within this 3D resolution map at (AN:MeOH) (25:75) (V:V) and pH 3.0 is shown in Table 2. Correlation was found to be excellent. The average difference between predicted an experimental retention times is approximately 0.1 min (6 s) and the largest difference is 0.23 min (14 s).

As mentioned in Section 1, further optimization of flow rate and gradient shape was carried out within the software. We are able

to transfer the chromatogram at the best set of conditions to the gradient editor and make further improvements such as changing gradient shape, changing starting or/and final % B, column length, ID, dwell volume or flow rate. To test this, we changed the flow rate by a factor 1.5 and decreased the gradient time by the same factor. At the same time gradient run time was reduced by a third with preservation of the separation selectivity. These results were predicted by the software and as seen in Fig. 9A and B, correlate excellently with experimental data.

4. Summary

A strategy for simultaneous multifactorial HPLC method optimization is presented in which automatic data generation, semi-automatic peak tracking, multidimensional retention modelling coupled with Design Space visualization and organized method documentation can be performed under the DryLab®2010 platform. The resulting method gave a good separation of all 14 peaks of a sample of 11 substances and their related compounds with a critical resolution well above base-line separation, with a run time of under 20 min. The method was proven to be robust with respect to four critical parameters. The methodology presented in this work is in accordance with Quality by Design (QbD) principles and results in the definition and visualization of the so-called Design Space and posterior application to finding multiple robust working conditions of HPLC methods.

HPLC computer simulation software has been shown to increase efficiency and to save time in routine method development and method transfer time and time again. Here it has been seen that other important factors such as building in flexibility and complying with regulatory demands can also be accomplished with the aid of an appropriate work flow and software. In this paper, a method and software are presented that locate the global optima for highly influential experimental parameters with respect to separation, analysis time and robustness. It has been seen that the graphical presentation of the critical parameters with an optimization Design Space helps considerably in judging the ruggedness of the separation system. Moreover inter-laboratory method transfer and documentation should be facilitated by this procedure.

Other potential applications of this methodology have also been touched upon. As well as its application within method development, transferral from one ternary eluent to another could also be assessed using this methodology. This could be particularly relevant at a time of inflated acetonitrile prices and an ever-increasing tendency towards greener solvents.

Acknowledgements

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