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Implementation of a generic liquid chromatographic method development workflow: Application to the analysis of phytocannabinoids and *Cannabis sativa* extracts

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ABSTRACT

A generic liquid chromatographic method development workflow was developed and successfully applied to the analysis of phytocannabinoids and *Cannabis sativa* extracts. Our method development procedure consists in four steps:

i) The screening of primary parameters (i.e. stationary phase nature, organic modifier nature and approximate mobile phase pH) was carried out with a generic gradient on a short narrow bore column, using a system able to accommodate numerous solvents/buffers and columns. Instead of complete peak tracking, the number of peaks which can be separated was considered as a response at this level, to save time.
ii) The optimization of secondary parameters (i.e. gradient conditions, mobile phase temperature and pH within a narrow range) requires only 12 initial experiments and the use of HPLC modeling software for data treatment. It allows to find out the best retention and selectivity for the selected compounds. Peak tracking was performed with a single quadruple mass detector in single ion recording mode, and UV detection (in a broad wavelength range).
iii) The refinement step allows to further adjust column efficiency, by tuning column length and mobile phase flow rate. This can also be done virtually using HPLC modeling software.
iv) The robustness testing step was also evaluated from a virtual experimental design. Success rate and regression coefficients were estimated in about 1 min, without the need to perform any real experiment.

At the end, this method development workflow was performed in less than 4 days and minimizes the costs of the method development in liquid chromatography.

1. Introduction

Liquid chromatographic (LC) method development includes the search for the optimal operating conditions (i.e. type of stationary phase, temperature, gradient steepness, *pH*, ionic strength, organic modifier nature . . .) resulting in the separation of a mixture into its individual components [1,2]. Because of the high probability of peak overlap in complex samples, the method development process is often tedious and time consuming in LC [3].

To find out the most appropriate stationary phase and mobile phase conditions, it might be useful to initially perform a screening process (often known as "scouting") as prior knowledge leads the choices [4–7]. Screening is typically performed on three or four selected columns possessing different chemistries, operating at three different mobile phase *pH* and with two different organic modifiers, using a generic gradient [4,5]. Then, the number of resolved peaks, peak shapes and elution windows are usually compared, to determine the best combination of stationary phase and mobile phase conditions.

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Next, an optimization step is required using the best conditions obtained during the screening process. A lot of trial-and-error type processes are still involved today in industrial laboratories, but software-assisted method optimization offers the potential to speed up the overall process, through retention modeling, based on a limited number of initial experiments [8–10]. During this optimization phase, accurate modeling of retention is required to find out the optimal separation conditions (errors as low as ~1–3% in retention time). Typically, the gradient steepness, mobile phase temperature and *pH* are selected as method variables to fine tune the separation [11–14]. Such software optimizes the design space (DS) mainly by measuring and visualizing the effects of the method variables, e.g. by mean of resolution map [15–17]. Besides speeding up the method development process, the systematic experimentation using modeling software packages also allows to meet Quality by Design (QbD) requirements in industrial laboratories, by providing a tool to improve the robustness of a chromatographic method. Finally, computer-assisted method development also reduces the solvent consumption by limiting the required number of experiments. Hence, it can be considered as a green strategy in liquid chromatography [18].

Analytical QbD is another useful approach for method development, which uses scientific knowledge to enhance understanding of the method, manage risks, and provide guarantee that the method is fit-for-purpose [19,20]. QbD strategy applied for LC method development is more than a simple optimization of a chromatographic separation. Indeed, its ultimate goal is to define a set of working conditions, namely the DS that guarantee the quality of separation with a sufficient probability. This is achieved by gaining strong scientific knowledge of the method. Combining the QbD approach with retention modeling enables to perform *in silico* robustness testing, and to simulate a few hundred experiments within only a few minutes [21]. Robustness is often defined as "The multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality" [22–24]. Therefore, it is obviously preferred to perform the robustness test during the method development. For that purpose, chromatographic modeling software offers a very efficient and straightforward way of testing robustness early in the method development process, based on retention modeling.

The aim of this work was to implement a generic method development workflow based on the combination of design of experiments (DoE) and chromatographic modeling simulation, including the (1) scouting phase, (2) optimization step, based on retention modeling and (3) robustness testing. For this purpose, a fast ultra-high performance LC (UHPLC) method was developed to separate eleven phytocannabinoids which are present in *Cannabis sativa* plant extracts [25]. This method is intended to be applied in routine laboratories, to distinguish legal-type (rich in CBD) and illegal-type products (rich in THC).

2. Experimental

2.1. Chemicals and samples

Acetonitrile (AcN), ethanol (EtOH), methanol (MeOH) and water were purchased from Fisher Scientific (Reinach, Switzerland). Formic acid (FA), and ammonium hydroxide (NH_4OH) were purchased from Sigma-Aldrich (Buchs, Switzerland). Acetic acid glacial was obtained from Biosolve (Dieuze, France).

The standard solutions of THCA (1 mg/mL in AcN), CBC, CBGA, THCV and CBDV (all 1 mg/mL in MeOH) were purchased from Sigma-Aldrich (Buchs, Switzerland) while CBD, CBN (both 1 mg/mL in MeOH), THC, Δ8-THC, CBG (all 1 mg/mL in EtOH) and CBDA (1 mg/mL in AcN) were from Lipomed (Arlesheim, Switzerland). Solutions of standards were prepared by dilution with MeOH. Table 1 lists the names and physico-chemical properties of the standard compounds.

Legal *Cannabis sativa* colas (Green Gold (GG)) was purchased on internet or in tobacco shop in Geneva, Switzerland. Illegal *Cannabis sativa* colas (SK) was obtained from the Geneva Police department, Switzerland. A standardized cannabis STUP218 sample (dosed at 5.5% of total THC with a reference method) was obtained from the School of Forensic sciences, University of Lausanne, Switzerland. All the cannabis materials were stored in a dark place to avoid light degradation.

2.2. Chromatographic system, software and columns

Measurements were performed on a Waters Acquity UPLC H-class system (Waters, Milford, MA, USA) equipped with a quaternary solvent manager, a sample manager with flow through needle (FTN) injector, a column manager, a photo diode array (PDA) detector and a single quadrupole mass detector (QDa®). Data acquisition, data handling and instrument control were performed with Empower™ 3 software (Waters).

The S40 pH-meter was purchased from Mettler-Toledo (Mettler-Toledo, Greifensee, Switzerland).

Modeling was carried out using DryLab v.4.0 and the quantitative robustness evaluation of generated models was performed with the latest DryLab Robustness Module v.1.0. (Molnár-Institute, Berlin, Germany).

Acquity UPLC BEH C18 1.7 μm, 2.1 × 50 mm, Acquity UPLC BEH Shield C18 1.7 μm, 2.1 × 50 mm and 2.1 × 100 mm, Acquity UPLC BEH Phenyl 1.7 μm, 2.1 × 50 mm, Acquity CSH Fluoro-Phenyl 1.7 μm, 2.1 × 50 mm and a Acquity UPLC BEH Shield RP18 guard pre-column 1.7 μm, 2.1 × 5 mm were all purchased from Waters (Milford, USA).

Table 1
Molar masses, pK_a, log *P* and log *D* at pH = 3, 5 and 9 of the 11 phytocannabinoid standards. (Calculated with Chemicalize software – https://chemicalize.com/#/).

Name (abbreviation)	Peak ID[a]	Molar mass (g/mol)	pK_a (acidic)			Log P	Log D (pH = 3)	Log D (pH = 5)	Log D (pH = 9)
Cannabichromene (CBC)	11	314.5	–	9.47	–	6.60	6.600	6.600	6.473
Cannabidiol (CBD)	6	314.5	–	9.13	10.58	6.33	6.325	6.325	6.081
Cannabidiolic acid (CBDA)	1	358.5	2.91	13.00	9.67	6.63	6.286	4.557	3.020
Cannabidivarin (CBDV)	4	286.4	–	9.13	10.58	5.44	5.436	5.436	5.192
Cannabigerol (CBG)	7	316.5	–	9.16	10.62	7.05	7.046	7.046	6.817
Cannabigerolic acid (CBGA)	2	360.5	2.92	13.07	9.70	7.35	7.009	5.282	3.747
Cannabinol (CBN)	8	310.4	–	9.32	–	6.41	6.413	6.413	6.244
Δ^9-Tetrahydrocannabinol (THC)	9	314.5	–	9.34	–	5.94	5.944	5.944	5.783
Δ^9-Tetrahydrocannabinolic acid (THCA)	3	358.5	2.89	12.21	–	6.25	5.890	4.151	2.723
Δ^9-Tetrahydrocannabivarin (THCV)	5	286.4	–	9.34	–	5.06	5.055	5.055	4.894
Δ^8-Tetrahydrocannabinol ($(\Delta^8$-)THC)	10	314.5	–	10.03	–	5.94	5.944	5.944	5.659

[a] Peak ID corresponds to chromatographic peak assignment on Figs. 5 and 8.

2.3. Sample and mobile phase preparation

The standard mixture included the eleven cannabinoids at a concentration of 0.09 mg/mL was diluted in MeOH.

The vegetal material, dried colas of *Cannabis sativa*, was grossly cleared of the stem and the seeds – if present. The cola was then grinded, put into a tube and homogenised using a grinder for 20 min at 6000 rpm with two glass beads of 6 mm diameter. Solid–liquid extraction was realised. 100 mg of the grinded cannabis was extracted with 1 mL of MeOH for 15 min in an ultrasonic bath at ambient temperature. The sample was vortexed at the beginning and at the end for 10 s. The extract was centrifuged for 1 min at 14500 rpm. The supernatant was filtered with a 0.2 μm filter. The extract was finally diluted with MeOH, and amber vials were used.

As mobile phase buffers (mobile phase "A"), 20 mM ammonium formate (pH = 2.7), 20 mM ammonium acetate (pH = 5.0) and 20 mM ammonia (pH = 8.9) were freshly prepared. AcN and EtOH were chosen as organic modifiers (mobile phase "B"). The choice of EtOH instead of MeOH was motivated by the more eco-friendly behaviour despite its higher viscosity [26]. Later on, during the optimization procedure, three pH values were set for the 20 mM ammonium acetate, as pH_1 = 4.2, pH_2 = 5.0 and pH_3 = 5.8.

2.4. Apparatus and methodology

2.4.1. Initial method screening step

A generic screening procedure was initially applied for the mixture of 11 standard compounds. Four different UHPLC columns of identical dimensions (50 × 2.1 mm, 1.7 μm) were used. In terms of column chemistries, three hybrid silica phases (BEH) bonded with (1) C18, (2) C18 with polar embedded groups (carbamate, known as shield C18) and (3) phenyl, were considered. A charged surface hybrid stationary phase (CSH) bonded with fluoro-phenyl moiety was also included in the screening, to tune selectivity. Because the compounds to separate possess a wide range of physico-chemical properties, three different volatile buffers (i.e., pH 2.7, 5.0 and 8.9) were investigated. Experiments were conducted using two organic modifiers, namely AcN (aprotic) and EtOH (protic). Based on preliminary studies and due to the high hydrophobicity of the tested molecules, a generic gradient from 45% to 100% B in 5 min at 0.35 mL/min flow rate was systematically employed. This gradient provided acceptable retention in all conditions. Mobile phase temperature was set at an intermediate value of 45 °C, which can be further optimized. Finally, this screening procedure is based on 24 experiments (4 columns × 3 pH × 2 organic modifiers).

Following these experiments, the number of peaks which can be separated was considered as a response. Moreover, elution windows and peak shapes were also took into account to select the most promising condition.

2.4.2. Retention modeling and optimization step

After the initial screening procedure, the gradient time (tG), mobile phase pH and temperature were further optimized using the most promising combination of stationary phase, buffer and organic modifier. For this purpose, a 3D retention model was built up based on twelve additional experiments ($tG \times T \times pH$). The following levels of the three variables were set: $pH_1 \sim 4.2$, $pH_2 \sim 5.0$ and $pH_3 \sim 5.8$, T_1 = 30 °C and T_2 = 70 °C and tG_1 = 4 min and tG_2 = 12 min. All these conditions were then combined in a full factorial experimental design (3 pH × 2 T × 2 tG = 12 runs).

In order to follow the peak movements and identify the compounds in different conditions, the peak tracking procedure was achieved by both UV and single quadrupole MS detection. In addition, for the isobaric cannabinoids, different concentration levels were set in the standard mixture, to further support peak tracking (by tracking peak intensity). Thus, for compounds possessing M = 314.5 g/mol, namely THC, CBC, CBD and Δ8-THC, their concentrations were set as 0.10, 0.08, 0.02 and 0.05 mg/mL, respectively. Similarly for THCA and CBDA having M = 358.5 g/mol, 0.08 and 0.05 mg/mL were set. Finally for THCV and CBDV having M = 286.4 g/mol, 0.10 and 0.08 mg/mL were considered. MS detection was performed in ESI- mode, using the single ion recording (SIR) mode.

2.4.3. Simulated method transfer (method refinement) and robustness testing

As the best final method only offers a critical resolution of $Rs_{,crit}$ = 1.5, the method was further transferred to a longer column (100 mm instead of 50 mm). On longer column, the resolution is expectedly to be higher (as it is a function of column length), but the analysis time is also doubled (when performing a pure geometrical scaling). Originally, on the 50 mm long column, the analysis time was 9 min and it should thus be equal to 18 min on the 100 mm long column. To decrease analysis time, a virtual method transfer was performed [27]. The flow rate was increased from 0.35 mL/min to 0.57 mL/min, which still resulted in acceptable pressure. The gradient program was scaled to the column length, but flow rate and system dwell volume were also taken into account. Finally, analysis time was decreased down to 11.6 min. All the method transfer procedure was performed *in silico*, and the simulated results were then experimentally verified.

As a last step of method development, a simulated robustness testing was performed. Besides the three model variables used for the optimization (tG, T, pH), the flow rate, as well as initial and final compositions (and additional time-points between the segments of the multi-linear gradient profile) of the mobile phase represent the investigated factors in the built-up robustness model.

The effect of nine factors was calculated at three levels (3^9 full factorial design corresponding to 17496 experiments) and their impact on critical resolution was calculated. The modeled deviations from the nominal values were the following: the gradient time was set to tG = 11.1, 11.6 and 12.1 min, temperature was set to 49, 50 and 51 °C, mobile phase pH was set to 5.7, 5.8 and 5.9, flow rate was set to 0.52, 0.57, 0.62 mL/min. The time of each gradient steps and their mobile phase compositions were set at ± 0.5 min and 0.5%B levels, respectively. Finally, the success rate and the regression coefficients were determined.

Fig. 1 summarizes the whole method development workflow.

2.4.4. Application

The final method was applied to the analysis of fresh *Cannabis sativa* extracts, using the 100 × 2.1 mm BEH Shield RP18 column. A guard column was also used (5 × 2.1 mm), when injecting the plant extracts. Mobile phase "A" consisted of 20 mM ammonium acetate (pH = 5.8), while mobile phase "B" was AcN. The column was operated at a flow rate of 0.57 mL/min and at T = 50 °C. The following multilinear gradient program was run: 45–61%B in 5.1 min, then 62%B at 7.2 min and finally 80%B at 11.6 min. The analysis was followed by a fast washing step and equilibration (45%B at 12.3 min and keeping till 16 min). Injection volume was 2 μL and detection was carried out at 220 nm.

3. Results and discussion

3.1. Scouting runs, method screening step

In the first step of the method development workflow, a full factorial design was created with three qualitative factors (i.e. column chemistry, mobile phase pH and organic modifier). The entire screening consisting in 24 experimental conditions was performed

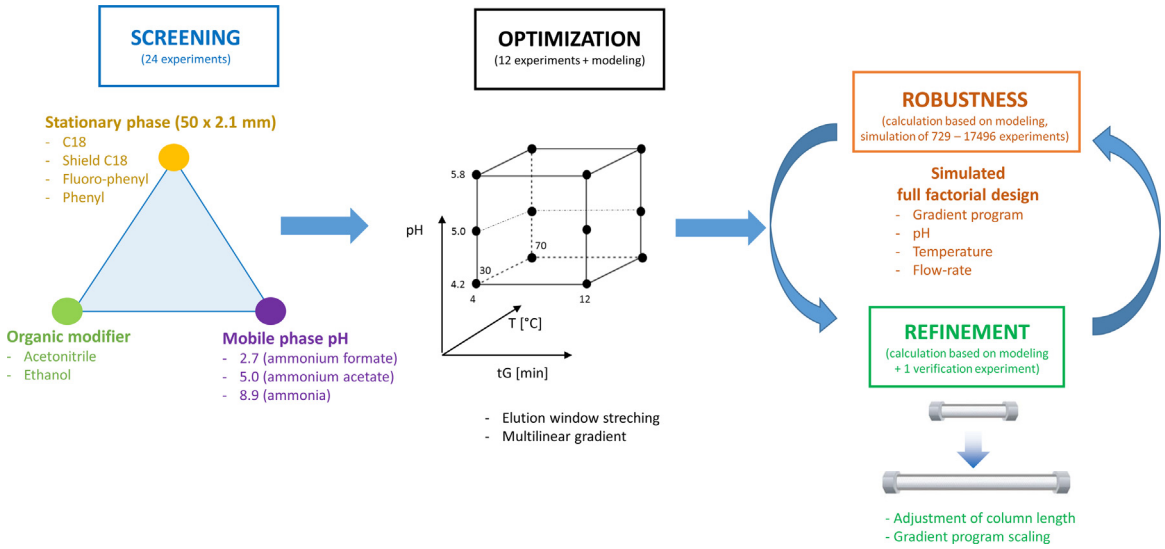

Fig. 1. Schematic view of the full method development workflow.

within two nights, thanks to the possibility offered by our low pressure UHPLC system to accommodate nine solvent lines and four columns in the oven compartment. Experiments were performed in a fully automated way. At this level, UV detection was used (from 210 to 400 nm), to help with the selection of the most appropriate wavelength for all compounds.

On these experiments, we focused on the number of peaks separated under the generic gradient, and the peak shapes. The advantage of this approach was to avoid a formal peak tracking of each compound, which could represent a time-consuming step when dealing with a relatively high number of compounds that have to be resolved. The elution window – determined by the first and last eluting compounds – is another important feature that was checked, as it controls the peak capacity, selectivity and the probability to separate a given number of peaks. The so-called elution window stretching and shifting method is indeed a promising approach, already applied for method screening purposes [28]. Fig. 2 shows the obtained chromatograms in the 24 conditions. The numbers on the top-right corners indicate the number of peaks which were at least partially separated (this value ranged between 7 and 11).

Working at *pH* = 2.7 never enabled the separation of the 11 compounds, moreover retention was quite high and the elution window was narrow. Low *pH* condition did not seem to be promising for this particular mixture of compounds.

At *pH* = 5.0, three columns were able to partially separate all the 11 compounds. Among them, the BEH Shield RP18 provided the most symmetrical peaks and the widest elution window, when using AcN as organic modifier. In addition, the peak distribution was also promising (the most equidistant).

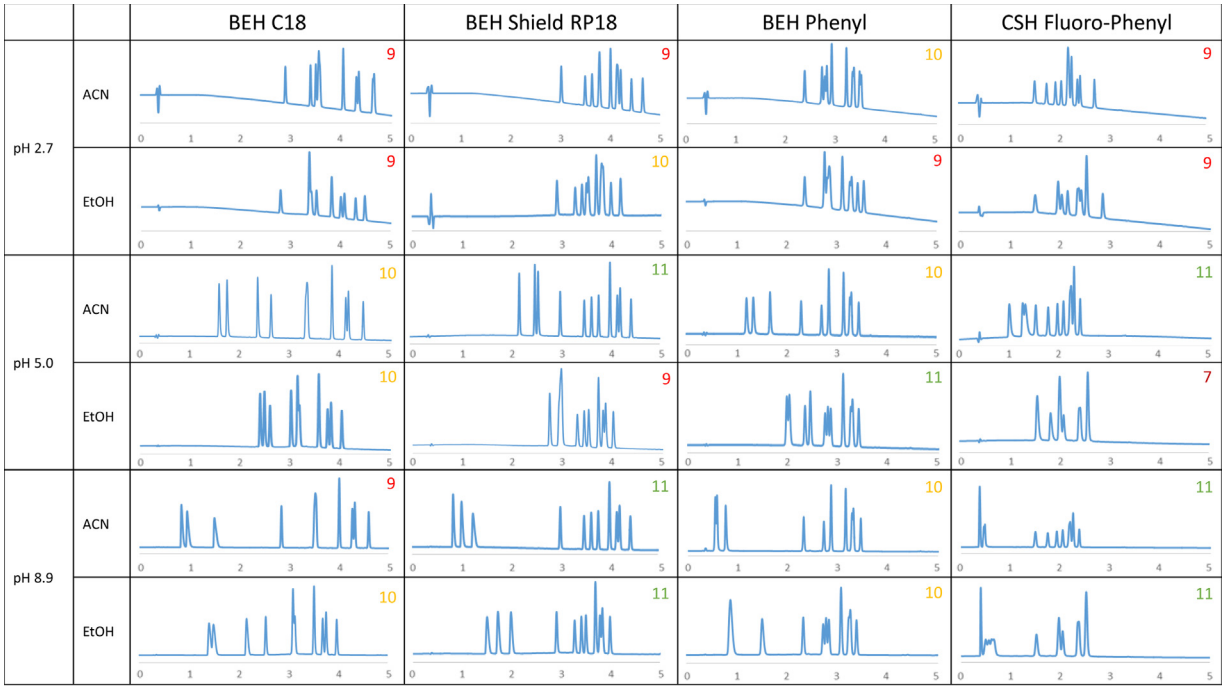

Fig. 2. Chromatograms obtained during the screening procedure, using four columns, three mobile phase pH and two organic modifiers, as indicated on the figure. The number of observed peaks was indicated on the top right of the chromatograms. Sample: standard mixture of 11 Cannabinoids.

At high *pH* (*pH* = 8.9), the three acidic compounds (i.e. CBDA, CBGA and THCA) eluted in wide and tailed peaks. Therefore, somewhat lower peak capacity was observed at *pH* = 8.9 compared to *pH* = 5.0. Both the BEH Shield RP18 and the CSH fluoro-phenyl phases could separate all the compounds, but the retention of the acidic compounds on the fluoro-phenyl phase was not appropriate (eluted close to the column dead time). Therefore, the BEH Shield RP18 column was selected for further experiments.

When comparing AcN and EtOH, the aprotic AcN always provided wider elution window than EtOH, and performed slightly higher peak capacity and thus was chosen as organic modifier for further optimization. It is true that EtOH is a "green" solvent but due to its higher viscosity the mass transfer kinetics is expectedly lower compared to less viscous modifiers (such as AcN). But it always worth trying since it may happen that EtOH provides more favourable selectivity than AcN and then this better selectivity can compensate for the lower kinetic performance.

Regarding detection, it was found that UV detection set at 220 nm provided acceptable sensitivity for all the 11 compounds.

3.2. Method optimization

To find out the optimum conditions to be used on a given column, a general methodology consists in simultaneously modeling the effect of mobile phase temperature and gradient steepness on selectivity and resolution [29,30]. Thanks to the recent developments in chromatographic modeling software, it is now possible to model the simultaneous effect of three variables on a given separation and also to calculate the effect of additional factors such as flow rate, column length, internal diameter, particle size, initial- and final mobile phase composition, gradient-step-points or dwell volume [31]. In our case, gradient steepness, temperature and mobile phase *pH* were selected as model variables to create a cube resolution map, which shows the critical resolution of the peaks to be separated against the three factors. Indeed, it appears that these three variables have the most significant effect on the selectivity and resolution for this type of analytes.

In reversed phase chromatography, the effect of temperature on retention factor (k) can generally be expressed by the van't Hoff equation (derived from the Gibbs free energy). When the logarithm of retention factor ($\log(k)$) is plotted against $1/T$, these plots follow a linear relationship for most solutes. However, quadratic dependence of $\log(k)$ versus $1/T$ over a wide range of temperature was also observed by several authors [32]. For the investigated compounds, a linear relationship was expected. Therefore, temperature was studied at two levels (T_1 = 30 °C and T_2 = 70 °C), when creating the three dimensional model. In most cases, two temperatures enables to build up an accurate retention model in the practically useful range.

In liquid chromatography, the linear solvent strength (LSS) model is the widely accepted theory which describes the analyte retention as a function of the volume fraction (Φ) of the B solvent. As Φ is determined by the gradient steepness (gradient time), it is useful to measure the dependence of gradient retention factor (k^*) on the gradient time (tG) [33,34]. For practical reasons, modeling software such as DryLab generally deal with transformed variables of k or k^* into $\log(k)$ or $\log(k^*)$, to build a mathematical model. The $\log(k^*)$ should follow a linear trend when plotted against the logarithm of gradient time (which is related to the gradient steepness) in case of "regular" solutes. Since the validity of this linear model was accepted in this study, the effect of gradient steepness (gradient time) was investigated at only two levels, namely tG_1 = 4 min and tG_2 = 12 min.

When separating ionizable compounds, *pH*-related changes in retention occur for *pH* values within ± 1.5 units of the pK_a value. Outside this range, the compound is considered as mostly ionized or non-ionized, and its retention is not significantly altered with *pH*. In a relatively small *pH* range, within the ± 1.5 units of the pK_a value, the dependence of retention on the mobile phase *pH* can be described with a quadratic model. Therefore, the effect of mobile phase *pH* has to be investigated at least at 3 levels. Since the pK_a values of the investigated compounds are quite diverse, it was important to cover a relatively large *pH* range (i.e. from pH 4.2–5.8). In the proposed final model, two variables were set at two levels while the third factor was set at three levels. Therefore, this full factorial experimental design required 12 experiments.

Peak tracking was supported by single quadrupole MS detector. The resolution map was built from the 12 initial experiments. It

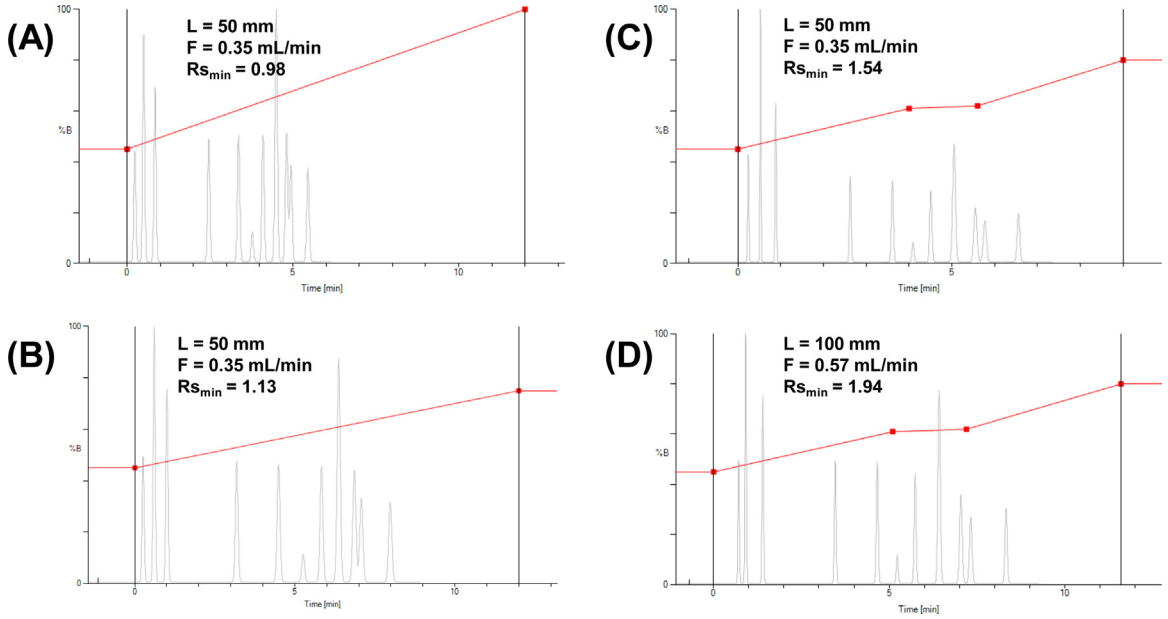

Fig. 3. Optimization of the gradient program (red curves), column length and flow rate. Column: 2.1 mm i.d. BEH Shield RP18 (1.7 μm), mobile phase "A": 20 mM ammonium acetate (*pH* = 5.8), mobile phase "B": AcN, T = 50 °C, injection volume: 2 μL, detection was carried out at 220 nm. Column length, flow rate and gradient programs were indicated on the panels. Sample: standard mixture of 11 Cannabinoids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggested that the highest resolution could be attained at a working point corresponding to $tG = 12$ min, $T = 50\,^{\circ}C$ and $pH \sim 5.8$. Then, by applying a linear gradient from 45 to 100%B, $Rs_{min} = 0.98$ could be achieved between the most critical peak pair, namely (Δ8-)THC and (Δ9-)THC (Fig. 3A). Since no peaks eluted in the second half of the chromatogram, the elution window could be stretched by decreasing $\Delta\Phi$. Running the linear gradient between 45 and 80%B within the same gradient time resulted in $Rs_{min} = 1.13$ and a wider elution window (Fig. 3B). This separation was still not acceptable and therefore a multilinear gradient was proposed to further improve resolution. By inserting a very flat gradient segment (almost isocratic step) between 4.0 and 5.6 min, a baseline separation was achieved between the critical peaks (Fig. 3C). This way, the highest possible resolution was attained on a 50 mm × 2.1 mm column within a reasonable analysis time ($tG = 9$ min). Considering probable differences between column batches and a potential loss of efficiency during the column lifetime, a longer column was tested in the next step.

3.3. Method refinement and robustness

To achieve rapidly the best possible separation on columns of 100 or 150 mm, some chromatograms were simulated and the method was virtually scaled (method refinement).

By simulating a pure geometrical method transfer on the 100 mm column length ($F = 0.35$ mL/min, $tG = 18$ min), the expected Rs_{min} was equal to 2.11. This separation quality was sufficient, but the total run time (including washing step and re-equilibration) was about 22 min. As this method is intended for routine application, the analysis time was an important criterion. To improve throughput, the flow rate was increased, even if a loss in resolution is expected, because of the higher mass transfer resistance (*C*-term of the van Deemter equation). However, on small particles ($d_p = 1.7$ μm), this loss is reasonable, as the *C*-term contribution depends on d_p^2. Increasing the flow rate to 0.57 mL/min (which lead to a pressure drop of $\Delta P \sim 700$ bar) and scaling the gradient program properly, resulted in $Rs_{min} = 1.94$ and analysis time of 16 min (Fig. 3D). To have the full picture of what can be achieved in terms of performance, a 150 mm long column was also simulated. Operating this column at 0.57 mL/min and adapting the gradient program properly could result in $Rs_{min} \sim 2.5$ but at the cost of analysis time (24 min). Finally, to verify the reliability of this simulated method refinement (virtual transfer between different lengths and flow rates), the three columns (50, 100 and 150 mm length) were experimentally tested with the optimized gradient program. The experimentally observed chromatograms are shown in Fig. 4. Excellent agreement was found between predicted and measured resolution values. Fig. 5 shows a face to face comparison of predicted and measured chromatograms for the 100 × 2.1 mm column (with the final conditions). The average error in retention time prediction was 1.61%, while it was equal to 11.81% for the resolution prediction.

The last step of the method development was the estimation of robustness. Fig. 6A and B shows the resolution cube for the final method and the design space where the $Rs_{min} > 1.5$ criterion was fulfilled, respectively. The small green circles indicate the working point. This visualization of the impact of the three main method variables on resolution gives an idea about robustness. As illustrated, working in the low pH range is risky as co-elution of peaks may happen (indicated by dark blue zones on Fig. 6A). In addition, working at higher pH resulted in better peak spacing and higher selectivity. Temperature seems to play a minor role on resolution. Working at higher temperature than ambient was found to be beneficial in UHPLC, to decrease pressure. However, at high temperature, the column lifetime can be reduced. Therefore, $T = 50\,^{\circ}C$

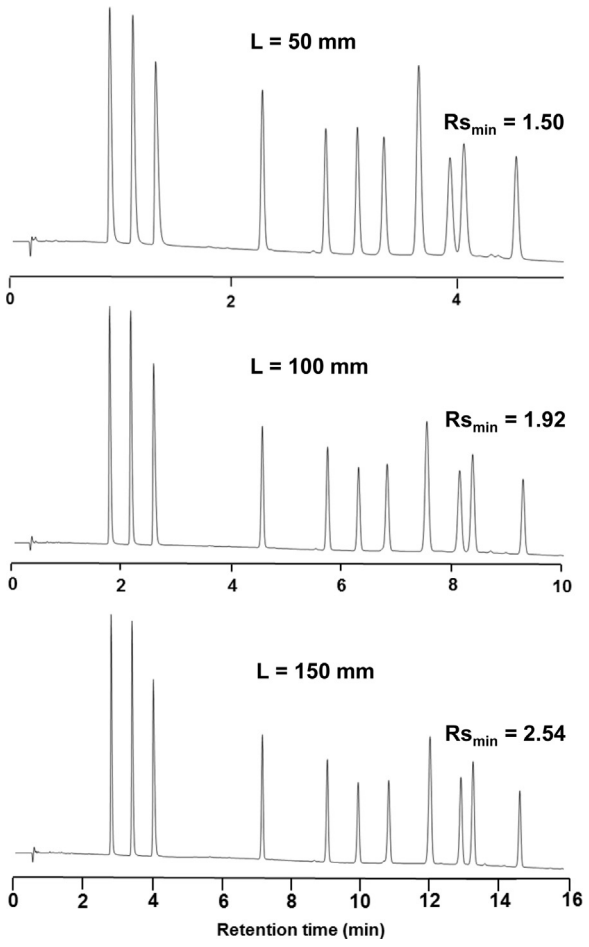

Fig. 4. Chromatograms of Cannabinoids standard mixture obtained on the 50, 100 and 150 mm long 2.1 mm i.d. BEH Shield RP18 (1.7 μm) columns, obtained by applying the optimized gradient. Other conditions are described in Section 3.3.

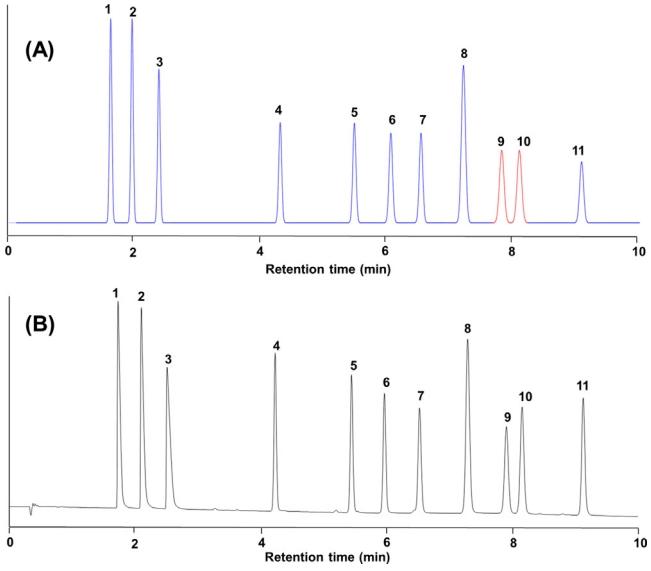

Fig. 5. Comparison of predicted (A) and measured (B) chromatograms of the standard mixture of 11 Cannabinoids. Column: 100 × 2.1 mm BEH Shield RP18 (1.7 μm), mobile phase "A": 20 mM ammonium acetate ($pH = 5.8$), mobile phase "B": can. The column was operated at 0.57 mL/min and $T = 50\,^{\circ}C$. Gradient program: 45–61%B in 5.1 min, then 62%B at 7.2 min and 80%B at 11.6 min. Injection volume was 2 μL and detection was carried out at 220 nm. For peak assignment please see Table 1.

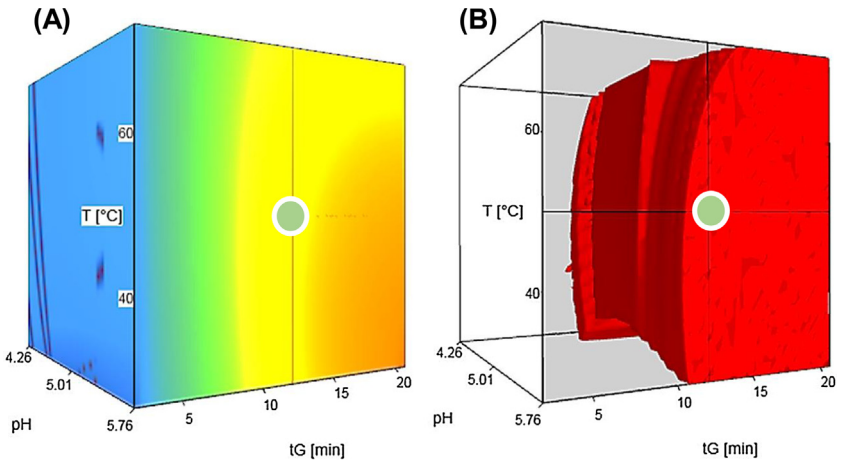

Fig. 6. Resolution cubes. (A) corresponds to the entire design space, while (B) shows only the parts of design space where *Rs* > 1.5 in red. The small green circles indicate the working point. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

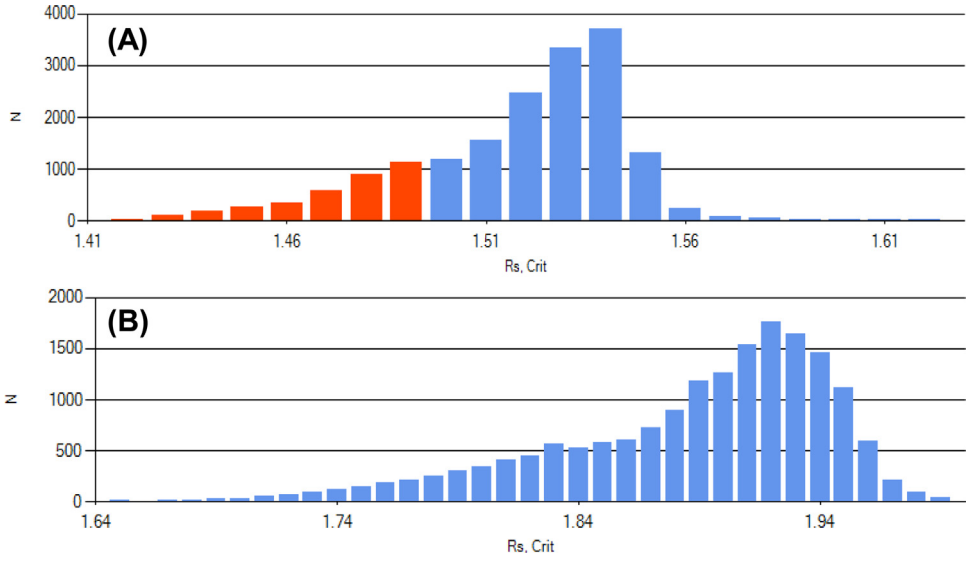

Fig. 7. Frequency of resolution values of the most critical peak pairs when using 50 mm (A) and 100 mm (B) long columns, based on 3^9 full factorial design, which corresponds to 17496 virtual experiments.

was selected as a good compromise. Regarding the gradient time, the longer the analysis time, the higher the resolution is.

As described in Section 2.4.3, a virtual robustness testing was performed on both the 50 and 100 mm long columns. We set a resolution limit at $Rs_{min} > 1.5$. Fig. 7 shows the results of the experiments expressed in frequency as a function of critical *Rs*. As illustrated, the most frequent resolution was $Rs_{min} = 1.54$ and 1.92 on the 50 and 100 mm long columns, respectively. The lowest resolutions were 1.41 and 1.64. With the 50 and 100 mm long columns, the failure rates ($Rs_{min} > 1.5$) were 21% and 0%, respectively. Then, the method developed on the 100 × 2.1 mm column can be considered as a robust one.

Among the studied variables during robustness testing (based on the calculated regression coefficients), the flow rate had the highest impact on the critical resolution. The gradient time and the length of the flat gradient segment also had significant impact on the resolution.

3.4. Application of the final method to real samples

Several non-decarboxylated extracts were analysed with the developed method. The tested samples included both legal (CBD-type) and illegal (THC-type) extracts of *Cannabis sativa*. Fig. 8 shows three representative chromatograms. The first one (Fig. 8A) was obtained from an extract of a fresh THC-type plant. As expected, THCA was present in significant amount. As shown in Fig. 8B, the extract of the CBD-type *Cannabis sativa* has a very different profile and contained predominantly CBDA and CBD. It is important to mention here that these two species (CBDA and CBD) cannot be discriminated with a GC method. Finally, the third chromatogram (Fig. 8C) was an extract of a standardized THC-type plant, which contains again some significant amount of THCA. However, this sample was older than the other ones, and this could be verified by the presence of CBN (degradation product of THC).

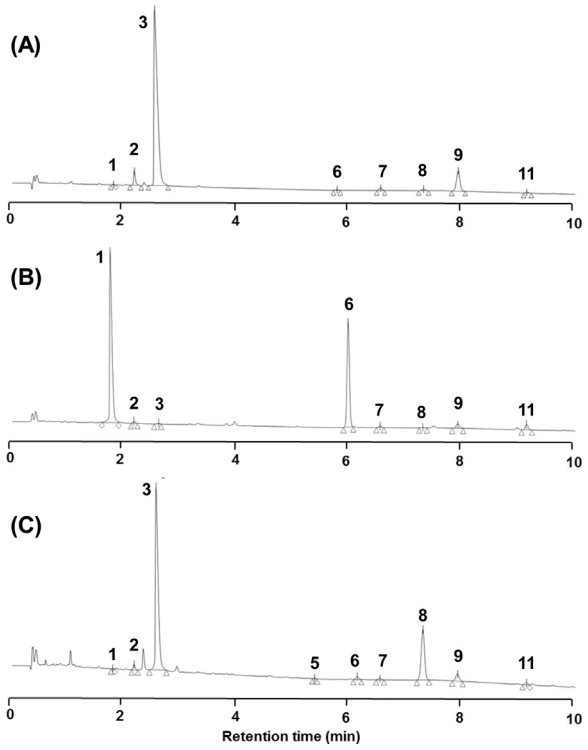

Fig. 8. Chromatograms of diluted extracts of *Cannabis sativa* colas (220 nm). Samples: SK (100) (A), GG (100) (B), STUP218 (100) (C). Chromatographic conditions were the same as described in the caption of Fig. 5, except that a 5 mm × 2.1 mm BEH Shield RP18 (1.7 μm) guard column was connected at the inlet of the analytical column. For peak assignment please see Table 1.

4. Conclusion

A generic liquid chromatographic method development workflow – including screening (1), optimization (2), virtual refinement (3) and virtual robustness testing (4) – was developed and applied to phytocannabinoids and *Cannabis sativa* extracts.

The screening process was based on around a 48 h long series of experiments to map 24 different chromatographic conditions. During the screening, the best combination of stationary phase, organic modifier nature and approximate pH was found. At this stage, short columns of 50 × 2.1 mm and fast generic gradients are suggested to save time.

In a second step (optimization), the effect of gradient program (i.e. linear, multilinear and gradient steepness), mobile phase pH and temperature was understood, by performing 12 systematic experiments. A retention model and resolution map can be built up and optimal conditions can be found rapidly. Again, small column dimension was employed for this retention modeling step to save time.

Once the best retention and selectivity have been found, it is still possible to increase the overall resolution by extending column length. Gradient program can be scaled using virtual experiments, and the analysis time can be reduced, by properly increasing the flow rate and adjusting the gradient program. Finally, the optimal column length can be determined (which fits to our expectations in terms of resolution and analysis time) and only one experiment has to be performed to verify the goodness of the final method.

Last but not least, robustness can also be evaluated by performing a virtual experimental design in various formats (e.g. 2^3, 3^3 and up to 3^9 factorial designs). Success rate and regression coefficients can be estimated in about 1 min, without the need to perform any real experiment. If robustness testing results in a low success rate, then the method can be further tuned (e.g. longer column) and robustness can be quickly re-evaluated (iteration between refinement and robustness).

This whole workflow took only 4 days of work and minimized the costs of the method development. This procedure was successfully applied for the analysis of *Cannabis sativa* extracts, but can also be easily used for any other kind of samples compatible with RPLC conditions.

References

[1] E. Tyteca, J.L. Veuthey, G. Desmet, D. Guillarme, S. Fekete, Computer assisted liquid chromatographic method development for the separation of therapeutic proteins, Analyst 141 (2016) 488–5501.
[2] S. Fekete, R. Kormany, D. Guillarme, Computer-assisted method development for small and large molecules, Recent Development in HPLC and UHPLC, LC-GC 2017, vol. June, 2–7.
[3] J.M. Davis, J.C. Giddings, Statistical theory of component overlap in multicomponent chromatograms, Anal. Chem. 55 (1983) 418–424.
[4] B. Debrus, D. Guillarme, S. Rudaz, Improved quality-by-design compliant methodology for method development in reversed-phase liquid chromatography, J. Pharm. Biomed. Anal. 84 (2013) 215–223.
[5] D. Spaggiari, F. Mehl, V. Desfontaine, A. Grand-Guillaume Perrenoud, S. Fekete, S. Rudaz, D. Guillarme, Comparison of liquid chromatography and supercritical fluidchromatography coupled to compact single quadrupole massspectrometer for targeted in vitro metabolism assay, J. Chromatogr. A 1371 (2014) 244–256.
[6] D.K. Appulage, K.A. Schug, Silica hydride based phases for small molecule separations using automated liquid chromatography–mass spectrometry method development, J. Chromatogr. A 1507 (2017) 115–123.
[7] D.K. Appulage, E.H. Wang, F. Carroll, K.A. Schug, Automated screening of reversed-phase stationary phases for small-molecule separations using liquid chromatography with mass spectrometry, J. Sep. Sci. 39 (2016) 1638–1647.
[8] J.L. Glajch, L.R. Snyder (Eds.), Computer-assisted method development for high performance liquid chromatography, Elsevier, 1990 (ISBN 0-444-88748-2, J. Chromatogr., 485, (1989), 1–640).
[9] J.W. Dolan, L.R. Snyder, M.A. Quarry, Computer simulation as a means of developing an optimized reversed-phase gradient-elution separation, Chromatographia 24 (1987) 261–276.
[10] I. Molnár, Computerized design of separation strategies by reversed-phase liquid chromatography: development of DryLab software, J. Chromatogr. A 965 (2002) 175–194.
[11] S. Fekete, S. Rudaz, J. Fekete, D. Guillarme, Analysis of recombinant monoclonal antibodies by RPLC: towards a generic method development approach, J. Pharm. Biomed. Anal. 70 (2012) 158–168.
[12] S. Fekete, I. Molnar, D. Guillarme, Separation of antibody drug conjugate species by RPLC: a generic method development approach, J. Pharm. Biomed. Anal. 137 (2017) 60–69.
[13] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, part I: salt gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 33–44.
[14] S. Fekete, A. Beck, J. Fekete, D. Guillarme, Method development for the separation of monoclonal antibody charge variants in cation exchange chromatography, part II: pH gradient approach, J. Pharm. Biomed. Anal. 102 (2015) 282–289.
[15] S. Fekete, J. Fekete, I. Molnár, K. Ganzler, Rapid high performance liquid chromatography method development with high prediction accuracy, using 5 cm long narrow bore columns packed with sub-2 μm particles and design space computer modeling, J. Chromatogr. A 1216 (2009) 7816–7823.
[16] A.H. Schmidt, I. Molnár, Using an innovative quality-by-design approach for development of a stability indicating UHPLC method for ebastine in the API and pharmaceutical formulations, J.Pharm. Biomed. Anal. 78–79 (78) (2013) 65–70.
[17] R. Kormány, H.J. Rieger, I. Molnár, Application of quality by design principles to a pharmaceutical sample using UHPLC method development with modeling technologies, LC–GC 31 (2013) 20–27.
[18] J. Plotka, M. Tobiszewski, A.M. Sulej, M. Kupska, T. Górecki, J. Namiesnik, Green chromatography, J. Chromatogr. A 1307 (2011) 1–20.
[19] P. Borman, K. Truman, D. Thompson, P. Nethercote, M. Chatfield, The application of quality by design to analytical methods, Pharm. Technol. 31 (10) (2007) 142–152.
[20] F.G. Vogt, A.S. Kord, Development of quality-by-design analytical methods, J. Pharm. Sci. 100 (3) (2011) 797–812.
[21] R. Kormány, J. Fekete, D. Guillarme, S. Fekete, Reliability of simulated robustness testing in fast liquid chromatography, using state-of-the-art column technology, instrumentation and modeling software, J. Pharm. Biomed. Anal. 89 (2014) 67–75.
[22] Guidelines of the international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use (ICH), Pharm. Dev. Q8 (R2) (2009) 1–24 (Step 4).
[23] B. Debrus, P. Lebrun, A. Ceccato, G. Caliaro, E. Rozet, I. Nistor, R. Oprean, F.J. Rupérez, C. Barbas, B. Boulanger, P. Hubert, Application of new methodologies based on design of experiments, independent component analysis and design

space for robust optimization in liquid chromatography, Anal. Chim. Acta 691 (2011) 33–42.
[24] B. Debrus, P. Lebrun, J.M. Kindenge, F. Lecomte, A. Ceccato, G. Caliaro, J. Mavar Tayey Mbay, B. Boulanger, R.D. Marini, E. Rozet, P.H. Hubert, Innovative highperformance liquid chromatography method development for the screening of 19 antimalarial drugs based on a generic approach, using design of experiments, independent component analysis and design space, J. Chromatogr. A 1218 (2011) 5205–5215.
[25] A. Leghissa, Z.L. Hildenbrand, K.A. Schug, J. Sep Sci, A review of methods for the chemical characterization of cannabis natural products, J. Sep. Sci. 41 (2018) 398–415.
[26] G. Vanhoenacker, P. Sandra, F. David, K. Sandra, A. Pereira, Green chromatography (Part 1): introduction and liquid chromatography, LCGC Europe 23 (2010) 242–259.
[27] R. Kormany, J. Fekete, D. Guillarme, S. Fekete, Reliability of computer-assisted method transfer between several column dimensions packed with 1.3–5 μm core–shell particles and between various instruments, J. Pharm. Biomed. Anal. 94 (2014) 188–195.
[28] E. Tyteca, A. Liekens, D. Clicq, A. Fanigliulo, B. Debrus, S. Rudaz, D. Guillarme, G. Desmet, Predictive elution window stretching and shifting as a generic search strategy for automated method development for liquid chromatography, Anal. Chem. 84 (2012) 7823–7830.
[29] J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, D.L. Saunders, L. Van Heukelem, T.J. Waeghe, Simultaneous variation of temperature and gradient steepness for reversed-phase high-performance liquid chromatography method development: I. Application to 14 different samples using computer simulation, J. Chromatogr. A 803 (1998) 1–31.
[30] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, second ed., John Wiley & Sons Inc., 1997.
[31] R. Kormány, I. Molnár, H.J. Rieger, Exploring better column selectivity choices in ultra-high performance liquid chromatography using Quality by Design principles, J. Pharm. Biomed. Anal. 80 (2013) 79–88.
[32] S. Heinisch, J.-L. Rocca, Sense and non-sense of high-temperature liquid chromatography, J. Chromatogr. A 1216 (2009) 642–658.
[33] J.W. Dolan, L.R. Snyder, Developing a gradient elution method for reversed-phase HPLC, LC-GC 5 (1988) 970–978.
[34] L.R. Snyder, Gradient elution, in: C. Horvath (Ed.), HPLC: Advances and Perspectives, Vol. 1, Academic Press, New York, 1980, pp. 208–316.