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Reversed-phase liquid chromatography column testing: robustness study of the test

K. Le Mapihan, J. Vial*, A. Jardy

Laboratoire "Environnement et Chimie Analytique", CNRS-UMR 7121, Ecole Supérieure de Physique et de Chimie Industrielles de la Ville de Paris 10, rue Vauquelin, 75005 Paris, France

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Abstract

Choosing the right RPLC column for an actual separation among the more than 600 commercially available ones still represents a real challenge for the analyst particularly when basic solutes are involved. Many tests dedicated to the characterization and the classification of stationary phases have been proposed in the literature and some of them highlighted the need of a better understanding of retention properties to lead to a rational choice of columns. However, unlike classical chromatographic methods, the problem of their robustness evaluation has often been left unaddressed. In the present study, we present a robustness study that was applied to the chromatographic testing procedure we had developed and optimized previously. A design of experiment (DoE) approach was implemented. Four factors, previously identified as potentially influent, were selected and subjected to small controlled variations: solvent fraction, temperature, pH and buffer concentration. As our model comprised quadratic terms instead of a simple linear model, we chose a D-optimal design in order to minimize the experiment number. As a previous batch-to-batch study [K. Le Mapihan, Caractérisation et classification des phases stationnaires utilisées pour l'analyse CPL de produits pharmaceutiques, Ph.D. Thesis, Pierre and Marie Curie University, 2004] had shown a low variability on the selected stationary phase, it was then possible to split the design into two parts, according to the solvent nature, each using one column. Actually, our testing procedure involving assays both with methanol and with acetonitrile as organic modifier, such an approach enabled to avoid a possible bias due to the column ageing considering the number of experiments required (16 + 6 center points). Experimental results were computed thanks to a Partial Least Squares regression procedure, more adapted than the classical regression to handle factors and responses not completely independent. The results showed the behavior of the solutes in relation to their physico-chemical properties and the relevance of the second term degree of our model. Finally, the robust domain of the test has been fairly identified, so that any potential user precisely knows to which extend each experimental parameter must be controlled when our testing procedure is to be implemented.

Keywords: Robustness; Experimental design; Stationary phases; Column characterization; Solvent effect; Basic compounds

1. Introduction

In the past two decades, reversed-phase liquid chromatography was revealed as the essential method to perform pharmaceutical analysis. Nevertheless, satisfactory separations may be difficult to obtain due to the basic properties of some compounds: the interactions with residual silanol groups are often invoked to explain peak tailing and poor resolution. Consequently, column manufacturers developed and combined different strategies to restrict the residual silanol access towards basic compounds in order to improve the separative power of their stationary phases, leading to the wide variety of available RPLC-phases. As a result, choosing the appropriate column among the more than 600 chromatographic sorbents remains a challenge for the analyst when a new separation has to be performed. Many chromatographic tests are dedicated to column characterization in the literature [2–17]. But

* Corresponding author. Tel.: +33 1 40794779; fax: +33 1 40794776.
E-mail address: jerome.vial@espci.fr (J. Vial).

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very few of them take into account the fact that the obtained classes are only based on estimates and not on true values. Like for chromatographic methods, the reliability of those tests should be evaluated. Nevertheless, few authors look into the repeatability of the test, the variability introduced by either the filling or the batch of the column (corresponding to column-to-column and batch-to-batch reproducibility) and the reproducibility of the test. Concerning column-to-column and batch-to-batch variabilities of stationary phases, the main study is to Kele and Guiochon's credit [18–24]. Other tests dealt also with this point, but more to visualize the impact of batch-to-batch dispersion on classifications [25–28] than for a quantitative and systematic concern. Neue also showed the great improvement made by manufacturers in batch-to-batch variability during the past 20 years [8]. Regarding test reproducibility, the best approach is to perform an interlaboratory trial [29]. A recent study was carried out for certifying an HPLC column as a reference material (certified reference material BCR-722), involving eight laboratories [30]. Thanks to a tightened protocol, fair reproducibilities were then obtained for shape and methylene selectivities. Another lab-to-lab comparison [31] studied the dispersions of numerous descriptors and confirmed the correlations shown in previous studies [13,15], strengthened by Neue [32]. In Kele and Guiochon' study, particular precautions were taken in order to minimize sources of error, like the use of a single preparation for the buffer. It is true that too flexible conditions lead to uncertainties, with a blurred effect [32] on classifications as inevitable consequence. If the influent factors for reversed-phase retention are clearly identified [24,30,33], their impact were frequently assessed with one-factor-at-a-time studies [9]. Nevertheless, such studies are not able to reveal potential interactions between factors, contrary to the design of experiment (DoE) methodology.

To demonstrate that our procedure is transferable, its ruggedness must be shown. According to ICH, *the robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, deliberate variations in method parameters and provides an indication of its reliability during normal usage*. To date, the robustness of chromatographic tests has been seldom taken into consideration. To our knowledge, only one attempt of robustness study by DoE has been evoked [28]. The aim of the present study is to evaluate the ruggedness of our testing procedure [34] and to define its robust domain thanks to a DoE procedure.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (MeCN, HPLC ultra gradient grade) and methanol (MeOH, HPLC gradient grade) were purchased from Mallinckrodt Baker B.V. (Deventer, Holland). Water was produced by a Milli-Q Plus ultrapure water purification system (Millipore, Molsheim, France). Sodium acetate and acetic acid volumetric standard (1.031 mol L^{-1}, $d = 1.010$) were obtained from Aldrich and used as received.

The test solutes were constituted of amiodarone hydrochloride (Sigma), ampicillin sodium salt (Fluka), atropine sulfate salt (Sigma), benzylamine hydrochloride (Sigma), *n*-butylbenzene (Aldrich), caffeine (Fluka), clofazimine (Sigma), cyanocobalamine (Sigma), digitoxin (Fluka), *n*-pentylbenzene (Aldrich), strychnine hemisulfate salt (Sigma), *o*-terphenyl (Fluka), triphenylene (Fluka), D-tubocurarine chloride (Sigma) and vancomycin hydrochloride (Sigma). The set of selected solutes had log *P* values distributed from −0.07 to 7.66, with molecular weights comprised between 92 and 1450 g mol^{-1} and acidity constants pK_a ranging from 1.9 to 10.0 if concern.

The robustness study was carried out with SymmetryShield RP 18 columns (3.5 μm, 150 mm × 4.6 mm i.d., Waters, Saint-Quentin en Yvelines, France).

2.2. Apparatus

The LC system consisted of a HP 1050 quaternary pump, a HP 1050 autosampler and a HP 1100 variable wavelength detector operated at 230 or 254 nm (see Table 1). The data acquisition was performed on a data station running under Chemstation 6.03 (Agilent Technologies, Waldbronn, Germany). The acquisition frequency was at least 25 Hz. Concerning temperature regulation, the tested columns were placed in an Alltech water jacket connected to a water bath set at 40 °C (±0.03 °C with a water bath Neslab RTE-101). All analyses were operated using a flow rate of 1 mL min^{-1}.

2.3. Separation protocol at nominal conditions

The protocol of the testing procedure was based on the previously described one [34]. As by definition, a close control of pH does not entail an accurate total concentration of the same

Table 1
Testing conditions

Common conditions	Solvent	Solvent fraction (%)	Solute
Acetate buffer 30 mM pH 5.00 at 25 °C	MeOH	70	Thiourea, digitoxin, clofazimine, amiodarone, butylbenzene*, pentylbenzene*, *o*-terphenyl*, triphenylene*
$T = 40$ °C	MeCN	59	
Flow rate = 1 mL min^{-1}	MeOH	15	Strychnine*, benzylamine*, caffeine*, D-tubocurarine, atropine, ampicillin, vancomycin, cyanocobalamin
	MeCN	9	

λ = 254 nm for solutes marked with (*); λ = 230 nm otherwise.

buffer, our protocol was slightly refined in order to control as accurately as possible these two factors simultaneously. Instead of adjusting the pH of the sodium acetate aqueous solution to the appropriate value with its concentrated conjugated acid, the 30 mM buffer was prepared by dissolving the appropriate weighted quantities of salt and acetic acid volumetric standard to reach a pH of 5.00, the value of which was checked a posteriori to validate the buffer preparation thanks to a pH-meter calibration taking into account temperature. All buffers were filtered through 0.45 μm HA type filters, (Millipore, Molsheim, France), before addition of the organic modifier. Mobile phases were freshly prepared just before use by weight for each experiment within the ratios indicated in Table 1, which summarizes the whole conditions of the test, including the detection conditions towards the corresponding solutes.

All compounds were injected at the following concentrations: 50 ppm for the majority of solutes except for *o*-terphenyl (12 ppm), triphenylene (3 ppm), benzylamine (600 ppm), atropine (400 ppm), ampicillin (200 ppm), strychnine (100 ppm) and D-tubocurarine (100 ppm). At least 1-h equilibration was performed for each mobile phase before the 10 μL injection of mixtures in duplicates. The column void volume was determined by the injection of thiourea (Aldrich) in the acetonitrile mobile phase. All samples were stored at 4 °C or less.

2.4. Softwares

JMP 4.0.5 (S.A.S. Institute Inc., Carry, NC, USA) was used to perform one-way analysis of variance (ANOVA) and to generate the design of experiments while coefficient calculations were carried out with MODDE 6.0 (Umetrics AB, Umeå, Sweden).

3. Design of experiments

3.1. Identification of factors and responses

Four factors were identified as potentially influent on the column testing procedure: the solvent fraction (%*S*) of the mobile phase, the concentration of the buffer (Conc.), its pH and the column temperature (*T*). All of them are quantitative process variables. Table 2 shows the levels chosen for these factors.

For solvent fraction, the applied variations could appear very small. However, we should remind that variations in a robustness study had to be of the same order of magnitude as variations that could occur accidentally in practice. In our case, they represented at least 10 times the potential weighting errors.

Three kinds of classical chromatographic parameters were recorded and considered as responses: retention factor (k), peak asymmetry (As' as previously described [35]) and reduced plate height (h), yielding 3 parameters × 15 solutes = 45 responses.

3.2. Model and experimental design selection

Two strategies can be considered concerning the robustness studies depending on the objective. If the investigation consists only in verifying that the study domain is robust, a screening design such as Plackett–Burmann or supersaturated ones can be sufficient. It generally occurs when robustness must be checked at the last step of method validation. In the case of the research of a model allowing the determination of a robust domain (tolerable variations), it is preferable to consider a more powerful tool. The present study lies within this last scope. As a robustness study must describe the response surface around the nominal conditions, at least a second-degree modeling must be used. The quadratic function cannot be obtained by neither fractional nor full factorial two-level designs and even less by screening designs. As second order interactions like $\%S \times T$ or pH × Conc. could be potentially influent, they were kept for the modeling and experimental responses could be described, using the following equation:

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i<j}^{4} \sum_{j=2}^{4} \beta_{ij} X_i X_j + \sum_{i=1}^{4} \beta_{ii} X_i^2 \qquad (1)$$

where β_0, β_i, β_{ij}, β_{ii}, stand, respectively, for the response mean, the coefficient of the main factors, the coefficient of the second order interactions and the coefficient of the quadratic terms.

As classical response surface designs such as central composite or Box–Behnken ones required too many assays, we used an asymmetric design to reduce the number of experiments (and consequently the duration of the study) and to avoid a potential bias due to column ageing [36]. Finally, we selected a D-optimal design constituted of 16 experimental

Table 2
Chromatographic parameter settings applied in the robustness investigation, corresponding to low (−1), central (0) and high (+1) levels

Factors	Low level (−1)	Center point (0)	High level (+1)
Solvent fraction (% *w*)	69.5 58.5 14.5 8.5	70 59 15 9	70.5 59.5 15.5 9.5
Temperature (°C)	39	40	41
pH	4.8	5.0	5.2
Buffer concentration (mM)	27	30	33

Table 3
Design table: experiments were randomized but with the constraints to perform center points at regular intervals

Run order	% Solvent	*T*	pH	Conc.
1	0	0	0	0
2	0	0	0	−1
3	−1	0	1	0
4	0	1	1	1
5	1	1	−1	−1
6	0	0	0	0
7	1	0	−1	1
8	−1	−1	−1	−1
9	1	−1	1	1
10	0	0	0	0
11	1	−1	−1	−1
12	1	1	0	0
13	−1	1	−1	1
14	−1	1	−1	−1
15	0	0	0	0
16	−1	1	1	−1
17	−1	−1	1	−1
18	0	−1	−1	0
19	−1	−1	0	1
20	0	0	0	0
21	1	1	1	−1
22	0	0	0	0

points and 6 center points. The experimental matrix is given in Table 3.

This design revealed a fair *G*-efficiency of 96.82. Positing the higher this criterion is, the lower the variance in the experimental domain, the chosen design could be considered efficient for the investigation of the neighborhood of the nominal conditions. The representation of experiments on Fig. 1 affords to visualize the selected design: it was based on a fractional factorial design (points of the extreme vertices) to which middle points of the edges were added, meaning that the final design was in a certain way derived from a Box–Behnken one.

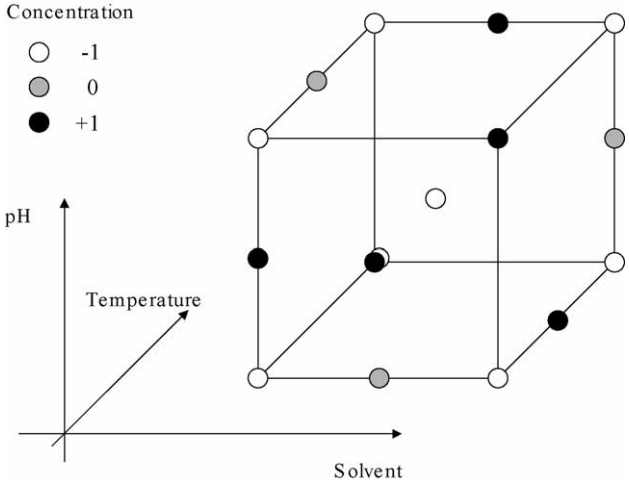

Fig. 1. Four-factor D-optimal design.

3.3. Carrying out of runs

All experiments were randomized to minimize the effects of uncontrolled factors that could affect the final results. However, as a potential drift resulting from the column ageing could not be excluded considering the duration of the study [36], we included the following constraints:

1. The set of randomized runs must include center points at regular intervals in addition to those at the beginning and at the end of the study: by this way, it was possible to detect any ageing of the column.
2. Two columns were used, one for each solvent, to allow a fair comparison of effects between organic modifiers without any bias due to ageing.

As we did not have two columns of the same batch, we chose two columns from different batches, given that a prior study carried out with three different batches of SymmetryShield RP18 had revealed a low batch-to-batch variability [1]. Besides, the ANOVA results of this study were consistent with those of Guiochon [18–23]. The investigations in methanol and in acetonitrile were performed with the same run order as reported in Table 3.

Experiments were performed as independently as possible. Eluents were prepared as previously described for the nominal conditions taking into account the changes for each level of each factor. For each experiment, chromatographic responses were obtained by the mean of the values resulting from duplicate injections.

3.4. Removal of poorly informative responses

The repeatability study at the center points constitutes a prerequisite for the robustness study. The objective of our repeatability study was to define a criterion under which a response will be stated non-informative and removed from the study. A classical statistical approach based only on the estimation of variance at center points presents a major drawback: poor repeatable responses could be discarded whereas they are yet informative and reveal the actual influence of a factor. The chosen alternative consists in taking into account both the repeatability and the information carried by the response: actually, an informative response with a poor repeatability can be proved more useful than a highly repeatable response with a poor informative power. The Information Index [37], defined by the following equation: Information Index = 1 − (Pure error variance/Total response variance), was used to that end. For each response, the pure error corresponds to the variation of the response at the center points.

The closer to 1 the Information Index is, the more informative the response is. Below the threshold value of 0.5, the response can be considered as poorly relevant and then can be removed from the study.

3.5. Calculation of coefficients and statistical interpretation

The coefficients of the polynomial model (given in Eq. (1)) are usually estimated by Multiple Linear Regression (MLR). However, one of the main pitfalls of this kind of regression consists in assuming the independence of factors. Actually, factors like temperature, pH and solvent fraction are well known to be interdependent. So, it seemed wiser to use Partial Least Squares (PLS) regression instead of MLR, because PLS regression does not need a nil covariance between factors and therefore deals far better with correlated factors than MLR does. PLS had been extensively described in the literature [38–43]. The responses were centered and scaled to unit variance. We used cross-validation to determine the number of significant PLS components. Nevertheless, classical statistical tools such as ANOVA were used to compute by excess estimates of confidence intervals used for determining significance of coefficients with $\alpha = 5\%$ (risk of type I error). An assessment of the quality of the fit can be performed thanks to two tools: the goodness of fit R^2 and the goodness of prediction Q^2 defined as follows: $R^2 = (SS_{REG}/SS)$ and $Q^2 = 1 - (PRESS/SS)$, where SS_{REG}, SS and PRESS represent, respectively, the sum of squares of Y explained by the model, the total sum of squares and the prediction error sum of squares. Finally, R^2 stands for the fraction of response variation explained by the model whereas Q^2 shows the fraction of response variation that can be predicted by the model. In the ideal case, values are close to 1 for both R^2 and Q^2, indicating a very good model with an excellent predictive power. These tools provide the best summary of the fit quality of the model [37]. In addition, R^2 overestimates and Q^2 underestimates the relevance of the model.

The raw coefficients were standardized as following: (Coefficient value/Mean value) × 100 for a more comprehensive comparison.

3.6. Robust domain construction

At this step, only responses with an acceptable Information Index have been kept. If no influence of factors was revealed, thus the response was fairly rugged on the experimental domain. At the opposite, if some coefficients were revealed influent, then the robust domain (within which the responses will be considered rugged) had to be delimited. For that purpose, a "tolerance" criterion was defined. Assuming that the batch-to-batch reproducibility of the studied stationary phase was satisfactory and acceptable for pharmaceutical method development, it was chosen as a general criterion to determine the spread of the robust domain of the test. Consequently, such conditions could not afford to discriminate between columns filled with different batches of the same stationary phase, provided that its batch-to-batch variability turned out to be smaller or equal to the one of SymmetryShield phase. Beforehand, the dispersions generated by the batch-to-batch reproducibility and the test repeatability had to be compared. If the batch-to-batch reproducibility was smaller, the tolerance criterion would be based on the test repeatability: the robust domain could never be smaller than the boundaries imposed by test repeatability (being the concern of purely experimental variations). Finally, to calculate the allowed intervals, the relative standard deviations were twice affected to either side of each response mean. As the responses were functions of the four studied factors, the 4D-response surface should be projected on the two-factor planes, defining so contour diagrams. The contour lines corresponding to the allowed limit values were figured on these response contour diagrams. For a comprehensive visualization, three kinds of contour diagrams would be drawn: $y = f(\%S, \text{pH})$, $y = f(\%S, T)$, and $y = f(\%S, \text{Conc.})$. Finally, six contour diagrams per response were displayed resulting from the presence of two organic modifiers (discontinuous variable). The final robust domain was then defined by the intersection of the valid domains found for all responses. Beyond these robust domains, the responses could not be regarded as rugged.

4. Results and discussion

4.1. Information Index and selection of responses

The Information Index values for all the chromatographic parameters are recorded on Table 4.

The Information Index revealed high values for all retention factors, meaning that all compounds were informative and should not be removed from the study. Concerning asymmetries and efficiencies, the number of valuable responses decreased significantly: only those of amiodarone, atropine and benzylamine were relevant and were prone to latter discussion.

The comparison between the relative dispersion of the solute retention factors caused on the one hand by the test repeatability and on the other hand by the batch-to-batch reproducibility previously studied is shown on Table 5.

Considering that the test repeatability was more comparable to a day-to-day uncertainty than the precision obtained in the best possible circumstances (like injection repeatability), the results were consistent with those of Visky et al. [31]. No harmful ageing of the columns was noticed. In addition, the dispersions resulting from the batch-to-batch variability were wider than that due to the test repeatability in most cases, except for cyanocobalamin, vancomycin and caffeine in acetonitrilic eluent only.

4.2. Estimates of the model coefficients and interpretation

In a general way, significant coefficients of the model (given in Eq. (1)) will be discussed according to organic modifier fractions and solute properties. First of all, the most influent coefficients corresponded to the main factors and very

Table 4
Information Index of the chromatographic parameters; up at high solvent fraction, down at low solvent fraction

Chromatographic parameter	Solvent	Digitoxin	Clofazimine	Amiodarone	Butylbenzene	*o*-Terphenyl	Pentylbenzene	Triphenylene
k	MeCN	0.985	0.981	0.997	0.971	0.972	0.973	0.970
	MeOH	0.989	0.996	1.000	0.994	0.996	0.997	0.996
As′	MeCN	−0.200	0.185	0.651	−0.200	−0.200	−0.200	−0.200
	MeOH	0.558	0.107	0.887	0.440	0.555	−0.200	−0.200
h	MeCN	0.123	−0.200	0.352	0.202	0.198	0.169	0.191
	MeOH	0.100	0.685	0.967	0.360	0.806	0.781	0.467

Chromatographic parameter	Solvent	Strychnine	D-Tubocurarine	Atropine	Ampicillin	Cyanocobalamin	Vancomycin	Caffeine	Benzylamine
k	MeCN	0.996	0.954	0.989	0.994	0.991	0.984	0.994	0.994
	MeOH	0.996	0.975	0.996	0.982	0.995	0.995	0.987	0.983
As′	MeCN	0.036	0.095	−0.200	−0.200	−0.200	−0.200	−0.200	−0.192
	MeOH	−0.148	−0.200	0.557	0.021	−0.143	0.252	−0.200	0.815
h	MeCN	0.381	−0.200	0.834	−0.014	−0.062	−0.011	0.069	0.792
	MeOH	0.030	−0.200	0.889	0.565	0.617	0.651	−0.200	0.792

few interactions were revealed significant. As a result, the final model for each response would be easier to understand and to link to retention mechanisms than with second-order interactions. The following discussion will be organized according to the content of organic modifier: high or low.

4.2.1. High contents of organic modifier

Table 6 gives the standardized coefficients for retention factors of hydrophobic compounds, respectively, in methanol and acetonitrile at high contents of organic modifier.

Concerning neutral compounds, i.e. digitoxine, butylbenzene, *o*-terphenyl, pentylbenzene and triphenylene, the goodness of fit was satisfactory for both organic modifiers. Solvent fraction and temperature were the only influent factors for all solutes in both solvents (except for digitoxine which was too weakly retained in acetonitrile), confirming an expected retention mechanism of partition. The effects were more pronounced in methanol than in acetonitrile while the solvent fraction appeared to be generally more than twice more influent than temperature, except for triphenylene. Concerning asymmetries and efficiencies, the modeling was not reliable (very low Q^2 values) and then the coefficients were not relevant in spite of an Informative Index greater than 0.5. Combined with the high variability observed at center points, this phenomenon confirmed the problem of reproducibility of such chromatographic parameters already encountered for efficiencies during the collaborative study of the EU project "HPLC column as a reference material" [30].

For basic compounds, i.e. clofazimine and amiodarone, the effect of solvent fraction was of the same order of magnitude as for neutrals. On the opposite, temperature was hardly revealed as a significant factor. As expected, the main in-

Table 5
RSD (%) on retention generated factors by batch-to-batch variability [1] and test repeatability

	MeOH		MeCN	
Solute	Repeatability	Batch-to-batch	Repeatability	Batch-to-batch
Digitoxin	0.50	1.57	0.29	2.99
Clofazimine	0.66	4.39	0.65	4.63
Amiodarone	0.37	3.28	0.29	3.41
Butylbenzene	0.29	1.00	0.47	1.03
Pentylbenzene	0.26	0.89	0.52	0.99
o-Terphenyl	0.27	0.90	0.50	1.02
Triphenylene	0.29	0.64	0.53	0.95
Strychnine	0.47	2.36	0.27	1.55
D-Tubocurarine	0.73	2.68	0.74	2.63
Atropine	0.50	2.76	1.65	1.74
Ampicillin	0.70	0.89	0.84	0.85
Cyanocobalamin	0.71	1.23	2.47	0.76
Vancomycin	0.78	1.83	3.70	2.11
Caffeine	0.54	1.15	0.60	0.51
Benzylamine	0.89	4.41	0.72	2.66

Table 6
Estimates of standardized coefficients for the modeling of retention factors at high level of solvent

Solvent	Clofazimine		Amiodarone		Digitoxin		Butylbenzene		Pentylbenzene		*o*-Terphenyl		Triphenylene	
	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN
Mean value (β_0)	2.98	4.06	10.07	7.15	1.65	0.64	5.26	5.39	7.67	7.69	7.33	6.73	15.62	9.86
%*S*	−3.57	−1.99	−3.45	−1.25	−4.26	−2.19	−3.44	−2.58	−3.84	−2.80	−3.98	−2.98	−3.79	−2.61
T	−1.11			1.42	−1.69		−1.53	−1.04	−1.72	−1.14	−1.77	−1.11	−2.46	−1.52
pH	10.08	10.89	18.05	19.39										
Conc.	−1.36		−1.10											
%*S* × %*S*														
T × *T*														
pH × pH			2.12	3.99										
Conc. × Conc.														
%*S* × *T*														
%*S* × pH	−0.54		−0.69											
%*S* × Conc.														
T × pH														
T × Conc.														
pH × Conc.														
R^2	1.00	0.99	1.00	1.00	0.99	0.99	1.00	0.99	1.00	0.99	1.00	0.99	1.00	0.99
Q^2	0.90	0.57	0.92	0.70	0.61	0.73	0.70	0.68	0.71	0.69	0.74	0.70	0.81	0.75

Only significant coefficients are given.

fluence was undoubtedly due to pH, by linear and quadratic terms 3–15 times more important than those for solvent. The magnitude of linear coefficients for pH was the same whatever the nature of the organic modifier. The appearance of a squared term (only for amiodarone) could be explained by the fact that the retention of the solute according to pH was no longer linear around the nominal conditions: such a behavior could be fairly approximated by a quadratic function [44]. As for temperature, buffer concentration was barely revealed as an influent factor in the methanol eluent. Because the repeatability of the test for basic compounds was not as good as for neutrals, factors of little influence like buffer concentration and temperature were partly masked. Consequently, the sign of the coefficients of such secondary factors was hazardous to interpret and would not be discussed. However, it should be underlined that the goodness of prediction was better in methanol than in acetonitrile, thanks to the supply of additional significant terms like concentration and interaction solvent × pH. Concerning peak asymmetry and efficiency of amiodarone (results not shown), pH was the main significant factor in both solvents, except for efficiency in acetonitrile mobile phase where no influent factors were revealed significant (but with poor R^2 and Q^2). The increase of pH induced a reduction both for peak asymmetry and reduced plate height, meaning a better efficiency. This phenomenon could be explained by the decrease of the protonated form fraction during the pH increase: on the premise that silanol groups were almost dissociated, the ion exchange part of the mixed retention mechanism decreased, involving a better symmetry of the peak and consecutively a better efficiency. In methanolic eluent, temperature showed also a slight positive impact on efficiency.

4.2.2. Low contents of organic modifier

Table 7 depicts the results obtained for the standardized coefficients of the retention factors of hydrophilic compounds eluted at low organic modifier contents.

Once again, the goodnesses of fit and prediction exhibited high values. Cyanocobalamin and caffeine behaved as neutral solutes, retention factor of which only depended on solvent fraction and temperature. Concerning the other compounds, pH could be considered as the other major factor. The relative influence of main factors depended on the solvent nature: in methanol, pH > %*S* > *T* > Conc. was the observed order for the factor magnitude whereas it was %*S* > pH > *T* > Conc. in acetonitrile. If %*S* affected more the hydrophilic solutes, pH was less influent than at high content of organic modifier: there was no more squared term, confirming that both the apparent pH and the pK_a of basic compounds were closer to the purely aqueous values. It indicated also that the retention mechanisms at high or low contents of organic modifier differed. Peak asymmetries for atropine and for benzylamine suffered from a too low goodness of fit to afford a rational discussion on significant coefficients. Concerning efficiencies, the main significant factor was again pH followed by buffer concentration.

Table 7
Estimates of standardized coefficients for the modeling of retention factors at low level of solvent

Solvent	Strychnine		D-Tubocurarine		Atropine		Ampicillin		Cyanocobalamin		Vancomycin		Caffeine		Benzylamine	
	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN	MeOH	MeCN
Mean value (β_0)	5.01	5.17	6.48	14.10	4.54	5.18	3.04	1.22	4.89	3.05	1.83	0.98	3.61	2.51	0.75	0.63
%*S*	−4.79	−8.69	−8.76	−13.92	−4.54	−8.49	−4.43	−10.02	−9.17	−18.06	−9.36	−18.25	−4.41	−7.43	−2.14	−4.48
T	−2.14	−2.33	−2.87	−2.97	−1.76	−1.65	−1.59	−0.49	−3.30	−2.54	−2.91	−1.99	−1.93	−1.79	−1.13	−1.46
pH	6.86	6.42	13.37	12.26	5.59	5.85	2.23	3.22			4.74	5.62			6.18	6.83
Conc.	0.89	0.84			1.19	1.02									1.42	1.26
%*S* × %*S*		0.86														
T × *T*																
pH × pH																
Conc. × Conc.																
%*S* × *T*																
%*S* × pH					−0.55											
%*S* × Conc.																
T × pH																
T × Conc.														0.55		
pH × Conc.			−1.57													
R^2	1.00	1.00	0.99	0.99	1.00	1.00	0.99	1.00	0.99	1.00	1.00	1.00	0.99	1.00	0.99	0.99
Q^2	0.89	0.88	0.68	0.57	0.85	0.88	0.79	0.79	0.68	0.85	0.78	0.81	0.72	0.80	0.84	0.70

Only significant coefficients are given.

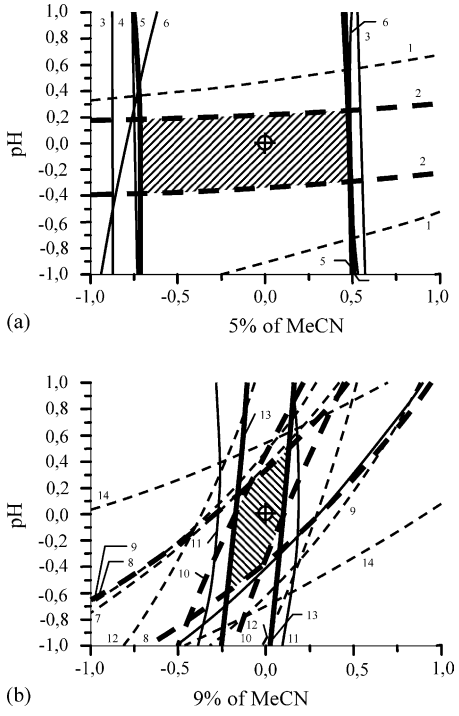

Fig. 2. Limiting contour lines of analytes according to pH and contents of acetonitrile: (a) high content, (b) low content: (1) clofazimine, (2) amiodarone, (3) butylbenzene, (4) pentylbenzene, (5) *o*-terphenyl, (6) triphenylene, (7) strychnine, (8) D-tubocurarine, (9) atropine, (10) ampicillin, (11) cyanocobalamin, (12) vancomycin, (13) caffeine, (14) benzylamine; the focal point stands for the nominal conditions.

4.3. Robustness domain

The final objective of this study was to define the confidence domain within which slight variations in experimental conditions will not affect the test results. As previously stated, the batch-to-batch reproducibility (given in Table 5) was chosen to determine the robust domain of the test. The procedure described in 3.6 was applied to all the responses at the rate of six diagrams per chromatographic parameter. The construction of one robust domain is illustrated by Fig. 2.

For the sake of readability, the construction was split into two steps according to organic modifier fraction. Numbered lines represent the limits obtained for the tolerance criterion applied to the retention factors of each solute. Thick lines indicate the contour lines that defined the robust domain (hatched region). As digitoxin was not limiting, its contour lines were not figured. As dotted lines stand for basic compounds, Fig. 2a shows that the robust domain boundaries depend on the physico-chemical properties of compounds: neutrals are limiting for solvent fraction whereas basics are limiting for pH. On the contrary, the diagonal contour lines on Fig. 2b underline the fact that both %*S* and pH were limiting for basic compounds. It confirmed the differences in retention mechanisms between high and low contents of organic modifier. Moreover, the tolerance domain was more restricted at low than at high content in this case. The final rugged domain resulted from the intersection of the two regions given

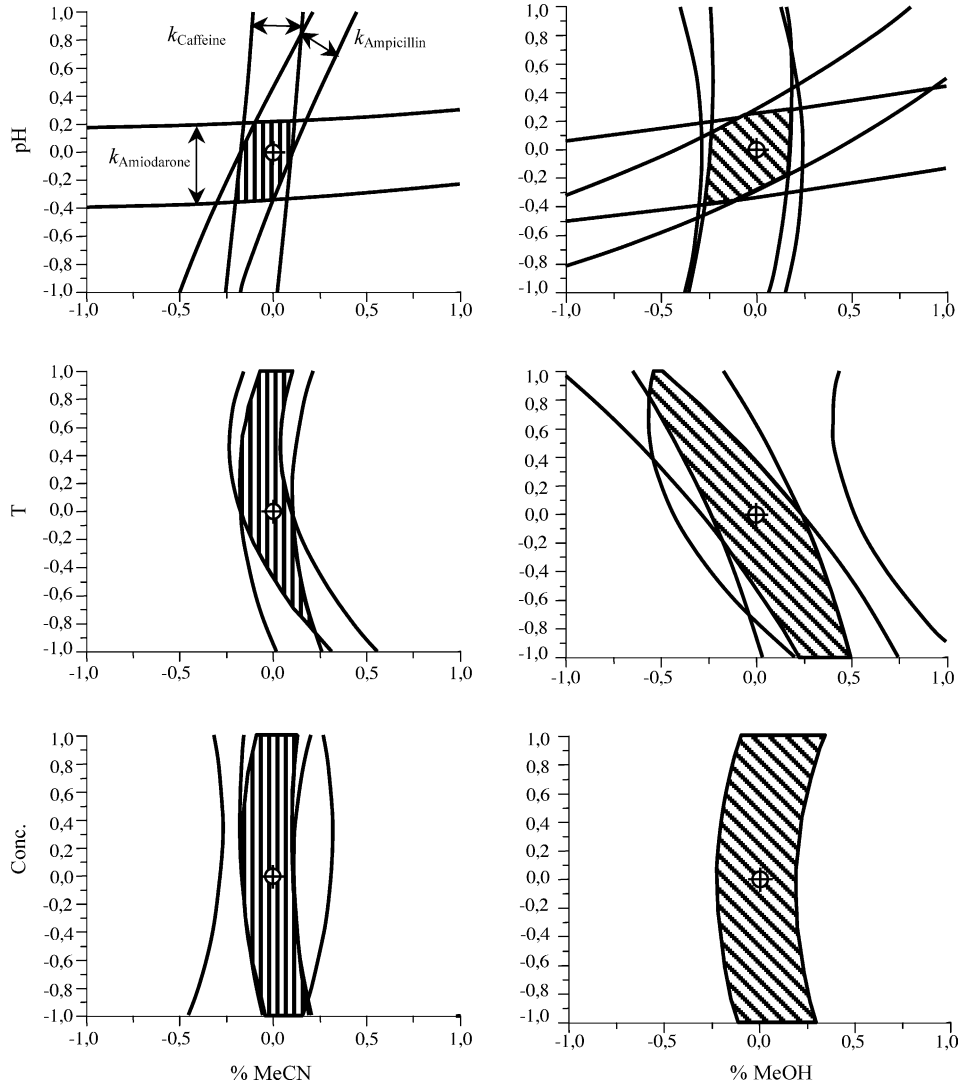

Fig. 3. Robust domains in acetonitrile (left) and in methanol (right) according to pH, temperature and buffer concentration; the focal point stands for the nominal conditions, hatched regions for robust domains.

in Fig. 2, as depicted by the diagram $y = f$(%MeCN, pH) in Fig. 3 (diagram at the top left), on which all the results are recorded. Three compounds were finally limiting concerning this example: amiodarone, ampicillin and caffeine.

First in Fig. 3, the nominal conditions were fairly centered in the 'confidence' domains, confirming the validity of our previous results. Second, the obtained regions were more tightened in acetonitrile mobile phases than in methanol eluents. This phenomenon could be explained by the masking effect of the latter organic modifier: methanol can interact with residual silanols and solutes via H bond interactions whereas acetonitrile cannot aspire to such a role. As a consequence, acetonitrile eluents are more prone to reveal potential interactions between solutes and supports, making the eluites more sensitive for probing. So, a stronger discrimination between chromatographic columns could be expected. Nevertheless, this could-be sensitive effect did not afford only advantages: to keep a similar confidence level in the test than in methanol eluents, the rugged domain was reduced, meaning more closely controlled running conditions. From a practical point of view, tolerance limits at nominal conditions are recorded in Table 8.

Table 8
Tolerance limits of nominal conditions

Test	Solvent fraction (w/w)	T (°C)	pH	Buffer concentration (mM)
MeCN	±0.05%	±0.4	±0.05	±3
MeOH	±0.08%	±0.4	±0.05	±3

Finally, the robust domains could be considered as rather restricted. However, one should remind that the deliberate variations for robustness studies had to be *controlled* and had to be compared with usual experimental errors. For example, let us consider the preparation of a 400 g eluent with 70% of methanol: making an error of 0.05% (the 10th of the

study domain) represents a weighting error of 0.2 g, which is within the characteristics of a classical precision balance. One should notice that this precision level could not have been reached with volumetric preparations for eluents, reinforcing our choice for the experimental protocol.

5. Conclusion

The robustness of our testing procedure has been assessed and the methodology we proposed exhibited different investigation abilities of DoE. It afforded to visualize the robust regions according to the solvent nature, temperature, pH and concentration of the aqueous buffer, and then the acceptable tolerances of the protocol when the testing procedure is to be implemented. At this stage, the confidence domain has been assessed. This step was essential before performing a reliable adding of new stationary phases to the column database and to gather columns with quite similar properties within clusters. This further database extension is necessary to confirm the characterization power of the test. It will afford to get comprehensive classifications of stationary phases for a rational column choice in method development. Moreover, if the discriminating power of the test is high enough, it will be then possible to follow the ageing of columns and define a "robust lifetime".

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