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Analysis of the retention of tetracyclines on reversed-phase columns: Chemometrics, design of experiments and quantitative structure-property relationship (QSPR) study for interpretation and optimization.

Claire Lafossas, Florence Benoit-Marquié, Jean Christophe Garrigues*

CNRS UMR 5623, UPS Laboratoire IMRCP, Université de Toulouse, 118 route de Narbonne, 31062
Toulouse, France

* Corresponding author E-mail: jean-christophe.garrigues@chimie.ups-tlse.fr ,

Phone number: +33 (0)5 61 55 61 51

E-mail addresses of other authors:

C. Lafossas: claire.lafossas@gmail.com

F. Benoit-Marquié: Florence@chimie.ups-tlse.fr

Abstract

In this study, design of experiments was applied for the analysis of 6 reversed phase U-HPLC columns used for the separation of four tetracyclines (TCs): tetracycline, doxycycline, chlortetracycline and oxytetracycline in different elution conditions. In a first part, a fractional factorial design (2^{4-1}) was used to study the influence of four chromatographic parameters: column temperature, pH, flow rate and composition of the mobile phase (i.e. nature of the solvent used as the organic modifier), on the quality of the separation, which was evaluated in terms of peak width and resolution between two pairs of TCs. This experimental design revealed that the nature of the solvent: acetonitrile (ACN) or methanol (MeOH), and the mobile phase flow rate were the two main factors actually having the most influence on the quality of the separation. Moreover, these two factors presented an antagonistic influence according to the response considered: peak width or peak resolution. In order to understand this behavior, a Doehlert design was performed in the second part. It consisted in modeling the evolution of responses as a function of the two main factors: nature of the composition of the mobile phase (mix of ACN and MeOH, from 100 % ACN

to 100 % MeOH) and mobile phase flow rate (from 0.3 to 0.8 mL.min⁻¹). For all the reversed phase columns studied, an inversion of the elution order of TCs and an increase of the retention factors was observed according to the composition of the organic mixture at the end of the gradient. To understand the modification of the interactions implied in the various retention modes related to the selectivity of the organic solvents used, a quantitative structure-property relationship (QSPR) study was achieved. In this final study, the molecular descriptors of each TCs were connected to its retention factor.

Keywords

- Tetracyclines
- Antibiotics
- U-HPLC
- Design of experiments
- Quality by design
- Quantitative structure-property relationship

Highlights

- Design of experiments was applied for the screening of 6 RP U-HPLC columns
- Interpretation of the separation of tetracycline, doxycycline, chlortetracycline, oxytetracycline.
- The robustness area is located in different specific areas
- The optimal conditions differ greatly for the columns tested.
- A QSPR study showed deep modification of the retention mechanisms.

Introduction

The use of veterinary antibiotics to treat diseases, to protect animals from infections or to improve feed efficiency is constantly increasing. Aquaculture is also involved, with its heavy use of

54 prophylactic antibiotics in the aquatic environment: rivers, lakes and oceans [1]. All these treatments
55 result in large amounts of antibiotics being released into various ecosystems [2]. The most used of the
56 broad class of antibiotics are tetracyclines (TCs), followed by β -lactamines, sulfonamides,
57 lincosamides, diaminopyrimidines and macrolides [3]. Since the late 1990s, the intense use and misuse
58 of antibiotics has received particular attention due to correlations with the numbers of resistant
59 pathogenic and commensal bacteria worldwide [4]. Studies of these effects on ecosystems and the
60 potential risks of these compounds for human health have involved the development of extraction [5]
61 and separation methods [6] for the identification and quantification of TCs. Because of the high
62 polarity, water solubility and non-volatility of many antibiotics, liquid chromatography, with diode-
63 array (DAD) [7], mass spectrometry (MS) or tandem MS [8] detection, is widely used for their
64 separation and quantification in complex matrices, such as surface water or wastewater [9]. In
65 particular, TCs have been separated on a variety of HPLC Reversed-phase (RP) columns with isocratic
66 or gradient elution. C18, C8, or phenyl selectivity has been used [10–12] with a great variety of
67 aqueous mobile phases using methanol (MeOH) or acetonitrile (ACN) as organic modifier and buffers
68 or acids such as oxalic acid, formic acid, oxalate buffers and citrate buffers. One of the problems
69 encountered with the separation of TCs is the interaction of these molecules with the residual silanols
70 of the stationary phases, leading to marked peak tailing. Zhu et al. [13] proposed the addition of EDTA
71 or oxalic acid to the mobile phase to limit this effect but the use of these agents decreases the
72 sensitivity of MS detection. Cinquina et al. [14] used a ternary mixture of oxalic acid in water (60%),
73 MeOH (25%) and ACN (15%). Skraskova et al. [15] showed the influence of different column
74 chemistries on the separation of TCs and used ACN or MeOH in optimized gradients. Nevertheless, it
75 is commonly accepted that some chromatographic conditions have antagonistic effects on
76 chromatographic quality. For example, conditions that lead to the narrowest peaks are sometimes very
77 different from those that improve the resolution. From this observation, the choice of mean conditions
78 with a mixture of organic solvents at the end of the gradient of elution are often used, without
79 optimization of the quality of separation. Consequently, it seems interesting to study the different
80 factors influencing the quality of TCs separation using chemometric tools and to connect these factors
81 with molecular interactions between the analytes, the stationary phase and the elution solvents.

Various chemometric methods are already being developed and applied in order to study and optimize the selectivity of the separation of antibiotics by liquid chromatography [10]. Design of experiments, DoE, is one such mathematical tool, which uses a multivariate approach for an exhaustive study of the factors influencing a phenomenon and / or the determination of predictive models. In the field of chromatographic methods, parameters influencing the quality of the separation are all closely linked [16], making these tools particularly effective. Numerous authors have shown the interest of this type of strategy for the analysis of pharmaceutical compounds using liquid chromatography [17–20].

Moreover, thanks to the determination of predictive models, DoE appears to be a very suitable strategy for performing desirability analysis [21] or predicting robustness areas (RAs) and thus finding the analytic optimum [22]. RAs are 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” [23]. They can therefore be considered as theoretical zones in which variations of the parameters of the analysis do not significantly alter separation quality.

In this context, the aim of the present work was to study and analyze the retention of four TCs: tetracycline (TC), doxycycline (DXC), chlortetracycline (CTC) and oxytetracycline (OTC) on six reversed phase U-HPLC columns by means of a methodological approach based on DoE and QSPR study. The chemical structures of the four TCs are given in Figure 1.

Two column technologies were evaluated: (i) the Core-Shell Technology, with three columns: ACQUITY CORTECS C18, 2.7 μm (Waters), KINETEX C18, 1.7 μm (Phenomenex) and NUCLEOSHELL RP18, 2.7 μm (Machery-Nagel) and (ii), for fully porous packings, different particle technologies and surface charges were evaluated with ACQUITY UPLC CSH C18, 1.7 μm (Waters), ACQUITY UPLC HSS T3, 1.8 μm (Waters), and ACQUITY UPLC BEH Shield RP18, 1.7 μm , (Waters). We analyzed the influence of: the selectivity of two organic solvents (ACN, MeOH) in gradient mode, the mobile phase flow rate, the mobile phase pH, and the temperature of the column, by means of experimental design.

First of all, preliminary experiments were used to find the most selective of the six columns. Then, for each column chosen, the chromatographic conditions were analyzed in two stages. Firstly, a Fractional Factorial Matrix was used to screen the four chosen analytical parameters: mobile phase composition, gradient time, mobile phase pH, and column temperature, and determine the influence of each of them on: peak width at 5% of the height of each TCs (W_i), and resolution of critical pairs (R_s). Secondly, the two most effective factors were studied more precisely thanks to a Doehlert Design (DD). The use of this kind of DoE led to the determination of models predicting W_i and R_s at any point of the experimental field. From these data, a robustness area was defined for each column and optimized chromatographic conditions were highlighted and tested. Finally, in order to understand the molecular mechanisms involved in the retention behavior of these TCs, a quantitative structure-property relationship (QSPR) study was carried out by using artificial neural networks (ANN). This QSPR study allowed a molecular-level analysis of the interactions related to the retention of TCs on the various stationary phases, according to the selectivity of the organic solvent used (ACN and MeOH) and the proportion of each one. This QSPR study enabled the modified order of elution of TCs to be analyzed according to the gradient conditions.

Materials and methods

Chemicals

Tetracycline hydrochloride, oxytetracycline, doxycycline and chlortetracycline were purchased from Sigma-Aldrich Co. (St. Quentin Fallavier, France). ACN and MeOH of HPLC grade and formic acid were also obtained from Sigma-Aldrich. All aqueous solutions were prepared using water purified with a milli-Q purification system (Millipore, St. Quentin Yvelines, France). The pH of the 0.1% (v:v) (pH = 2.2) or 0.01% (v:v) (pH = 3.3) aqueous solution of formic acid was measured with a pH-meter (Sartorius, Palaiseau, France).

U-HPLC equipment and separation conditions

U-HPLC analyses were performed using an Acquity® H-Class UPLC system composed of a quaternary solvent manager, a sample FTN manager and a PDA detector ($\lambda=254$ nm) (Waters Corporation, Milford, MA, USA). Analyses were monitored by Empower 3 software (Waters Co.). Eluents were filtered with 47 mm, 0.45 μ m nylon membrane discs (Waters Co.).

The samples (10^{-3} mol.L⁻¹) were prepared in pure water and stored at 4°C, in the dark. Each TCs was injected (2 μ L) separately, as well as in the mixture, for LC analysis and precise determination of the chromatographic parameters. For these analyses, a gradient mode was programmed with all initial conditions t_0 : 100% aqueous solution of formic acid (pH 2.2 or 3.2) and all final conditions t_{end} : 100% organic solvent (MeOH or ACN), with a run time of 3 minutes, a column temperature of 40 °C when using MeOH or 30 °C with ACN as the organic solvent, and a flow rate of 0.6 mL.min⁻¹.

These conditions were applied to 6 reversed phase columns: ACQUITY UPLC BEH Shield RP18, 1.7 μ m, 2.1 x 50 mm (Waters); ACQUITY CORTECS C18, 2.7 μ m, 2.1 x 100 mm (Waters); ACQUITY UPLC CSH C18, 1.7 μ m, 2.1 x 50 mm (Waters); ACQUITY UPLC HSS T3, 1.8 μ m, 2.1 x 50 mm; KINETEX C18, 1.7 μ m, 3 x 50 mm (Phenomenex, Torrance, CA, USA); NUCLEOSHELL RP18, 2.7 μ m, 2 x 100 mm (Macherey-Nagel GmbH, Düren, Germany).

Optimized separation conditions

The separations were carried out on the H-Class UPLC system described previously, on two reversed phase columns: ACQUITY CORTECS C18, 2.7 μ m, 2.1 x 100 mm (Waters) and ACQUITY CSH C18, 1.7 μ m, 2.1 x 50 mm (Waters). Each TCs (10^{-3} mol.L⁻¹) was injected (2 μ L) separately as well as in the mixture, for LC analysis, in gradient mode with all initial conditions (% vol.) t_0 : 100% aqueous solution of formic acid (pH 2.2) and final conditions: UPLC a t_{3min} :25% MeOH, 75% ACN, flow 0.6 mL.min⁻¹; UPLC b t_{3min} :88% MeOH, 12% ACN, flow 0.65 mL.min⁻¹; UPLC c t_{3min} :5% MeOH, 95% ACN, flow 0.5 mL.min⁻¹. UPLC a was applied with the ACQUITY CORTECS C18 column, and UPLC b and UPLC c were applied with the CSH C18 column. For all conditions, the run time was 3 minutes and the column temperature was 40 °C.

Design of Experiments (DoE)

Selection of responses and experimental factors

The first step of a DoE approach is the selection of the chromatographic parameters to be optimized.

These parameters are named responses. The present case focused on two responses:

- Peak width at 5% of the height (W_i) calculated for the TCs injected one by one, expressed in seconds
- Resolution (R_s) of the TC pairs: TC and OTC on the one hand, and CTC and DXC on the other (eq. 1)

$$R_s = 2 \left(\frac{t_{r(i)} - t_{r(j)}}{W_i + W_j} \right) \quad \text{eq. 1}$$

with $t_{r(i)}$ the retention time of the second eluted TCs of the pair and $t_{r(j)}$ the retention time of the first eluted TCs.

The second step consisted in evaluating the experimental parameters likely to significantly affect responses. These parameters are named factors and noted U_i . In the present study, the selected factors were:

- U_1 : Initial mobile phase pH (i.e. formic acid concentration): $\text{pH}_i = 2.2$, i.e. $[\text{HCOOH}] = 0.1 \%$ (v:v), or $\text{pH}_i = 3.2$, $[\text{HCOOH}] = 0.01 \%$ (v:v) corresponding to low and high pH with values lower than and close to the smallest pK_a of the 4 TCs [15].
- U_2 : Type of organic solvent used as modifier in the gradient of elution: pure acetonitrile (ACN), pure methanol (MeOH) or different mixes of the two. These solvents were selected for their differences of polarity, selectivity, and hydrogen bond acidity or basicity, according to the Snyder and Rutan classifications [24].
- U_3 : Mobile phase flow rate: from $Q = 0.3 \text{ mL} \cdot \text{min}^{-1}$ to $Q = 0.8 \text{ mL} \cdot \text{min}^{-1}$, corresponding to a low and high linear velocity for all the column geometries tested.

- U₄: Column temperature: T = 30 °C or T = 40 °C corresponding to a low and high value acting on the viscosity of the mobile phase [25].

DoE selection

The most influential factors among the four mentioned above were selected by using a factorial fractional design. This well-known design was chosen to investigate the effect of each factor, and also had the advantage of highlighting the possible interactions between factors.

DoE construction

Factorial fractional design (FFD)

An FFD determines the most influential factors, that is to say, factors that have the highest effect on the selected response.

The domains of pH_i (U₁); Solvent type (U₂); flow rate (U₃) and temperature (U₄) were 3.2-2.2; Acetonitrile (ACN)-Methanol (MeOH); 0.3-0.8 mL.min⁻¹; 30-40 °C, respectively.

The relation between the selected response Y and the coded variables X_i that are related to the natural variables U_i was expressed as eq. 2:

$$Y = b_0 + \sum_{i=1}^4 b_i X_i + \sum_{i=1}^3 \sum_{\substack{j=2 \\ i < j}}^4 b_{ij} X_i X_j + \sum_{i=1}^2 \sum_{\substack{j=2 \\ i < j}}^3 \sum_{k=3}^4 b_{ijk} X_i X_j X_k + b_{ijkl} X_1 X_2 X_3 X_4 \quad \text{eq. 2}$$

where b_i represents the estimation of the main effect of factor i , b_{ij} the estimation of the effect of the first order interaction between factor i and factor j , and b_{ijk} , b_{ijkl} and b_{ijklm} the estimation of the effect of the interactions of order two, three and four respectively. The aim is to calculate these effects in order to evaluate the influence of each parameter on the response.

Based on this modeled equation, an experimental matrix consisting of 16 different experiments could be generated. However, to reduce the number of experiments required, it was decided to fractionalize the experimental matrix, i.e. to perform only 8 of the 16 experiments. In such cases, it is assumed that some interactions are negligible and consequently can be confounded with significant factors or interactions, resulting in a reduction of the number of experiments required. By this operation, the

205 results obtained are combinations of significant and negligible effects. These combinations are named
 206 aliases, noted I_i . By analyzing the significance of each alias, it is possible to get back to the
 207 significance of main effects and first order interaction effects.

208 Here, it was unlikely that there would be an interaction between the initial pH (represented by X_1), the
 209 composition (represented by X_2) and the flow rate (represented by X_3) of the mobile phase.

210 Consequently, the interaction $X_1X_2X_3$ could be considered negligible and confounded with another
 211 factor or interaction. In our case, it was assumed that: $X_4 = X_1X_2X_3$. From this equation, the defining
 212 relation could be identified as: $I = 1234$.

213

214 The defining relation was then used:

- 215 • To develop the complete alias structure (Table 1).
- 216 • To design the experimental matrix (Table 2)

217

218 Additional runs (1^a to 1^e) were carried out to validate the statistical significance of the results through
 219 an Analysis of Variance model (ANOVA).

220 Table 1: Structure of aliases (FFD)

$I_0 = b_0 + b_{1234}$	$I_4 = b_4 + b_{123}$
$I_1 = b_1 + b_{234}$	$I_5 = b_{12} + b_{34}$
$I_2 = b_2 + b_{134}$	$I_6 = b_{13} + b_{24}$
$I_3 = b_3 + b_{124}$	$I_7 = b_{23} + b_{14}$

Table 2: Experimental matrix with the pH of the aqueous solution (pH_i), organic solvent used in the gradient, flow rate of the mobile phase, column temperature and effect matrix (FFD)

Run	Experimental plan			
	U ₁	U ₂	U ₃	U ₄
	pH _i	Solvent	Flow rate	Temperature
			mL.min ⁻¹	°C
1 ^a	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 ^b	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 ^c	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 ^d	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 ^e	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
2	2.2 (+1)	ACN (-1)	0.3 (-1)	40 (+1)
3	3.2 (-1)	MeOH (+1)	0.3 (-1)	40 (+1)
4	2.2 (+1)	MeOH (+1)	0.3 (-1)	30 (-1)
5	3.2 (-1)	ACN (-1)	0.8 (+1)	40 (+1)
6	2.2 (+1)	ACN (-1)	0.8 (+1)	30 (-1)
7	3.2 (-1)	MeOH (+1)	0.8 (+1)	30 (-1)
8	2.2 (+1)	MeOH (+1)	0.8 (+1)	40 (+1)

Doehlert design (DD)

A DD was performed to model the evolution of peak widths and peak resolutions as a function of the organic solvent in the gradient of elution (named U₁ here) and the mobile phase flow rate (named U₂ here). The aim of this experimental design was to create a surface of response in order to capture the possible nonlinear effects and curvatures in the domain and consequently forecast the response at any point of the domain.

The postulated model is the quadratic equation given by eq. 4:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1X_1 + b_{22}X_2X_2 + b_{12}X_1X_2 \quad \text{eq. 4}$$

where Y represents the selected response, X_1 and X_2 the coded forms of the factors U_1 and U_2 respectively, b_1 and b_2 the estimation of the main effect of factors 1 and 2 respectively, b_{12} the estimation of the effect of the first order interaction between factor U_1 and factor U_2 , b_{11} and b_{22} the estimation of the quadratic effect of the factors U_1 and U_2 respectively.

$X_1 = (U_1 - U_1^0) / \Delta U_1$; $X_2 = (U_2 - U_2^0) / \Delta U_2$, where the value at the center of the experimental domain was 50% for U_1^0 and 0.55 mL.min⁻¹ for U_2^0 , and the variation steps ΔU_1 and ΔU_2 were 25% and 0.25 mL.min⁻¹, respectively. It should be noted that the solvent composition is expressed in terms of %MeOH at the end of the gradient of elution (for example, 25%MeOH means that the solvent composition is MeOH 25% and ACN 75%).

The experimental matrix is given in Table 3. Three additional runs were performed to validate the statistical significance of the results through an Analysis of Variance (ANOVA). For these experiments, pH_i was fixed at 3.2 and the column temperature at 40 °C.

244 Table 3: Experimental matrix with the organic mixture composition at the end of the gradient
 245 (%MeOH), flow rate and effect matrix (DD). The mixture is composed of %MeOH and 100 - %
 246 MeOH for the ACN proportion.

Experimental plan		
Run	U ₁	U ₂
	% MeOH	Flow rate
		mL.min ⁻¹
1	100 (+1)	0.55 (0)
2	0 (-1)	0.55 (0)
3	75 (+0.5)	0.799 (+0.87)
4 ^a	25 (-0.5)	0.301 (-0.87)
4 ^b	25 (-0.5)	0.301 (-0.87)
4 ^c	25 (-0.5)	0.301 (-0.87)
5	75 (+0.5)	0.301 (-0.87)
6	25 (-0.5)	0.799 (+0.87)
7	50 (0)	0.55 (0)

247

248 *DoE analysis*

249 Experimental results obtained from the different designs were analyzed by the statistical graphic
 250 software NemrodW® [26], which was used for the estimation of regression coefficients, statistical
 251 tests, and graphical analysis. The statistical significance was calculated using a classical Analysis of
 252 Variance (ANOVA) model.

253 QSPR study

254 The concept of QSPR studies was proposed in the work of Hammett, Taft, Hanch, Fujita, Free and
 255 Wilson [27–30]. ANNs are widely used for variable selection and model construction in QSAR or

QSPR studies [31]. The multilayer feed forward ANNs associated with a back propagation learning algorithm, proposed by Rumelhart et al. in 1986 [32], were used in this study, with independent input and output layers [33].

The 3D structures of the TCs were optimized using semi-empirical AM1 quantum mechanical calculations of the MOPAC application, in Chem 3D Pro version 14.0 software (CambridgeSoft). An RMS gradient of 0.100 was used to minimize energy for all compounds. The optimized structures were used to calculate 2D and 3D molecular descriptors in the Chem 3D Pro 14.0 and Chem 3D Ultra 9.0 software (CambridgeSoft). Four classes of descriptors were calculated: thermodynamic (boiling point, Bp; critical pressure, Cp; critical temperature, Ct; critical volume, Cv; Gibbs free energy, G; heat of formation, H; Henry's law constant, Hc; ideal gas thermal capacity, Ctc; octanol–water partition coefficient, log P; molar refractivity, MR; melting point, Mp; freezing point, Fp; vapor pressure, Vp; water solubility, log S), topological (Balaban index, J; cluster count, CC; molecular topological index, MTI; Wiener index, W), steric (ovality, O; Connolly accessible area, CAA), and electrostatic (dipole moment, μ ; dipole length, dl; total valence connectivity, Tvc; total connectivity Tcn). An ANN was created using the Stuttgart Neural Network Simulator version 4.2 (University Of Stuttgart, Germany) with a sigmoid activation function and a back-propagation learning algorithm. This ANN was structured with an input layer connected to each molecular descriptor and an output layer linked to the retention factor (k) of each TCs.

Results and discussion

U-HPLC separation of TCs on the 6 RP columns

The typical chromatograms obtained with the 6 RP columns are given in Figure S1 and the retention parameters calculated are given in Table S1.

For all the chromatographic conditions, the four TCs were separated into two groups: OTC and TC in a first group of peaks, followed by CTC and DXC. (Figure S1). Retention times were always increased

by using MeOH as the organic solvent in the gradient of elution and the fastest separation was carried out in 1.58 min with an ACQUITY UPLC CSH C18 column using ACN as the organic solvent in the gradient of elution (Figure S1). In contrast, the results show that the peak width of all the TCs decreased when ACN was used. In fact, the lowest values of W_i were obtained with MeOH on the ACQUITY UPLC BEH Shield RP18, ACQUITY CORTECS C18 and ACQUITY UPLC CSH C18 columns (Table S1). The tailing factor values were close to 1 for the ACQUITY CORTECS C18 and the ACQUITY UPLC BEH Shield RP18 columns for both ACN and MeOH.

The use of a linear gradient varying from 100% aqueous solution at initial conditions to 100% MeOH or ACN after 3 minutes allowed us to identify the column that satisfied the selected criteria of effectiveness with the smallest peak widths, the shortest retention time and a symmetry of peak close to 1. The polarity and similar chemical structures of TCs led to very different chromatographic developments, including simple linear gradients from 100% aqueous to 100% organic with MeOH and ACN [34]. On the basis of these criteria, we chose a fully porous column: ACQUITY UPLC CSH C18, which gave the lowest W_i with ACN, and a Core-Shell column: ACQUITY CORTECS C18, which gave a small value for W_i and a tailing factor close to 1 for ACN and MeOH. These two columns were used in a DoE optimization in order to identify the ideal conditions of retention for these TCs. The analysis of the retention parameters showed great variability of W_i and R_s for the two selected columns, according to whether ACN or MeOH was used (Table S1). For t_f , the variation was not representative when the values obtained with ACN or MeOH were compared for the two selected columns (Table S1). In the DoE phase, the selected criteria were thus W_i and R_s .

For all the columns tested, the order of elution was identical when ACN was used as the organic solvent in the gradient of elution (OTC, TC, CTC then DXC) whereas this order was modified for all the tested columns when MeOH was used (TC eluted before OTC) (Figure S1). A study of the chromatograms showed that this peak inversion was accompanied by an increase in retention times for all the TCs and all the columns tested (Figure S1).

Fractional factorial design

First, a factorial fractional design was performed to highlight the chromatographic factors that had the most influence, for the two columns selected (ACQUITY CORTECS C18 and ACQUITY UPLC CSH C18), that is to say, the factors that had the greatest effect on the selected responses. Responses were peak widths (W_{TC} , W_{OTC} , W_{CTC} , W_{DXC}) and peak resolutions ($R_{S_{TC/OTC}}$, $R_{S_{CTC/DXC}}$). The choice of these two complementary chromatographic parameters allowed the width of each chromatographic peak to be optimized, firstly by considering the effect of the analytical conditions over this width. The resolution calculation improved the separation analysis of the four peaks. The chosen factors and their values are detailed in part 2.4.1. In each case, the two levels had to be sufficiently different to induce response variations.

The experiments described in the experimental matrix (Table 2) were performed with the two columns. The chromatographic results: peak widths and resolutions, are given in the complementary data for each column (Table S1). Aliases l_1 , l_2 , l_3 , l_4 , l_5 , l_6 and l_7 , were calculated by least squares linear regressions of the experimental results, together with the margin of error (by means of a Student test), by the NemrodW software, for the 6 responses of each column.

For all the responses studied, according to the structure of the aliases (Table 1), and considering that interactions between three or more factors were irrelevant, (i) aliases l_1 , l_2 , l_3 , l_4 were almost equal to the corresponding factor effect, respectively b_1 , b_2 , b_3 , b_4 , and (ii) l_5 , l_6 and l_7 represented combinations of interaction effects, respectively $b_{12} + b_{34}$, $b_{13} + b_{24}$, $b_{23} + b_{14}$. For all TCs, the initial pH of the mobile phase, the solvent type in the gradient of elution and the mobile phase flow rate had significant effects on peak width. The values of aliases l_2 and l_3 were the highest, showing clearly that the two most influential factors were the solvent nature and the mobile phase flow rate.

The study of the interaction effects contained in the l_6 and l_7 aliases revealed that interactions between the initial pH of the mobile phase and the solvent type in the gradient of elution (b_{12}), and between the mobile phase flow rate and temperature (b_{34}) did not have significance influence. In contrast, interactions between initial pH and flow rate (b_{13}), solvent type and temperature (b_{24}), solvent type and

flow rate (b_{23}) and initial pH and temperature (b_{14}) seemed to have significant influence on the TCs peak widths. Nevertheless, as the temperature did not have significant influence, it can reasonably be considered that interactions including this factor were not significant. Thus, interactions b_{24} and b_{14} can be judged non-significant. The other two interactions b_{13} and b_{23} need to be studied more precisely through their interaction diagrams (Figure 2).

Each diagram represents the four different combinations of the two factors tested in the experimental plan (i.e. for the X_1X_3 diagram: $X_1 = +1$ and $X_3 = +1$, $X_1 = +1$ and $X_3 = -1$, $X_1 = -1$ and $X_3 = +1$ and $X_1 = -1$ and $X_3 = -1$) and the average of the experimental responses obtained for each combination. From these diagrams, it is possible to find the combination that gives the best condition relative to the chosen response. In the case of the response W_{DXC} , peak width has to be as small as possible, (red circle in Figure 2). Consequently, the best conditions for W_{DXC} are: $X_1 = +1$, $X_2 = -1$ and $X_3 = +1$, i.e. $pHi = 2.2$, solvent = ACN and $Q = 0.8 \text{ mL} \cdot \text{min}^{-1}$.

Eventually, from the analysis of the Pareto graph and the interaction diagrams it is possible to conclude that (i) the two most influential factors are the type of solvent and the mobile phase flow rate and (ii) the best conditions to obtain the smallest DXC peak width are:

- Organic solvent in the gradient of elution : pure ACN
- Mobile phase flow rate: $Q = 0.8 \text{ mL} \cdot \text{min}^{-1}$
- Initial mobile phase pH: $pHi = 2.2$ (i.e. $[\text{HCOOH}] = 0.1 \text{ \% (v:v)}$)
- Column temperature: N.S.

The same analysis was repeated for the other responses and for the two columns. It should be noted that the peak width needs to be as low as possible and the resolution between two peaks as close to two as possible. The best conditions obtained in each case are summarized in Table 4 for the two columns.

356

357 Table 4: Results from analysis of Student test and interaction diagrams for the 6 responses and the two
 358 different columns

Factors	Results											
	ACQUITY UPLC CSH C18 column						ACQUITY CORTECS C18 column					
	Peak width				Peak resolution		Peak width				Peak resolution	
	W _{TC}	W _{DXC}	W _{CTC}	W _{OTC}	Rs	Rs	W _{TC}	W _{DXC}	W _{CTC}	W _{OTC}	Rs	Rs
					TC/OTC	CTC/DXC					TC/OTC	CTC/DXC
pH _i	2.2	2.2	2.2	2.2	N.S.	2.2	2.2	2.2	2.2	2.2	2.2	2.2
Solvent	ACN	ACN	ACN	ACN	MeOH	MeOH	ACN	ACN	ACN	ACN	MeOH	MeOH
Flow rate	0.8	0.8	0.8	0.8	0.8	0.3	0.8	0.8	0.8	0.8	0.3	0.3
Temperature	N.S.	N.S.	N.S.	N.S.	40	30	N.S.	N.S.	N.S.	N.S.	40	30

359

360 From these results, it is possible to conclude, firstly, that the most influential factors were the type of
 361 solvent in the gradient of elution and the mobile phase flow rate for the two columns and the six
 362 different responses. In the same way, all the responses were enhanced when the mobile phase had a pH
 363 = 2.2. It appears that the effect of the temperature on W_i was not significant, in the selected range, for
 364 either of the columns. For Rs, the temperature had a different effect according to the peak pairs
 365 TC/OTC and CTC/DXC. The effect was greater with the flow rate of 0.8 mL/min and with MeOH
 366 used as the organic solvent in the gradient (Table 4). The temperature applied for the optimized
 367 conditions will be 40 °C to take account of the viscosity of MeOH used as organic solvent. The
 368 conditions required to optimize the peak widths were the same for the four TCs and for both columns:
 369 a mobile phase consisting of acetonitrile as solvent in the gradient of elution with a pH of 2.2, and a
 370 flow rate of 0.8 mL.min⁻¹. However, the conditions to obtain the best resolution of peaks presented

some differences from one column to the other and from one critical pair to the other. Globally, the resolution of the two pairs was enhanced when the mobile phase was MeOH with a pH of 2.2, and a flow rate of 0.3 mL.min⁻¹.

These observations revealed that the two main factors, the type of solvent in the gradient of elution and the mobile phase flow rate, had opposite influences according to the response considered: peak width or peak resolution. In order to understand this behavior and find optimal conditions whatever the response, a Doehlert design was performed, modeling the evolution of the six responses as a function of the two main factors.

Doehlert design

The Doehlert design was performed to model the evolution of peak widths and peak resolutions as a function of organic solvent composition at the end of the gradient of elution, and the mobile phase flow rate. It should be noted that the organic solvent was a mix of ACN and MeOH. It is expressed as %MeOH. For example 25%MeOH means that the solvent composition was MeOH 25% and ACN 75%.

Experiments described in the experimental matrix (Table 3) were performed with the two columns (ACQUITY CORTECS C18 and ACQUITY UPLC CSH C18). Model coefficients b_1 , b_2 , b_{11} , b_{22} , and b_{12} were calculated by the NemrodW software and validated through ANOVA tests for the six responses and the two columns. Finally, model equations were expressed in the form of surface responses where the evolution of one response was plotted as a function of the two selected factors. Figure S2 shows the isoresponse curves for W_{TC} (Figure S2a₁), W_{OTC} (Figure S2b₁), W_{CTC} (Figure S2c₁), W_{DXC} (Figure S2d₁), $Rs_{TC/OTC}$ (Figure S2e₁) and $Rs_{CTC/DXC}$ (Figure S2f₁) on the ACQUITY CORTECS C18 column, depending on the organic solvent composition and the mobile phase flow rate. In order to find the optimized conditions, an area of interest was chosen for each response. This area fitted the smallest peak widths and the highest peak resolution. Consequently, only peak widths

smaller than 1.5 (Figure S2a₂, Figure S2b₂, Figure S2c₂ and Figure S2d₂) and resolutions higher than 2 (Figure S2e₂ and Figure S2f₂) were considered.

In order to define the robustness area for the ACQUITY CORTECS C18 column, all areas of interest were superimposed in the same graph, resulting in Figure 3. The same analysis is shown for the ACQUITY UPLC CSH C18 column in Figure 4. In these graphs, the experimental area studied by the Doelhart design is represented by a red circle.

The hatched area is the overlap of the robustness areas of all responses, it indicates the best operating conditions for the separation of these four TCs in U-HPLC using a given column. Thus, the best separation conditions are obtained, for the ACQUITY CORTECS C18 column, with a mobile phase mainly composed of ACN at the end of the gradient of elution and a flow rate of around 0.6 mL.min⁻¹, whereas, for the ACQUITY UPLC CSH C18 column, the mobile phase at the end of the gradient needs to be either pure MeOH with a flow rate higher than 0.65 mL.min⁻¹ or pure ACN with a flow rate around 0.5 to 0.6 mL.min⁻¹.

Optimized separation of the TCs

From these results, the optimized conditions were defined for the 2 columns. For the ACQUITY CORTECS C18 column, the robustness area shows only one optimized zone, close to 25% MeOH, 75% ACN at the end of the gradient of elution. For the ACQUITY UPLC CSH C18 column, two conditions can be applied, with either 88 % MeOH, 12 % ACN or 5 % MeOH, 95% ACN at the end of the gradient of elution. These three conditions were applied to the separation of the four TCs, as three test points. The chromatograms obtained with the two columns are given in Figure 5 and the calculated retention parameters in Table 5.

Table 5 : Retention parameters calculated from U-HPLC optimized chromatograms of TCs solutions: tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DXC) obtained on ACQUITY CORTECS C18, 2.7 µm, 2.1 x 100 mm column, with 0.6 mL.min⁻¹, 25% MeOH, 75% ACN at the end of the gradient (a1), and ACQUITY UPLC CSH C18, 1.7 µm, 2.1 x 50 mm with 0.5 mL.min⁻¹, 5% MeOH, 95% ACN at the end of the gradient (a2) or with 0.65 mL.min⁻¹, 88%

422 MeOH, 12% ACN at the end of the gradient (a3) as the organic solvent in gradient mode. tr: retention
423 time, k' : retention factor, W_i : peak width at 5%, tf: tailing factor, Rs: resolution.

Organic Solvent (end of gradient): 25% MeOH, 75% ACN, Column: ACQUITY CORTECS C18, chromatogram a1, Figure 5.

Compound	tr (min)	k'	W _i (x10 ⁻² min.)	tf	Rs
OTC	1.59	3.0	2.05	1.20	3.86
TC	1.67	3.2	2.03	1.28	
CTC	1.79	3.5	2.20	0.95	2.95
DXC	1.86	3.7	2.06	0.77	

Organic Solvent (end of gradient): 5% MeOH, 95% ACN, Column: CSH C18, chromatogram a2, Figure 5.

Compound	tr (min)	k'	W _i (x10 ⁻² min.)	tf	Rs
OTC	1.40	3.87	2.01	1.07	2.08
TC	1.44	4.00	1.82	1.10	
CTC	1.60	4.57	2.01	0.62	2.54
DXC	1.65	4.71	1.84	0.61	

Organic Solvent (end of gradient): 88% MeOH, 12% ACN, Column: CSH C18, chromatogram a3, Figure 5.

Compound	tr (min)	k'	W _i (x10 ⁻² min.)	tf	Rs
TC	1.36	5.41	2.04	0.87	2.33
OTC	1.41	5.20	1.97	1.02	
CTC	1.63	6.41	2.75	0.62	6.22
DXC	1.80	7.21	2.87	0.60	

Two DoE tools were used to determine optimized conditions for the separation of TCs on two RP18 columns. For the three test points, the objective was achieved for the 4 TCs. The choice of a simple pH modifier (0.1% formic acid in water) and one or two organic solvents at the end of the gradient, revealed one or more robustness zones for the two selected columns. The resolution $R_s > 2$ was obtained with a separation of less than two minutes for the four analytes. The peak widths were also optimized. The lowest values ($\approx 2 \times 10^{-2}$ min) were obtained with the ACQUITY CORTECS C18 column and the gradient conditions UPLC a (25% MeOH, 75% ACN, end of gradient) and for the ACQUITY CSH C18 column with the gradient conditions UPLC c (5% MeOH, 95% ACN, end of gradient). For all the optimized conditions, k' factors were not controlled and varied from 0.60 to 1.28 (Table 5).

The chromatogram for the optimized conditions with 88% MeOH, 12% ACN at the end of gradient, used with the ACQUITY CSH C18 column, showed a modified order of elution for the TCs (Figure 5, a3). This inversion in elution order of TCs was also observed in the screening phase of the six reversed phase columns, when MeOH was used as the organic solvent to replace ACN (Figure S1). With a majority of ACN at the end of the gradient, the order of the first pair of TCs was OTC-TC (Figure 5, a2). In majority presence of MeOH at the end of the gradient, this order changed to TC-OTC (Figure 5, a3). For the ACQUITY CORTECS C18 column, the mixture used at the end of the gradient, 75% ACN, 25% MeOH, showed the OTC-TC order of elution (Figure 5, a1). To study these retention mechanisms and this selectivity related to the solvent used in the end of gradient, we undertook a QSPR study with ANNs, in order to connect the molecular descriptors of each TC to its retention factor, k' . The weights with absolute value > 1 of nodes linked to molecular descriptors of the TCs are given in Figure 6. It appears that, in the presence of ACN-rich mixture (75% ACN; 25% MeOH or 95% ACN; 5% MeOH), only two molecular descriptors have a significant weight: log P, linked to hydrophobicity; and total valence connectivity (T_{vc}), related to the steric structure of the molecule. With ACN-rich mixture, k' is not directly linked to the calculated log P for all TCs and the elution order is: OTC, log P: -4.53; TC, log P: -3.76; CTC, log P: -3.24; DXC, log P: -3.41. DXC with a lower log P is eluted after CTC. The steric parameter T_{vc} shows an important influence in the elution of

CTC/DXC, and shows less influence in the elution of OTC/TC. Koopmans et al. [35] showed the importance of calculated log P in the analysis of the retention of polyaromatics using ACN as organic modifier. In their study, the parameter of connectivity chosen (Tcn) did not bring extra information. In the case of TCs, calculated log P does not predict or explain the order of elution of the compounds. For this, it is necessary to add a steric parameter Tvc or Tcn.

With MeOH-rich mixture, log P is the descriptor of strongest weight (5.64) and 10 other molecular descriptors are linked to the retention factor of the TCs (Figure 6). Four descriptors have very significant weights >3: log P, Balaban and Tcn; two topological parameters; and a thermodynamic descriptor, critical pressure. With a MeOH-rich mixture, the influence of the steric parameters is very marked for the elution of OTC/TC. The QSPR study showed that the retention of the four TCs was controlled by log P and one steric parameter when ACN was mainly used on all the stationary phases retained in our work. In this case, the retention factors, k' , were smaller and lay between 3 and 4.71 under the optimized conditions (Table 5, a1, a2). In presence of a majority of MeOH, the retention was controlled by 10 molecular descriptors, including log P, 2 steric descriptors and 1 thermodynamic descriptor for the four having very strong influence (Figure 6). The mechanisms of retention were then significantly modified, with an increase of k' for the four TCs (range 5.41-7.21, Table 5, a3). This diversity of molecular interactions was also connected with an inversion of the OTC/TC elution order (Figure 5, a3) and no modification of the elution order for CTC/DXC. The stationary phases using charged surface hybrid (CSH) technology showed optimized separation with $R_s > 2$ in 2 different elution systems: using 95% ACN and 5% MeOH or 88% MeOH and 12% ACN at the end of the gradient. With CSH columns, the silica particles are covered with positive charges, allowing an electrostatic repulsion of TCs from free silanols, which are separated by hydrophobic and steric interactions (Figure 6). This type of column was used by K. Skraskova et al. for the separation of TCs in surface water [15]. The authors used ACN or MeOH alone to optimize the retentions, without evaluating the optimization of MeOH/ACN mixtures. A low flow of 0.2 mL/min. was applied by Jia et al. for the separation of TCs on a BEH C18 U-HPLC column by using ACN as organic solvent in a gradient mode [36]. MeOH was used in gradient mode for the separation of TCs on a Luna C18

column [37]. In a recent review of analytical methods used for the separation of TCs, Kim et al. have shown that ACN used in gradient mode with aqueous formic acid is the preferred mobile phase [38]. However, the influence of each of the organic solvents, ACN and MeOH or mixture, is not analyzed on the separation performances. In this study, we show that it is necessary to carry out 7 experiments (Table 3) to identify the flow and the proportion of ACN/MeOH mixture at the end of gradient to obtain a $R_s > 2$ for the 4 TCs on a U-HPLC RP18 column. It is the chemistry of the stationary phase that determines the optimal proportions of the organic solvent of the gradient mobile phase. For the ACQUITY CORTECS C18 column, the mixture at the end of the gradient must contain less than 30% of MeOH and between 90% and 70% of ACN (Figure 3). For the CSH C18 column, 2 different areas allowing optimized separation are identified: one containing 100% ACN or a mixture of more than 90% of ACN with less than 10% of MeOH at the end of the gradient, and the other containing between 88% and 95% of MeOH and less than 5% of ACN at the end of the gradient. It is important to note that, when using MeOH as organic solvent, it is necessary to mix it with between 2% and 5% of ACN to optimize the resolution of the 4 TCs (Figure 4). The QSRR study shows that the mechanisms of retention are equivalent on both ACQUITY CORTECS C18 and ACQUITY CSH C18 columns and implies hydrophobic and steric interactions (Figure 6, a1, a2) when ACN is used as the predominant organic solvent at the end of the gradient. The selectivity, solvent strength, acidity or basicity of ACN and MeOH used as the mobile phase was studied and modeled by Snyder [39] and Glajch [40]. For the 4 TCs, the QSRR study did not show any influence of a molecular parameter linked to proton exchange, or electronic or polar structure of the analytes (Figure 6). The elution order of the 4 TCs was not directly correlated to log P. The mechanism of retention involved hydrophobic and steric interactions as shown in Figure 6. By molecular simulation, Rafferty et al. [41] showed that ACN/water mixture could interact with the alkyl chains of the column at different levels. ACN largely penetrates the alkyl chains, close to the silica substrate. The result is an increase in the alkyl chain order with the use of ACN. With the use of MeOH, the structure of the alkyl chains is more flexible, in connection with an increased weight of the steric parameters, controlling the retention of the TCs in association with hydrophobic interactions.

Conclusion.

This study has shown that the robustness area of some particular columns, such as the ACQUITY UPLC CSH C18, is located in different specific areas corresponding to the use of pure organic modifier or a mixture, depending on the chemistry and type of reversed phase column. These optimal conditions are very different from those more currently used in the literature since most of those studies are performed with average conditions, without robustness optimization. In the case presented here, the antagonistic effects of the retention parameter quality are avoided. Moreover, the optimal areas highlighted in this study are very thin and thus could not be found without the methodology used here, and particularly without the use of a Doelhart network. With this optimization methodology, four Tcs could be separated in less than 2 min. with a resolution $R_s > 2$. Simultaneously with the optimization step, it was possible to study the mechanisms of retention at the molecular level. The QSPR study showed the deep modification of the mechanisms involved with the use of ACN or MeOH as the organic modifier. The increase in the factors of retention, k' , observed with the use of MeOH is connected with the number and the diversity of the molecular descriptors involved in the chromatographic retention. The mechanism of retention involves hydrophobic and steric interactions and is not directly correlated with oil/water partitioning ($\log P$).

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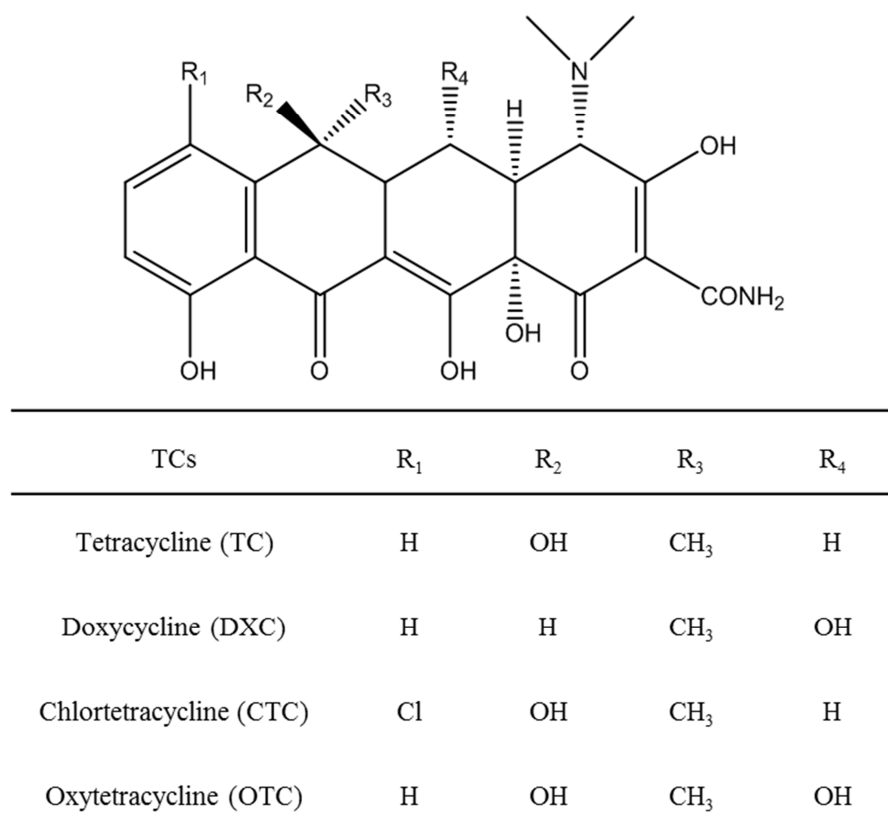


Figure 1: Molecular structures of the TCs analyzed by RP U-HPLC

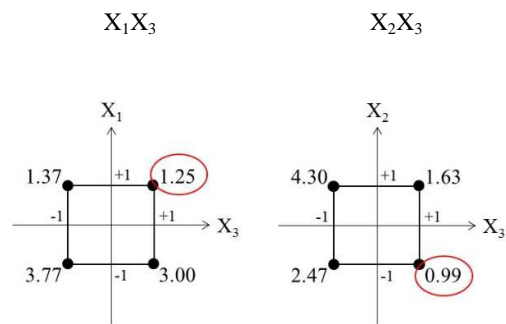


Figure 2: Interaction diagrams X_1X_3 and X_2X_3 , for the W_{DTC} response on the CORTECS C18 column

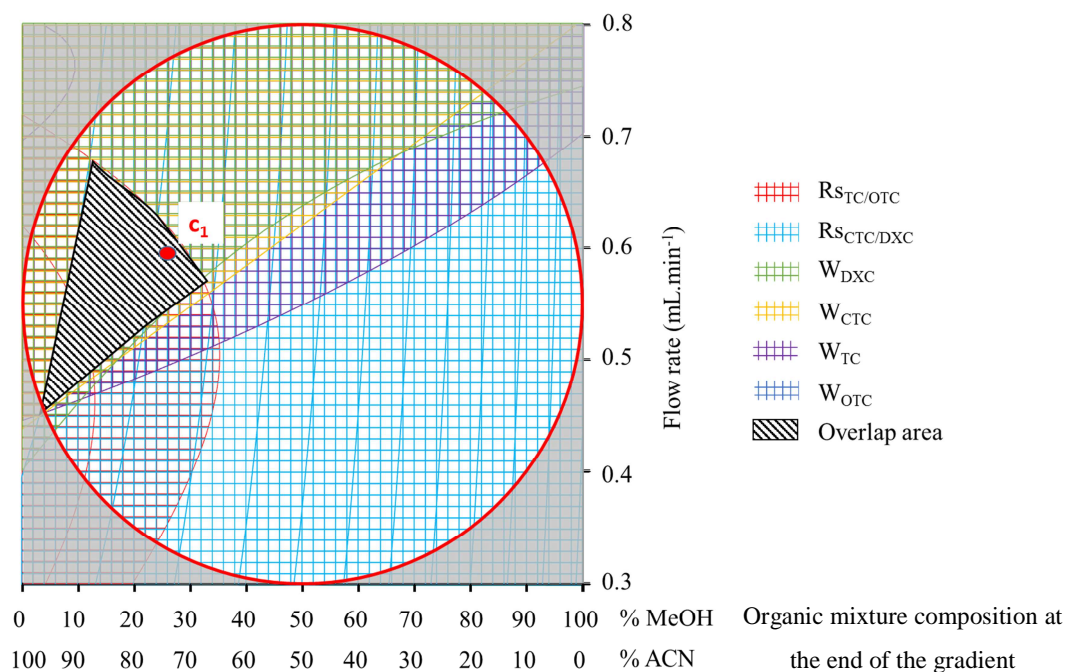


Figure 3: CORTECS C18 column: Overlap of areas of interest showing $Rs_{TC/OTC}$, $Rs_{CTC/DXC}$, W_{TC} , W_{OTC} , W_{CTC} , and W_{DXC} in relation with the flow rate and organic mixture composition (%MeOH and %ACN) at the end of the gradient. The typical point C_1 corresponds to a gradient: t_0 : 100% aqueous solution of formic acid (pH 2.2) and final conditions: t_{3min} : 25% MeOH, 75% ACN, flow 0.6 mL.min⁻¹. W: peak width at 5%, Rs: resolution.

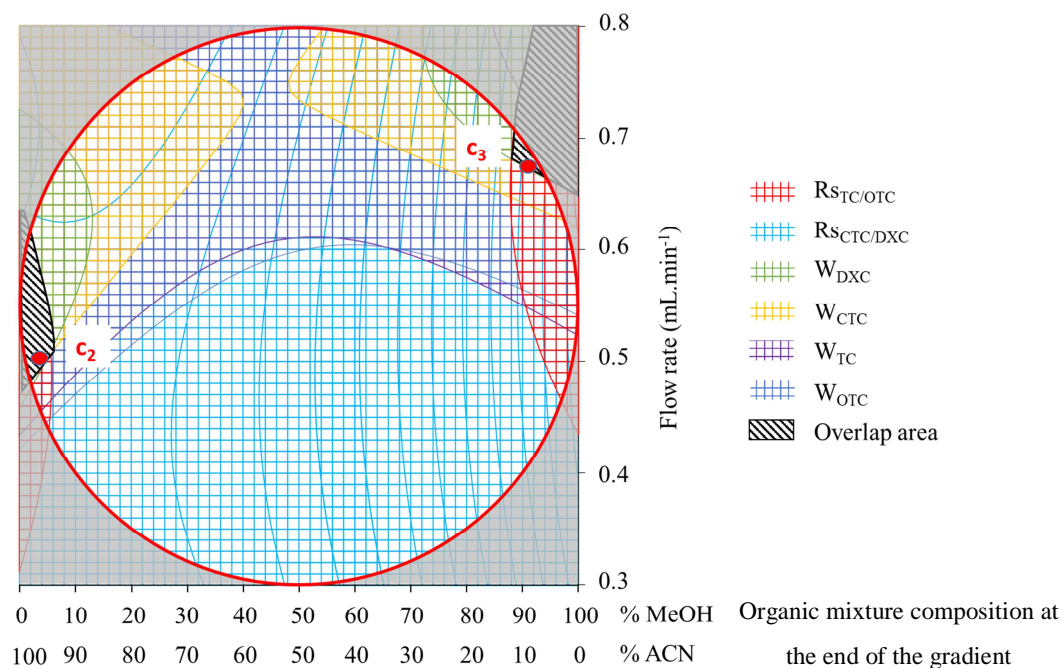


Figure 4: ACQUITY UPLC CSH C18 column: Overlap of areas of interest showing $Rs_{TC/OTC}$, $Rs_{CTC/DXC}$, W_{TC} , W_{OTC} , W_{CTC} , and W_{DXC} in relation with the flow rate and organic mixture composition (%MeOH and %ACN) at the end of the gradient. The typical points correspond to a gradient: t_0 : 100% aqueous solution of formic acid (pH 2.2) and final conditions for C_2 : t_{3min} : 5% MeOH, 95% ACN, flow 0.5 mL.min⁻¹, and for C_3 : t_{3min} : 88% MeOH, 12% ACN, flow 0.65 mL.min⁻¹. W: peak width at 5%, Rs: resolution.

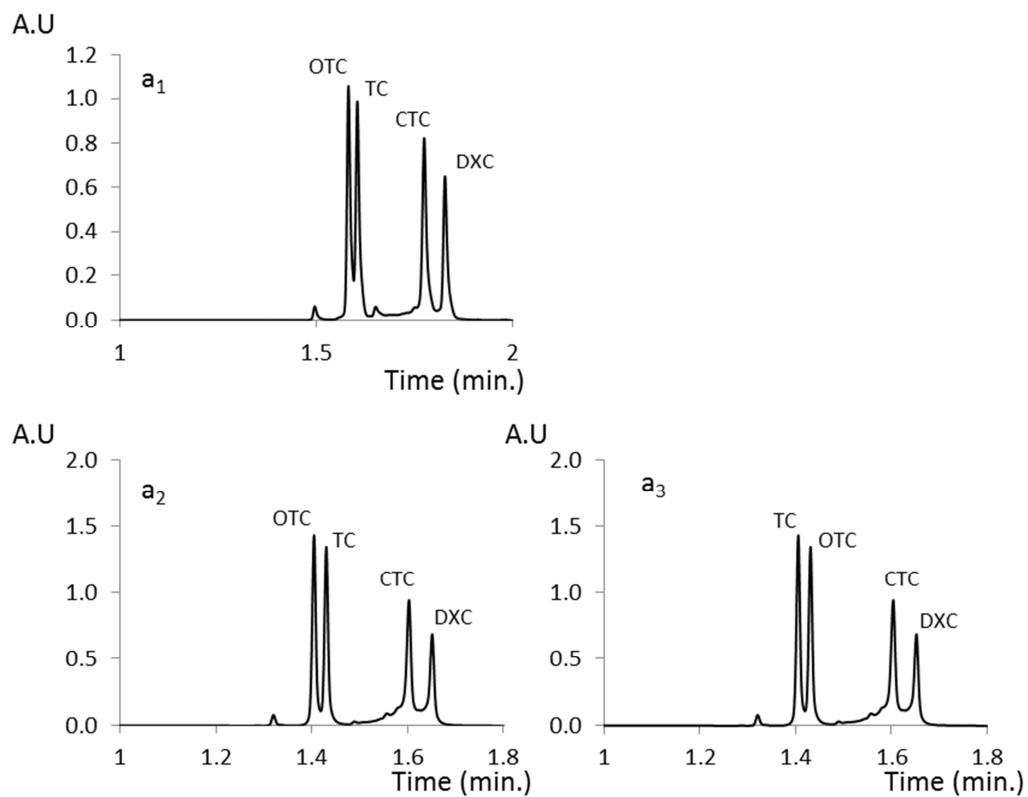


Figure 5: Optimized U-HPLC chromatograms of TC solutions: tetracycline (T), oxytetracycline (O), chlortetracycline (C) and doxycycline (D) obtained on CORTECS C18, 2.7 μm, 2.1 x 100 mm column, with flow rate 0.6 mL.min⁻¹, pH t_0 =2.2, 25% MeOH, 75% ACN at the end of the gradient (a₁), and ACQUITY UPLC CSH C18, 1.7 μm, 2.1 x 50 mm with flow rate 0.5 mL.min⁻¹, 5% MeOH, 95% ACN at the end of the gradient (a₂) or with flow rate 0.65 mL.min⁻¹, 88% MeOH, 12% ACN at the end of the gradient (a₃) as the organic solvent in gradient mode

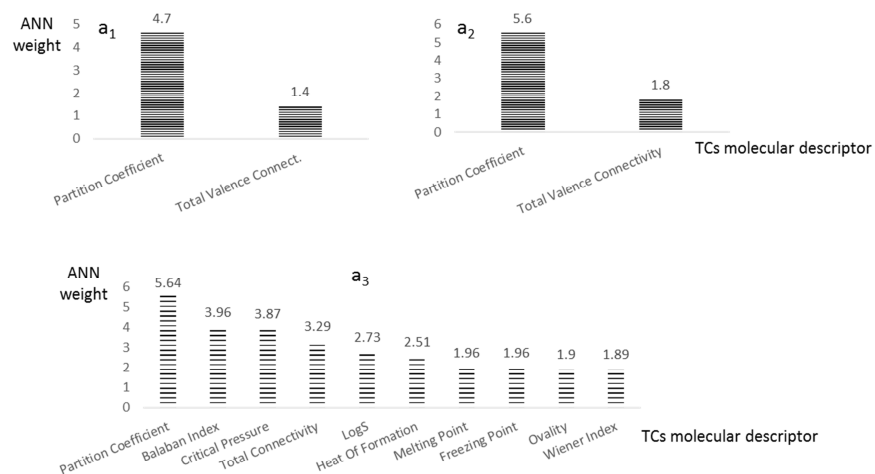
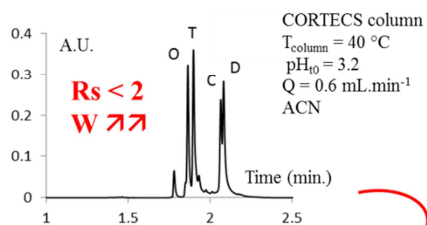
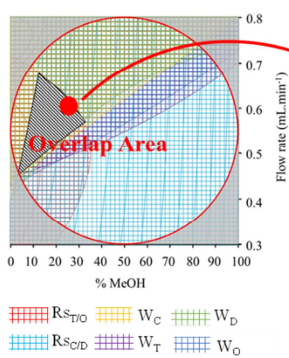


Figure 6: Weight (absolute value) of the key molecular parameters connecting the structures of TCs to their retention factor (k') for the CORTECS column with 25%MeOH, 75%ACN end of gradient (a1), CSH C18 column with 95% ACN, 5% MeOH end of gradient (a2) and 88% MeOH, 12% ACN end of gradient (a3)



Fractional factorial design (2^{4-1})

Factors	Exp region	
pH_{10}	3.2	2.2
Solvent	ACN	MeOH
$Q\text{ (mL}\cdot\text{min}^{-1}\text{)}$	0.3	0.8
$T_{\text{column}}\text{ (}^{\circ}\text{C)}$	30	40



Doehlert Designs

