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### ► To cite this version:

Claire Lafossas, Florence Benoît-marquié, Jean-Christophe Garrigues. 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. *Talanta*, 2019, 198, pp.550-559. 10.1016/j.talanta.2019.02.051 . hal-02194427

**HAL Id: hal-02194427**

**<https://hal.science/hal-02194427>**

Submitted on 22 Oct 2021

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

4  
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13

14 **Abstract**

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

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

35

### 36 **Keywords**

- 37 • Tetracyclines
- 38 • Antibiotics
- 39 • U-HPLC
- 40 • Design of experiments
- 41 • Quality by design
- 42 • Quantitative structure-property relationship

43

### 44 **Highlights**

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

50

### 51 **Introduction**

52 The use of veterinary antibiotics to treat diseases, to protect animals from infections or to improve  
53 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  $\beta$ -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.

82 Various chemometric methods are already being developed and applied in order to study and optimize  
83 the selectivity of the separation of antibiotics by liquid chromatography [10]. Design of experiments,  
84 DoE, is one such mathematical tool, which uses a multivariate approach for an exhaustive study of the  
85 factors influencing a phenomenon and / or the determination of predictive models. In the field of  
86 chromatographic methods, parameters influencing the quality of the separation are all closely linked  
87 [16], making these tools particularly effective. Numerous authors have shown the interest of this type  
88 of strategy for the analysis of pharmaceutical compounds using liquid chromatography [17–20].

89 Moreover, thanks to the determination of predictive models, DoE appears to be a very suitable strategy  
90 for performing desirability analysis [21] or predicting robustness areas (RAs) and thus finding the  
91 analytic optimum [22]. RAs are defined as “the multidimensional combination and interaction of input  
92 variables (e.g. material attributes) and process parameters that have been demonstrated to provide  
93 assurance of quality” [23]. They can therefore be considered as theoretical zones in which variations  
94 of the parameters of the analysis do not significantly alter separation quality.

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

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

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

## 122 **Materials and methods**

### 123 **Chemicals**

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

### 130 **U-HPLC equipment and separation conditions**

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

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

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

#### 146 Optimized separation conditions

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

156 Design of Experiments (DoE)

157 *Selection of responses and experimental factors*

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

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

- 160
- Peak width at 5% of the height ( $W_i$ ) calculated for the TCs injected one by one, expressed in  
161 seconds
  - Resolution ( $R_s$ ) of the TC pairs: TC and OTC on the one hand, and CTC and DXC on the  
162 other (eq. 1)  
163

164

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

165

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

168

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

- 172
- $U_1$ : Initial mobile phase pH (i.e. formic acid concentration):  $\text{pH}_i = 2.2$ , i.e.  $[\text{HCOOH}] = 0.1 \%$   
173 (v:v), or  $\text{pH}_i = 3.2$ ,  $[\text{HCOOH}] = 0.01 \%$  (v:v) corresponding to low and high pH with values  
174 lower than and close to the smallest  $\text{pK}_a$  of the 4 TCs [15].
  - $U_2$ : Type of organic solvent used as modifier in the gradient of elution: pure acetonitrile  
175 (ACN), pure methanol (MeOH) or different mixes of the two. These solvents were selected for  
176 their differences of polarity, selectivity, and hydrogen bond acidity or basicity, according to  
177 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  
178  
179 low and high linear velocity for all the column geometries tested.  
180



181 • U<sub>4</sub>: Column temperature: T = 30 °C or T = 40 °C corresponding to a low and high value acting  
182 on the viscosity of the mobile phase [25].

183

#### 184 *DoE selection*

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

#### 188 *DoE construction*

##### 189 Factorial fractional design (FFD)

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

192 The domains of pH<sub>1</sub> (U<sub>1</sub>); Solvent type (U<sub>2</sub>); flow rate (U<sub>3</sub>) and temperature (U<sub>4</sub>) were 3.2-2.2;  
193 Acetonitrile (ACN)-Methanol (MeOH); 0.3-0.8 mL.min<sup>-1</sup>; 30-40 °C, respectively.

194 The relation between the selected response Y and the coded variables X<sub>i</sub> that are related to the natural  
195 variables U<sub>i</sub> was expressed as eq. 2:

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

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

200 Based on this modeled equation, an experimental matrix consisting of 16 different experiments could  
201 be generated. However, to reduce the number of experiments required, it was decided to fractionalize  
202 the experimental matrix, i.e. to perform only 8 of the 16 experiments. In such cases, it is assumed that  
203 some interactions are negligible and consequently can be confounded with significant factors or  
204 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  $l_i$ . 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)

---

$l_0 = b_0 + b_{1234}$	$l_4 = b_4 + b_{123}$
$l_1 = b_1 + b_{234}$	$l_5 = b_{12} + b_{34}$
$l_2 = b_2 + b_{134}$	$l_6 = b_{13} + b_{24}$
$l_3 = b_3 + b_{124}$	$l_7 = b_{23} + b_{14}$

---

221 Table 2: Experimental matrix with the pH of the aqueous solution (pH<sub>i</sub>), organic solvent used in the  
 222 gradient, flow rate of the mobile phase, column temperature and effect matrix (FFD)

Experimental plan				
Run	U <sub>1</sub>	U <sub>2</sub>	U <sub>3</sub>	U <sub>4</sub>
	pH <sub>i</sub>	Solvent	Flow rate mL.min <sup>-1</sup>	Temperature °C
1 <sup>a</sup>	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 <sup>b</sup>	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 <sup>c</sup>	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 <sup>d</sup>	3.2 (-1)	ACN (-1)	0.3 (-1)	30 (-1)
1 <sup>e</sup>	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)

223

224 Doehlert design (DD)

225 A DD was performed to model the evolution of peak widths and peak resolutions as a function of the  
 226 organic solvent in the gradient of elution (named U<sub>1</sub> here) and the mobile phase flow rate (named U<sub>2</sub>  
 227 here ). The aim of this experimental design was to create a surface of response in order to capture the  
 228 possible nonlinear effects and curvatures in the domain and consequently forecast the response at any  
 229 point of the domain.

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

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

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

235  $X_1 = (U_1 - U_1^0) / \Delta U_1$  ;  $X_2 = (U_2 - U_2^0) / \Delta U_2$ , where the value at the center of the experimental domain  
236 was 50% for  $U_1^0$  and 0.55 mL.min<sup>-1</sup> for  $U_2^0$ , and the variation steps  $\Delta U_1$  and  $\Delta U_2$  were 25% and  
237 0.25 mL.min<sup>-1</sup>, respectively. It should be noted that the solvent composition is expressed in terms of  
238 %MeOH at the end of the gradient of elution (for example, 25%MeOH means that the solvent  
239 composition is MeOH 25% and ACN 75%).

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

243

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 <sub>1</sub>	U <sub>2</sub>
	% MeOH	Flow rate mL.min <sup>-1</sup>
1	100 (+1)	0.55 (0)
2	0 (-1)	0.55 (0)
3	75 (+0.5)	0.799 (+0.87)
4 <sup>a</sup>	25 (-0.5)	0.301 (-0.87)
4 <sup>b</sup>	25 (-0.5)	0.301 (-0.87)
4 <sup>c</sup>	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

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

259 The 3D structures of the TCs were optimized using semi-empirical AM1 quantum mechanical  
260 calculations of the MOPAC application, in Chem 3D Pro version 14.0 software (CambridgeSoft). An  
261 RMS gradient of 0.100 was used to minimize energy for all compounds. The optimized structures  
262 were used to calculate 2D and 3D molecular descriptors in the Chem 3D Pro 14.0 and Chem 3D Ultra  
263 9.0 software (CambridgeSoft). Four classes of descriptors were calculated: thermodynamic (boiling  
264 point, Bp; critical pressure, Cp; critical temperature, Ct; critical volume, Cv; Gibbs free energy, G;  
265 heat of formation, H; Henry's law constant, Hc; ideal gas thermal capacity, Ctc; octanol-water  
266 partition coefficient, log P; molar refractivity, MR; melting point, Mp; freezing point, Fp; vapor  
267 pressure, Vp; water solubility, log S), topological (Balaban index, J; cluster count, CC; molecular  
268 topological index, MTI; Wiener index, W), steric (ovality, O; Connolly accessible area, CAA), and  
269 electrostatic (dipole moment,  $\mu$ ; dipole length, dl; total valence connectivity, Tvc; total connectivity  
270 Tcn). An ANN was created using the Stuttgart Neural Network Simulator version 4.2 (University Of  
271 Stuttgart, Germany) with a sigmoid activation function and a back-propagation learning algorithm.  
272 This ANN was structured with an input layer connected to each molecular descriptor and an output  
273 layer linked to the retention factor (k) of each TCs.

274

## 275 **Results and discussion**

276 U-HPLC separation of TCs on the 6 RP columns

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

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

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

288

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

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

307 Fractional factorial design

308 First, a factorial fractional design was performed to highlight the chromatographic factors that had the  
309 most influence, for the two columns selected (ACQUITY CORTECS C18 and ACQUITY UPLC CSH  
310 C18), that is to say, the factors that had the greatest effect on the selected responses. Responses were  
311 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  
312 two complementary chromatographic parameters allowed the width of each chromatographic peak to  
313 be optimized, firstly by considering the effect of the analytical conditions over this width. The  
314 resolution calculation improved the separation analysis of the four peaks. The chosen factors and their  
315 values are detailed in part 2.4.1. In each case, the two levels had to be sufficiently different to induce  
316 response variations.

317 The experiments described in the experimental matrix (Table 2) were performed with the two  
318 columns. The chromatographic results: peak widths and resolutions, are given in the complementary  
319 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  
320 regressions of the experimental results, together with the margin of error (by means of a Student test),  
321 by the NemrodW software, for the 6 responses of each column.

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

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



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

338 Each diagram represents the four different combinations of the two factors tested in the experimental  
339 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  $X_1$   
340  $= -1$  and  $X_3 = -1$ ) and the average of the experimental responses obtained for each combination. From  
341 these diagrams, it is possible to find the combination that gives the best condition relative to the  
342 chosen response. In the case of the response  $W_{DXC}$ , peak width has to be as small as possible, (red  
343 circle in Figure 2). Consequently, the best conditions for  $W_{DXC}$  are:  $X_1 = +1$ ,  $X_2 = -1$  and  $X_3 = +1$ , i.e.  
344  $pHi = 2.2$ , solvent = ACN and  $Q = 0.8 \text{ mL}\cdot\text{min}^{-1}$ .

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

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

352 The same analysis was repeated for the other responses and for the two columns. It should be noted  
353 that the peak width needs to be as low as possible and the resolution between two peaks as close to  
354 two as possible. The best conditions obtained in each case are summarized in Table 4 for the two  
355 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	
	$W_{TC}$	$W_{DXC}$	$W_{CTC}$	$W_{OTC}$	$R_s$ TC/OTC	$R_s$ CTC/DXC	$W_{TC}$	$W_{DXC}$	$W_{CTC}$	$W_{OTC}$	$R_s$ TC/OTC	$R_s$ CTC/DXC
pH <sub>i</sub>	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  $R_s$ , 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<sup>-1</sup>. However, the conditions to obtain the best resolution of peaks presented

371 some differences from one column to the other and from one critical pair to the other. Globally, the  
372 resolution of the two pairs was enhanced when the mobile phase was MeOH with a pH of 2.2, and a  
373 flow rate of 0.3 mL.min<sup>-1</sup>.

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

379

#### 380 Doehlert design

381 The Doehlert design was performed to model the evolution of peak widths and peak resolutions as a  
382 function of organic solvent composition at the end of the gradient of elution, and the mobile phase  
383 flow rate. It should be noted that the organic solvent was a mix of ACN and MeOH. It is expressed as  
384 %MeOH. For example 25%MeOH means that the solvent composition was MeOH 25% and ACN  
385 75%.

386 Experiments described in the experimental matrix (Table 3) were performed with the two columns  
387 (ACQUITY CORTECS C18 and ACQUITY UPLC CSH C18). Model coefficients  $b_1$ ,  $b_2$ ,  $b_{11}$ ,  $b_{22}$ , and  
388  $b_{12}$  were calculated by the NemrodW software and validated through ANOVA tests for the six  
389 responses and the two columns. Finally, model equations were expressed in the form of surface  
390 responses where the evolution of one response was plotted as a function of the two selected factors.  
391 Figure S2 shows the isoresponse curves for  $W_{TC}$  (Figure S2a<sub>1</sub>),  $W_{OTC}$  (Figure S2b<sub>1</sub>),  $W_{CTC}$  (Figure  
392 S2c<sub>1</sub>),  $W_{DXC}$  (Figure S2d<sub>1</sub>),  $Rs_{TC/OTC}$  (Figure S2e<sub>1</sub>) and  $Rs_{CTC/DXC}$  (Figure S2f<sub>1</sub>) on the ACQUITY  
393 CORTECS C18 column, depending on the organic solvent composition and the mobile phase flow  
394 rate. In order to find the optimized conditions, an area of interest was chosen for each response. This  
395 area fitted the smallest peak widths and the highest peak resolution. Consequently, only peak widths

396 smaller than 1.5 (Figure S2a<sub>2</sub>, Figure S2b<sub>2</sub>, Figure S2c<sub>2</sub> and Figure S2d<sub>2</sub>) and resolutions higher than 2  
397 (Figure S2e<sub>2</sub> and Figure S2f<sub>2</sub>) were considered.

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

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

#### 409 Optimized separation of the TCs

410 From these results, the optimized conditions were defined for the 2 columns. For the ACQUITY  
411 CORTECS C18 column, the robustness area shows only one optimized zone, close to 25% MeOH,  
412 75% ACN at the end of the gradient of elution. For the ACQUITY UPLC CSH C18 column, two  
413 conditions can be applied, with either 88 % MeOH, 12 % ACN or 5 % MeOH, 95% ACN at the end of  
414 the gradient of elution. These three conditions were applied to the separation of the four TCs, as three  
415 test points. The chromatograms obtained with the two columns are given in Figure 5 and the  
416 calculated retention parameters in Table 5.

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

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

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

Compound	tr (min)	k'	W <sub>i</sub> (x10 <sup>-2</sup> 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 <sub>i</sub> (x10 <sup>-2</sup> 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 <sub>i</sub> (x10 <sup>-2</sup> 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	

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

435 The chromatogram for the optimized conditions with 88% MeOH, 12% ACN at the end of gradient,  
436 used with the ACQUITY CSH C18 column, showed a modified order of elution for the TCs (Figure 5,  
437 a3). This inversion in elution order of TCs was also observed in the screening phase of the six reversed  
438 phase columns, when MeOH was used as the organic solvent to replace ACN (Figure S1). With a  
439 majority of ACN at the end of the gradient, the order of the first pair of TCs was OTC-TC (Figure 5,  
440 a2). In majority presence of MeOH at the end of the gradient, this order changed to TC-OTC (Figure  
441 5, a3). For the ACQUITY CORTECS C18 column, the mixture used at the end of the gradient, 75%  
442 ACN, 25% MeOH, showed the OTC-TC order of elution (Figure 5, a1). To study these retention  
443 mechanisms and this selectivity related to the solvent used in the end of gradient, we undertook a  
444 QSPR study with ANNs, in order to connect the molecular descriptors of each TC to its retention  
445 factor,  $k'$ . The weights with absolute value  $> 1$  of nodes linked to molecular descriptors of the TCs are  
446 given in Figure 6. It appears that, in the presence of ACN-rich mixture (75% ACN; 25% MeOH or  
447 95% ACN; 5% MeOH), only two molecular descriptors have a significant weight: log P, linked to  
448 hydrophobicity; and total valence connectivity ( $T_{vc}$ ), related to the steric structure of the molecule.  
449 With ACN-rich mixture,  $k'$  is not directly linked to the calculated log P for all TCs and the elution  
450 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  
451 log P is eluted after CTC. The steric parameter  $T_{vc}$  shows an important influence in the elution of

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

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



479 column [37]. In a recent review of analytical methods used for the separation of TCs, Kim et al. have  
480 shown that ACN used in gradient mode with aqueous formic acid is the preferred mobile phase [38].  
481 However, the influence of each of the organic solvents, ACN and MeOH or mixture, is not analyzed  
482 on the separation performances. In this study, we show that it is necessary to carry out 7 experiments  
483 (Table 3) to identify the flow and the proportion of ACN/MeOH mixture at the end of gradient to  
484 obtain a  $R_s > 2$  for the 4 TCs on a U-HPLC RP18 column. It is the chemistry of the stationary phase  
485 that determines the optimal proportions of the organic solvent of the gradient mobile phase. For the  
486 ACQUITY CORTECS C18 column, the mixture at the end of the gradient must contain less than 30%  
487 of MeOH and between 90% and 70% of ACN (Figure 3). For the CSH C18 column, 2 different areas  
488 allowing optimized separation are identified: one containing 100% ACN or a mixture of more than  
489 90% of ACN with less than 10% of MeOH at the end of the gradient, and the other containing between  
490 88% and 95% of MeOH and less than 5% of ACN at the end of the gradient. It is important to note  
491 that, when using MeOH as organic solvent, it is necessary to mix it with between 2% and 5% of ACN  
492 to optimize the resolution of the 4 TCs (Figure 4). The QSRR study shows that the mechanisms of  
493 retention are equivalent on both ACQUITY CORTECS C18 and ACQUITY CSH C18 columns and  
494 implies hydrophobic and steric interactions (Figure 6, a1, a2) when ACN is used as the predominant  
495 organic solvent at the end of the gradient. The selectivity, solvent strength, acidity or basicity of ACN  
496 and MeOH used as the mobile phase was studied and modeled by Snyder [39] and Glajch [40]. For the  
497 4 TCs, the QSRR study did not show any influence of a molecular parameter linked to proton  
498 exchange, or electronic or polar structure of the analytes (Figure 6). The elution order of the 4 TCs  
499 was not directly correlated to log P. The mechanism of retention involved hydrophobic and steric  
500 interactions as shown in Figure 6. By molecular simulation, Rafferty et al. [41] showed that  
501 ACN/water mixture could interact with the alkyl chains of the column at different levels. ACN largely  
502 penetrates the alkyl chains, close to the silica substrate. The result is an increase in the alkyl chain  
503 order with the use of ACN. With the use of MeOH, the structure of the alkyl chains is more flexible, in  
504 connection with an increased weight of the steric parameters, controlling the retention of the TCs in  
505 association with hydrophobic interactions.

506 **Conclusion.**

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

522 **References.**

- 523 [1] F.C. Cabello, Heavy use of prophylactic antibiotics in aquaculture: a growing problem for  
524 human and animal health and for the environment., *Environ. Microbiol.* 8 (2006) 1137–1144.  
525 doi:10.1111/j.1462-2920.2006.01054.x.
- 526 [2] J.L. Martinez, Environmental pollution by antibiotics and by antibiotic resistance determinants,  
527 *Environ. Pollut.* 157 (2009) 2893–2902. doi:10.1016/j.envpol.2009.05.051.
- 528 [3] A.K. Sarmah, M.T. Meyer, A.B.A. Boxall, A global perspective on the use, sales, exposure  
529 pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment,  
530 *Chemosphere.* 65 (2006) 725–759. doi:10.1016/j.chemosphere.2006.03.026.
- 531 [4] T.M. Barbosa, S.B. Levy, The impact of antibiotic use on resistance development and

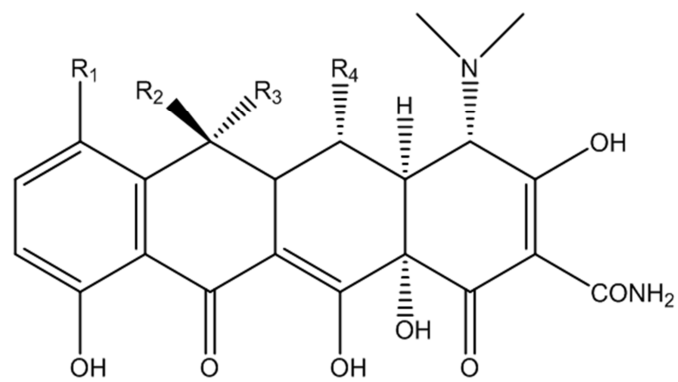
- 532 persistence, *Drug Resist.* 3 (2000) 303–311. doi:10.1054/drup.2000.0167.
- 533 [5] B. Subedi, L. Aguilar, E.M. Robinson, K.J. Hageman, E. Björklund, R.J. Sheesley, et al.,  
534 Selective pressurized liquid extraction as a sample-preparation technique for persistent organic  
535 pollutants and contaminants of emerging concern, *TrAC - Trends Anal. Chem.* 68 (2015) 119–  
536 132. doi:10.1016/j.trac.2015.02.011.
- 537 [6] M.S. Díaz-Cruz, D. Barceló, Recent advances in LC-MS residue analysis of veterinary  
538 medicines in the terrestrial environment, *TrAC - Trends Anal. Chem.* 26 (2007) 637–646.  
539 doi:10.1016/j.trac.2007.04.004.
- 540 [7] J. Tuerk, M. Reinders, D. Dreyer, T.K. Kiffmeyer, K.G. Schmidt, H.M. Kuss, Analysis of  
541 antibiotics in urine and wipe samples from environmental and biological monitoring -  
542 Comparison of HPLC with UV-, single MS- and tandem MS-detection, *J. Chromatogr. B Anal.*  
543 *Technol. Biomed. Life Sci.* 831 (2006) 72–80. doi:10.1016/j.jchromb.2005.11.030.
- 544 [8] L.J. Zhou, G.G. Ying, S. Liu, J.L. Zhao, F. Chen, R.Q. Zhang, et al., Simultaneous  
545 determination of human and veterinary antibiotics in various environmental matrices by rapid  
546 resolution liquid chromatography-electrospray ionization tandem mass spectrometry, *J.*  
547 *Chromatogr. A.* 1244 (2012) 123–138. doi:10.1016/j.chroma.2012.04.076.
- 548 [9] S. Babić, D. Ašperger, D. Mutavdžić, A.J.M. Horvat, M. Kaštelan-Macan, Solid phase  
549 extraction and HPLC determination of veterinary pharmaceuticals in wastewater, *Talanta.* 70  
550 (2006) 732–738. doi:10.1016/j.talanta.2006.07.003.
- 551 [10] S. Sczesny, H. Nau, G. Hamscher, Residue analysis of tetracyclines and their metabolites in  
552 eggs and in the environment by HPLC coupled with a microbiological assay and tandem mass  
553 spectrometry, *J. Agric. Food Chem.* 51 (2003) 697–703. doi:10.1021/jf0258407.
- 554 [11] B.F. Spisso, A.L. de Oliveira e Jesus, M.A.G. de Araújo Júnior, M.A. Monteiro, Validation of  
555 a high-performance liquid chromatographic method with fluorescence detection for the  
556 simultaneous determination of tetracyclines residues in bovine milk, *Anal. Chim. Acta.* 581

- 557 (2007) 108–117. doi:10.1016/j.aca.2006.08.004.
- 558 [12] M.J. Schneider, S.E. Braden, I. Reyes-Herrera, D.J. Donoghue, Simultaneous determination of  
559 fluoroquinolones and tetracyclines in chicken muscle using HPLC with fluorescence detection,  
560 *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 846 (2007) 8–13.  
561 doi:10.1016/j.jchromb.2006.08.005.
- 562 [13] J. Zhu, D.D. Snow, D. a. Cassada, S.J. Monson, R.F. Spalding, Analysis of oxytetracycline,  
563 tetracycline, and chlortetracycline in water using solid-phase extraction and liquid  
564 chromatography–tandem mass spectrometry, *J. Chromatogr. A.* 928 (2001) 177–186.  
565 doi:10.1016/S0021-9673(01)01139-6.
- 566 [14] A.L. Cinquina, F. Longo, G. Anastasi, L. Giannetti, R. Cozzani, Short communication  
567 Validation of a high-performance liquid chromatography method for the determination of  
568 oxytetracycline , tetracycline , chlortetracycline and doxycycline in bovine milk and muscle, *J.*  
569 *Chromatogr. A.* 987 (2003) 227–233.
- 570 [15] K. Škrášková, L.H.M.L.M. Santos, D. Šatínský, A. Pena, M.C.B.S.M. Montenegro, P. Solich,  
571 et al., Fast and sensitive UHPLC methods with fluorescence and tandem mass spectrometry  
572 detection for the determination of tetracycline antibiotics in surface waters, *J. Chromatogr. B*  
573 *Anal. Technol. Biomed. Life Sci.* 927 (2013) 201–208. doi:10.1016/j.jchromb.2012.12.032.
- 574 [16] A.M. Siouffy, R. Phan-Tan-Luu, Optimization methods in chromatography and capillary  
575 electrophoresis, *J. Chromatogr. A.* 892 (2000) 75–106.
- 576 [17] B. V. Fisher, R. Jones, chemometrics in pharmaceutical analysis, *J. Pharm. Biomed. Anal.* 5  
577 (1987) 455–467.
- 578 [18] D.B. Hibbert, Experimental design in chromatography: A tutorial review, *J. Chromatogr. B*  
579 *Anal. Technol. Biomed. Life Sci.* 910 (2012) 2–13. doi:10.1016/j.jchromb.2012.01.020.
- 580 [19] J.L. Glajch, J.J. Kirkland, Method development in high-performance liquid chromatography  
581 using retention mapping and experimental design techniques, *J. Chromatogr.* 485 (1989) 51–

- 582 63.
- 583 [20] N. Kumar, A. Bansal, G.S. Sarma, R.K. Rawal, Chemometrics tools used in analytical  
584 chemistry: An overview, *Talanta*. 123 (2014) 186–199. doi:10.1016/j.talanta.2014.02.003.
- 585 [21] L. Ferey, N. Delaunay, D.N. Rutledge, A. Huertas, Y. Raoul, P. Gareil, et al., An experimental  
586 design based strategy to optimize a capillary electrophoresis method for the separation of 19  
587 polycyclic aromatic hydrocarbons, *Anal. Chim. Acta*. 820 (2014) 195–204.  
588 doi:10.1016/j.aca.2014.02.040.
- 589 [22] J.K. Mbinze, A. Dispas, P. Lebrun, J.M.T. Mbay, V. Habyalimana, N. Kalenda, et al.,  
590 Application of an innovative design space optimization strategy to the development of LC  
591 methods for the simultaneous screening of antibiotics to combat poor quality medicines, *J.*  
592 *Pharm. Biomed. Anal.* 85 (2013) 83–92. doi:10.1016/j.jpba.2013.06.036.
- 593 [23] Ich, Pharmaceutical Development Q8, ICH Harmon. Tripart. Guidel. 8 (2009) 1–28.
- 594 [24] V. Barwick, Strategies for solvent selection—a literature review, *Trends Anal. Chem.* 16  
595 (1997). <http://www.sciencedirect.com/science/article/pii/S0165993697000393>.
- 596 [25] E. Katz, K. Ogan, R.P.W. Scott, Effect of pressure on solute diffusivity, solvent viscosity and  
597 column temperature in liquid chromatography, *J. Chromatogr. A*. 260 (1983) 277–295.  
598 doi:10.1016/0021-9673(83)80037-5.
- 599 [26] D. Mathieu, J. Nony, R. Phan-Tan-Luu, W. Nemrod, new efficient methodology for research  
600 using optimal design (nemrod) software, LPRAI, Marseille, France, (2000).
- 601 [27] L. P. Hammett, Some relations between reaction rates and equilibrium constants, *Chem. Rev.*  
602 (1935) 125–136.
- 603 [28] R.W. Taft, Polar and Steric Substituent Constants for Aliphatic and o-Benzoate Groups from  
604 Rates of Esterification and Hydrolysis of Esters, *J. Am. Chem. Soc.* 74 (1952) 3120–3128.  
605 doi:10.1021/ja01132a049.

- 606 [29] C. Hansch, T. Fujita,  $\rho$ - $\sigma$ - $\pi$  Analysis. A Method for the Correlation of Biological Activity and  
607 Chemical Structure, *J. Am. Chem. Soc.* 86 (1964) 1616–1626. doi:10.1021/ja01062a035.
- 608 [30] S.M. Free, J.W. Wilson, a Mathematical Contribution To Structure-Activity Studies., *J. Med.*  
609 *Chem.* 7 (1964) 395–399. doi:10.1021/jm00334a001.
- 610 [31] S. Yousefinejad, B. Hemmateenejad, Chemometrics tools in QSAR/QSPR studies: A historical  
611 perspective, *Chemom. Intell. Lab. Syst.* 149 (2015) 177–204.  
612 doi:10.1016/j.chemolab.2015.06.016.
- 613 [32] D.E. Rumelhart, G.E. Hinton, R.J. Williams, Learning representations by back-propagating  
614 errors, *Nature.* 323 (1986) 533–536. doi:10.1038/323533a0.
- 615 [33] T.Y. Pai, P.Y. Yang, S.C. Wang, M.H. Lo, C.F. Chiang, J.L. Kuo, et al., Predicting effluent  
616 from the wastewater treatment plant of industrial park based on fuzzy network and influent  
617 quality, *Appl. Math. Model.* 35 (2011) 3674–3684. doi:10.1016/j.apm.2011.01.019.
- 618 [34] H. Oka, Y. Ito, H. Matsumoto, Chromatographic analysis of tetracycline antibiotics in foods, *J.*  
619 *Chromatogr. A* 882 (2000) 109-133. doi:10.1016/S0021-9673(99)01316-3.
- 620 [35] R.E. Koopmans, R.F. Rekker, High-performance liquid chromatography of alkylbenzenes:  
621 Relationship with lipophilicities as determined from octanol-water partition coefficients or  
622 calculated from hydrophobic fragmental data and connectivity indices; lipophilicity predictions  
623 for polyaromatics, , *J. Chromatogr. A* 285 (1984) 267-279. doi:10.1016/S0021-9673(01)87768-  
624 2.
- 625 [36] A. Jia, Y. Xiao, J. Hu, M. Asami, S. Kunikane, Simultaneous determination of tetracyclines and  
626 their degradation products in environmental waters by liquid chromatography–electrospray  
627 tandem mass spectrometry, *J. Chromatogr. A* 1216 (2009) 4655-5662.  
628 doi:10.1016/j.chroma.2009.03.073

- 629 [37] A. Pamreddy, M. Hidalgo, J. Havel, V. Salvadó, Determination of antibiotics (tetracyclines and  
630 sulfonamides) in biosolids by pressurized liquid extraction and liquid chromatography–tandem  
631 mass spectrometry, *J. Chromatogr. A* 1298 (2013) 68-75. doi:10.1016/j.chroma.2013.05.014
- 632 [38] C. Kim, H.D. Ryu, E.G. Chung, Y. Kim, J.K. Lee, A review of analytical procedures for the  
633 simultaneous determination of medically important veterinary antibiotics in environmental  
634 water: Sample preparation, liquid chromatography, and mass spectrometry, *J. Environ. Manage*  
635 217 (2018) 629-645. doi:10.1016/j.jenvman.2018.04.006
- 636 [39] L.R. Snyder, M.A. Quarry, J.L. Glajch, Solvent-Strength Selectivity in Reversed-Phase HPLC,  
637 *Chromatographia* 24 (1987) 33-44. doi:10.1007/BF02688465
- 638 [40] J.L.Glajch, J.J.Kirkland, Method development in high-performance liquid chromatography using  
639 retention mapping and experimental design techniques, *J. Chromatogr. A* 485 (1989) 51-63.  
640 doi:10.1016/S0021-9673(01)89132-9
- 641 [41] J.L. Rafferty, J.I. Siepmann, M.R. Schure, Mobile phase effects in reversed-phase liquid  
642 chromatography: A comparison of acetonitrile/water and methanol/water solvents as studied by  
643 molecular simulation, *J. Chromatogr. A* 1218 (2011) 2203-2213.  
644 doi:10.1016/j.chroma.2011.02.012



TCs	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>
Tetracycline (TC)	H	OH	CH <sub>3</sub>	H
Doxycycline (DXC)	H	H	CH <sub>3</sub>	OH
Chlortetracycline (CTC)	Cl	OH	CH <sub>3</sub>	H
Oxytetracycline (OTC)	H	OH	CH <sub>3</sub>	OH

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



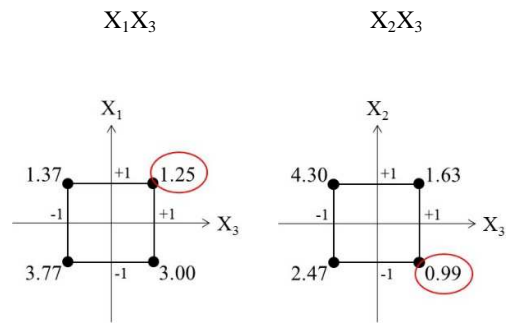


Figure 2: Interaction diagrams  $X_1X_3$  and  $X_2X_3$ , for the  $W_{DTC}$  response on the CORTECS C18 column

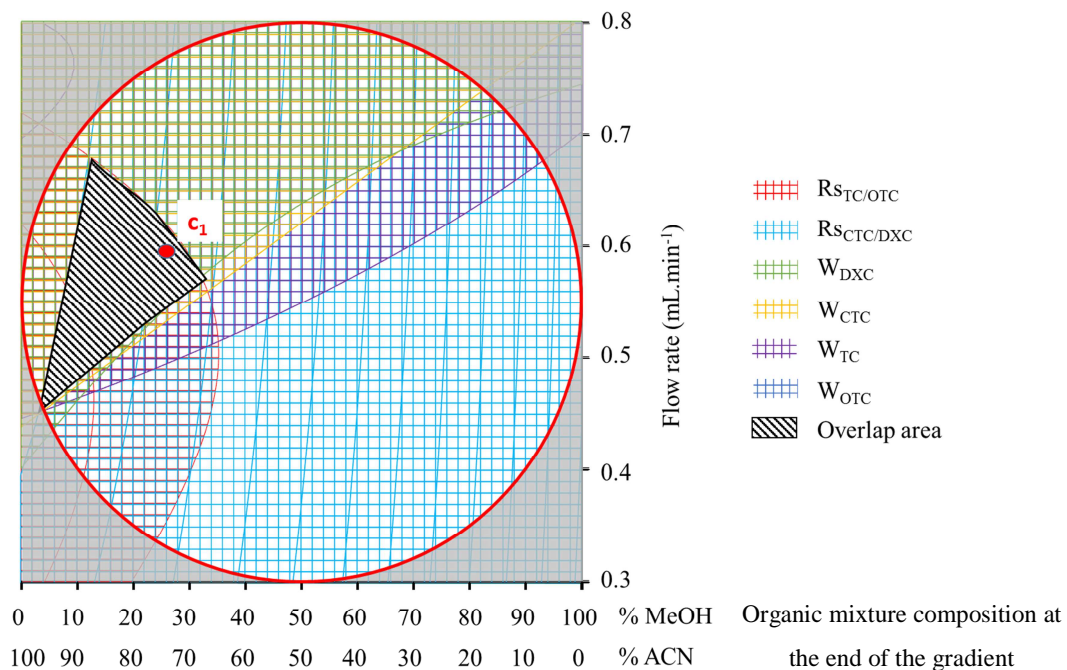


Figure 3: CORTECS C18 column: Overlap of areas of interest showing  $Rs_{TC/OTC}$ ,  $Rs_{CT/DXC}$ ,  $W_{TC}$ ,  $W_{OTC}$ ,  $W_{CTC}$ , and  $W_{DXC}$  in relation with the flow rate and organic mixture composition (% MeOH and % ACN) at the end of the gradient. The typical point  $C_1$  corresponds 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<sup>-1</sup>. W: peak width at 5%, Rs: resolution.

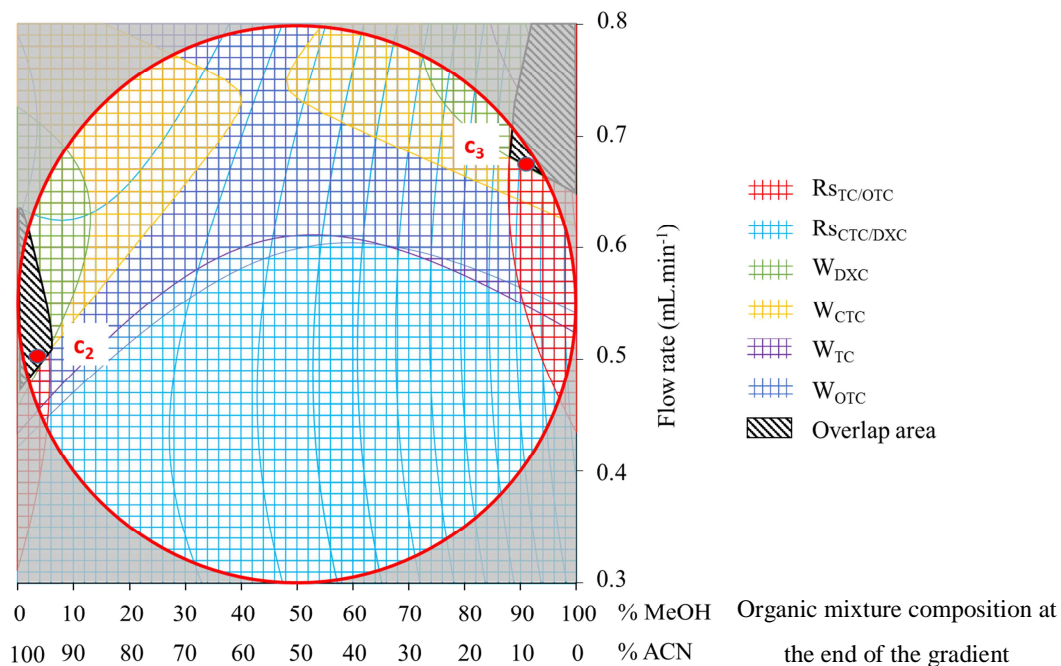


Figure 4: ACQUITY UPLC CSH C18 column: Overlap of areas of interest showing  $Rs_{TC/OTC}$ ,  $Rs_{CTC/DXC}$ ,  $W_{TC}$ ,  $W_{OTC}$ ,  $W_{CTC}$ , and  $W_{DXC}$  in relation with the flow rate and organic mixture composition (%MeOH and %ACN) at the end of the gradient. The typical points correspond 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<sup>-1</sup>, and for  $C_3$ :  $t_{3min}$ :88% MeOH, 12% ACN, flow 0.65 mL.min<sup>-1</sup>.W: peak width at 5%, Rs: resolution.

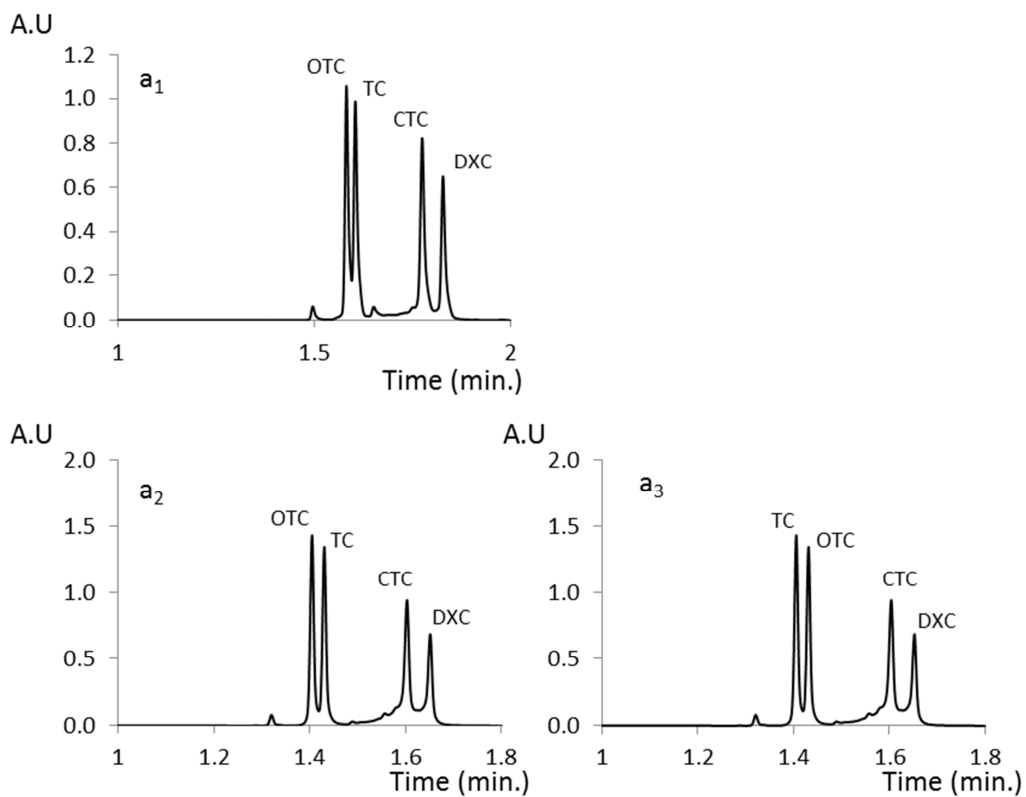


Figure 5: Optimized U-HPLC chromatograms of TC solutions: tetracycline (T), oxytetracycline (O), chlortetracycline (C) and doxycycline (D) obtained on CORTECS C18, 2.7  $\mu\text{m}$ , 2.1 x 100 mm column, with flow rate 0.6 mL.min<sup>-1</sup>, pH  $t_0=2.2$ , 25% MeOH, 75% ACN at the end of the gradient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

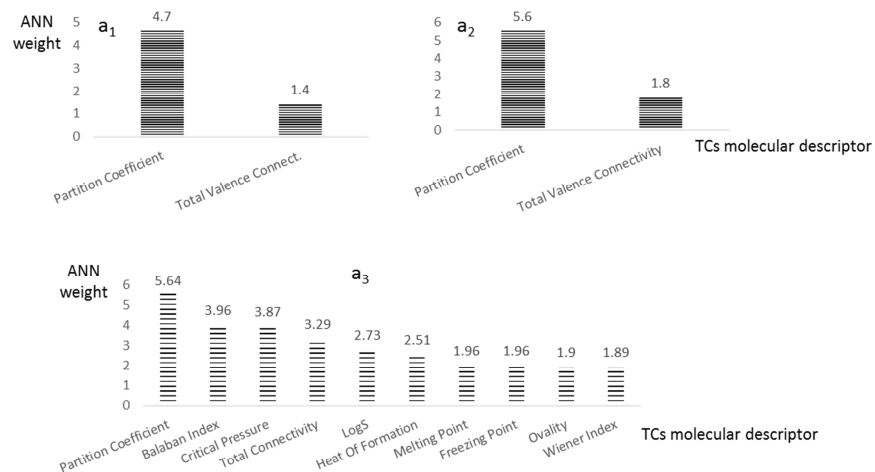
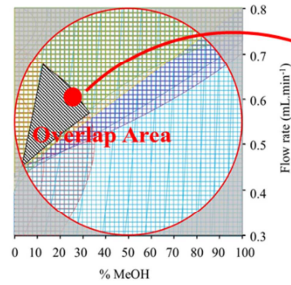
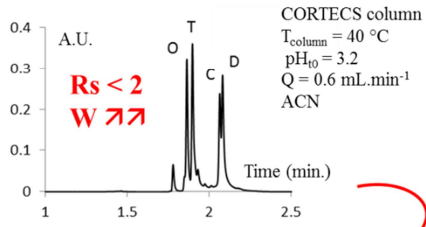


Figure 6: Weight (absolute value) of the key molecular parameters connecting the structures of TCs to their retention factor ( $k'$ ) for the CORTECS column with 25%MeOH, 75%ACN end of gradient (a<sub>1</sub>), CSH C18 column with 95% ACN, 5% MeOH end of gradient (a<sub>2</sub>) and 88% MeOH, 12% ACN end of gradient (a<sub>3</sub>)



**Doehlert Designs**

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

Factors	Exp region	
pH <sub>10</sub>	3.2	2.2
Solvent	ACN	MeOH
Q (mL.min <sup>-1</sup> )	0.3	0.8
T <sub>column</sub> (°C)	30	40

CORTECS column  
T<sub>column</sub> = 40 °C  
pH<sub>10</sub> = 2.2  
Q = 0.6 mL.min<sup>-1</sup>  
25%MeOH – 75% ACN  
end of the gradient

