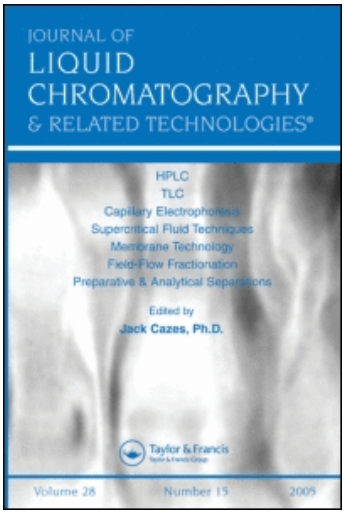

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Finding an Alternative Column for the Separation of Antibiotics on XTerra RP using a Column Classification System

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Abstract: A project to develop a test procedure to characterize reversed-phase liquid chromatographic C_{18} columns was started earlier and resulted in a fast, simple, repeatable, and reproducible test procedure. In this paper, the separations of two antibiotics (erythromycin and tetracycline) from their respective impurities were examined. Both methods were developed on XTerra RP, a hybrid column. The performance of the column classification system was evaluated by finding an alternative column for the XTerra RP column. The column classification system was helpful in the selection of a suitable column for the separation of erythromycin, but also showed its limitations towards the separation of tetracycline. The addition of an efficiency parameter and/or omission of one of the original parameters did not improve the results.

Keywords: Test procedure, Reversed-phase liquid chromatographic C_{18} columns, Erythromycin, Tetracycline, XTerra RP

INTRODUCTION

Official compendia like the European Pharmacopoeia (Ph. Eur.)[1] and the United States Pharmacopeia (USP)[2] mostly prescribe reversed-phase liquid

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chromatography (RP-LC) as an analysis technique in their monographs. In these monographs, the mobile phase is exactly described, whereas the stationary phase is only vaguely mentioned in terms like chain length, end-capping, base-deactivation, particle size, and sometimes pore size and specific surface. This description is not sufficient for an analyst to select a suitable column from the huge number of commercially available RP-LC columns. For recently developed monographs, usually more information about the suitable stationary phases can be found on the Ph. Eur. website, under the box "knowledge database."[3] The information on the development column for the USP is provided in their chromatographic reagents book and in the electronic version of USP.[4] However, it is possible that the reported column is missing in the laboratory of the analyst. Which column to select as a suitable alternative therefore may remain a problem? The description provided by column manufacturers are non homogeneous and insufficient to get out of this complex situation. A column characterisation and classification study of our laboratory led to a tool that can help analysts to choose a similar or dissimilar column compared to a reference one. This system was already tested successfully for several general separations.[5–15]

The development of an LC method requires, at the first place, the choice of an analytical column. Instead of a typical C_{18} column, sometimes an innovative column with special properties can be chosen, like in the analysis of erythromycin or tetracycline where an XTerra RP was chosen.[16,17] Before 2000, silica based packings were predominantly used in RP-LC because of their mechanical strength, chromatographic efficiency, and ease to change their bonding chemistry. However, a serious drawback was the incompatibility of these phases with extreme pH values; low pH causing acidic hydrolysis, and high pH leading to dissolution of the silica, loss of efficiency and voids in the packed bed. The first problem was partially solved by new bonding technologies like trifunctional or sterically hindered monofunctional silanes. The XTerra RP column with Hybrid Particle Technology (HPT) was rather produced to overcome the latter problem. In HPT, one of every three silanols is replaced with a methyl group. This hydrophobic character is present throughout the entire structure of the particle backbone. The result is a rugged (inorganic/organic) particle that can be operated at high speed, high temperature, and high pH. The presence of 33% fewer residual silanols after endcapping and bonding also means that the XTerra column is claimed to give very sharp, high efficiency peaks for basic compounds. XTerra RP combines its HPT with Shield Technology by introducing carbamate groups between the backbone structure and the organosiloxane side chain substituents, which shield remaining free surface silanol groups and enhance the wettability of the packing material, even in 100% aqueous mobile phases.[18] XTerra RP is reported to exhibit high efficiency compared to classical silica based or polymeric packings, which are now prescribed by several official LC methods.

In this paper, it was investigated whether the column ranking as developed in our lab can also be useful to select alternative columns for the analyses of erythromycin or tetracycline, which were originally developed on the special stationary phase XTerra RP.[16,17] These two separations are quite complicated in terms of the high number of potential components, many of which were available in our laboratory. Each separation was examined on 65 different stationary phases. The separation of erythromycin from its related substances uses isocratic elution, while tetracycline requires a gradient mode.

COLUMN CLASSIFICATION SYSTEM

Many papers describing methods to characterise columns were published, but only the more recent ones are cited here.[19–34]

In recent years, a simple chromatographic test procedure to characterise and classify RP-LC C_{18} columns has been developed by Hoogmartens and co-workers.[5–15] First of all, 36 test parameters were selected from literature and applied to 69 RP columns. The repeatability and reproducibility was checked in several laboratories and 24 of the test parameters complied.[5–7] These 24 parameters were reduced to a final set of 4 parameters: the retention factor of amylbenzene, $k'_{\text{amylbenzene}}$ (k'_{amb}), the relative retention factor benzylamine/phenol at pH 2.7, $rk'_{\text{benzylamine/phenol}}$ ($rk'_{ba/ph\ 2.7}$), the retention factor of 2,2′-dipyridyl, $k'_{2,2'\text{-dipyridyl}}$ ($k'_{2,2'\text{-}dip}$), and the relative retention factor triphenylene/o-terphenyl, $rk'_{\text{triphenylene/o-terphenyl}}$ ($rk'_{tri/o\text{-}ter}$).[8]

Next, a ranking system based on the F-values was introduced, which starts with the selection of 4 reference parameters, corresponding to a freely chosen reference column. The F-value for a column i is calculated as:

$$F = (k'_{amb,ref} - k'_{amb,i})^2 + (rk'_{ba/ph\,2.7,\,ref} - rk'_{ba/ph\,2.7,\,i})^2 + (k'_{2,2'\text{-}dip,ref} - k'_{2.2'\text{-}dip,i})^2 + (rk'_{tri/o-ter,\,ref} - rk'_{tri/o-ter,\,i})^2 \quad (1)$$

The F-value of a column i equals the sum of squares of the differences between each parameter value of the reference column and of a column i. The smaller the F-value, the more similar is column i to the reference column and the higher is column i found in the ranking (high ranked columns). Before being introduced in Eq. (1), the parameters are autoscaled:

$$\frac{x_{ij} - \bar{x}_j}{s_j} \quad (2)$$

where x_{ij} is the value of parameter j on column i, $\bar{x}_j$ is the mean of parameter j on all tested columns, and s_j is the standard deviation for parameter j.

The next step was to check the correlation of the classification system with real separations. Previous studies included the separation of

acetylsalicylic acid, clindamycin hydrochloride, buflomedil hydrochloride, chloramphenicol sodium succinate, nimesulide, phenoxymethylpenicillin, dihydrostreptomycin sulphate, vancomycin, fluoxetine, and gemcitabine from their respective impurities.[9–13,15] After testing 69 columns, a nice relationship between the ranking of the columns and the selectivity found in the separations of the pharmaceuticals was demonstrated and it was concluded that the column classification system was a helpful tool for analysts in the selection of a suitable RP-LC C_{18} column for the drugs investigated. The parameters of the used columns are freely accessible on a website,[35] where anyone can freely define a reference column or reference parameters to easily obtain the column ranking based on the F-values. In order to evaluate the chromatograms, the chromatographic response function (CRF), which is a measure for the overall selectivity, was applied. The CRF was calculated as:

$$CRF = \prod_{i=1}^{n-1} \frac{f_i}{g_i} \tag{3}$$

where n is the total number of solutes, g the interpolated peak height (i.e., the distance between the baseline and the line connecting the two peak tops) at the location of the valley, and f the depth of the valley, measured from the line connecting the two peak tops. It follows that a baseline separated peak pair has an f/g ratio of 1.00, a non-separated pair has a value of 0.00, and in case of partial co-elution an intermediate value. Columns with CRF = 1.00 show baseline separation for all peaks, but this does not mean that the separation is identical or column properties are exactly the same. It only indicates that these columns are suitable for that certain separation.

The column ranking procedure starts with the selection of all columns yielding a sufficient separation (e.g., columns with a CRF = 1.00) for the concerned separation. Each of these columns was characterised previously with 4 parameters. In order to find a virtual column, which can be considered as ideal for the given separation, the mean value for each of the 4 parameters is calculated after checking for eventually outlying values using a Grubbs' test ($\alpha = 0.05$). If a column has one or more outliers, all of its four parameters are omitted for the calculation of the mean values. Finally, F-values for all columns are calculated versus the reference parameters obtained.

EXPERIMENTAL

Chromatographic Tests and Tested Columns

General information concerning the column test methods resulting in the four final parameters, was published earlier.[8–10] An adaptation of the method was published recently, using a buffered mobile phase for the determination of

2,2′-dipyridyl and using the dead volume determination from the third method to facilitate the calculations.[14] For the present analysis, 65 new C_{18} columns were used (Table 1).

Samples and Reagents

Erythromycin A (EA, main component), erythromycin A N-oxide (EANO), erythromycin E (EE), erythromycin F (EF), N-demethyl erythromycin A (NdMeEA), anhydroerythromycin A (AEA), pseudoerythromycin A enol ether (PsEAEN), and erythromycin A enol ether (EAEN) were house standards, while erythromycin B (EB) and erythromycin C (EC) were purchased from the Ph. Eur. Laboratory (Strasbourg, France).

Reference substances of tetracycline (TC), 4-epitetracycline (ETC), anhydrotetracycline (ATC), chlortetracycline (CTC), 4-epianhydrotetracycline (EATC), and oxytetracycline (OTC) were available from Acros Organics (Geel, Belgium) and ADTC was obtained from Pfizer (Brussels, Belgium).

All solvents and reagents were of Ph. Eur. quality. Acetonitrile (Acros Organics) and methanol (Prolabo, Paris, France) were of LC grade, other chemicals of AR grade. Triethylamine and tetrahydrofuran were purchased from Acros Organics and dipotassium hydrogen phosphate from AppliChem (Darmstadt, Germany). Sodium dihydrogen phosphate was from Fluka (Buchs, Switzerland) and phosphoric acid from Sigma-Aldrich (Seelze, Germany). Water was purified (Milli-Q50, Millipore, Milford, MA, USA) before use.

Tetrabutyl ammonium hydrogen sulphate (TBA) and disodium ethylenediaminetetraacetic acetate (EDTA) reagent grade were purchased from Acros Organics. Concentrated ammonia was obtained from BDH (Poole, UK). Hydrochloric acid was acquired from Chem-Lab (Zedelgem, Belgium).

Chromatographic Conditions

Analyses were carried out using a Varian (Walnut Creek, CA, USA) 9010 LC pump, a 9100 autosampler, and a 9050 UV-Vis detector with ChromPerfect 4.4.0 software (Justice Laboratory Software, Fife, UK) for data acquisition. The columns were immersed in a water bath heated by a Julabo EC thermostat (Julabo, Seelbach, Germany) with $\pm$ 0.1°C accuracy.

The chromatographic procedures for tetracycline and erythromycin were performed according to methods from literature.[14,15] The nomenclature of the Ph. Eur. was used. Since the elution order of the peaks might change on different stationary phases, for each antibiotic a test mixture was prepared in a way that each compound had different areas in the chromatogram in order to facilitate peak identification.

The chromatographic conditions used are summarised below.

Table 1. List of C_{18} RP-LC columns examined and their properties as provided by the manufacturers

Column number	Name of the column	Length (mm)	Internal diameter (mm)	Particle size (μm)	Pore size (Å)	Manufacturer/ Supplier
1	Acclaim 3 μm	150	4.6	3	300	Dionex
2	Acclaim 5 μm	250	4.6	5	120	Dionex
3	ACE 5 C18	250	4.6	5	100	Achrom
4	Alltima AQ	250	4.6	5	100	Alltech
5	Alltima C18	250	4.6	5	117	Alltech
6	Alltima HP C18	250	4.6	5	100	Alltech
7	Alltima HP C18 Amide	250	4.6	5	100	Alltech
8	Brava BDS C18	250	4.6	5	145	Alltech
9	Capcell Pak C18 ACR	250	4.6	5	80	Shiseido Fine Chemicals
10	Capcell Pak C18 AQ	250	4.6	5	80	Shiseido Fine Chemicals
11	Capcell Pak C18 MG	250	4.6	5	90	Shiseido Fine Chemicals
12	Capcell Pak C18 UG120	250	4.6	5	120	Shiseido Fine Chemicals
13	Chromolith Performance	100	4.6	—	20000/ 130[a]	Merck
14	Discovery C18	250	4.6	5	180	Supelco
15	Discovery HS C18	250	4.6	5	120	Supelco
16	Exsil ODS 5 μm	250	4.6	5	80	SGE
17	Hamilton Hx Sil C18	250	4.6	5	312	Hamilton
18	Hydrospher C18	250	4.0	5	120	YMC
19	HyPURITY Advance	250	4.6	5	190	Thermo Electron Corp.
20	HyPURITY Aquastar	250	4.6	5	190	Thermo Electron Corp.
21	HyPURITY C18	250	4.6	5	190	Thermo Electron Corp.
22	Inertsil ODS-2	250	4.6	5	150	GL Sciences Inc.
23	Inertsil ODS-3	250	4.6	5	100	GL Sciences Inc.
24	Inertsil ODS-80A	250	4.6	5	80	GL Sciences Inc.
25	Inertsil ODS-P	250	4.6	5	100	GL Sciences Inc.

(*continued*)

Table 1. Continued

Column number	Name of the column	Length (mm)	Internal diameter (mm)	Particle size (μm)	Pore size (Å)	Manufacturer/ Supplier
26	Kromasil KR100-5C18	250	4.6	5	100	EKA Chemicals
27	LiChrosorb RP-18	250	4.6	5	100	Merck
28	LiChrospher 100 RP-18	250	4.6	5	100	Merck
29	MP-Gel ODS-5	250	4.0	5	120	YMC/Omni Chrom
30	Omnispher 5 C18	250	4.6	5	110	Varian
31	Platinum C18	250	4.6	5	100	Alltech
32	Platinum EPS C18	250	4.6	5	100	Alltech
33	Polaris 5u C18-A	250	4.6	5	180	Varian
34	Prevail Amide	250	4.6	5	190	Alltech
35	Prevail C18	250	4.6	5	110	Alltech
36	Prevail Select C18	250	4.6	5	120	Alltech
37	Prontosil 120 5 C18 AQ	250	4.6	5	120	Bischoff
38	Prontosil 120 5 C18 AQ PLUS	250	4.6	5	120	Bischoff
39	Prontosil 120 5 C18 ace EPS	250	4.6	5	120	Bischoff
40	Prontosil 120 5 C18 H	250	4.6	5	120	Bischoff
41	Prontosil 120 5 C18 SH	250	4.6	5	120	Bischoff
42	Prontosil 60 5 C18 H	250	4.6	5	60	Bischoff
43	Purospher RP-18e	250	4.6	5	90	Merck
44	Purospher Star RP-18	250	4.6	5	120	Merck
45	Pursuit 5 C18	250	4.6	5	180	Varian
46	Restek Allure C18	250	4.6	5	60	Restek
47	Restek Pinnacle DB C18	250	4.6	5	140	Restek

(*continued*)

Table 1. Continued

Column number	Name of the column	Length (mm)	Internal diameter (mm)	Particle size (μm)	Pore size (Å)	Manufacturer/ Supplier
48	Restek Pinnacle II C18	250	4.6	5	110	Restek
49	Restek Ultra C18	250	4.6	5	100	Restek
50	Supelcosil LC-18	250	4.6	5	100	Supelco
51	Supelcosil LC-18 DB	250	4.6	5	100	Supelco
52	Superspher 100 RP-18	250	4.6	5	100	Merck
53	Uptisphere 5 HDO-25QS	250	4.6	5	120	Interchrom/ Achrom
54	Uptisphere 5 ODB-25QS	250	4.6	5	120	Interchrom/ Achrom
55	Wakosil II 5 C18 RS	250	4.6	5	120	SGE
56	Xterra MS C18	250	4.6	5	125	Waters
57	Xterra RP C18	250	4.6	5	125	Waters
58	YMC-Pack Pro 3 C18	250	4.6	3	120	YMC
59	YMC-Pack Pro 5 C18	250	4.6	5	120	YMC
60	YMC-Pack Pro C18 RS	250	4.6	5	80	YMC
61	ZirChrom PS 3 μm	150	4.6	3	300	ZirChrom
62	Zorbax Eclipse XDB - C18	250	4.6	5	80	Agilent
63	Zorbax Extend - C18	250	4.6	5	80	Agilent
64	Zorbax SB - Aq	250	4.6	5	80	Agilent
65	Zorbax SB - C18	250	4.6	5	80	Agilent

[a]Macropores/mesopores.

Analysis of Erythromycin

The mobile phase consisted of acetonitrile-0.2mol/L potassium phosphate pH 7.5-water (35:5:65 v/v/v). The mixture was sparged with helium. The column was maintained at 65°C by immersion in a water bath and the detection wavelength was set at 210 nm. Adequate amounts of related substances were

dissolved in ethanol and the solvent was evaporated under vacuum. This mixture contained about 70% EA, 10% EB, 5% EC, 2% EE, 1% EF, 6% AEA, 3% EANO, 3% NdMeEA, 0.2% PsEAEN, and 0.2% EAEN. 18 mg of this sample was directly weighed in a 2.0 mL vial and dissolved in 1.5 mL of acetonitrile-0.2mol/L potassium phosphate buffer pH 7.0 (3:7 v/v). To avoid excessive degradation, the dissolved sample was kept in the dark and used for a maximum of 2 days. A 1.0 mL/min flow rate was employed and 100 μL were injected. For each column, the solution was analysed two times and the mean results were taken for further calculations.

Analysis of Tetracycline

The mobile phase consisted of acetonitrile-0.3mol/L TBA pH 7.5 −0.3 mol/L EDTA pH 7.5-water for the mobile phases: A) (12:35:35:18 v/v/v/v) and B) (30:35:35:0 v/v/v/v). Gradient program used: 0–15 min, 5% of B (isocratic); 15–45 min, 5 to 75% of B (linear gradient), and 45–65 min, 75% of B (isocratic). The EDTA and TBA solutions were adjusted to the required pH with concentrated ammonia before bringing up to volume. The mobile phases were sparged with helium. Since a small change in the temperature does not affect the separation of the critical pair TC-ADTC,[17] the temperature was lowered from 40°C to 35°C, which is a more suitable temperature to protect the columns. Additionally, as the run time for TC on all the columns could not be predicted, the last isocratic step was prolonged to 65 minutes instead of the described 45 minutes. Other chromatographic conditions were kept the same. UV-detection was performed at 280 nm. As for erythromycin, adequate amounts of related substances were dissolved in ethanol and the solvent was evaporated under vacuum. The mixture contained about 85% TC, 3% ATC, 1% ADTC, 2% EATC, 2% ETC, 5% OTC and 2% CTC. Of the sample, 10 mg was dissolved and diluted to 10 mL with 0.01 mol/L hydrochloric acid. To avoid excessive degradation, the final solution was kept in the dark and used for a maximum of 2 days. The mobile phase and the gradient were the same for all columns; no adaptation was made in order to obtain comparable retention times on all columns. A flow rate of 1 mL/min was used and 20 μL of the sample solution were injected. The run time was about 70 min.

RESULTS AND DISCUSSION

Separation of Erythromycin A and its Related Compounds

A typical chromatogram of the erythromycin separation is presented in Figure 1. It shows 10 peaks corresponding to known substances, as well as some unidentified degradation products. To keep the latter at a low level, every two days a fresh sample solution was prepared.

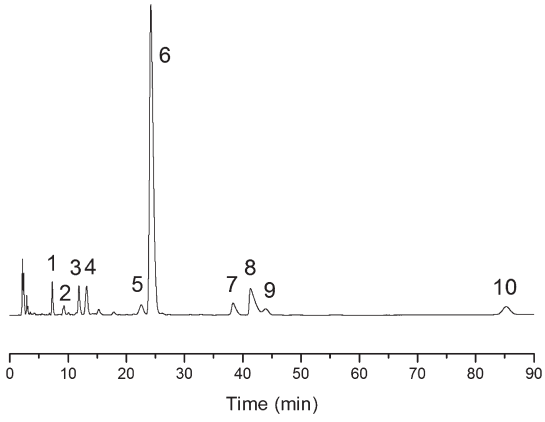

Figure 1. Separation of erythromycin A (EA) and its related compounds. 1 = EANO, 2 = EF, 3 = NdMeEA, 4 = EC, 5 = EE, 6 = EA, 7 = AEA, 8 = EB, 9 = PsEAEN, 10 = EAEN. Column: XTerra RP C_{18} (No. 57).

The parameter values of a virtual ideal column were calculated using all the 23 columns giving a separation with CRF = 1.00. The mean of each chromatographic parameter was calculated and the results were used as reference values to calculate the F-values for all the columns. The ranking according to these F-values is shown in Table 2. Of the high ranked columns, 54% (21/39) with $F < 2$ show baseline separation (CRF = 1.00), compared to 9% (2/22) of the intermediate group ($2 < F < 6$) and 0% (0/4) of the low ranked ($F > 6$) columns.

The XTerra RP column, on which the method was developed, is ranked in the second group and appears not to be the best column for this separation, since it has a CRF value of 0.75. This corresponds to the fact that peaks 8 and 9 (Figure 1) are not completely separated. When using the XTerra RP column as a reference column, only 5 of the 30 columns (17%) in the range $F < 2$ had a CRF = 1.00. For the range $2 < F < 6$, the ratio was 12 out of 18 columns (67%) and for the range $F > 6$, it was 6 out of 17 (35%). This may be explained by the fact that the CRF value of XTerra column is only 0.75. Since the columns are ranked based on similar characteristics, more columns with lower CRF values will be found high in the ranking when a column with an incomplete separation is taken as reference. As a consequence, it is better not to use a reference column with CRF < 1.00.

It has to be remarked that the analysis time of 90 minutes, associated with the XTerra RP column, was much shorter than all other columns with CRF = 1.00, for which the average analysis time was 250 minutes with a maximum value of 500 min. However, the length of an analysis was not taken into consideration here.

It can be concluded that the ranking system is most useful to analysts when the reference parameter values are calculated using columns with CRF = 1.00.

Table 2. Column ranking based on the F-values, relative to virtual, ideal column values (k'_{amb}: 0.689, $rk'_{ba/ph\ 2.7}$: −0.140, $k'_{2,2'\text{-dip}}$: −0.047, $rk'_{tri/o\text{-}ter}$: −0.140) for the separation of erythromycin

No.	Column name	k'_{amb}	$rk'_{ba/ph}$	$k'_{2,2'\text{-dip}}$	$rk'_{tri/o\text{-}ter}$	F-value	CRF
52	Superspher 100 RP-18	0.692	−0.122	0.025	−0.123	0.006	1.00
11	Capcell Pak C18 MG	0.713	−0.135	0.041	−0.158	0.009	1.00
54	Uptisphere 5 ODB-25QS	0.595	−0.133	−0.097	−0.149	0.012	0.96
58	YMC-Pack Pro 3 C18	0.570	−0.151	−0.079	−0.158	0.016	1.00
30	Omnispher 5 C18	0.680	−0.134	−0.204	−0.117	0.025	1.00
15	Discovery HS C18	0.815	−0.137	−0.160	−0.134	0.028	1.00
22	Inertsil ODS-2	0.452	−0.161	−0.215	−0.120	0.085	1.00
63	Zorbax Extend - C18	0.649	−0.145	−0.343	−0.137	0.089	1.00
2	Acclaim 5 μm	0.984	−0.133	0.024	−0.150	0.092	1.00
53	Uptisphere 5 HDO-25QS	0.387	−0.133	−0.016	−0.160	0.093	0.56
9	Capcell Pak C18 ACR	0.403	−0.144	−0.252	−0.130	0.124	1.00
55	Wakosil II 5 C18 RS	0.333	−0.148	−0.034	−0.166	0.128	1.00
44	Purospher Star RP-18	0.655	−0.142	0.342	−0.125	0.153	1.00
26	Kromasil KR100-5C18	1.163	−0.129	0.047	−0.131	0.233	1.00
59	YMC-Pack Pro 5 C18	0.298	−0.159	−0.333	−0.158	0.236	1.00
41	Prontosil 120 5 C18 SH	0.410	−0.130	0.351	−0.137	0.236	0.00
62	Zorbax Eclipse XDB - C18	0.353	−0.136	−0.422	−0.158	0.254	0.00
29	MP-Gel ODS-5	0.247	−0.144	0.255	−0.106	0.288	0.21
17	Hamilton Hx Sil C18	0.348	−0.128	0.406	−0.136	0.321	0.00
48	Restek Pinnacle II C18	0.170	−0.114	−0.398	−0.136	0.393	1.00
49	Restek Ultra C18	1.348	−0.136	0.101	−0.131	0.457	1.00
23	Inertsil ODS-3	1.169	−0.154	0.461	−0.155	0.489	1.00
39	Prontosil 120 5 C18 ace EPS	0.069	−0.164	−0.485	−0.089	0.579	0.00
40	Prontosil 120 5 C18 H	−0.065	−0.129	−0.261	−0.144	0.615	0.00
37	Prontosil 120 5 C18 AQ	−0.106	−0.125	−0.189	−0.156	0.653	0.79
65	Zorbax SB - C18	−0.160	−0.113	−0.236	−0.159	0.758	0.00
12	Capcell Pak C18 UG120	−0.067	−0.150	−0.551	−0.155	0.826	1.00
5	Alltima C18	0.760	−0.134	0.865	−0.127	0.838	0.00
18	Hydrospher C18	−0.197	−0.155	−0.297	−0.168	0.849	0.60
43	Purospher RP-18e	1.044	−0.147	0.990	−0.100	1.203	1.00
3	ACE 5 C18	−0.248	−0.129	−0.706	−0.133	1.312	0.00
56	Xterra MS C18	−0.404	−0.134	−0.432	−0.170	1.344	1.00
50	Supelcosil LC-18	−0.321	0.245	−0.553	−0.145	1.426	0.00
47	Restek Pinnacle DB C18	−0.354	−0.111	−0.684	−0.134	1.495	1.00

(*continued*)

Table 2. Continued

No.	Column name	k'_{amb}	$rk'_{ba/ph}$	$k'_{2,2'\text{-dip}}$	$rk'_{tri/o\text{-}ter}$	F-value	CRF
45	Pursuit 5 C18	−0.375	−0.133	−0.672	−0.150	1.521	1.00
10	Capcell Pak C18 AQ	−0.536	−0.153	0.164	−0.150	1.546	0.94
24	Inertsil ODS-80A	1.630	−0.151	0.853	−0.154	1.695	0.00
51	Supelcosil LC-18 DB	−0.579	−0.100	−0.593	−0.154	1.906	0.00
38	Prontosil 120 5 C18 AQ PLUS	0.289	−0.153	1.295	−0.105	1.963	0.00
14	Discovery C18	−0.669	−0.135	−0.827	−0.138	2.452	0.86
6	Alltima HP C18	−0.770	−0.128	−0.827	−0.152	2.736	0.00
42	Prontosil 60 5 C18 H	2.173	−0.152	0.748	−0.149	2.835	0.00
33	Polaris 5u C18-A	−0.813	−0.134	−0.828	−0.112	2.868	0.95
21	HyPURITY C18	−0.807	−0.132	−0.900	−0.130	2.967	0.00
36	Prevail Select C18	−0.838	−0.205	−0.852	−0.040	2.996	0.00
60	YMC-Pack Pro C18 RS	2.368	−0.160	0.382	−0.150	3.003	1.00
8	Brava BDS C18	−0.984	−0.109	−0.503	−0.127	3.009	0.00
16	Exsil ODS 5 μm	−0.120	−0.085	1.614	−0.102	3.419	0.00
13	Chromolith Performance	−1.117	−0.144	−0.851	−0.137	3.909	0.00
57	Xterra RP C18	−1.107	−0.146	−0.949	−0.093	4.040	0.75
4	Alltima AQ	−0.340	−0.126	1.698	−0.035	4.117	0.76
46	Restek Allure C18	2.543	−0.140	0.858	−0.136	4.256	1.00
35	Prevail C18	−0.269	−0.130	1.885	−0.041	4.662	0.00
27	LiChrosorb RP-18	−0.425	0.116	1.784	−0.093	4.663	0.00
1	Acclaim 3 μm	−1.252	−0.124	−1.117	−0.155	4.915	0.93
31	Platinum C18	−1.451	−0.003	−0.659	−0.147	4.975	0.00
20	HyPURITY Aquastar	−1.537	−0.092	−0.360	0.025	5.083	0.00
32	Platinum EPS C18	−1.568	0.092	0.096	−0.066	5.174	0.44
34	Prevail Amide	−1.452	−0.215	−0.848	−0.080	5.238	0.00
7	Alltima HP C18 Amide	−1.321	−0.178	−1.191	−0.023	5.366	0.00
7	Alltima HP C18 Amide	−1.321	−0.178	−1.191	−0.023	5.366	0.00
28	LiChrospher 100 RP-18	0.529	−0.065	2.277	−0.101	5.433	0.00
64	Zorbax SB - Aq	−1.744	−0.103	−0.369	−0.182	6.025	0.00
19	HyPURITY Advance	−1.906	−0.286	−1.435	−0.072	8.688	0.00
25	Inertsil ODS-P	1.032	−0.144	4.734	−0.026	22.993	0.00
61	ZirChrom PS 3 μm	−1.968	7.918	−1.566	7.932	139.452	0.00

Separation of Tetracycline and its Related Compounds

A typical chromatogram of the tetracycline separation is presented in Figure 2. Here, 7 major peaks can be observed. Especially CTC and EATC were frequently co-eluted, resulting in many columns with CRF = 0.00. Another problem was the calculation of the CRF value (Figure 3) since for the peak

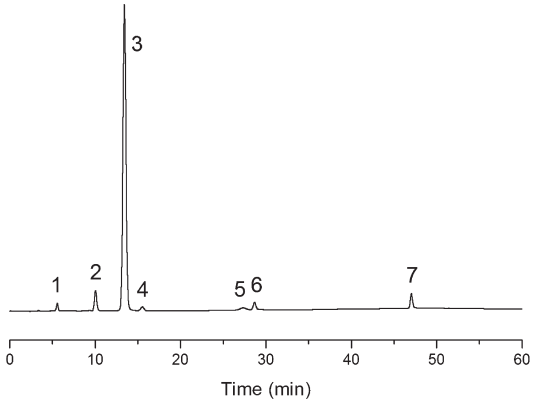

Figure 2. Separation of tetracycline (TC) and its related compounds. 1 = ETC, 2 = OTC, 3 = TC, 4 = ADTC, 5 = CTC, 6 = EATC, 7 = ATC. Column: XTerra RP C_{18} (No. 57).

pair TC-ADTC, the large peak of TC had to be connected with the much smaller peak of ADTC. This was resolved by using a horizontal line to find the *f* and *g* values, instead of a line connecting the peak tops, as can be seen on Figure 4. Only 2 columns gave baseline separation: the Zorbax SB-Aq and the XTerra RP. The chromatogram on Zorbax SB-Aq is shown in Figure 5. This indicates that complete separation of the tetracycline derivatives is not easy to obtain.

Since a separation with CRF = 0.80 is still acceptable, all eight columns with CRF $\geq$ 0.80 were used to calculate average reference parameter values for a virtual ideal column. The ranking in Table 3 shows that of the 8 columns with CRF $\geq$ 0.80, 5 are present in the range F $<$ 2 and 3 in the range $2 < F < 6$. In the range F $<$ 2, 31 out of the 45 columns had CRF = 0.00 and 9 out of 15 in the range $2 < F < 6$. When using only the XTerra RP as reference column, of the 8 columns with CRF $\geq$ 0.80, 7 are in the range F $<$ 6. When comparing the separation on the two columns with CRF = 1.00 (XTerra RP and Zorbax SB-Aq, Figures 2 and 5, respectively),

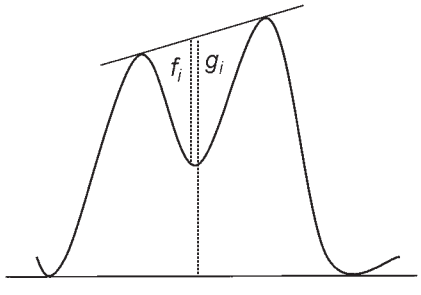

Figure 3. Determination of CRF value.

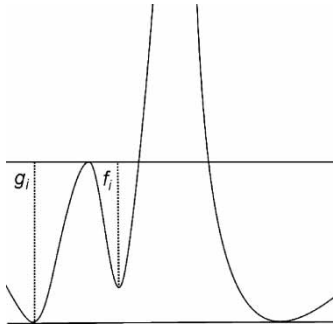

Figure 4. Determination of CRF value for a difficult peak pair, e.g. TC-ADTC.

the elution order is the same, but the separation of peak pairs TC-ADTC and CTC-EATC is better for the Zorbax SB-Aq column.

For tetracycline, the column classification system gave less good discrimination between suitable and non-suitable columns. Therefore, it was investigated whether the column classification system could be improved by adding an additional parameter.

Column Classification System using an Additional Parameter: Column Efficiency

Efficiency as a parameter was not included in the 4 originally chosen parameters. Claessens and co-workers found that column efficiency and

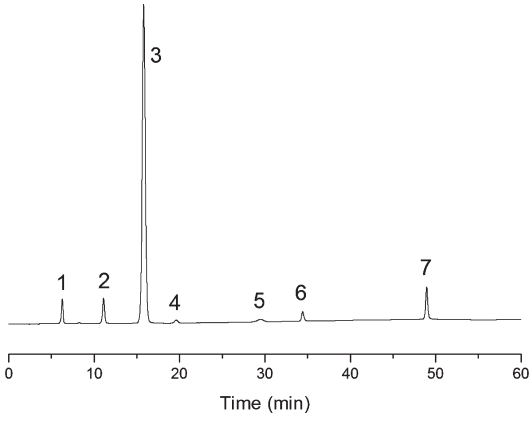

Figure 5. Separation of tetracycline (TC) and its related compounds. 1 = ETC, 2 = OTC, 3 = TC, 4 = ADTC, 5 = CTC, 6 = EATC, 7 = ATC. Column: Zorbax SB-Aq (No. 64).

Table 3. Column ranking based on the F-values, relative to virtual, ideal column values (k'_{amb}: -0.346, $rk'_{ba/ph\ 2.7}$: −0.130, $k'_{2,2'\text{-dip}}$: −0.124, $rk'_{tri/o\text{-}ter}$: −0.130) for the separation of tetracycline

No.	Name	k'_{amb}	$rk'_{ba/ph}$	$k'_{2,2'\text{-dip}}$	$rk_{tri/o\text{-}ter}$	F-value	CRF
10	Capcell Pak C18 AQ	−0.536	−0.153	0.164	−0.150	0.039	0.95
37	Prontosil 120 5 C18 AQ	−0.106	−0.125	−0.189	−0.156	0.156	0.00
65	Zorbax SB - C18	−0.160	−0.113	−0.236	−0.159	0.165	0.89
18	Hydrospher C18	−0.197	−0.155	−0.297	−0.168	0.201	0.00
40	Prontosil 120 5 C18 H	−0.065	−0.129	−0.261	−0.144	0.228	0.70
56	Xterra MS C18	−0.404	−0.134	−0.432	−0.170	0.314	0.00
29	MP-Gel ODS-5	0.247	−0.144	0.255	−0.106	0.369	0.70
55	Wakosil II 5 C18 RS	0.333	−0.148	−0.034	−0.166	0.487	0.00
12	Capcell Pak C18 UG120	−0.067	−0.150	−0.551	−0.155	0.534	0.00
48	Restek Pinnacle II C18	0.170	−0.114	−0.398	−0.136	0.540	0.00
39	Prontosil 120 5 C18 ace EPS	0.069	−0.164	−0.485	−0.089	0.546	0.63
53	Uptisphere 5 HDO-25QS	0.387	−0.133	−0.016	−0.160	0.557	0.00
17	Hamilton Hx Sil C18	0.348	−0.128	0.406	−0.136	0.561	0.31
51	Supelcosil LC-18 DB	−0.579	−0.100	−0.593	−0.154	0.569	0.00
50	Supelcosil LC-18	−0.321	0.245	−0.553	−0.145	0.601	0.00
41	Prontosil 120 5 C18 SH	0.410	−0.130	0.351	−0.137	0.623	0.89
59	YMC-Pack Pro 5 C18	0.298	−0.159	−0.333	−0.158	0.624	0.00
45	Pursuit 5 C18	−0.375	−0.133	−0.672	−0.150	0.634	0.00
47	Restek Pinnacle DB C18	−0.354	−0.111	−0.684	−0.134	0.653	0.00
3	ACE 5 C18	−0.248	−0.129	−0.706	−0.133	0.698	0.36
9	Capcell Pak C18 ACR	0.403	−0.144	−0.252	−0.130	0.703	0.00
22	Inertsil ODS-2	0.452	−0.161	−0.215	−0.120	0.752	0.00
62	Zorbax Eclipse XDB - C18	0.353	−0.136	−0.422	−0.158	0.788	0.00
8	Brava BDS C18	−0.984	−0.109	−0.503	−0.127	0.801	0.74
58	YMC-Pack Pro 3 C18	0.570	−0.151	−0.079	−0.158	0.881	0.00
54	Uptisphere 5 ODB-25QS	0.595	−0.133	−0.097	−0.149	0.934	0.00
14	Discovery C18	−0.669	−0.135	−0.827	−0.138	1.009	0.00
44	Purospher Star RP-18	0.655	−0.142	0.342	−0.125	1.050	0.00
6	Alltima HP C18	−0.770	−0.128	−0.827	−0.152	1.085	0.00

(*continued*)

Table 3. Continued

No.	Name	k'_{amb}	$rk'_{ba/ph}$	$k'_{2,2'\text{-dip}}$	$rk_{tri/o\text{-}ter}$	F-value	CRF
52	Superspher 100 RP-18	0.692	−0.122	0.025	−0.123	1.088	0.00
33	Polaris 5u C18-A	−0.813	−0.134	−0.828	−0.112	1.125	0.21
11	Capcell Pak C18 MG	0.713	−0.135	0.041	−0.158	1.130	0.00
30	Omnispher 5 C18	0.680	−0.134	−0.204	−0.117	1.161	0.00
63	Zorbax Extend - C18	0.649	−0.145	−0.343	−0.137	1.209	0.00
36	Prevail Select C18	−0.838	−0.205	−0.852	−0.040	1.210	0.00
21	HyPURITY C18	−0.807	−0.132	−0.900	−0.130	1.262	0.00
15	Discovery HS C18	0.815	−0.137	−0.160	−0.134	1.427	0.00
13	Chromolith Performance	−1.117	−0.144	−0.851	−0.137	1.546	0.00
32	Platinum EPS C18	−1.568	0.092	0.096	−0.066	1.547	0.00
20	HyPURITY Aquastar	−1.537	−0.092	−0.360	0.025	1.678	0.00
57	Xterra RP C18	−1.107	−0.146	−0.949	−0.093	1.731	1.00
5	Alltima C18	0.760	−0.134	0.865	−0.127	1.771	0.00
38	Prontosil 120 5 C18 AQ PLUS	0.289	−0.153	1.295	−0.105	1.775	0.60
2	Acclaim 5 μm	0.984	−0.133	0.024	−0.150	1.780	0.81
31	Platinum C18	−1.451	−0.003	−0.659	−0.147	1.851	0.37
34	Prevail Amide	−1.452	−0.215	−0.848	−0.080	2.180	0.88
64	Zorbax SB - Aq	−1.744	−0.103	−0.369	−0.182	2.201	1.00
16	Exsil ODS 5 μm	−0.120	−0.085	1.614	−0.102	2.273	0.00
26	Kromasil KR100-5C18	1.163	−0.129	0.047	−0.131	2.282	0.00
1	Acclaim 3 μm	−1.252	−0.124	−1.117	−0.155	2.364	0.00
23	Inertsil ODS-3	1.169	−0.154	0.461	−0.155	2.410	0.58
4	Alltima AQ	−0.340	−0.126	1.698	−0.035	2.487	0.76
43	Purospher RP-18e	1.044	−0.147	0.990	−0.100	2.683	0.00
7	Alltima HP C18 Amide	−1.321	−0.178	−1.191	−0.023	2.694	0.00
27	LiChrosorb RP-18	−0.425	0.116	1.784	−0.093	2.823	0.00
49	Restek Ultra C18	1.348	−0.136	0.101	−0.131	2.871	0.00
35	Prevail C18	−0.269	−0.130	1.885	−0.041	3.114	0.82
24	Inertsil ODS-80A	1.630	−0.151	0.853	−0.154	4.435	0.00
19	HyPURITY Advance	−1.906	−0.286	−1.435	−0.072	4.894	0.46
28	LiChrospher 100 RP-18	0.529	−0.065	2.277	−0.101	5.404	0.00
42	Prontosil 60 5 C18 H	2.173	−0.152	0.748	−0.149	6.736	0.11
60	YMC-Pack Pro C18 RS	2.368	−0.160	0.382	−0.150	7.433	0.00
46	Restek Allure C18	2.543	−0.140	0.858	−0.136	8.884	0.42
25	Inertsil ODS-P	1.032	−0.144	4.734	−0.026	23.163	0.29
61	ZirChrom PS 3 μm	−1.968	7.918	−1.566	7.932	135.251	0.00

hydrophobicity are usually interchangeable and that column classifications using these parameters would provide similar patterns.[36] Walters included efficiency as a test parameter.[37] Vervoort and co-workers reported that efficiency is an important parameter for column comparison.[38] In previous work on the separation of fluoxetine and gemcitabine, the column efficiency measured on the main peak was examined for improvement of the column classification.[15] Column efficiency is expressed as the height equivalent of the theoretical plate (HETP or H) or as the number of theoretical plates (N). It provides a measure of how peaks broaden while they pass through a chromatographic column. It is a function of particle size and shape, uniformity of the stationary phase, diffusion coefficients of the analyte in the mobile and stationary phases, solvent viscosity, and flow rate. However, it is not possible for an analyst to determine for every single separation the efficiency on 65 columns and then to select the best one. Therefore, it would be more convenient if the efficiency could be determined from an efficiency parameter independent of the separation.

Euerby and Petersson implemented efficiency in their column classification system by measuring the number of theroretical plates on n-pentylbenzene.[39,40] In order not to make the test procedure more difficult, in this study the efficiency of amylbenzene (N_{amb}) and benzylamine (N_{ba}) was calculated from the already obtained data.

The efficiency values (N) for amylbenzene and benzylamine are plotted versus each other in Figure 6. As can be seen, little correlation was observed. Although this parameter was determined using different mobile phases, it was expected to show the same tendency. This was not observed,

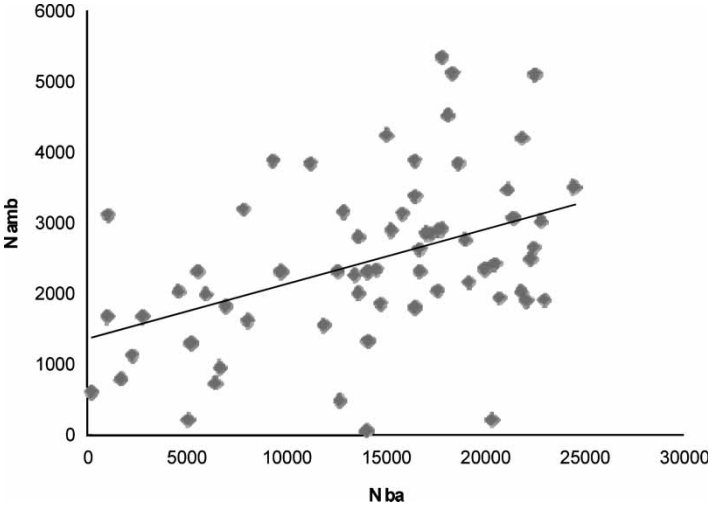

Figure 6. Plot of the efficiency of amylbenzene versus benzylamine.

Table 4. Overview of the ratio of columns with CRF $\geq$ 0.80 for the separation of tetracycline in the different ranges ($F < 2$, $2 < F < 6$, $F > 6$) when adding efficiency or omitting one of the 4 original parameters (k'_{amb}, $rk'_{ba/ph\ 2.7}$, $k'_{2,2'\text{-dip}}$, $rk'_{tri/o\text{-}ter}$) in the column classification system

Reference column	Added parameter:	—	$+N_{amb}$	$+N_{amb}$	$+N_{amb}$	$+N_{amb}$	$+N_{ba}$	$+N_{ba}$	$+N_{ba}$	$+N_{ba}$	Added parameter:	$+N_{amb}$	$+N_{ba}$
	Omitted parameter: range	—	$-k'_{amb}$	$-rk'_{ba}$	$-k'_{2,2'\text{-dip}}$	$-rk'_{tri/o\text{-}ter:}$	$-k'_{amb}$	$-rk'_{ba}$	$-k'_{2,2'\text{-dip}}$	$-rk'_{tri/o\text{-}ter}$	Omitted parameter: range	—	—
Virtual, ideal column	$F < 2$	5/45	6/32	5/24	6/33	3/19	5/44	4/29	5/38	3/30	$F < 2.5$	5/29	4/37
	$2 < F < 6$	3/15	2/31	2/31	1/24	4/37	3/15	4/24	3/20	5/25	$2.5 < F < 7.5$	3/30	4/21
	$F > 6$	0/5	0/2	1/10	1/8	1/9	0/6	0/12	0/7	0/10	$F > 7.5$	0/6	0/7
XTerra RP	$F < 2$	5/30	4/33	2/20	5/25	2/20	4/40	2/17	3/22	1/17	$F < 2.5$	4/25	3/22
	$2 < F < 6$	2/18	3/25	4/26	2/31	4/26	3/16	4/28	5/33	4/17	$2.5 < F < 7.5$	2/23	4/27
	$F > 6$	1/17	1/17	2/19	1/4	2/19	1/9	2/20	0/10	2/21	$F > 7.5$	2/17	1/16

Table 5. Overview of the ratio of columns with CRF = 1.00 for the separation of erythromycin in the different ranges ($F < 2$, $2 < F < 6$, $F > 6$) when adding efficiency or omitting one of the 4 original parameters (k'_{amb}, $rk'_{ba/ph\ 2.7}$, $k'_{2,2'\text{-dip}}$, $rk'_{tri/o\text{-}ter}$) in the column classification system

Reference column	Added parameter: / Omitted parameter: range	—	$+N_{amb}$	$+N_{amb}$	$+N_{amb}$	$+N_{amb}$	$+N_{ba}$	$+N_{ba}$	$+N_{ba}$	$+N_{ba}$	Added parameter: / Omitted parameter: range	$+N_{amb}$	$+N_{ba}$
		—	$-k'_{amb}$	$-rk'_{ba}$	$-k'_{2,2'\text{-dip}}$	$-rk'_{tri/o\text{-}ter}$	$-k'_{amb}$	$-rk'_{ba}$	$-k'_{2,2'\text{-dip}}$	$-rk'_{tri/o\text{-}ter}$		—	—
Virtual, ideal column	$F < 2$	21/39	23/45	20/28	20/32	20/28	22/47	19/32	20/36	20/32	$F < 2.5$	21/31	21/36
	$2 < F < 6$	2/22	0/12	3/24	3/25	3/24	1/13	4/19	4/21	4/19	$2.5 < F < 7.5$	2/25	2/20
	$F > 6$	0/4	0/8	0/13	0/8	0/13	0/5	0/14	0/8	0/14	$F > 7.5$	0/9	0/9
XTerra RP	$F < 2$	5/30	10/33	4/20	4/25	4/20	19/40	3/17	4/22	3/17	$F < 2.5$	4/25	6/22
	$2 < F < 6$	12/18	13/25	11/26	13/31	11/26	4/16	14/28	16/33	14/27	$2.5 < F < 7.5$	12/23	12/27
	$F > 6$	6/17	0/7	8/19	6/9	8/19	0/9	6/20	3/10	6/21	$F > 7.5$	7/17	5/16

as the coefficient of correlation was 0.191 with an equation of $y = 0.078x + 1347$. Therefore, both efficiency values were investigated.

Each of the efficiency values was added to the original 4 parameters and the column ranking was calculated. Instead of the ranges $F < 2{,}2 < F < 6$ and $F > 6$ for 4 parameters, now ranges $F < 2.5$, $2.5 < F < 7.5$ and $F > 7.5$ were used for 5 parameters. The influence of omitting one of the 4 original parameters, in order to come again to a total of 4 parameters, was also examined. As reference column, the virtual ideal column and the XTerra RP column were used. Table 4 gives an overview of the number of columns with CRF ≥ 0.80 in the three different ranges ($F < 2$, $2 < F < 6$ and $F > 6$). As can be seen, adding the efficiency or replacing one of the original parameters by the efficiency does not improve the classification.

Similar calculations, as shown in Table 4, were also performed for the erythromycin results (Table 5). Now, the ratio of columns with CRF = 1.00 is reported. When using the virtual column as reference column, it was observed that the ranking did not change much. Using the XTerra RP column as reference column, the ranking observed with the original parameters was also here not improved, since most columns with CRF = 1.00 could be found in the $F > 2.5$ part. Therefore, it was decided not to change the original combination of the 4 parameters.

CONCLUSION

This paper focuses on the performance of a column classification system when applied to two pharmaceutical separations, developed on a specific column, the XTerra RP. The columns were ranked according to their F value, which is calculated from 4 chromatographic parameters. In the two examples described, a virtual ideal column was used as a reference column. The chromatographic parameters for this virtual column were calculated as the means of the parameters of the columns giving sufficient separation after correcting for outliers. The relationship between the ranking of the columns and their separation performance was investigated. In this study, the CRF value was used to evaluate the separations. For erythromycin, a CRF = 1.00 was used as criterion. Columns were classified in three arbitrary groups: $F < 2$, $2 < F < 6$, and $F > 6$. Columns with $F < 2$ show a higher probability to be suitable: 57% compared to only 12% for columns with $2 < F < 6$. For tetracycline, CRF ≥ 0.80 was used as criterion and the ranking results were less discriminating than for erythromycin. It was, therefore, checked if addition of efficiency as parameter and/or omitting one of the original parameters would improve the system. This was not the case, so no changes to the original classification system were applied. The described column classification system has proven to be a helpful tool to find a suitable column in several separations, but it is also shown to be of more limited value in complex situations as the tetracycline separation.

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