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Experimental design on liquid chromatographic parameters in the analysis of tetracycline on poly(styrene–divinylbenzene)*

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Abstract: A previously described isocratic liquid chromatographic (LC) method for the analysis of tetracycline on poly(styrene–divinylbenzene) allows the complete separation and resolution of tetracycline (TC), 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC). By means of a half-fraction factorial design, the importance of the individual chromatographic parameters and parameter interactions of this LC method was examined. The influence on the retention of ETC, TC and EATC is measured. A mathematical regression model was derived which predicts retention times with good reliability. Both the retentions of ETC and EATC are strongly affected by chromatographic parameters and parameter interactions, which only slightly affect the retention of TC. Adequate combination of the most relevant of these parameters enables optimization of the chromatographic separation if necessary.

Keywords: *Liquid chromatography; experimental design; tetracycline; poly(styrene–divinylbenzene); regression modelling; response surface plot.*

Introduction

Recently an isocratic liquid chromatographic (LC) method for quantification of tetracycline (TC) using a poly(styrene–divinylbenzene) copolymer (PSDVB) column was published [1]. This method allowed the complete separation and resolution of TC, 4-epitetracycline (ETC), anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC). A fermentation impurity, 2-acetyl-2-decarboxamidotetracycline (ADTC), also was resolved from tetracycline. The mobile phase contained 2-methyl-2-propanol as the organic modifier, water and phosphate buffer, tetrabutylammonium sulphate and sodium edetate at a pH of 9.0. The column was heated at 60°C.

A collaborative study in five different laboratories with a total of seven PSDVB columns, confirmed the fair reproducibility (1.5%) of the method for tetracycline content measurements in bulk material [2]. Laboratory-packed and prepacked columns as well as wide pore (1000 Å) and narrow pore (100 Å) material were involved.

This method is suitable for the assay and purity control of TC, and as a consequence it was adopted in the monographs of TC and tetracycline hydrochloride (TC·HCl) of the European Pharmacopoeia (Ph.Eur.). However, in the collaborative study diverging resolutions between ETC and TC (ranging from 3.0 to 4.5) and between TC and EATC (ranging from 8.6 to 14.1) as well as different capacity factors for TC (ranging from 0.5 to 2.7) were observed on the seven PSDVB columns involved. Only slightly different isocratic mobile phases were used, in which the percentage of organic modifier ranged from 7.7 to 8.5%, while all other chromatographic parameter settings were kept identical for each column.

In this study the relative influence of each of the five most important chromatographic parameters (variables), governing the separation process, was examined, applying a half-fraction factorial design at two levels. This involves at least 2^5: 2 = 16 different experimental measurements, combining the five parameters examined at two previously fixed

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extreme levels for each parameter. Three repeated central level combinations were included in the design and so 19 measurements had to be performed.

The most relevant chromatographic parameters examined as variables were:

(1) the concentration of 2-methyl-2-propanol (MPOH) as organic modifier in the mobile phase;
(2) the concentration of tetrabutylammonium (TBA);
(3) the concentration of sodium edatate (EDTA);
(4) the pH of the mobile phase;
(5) the column temperature.

The measured response variables were the retention times of TC, ETC and EATC.

Several other factorial designs have been described previously to investigate and optimize LC systems. Lindberg *et al.* used a factorial design to study the effects of four parameters for the optimization of a separation in ion-pairing LC [3]. Cotton and Down employed a factorial design, not to find the optimum, but rather to describe the response surfaces surrounding a known optimum condition [4]. Wester *et al.* selected LC variables to be optimized by a reduced factorial design and performed a detailed study of these variables by a complete factorial design [5]. Yuzhu Hu and Massart proposed a Doehlert matrix design for LC optimization [6]. A nice and comprehensive tutorial article on experimental designs was written by Morgan *et al.* [7]. A more mathematical and chemometric approach of experimental design was presented by Deming and Morgan [8] and by Box *et al.* [9].

Experimental

Apparatus and column

The LC equipment was build up with a Model 600 multisolvent delivery system (Millipore-Waters, Milford, MA, USA), allowing mobile phase mixing from four separate reservoirs, continuously degassed with helium. The pump system was provided with a built-in "silk" device, reducing baseline noise. Samples were injected with a Marathon autosampler (Spark Holland, Emmen, The Netherlands) equipped with a loop of about 20 μl. The Waters 990 photodiode array detector was linked to a Nec Powermate 386/33i data station. Two-dimensional chromatograms were recorded at 254 nm. Peek tubing was used to connect column and injector, and column and detector. A flow rate of 1.0 ml min^{-1} was used throughout this study. The 25 × 0.46 cm i.d. LC column was packed with PRP-1 10 μm (Hamilton, Reno, NV, USA) and was immersed in a thermostatically controlled water bath.

Samples, chemicals and solvents

The reference samples used were available from Janssen Chimica (Beerse, Belgium): tetracycline hydrochloride (TC·HCl), 4-epi-tetracycline hydrochloride (ETC·HCl), 4-epi-anhydrotetracycline hydrochloride (EATC·HCl) and anhydrotetracycline hydrochloride (ATC·HCl). Chemicals and solvents were of *pro analysi* quality. Water was prepared with a Milli Q system (Millipore, Milford, MA, USA). Hydrochloric acid (HCl) 0.01 M was used as the solvent for the samples.

Mobile phase composition

Four separate buffered solutions of each mobile phase component were prepared for each examined pH-level. Each of these four solutions was stored in each of the four available reservoirs A, B, C and D of the multisolvent delivery system. The examined pH-levels were obtained with a phosphate buffer of pH 9.0 (central level), 8.0 and 10.0. Phosphoric acid (10%, m/v) or sodium hydroxide solution (8.5%, m/v) were used to adjust the pH.

Reservoir A. This contained a mixture of 58.0 g of MPOH and 50.0 ml of a 3.5% (m/v) dipotassium hydrogen phosphate buffer solution, previously adjusted to the required pH-level and diluted to 500 ml with water.

Reservoir B. This contained a mixture of 9.5 g of TBA and 50.0 ml of a 3.5% (m/v) dipotassium hydrogen phosphate buffer solution, adjusted to the required pH-level and diluted to 500 ml with water.

Reservoir C. This contained a mixture of 10.0 g of EDTA and 25.0 ml of a 3.5% (m/v) dipotassium hydrogen phosphate buffer solution. The pH was adjusted to the required level before dilution to 250 ml with water.

Reservoir D. This contained a solution of 50.0 ml of a 3.5% (m/v) dipotassium hydrogen phosphate buffer solution, previously adjusted

to the required pH-level and diluted to 500 ml with water.

The amount (%, v/v) used from reservoirs A, B and C was as described in the different steps of the design, and reservoir D was used to adjust the total volume to 100% (v/v). Because the total buffer concentration was kept constant in each solvent combination, the buffer concentration was not to be considered as a variable.

Sample preparation

The solvent used was 0.01 M HCl. Because the response variables, measured during the 19 experiments of the half-fraction factorial design, were the retention times (R_t) of TC, ETC and EATC, a solution of these three substances was prepared by diluting a mixture of 1.0 ml of 25.0 mg TC·HCl in 25.0 ml, 2.0 ml of 12.5 mg ETC·HCl in 50.0 ml and 5.0 ml of 10.0 mg EATC·HCl in 50.0 ml to 25.0 ml. In this solution ATC·HCl was not included because of its very high retention times in some of the 19 runs.

The reliability of optimized chromatographic conditions was controlled by analysis of a mixture of 5.0 ml of 10.0 mg ATC·HCl in 50.0 ml, 1.0 ml of 25.0 mg TC·HCl in 25.0 ml, 2.0 ml of 12.5 mg ETC·HCl in 50.0 ml and 5.0 ml of 10.0 mg EATC·HCl in 50.0 ml, diluted to 25.0 ml, and by analysis of a solution of 25.0 mg TC·HCl in 25 ml. The solutions were considered to be stable for 12 h at about 5°C [1, 2].

Analysis of results

The set-up of the applied half-fraction factorial design, together with the analysis of the measured response variables and the multivariate regression calculation, was supported by the statistical graphics software system 'STATGRAPHICS' version 5.0 (STSC Inc., Rockville, MD, USA).

Practical execution of the half-fraction factorial design

In the standard isocratic LC method for the quantification of (TC) on the PSDVB column [1, 2], the values for the examined chromatographic variables were as follows: the concentration of MPOH was 8.0% (m/v), the pH of the 0.35% (m/v) phosphate buffer was 9.0, the concentration of TBA (pH 9.0) was 0.2% (m/v), the concentration of EDTA (pH 9.0) was 0.04% (m/v) and the column temperature was maintained at 60°C. This mobile phase composition was obtained with the multisolvent delivery system by mixing 70.0% solvent of reservoir A, 10.0% solvent of reservoir B, 1.0% solvent of reservoir C and 19% solvent of reservoir D. These standard LC conditions were considered as the central values in the half-fraction factorial design.

The values for the design were chosen as follows:

Chromatographic variable		Low value (−1)	Central value (0)	High value (+1)
MPOH	(res. A)	60 vol	70 vol	80 vol
TBA	(res. B)	5 vol	10 vol	15 vol
EDTA	(res. C)	0 vol	1 vol	2 vol
pH		8.0	9.0	10.0
Column temperature		50°C	60°C	70°C

The worksheet of the applied design is reproduced in Table 1.

Results

The measured response variables were the retention times of TC, ETC and EATC. The values measured are represented in Table 2 (runs 1–19). Results of duplicate experiments (20–38) are reported in parentheses. The retention time of EATC in run 13 was omitted,

Table 1
Applied half-fraction factorial design

Run	MPHO	pH	Temp.	TBA	EDTA
1	0	0	0	0	0
2	−1	−1	−1	−1	1
3	1	−1	−1	−1	−1
4	−1	1	−1	−1	−1
5	1	1	−1	−1	1
6	−1	−1	1	−1	−1
7	1	−1	1	−1	1
8	−1	1	1	−1	1
9	1	1	1	−1	−1
10	0	0	0	0	0
11	−1	−1	−1	1	−1
12	1	−1	−1	1	1
13	−1	1	−1	1	1
14	1	1	−1	1	−1
15	−1	−1	1	1	1
16	1	−1	1	1	−1
17	−1	1	1	1	−1
18	1	1	1	1	1
19	0	0	0	0	0

The 0 corresponds to the central values, −1 to the low level values and 1 to the high level values of the varying chromatographic parameters (see text).

Runs 1–19 are repeated as runs 20–38 for regression modelling.

Table 2
Measured response variables: retention times in minutes

Run	TC	ETC	EATC
1	5.54	4.10	16.12
(20)	(5.53)	(4.09)	(16.09)
2	8.82	3.80	10.91
(21)	(8.12)	(3.71)	(10.91)
3	4.57	2.84	5.08
(22)	(4.66)	(2.85)	(5.24)
4	6.49	5.05	26.27
(23)	(6.50)	(4.96)	(26.18)
5	3.42	3.06	8.13
(24)	(3.54)	(3.10)	(8.22)
6	6.10	3.41	7.63
(25)	(6.02)	(3.36)	(7.55)
7	3.63	2.76	3.85
(26)	(3.66)	(2.76)	(3.91)
8	4.66	4.09	13.21
(27)	(4.70)	(4.16)	(13.79)
9	3.41	2.93	5.18
(28)	(3.23)	(2.75)	(4.89)
10	5.52	4.20	16.15
(29)	(5.45)	(4.15)	(16.20)
11	12.16	4.77	24.45
(30)	(12.55)	(4.86)	(24.35)
12	5.73	3.33	8.22
(31)	(5.81)	(3.32)	(8.12)
13	10.48	10.14	(>60)*
(32)	(10.36)	(10.10)	—
14	5.20	4.29	19.45
(33)	(5.22)	(4.29)	(19.45)
15	8.41	4.38	16.09
(34)	(8.23)	(4.29)	(15.81)
16	4.64	3.24	6.30
(35)	(4.66)	(3.25)	(6.35)
17	8.29	7.17	36.71
(36)	(8.18)	(7.22)	(36.52)
18	3.83	3.83	10.44
(37)	(3.81)	(3.81)	(10.43)
19	5.63	4.20	16.40
(38)	(5.66)	(4.13)	(16.50)

*Value omitted.
Repeated measurements are given in parentheses.

because of its extreme high value (>60 min). This value seems unsuitable to fit in the calculated regression model. With the measured values of runs 1–19 for TC, ETC and EATC as response variables, covering the half-fraction factorial design, preliminary data analysis can be performed. This includes estimation of the effect of each chromatographic parameter, an ANOVA table calculation and a standardized pareto chart for each compound.

To fit the experimental values of each response variable in a multivariate regression model and to compare observed values with fitted values, results of duplicate experiments (runs 20–38) were used. The highest order of variable interactions, included in the data analysis, is 2. Some additional chromatographic parameter combinations (Table 3, runs 39–45) were included to check whether retention time prediction also could be accomplished here with the same regression model. The values measured also are included in Table 3.

Estimation of the chromatographic parameter effects

The effect of an individual chromatographic parameter on the response variables is the mean response value at the high level minus the mean response at the low level. The effects of parameter interactions are obtained by subtracting the mean values of their positive and the mean values of their negative products. An estimate of the error is given by the

Table 3
Additional variable parameter combinations with corresponding response variable measurements: retention times in minutes

Run	MPOH	pH	Temp.	TBA	EDTA	TC	ETC	EATC
39	0	−1	0	†	0	6.17	3.56	9.26
						6.91*	3.66*	11.39*
40	0	−1	1	1	0	5.74	3.55	9.20
						6.68*	3.73*	10.63*
41	−1	−1	0	0	0	8.17	4.01	13.45
						8.33*	4.03*	14.53*
42	−1	−1	0	†	0	8.68	4.20	15.56
						9.02*	4.37*	16.85*
43	−1	−1	0	−1	0	6.54	3.45	8.35
						6.62*	3.28*	8.72*
44	−1	−1	1	−1	0	5.89	3.33	7.60
						5.72*	3.06*	6.45*
45	−1	−1	1	‡	0	4.45	3.01	4.45
						4.60*	2.64*	3.44*

*Fitted values in the regression model.
†Corresponds to 12.0 vol %.
‡Corresponds to 2.0 vol %.

standard error of the estimate for each effect and by a *t*-statistic for a two-tailed test with alpha at 0.05. A detailed explanation of these statistical calculations is described by Massart *et al.* [10]. The found estimated effects of the five chromatographic parameters with their second-order interactions on the retention times of TC, ETC and EATC as response variables, and their standard errors, are given in Table 4.

ANOVA tables

The analysis of variance (ANOVA) tables include the calculation of the sum of squares, degrees of freedom (d.f.) and mean square for each of the chromatographic parameters and their second-order interactions. From the estimated effects tables, the sum of squares for each parameter or interaction can be calculated. The ANOVA tables calculated from the retention times of TC, ETC and EATC as the response variables are reproduced in Tables 5a–5c.

In this experimental design an estimate of the error is available, so that the ANOVA tables also give the *F*-ratios and the significance level for each parameter, expressed as the *P*-value.

Table 4
Estimated effects on retention times of TC, ETC and EATC

Parameter	TC	ETC	EATC
(A) MPOH	−3.7975	−2.06625	−15.7842
(B) pH	−0.96	1.50375	11.8142
(C) Temp.	−1.6625	−0.68375	−7.59417
(D) TBA	2.28	1.65125	12.3817
(E) DTA	−0.31	0.21125	−0.32083
AB	0.2825	−1.01875	−6.87667
AC	0.81	0.49357	3.81667
AD	−1.1875	−0.87625	−6.83917
AE	0.0075	−0.29125	−1.02167
BC	0.3125	−0.44625	−3.89667
BD	0.175	0.92375	5.48412
BE	0.06	0.20875	0.77667
CD	−0.4375	−0.29375	−2.46417
CE	−0.1675	−0.63375	−2.73667
DE	−0.15	0.34125	1.69417

Stand. error of the effect on TC ret. times = ±0.29424
Stand. error of the effect on ETC ret. times = ±0.07338
Stand. error of the effect on EATC ret. times = ±0.208153
Stand. error estimated from total error with 3 d.f. (t = 3.1824)
Stand. error estimated from total error with 2 d.f. (t = 4.3026)

Standardized pareto charts

A standardized pareto chart consists of bars, the lengths of which are proportional to the absolute value of the estimated effects, divided by its standard error. Charts for the retention times of TC, ETC and EATC as response variables are depicted in Figs 1a–1c. The bars are displayed in order of the size of the effects, with the largest effects on top. The chart includes a vertical line at the critical *t*-value for an alpha of 0.05.

Discussion

From Table 4, Tables 5a–5c and Figs 1a–1c, it is clear that the chromatographic retention of TC, ETC and EATC in this LC system is principally influenced by the percentage of the organic modifier (MPOH) in the mobile phase. As expected, an increase of the concentration of the organic modifier shortens the retention times. The second most important chromatographic parameter, by which the retention times of the three compounds are influenced, is the concentration of TBA in the mobile phase. Retention times increase with increasing TBA concentration. This can be explained by interaction between the positively charged TBA and the negatively charged tetracyclines. A third chromatographic parameter which influences the retention of the three examined compounds, is the pH of the mobile phase. For ETC and EATC, its influence is of about the same weight as the TBA concentration. A mobile phase pH rise from 8.0 to 10.0 causes retention increases for both compounds. On the contrary, the retention time of TC is slightly reduced by a pH increase. This means that the resolution between ETC and TC, and between TC and EATC is most affected by the mobile phase pH. Reducing the pH not only lowers the retention times of ETC and EATC, but also may improve the resolution between ETC and TC, while that between TC and EATC is reduced.

The column temperature has a less important but significant effect on the retention times of the three compounds. It also influences the efficiency of the system. The EDTA concentration in the mobile phase has no significant impact on the retention times of the examined compounds. However, EDTA ensures peak symmetry by preventing complexation of tetracyclines with metal ions. This means that EDTA may be omitted in the half-fraction

Table 5a
ANOVA for retention times of TC (five factor study)

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
(A) MPOH	57.68402	1	57.68402	166.56	0.0010
(B) pH	3.6864	1	3.6864	10.64	0.047
(C) Temp.	11.05562	1	11.05562	31.92	0.11
(D) TBA	20.7936	1	20.7936	60.04	0.0045
(E) EDTA	0.3844	1	0.3844	1.11	0.3695
AB	0.31922	1	0.31922	0.92	0.4173
AC	2.6244	1	2.6244	7.58	0.0706
AD	5.640625	1	5.640625	16.29	0.0274
AE	0.000225	1	0.000225	0	0.9815
BC	0.390625	1	0.390625	1.13	0.3661
BD	0.1225	1	0.1225	0.35	0.5997
BE	0.0144	1	0.0144	0.04	0.8535
CD	0.765625	1	0.765625	2.21	0.2338
CE	0.112225	1	0.112225	0.32	0.6146
DE	0.09	1	0.09	0.26	0.6504
Total error	1.038952	3	0.346318		

*Total (corr.) 104.7228, 18 d.f.

Table 5b
ANOVA for retention times of ETC (five factor study)

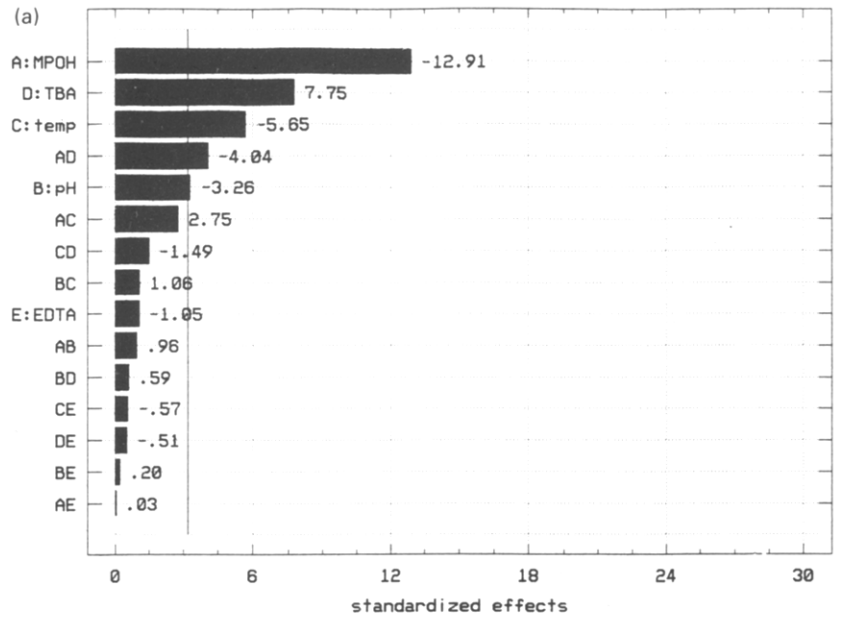
Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
(A) MPOH	17.07755	1	17.07755	792.84	0.0001
(B) H	9.0450	1	9.0450	419.92	0.0003
(C) Temp.	1.870056	1	1.870056	86.82	0.0026
(D) TBA	10.9065	1	10.9065	506.34	0.0002
(E) EDTA	0.178506	1	0.178506	8.29	0.0636
AB	4.151406	1	4.151406	192.73	0.0008
AC	0.97515	1	0.97515	45.27	0.0067
AD	3.071256	1	3.071256	142.59	0.0013
AE	0.339306	1	0.339306	15.75	0.0286
BC	0.796556	1	0.796556	36.98	0.0089
BD	3.41325	1	3.41325	158.46	0.0011
BE	0.17430	1	0.17430	8.09	0.0654
CD	0.345156	1	0.345156	16.02	0.0280
CE	1.606556	1	1.606556	74.59	0.0033
DE	0.465806	1	0.465806	21.63	0.0188
Total error	0.064619	3	0.021540		

*Total (corr.) 54.48106, 18 d.f.

Table 5c
ANOVA for retention times of ETC (five factor study)

Effect	Sum of squares	d.f.	Mean square	*F*-ratio	*P*-value
(A) MPOH	135.8945	1	135.8945	5750.12	0.0002
(B) pH	76.13156	1	76.13156	3221.36	0.0003
(C) Temp.	31.45711	1	31.45711	1331.05	0.0008
(D) TBA	83.62127	1	83.62127	3538.28	0.0003
(E) EDTA	0.056146	1	0.056146	2.38	0.2632
AB	25.79375	1	25.79375	1091.41	0.0009
AC	7.94561	1	7.94561	336.20	0.0030
AD	25.5132	1	25.5132	1079.54	0.0009
AE	0.569347	1	0.569347	24.09	0.0391
BC	8.282188	1	8.282188	350.45	0.0028
BD	16.4051	1	16.4051	694.15	0.0014
BE	0.32902	1	0.32902	13.92	0.0649
CD	3.312064	1	3.312064	140.14	0.0071
CE	4.085097	1	4.085097	172.85	0.0057
DE	1.565564	1	1.565564	66.24	0.0148
Total error	0.047267	2	0.02363		

*Total (corr.) 1275.2958, 17 d.f.



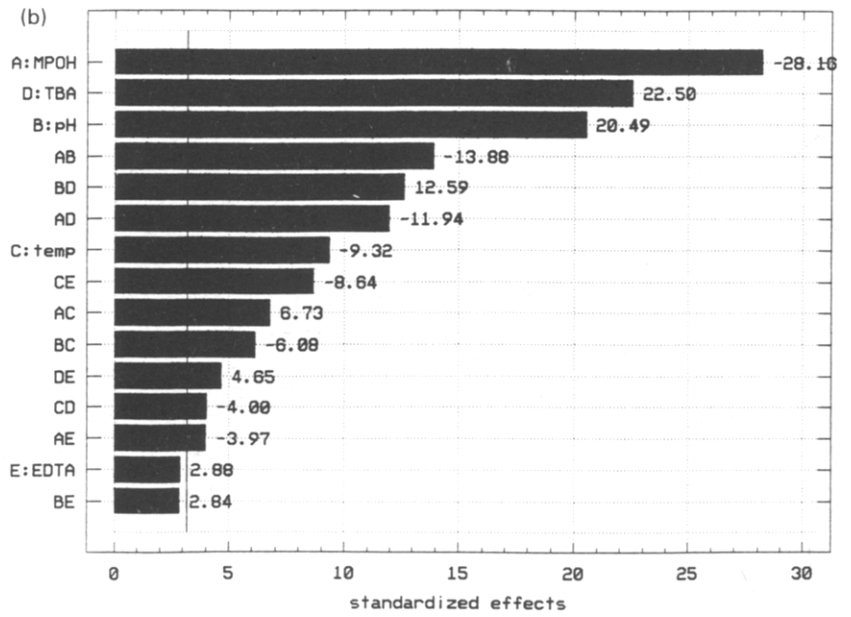


Figure 1a
Standardized pareto chart, representing the estimated effects of parameters and parameter interactions on the retention time of TC.

Figure 1b
Standardized pareto chart, representing the estimated effects of parameters and parameter interactions on the retention time of ETC.

factorial design as one of the variable chromatographic parameters.

From Tables 4 and 5 and Figs 1a–1c some important chromatographic parameter interactions also are revealed, especially with respect to the retention of ETC and EATC. These interactions are established between the organic modifier concentration and the pH of the mobile phase (negative interaction), between the organic modifier and the TBA concentrations (negative interaction) and between the TBA concentration and the pH (positive interaction). This means that a pH increase of the mobile phase enhances the ETC and EATC retentions much more at lower organic modifier concentrations than at higher

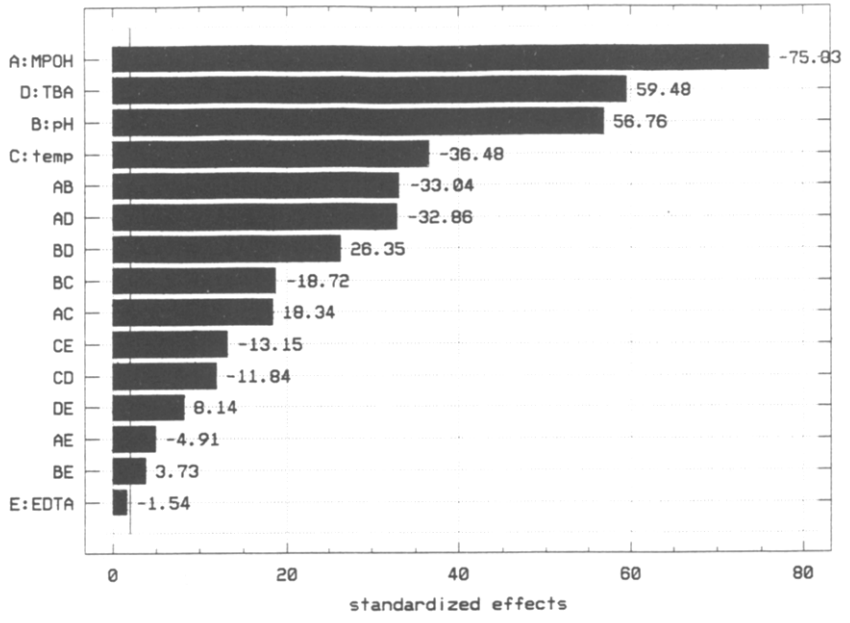


Figure 1c
Standardized pareto chart, representing the estimated effects of parameters and parameter interactions on the retention time of EATC.

modifier concentrations. An increase of TBA concentration in the mobile phase increases the ETC and EATC retentions much more at lower organic modifier concentrations than at higher modifier concentrations. On the contrary, an increase of TBA concentration in the mobile phase raises the ETC and EATC retentions much more at a higher mobile phase pH than at a lower mobile phase pH. As to the chromatographic retention of TC, interactions between chromatographic parameters seem rather insignificant. These findings fulfil what may be expected from the chemical properties of ETC, TC and EATC in this chromatographic model.

Since EDTA may be omitted in the half-fraction design, now a full factorial design of four variables, which needs at least 16 experiments, is available. As a consequence, estimation of regression models, fitting the original experimental data, can be performed. It is evident that non-significant parameters or parameter interactions will be excluded from estimation of the regression models for the examined response variables.

Regression modelling

Two-level factorial designs can only estimate the main effects and their interactions. In a 2^4 design any response in any parameter combination is modelled as:

$$Y = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_4x_4 + B_{12}x_1x_2 + B_{13}x_1x_3 + B_{14}x_1x_4 + B_{23}x_2x_3 + B_{24}x_2x_4 + B_{34}x_3x_4 + \text{error},$$

where Y is the measured retention time, B_0 is the intercept, B_1 to B_4 are the slopes in the directions x_{1-4}, and B_{12} . . . B_{34} are the interaction coefficients.

The total of 45 (43 for EATC) retention times as response variables for the examined compounds TC, ETC and EATC (Tables 2 and 3), are fitted to such a regression model, where the independent variables are the significant chromatographic parameters. Only significant parameter interactions are included in the linear regression model. This significance is based upon the critical t-values for an alpha of 0.05 in the respective standardized pareto charts (Figs 1a–1c). Thus, the retention times of, for example, TC as a response variable are fitted to a model with the four significant chromatographic parameters and the only significant interaction term.

The fair agreement between observed and fitted values is visualized in Figs 2a–2c. Here the fitted and the observed retention times of TC, ETC and EATC as response variables and their 99% intervals for the means are plotted against each other. Only three values exceed 3 standard deviations.

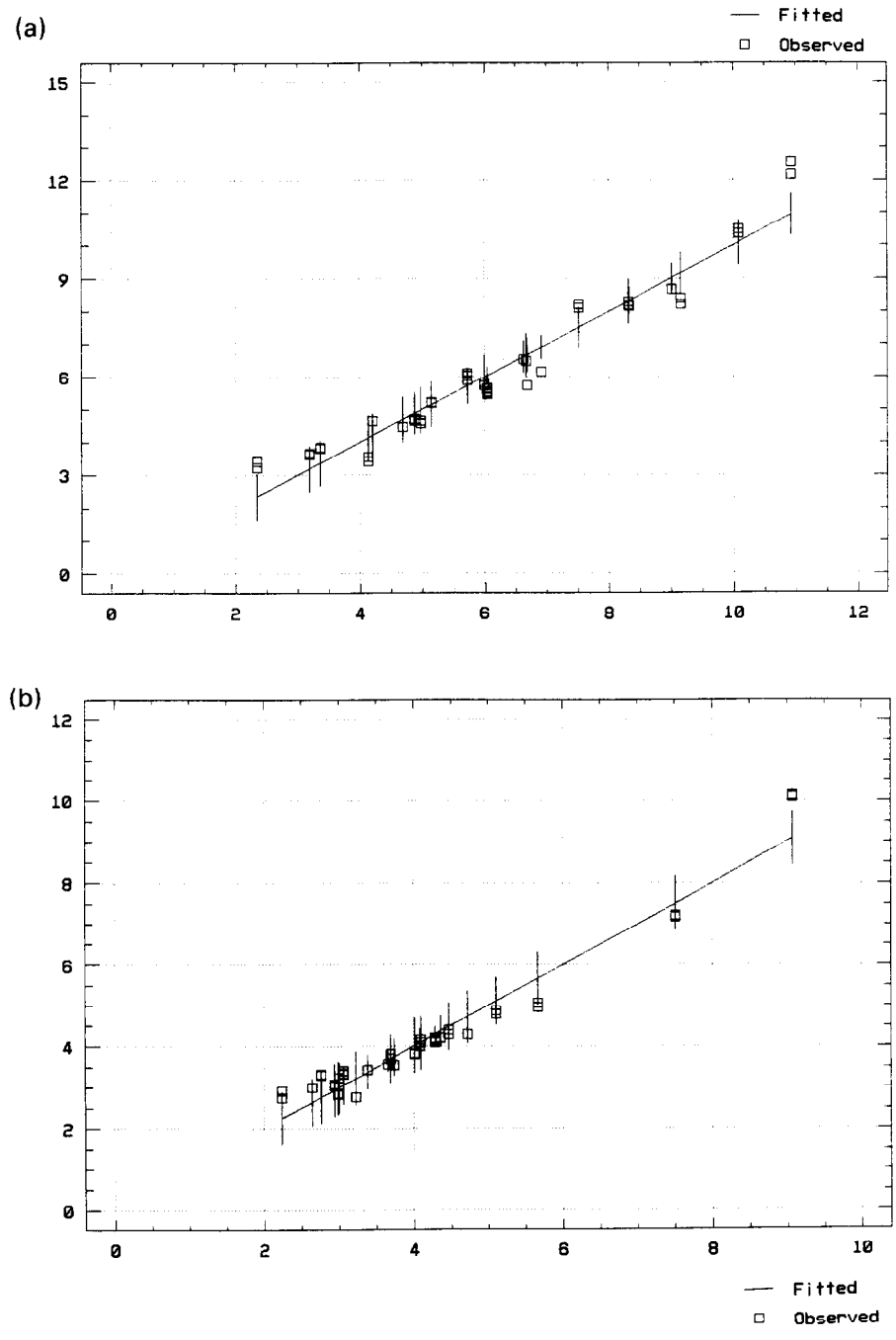


Figure 2a
Observed retention times versus predicted retention times of TC with 99% confidence limits for the means.

Figure 2b
Observed retention times versus predicted retention times of ETC with 99% confidence limits for the means.

Response surface plots

The object of an experimental design is also to discover the combination of independent variable levels which may optimize the response. Here, an optimized response results in a chromatogram, characterized by complete peak resolutions within the shortest possible time. Once the estimates of all significant coefficients in the regression models are known, the response variables may be modelled for each combination of the independent explanatory variables and a

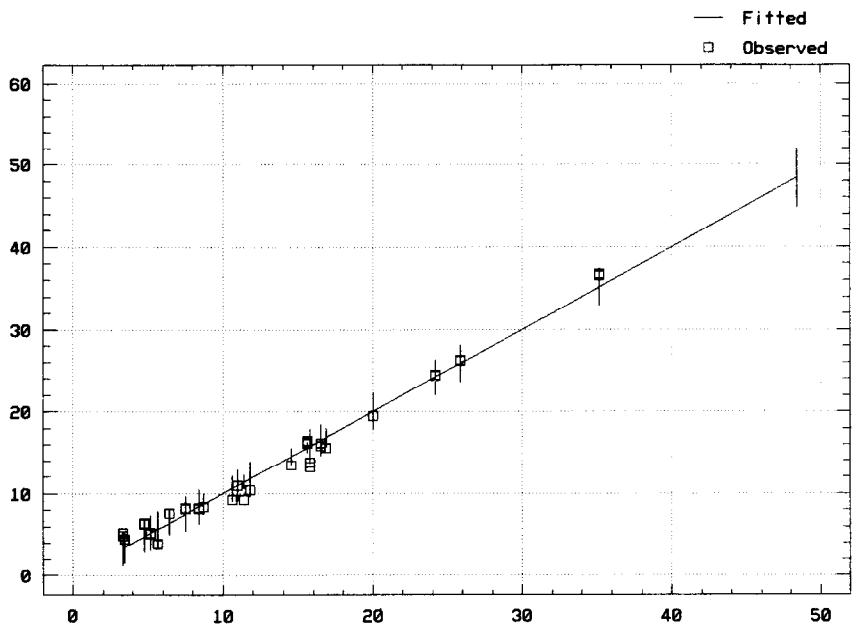


Figure 2c
Observed retention times versus predicted retention times of EATC with 99% confidence limits for the means.

response surface can be constructed. No more additional experiments are needed, which results in an important gain of time.

In Fig. 3 estimated response surface plots show how the retention times of ETC and TC, as well as these of TC and EATC, vary with regard to each other as a function of the organic modifier concentration and the pH of the mobile phase. The column temperature and the TBA concentration in the mobile phase were kept constant at 70°C and 15 vol parts, respectively. An optimized chromatogram, obtained from this response surface plot in Fig. 3, is represented in Fig. 4 for a TC·HCl sample (see *Sample preparation*), together with a chromatogram of the same sample, obtained on the same column with the chromatographic conditions as described in the protocol of the collaborative study [2].

Conclusions

This study of a half-fraction factorial design, applied to a reference LC method for analysis of tetracycline and its related substances, enables the estimation of the impact of the individual chromatographic parameters and their interactions on the chromatographic behaviour of tetracycline and its related substances on a PSDVB column.

It is revealed that the organic modifier in the mobile phase is the most important chromatographic variable with respect to the retention of the studied substances TC, ETC and EATC. The effects of the other parameters, like the pH of the mobile phase and its TBA concentration, however, are also significant. They interact with the organic modifier. A particular finding is that the retention of TC is not very sensitive to small mobile phase pH fluctuations, in contrast to its related substances ETC and EATC. This means that, for example, a small pH increase of the mobile phase may lower the required chromatographic resolution and extend unnecessarily the time for a complete chromatographic run.

A regression model with the significant chromatographic parameters as independent variables and the retention times as response variables, can be estimated. From the found regression model, a response surface plot can be constructed, which can help to select those chromatographic parameter combinations, that ensure optimized chromatographic separations. Such a response surface plot may be helpful not only to discovery optimum combinations of chromatographic parameters, but also to modify the prescribed chromatographic parameters in order to meet the requirements of the system suitability tests.

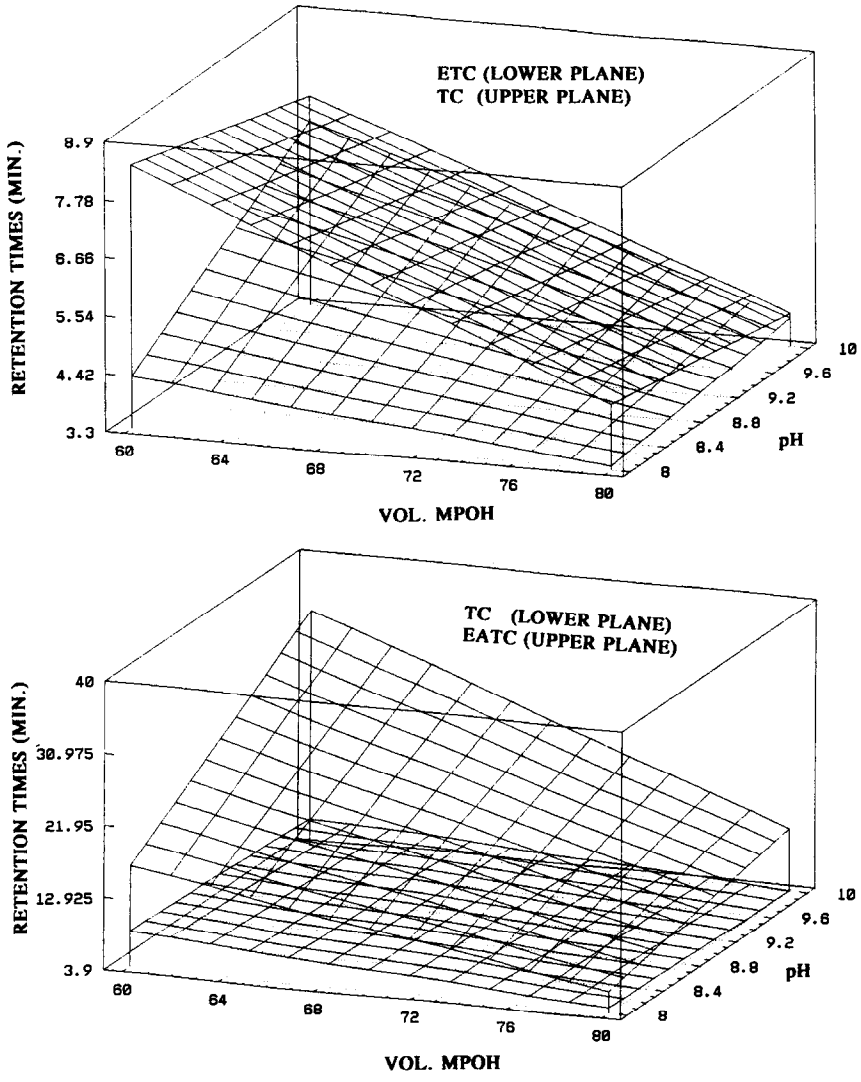


Figure 3
Estimated response surface plots for ETC (lower plane) and TC (upper plane) and for TC (lower plane) and EATC (upper plane). Temp. = 70°C. TBA = 15 vol.

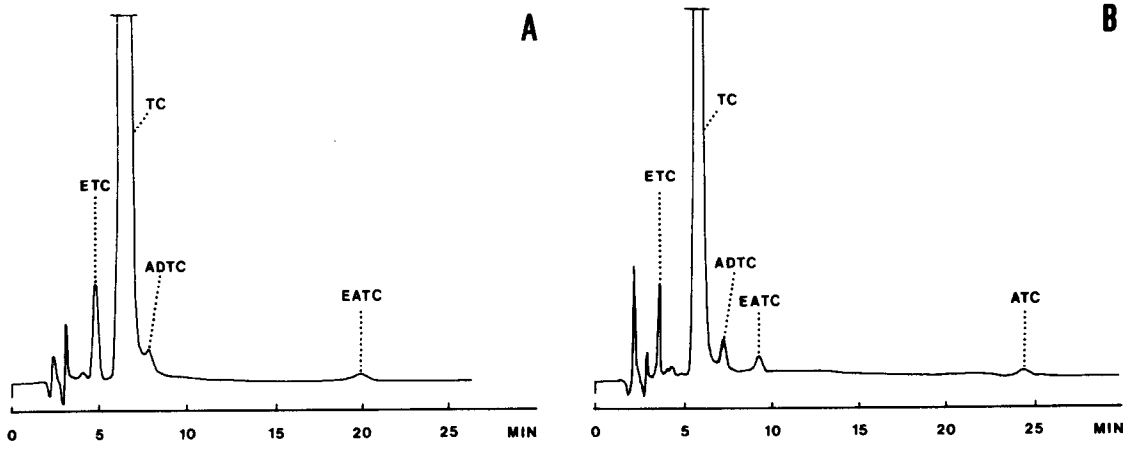


Figure 4
Liquid chromatograms of a TC·HCl sample, obtained on the same column with (A) the standard conditions (central values Table 2), and (B) with the optimized conditions (run 40 Table 3).

Experimental design may also enable global optimization of the HPLC separation of tetracycline and its related substances on a PSDVB column with a minimum of experiments. This study confirms findings of previous work with less experiments.

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