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Robustness testing of a reversed-phase high-performance liquid chromatographic assay: comparison of fractional and asymmetrical factorial designs

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Abstract

Robustness tests were performed on a reversed-phase HPLC assay for triadimenol. Different experimental designs were compared. Two-level fractional factorial designs with different resolutions were used to study the influence of procedure-related factors. The factors chromatographic column manufacturer at four levels and instrument at three levels were stepwise included in the study using asymmetrical factorial designs. The significance of the factor effects was determined statistically, using two types of error estimates in the calculation of critical effects, and graphically, by means of half-normal plots. The asymmetrical designs turned out to be an efficient and economic method to examine the influence of factors at different numbers of levels in the robustness testing of analytical methods.

Keywords: Robustness test; Fractional factorial design; Half-normal plots; Critical effects

1. Introduction

Robustness tests are performed at the end of method development to test the susceptibility of an analytical procedure to small changes in the experimental conditions [1]. The test simulates the changes that can be expected when transferring the method between laboratories, instruments and/or operators.

The robustness test starts with the selection of the factors that might influence the performance of the method. An experimental design approach is then used to evaluate their influence on the responses [2–6]. The factors can be divided into two groups: procedure-related factors and non-procedure-related factors. Procedure-related factors are specific for a given assay, usually explicitly prescribed in the protocol, and in general comprise quantitative factors, e.g. the pH of an eluent, the column or reaction temperature, the concentration of a reagent or the fraction of a solvent in a mixture. Non-procedure-related factors are frequently qualitative factors, e.g. a batch of reagent, a column or instrument manufacturer. For the latter type of factor, the protocol often allows to use, for instance, a column or instrument with similar characteristics as the one described.

In a robustness test no or only a small number of significant main effects is expected at least on the

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content determination of the sample [2]. In most robustness tests, only procedure-related factors are examined [3,7–10]. Two-level designs (low/high level) can be applied because the response functions of most of the factors are monotonously in- or decreasing in the small intervals studied. Often, Plackett–Burman [11] or fractional factorial designs [12] are used.

For chromatographic methods, consideration of non-procedure-related factors, such as the chromatographic column manufacturer, in the robustness test seems interesting. More than two levels should preferably be considered for these factors because an examination at two levels is not representative for their population. This is, however, not possible with the above mentioned screening designs. As an alternative, the asymmetrical factorial designs proposed by Addelman can be applied [13]. These screening designs allow a fast examination of factors at different numbers of levels.

The effect of an examined factor on the considered response is statistically non-significant if its absolute value does not exceed a critical effect, which is derived from an error estimate. Effects that beforehand are considered to be negligible, for instance from dummies (imaginary variables) or from two-factor interactions, can be used to estimate the experimental error for the calculation of the critical effects [3–5]. Besides, the distribution of the estimated effects themselves can also be used to decide which effects can be considered negligible and therefore allows another error estimate [14,15]. Furthermore, normal [16] and half-normal plots [17,18] can be applied as a graphical tool to decide visually on the significance of the effects.

In this paper, the robustness of a reversed-phase high-performance liquid chromatographic (RP-HPLC) assay for the fungicide triadimenol in technical products is studied. Fractional factorial designs are applied to examine the procedure-related factors at two levels. The factors column manufacturer at four levels and instrument at three levels are included subsequently using two of Addelman's asymmetrical designs. It is studied whether the examination of these non-procedure-related factors reveals further information. A comparison of the asymmetrical designs with each other and with the fractional factorial designs is made.

2. Theory

2.1. Fractional factorial designs

Fractional factorial designs are derived from full factorial designs [12,19,20]. The program RTS [6] was used to set up the fractional factorial designs for the robustness tests. The first design applied is an eighth-fraction factorial design for six factors at two levels (2^{6-3}). The generators for this design, that has resolution III, were $D=-AB$, $E=-AC$ and $F=-BC$ (Table 1). Secondly, a fractional factorial design with a higher resolution, namely IV, is applied. This fourth-fraction design (2^{6-2}) is char-

Table 1
Design and results of the 2^{6-3} fractional factorial design with generators $D=-AB$, $E=-AC$ and $F=-BC$

Experimental	Factors						Responses		
	Temp. (A)	Flow (B)	μ (C)	pH (D)	%B (E)	λ (F)	Content (%)	Area (V/min)	Resolution
1	−1	−1	−1	−1	−1	−1	97.64	3.62	1.68
2	1	−1	−1	1	1	−1	97.49	3.49	1.14
3	−1	1	−1	1	−1	1	96.91	1.39	1.63
4	1	1	−1	−1	1	1	97.12	1.33	1.02
5	−1	−1	1	−1	1	1	96.95	1.77	1.13
6	1	−1	1	1	−1	1	97.36	1.79	1.51
7	−1	1	1	1	1	−1	97.80	2.70	1.16
8	1	1	1	−1	−1	−1	97.74	2.75	1.49

Table 2
Design and results of the 2^{6-2} fractional factorial design with resolution IV (generators: $E=BCD$, $F=ACD$)

Experimental	Factors						Responses		
	Temp. (A)	Flow (B)	μ (C)	pH (D)	%B (E)	λ (F)	Content (%)	Area (V/min)	Resolution
1	−1	−1	−1	1	1	1	97.33	1.75	1.22
2	1	−1	−1	−1	−1	1	97.12	1.79	1.50
3	−1	1	−1	−1	1	−1	97.92	2.70	1.19
4	1	1	−1	1	−1	−1	97.16	2.77	1.47
5	−1	−1	1	1	−1	−1	97.91	3.63	1.66
6	1	−1	1	−1	1	−1	97.40	3.49	1.13
7	−1	1	1	−1	−1	1	97.04	1.42	1.54
8	1	1	1	1	1	1	97.06	1.33	1.05
9	−1	−1	−1	−1	−1	−1	97.64	3.62	1.68
10	1	−1	−1	1	1	−1	97.49	3.49	1.14
11	−1	1	−1	1	−1	1	96.91	1.39	1.63
12	1	1	−1	−1	1	1	97.12	1.33	1.02
13	−1	−1	1	−1	1	1	96.95	1.77	1.13
14	1	−1	1	1	−1	1	97.36	1.79	1.51
15	−1	1	1	1	1	−1	97.80	2.70	1.16
16	1	1	1	−1	−1	−1	97.74	2.75	1.49

acterized by the generators $E=BCD$ and $F=ACD$ and consists of 16 experiments (Table 2).

2.2. Asymmetrical factorial designs [13]

The factorial plans usually used are symmetrical since they contain only factors with the same number of levels. An asymmetrical design, which is a design where the factors have a different number of levels, could be obtained from a combination of two or more symmetrical plans. However, most of the experimental designs obtained in this way require a large number of experiments. Asymmetrical designs requiring a minimum number of experiments can be constructed using the collapsing principle of Addelman [13]. However, to facilitate the construction of asymmetrical designs, Addelman proposed seven basic orthogonal main-effect plans. These basic plans consist of different designs. The condition to execute Addelman's designs, namely that interactions should be negligible can be assumed fulfilled in robustness tests. The basic plan used for our experiments is given in Table 3a. It consists of three plans: one for four-level, one for three-level and one for two-level factors in 16 trials. To derive, e.g., a design with one factor at four levels, one factor at three levels and nine factors at two levels ($4\cdot3\cdot2^9$), one selects one column of the first, one of the second and nine columns of the third plan, taking into account that certain combinations are not allowed. These combinations are summarized in Table 3b. Therefore, if, e.g., column 2 is selected from the four-level plan, this means, that column 2 from the three-level plan and columns 4 to 6 from the two-level plan cannot be used in the construction of the asymmetrical design.

For the robustness tests two different asymmetrical designs were applied. As indicated at the bottom of Table 3a, the $4\cdot2^{12}$ design was obtained by combining column 1 of the four-level plan with columns 4 to 15 of the two-level plans. The $4\cdot3\cdot2^9$ design was obtained by combining column 1 of the four-level plan with column 2 of the three-level plan and with columns 7 to 15 of the two-level plan.

When the number of factors to be examined is lower than the number of columns in the design, the remaining columns of the design are defined as dummy factors (see below) as is also the case in Plackett–Burman designs [11].

Table 3
(a) Addelman's basic plan: 4^5, 3^5, 2^{15}/16 trials, (b) combinations that cannot be used in the construction of asymmetrical designs from the basic plan 4^5 3^5, 2^{15}/16 trials

(a)

	Plan for four levels					Plan for three levels					Plan for two levels														
Factor experiment	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	−2	−2	−2	−2	−2	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1
2	−2	−1	−1	1	2	−1	0	0	1	0	−1	−1	−1	−1	1	1	−1	1	1	1	−1	1	1	1	−1
3	−2	1	1	2	−1	−1	1	1	0	0	−1	−1	−1	1	−1	1	1	−1	1	1	1	−1	−1	1	1
4	−2	2	2	−1	1	−1	0	0	0	1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1	−1	1
5	−1	−2	−1	−1	−1	0	−1	0	0	0	−1	1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	1	1
6	−1	−1	−2	2	1	0	0	−1	0	1	−1	1	1	−1	1	1	−1	−1	−1	1	1	−1	1	−1	1
7	−1	1	2	1	−2	0	1	0	1	−1	−1	1	1	1	−1	1	1	1	−1	1	−1	1	−1	−1	−1
8	−1	2	1	−2	2	0	0	1	−1	0	−1	1	1	1	1	−1	1	−1	1	−1	−1	−1	1	1	−1
9	1	−2	1	1	1	1	−1	1	1	1	1	−1	1	−1	−1	−1	1	−1	1	1	−1	1	1	−1	1
10	1	−1	2	−2	−1	1	0	0	−1	0	1	−1	1	−1	1	1	1	1	−1	−1	−1	−1	−1	1	1
11	1	1	−2	−1	2	1	1	−1	0	0	1	−1	1	1	−1	1	−1	−1	−1	−1	1	1	1	1	−1
12	1	2	−1	2	−2	1	0	0	0	−1	1	−1	1	1	1	−1	−1	1	1	1	1	−1	−1	−1	−1
13	2	−2	2	2	2	0	−1	0	0	0	1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	1	1	−1
14	2	−1	1	−1	−2	0	0	1	0	−1	1	1	−1	−1	1	1	1	−1	1	−1	1	1	−1	−1	−1
15	2	1	−1	−2	1	0	1	0	−1	1	1	1	−1	1	−1	1	−1	1	1	−1	−1	−1	1	−1	1
16	2	2	−2	1	−1	0	0	−1	1	0	1	1	−1	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1
Columns used in the asymmetrical designs																									
$4\cdot2^{12}$	x													x	x	x	x	x	x	x	x	x	x	x	x
$4\cdot3\cdot2^{9}$	x						x										x	x	x	x	x	x	x	x	x

Combinations that cannot be used in the construction of asymmetrical designs from the basic plan, 4^5, 3^5, 2^{15}/16 trials

(b)

Column of the four-level plan	Column of the three-level plan	Columns of the two-level plan		
1	1	1	2	3
2	2	4	5	6
3	3	7	8	9
4	4	10	11	12
5	5	13	14	15

2.3. Calculation of the factor effects

The effect of a factor X is calculated as:

$$E_X = \frac{\sum Y(i)}{n_i} - \frac{\sum Y(j)}{n_j} \quad (1)$$

with $\Sigma\ Y(i)$ and $\Sigma\ Y(j)$ the sums of the responses where factor X is at levels (i) and (j), respectively; n_i and n_j the number of runs from the design with the factor at levels (i) and (j), respectively. For two-level factors, one effect can be estimated, while for factors at more levels, all possible differences between two levels can be calculated.

2.4. Evaluation of the significance of the effects

Different methods can be used to decide on the significance of the effects; they can be either statistical or graphical. The methods used are shortly explained below.

2.4.1. Comparison with a critical effect

The significance of the effects is often tested by comparison with a critical effect derived from a t-test [3–5,14,15,22]:

$$E_{\text{critical}} = t_{\text{critical}} \cdot (SE)_{\text{est}} \quad (2)$$

with $E_{critical}$ the critical effect, $(SE)_{est}$ the estimated standard error on an effect and $t_{critical}$ the critical t-value. An effect is considered significant if its absolute value is larger than the critical effect. The standard error can be estimated in several ways, e.g. from factor effects considered negligible by definition, such as dummy factor effects [3–5,21] or interaction effects [3,4]. It can also be obtained from the distribution of the estimated effects themselves [14,15,22] or from additional replicated experiments, e.g. at the nominal levels of the factors [3,22]. The latter approach was however not considered here.

2.4.1.1. Critical effects derived from negligible factor effects

In fractional factorial designs, two-factor interactions are either confounded with main effects, higher-order interactions and/or with each other. In robustness tests, one assumes that they are negligible [3,21,22]. Dummy factor effects are effects that are not assigned to chemical or physical modifications, i.e. main effects from imaginary variables. They are however confounded with two-factor and higher-order interaction effects and were used to complete the asymmetrical factorial designs (Tables 4 and 5). The two-factor interaction effects, not confounded with main effects, and the dummy effects were used to estimate the standard error from the fractional factorial designs and the asymmetrical factorial designs, respectively.

The standard error from these negligible factor effects is calculated as:

$$(SE)_{est} = \sqrt{\frac{\sum E^2_{X_iX_j}}{n_{X_iX_j}}} \qquad (3)$$

where $E_{X_iX_j}$ is either the effect of a two-factor interaction or of a dummy factor and denotes the number of negligible factor effects, which are used for the estimation of the standard error. The number of degrees of freedom for $t_{critical}$ in Eq. (2) corresponds to the number of negligible factor effects considered. The use of at least three negligible interactions or dummies is recommended in order to obtain acceptable estimates for $(SE)_{est}$ and not to have to work with too high $t_{critical}$-values [23].

2.4.1.2. Critical effects derived from the distribution of the effects [14,15]

The critical effects can also be derived from the distribution of the estimated effects [14,15]. In a first step, one assumes only non-significant effects, so that all effects belong to a normal distribution around zero: $N(0, \sigma)$. Accordingly, a first estimate of the standard deviation σ is given by:

$$s_0 = 1.5 \cdot \underset{i}{\text{median}} \, |E_i| \qquad (4)$$

Table 4
Design and results of the $4 \cdot 2^{12}$ design (dummy factors: d_1 to d_6)

Exp.	Column	d_1	d_2	d_3	Temp. (*A*)	Flow (*B*)	μ (*C*)	pH (*D*)	%*B* (*E*)	*d* (*F*)	d_4	d_5	d_6	Content (%)	Area (V/min)	Resolution
1	−2	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	97.55	2.875	1.73
2	−2	−1	1	1	−1	1	1	1	−1	1	1	1	−1	96.69	1.463	1.72
3	−2	1	−1	1	1	−1	1	1	1	−1	−1	1	1	96.72	2.789	1.09
4	−2	1	1	−1	1	1	−1	−1	1	1	1	−1	1	98.16	1.412	1.06
5	−1	−1	−1	−1	−1	1	1	−1	1	1	−1	1	1	97.86	1.395	1.57
6	−1	−1	1	1	−1	−1	−1	1	1	−1	1	−1	1	97.13	2.757	1.58
7	−1	1	−1	1	1	1	−1	1	−1	1	−1	−1	−1	97.11	1.435	2.00
8	−1	1	1	−1	1	−1	1	−1	−1	−1	1	1	−1	98.19	2.820	2.01
9	1	−1	−1	−1	1	−1	1	1	−1	1	1	−1	1	97.15	1.620	1.42
10	1	−1	1	1	1	1	−1	−1	−1	−1	−1	1	1	**95.52**	2.533	1.42
11	1	1	−1	1	−1	−1	−1	−1	1	1	1	1	−1	97.24	1.587	1.05
12	1	1	1	−1	−1	1	1	1	1	−1	−1	−1	−1	97.53	2.399	1.26
13	2	−1	−1	−1	1	1	−1	1	1	−1	1	1	−1	98.32	2.466	1.09
14	2	−1	1	1	1	−1	1	−1	1	1	−1	−1	−1	96.55	1.647	1.06
15	2	1	−1	1	−1	1	1	−1	−1	−1	1	−1	1	96.93	2.536	1.60
16	2	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1	98.13	1.687	1.64

Table 5
Results of the $4 \cdot 3 \cdot 2^9$ design (dummy factors: d_1 to d_3)

Exp.	Column	Instrument	Temp. (*A*)	Flow (*B*)	μ (*C*)	pH (*D*)	%*B* (*E*)	*d* (*F*)	d_1	d_2	d_3	Content (%)	Resolution
1	−2	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	96.95	1.82
2	−2	0	−1	1	1	1	−1	1	1	1	−1	96.69	1.72
3	−2	1	1	−1	1	1	1	−1	−1	1	1	98.51	1.18
4	−2	0	1	1	−1	−1	1	1	1	−1	1	98.16	1.06
5	−1	−1	−1	1	1	−1	1	1	−1	1	1	97.20	1.59
6	−1	0	−1	−1	−1	1	1	−1	1	−1	1	97.13	1.58
7	−1	1	1	1	−1	1	−1	1	−1	−1	−1	98.28	2.14
8	−1	0	1	−1	1	−1	−1	−1	1	1	−1	98.19	2.01
9	1	−1	1	−1	1	1	−1	1	1	−1	1	95.94	1.59
10	1	0	1	1	−1	−1	−1	−1	−1	1	1	95.52	1.42
11	1	1	−1	−1	−1	−1	1	1	1	1	−1	96.35	1.20
12	1	0	−1	1	1	1	1	−1	−1	−1	−1	97.53	1.26
13	2	−1	1	1	−1	1	1	−1	1	1	−1	96.21	1.20
14	2	0	1	−1	1	−1	1	1	−1	−1	−1	96.55	1.06
15	2	1	−1	1	1	−1	−1	−1	1	−1	1	95.48	1.79
16	2	0	−1	−1	−1	1	−1	1	−1	1	1	98.13	1.64

In practice, the condition that all effects are non-significant is frequently not fulfilled. In the presence of potentially significant effects, s_0 will be an overestimation of σ. To obtain a more reliable estimate of the standard error, only the potentially non-significant effects are considered in a second estimation of σ. Therefore, the method only uses the effects that in absolute value do not exceed 2.5 times the first estimate s_0 to obtain a second estimate of σ:

$$s_1 = \sqrt{m_{in}^{-1} \cdot \sum_{|E_i| < 2.5 s_0} E_i^2} \quad (5)$$

with m_{in} the number of effects, which are in absolute value smaller than 2.5 times the estimated standard error s_0 of Eq. (4). The corresponding critical effect is calculated according to Eq. (2), with m_{in} as the number of degrees of freedom:

$$E_{\text{critical(ME)}} = t_{(1-\alpha, m_{in})} \cdot s_1 \quad (6)$$

2.4.2. Half-normal plots [17,18]

Besides these algorithms, graphical methods such as the normal [16] and the half-normal plots [17,18,22] allow distinguishing random and significant effects. The half-normal plot leads to a straight line for non-significant effects, i.e. effects with a normal distribution around zero. Therefore, a deviation from this straight line indicates that the corresponding effect belongs to another distribution and has to be considered significant. Moreover, the slope of the straight line through the non-significant effects is a measure for the standard deviation of their distribution.

3. Experimental

3.1. Analyte

Triadimenol is a fungicide with molecular mass 295.8 g/mol. The structural formula is given in Fig. 1. Products of technical grade active ingredient were analysed.

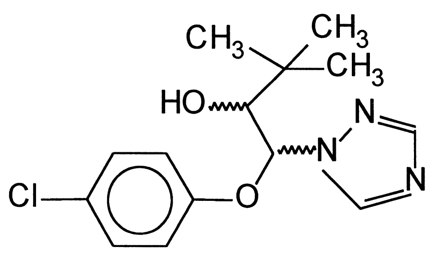

Fig. 1. Triadimenol, $C_{14}H_{18}ClN_3O_2$.

Table 6
Nominal chromatographic conditions of the RP-HPLC analysis method for triadimenol

Chromatograph	Shimadzu SCL-10A with SPD-10A detector (or another instrument with similar characteristics)
Column	Merck LiChrospher 100 RP8, 125 mm length, 4 mm internal diameter, parts with 5 μm diameter (or another column with similar characteristics)
Eluent A	Phosphate buffer at pH = 3 with ionic strength given by 1.5 g/l $NaH_2PO_4H_2O$
Eluent B	Acetonitrile
Mixture of the eluents	A:B 55:45 v/v
Modus	Isocratic
Column temp.	40°C
Flow rate	1.5 ml/min
Detection	UV-detection at 230 nm
Injection volume	5 μl
Concentration range	600 mg/l of either standard or technical product
Solvent	Acetonitrile
Pretreatment	15 min treatment in an ultrasonic bath

3.2. Nominal chromatographic conditions

The conditions of the RP-HPLC assay with external standard are given in Table 6.

3.3. Designs and factors chosen

From the nominal chromatographic conditions, six procedure-related factors (Table 7) that might affect the outcome of the assay are selected: column temperature, flow-rate of the eluent, ionic strength μ of the phosphate buffer given by the concentration of $NaH_2PO_4 \cdot H_2O$, pH of the phosphate buffer, fraction of acetonitrile (%B) in the eluent and detection wavelength. Table 7 also shows the levels applied. The same procedure-related factors as with the fractional factorial designs are studied in the asymmetrical designs, the levels for the factors flow-rate and wavelength were, however, modified based on the results of the fractional factorial designs (Table 7). Additional factors examined in the asymmetrical designs are columns (Table 4) as well as columns and instruments (Table 5). Four columns and three instruments were used. Mono-octylsilyl silica gel (5 μm) was the bonded phase of all columns. They had a length of 125 mm and an internal diameter of 4 mm. In the fractional factorial designs, a Lichrospher 100 column (Macherey–Nagel, Düren, Germany) packed with material from Merck (Darmstadt, Germany) was used. Besides this column, a Lichrospher 100 (Merck; level -2), a CS-04 Kromasil 1C (Bischoff, Leonberg, Germany; level -1) and a Hypersil column (Grom, Ammerbuch, Germany; level 1) were examined in the

Table 7
Factors and levels chosen for the fractional factorial designs, B' and F' indicate the modification of the levels for the asymmetrical factorial designs

Factor	Description	−1	Nominal	+ 1
A	Column temperature	36°C	40°C	44°C
B	Flow rate	1.3 ml/min	1.5 ml/min	1.7 ml/min
C	Conc. phosphate (μ)	1.4 g/l	1.5 g/l	1.6 g/l
D	pH eluent A	2.8	3.0	3.2
E	Ratio eluents A:B	58:42	55:45	52:48
F	λ	227 nm	230 nm	233 nm
B′	Flow rate	1.4 ml/min	1.5 ml/min	1.6 ml/min
F′	λ	228 nm	230 nm	232 nm

asymmetrical factorial designs. The Macherey–Nagel (MN) column got level 2. The Hypersil column was a used one while the others were new.

The fractional factorial and the $4 \cdot 2^{12}$ factorial designs were performed on a Shimadzu series 10 instrument (Shimadzu, Kyoto, Japan). It consisted of two LC-10 AT pumps, a CTO-10A column oven, a SPD-10A UV spectrophotometer, an autosampler, a SCL-10A system controller, a Class VP Chromatography Data System version 4.2 and a Gastorr 102 degasser (Flom, Kyoto, Japan). In the $4 \cdot 3 \cdot 2^9$ design, this instrument was coded as 0. Furthermore, another Shimadzu instrument (coded as 1) and a HP (Hewlett-Packard, Waldbronn, Germany) instrument (coded as 1) were used. In the level (1) Shimadzu instrument, Series 6 and 10 instruments were combined: two LC-10 AS pumps, a CTO-10A column oven, a SPD-6A UV spectrophotometric detector, an autosampler, a SCL-10A system controller and a Class VP Chromatography data system, version 4.2. No degasser was connected. The HP instrument used two G1312A binary pumps, a G1322A degasser, a G1313A auto sampler, a G1316 column oven, a G1315A DAD detector and a HP1100 3D data system.

3.4. Reagents and solutions

Analytical grade sodium dihydrogenphosphate (Merck) and milli-Q-purified water (Millipore, Bedford, MA) were used for the preparation of the phosphate buffer; the pH of the phosphate buffer was adjusted with ortho-H_3PO_4 (85%) p.a. plus (Riedel–de Haën, Seelze, Germany). A Knick pH-meter, type 647 (Knick, Berlin, Germany) was calibrated with buffer-solutions at pH 7 and 4. The organic modifier was acetonitrile Chromasolv G (Riedel–de Haën), which was also used to prepare sample and calibration solutions. Both sample and calibration substance were available in the laboratory of Bayer (Dormagen, Germany). Dissolution of the samples was achieved by 15 min treatment in a Branson 8200 ultrasonic bath (Branson Ultrasonics B.V., Soest, The Netherlands). A stream of helium was used to degass the eluents.

3.5. Sequence of measurements

In the fractional factorial designs, the experiments were executed in a random order. In the $4 \cdot 2^{12}$ design, the experiments were blocked according to the columns. Within such a block, the order was random. In the $4 \cdot 3 \cdot 2^9$ design, randomization was applied within the different instruments and columns.

Both the calibration and the sample measurements are based on the average result of two injections of the same solution from individual vials.

3.6. Responses studied

The responses studied were the total content of triadimenol, the total peak area and the peak resolution.

The total content (C_{Sa}) of triadimenol is calculated by

$$C_{Sa} = \frac{A_{Sa} \cdot C_S}{A_S} \quad (7)$$

where A_{Sa} and A_S are the sums of the areas of the diastereomers triadimenol *A* and *B* (see Fig. 2) from the sample and the standard, respectively, and C_S is the total content of triadimenol *A* and *B* in the calibration standard.

The resolution was calculated as:

$$R = 1.17 \cdot (t_B - t_A)/(W_A + W_B) \quad (8)$$

with t_B and t_A the retention times for triadimenol *B* and triadimenol *A*, respectively, and W_i the peak width at half-height for the corresponding peak.

4. Results and discussion

Triadimenol consists of four isomers. Two pairs of diastereomers, labeled as *A* and *B*, respectively, show different retention, as can be seen from the chromatogram shown in Fig. 2. Measurement at nominal conditions does not lead to a baseline separation of these two peaks, except on the Bischoff column. The protocol prescribes the use of the sums of the peak areas for the determination of the content.

The results of the 2^{6-3} fractional factorial design are given in Table 1, while Table 2 summarises those of the 2^{6-2} design. The results from the asymmetrical designs are shown in Tables 4 and 5.

In the $4 \cdot 2^{12}$ design four chromatographic columns allow deriving six linear-dependent column effects

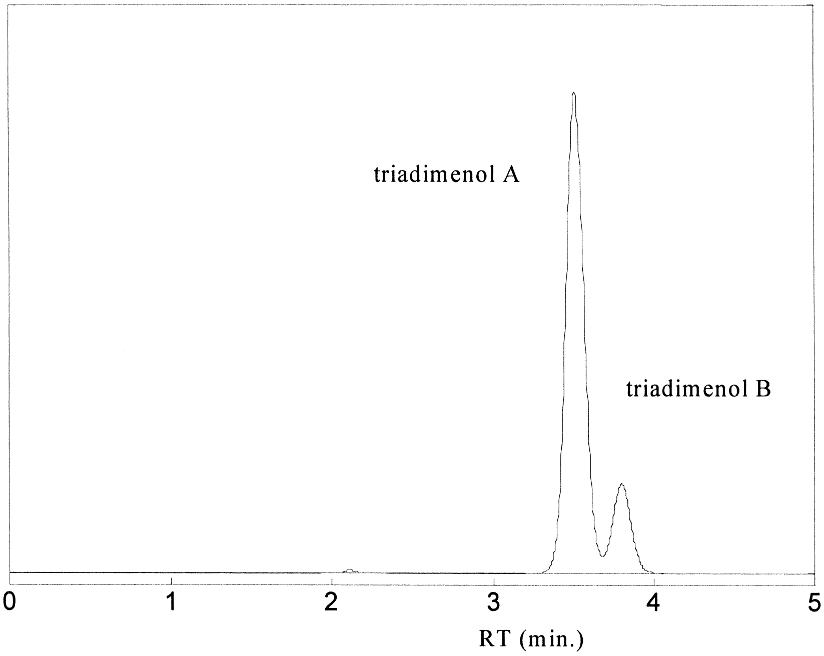

Fig. 2. Chromatogram of triadimenol at nominal conditions.

(C_1–C_6, Table 8). Experiment 10 of Table 4 resulted in a value for content that is lower than the results of the other experiments. According to the method of Goupy [24] however, it is not considered as an outlier. The three instruments considered in the $4 \cdot 3 \cdot 2^9$ design allow calculating three linear-dependent instrument effects (I_1–I_3, Table 8).

The effects calculated from the different designs are summarised for the content, peak area and the resolution in Tables 9, 10 and 11, respectively. The corresponding critical effects and their number of degrees of freedom are shown as well. They were calculated with *t*-values for a one-sided confidence level of 95%. The half-normal plots for the responses content, peak area and resolution for all designs are shown in Figs. 3–5, respectively. The plots show the effects as a function of the *z*-values, which correspond to the relative cumulative probabilities. Significant and non-significant effects were distinguished by visual inspection of the graphs. The regression lines shown are based on the effects considered non-significant. Since the effects observed for factors *B* and *F* on peak area are very large in all three designs considered (Table 10), the corresponding half-normal plot zooms in on the remaining effects (Fig. 4).

To facilitate the comparison between the designs, the significance of the effects in the respective designs is also indicated in Tables 9–11. The letter ‘‘a’’ behind an effect denotes that it is deviating from the line of non-significant effects in the half-normal plot (visually identified significant). The significance of an effect relative to the critical effect derived from the negligible factor effects is marked with ‘‘b’’. An effect exceeding the criterion based on the distribution of the effects [14,15] was identified by ‘‘c’’.

Table 8
Confounding of two-factor interactions with column effects (in both asymmetrical designs) or with instrumental effects ($4 \cdot 3 \cdot 2^9$ design only) (+: confounding, −: no confounding)

	AE	*BF*	*CD*	*AD*	*BE*	*CF*
Merck–Bischoff (C_1)	+	−	+	−	−	−
Merck–Grom (C_2)	+	+	−	−	−	−
Merck–MN (C_3)	−	+	+	−	−	−
Bischoff–Grom (C_4)	−	+	+	−	−	−
Bischoff–MN (C_5)	+	+	−	−	−	−
Grom–MN (C_6)	+	−	+	−	−	−
−1–0 (I_1)	−	−	−	+	−	−
−1–1(I_2)	−	−	−	−	+	+
0–1 (I_3)	−	−	−	+	−	−

Table 9
Comparison of the estimated effects on the response content

	Fractional factorial designs				Asymmetrical designs			
	2^{6-3}		2^{6-2}		$4\cdot2^{12}$		$4\cdot3\cdot2^{9}$	
	Effect	Significance	Effect	Significance	Effect	Significance	Effect	Significance
I_1 Instrument							−0.67	
I_2 effects							−0.58	
I_3							0.09	
C_1					−0.29		−0.13	
C_2					0.42		1.24	a,b,c
C_3 Column					−0.20		0.99	a,b,c
C_4 effects					0.71	a,c	1.37	a,b,c
C_5					0.09		1.11	a,b,c
C_6					−0.62	a,c	−0.26	
A (Temp)	0.11		−0.13		−0.17		0.24	
B (Flow)	0.03		−0.05		−0.07		−0.34	
C (μ)	0.17		0.07		−0.19		−0.08	
D (pH)	0.03		0.01		0.10		0.50	
E (%*B*)	−0.07		0.02		0.28		0.31	
F (λ)	−0.58	a,c	−0.52	a,b,c	0.12		0.22	
AB			$-0.02^{(2)}$					
AC			$0.10^{(3)}$					
AD			$-0.09^{(4)}$		0.12			
AE			$-0.10^{(5)}$					
AF	$0.21^{(1)}$		$0.24^{(6)}$	c	−0.07		−0.10	
BC			$0.06^{(7)}$					
BD			$-0.23^{(8)}$	c	0.20		0.08	
BE	$0.21^{(1)}$		$0.24^{(6)}$	c	1.13	a,b,c		
BE			$-0.10^{(5)}$					
CD	$0.21^{(1)}$		$0.24^{(6)}$	c				
CE			$-0.23^{(8)}$	c	−0.36		0.56	
CF			$-0.09^{(4)}$		−0.41			
DE			$0.06^{(7)}$					
DF			$0.10^{(3)}$					
EF			$-0.02^{(2)}$					
d.f. (negl. eff.)		1		7		6		3
E_{crit} (negl. eff.)		1.31		0.27		1.01		0.78
d.f. (distrib.)		6		12		17		16
E_{crit} (distrib.)		0.24		0.21		0.55		0.87
$(SE)_{est}$ (negl. eff.)		0.21		0.14		0.52		0.33
s_1 (distrib.)		0.12		0.12		0.32		0.50
b_i (half-n. plot)		0.17		0.14		0.34		0.56

a: Significant effects from the half-normal plot.
b: Significant effects from comparison with the critical effect from negligible effects.
c: Significant effects from the effects' distribution; (1), (2), (3), (4), (5), (6), (7) and (8): interaction effects confounded with each other within a given design. Example: $AF+BE+CD$ are confounded in the 2^{6-3} and, among others, $AB+EF$ are confounded in the 2^{6-2} fractional factorial design.

Furthermore, the estimated standard error s_1 calculated in the latter method as well as $(SE)_{est}$, derived from the negligible factor effects, and b_i, the slopes of the half-normal plots, which are also estimates of the standard deviation, are shown. In the $4\cdot2^{12}$ asymmetrical design, the dummy factor effects are confounded with the interactions *AD*, *AF*, *BD*, *BE*, *CE* and *CF*, whereas *AF*, *BD* and *CE* are aliased

Table 10
Comparison of the estimated effects on the response peak area

	Fractional factorial designs				Asymmetrical designs	
	2^{6-3}		2^{6-2}		$4\cdot2^{12}$	
	Effect	Significance	Effect	Significance	Effect	Significance
C_1 Column effects					0.033	b
C_2					0.100	a,b,c
C_3					0.051	b
C_4					0.067	b,c
C_5					0.018	b
C_6					−0.049	b
A (Temp)	−0.031		−0.031	c	0.003	
B (Flow)	−0.624	a,b,c	−0.617	a,b,c	−0.268	a,b,c
C (μ)	−0.204	a,b,c	0.005		−0.010	
D (pH)	−0.026		−0.002		−0.024	b
E (%*B*)	−0.067	b	−0.075	a, (b)*, c	−0.065	b,c
F (λ)	−1.568	a,b,c	−1.570	a,b,c	−1.116	a,b,c
AB			0.024(2)			
AC			−0.008(3)			
AD			0.007(4)		−0.002	
AE			0.209(5)			
AF	$0.010^{(1)}$		0.011(6)		−0.007	
BC			−0.001(7)			
BD			0.001(8)		−0.005	
BE	$0.010^{(1)}$		0.011(6)		−0.009	
BF			0.209(5)	a,b,c		
CD	$0.010^{(1)}$		0.011(6)			
CE			0.001(8)		0.012	
CF			0.007(4)		0.011	
DE			−0.001(7)			
DF			−0.008(3)			
EF			0.024(2)			
d.f. (negl. eff.)		1	$6^{(\bullet)}$	7		6
E_{crit} (negl. eff.)		0.063	$0.021^{(\bullet)}$	0.151		0.017
d.f. (distrib.)	$4^{(+)}$	5		9		15
E_{crit} (distrib.)	$0.084^{(+)}$	0.197		0.026		0.058
$(SE)_{est}$ (negl. eff.)		0.010	$0.012^{(\bullet)}$	0.080		0.008
s_1 (distrib.)	$0.040^{(+)}$	0.098		0.014		0.033
b_i (half-n. plot)		0.092		0.027		0.048

a: Significant effects from the half-normal plot.
b: Significant effects from comparison with the critical effect from negligible effects.
c: Significant effects from the effects' distribution; (1), (2), (3), (4), (5), (6), (7) and (8): interaction effects confounded with each other within a given design (see Table 9); (b)*: Significantly larger than the critical effect derived from the interaction effects omitting interaction *BF*; (●): without *BF*; (+): without *C* + *AE* + *BF*.

with the dummy factors in the $4\cdot3\cdot2^9$ asymmetrical design. The dummy factor effects were therefore indicated in the corresponding two-factor interaction cells in Tables 9–11. Empty cells indicate that the corresponding two-factor interactions are confounded with main effects or that the main effects could not be estimated.

4.1. Statistical evaluation

For the response **content**, the critical effects are quite different for the different designs. In general, the limits are higher in the asymmetrical than in the fractional factorial designs. This coincides with the steeper slopes for the asymmetrical designs in the

Table 11
Comparison of the estimated effects on the response resolution

		Fractional factorial designs				Asymmetrical designs			
		2^{6-3}		2^{6-2}		$4\cdot2^{12}$		$4\cdot3\cdot2^{9}$	
		Effect	Significance	Effect	Significance	Effect	Significance	Effect	Significance
I_1	Instrument							0.082	
I_2	effects							−0.028	
I_3								−0.110	
C_1						−0.391	a,b,c	−0.384	a,b,c
C_2						0.109	b,c	0.077	
C_3	Column					0.052		0.024	
C_4	effects					0.500	a,b,c	0.461	a,b,c
C_5						0.443	a,b,c	0.408	a,b,c
C_6						−0.057		−0.053	
A (Temp)		−0.109	a,b,(c)*	−0.111	a,b,c	−0.124	b,c	−0.118	b,c
B (Flow)		−0.039	b	−0.053	b,c	0.018		0.013	
C (μ)		−0.043	b	−0.022		0.019		0.018	
D (pH)		0.029		0.019		0.038		0.045	
E (%*B*)		−0.460	a,b,c	−0.428	a,b,c	−0.472	a,b,c	−0.500	a,b,c
F (λ)		−0.046	b	−0.041	b	−0.034		−0.036	
AB				$-0.010^{(2)}$					
AC				$0.032^{(3)}$	b				
AD				$-0.014^{(4)}$		−0.025			
AE				$0.021^{(5)}$					
AF		$0.032^{(1)}$		$0.003^{(6)}$		0.017		0.044	
BC				$0.005^{(7)}$					
BD				$-0.002^{(8)}$		0.067		0.068	
BE		$0.032^{(1)}$		$0.003^{(6)}$		0.035			
BE				$0.021^{(5)}$					
CD		$0.032^{(1)}$		$0.003^{(6)}$					
CE				$-0.002^{(8)}$		0.027		−0.006	
CF				$-0.014^{(4)}$		−0.017			
DE				$0.005^{(7)}$					
DF				$0.032^{(3)}$	b				
EF				$-0.010^{(2)}$					
d.f. (negl. eff.)			1		7		6		3
E_{crit} (negl. eff.)			0.032		0.030		0.069		0.110
d.f. (distrib.)		$5^{(+)}$	6		11		14		14
E_{crit} (distrib.)		$0.074^{(+)}$	0.116		0.046		0.099		0.108
$(SE)_{est}$ (negl. eff.)			0.005		0.016		0.036		0.047
s_1 (distrib.)		$0.037^{(+)}$	0.056		0.026		0.056		0.062
b_i (half-n. plot)			0.074		0.036		0.090		0.097

a: Significant effects from the half-normal plot.
b: Significant effects from comparison with the critical effect from negligible effects.
c: Significant effects from the effects' distribution; (1), (2), (3), (4), (5), (6), (7) and (8): interaction effects confounded with each other within a given design (see Table 9); (c)*: Significantly larger than the critical effect derived from the effect-distribution omitting factor A; (+): without A.

half-normal plots (Fig. 3). These differences are not illogical since the variations introduced in the asymmetrical designs are more diverse than those in the fractional factorial ones. By examining several columns and instruments, one already has a situation simulating somewhat reproducibility. Superposed on these conditions, variations in the procedure-related factors are introduced. In contrast, in the fractional factorial designs, only the latter is done. Therefore, larger experimental error could be expected in the

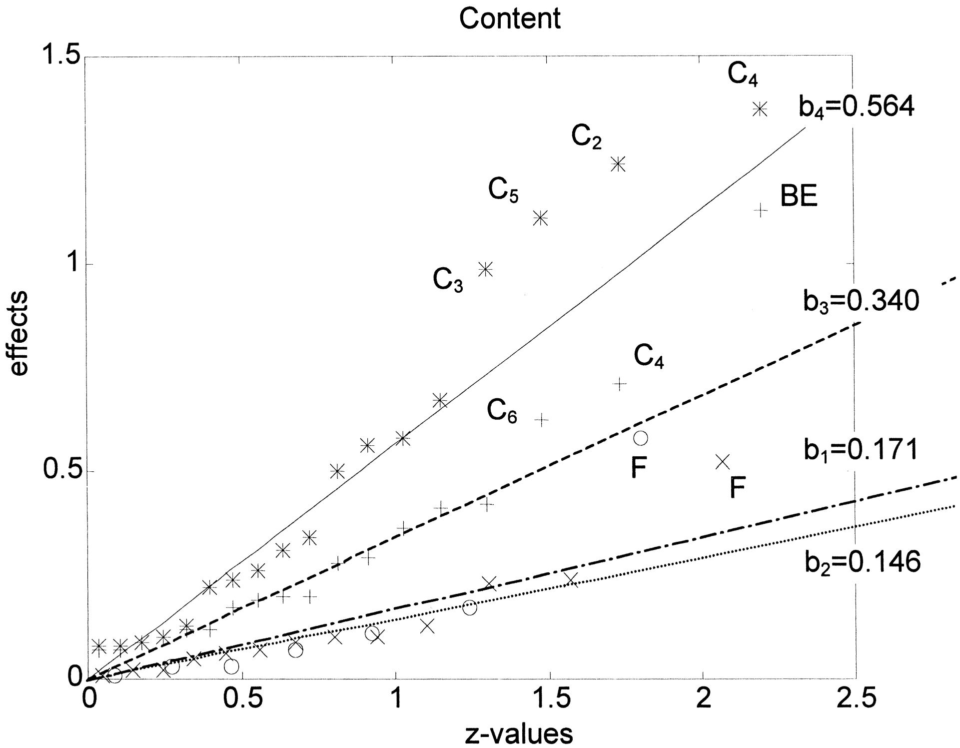

Fig. 3. Half-normal plots for **content** (○: 2^{6-3} fractional factorial design (slope b_1) ×: 2^{6-2} fractional factorial design (slope b_2), +: $4\cdot2^{12}$ design (slope b_3), *: $4\cdot3\cdot2^{9}$ design (slope b_4)).

asymmetrical designs, as was observed here. In all designs, the s_1 according to Dong [14] shows a good agreement with the b_i-values of the half-normal plot (Table 9). Both are smaller than $(SE)_{est}$ derived from the negligible factor effects except in the $4\cdot3\cdot2^9$ design. These differences can be explained as follows. $(SE)_{est}$ is estimated with much less degrees of freedom than s_1 and b_i. Moreover, the effects used to calculate $(SE)_{est}$ could on average be relatively high compared to those used to estimate s_1 and b_i, since one has already defined beforehand which ones will be used to estimate $(SE)_{est}$ without considering their magnitude. Some relatively large effects could have been excluded in the estimation of s_1 and b_i, but used in $(SE)_{est}$, leading to higher $(SE)_{est}$-values. On the other hand, the situation in which the effects to estimate $(SE)_{est}$ are a subset of the ones used to calculate s_1 and b_i, is also possible. This would then lead to smaller estimates for $(SE)_{est}$.

The factor F (detection wavelength) shows the largest and similar effects in both fractional factorial designs. This factor is always significant except for using the criterion with the negligible effects in the 2^{6-3} design since there the number of degrees of freedom to estimate E_{crit} is too low. The interval between the factor levels for F is reduced in the asymmetrical factorial designs (Table 7), and the effects decreased as expected. Non-significant effects for F result. In both asymmetrical factorial designs, the column effect C_4 deviates from the line of normality in the half-normal plot and exceeds at least the critical effect derived from the effect-distribution and can be considered significant, even though the magnitude of the effects is considerably different in both designs. Larger effects for the columns might be expected in the $4\cdot3\cdot2^9$ asymmetrical design given the more diverse situations under which they are tested. This was indeed observed and lead to a total of four significant column effects. In the $4\cdot2^{12}$ design, the two-factor interaction BE results in the largest effect observed, although no physical explanation for it can be given. When a large number of

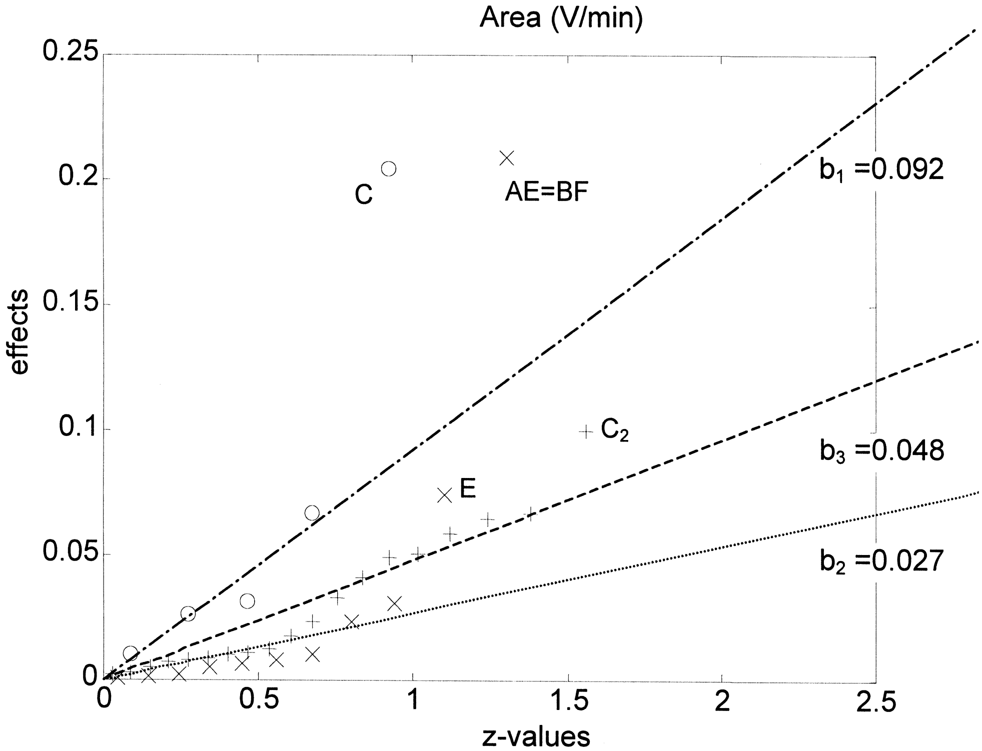

Fig. 4. Zoomed-in half-normal plots for **peak area**; effects B and F are omitted in all designs (○: 2^{6-3} fractional factorial design (slope b_1) ×: 2^{6-2} fractional factorial design (slope b_2) +: $4 \cdot 2^{12}$ design (slope b_3)).

effects are considered, one always has the possibility that a non-significant one will show a large value and will be considered significant, even though it is not (α-error).

As for the content, the critical effects for the **peak area** are quite different (Table 10).

The E_{crit} from the negligible factors is high in the 2^{6-2} design, while E_{crit} from the effect-distribution shows a high value in the 2^{6-3} design. To estimate E_{crit} from two-factor interactions, it is a priori assumed that these effects are negligible. The result for the interaction $BF + AE$ in the 2^{6-2} design shows that this condition is not always fulfilled (see below). Occasional significant interaction and dummy effects should be excluded from the estimation of the experimental error, which can be done by looking at the half-normal plots [4]. Such an interpretation leads to the exclusion of the interaction $BF + AE$ in the E_{crit} (negl. eff.) estimation in the 2^{6-2} design. Omitting this value in the calculation reduces the estimate of the standard error from 0.080 to 0.012, which corresponds to s_1 in this design and to $(SE)_{est}$ in the other designs. In the method according to Dong [14], which estimates the significant effects from their own distribution, the use of the median should prevent from considering significant effects in the estimation of s_1. Nevertheless, due to the small number of effects considered, the large effect $C + AE + BF$ is included in this estimation in the 2^{6-3} design leading to a large E_{crit} (distrib.). Setting the limit for the estimation to for instance $2s_0$ instead of $2.5s_0$ would exclude this factor from the calculation. Such a reduction decreases the risk of including significant effects in the estimation of s_1, but of course increases the risk that some of the non-significant effects might also be eliminated. A comparably large value for the error-estimate b_i as for s_1 is obtained from the half-normal plot even though the effect $C + AE + BF$ was excluded in the regression.

In both fractional factorial designs, the factors F (detection wavelength) and B (flow-rate) show the

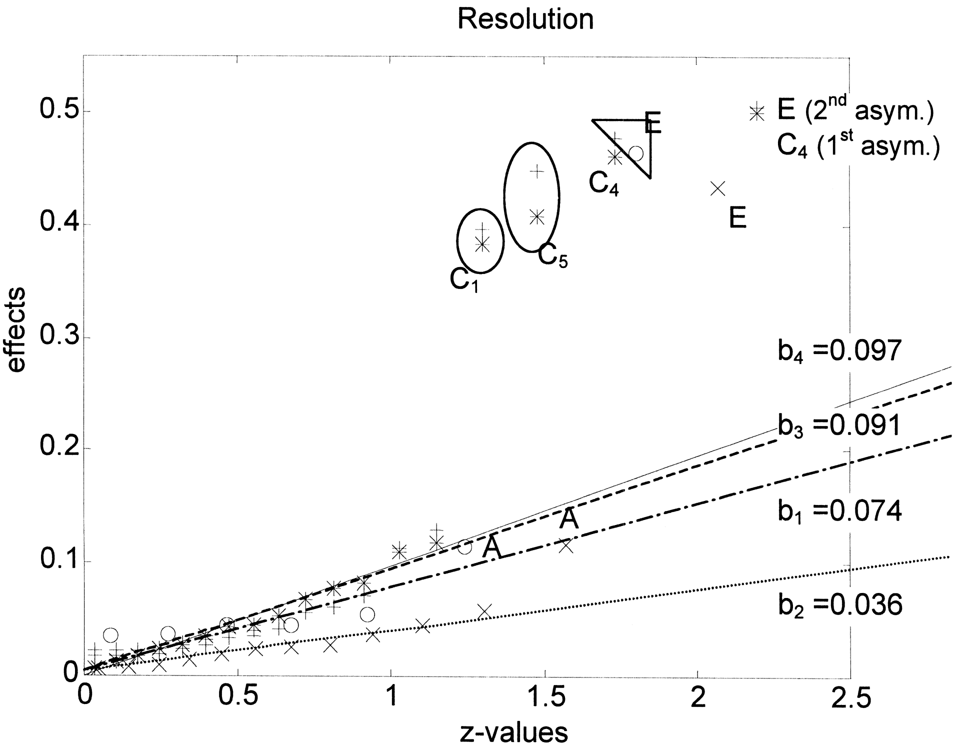

Fig. 5. Half-normal plots for **peak resolution** (○: 2^{6-3} fractional factorial design (slope b_1), ×: 2^{6-2} fractional factorial design (slope b_2), +: $4\cdot2^{12}$ design (slope b_3), *: $4\cdot3\cdot2^{9}$ design (slope b_4)).

largest effects. For each individual factor, they are similar in magnitude and significant. In the 2^{6-3} fractional factorial design, the main effect *C* (ionic strength) is also significant (Fig. 4, Table 10). In contrast, in the 2^{6-2} fractional factorial design, it is clearly non-significant. Instead, the confounded interaction-effect $AF+BF$ and the main effect *E* (%*B*) deviate from the line of non-significant effects. The confounding pattern of the 2^{6-3} fractional factorial design reveals that factor *C* is confounded with the interactions *AE* and *BF*. Since the main effects *F* and *B* are clearly significant, one can assume that their interaction *BF* causes the significance of the effect $C+AE+BF$ in the 2^{6-3} design. This is confirmed by the results of the 2^{6-2} design. In the 2^{6-3} design, the effect of factor *E* (percentage of organic modifier in the eluent) does not exceed the E_{crit} (distrib.). The latter is probably overestimated since $C+AE+BF$ is included, so that factor *E* might be considered significant.

Because of the large interaction $BF+AE$, it was decided to reduce the intervals between the factor levels for factors *B* and *F* in the asymmetrical designs. As can be seen from Table 7, for factor *B*, it was reduced by 50% and for factor *F* by one third. The comparison of the corresponding effects between the fractional factorial and the asymmetrical design reflects the reduction of the intervals. The column effect C_2 exceeds all critical limits in the asymmetrical factorial design. However, in this design the significance of effect C_2 has to be questioned since it is confounded with the interaction *BF*. In Table 8, it is indicated which interactions are confounded with the column effects (and also with the instrument effects). Due to this confounding, no estimation for these interactions can be made. Because of the significance of *BF* in the fractional factorial designs, the column effects confounded with *BF* have to be interpreted with care.

For **peak resolution**, the critical effects are again very different. The critical effects derived from the negligible effects are smaller in the fractional factorial designs than in the asymmetrical ones, as was also observed for the content. The same tendency can be seen among the error estimates s_1 and b_i. In the 2^{6-3} design, this is superposed by a further effect: the method according to Dong [14] includes factor A in the estimation of the standard error, so that a larger E_{crit} (distrib.) results. This confirms – as already seen from peak area – that the method of Dong can provide problems if the total number of factor effects is relatively small.

The factors E (%B) and A (column temperature) lead to comparable effects in all designs for the response peak resolution. While E is clearly significant using any method, the decision on the significance of A is not straightforward. Taking into account that E_{crit} (distrib.) is probably overestimated in the 2^{6-3} design, this factor might be significant.

The same column effects are significant in both asymmetrical designs; they also show comparable magnitude: $C_4 > C_5 > C_1$.

The main effects B (flow-rate) and C (ionic strength) as well as the interaction effect $AC + DF$ slightly exceed some critical effects in at least one of the fractional factorial designs. In contrast, they are non-significant in the half-normal plots. This is confirmed in the asymmetrical designs, so that they are probably non-significant.

4.2. Chromatographic interpretation

In the fractional factorial designs, one clearly significant effect is observed on the determination of the **content**, namely from the detection wavelength (Table 9). Since the influence of the detection wavelength on both diastereomers as well as on the calibration and sample measurements should be comparable, a non-significant effect is to be expected. The operation procedure prescribes the measurements in a spectral region with a steep slope (Fig. 6). The spectral behaviour observed for both

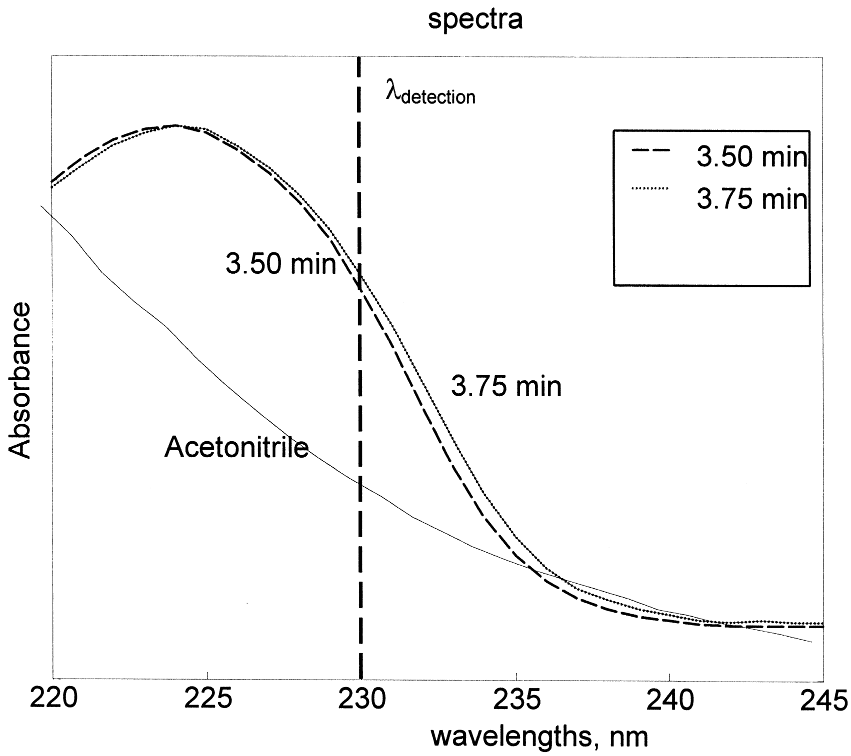

Fig. 6. DAD-spectra recorded at 3.50 and 3.75 min, and spectrum of the solvent, acetonitrile. Remark: spectra are recorded at different absorbance scales. Spectra of triadimenol A and B were scaled to have equal $A_{\lambda_{max}}$ values.

isomers seems similar but not identical. However, calculation of the first derivatives of the spectra indicates a wavelength shift of 0.2 to 0.3 nm, which cannot be considered larger than the uncertainty in the wavelength. The steep slope in the measurement region together with the wavelength uncertainty might cause the significant effect of wavelength. Reduction of the interval of the factor levels, as was done in the asymmetrical designs, reduces the observed effect on the content determination to a non-significant value. The above at least indicates that the prescribed detection wavelength of the method was not optimal.

The column selected can also significantly influence the determination of the content as can be seen in Table 9. This could, in addition to the detector uncertainty discussed above partly be explained by the differences in resolution (Table 11) observed on the different columns. The $4\cdot3\cdot2^9$ design revealed that the use of different instruments does not affect the performance of the method.

For the response **peak area**, two main effects are clearly significant: the detection wavelength and the flow-rate of the eluent. The large negative effect of the detection wavelength could be expected from the steep ascent in the spectra of the triadimenol diastereomers (see Fig. 6). A higher flow-rate reduces the remaining time of the analyte in the detection cell. The peak width is therefore decreased while the peak height remains unaffected. As a consequence, a smaller peak area results [25].

A chromatographic interpretation of the effect of factor E (%B) is not evident. It could again, to a certain extent, be related to differences in resolution combined with the spectral uncertainty.

The chromatographic reason for the observed column effects again could be searched in the differences in resolution observed on certain columns, as was also the case for factor E (%B).

A large agreement can be found between the designs for the response **peak resolution**. The portion of organic modifier always leads to large effects. A higher percentage of acetonitrile increases the solvent strength of the mobile phase. This causes faster elution and a lower peak resolution.

A higher column temperature (factor A) reduces the solvent viscosity. This leads to an increase in solute diffusion and thus to a reduction in retention. Therefore, a higher column temperature can lead to a worse peak resolution.

As to the column effects, the asymmetrical designs show three clearly significant effects (C_4, C_5 and C_1). The Bischoff Kromasil column always yields a significantly better resolution than the other columns. It leads to the most symmetrical peaks and to the best resolution too. The fact that the Hypersil column was already used before the robustness tests probably explains its poor performance. The number of theoretical plates (3100) measured for this column in the $4\cdot2^{12}$ design is considerably lower than the ones of the other columns (e.g. 4200 for Merck).

4.3. Benefits and drawbacks of the different designs

The low resolution of the $\mathbf{2^{6-3}}$ **fractional factorial design** can complicate the evaluation of main effects if large interaction effects (e.g. BF) occur. However, such large interactions are usually not expected for the most important response, the determination of the content. The main drawback of this design is that it has only one interaction effect that can be calculated besides the main effects, which gives a weak statistical base for the decision on significant effects. Accordingly, the critical effects from this effect are usually less sensitive than the ones in the fractional factorial designs with higher resolution.

Due to the small number of effects in the 2^{6-3} design, the critical effect (E_{crit} (distrib.)) can be overestimated in the method according to Dong if significant effects are included in the estimation of the standard error, as was shown for the responses peak area and peak resolution.

The **fractional factorial design** $\mathbf{2^{6-2}}$ showed none of theses disadvantages, it requires, however, the double amount of experiments. The half-normal plot should be used to exclude potentially significant interaction effects from the error-estimation.

The fact that for some factors (detection wavelength and flow-rate) the interval between the levels considered has been decreased in the asymmetrical design has to be taken into consideration while comparing the fractional factorial and the **asymmetrical designs**, since this largely influences the significance of some effects.

Nevertheless, for the effects other than the detection wavelength and flow-rate, the results are rather comparable between both types of designs.

The $4 \cdot 3 \cdot 2^9$ design revealed that the difference between the instruments is not significant. Moreover, it confirmed the results of the $4 \cdot 2^{12}$ design to a large degree. This shows that the consideration of factors at more than two levels can indeed provide useful information.

The asymmetrical designs have resolution III, so that the confounding between the two-factor interactions and main effects can cause similar problems as in the 2^{6-3} design (see, e.g. the confounding between column effects and *BF*).

5. Conclusion

The fractional factorial design with resolution III can be applied in robustness tests if one can exclude that large interaction effects are observed and if only procedure-related factors are of interest.

Since the fractional factorial designs with resolution IV and the asymmetrical designs considered require the same number of experiments, it seems favourable to screen procedure-related factors at two levels and non-procedure-related factors at more than two levels simultaneously. For the method examined, it is experienced that the non-procedure-related effects could indeed also affect the results. The low resolution of the asymmetrical designs (III) can lead to problems in the interpretation if large interaction effects occur.

Simultaneous consideration of half-normal plots and critical effects facilitates the decision on the significance of effects. Nevertheless, a thorough consideration of chromatographic aspects in the interpretation of the results is recommended.

From the results of the different designs, it follows that the method evaluated cannot really be considered robust. The detection wavelength chosen is not optimal. Detection at λ_{max} (224 nm) seems also to be preferable, since at that wavelength the slope of the extinction curve ($d\varepsilon/d\lambda$) of triadimenol (both diastereomers) is considerably lower than at the wavelength prescribed (see Fig. 6). No problems have to be expected because of the higher absorption of acetonitrile at this lower wavelength, because the method is isocratic.

The asymmetrical designs showed that the use of different columns can significantly influence the robustness of the method. Therefore, it is recommendable to establish system suitability (SST) criteria, e.g. for the resolution.

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