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Review

Experimental designs and their recent advances in set-up, data interpretation, and analytical applications

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ABSTRACT

In this review, the set-up and data interpretation of experimental designs (screening, response surface, and mixture designs) are discussed. Advanced set-ups considered are the application of D-optimal and supersaturated designs as screening designs. Advanced data interpretation approaches discussed are an adaptation of the algorithm of Dong and the estimation of factor effects from supersaturated design results. Finally, some analytical applications in separation science, on the one hand, and formulation-, product-, or process optimization, on the other, are discussed.

Contents

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1. Introduction

Optimization strategies are procedures followed when attempting to optimize, for instance, a formulation, product, process, or an analytical method, e.g. a chromatographic method to separate components in a given matrix. In an optimization, one tries to find the optimal settings or conditions for a number of factors. Factors are parameters than can be set and reset at given levels, e.g. temperature, pH, reagens concentration, reaction time, etc., and that affect the responses or the outcome of a method or procedure. The factors and their level ranges form the experimental domain within which one tries to find the global optimum, i.e. the overall best conditions. Factors also might 'interact', for instance, a two-factor interaction occurs when the influence of one factor on the response is different at different levels of the second factor.

In case only one factor needs to be optimized, a simple univariate procedure is performed. However, usually two or more factors are studied. This can be done using either univariate or multivariate optimization strategies [1,2]. The different strategies are represented in Fig. 1.

A classically applied *univariate* procedure is the one-variable-at-a-time (OVAT) approach, where only one factor at a time is varied and optimized. The OVAT procedure, however, has some disadvantages, i.e. interactions between factors are not taken into account, many experiments are needed when the number of factors increases, only a small part of the experimental domain is examined, the global optimum might not be found, and the found optimal conditions might depend on the starting conditions [1,2].

On the other hand, a multivariate approach varies several factors simultaneously. *Multivariate* approaches are subdivided into sequential and simultaneous ones [1,2]. Sequential procedures conduct a few initial experiments and use their results to define the following experiment(s) [3]. Sequential approaches can be applied when the experimental domain containing the optimum is a priori unknown, but are limited to the optimization of only one response. Simultaneous procedures perform a predefined number of experiments, according to a well-defined experimental set-up, e.g. an experimental design [1,2,4–6].

An experimental design is an experimental set-up to simultaneously evaluate several factors at given numbers of levels in a predefined number of experiments. Roughly, experimental designs can be divided into screening designs (e.g. full factorial, fractional factorial, and Plackett–Burman designs), response surface designs and mixture designs. Screening designs allow screening a relatively large number of factors in a relatively small number of experiments. They are used to identify the most influencing factors. Typically, the factors are evaluated at two levels in these designs. Response surface designs are used to find the optimal levels of the most important factors (which occasionally are selected from a screening design approach). In these designs, factors are examined at least

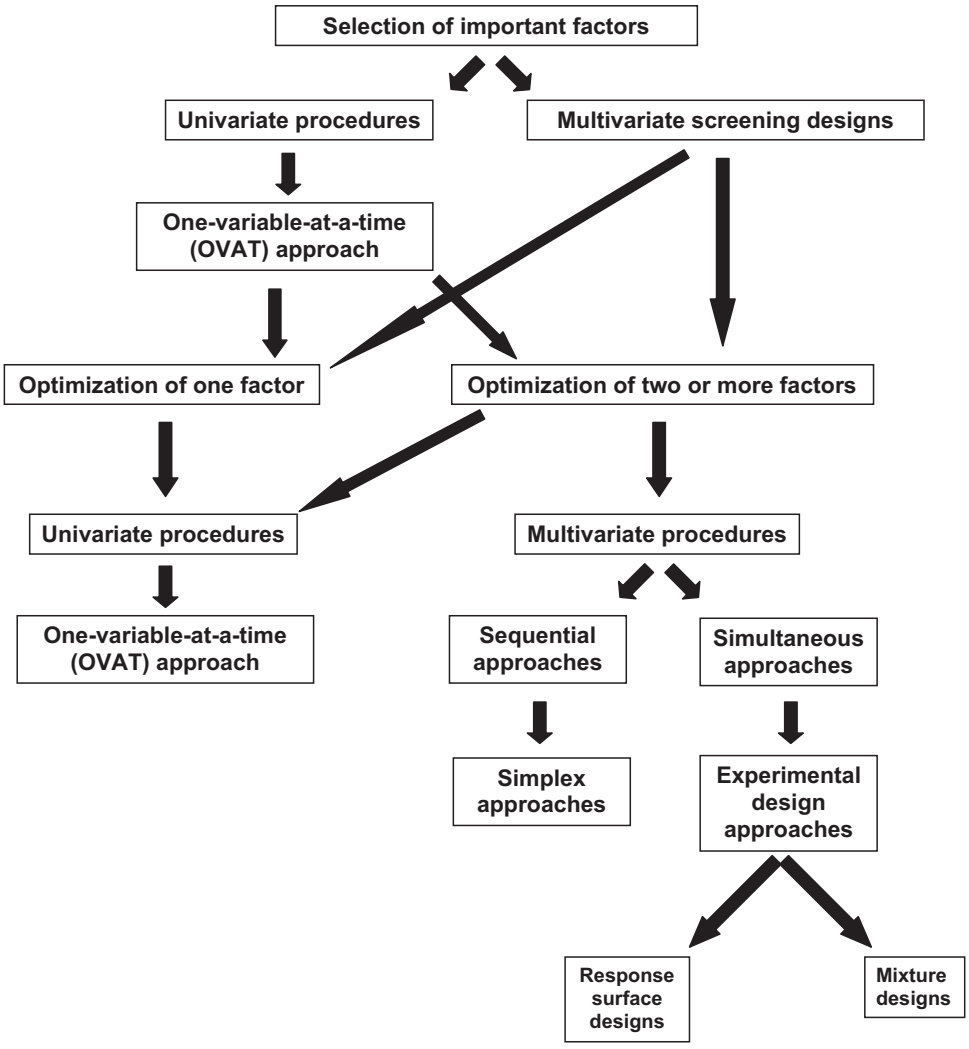

Fig. 1. Optimization strategies.

at three levels. The optimal conditions are usually derived from response surfaces build with the design results. Mixture designs are response surface designs used when all factors examined are mixture-related, i.e. factors representing the fraction of a given component in a mixture. Examples of such factors are the organic modifiers in a mobile phase in chromatography, or the excipients in a tablet or another pharmaceutical formulation. Which designs finally are applied depends on the number and type of factors to be examined, on the purpose of their use, and on the preference of the analyst.

Experimental designs are applied in many different fields and sciences. Within this paper, two application areas from pharmaceutical sciences will be considered, i.e. the optimization of separation methods, on the one hand, and the optimization of formulations, products, or processes, on the other.

Our first application area is thus the use of experimental designs in separation science. To develop an analytical assay to separate and quantify components in a given matrix, different steps are distinguished, i.e. the selection of the technique, the optimization of the method, and its validation [2,4,5]. The *technique selection* is mainly based on the properties of the components to be analyzed and on its availability in the development laboratory. As techniques to separate compounds in various matrices, high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and gas chromatography (GC) are frequently applied.

The *optimization of a method* is often split into a screening and an optimization phase. During the screening phase, all factors, potentially influencing the responses of interest, are tested in order to indicate those with the largest effects. These most important factors are then further explored in the optimization phase, where their best settings, i.e. the best conditions, are determined. In the above steps, screening and response surface designs, respectively, are applied. For the optimization of mixtures of solvents, e.g. the mobile phase composition, mixture designs are used [1,2].

After optimization, the *method* should be *validated*, i.e. evaluated whether it can be applied for its intended purpose(s). One of the method validation items is robustness testing, which evaluates the effects of small changes in the factors on the considered responses, and which applies screening designs for this purpose [7].

The second application area considered is the optimization of formulations, products, or processes, i.e. pharmaceutical manufacturing. Classically, batch processing with off-line testing of randomly collected samples of intermediate and/or end product(s) is performed in order to evaluate the quality of the product. Nowadays, industries are encouraged to implement a Process Analytical Technology (PAT) approach in their production processes, as proposed by a guideline of the Food and Drug Administration (FDA) [8]. By implementing PAT, the focus changes from off-line testing to real-time on-line testing of the intermediate and/or end product(s).

Also here, several experimental design approaches can be applied [9]. Screening designs are used to indicate the most important of all factors, potentially influencing the formulation, product, or process. Response surface designs are again applied to find the optimal factor settings, and mixture designs to optimize, for instance, the excipients composition in formulations.

In this review, advances in experimental set-up and their data interpretation are described. First, the classic screening, response surface, and mixture designs are discussed. Advances in experimental set-ups discussed are the use of supersaturated and D-optimal designs as screening designs. Advances in data interpretation considered are the adapted algorithm of Dong used to indicate significant effects in classic screening designs in cases when many significant factors are present, and a newly proposed method for the estimation and statistical evaluation of effects from a supersaturated design. Finally, some analytical applications are presented, either in the context of optimizing separation techniques, or of formulations, products, and processes.

2. Experimental set-up

2.1. Screening designs

Screening designs are used to indicate the most important factors from those potentially influencing the considered responses. They are applied in the context of optimizing separation techniques during screening and in robustness testing, and in the context of optimizing formulations, products, or processes. Most often, two-level screening designs, such as fractional factorial or Plackett–Burman designs, are used [2,4–7,9–11], which allow examining a relatively large number of factors f at $L = 2$ levels in a relatively small number of experiments ($N \geq f + 1$). When f is small, two-level full factorial designs might also be applied for screening purposes [2,4,5].

These designs allow the simultaneous examination of qualitative (changing on a discrete scale), quantitative (varying on a continuous scale), and mixture-related factors. For the latter, all but one component of a mixture maximally can be examined in one design.

2.1.1. Two-level full factorial designs

A two-level full factorial design contains all possible combinations between the f factors and their $L = 2$ levels, leading to $N = L^f = 2^f$ experiments to be performed (Table 1). These designs allow estimating all main (i.e. of the factors) and interaction effects between the considered factors [4–6,9]. The interaction effects are calculated from the columns of contrast coefficients (Table 1).

2.1.2. Two-level fractional factorial designs

A two-level fractional factorial $2^{f-\nu}$ (FF) design contains a fraction of the full factorial design, and allows examining f factors at two levels in $N = 2^{f-\nu}$ experiments, with $1/2^{\nu}$ representing the fraction of the full factorial ($\nu = 1, 2, 3, \ldots$) (Table 2) [4–6,9]. For FF designs, N is a power of two ($N = 8, 16, 32, \ldots$). Because only a fraction of a full factorial design is performed, some information is lost.

Table 1
(a) 2^3 two-level full factorial design for 3 factors, and (b) the columns of contrast coefficients for the interactions.

(a)

Experiment	Factors		
	A	*B*	*C*
1	−1	−1	−1
2	1	−1	−1
3	−1	1	−1
4	1	1	−1
5	−1	−1	1
6	1	−1	1
7	−1	1	1
8	1	1	1

(b)

Experiment	Contrast coefficients			
	AB	AC	BC	ABC
1	1	1	1	−1
2	−1	−1	1	1
3	−1	1	−1	1
4	1	−1	−1	−1
5	1	−1	−1	1
6	−1	1	−1	−1
7	−1	−1	1	−1
8	1	1	1	1

Table 2
2^{4-1} fractional factorial design for 4 factors, and the columns of contrast coefficients that still can be constructed. A = BCD, B = ACD, C = ABD, D = ABC, I_1 = AB + CD, I_2 = AC + BD, I_3 = AD + BC.

Experiment	Factors				Contrast coefficients		
	A	*B*	*C*	*D*	I_1	I_2	I_3
1	−1	−1	−1	−1	1	1	1
2	1	−1	−1	1	−1	−1	1
3	−1	1	−1	1	−1	1	−1
4	1	1	−1	−1	1	−1	−1
5	−1	−1	1	1	1	−1	−1
6	1	−1	1	−1	−1	1	−1
7	−1	1	1	−1	−1	−1	1
8	1	1	1	1	1	1	1

Table 3
Plackett–Burman design for 7 factors.

Experiment	Factors						
	A	*B*	*C*	*D*	*E*	*F*	*G*
1	1	1	1	−1	1	−1	−1
2	−1	1	1	1	−1	1	−1
3	−1	−1	1	1	1	−1	1
4	1	−1	−1	1	1	1	−1
5	−1	1	−1	−1	1	1	1
6	1	−1	1	−1	−1	1	1
7	1	1	−1	1	−1	−1	1
8	−1	−1	−1	−1	−1	−1	−1

Not all main and interaction effects can be estimated separately anymore. Some effects are confounded, meaning that they are estimated together (Table 2). For instance, in a half-fraction factorial design, each estimated effect is a confounding of two effects.

2.1.3. Two-level Plackett–Burman designs

A Plackett–Burman (PB) design allows examining maximally $f = N - 1$ factors in N experiments, where N is a multiple of four (N = 8, 12, 16, 20, ...) (Table 3) [4,5,12]. When f exceeds the number of real factors to be examined, the remaining columns of the PB design are defined as dummy factor columns, for which a change between the levels −1 and +1 has no physicochemical meaning.

2.1.4. Three-, more- or mixed-level screening designs

When it is expected that the effects between [−1,0] differ from those between [0,+1] (non-linear behavior of response as function of factor levels), it might be interesting to screen the factors at three levels (−1,0,+1), instead of only at two (−1,+1). This can be done using so-called reflected designs [13], which are in fact duplicated two-level designs. The two-level designs are executed once with the factor levels [−1,0], and once with [0,+1]. The reflected design examines f factors in $2N - 1$ experiments (Table 4).

Table 4
Reflected Plackett–Burman design to examine 7 factors at three levels.

Experiment	Factors						
	A	*B*	*C*	*D*	*E*	*F*	*G*
1	1	1	1	0	1	0	0
2	0	1	1	1	0	1	0
3	0	0	1	1	1	0	1
4	1	0	0	1	1	1	0
5	0	1	0	0	1	1	1
6	1	0	1	0	0	1	1
7	1	1	0	1	0	0	1
8	0	0	0	0	0	0	0
9	−1	−1	−1	0	−1	0	0
10	0	−1	−1	−1	0	−1	0
11	0	0	−1	−1	−1	0	−1
12	−1	0	0	−1	−1	−1	0
13	0	−1	0	0	−1	−1	−1
14	−1	0	−1	0	0	−1	−1
15	−1	−1	0	−1	0	0	−1

Besides the reflected designs, some other three-level screening designs are discussed in the literature [13]. In refs. [6,14–16], asymmetrical or mixed-level factorial designs (Table 5) were used to screen different factors at different numbers of levels. In ref. [14], Addelman described a procedure to construct more- or mixed-level screening designs.

2.1.5. D-optimal designs as screening designs

Besides using D-optimal designs as response surface designs (see Section 2.2), they can also be used in the context of screening [17]. However, their application as screening design in pharmaceutical analysis is not very frequent. Nevertheless, to examine a given number of factors these designs require less experiments than the higher discussed screening designs.

To construct a D-optimal design to examine f factors, first the type of model to be build, which requires minimal N_{min} experiments to enable estimating the model coefficients, is defined (see further, Eq. (1) for a screening design or Eq. (2) for a response surface design). Secondly, the number of experiments, N, to be performed is defined ($N \geq N_{min}$). The experimental domain is represented by a number of candidate experiments (N_{grid}) forming a grid over the domain. The N experiments of the D-optimal design are selected as that combination with the maximal determinant for $\mathbf{X^T X}$ (=D-optimality), with $\mathbf{X^T}$ the transpose of the model matrix $\mathbf{X}$.

Table 5
$4^1 2^{12}$ mixed-level asymmetrical design, constructed according to Addelman [14], to examine one factor at four levels and 12 factors at two levels in 16 experiments [16].

Experiment	Factors												
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*
1	−2	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1
2	−2	−1	1	1	−1	1	1	1	−1	1	1	1	−1
3	−2	1	−1	1	1	−1	1	1	1	−1	−1	1	1
4	−2	1	1	−1	1	1	−1	−1	1	1	1	−1	1
5	−1	−1	−1	−1	−1	1	1	−1	1	1	−1	1	1
6	−1	−1	1	1	−1	−1	−1	1	1	−1	1	−1	1
7	−1	1	−1	1	1	1	−1	1	−1	1	−1	−1	−1
8	−1	1	1	−1	1	−1	1	−1	−1	−1	1	1	−1
9	1	−1	−1	−1	1	−1	1	1	−1	1	1	−1	1
10	1	−1	1	1	1	1	−1	−1	−1	−1	−1	1	1
11	1	1	−1	1	−1	−1	−1	−1	1	1	1	1	−1
12	1	1	1	−1	−1	1	1	1	1	−1	−1	−1	−1
13	2	−1	−1	−1	1	1	−1	1	1	−1	1	1	−1
14	2	−1	1	1	1	−1	1	−1	1	1	−1	−1	−1
15	2	1	−1	1	−1	1	1	−1	−1	−1	1	−1	1
16	2	1	1	−1	−1	−1	−1	1	−1	1	−1	1	1

Table 6
D-optimal screening design to examine 9 factors in 10 experiments [17].

Experiment	Factors								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
1	1	−1	1	−1	1	−1	−1	−1	−1
2	1	1	−1	−1	−1	−1	1	−1	−1
3	−1	−1	−1	1	1	1	1	−1	−1
4	1	−1	−1	1	−1	−1	−1	1	−1
5	−1	1	1	−1	−1	1	−1	1	−1
6	−1	1	−1	1	1	−1	−1	−1	1
7	1	−1	−1	−1	−1	1	−1	−1	1
8	−1	−1	1	1	−1	−1	1	−1	1
9	−1	−1	−1	−1	1	−1	1	1	1
10	1	1	1	1	1	1	1	1	1

However, different to the use of D-optimal designs as response surface designs, in the case of screening, usually more than two or three factors are examined, the number of factor levels is restricted to two or three, and the examined domain is symmetrical. Most often, only the coefficients for the main factors are included in the regression model (Eq. (1)),

$$y = \beta_0 + \sum_{i=1}^{f} \beta_i x_i \quad (1)$$

where y is the response, β_0 the intercept, and β_i the main regression coefficients.

In Table 6, a D-optimal design is shown that allows examining 9 factors in 10 experiments [17].

2.1.6. Supersaturated designs as screening designs

Supersaturated designs are discussed here, not because they are recently developed designs, but because their application as screening design in pharmaceutical analysis is not very common. To examine a given number of factors, SS designs require less experiments than the regular screening designs.

As already mentioned, usually two-level screening designs, such as FF and PB designs, are applied for screening purposes. However, in cases where many factors need to be examined, still their number of experiments might be considered unfeasibly high. Or else, one wants to perform an absolutely minimal number of experiments. Moreover, from all examined factors during screening, usually most are found to be unimportant, while only a few have a significant influence on the method response(s). This is called the 'effect sparsity principle' [18].

Generally, SS designs examine more than $N_{SS} - 1$ factors (at least N_{SS}) in N_{SS} experiments. As a consequence, in these designs, the main effects are confounded and cannot be estimated unconfounded anymore (see Section 3.1) [18–22]. There are various methods to construct two-level, multi-level or mixed-level SS designs [18].

Two-level SS designs examine minimally N_{SS} factors at two levels in N_{SS} experiments. Two-level SS designs can be constructed randomly [23]; systematically, i.e. using a specific optimality criterion in order to approach orthogonality as much as possible [24–26]; or as half-fractions of Plackett–Burman designs [27]. The latter method uses one column of a (N, f) PB design as branching column to construct two supersaturated designs. All experiments with the branching column either at high or low level are selected and then the branching column is deleted, resulting in two supersaturated designs with $f_{SS} = f - 1$ factors and $N_{SS} = N/2$ experiments (Table 7).

Other construction methods apply columnwise–pairwise algorithms based on D-optimal design searches (Table 8) [28], are based on evolutionary or genetic algorithms [29], on the Galois field theory [30], on cyclic balanced incomplete block designs [31,32], apply an optimal foldover plan [33], or use Bayesian D-optimality

Table 7
$N = 12$, $f = 11$ Plackett–Burman design, and two $N_{SS} = 6$, $f_{SS} = 10$ supersaturated designs, constructed according to Lin [27] using column *J* of the PB design as branching column.

Plackett–Burman design ($N = 12, f = 11$)

Experiment	Factors										
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*
1	1	1	−1	1	1	1	−1	−1	−1	1	−1
2	−1	1	1	−1	1	1	1	−1	−1	−1	1
3	1	−1	1	1	−1	1	1	1	−1	−1	−1
4	−1	1	−1	1	1	−1	1	1	1	−1	−1
5	−1	−1	1	−1	1	1	−1	1	1	1	−1
6	−1	−1	−1	1	−1	1	1	−1	1	1	1
7	1	−1	−1	−1	1	−1	1	1	−1	1	1
8	1	1	−1	−1	−1	1	−1	1	1	−1	1
9	1	1	1	−1	−1	−1	1	−1	1	1	−1
10	−1	1	1	1	−1	−1	−1	1	−1	1	1
11	1	−1	1	1	1	−1	−1	−1	1	−1	1
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1

First supersaturated design ($N_{SS} = 6, f_{SS} = 10$)

Experiment	Factors									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*
2	−1	1	1	−1	1	1	1	−1	−1	1
3	1	−1	1	1	−1	1	1	1	−1	−1
4	−1	1	−1	1	1	−1	1	1	1	−1
8	1	1	−1	−1	−1	1	−1	1	1	1
11	1	−1	1	1	1	−1	−1	−1	1	1
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1

Second supersaturated design ($N_{SS} = 6, f_{SS} = 10$)

Experiment	Factors									
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*K*
1	1	1	−1	1	1	1	−1	−1	−1	−1
5	−1	−1	1	−1	1	1	−1	1	1	−1
6	−1	−1	(1	1	−1	1	1	−1	1	1
7	1	−1	−1	−1	1	−1	1	1	−1	1
9	1	1	1	−1	−1	−1	1	−1	1	−1
10	−1	1	1	1	−1	−1	−1	1	−1	1

[34]. In ref. [35], SS designs are constructed in such a way that stepwise regression should be more effective in finding the active factors. New design criteria are proposed, based on the adaptation of Bayesian approaches [36], and applied to the context of optimal design construction and method evaluation [35].

Multi-level SS designs examine in N_{SS} experiments minimally N_{SS} factors at minimally three levels ($L \geq 3$), and mixed-level SS designs at minimally two levels ($L \geq 2$) but with different numbers for some factors. Multi-level SS designs are constructed according to the approaches described in refs. [37–41], and mixed-level according to those in refs. [42–45].

2.2. Response surface designs

The most important factors, either found from screening or known from experience, are examined in more detail using response surface designs. These in fact are used to determine the optimal conditions for the factors. In these designs, only quantitative and mixture-related factors are examined. The reason is that the responses considered are modeled as a function of the factors. These response surfaces are then visualized. Most often, only two or three factors are further explored. There are several reasons for that. Examining more factors usually requires a too high number of experiments. Secondly, from three factors on, the entire response surface cannot be visualized anymore, which makes it difficult to determine the optimal conditions. For more than two factors, only fractions of the entire response surface are visualized. For mixture-

Table 8
$N_{SS} = 12$ $f_{SS} = 16$ supersaturated design, constructed according to Li and Wu [28].

Experiment	Factors															
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*
1	−1	−1	1	1	1	1	−1	−1	−1	1	1	−1	1	1	−1	1
2	−1	1	−1	1	1	1	−1	1	1	−1	−1	1	−1	1	1	1
3	1	−1	1	−1	1	−1	−1	1	1	−1	−1	−1	1	−1	1	1
4	−1	1	1	1	1	−1	1	1	−1	1	−1	1	1	−1	−1	−1
5	−1	1	1	−1	−1	1	−1	−1	1	1	1	1	1	−1	1	−1
6	1	1	−1	1	−1	−1	1	−1	1	−1	1	1	1	−1	−1	1
7	1	−1	1	1	−1	−1	−1	1	−1	−1	1	1	−1	1	1	−1
8	1	−1	1	−1	−1	1	1	−1	1	1	−1	1	−1	1	−1	1
9	−1	1	−1	−1	−1	−1	1	1	−1	1	1	−1	−1	1	1	1
10	1	−1	−1	1	1	1	1	1	1	1	1	−1	−1	−1	−1	−1
11	1	1	−1	−1	1	1	1	−1	−1	−1	−1	−1	1	1	1	−1
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1

Table 9
Three-level full factorial design for 2 factors. Etc. refers to possible replicates of the centre point.

Experiment	Factors	
	A	*B*
1	−1	−1
2	−1	1
3	−1	0
4	1	−1
5	1	1
6	1	0
7	0	−1
8	0	1
9, etc.	0	0

related factors, again all but one component can be selected as factor. The response surface designs can be divided into symmetrical and asymmetrical designs, depending on their appropriateness to be used in an asymmetrical domain [1,4–6,9].

2.2.1. Symmetrical designs

Symmetrical designs cover a symmetrical experimental domain. They contain, for instance, three-level full factorial, central composite, Box–Behnken, and Doehlert designs. Often in these designs the centre point is replicated 3–5 times, usually to estimate the experimental error.

A three-level full factorial design contains all possible combinations between the f factors and their $L = 3$ levels, leading to $N = L^f = 3^f$ experiments, including one centre point. Thus for two factors, 9 experiments are needed (Table 9), while for three factors, it are already 27 experiments (Fig. 2).

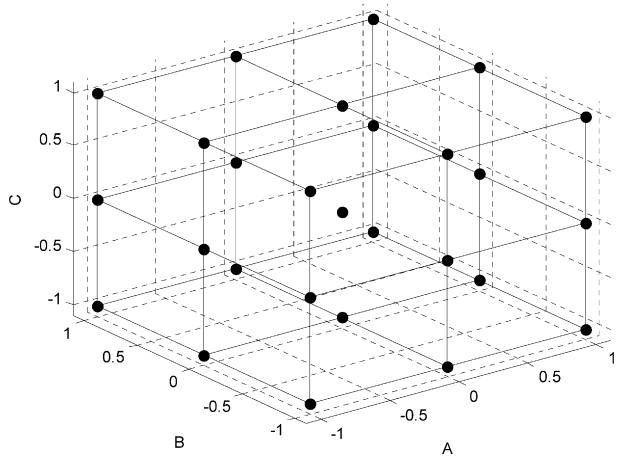

Fig. 2. Three-level full factorial design to examine three factors in 27 experiments.

A central composite design (CCD) contains a two-level full factorial design (2^f experiments), a star design ($2f$ experiments) and a centre point, requiring $N = 2^f + 2f + 1$ experiments to examine f factors [1,4–6,9]. Thus for two factors, 9 experiments are needed, while for three factors, 15 are (Table 10 and Fig. 3) needed. The points of the full factorial design are situated at the factor levels −1 and +1, those of the star design at the factor levels 0, $-\alpha$ and $+\alpha$, and the centre point at the factor levels 0. Depending on the α value, two CCD's exist, i.e. a face-centred CCD (FCCD) with $|\alpha| = 1$ examining the factors at three levels, and a circumscribed CCD (CCCD) with $|\alpha| > 1$ examining the factors at five levels. For a so-called rotatable CCCD, the α level should be $|\alpha| = (2^f)^{1/4}$, i.e. 1.41 and 1.68 for 2 and 3 factors, respectively [1].

A Box–Behnken design contains $N = (2f(f-1)) + 1$ experiments, of which one centre point [46]. For two factors, no design is described. For three factors, 13 experiments are described to be performed (Table 11 and Fig. 4).

A Doehlert (uniform shell) design has equal distances between all neighbouring experiments [47]. The Doehlert design for two factors consists of the six vertices of a hexagon with a centre point, requiring $N = 7$ experiments. The design for three factors consists of a centred dodecahedron, needing $N = 13$ experiments (Table 12 and Fig. 5). Contrary to the above response surface designs, the factors are varied at different numbers of levels, e.g. one at three and one at five levels in the two-factor design, and one at three, one at five, and one at seven levels in the three-factor design.

2.2.2. Asymmetrical designs

When an asymmetrical domain should be examined, asymmetrical designs, such as D-optimal designs or designs constructed

Table 10
Central composite design for 3 factors. Etc. see Table 9.

Experiment	Factors		
	A	*B*	*C*
1	−1	−1	−1
2	1	−1	−1
3	−1	1	−1
4	1	1	−1
5	−1	−1	1
6	1	−1	1
7	−1	1	1
8	1	1	1
9	$-\alpha$	0	0
10	$+\alpha$	0	0
11	0	$-\alpha$	0
12	0	$+\alpha$	0
13	0	0	$-\alpha$
14	0	0	$+\alpha$
15, etc.	0	0	0

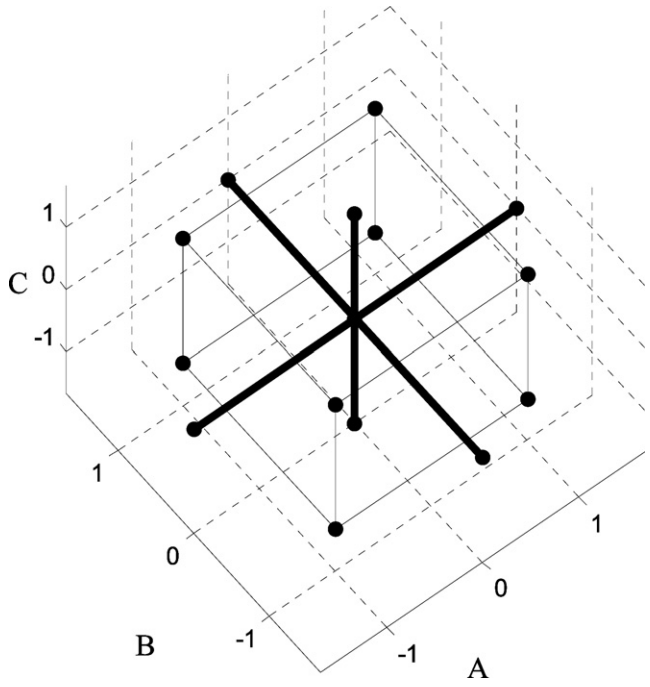

Fig. 3. Circumscribed central composite design to examine three factors in 15 experiments.

Table 11
Box–Behnken design for 3 factors. Etc. see Table 9.

Experiment	Factors		
	A	*B*	*C*
1	1	1	0
2	1	−1	0
3	−1	1	0
4	−1	−1	0
5	1	0	1
6	1	0	−1
7	−1	0	1
8	−1	0	−1
9	0	1	1
10	0	1	−1
11	0	−1	1
12	0	−1	−1
13, etc.	0	0	0

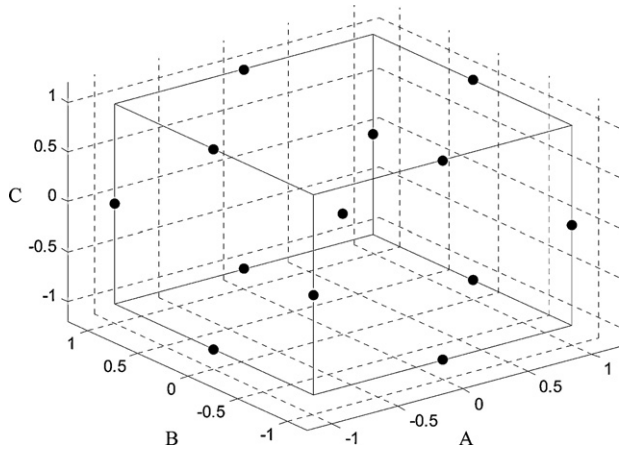

Fig. 4. Box–Behnken design to examine three factors in 13 experiments.

Table 12
Doehlert designs for 3 factors. Etc. see Table 9.

Experiment	Factors		
	A	*B*	*C*
1	1	0	0
2	0.5	0.866	0
3	0.5	0.289	0.816
4	−1	0	0
5	−0.5	−0.866	0
6	−0.5	−0.289	−0.816
7	0.5	−0.866	0.000
8	0.5	−0.289	−0.816
9	0	0.577	−0.816
10	−0.5	0.866	0
11	−0.5	0.289	0.816
12	0	(0.577	0.816
13, etc.	0	0	0

with the uniform mapping algorithm of Kennard and Stone, can be applied [1,5,9,48,49]. These designs are called asymmetrical because when plotting their experiments, they take an asymmetrical shape when an asymmetrical domain is examined. These designs can also be used in a symmetrical domain, and then a symmetric shape may be obtained.

Asymmetric designs are used because symmetric designs in an asymmetric domain are problematic. Either they are too large and require experiments in an impossible area or they are too small and then a considerable part of the domain is not covered (Fig. 6a and b).

To construct a D-optimal design for f factors, the method described above in Section 2.1.5 is used. In Fig. 6, this is represented for two factors. From the candidate points forming a grid over the asymmetrical domain (Fig. 6c), the N experiments forming the D-optimal design are selected (Fig. 6d).

Most often, from a response surface design, the general model build for f factors is as follows,

$$y = \beta_0 + \sum_{i=1}^{f} \beta_i x_i + \sum_{1 \le i < j}^{f} \beta_{ij} x_i x_j + \sum_{i=1}^{f} \beta_{ii} x_i^2 \quad (2)$$

where y is the response, β_0 the intercept, β_i the main coefficients, β_{ij} the two-factor interaction coefficients, and β_{ii} the quadratic

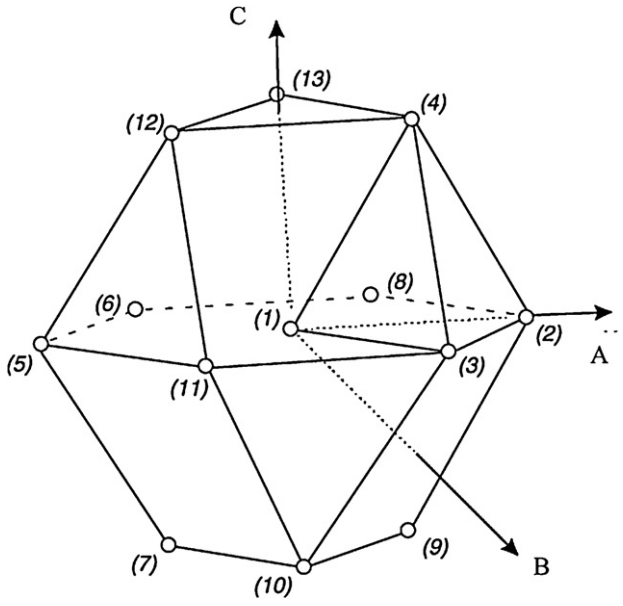

Fig. 5. Doehlert design to examine three factors in 13 experiments.

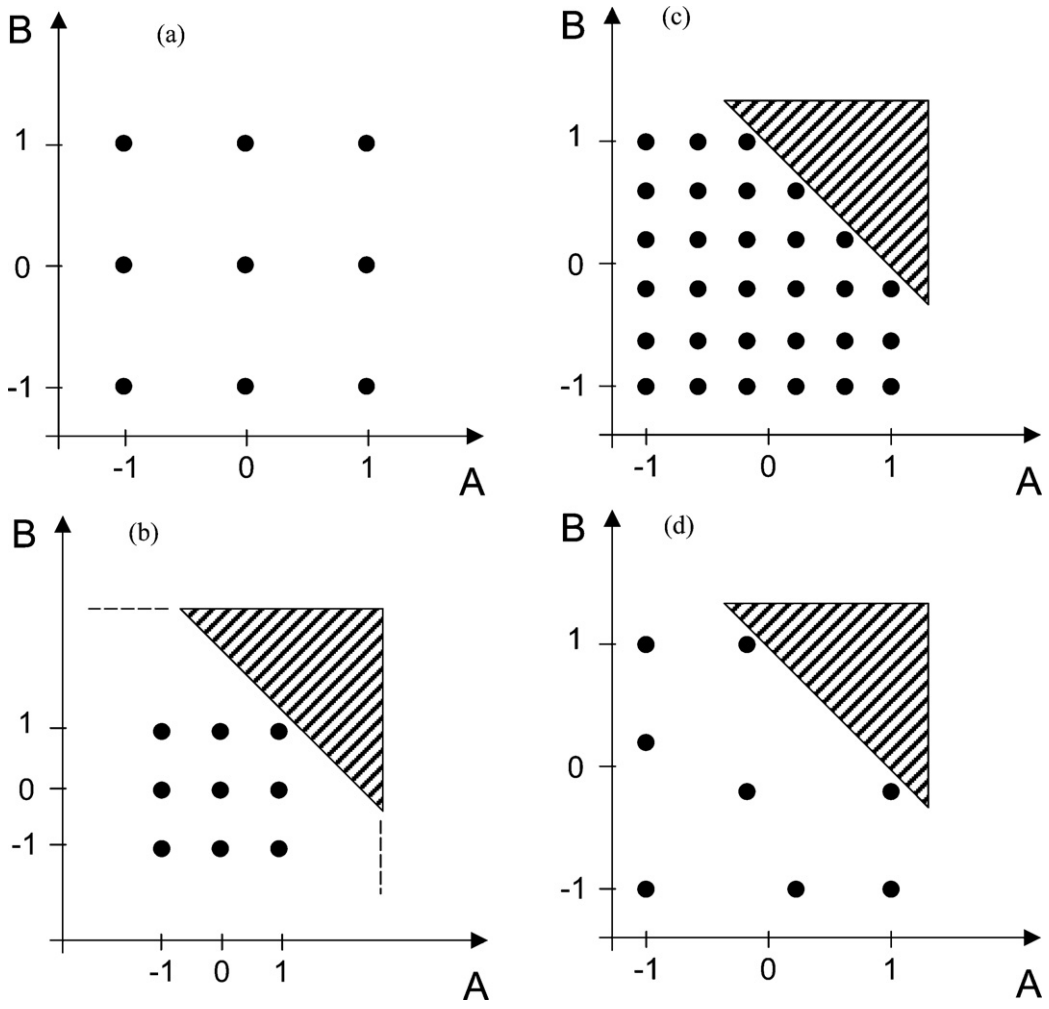

Fig. 6. (a) A 3^2 full factorial design in a rectangular symmetrical domain, (b) a restricted 3^2 full factorial design in an asymmetrical domain, (c) the candidate points of the grid in the asymmetrical domain, and (d) the selected points constructing an 8-experiments D-optimal design (possible or selected experiments (●)).

coefficients. Occasionally, the interaction terms are restricted to two-factor interactions ($x_i x_j$) and the higher-order interactions neglected, as in Eq. (2). Occasionally the non-significant terms of the model are deleted after a statistical analysis.

The experiments selected using the Kennard and Stone algorithm cover the experimental domain as uniformly as possible, and are situated as far as possible from each other. This is obtained by maximizing the minimal Euclidean distance of a new experiment to those previously selected. The algorithm can be initiated in two ways, i.e. either earlier performed experiments are included (Fig. 7a) or not (Fig. 7b). In this approach, the points are sequentially selected, e.g. the 9-experiments design equals that with eight plus the next selected experiment. In D-optimal designs, this is not the case. The designs with 8 and 9 experiments are different selections from the grid points.

2.3. Mixture designs

Mixture designs are response surface designs studying only mixture variables, and are applied to optimize the composition of mixtures, such as of solvents, e.g. the mobile phase during optimization of separation techniques, or of excipients in formulations (e.g. tablets) in pharmaceutical manufacturing. Here all mixture components can be examined in one design. For instance, to examine a three-components mixture, either a (3,1) simplex lattice mixture design with 3 experiments (exp 1–3), a (3,2) simplex lattice mixture design with 6 experiments (exp 1–6), a (3,3) simplex lattice–centroid mixture design with 7 experiments (exp 1–7), or an augmented simplex lattice–centroid mixture design with 10 experiments (exp 1–10) can be selected (Table 13 and Fig. 8).

For mixtures, very often limitations are defined for some of the components. For instance, suppose one is preparing a tablet and wants to optimize the composition of three excipients. Most often all three need to be present. Thus the vertices on the sides of the

Table 13
Mixture designs.

Experiment	Factors			Response
	A	*B*	*C*	
1	1	0	0	y_1
2	0	1	0	y_2
3	0	0	1	y_3
4	0.5	0.5	0	y_{12}
5	0.5	0	0.5	y_{13}
6	0	0.5	0.5	y_{23}
7	0.333	0.333	0.333	y_{123}
8	0.670	0.165	0.165	y_8
9	0.165	0.670	0.165	y_9
10	0.165	0.165	0.670	y_{10}

(3,1) simplex lattice design with 3 experiments (exp 1–3); (3,2) simplex lattice design with 6 experiments (exp 1–6); (3,3) simplex lattice–centroid design with 7 experiments (exp 1–7); or augmented simplex lattice–centroid design with 10 experiments (exp 1–10).

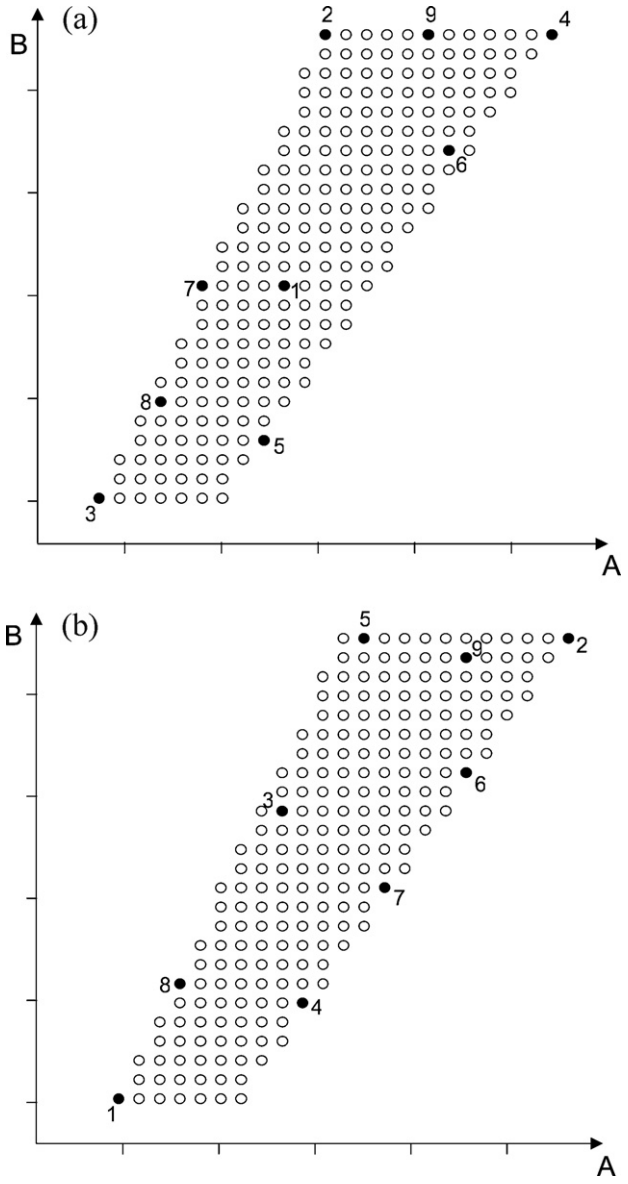

Fig. 7. Nine-experiments design: experiments selected by the uniform mapping algorithm of Kennard and Stone: (a) with the requirement that a central point was the first selected, and (b) without requirements.

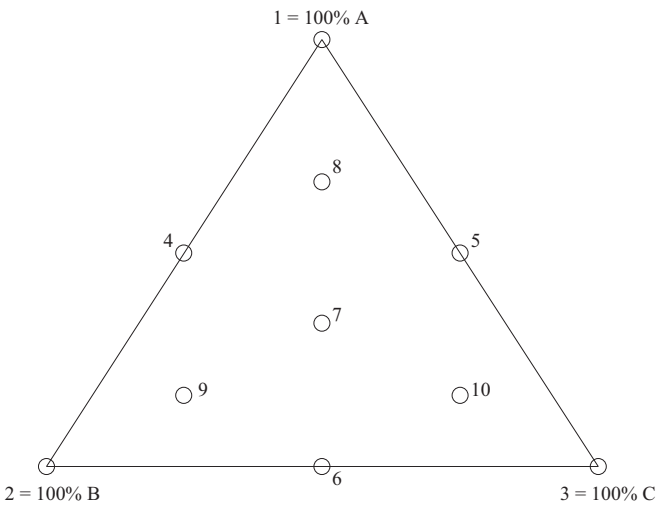

Fig. 8. Mixture designs: three-component (3,1) simplex lattice design with 3 experiments (exp 1–3); (3,2) simplex lattice design with 6 experiments (exp 1–6); (3,3) simplex lattice–centroid design with 7 experiments (exp 1–7); or augmented simplex lattice–centroid design with 10 experiments (exp 1–10).

triangle do not result in a tablet. One has to limit to given regions within the triangular domain of Fig. 8. Depending on the restrictions, the remaining domain is either another triangle (within the larger one), or irregular. In the former case, the above mixture designs can be applied in the smaller triangle, and in the latter, the earlier discussed asymmetrical designs (D-optimal, Kennard and Stone) can be constructed.

3. Data interpretation

3.1. Screening designs

From the *results of a full factorial*, *FF* or *PB design*, the effect of each factor X on each response Y is estimated as follows

$$E_X = \frac{\sum Y(+1) - \sum Y(-1)}{N/2} \quad (3)$$

$\sum Y(+1)$ and $\sum Y(-1)$ represent the sums of the responses where factor X is at (+1) and (−1) level, respectively, and N the number of design experiments [4,5].

An alternative is to estimate the coefficients of the regression model [1,4–6], given earlier in Eq. (1). The latter approach is mandatory to analyze the *results of a D-optimal screening design*.

In general, a regression model estimates the relation between the $N \times 1$ response vector $\mathbf{y}$, and the $N \times t$ model matrix $\mathbf{X}$ (Eq. (4)), with N being the number of design experiments, and t the number of terms included in the model. The model matrix $\mathbf{X}$ is obtained by adding a column of ones before the $t-1$ design matrix columns, which consists of the coded factor levels (e.g. −1 and +1) and the columns of contrast coefficients, as defined by the considered experimental design.

$$\mathbf{y} = (\mathbf{X}\boldsymbol{\beta}) + \boldsymbol{\varepsilon} \quad (4)$$

$\boldsymbol{\beta}$ is the $t \times 1$ vector of regression coefficients and $\boldsymbol{\varepsilon}$ is an $N \times 1$ error vector. The regression coefficients $\mathbf{b}$ are usually calculated using least squares regression,

$$\mathbf{b} = (\mathbf{X}^\mathbf{T}\mathbf{X})^{-1}\mathbf{X}^\mathbf{T}\mathbf{y} \quad (5)$$

where $\mathbf{X}^\mathbf{T}$ is the transposed matrix of $\mathbf{X}$.

Because effects estimate the change in response when changing the factor levels from −1 to +1, and coefficients that between levels 0 and +1, both are related as follows.

$$E_X = 2b_X \quad (6)$$

Usually a graphical and/or statistical interpretation of the estimated effects is done to determine their significance. Graphically, normal probability or half-normal probability plots (Fig. 9) can be drawn [4,5]. On these plots, the unimportant effects are found on a straight line through zero, while the important deviate from this line.

The statistical interpretations usually calculate a t-test statistic for the factors and compare either this t-value or the effect E_X with a limit value, t_{critical}, or a critical effect, E_{critical}, respectively. All effects that in absolute value are larger than or equal to this E_{critical} are then considered significant [4,5].

$$t = \frac{|E_X|}{(\text{SE})_\text{e}} \Leftrightarrow t_{\text{critical}} \quad (7)$$

with $(\text{SE})_\text{e}$ being the standard error of an effect. The critical t-value, t_{critical}, depends on the number of degrees of freedom associated

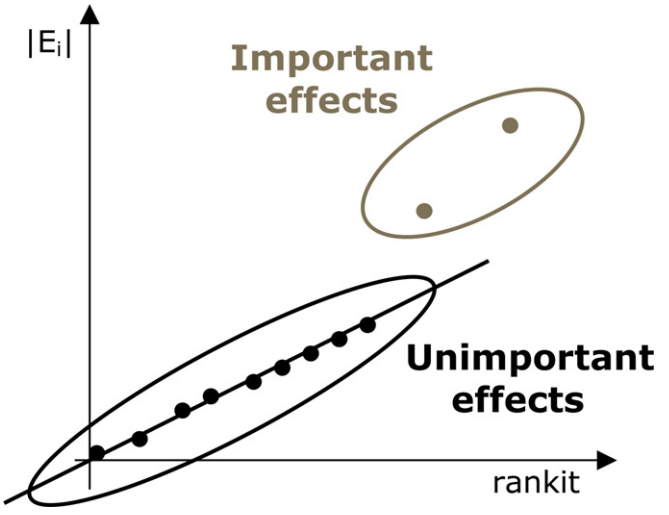

Fig. 9. Half-normal probability plot for 11 effects.

with $(SE)_e$ and on the significance level, usually $\alpha = 0.05$ (Eq. (7)). The critical effect, $E_{critical}$ (Eq. (8)), is then obtained as follows.

$$E_{critical} = t_{critical}(SE)_e \Leftrightarrow |E_X| \quad (8)$$

$(SE)_e$ can be estimated from different data: from the variance of replicated experiments, from a priori declared negligible effects (Eq. (9)), and from a posteriori defined negligible effects (Eqs. (10–12)). We consider the last two approaches as most appropriate to properly estimate $(SE)_e$. They are discussed below.

$(SE)_e$ can be derived from n_N a priori declared negligible effects, E_N, being either (two-factor) interaction or dummy effects in FF and PB designs, respectively (Eq. (9)). It is recommended that at least three negligible effect estimates are available to properly estimate the error.

$$(SE)_e = \sqrt{\frac{\sum E_N^2}{n_N}} \quad (9)$$

$(SE)_e$ can also be derived from a posteriori defined negligible effects by using, for example, the algorithm of Dong [50]. Even for a minimal screening design, this algorithm can be applied. An initial error estimate based on the median of the absolute effects, s_0 (Eq. (10)), is then used to make a final error estimate, $(SE)_e$ (Eq. (11)), based on the m effects E_k that are considered unimportant, i.e. for which $|E_k| \leq 2.5 s_0$.

$$s_0 = 1.5 \times \text{median}\,|E_X| \quad (10)$$

$$(SE)_e = \sqrt{\frac{\sum E_k^2}{m}} \quad (11)$$

The algorithm of Dong requires effect sparsity, i.e. ≪50% significant effects. Problems of detecting the significant effects correctly occur in situations where the effect sparsity principle is violated and the number of significant effects approaches 50%. The algorithm then overestimates $(SE)_e$ (Eqs. (10) and (11)) and thus also $E_{critical}$ (Eq. (8)), resulting in significant effects incorrectly considered non-significant [51,52].

An alternative for these situations is to use an adaptation to the algorithm of Dong as suggested in [52]. The adapted approach recommends applying the 75% lowest absolute effects, $|E_{75\%}|$, for the initial error estimation s_0 (Eq. (12)).

$$s_0 = 1.5 \times \text{median}\,|E_{75\%}| \quad (12)$$

To analyze *SS screening design results*, many different interpretation approaches have been proposed [18]. However, because of the confounding of main effects, it is not evident to properly estimate them. Cela et al. [53] proposed solving the results of SS designs, constructed according to reference [29], applying genetic algorithm-based regression (Supersat® software, freely downloadable at http://www.usc.es/gcqprega/).

Phan-Tan-Luu and co-workers [54] also described an approach to indicate the most significant effects from SS design results. The method is included in their Nemrod® software (http://www.nemrodw.com/), and represented in Fig. 10.

In a first step, a coefficient is estimated for all factors of the design matrix (N_{SS}, f_{SS}) using ridge regression (Fig. 10a). The second step uses the first f_1 factors with the largest absolute coefficients ($f_1 < f_{SS}$ and $f_1 \approx 2f_{SS}/3$) to estimate a new coefficient for, using the reduced matrix (N_{SS}, f_1) (Fig. 10b). Stepwise regression and all-subset regression are used to indicate, from these f_1 factors, the f_2 most important with $1 \leq f_2 \leq 6$. The latter decision is based on the determination coefficient, r^2, and the residual variance, s^2, of the models. In a third step, a coefficient is estimated for all factors of the matrix (N_{SS}, f_3), containing $f_3 = f_{SS} - f_1 + f_2$ factors (Fig. 10c). This way, the factors that were discarded ($f_{SS} - f_1$) and those selected (f_2) are re-evaluated. This allows reconfirming the f_2 important factors and to ensure that no important factors have been incorrectly excluded. Then, from these f_3 factors, again a number of factors ($1 \leq f_4 \leq 6$) is retained using stepwise regression and all-subset regression, for which the decision is again based on the determination coefficient, r^2, and the residual variance, s^2, of the models. In a final step, the initial design is projected into a smaller non-saturated design, only containing the f_4 active factors (Fig. 10d). Then classic tools of regression analysis can be used to estimate the coefficients of these most active factors.

Our group developed a method, called the Fixing Effects and Adding Rows (FEAR) method, to estimate all factor effects from supersaturated design results [21,22]. The supersaturated designs studied so far are the half-fractions of PB designs. The key ideas in this approach are that SS designs possess too few experiments, i.e. equations, for an accurate estimation of all factor coefficients, and that effect sparsity occurs. The FEAR method is represented in Fig. 11.

In a preliminary step (step 0), the factor effects are estimated in the classic way using Eq. (1) (Fig. 11a). These estimates are inaccurate, because main effects are confounded. They will be reconsidered later in the procedure. In a first step (step 1), a number of zero effect rows, i.e. situations where the coefficient of a given factor arbitrarily is defined to be zero, is added to the model matrix (Fig. 11b). From this combined matrix, the coefficients are estimated using least squares. This is repeated for a large number of different combinations of added zero effect rows, i.e. either the $f_{SS}!/((f_{SS}+1-N_{SS})!\,(N_{SS}-1)!)$ possible combinations or a maximum defined number, e.g. 20,000, for computational reasons. From the distributions of the estimated least squares regression coefficients (effects) for the different factors, the most important factor is defined, its coefficient estimated and fixed (Fig. 11b). In the histogram, one observes also the situations where the coefficient (effect) had been fixed at zero. These should be ignored in the estimation of the coefficient/effect. This is called one FEAR step, which is then repeated, but with the largest coefficient fixed. Using a sequential approach, the FEAR method thus estimates consecutively the largest coefficients and removes their confounding from the other estimated factor coefficients (Fig. 11c). Finally, after adding all rows with fixed effects, the factor coefficients are calculated from the complete matrix using least squares (Fig. 11d). In each step, the factor effects are estimated from the factor coefficients (Eq. (6)) and their significance is then determined using the algorithm of Dong [50] at significance level $\alpha = 0.05$.

However, fixing too many factor coefficients usually results in an overcorrection. Thus one should stop when all important

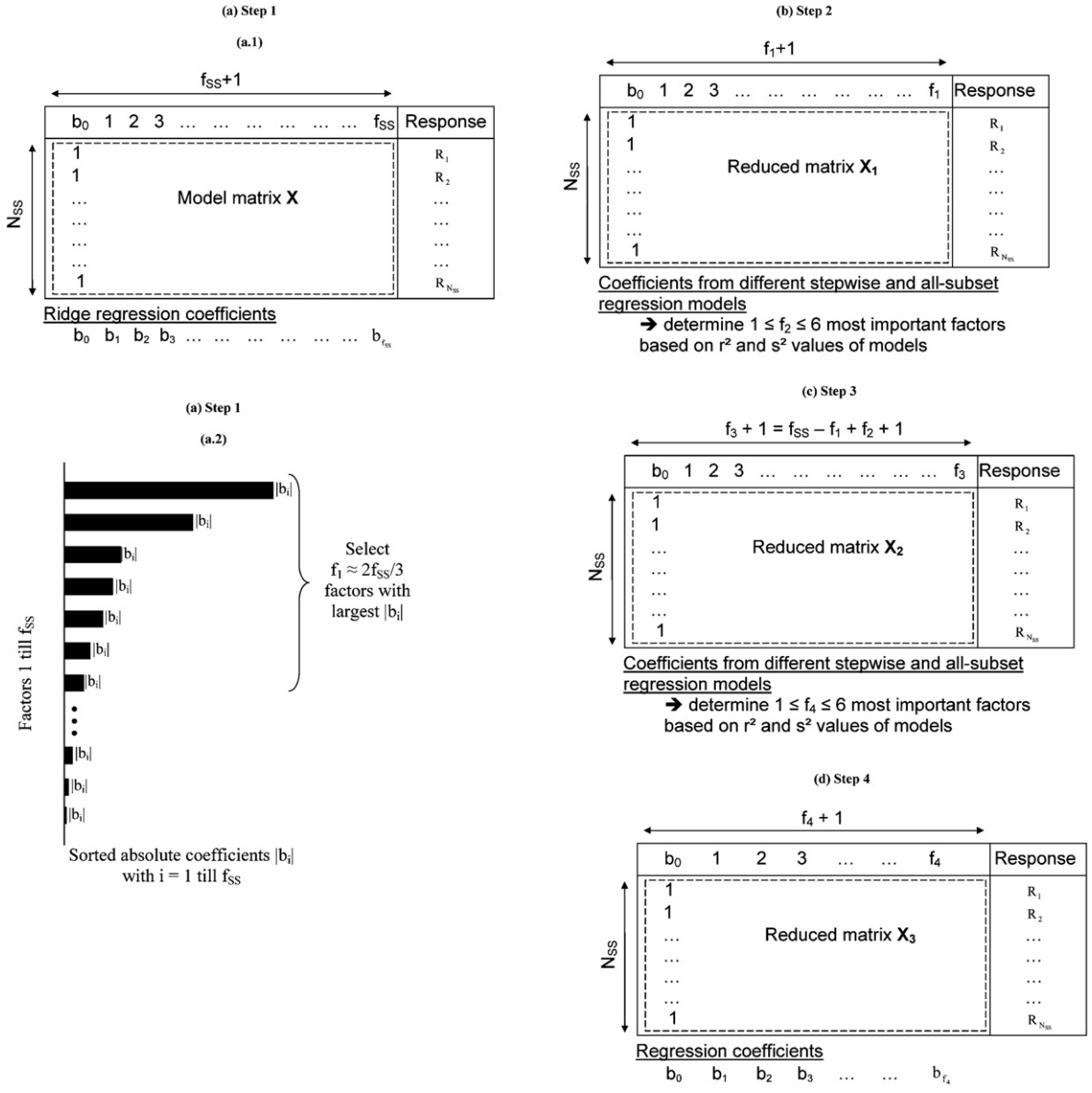

Fig. 10. Phan-Tan-Luu and Sergent's approach to estimate the most significant factors in supersaturated designs. (a) Step 1: (a.1) the $N_{SS} \times (f_{SS} + 1)$ supersaturated model matrix **X** (dotted box), and its ridge regression coefficients. (a.2) Selection of the $f_1 \approx 2f_{SS}/3$ factors with the largest absolute coefficients. (b) Step 2: the $N_{SS} \times (f_1 + 1)$ reduced matrix $\mathbf{X_1}$ (dotted box), its stepwise and all-subset regression coefficients, and selection of the $1 \leq f_2 \leq 6$ most important factors based on the r^2 and s^2 values of the models. (c) Step 3: the $N_{SS} \times (f_3 + 1)$ reduced matrix $\mathbf{X_2}$ (dotted box) with $f_3 = f_{SS} - f_1 + f_2$, its stepwise and all-subset regression coefficients, and selection of the $1 \leq f_4 \leq 6$ most important factors based on the r^2 and s^2 values of the models. (d) Step 4: the $N_{SS} \times (f_4 + 1)$ reduced matrix $\mathbf{X_3}$ (dotted box), and its classically estimated regression coefficients.

effects are fixed. Therefore, to select this step with the 'best estimated' effects, the critical effects from Dong's approach or from the adapted Dong's approach are plotted as a function of the FEAR steps. A large decrease in the critical effect or the beginning of a series of critical effects that do not decrease largely anymore indicates the step to be chosen (Fig. 11e). It indicates the step where important effects were estimated, fixed, and removed from the matrix. At that step, the factor effects seem to be estimated best.

3.2. Response surface designs

With the experimental results of a response surface design, a polynomial model, describing the relation between a response and the considered factors, is build. Usually a second-order polynomial model (Eq. (2)) is constructed.

Afterwards, the model can be interpreted graphically and/or statistically. Graphically, the model is visualized by drawing 2D contour plots or 3D response surface plots. A 2D contour plot (Fig. 12a) shows the isoresponse lines as a function of the levels of two factors, while a 3D response surface plot (Fig. 12b) represents the response in a third dimension. From such plots, often the best or optimal conditions are derived. However, one should be aware that, in case three or more factors are considered, a plot as in Fig. 12 only represents a part (occasionally a very small) of the entire response surface in the examined domain.

The fit of the model to the data can be evaluated statistically applying either Analysis of Variance (ANOVA), a residual analysis, or an external validation using a test set [1,4–6]. One also can determine the significance of the **b** coefficients in the above model and then eliminate the non-significant ones, for instance, sequentially. Because most often, in practice, the optimum is not one point but a region with acceptable performance, the quadratic model without statistical analysis performs acceptably well. The model is used to find the proper conditions and not for predictive purposes as

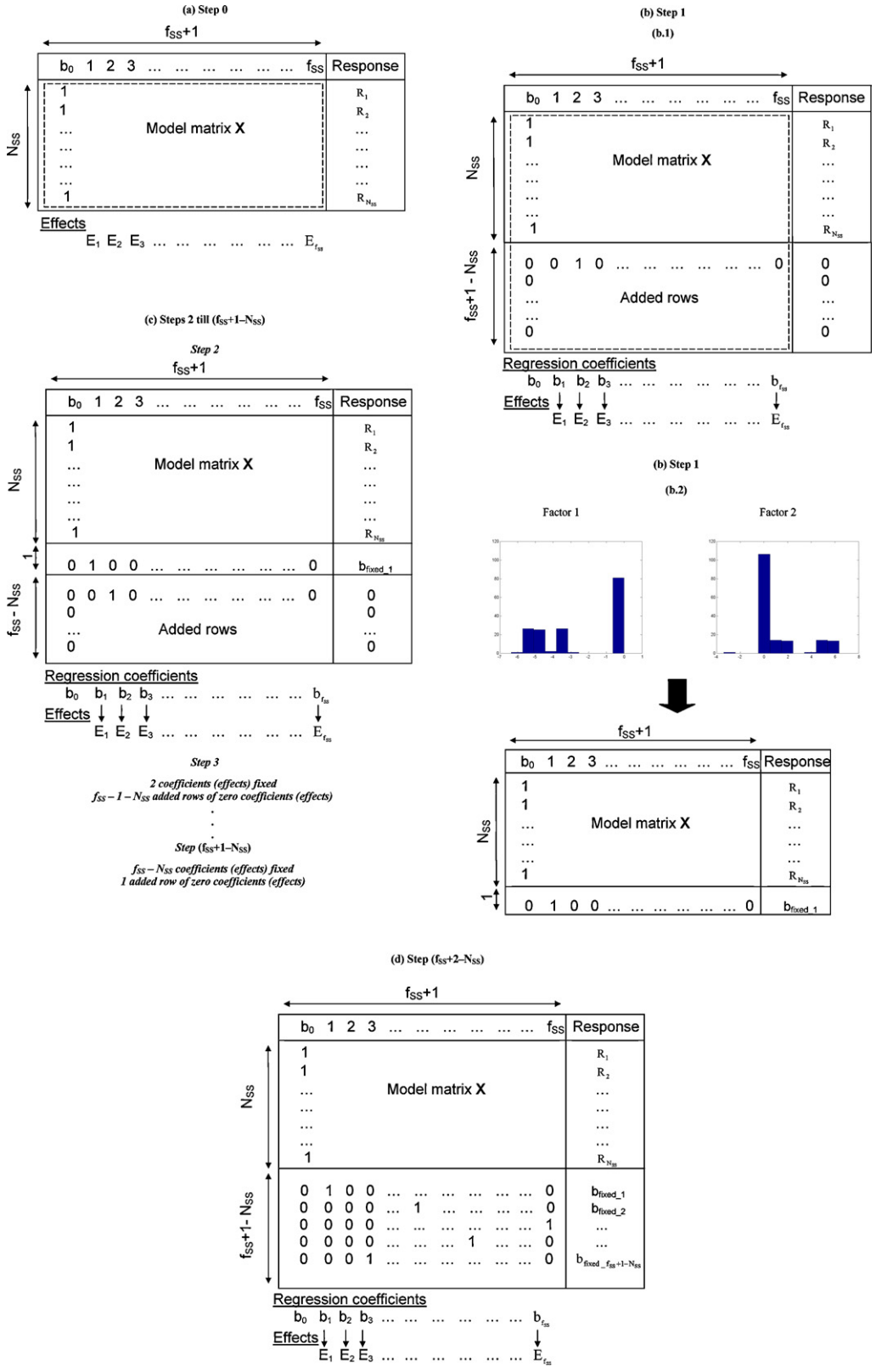

Fig. 11. The FEAR method to estimate factor effects in supersaturated designs. (a) Step 0: the $N_{SS} \times (f_{SS}+1)$ supersaturated model matrix **X** (dotted box), and its classically estimated factor effects, (b) step 1: (b.1) the $N_{SS} \times (f_{SS}+1)$ model matrix with $f_{SS}+1-N_{SS}$ added rows. In each added row, one effect is randomly defined as zero. Regression coefficients for the combinations with $\det(\mathbf{Z}^T\mathbf{Z}) \neq 0$ are calculated. Here the dotted box represents **Z**. (b.2) Some histograms of estimated coefficients used to derive the most important factor, and the $(N_{SS}+1) \times (f_{SS}+1)$ matrix with a fixed largest coefficient, (c) steps 2 till $(f_{SS}+1-N_{SS})$: add $f_{SS}-N_{SS}$ more rows of zero effects in step 2 and fix the largest coefficient again as in step 1. Then iteratively apply the procedure (each time with one row less of zero effects added) till all $(f_{SS}+1-N_{SS})$ added rows contain a fixed estimated coefficient, (d) step $(f_{SS}+2-N_{SS})$: estimate the least squares regression coefficients from the $(f_{SS}+1) \times (f_{SS}+1)$ matrix, (e) critical effects as a function of the different steps. Graphic approaches to indicate at which step of the iterative process the best effect estimates are obtained.

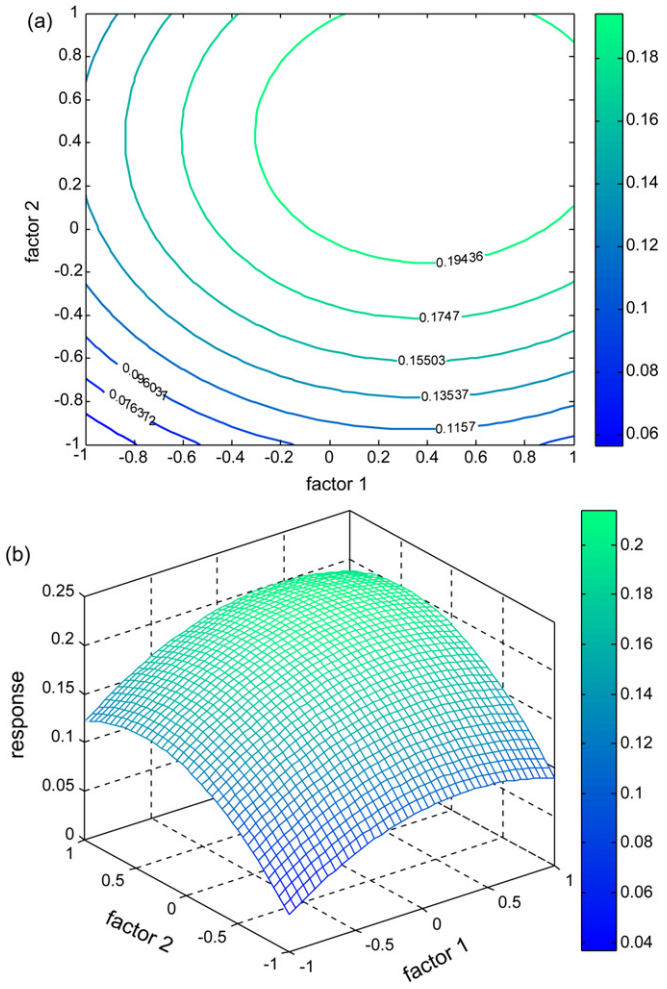

Fig. 12. (a) 2D contour plot, and (b) 3D response surface plot.

multivariate calibration models are. Therefore less effort can be spent in finding the best model and the quadratic one usually fits the data acceptably good.

3.3. Mixture designs

As already mentioned, to examine three components of a mixture, either a mixture design with three, six, seven or 10 points can be used (see Table 13 and Fig. 8). A three-experiments (3,1) simplex lattice design (Table 13, exp 1–3) is used to estimate the coefficients of the model.

$$y = b_1x_1 + b_2x_2 + b_3x_3 \quad (13)$$

A six-experiments (3,2) simplex lattice design (Table 13, exp 1–6) is used to estimate the coefficients of the model.

$$y = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (14)$$

A seven-experiments (3,3) simplex lattice–centroid mixture design (Table 13, exp 1–7) is used to estimate the coefficients of the following reduced cubic model

$$y = b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{123}x_1x_2x_3 \quad (15)$$

In Eqs. (13)–(15), y is the modeled response, x_1, x_2, and x_3 are the fractions of the first, second, and third mixture component, respectively, and b_i, b_{ij} and b_{ijk} are the model coefficients. The above situations require no regression and the model coefficients can simply be estimated from one or some measured results (Eqs. (16)–(22)).

$$b_1 = y_1 \quad (16)$$

$$b_2 = y_2 \quad (17)$$

$$b_3 = y_3 \quad (18)$$

$$b_{12} = 4y_{12} - 2(y_1 + y_2) \quad (19)$$

$$b_{13} = 4y_{13} - 2(y_1 + y_3) \quad (20)$$

$$b_{23} = 4y_{23} - 2(y_2 + y_3) \quad (21)$$

$$b_{123} = 27y_{123} - 12(y_{12} + y_{13} + y_{23}) + 3(y_1 + y_2 + y_3) \quad (22)$$

It can be demonstrated that the model of Eq. (15) is a representation of the above mentioned cubic model (Eq. (2)), but only applicable for mixtures. When performing experiments 1–10 (Table 13), which constitutes another mixture design, then the coefficients of Eq. (15) need to be estimated applying a regression. The model of Eq. (15)) can also be used to draw 2D contour plots or 3D response surface plots for the triangular domain of Fig. 8 or for parts of this domain in case one works in a restricted domain within the triangle.

4. Analytical applications

4.1. Some classic applications

In the context of optimizing and validating separation methods, the application of screening and response surface designs has already been discussed and reviewed frequently [5,7,11,55,56]. For screening, two-level full factorial [57–60], fractional factorial [61–66], Plackett–Burman [67–72], and three-level [73–75] screening designs were mainly applied. For the actual optimization of important factors, response surface designs, such as three-level full factorial [76,77], central composite [62,66,78–80], Box–Behnken [81], Doehlert [67,68,82], D-optimal designs [69,83,84], or designs constructed using the uniform mapping algorithm of Kennard and Stone [85,86], were used.

Applications of designs were reviewed in the context of method optimization (both screening and optimization) [5] and robustness testing [5,7] of CE methods. For robustness testing of HPLC, CE, GC, supercritical fluid chromatography (SFC), ultra-performance liquid chromatography (UPLC) methods, experimental design approaches were discussed in ref. [11]. Applications of response surface designs to optimize analytical methods, e.g. HPLC, CE, GC, atomic absorption spectrophotometric (AAS), atomic emission spectrophotometric (AES), amperometric, voltammetric, spectroscopic, and capillary chromatographic methods, are described in ref. [55].

The use of Doehlert designs was reviewed in ref. [56]. Optimized methods were AAS, AES, voltammetric, polarographic, SFC, GC, capillary chromatographic, and HPLC ones. Reference [87] describes some practical problems that can occur during method optimization of chromatographic methods in pharmaceutical analysis.

In ref. [88], the HPLC assay of the active ingredients of St. John's Wort, described in a monograph [89], was optimized. An asymmetrical mixed-level $2^3\,3^1\,4^1$ screening design was constructed according to Addelman [14] and it examined in 16 experiments the effects of three factors at two levels, one at three levels, and one at four levels on the hypericin concentration. Based on the results, it was decided to adapt the monograph method. Therefore, an 18-factors 20-experiments two-level PB design was used to evaluate the new sample pretreatment of this assay.

In the context of optimizing formulations, products, or processes, two-level screening designs, such as full factorial [90],

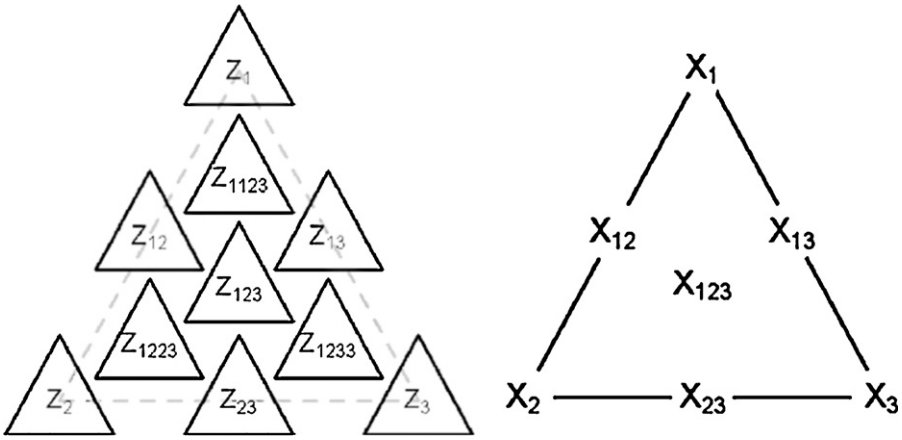

Fig. 13. Composite simplex centroid (10 experiments)–simplex centroid (7 experiments mixture design for the simultaneous optimization of the extraction solution and the mobile phase composition, respectively.
Reproduced with permission from [102].

fractional factorial [91,92], and Plackett–Burman [93–95] designs were applied. Response surface designs, such as three-level full factorial [96–98], central composite [93,94], Box–Behnken [91], and Doehlert [99,100] designs, were also used to optimize the most important factors.

Mixture designs were applied to optimize the mobile phase composition [101–103] in chromatographic techniques, the extraction solution [102,103], as well as formulations, products, or processes [104–106]. In ref. [104], a micro-emulsion formulation was optimized, in ref. [105], the size of amphiphilic cyclodextrin nanoparticles, and in ref. [106] a pharmaceutical gel formulation. A (3,3) simplex lattice–centroid design with seven experiments (1–7 in Table 13) was applied in ref. [104], and an augmented simplex lattice–centroid design with 9 experiments (1–9 in Table 13) in ref. [101]. In ref. [105], a D-optimal approach was used to select 12 experiments in a triangular domain. In ref. [106], also a D-optimal approach selected 18 experiments in a tetrahedron-shaped domain with constraints for the factors.

In ref. [102], a composite simplex centroid–simplex centroid mixture design (Fig. 13) is applied for the simultaneous optimization of the extraction solution and the mobile phase composition, respectively. The first design has 10 experiments (1–10 in Table 13) and was used to optimize the extraction solution. This design was performed at each experiment of the second design, which has seven experiments (1–7 in Table 13) and was used for the optimization of the mobile phase composition. This leads to 70 different combinations, which does not seem very economical. However, it can also be represented as analyzing 10 samples at the 7 mobile phases of the 7-point design.

In ref. [103], a crossed mixture design and a simplex centroid (Fig. 14) were used to respectively optimize the mobile phase composition and the extraction solution simultaneously. The first design has 9 experiments in an asymmetric part of the triangle and was used to optimize the mobile phase composition. At each of these experiments, the second design was performed. The second design has 10 experiments (1–10 in Table 13) and was used to optimize the extraction solution. This leads to 90 different combinations, for which a similar comment as above can be made.

A rather new application of classic experimental designs is seen in PAT-related research. Full factorial designs for three (2^3) [107] and four factors (2^4) [108–110] were applied to examine the in-line monitoring and understanding of the homogenization process of a pharmaceutical suspension [108], of a freeze-drying process [109], of a powder-blending process [107], and of a fluid-bed granulation process [110].

4.2. Advanced analytical applications

4.2.1. Method development for drug impurity profiling (or mixtures of (drug) substances in general)

As already mentioned, to optimize separations in chromatography, electrophoresis, or electrochromatography, often the screening is not needed because the importance of the factors on the selectivity is known. For instance, in reversed-phase high-performance liquid chromatography (RPLC), the stationary phase, mobile phase pH, organic modifier composition, gradient slope, and column temperature are, in decreasing order, important for the selectivity. These most important factors then are optimized using a sequential approach, in which experimental designs also can be included to optimize a couple of factors simultaneously. Such approach can, for example, be applied to develop drug impurity profiles.

Impurities in drugs should be identified, qualified, and/or quantified, depending whether or not certain threshold (concentration) limits are exceeded [111]. Therefore, pharmaceutical companies

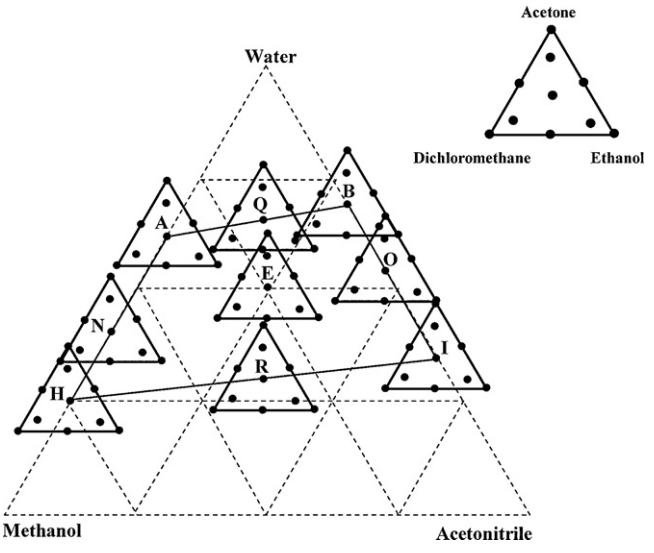

Fig. 14. Crossed mixture design (9 experiments) and a simplex centroid (10 experiments) for the optimization of the mobile phase composition and the extraction solution, respectively.
Reproduced with permission from [103].

develop a drug impurity profile, i.e. '*a description of the identified and unidentified impurities present in a new drug substance*' [111].

Often, chromatographic impurity profiles are developed using RPLC, and should allow detecting and separating all (un)identified impurities in each new drug. In ref. [112], various HPLC mass spectrometry (HPLC–MS) methods were screened in order to obtain a generic impurity profiling approach. Finally, four methods, using different stationary and mobile phases, and gradient elution procedures, were selected as orthogonal or dissimilar systems.

In refs. [113–115], a sequential approach is defined to develop a chromatographic drug impurity profile. The factors are optimized in the sequence of importance on the selectivity. The steps distinguished are (1) selection of dissimilar columns, i.e. with different selectivities, (2) selection of a suitable column and optimization of the mobile phase pH, (3) optimization of the organic modifier composition, and (4) optimization of the gradient slope and the column temperature [113,114].

The drug impurities mixture is screened on the selected columns at different mobile phase pH values and/or different organic modifier compositions [113,114]. These latter parameters can be optimized sequentially or simultaneously. In the sequential approach, the pH is optimized first, resulting in the selection of the most suitable column and pH [113,114]. Then the organic modifier composition is optimized on the above column and at the selected pH, for instance, in a mixture design-based approach [115]. Finally, occasionally as fine-tuning of the method, parameters with less influence on the selectivity, such as the gradient slope profile and the temperature, can be optimized, for instance, in a response surface design-based approach [115].

In chromatography, it is not suitable to model selectivity factors or resolutions, i.e. parameters describing the separation, as a function of the examined factor(s). For each impurity a model is build, relating its retention to the evaluated parameters. This allows predicting the retention of each impurity at intermediate parameter values. Then, for each composition, the predicted retentions and corresponding peak widths (considered constant in gradient elution and modeled for isocratic elution) are sorted, enabling the calculation of the separation responses. The optimal conditions are those where the separation responses are highest, i.e. where the worst separated peak pair is separated best [114,115].

Impurity profiles can also be developed using electrophoretic or electrochromatographic methods. Electrophoretic profiles were developed using capillary zone electrophoresis (CZE) [116] and non-aqueous capillary electrophoresis (NACE) [117], both coupled to electrospray ionization mass spectrometry (ESI–MS). Three CZE–ESI–MS methods and one NACE–ESI–MS method were selected as being orthogonal. Electrochromatographic impurity profiles were developed using open-tubular capillary electrochromatography (OTCEC) coupled to ESI–MS, and two methods were chosen as orthogonal [117]. The orthogonality or dissimilarity of four HPLC–MS [112], three CZE–ESI–MS [116], one NACE–ESI–MS [117], and two OTCEC–ESI–MS [117] methods was evaluated, in order to define a generic approach for impurity profiling.

4.2.2. Supersaturated designs as screening designs

To our knowledge, only a few research groups examined the use of supersaturated designs in analytical chemistry, i.e. these of Cela, Phan-Tan-Luu/Sergent, and our own. Analytical applications considered are screening for contaminants in composite samples [118–123], screening for important factors in the context of optimizing formulations [124] or processes [54], and method optimization or robustness testing of pharmaceutical assays [125].

The group of Cela has several publications applying supersaturated designs [118–123]. The designs were used in the development of a new procedure for sample composition determination, called the strategic sample composition (SSC), which is to be applied in environmental or food safety screening campaigns. To test for contaminants, usually, conventional composite samples, obtained by mixing several individual samples, are analyzed. If such composite sample is found to be 'positive', all original samples in the composite must be analyzed individually to identify only those that are really above the threshold limit of the considered contaminant, in order to locate the source of contamination. In SSC, a SS design is used to define the composition of a set of samples. The rows represent the composite samples constitution, and the columns the original samples. The (−1) and (+1) levels in the two-level SS designs indicate the absence or presence of the original sample, respectively. First, the conventional composition sample with all original samples present (all at (+1) level) is analyzed. When a 'negative' result is obtained, the evaluation is ended. On the other hand, when obtaining a 'positive' result, the composite samples, defined by the SS design, are analyzed. Then the obtained data are processed using genetic algorithm-based regression [53] to indicate those original samples that are above the threshold limit and to estimate the analyte concentrations in these samples. SSC was already applied to the screening of trace metals [118], polycyclic aromatic hydrocarbons (PCB's) [119], anti-inflammatory drug residues [120], pesticides [121], and polychlorinated biphenyls [122] in water samples, and of PCB's in milk samples [123].

The group of Phan-Tan-Luu and Sergent also presented some analytical applications [54,124]. To optimize a wet granulation process, a supersaturated design (N_{SS} = 16, f_{SS} = 30) was constructed as a half-fraction of a 32-experiments PB design. The design was used to screen 28 factors potentially influencing the results of the granulation process [124]. The method described higher in Section 3.1 (Fig. 10) was applied to analyze the results, and six factors were found important.

In ref. [54], a supersaturated design (N_{SS} = 18, f_{SS} = 31) was constructed as a half-fraction of a 36-experiments PB design, and used to screen factors influencing the preparation of sulfated amides from olive pomace oil fatty acids. The conversion from olive pomace oil fatty acids to sulfated amides involves the following process steps: saponification, hydrolysis, esterification, amidation, and sulfation. These sulfated amides are then applied as lime soap dispersant. Six factors were found to influence the reaction yield most, and three intermediately, after applying the above discussed data analysis (Section 3.1).

Our group performed several robustness tests on an optimized Flow Injection Analysis method to assay *L*–*N*-monomethylarginine. Several designs, containing different numbers of experiments, were compared [125]. Both PB (N = 8, f = 7 or N = 12, f = 11) and SS (N_{SS} = 6, f_{SS} = 10, constructed as a half-fraction of a 12-experiments PB design) designs were examined. It was evaluated whether reducing the number of experiments from 12 to 8 or 6, leads to similar factor effect and critical effect estimates, and whether the same effects are considered (non-)significant. To estimate the factor effects from the SS designs, the FEAR method, described above in Section 3.1 (Fig. 11), was used.

Generally, the estimated (critical) effects were similar for all designs, although those from the SS designs tended to be somewhat overestimated. From all designs, the method was considered robust, since no significant effects were found for the response describing the quantitative aspect of the method. For other responses, such as peak height and residence time, significant effects occurred. For these responses, the most important factors were indicated as significant from all applied designs.

5. Conclusions

This review gave an overview of both classic and advanced experimental design set-ups and their data interpretation. Rather uncommon experimental set-ups, such as D-optimal designs or

supersaturated designs as screening designs, were considered. The advanced or adapted data interpretations where we focused on were the adapted algorithm of Dong and the estimation of factor effects from supersaturated design results. As analytical applications, first a short overview is given of applications with classic experimental designs. This is followed by a discussion on the development of drug impurity profiles, and one about applications using supersaturated designs as screening designs.

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