Application of statistical 'design of experiments' methods in drug discovery

Heather Tye

The use of 'design of experiments' (DoE) is a revolutionary approach to optimisation and screening of experimental parameters. Simple experimental designs and statistical tools for data analysis can provide much information about the system under investigation after only a few experiments. Such information can be key in decision-making for further experiments and can enable the development of robust and reliable protocols for chemical synthesis, analytical methods or biological assays. Coupling of design of experiments with modern high-throughput automation systems has the potential to maximise the capabilities of these systems and give increased productivity for many drug discovery applications.

Heather Tye
Evotec OAI
151 Milton Park
Abingdon
Oxfordshire
UK OX14 4SD
e-mail:
heather.tye@evotecoai.com

▼ Statistical methods have become increasingly prevalent in the drug discovery arena. Most researchers are familiar with the many QSAR (quantitative structure–activity relationship) tools, used extensively for drug design and prediction of ADME-Tox properties of target compounds [1]. Fewer researchers will be familiar with the use of statistical tools for experimental design and optimisation (so-called 'DoE' methods); however, DoE was first exploited in 1958 by Fisher, and has since been used extensively in agriculture and engineering, among other disciplines [2,3]. Nevertheless, we must consider that scientists are creatures of habit and, whereas the advent of QSAR methods created a new paradigm, DoE methods challenge an existing one that is well-entrenched in our discipline(s).

Why should researchers consider using DoE and changing the way we approach experimental design and protocol optimisation [4]? The use of an intuitive approach to optimisation, 'changing one separate factor at a time' (COST), often does not lead to the real optimum and gives different implications, depending on the starting point. The COST approach also requires many experiments for little gain in information about the system under investigation. In contrast to this, DoE offers an organized approach that connects experiments in a rational manner, giving more precise information from fewer experiments. Use of DoE also allows the signal to be decoupled from background noise, enabling an estimation of the inherent error. One useful output of DoE is a response surface map of the experimental region, which facilitates the decision-making process when determining the next most appropriate phase of experimentation.

Why not use DoE? One of the main reasons for not using DoE is based on a fear of statistics, which many researchers consider to be a complication. With the advent of sophisticated computer packages that are specially designed for DoE, this is no longer a valid excuse. Although an appreciation of the meaning behind several statistical tests is needed to successfully conduct DoE, there is no requirement for an in-depth knowledge of statistics. Indeed, many of the software packages lead the user through the data analysis without a statistical equation in sight! Several DoE packages are commercially available, four of the most commonly used being MODDE from Umetrics (http://www.umetrics.com), Design Expert from Statease (http://www.statease.com), Fusion Pro from S-Matrix (http://www.smatrix.com) and JMP from SAS Institute (http://www.jmp.com). All of these packages offer a suite of experimental designs for screening, optimisation, response surface modelling and robustness testing. In each case, the user is taken through the design and

1359-6446/04/$ – see front matter PII: S1359-6446(04)03086-7

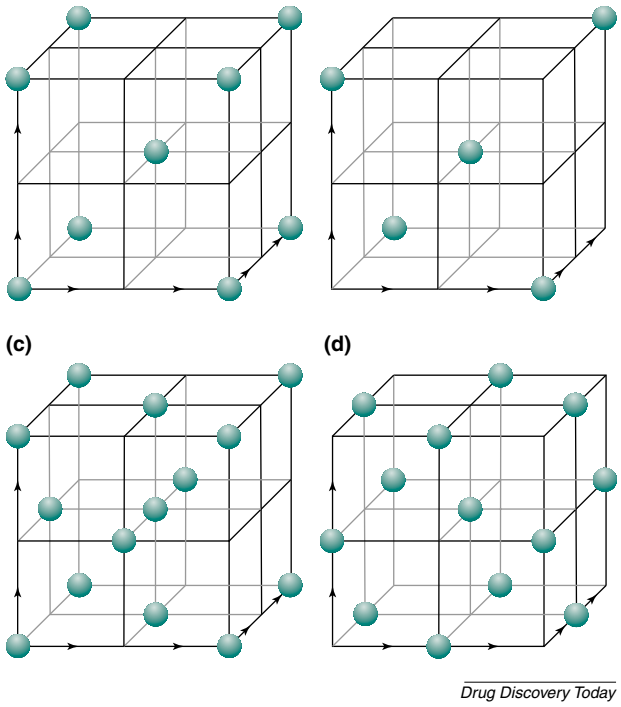

Figure 1. Design regions for different types of design. The examples shown are for two-level designs, using three factors (variables) and can be modelled on a cube to represent the experimental region being explored. **(a)** Full factorial design (used for screening), in which each corner of the cube represents a combination of the factors used in an experiment. A centre point is included and is usually replicated three times to determine reproducibility and detect non-linear responses. **(b)** Fractional factorial design (used for screening), which is based on the full factorial design but with two of the corner points omitted to cut down on the number of experiments conducted. **(c)** Central composite CCF design (used for response surface modelling) is derived from the full factorial design with additional points at the centres of the faces to approximate a sphere. **(d)** Box-Benhken design (used for response-surface modelling) is an alternative to the CCF design, where the centres of the cube edges are sampled again to approximate a sphere.

analysis phases in a logical way, and the results can be presented in several diagrammatic formats.

A further reason for not using DoE is the number of experiments that have to be performed in parallel; these can be reasonably large, depending on the complexity of the design. However, this has been countered by the advent of highly automated systems for the performance of experiments and, in many cases, on-line data acquisition and analysis. The coupling of these automated systems with DoE provides a powerful tool for protocol development.

The phases to the DoE approach

There are several phases, as listed here:

- Identification of factors that may affect the outcome of the experiment and responses that give a measure of the outcome.
- Choosing an appropriate experimental design, either for screening or response surface modelling.
- Generation of a design matrix, determining which experiments will be conducted.
- Conducting the experiments.
- Fitting the data and generating plots that describe the trends in the results.
- Drawing conclusions and planning the next step.

There are many types of experimental design (Figure 1 gives some examples). The most commonly used and simplest to understand are those that are based on factorial designs. Take, for example, a set of experiments in which three factors are thought to be important. A two-level factorial design can be generated, for which a low and high value of each factor is used. The design can be envisaged as a cube, and the possible combinations of the factor levels (low or high) are at the corners of this cube. The cube is, thus, a representation of the experimental region being explored. Many other design types are based on this cubic model with experimental points at corners, centres of faces, centres of edges and so forth. For designs in which more than three factors are adjusted, the same concepts apply except that, in these cases, a hyper-cube represents the experimental region (although this is not so easy to envisage). Such cubic designs are popular because they are symmetrical and therefore straight-forward to model using statistical methods, which, in turn, means that trends in the results are easier to interpret.

The choice of design is usually a compromise between the information required and the number of experiments to be conducted. For simpler screening of experimental parameters, low resolution designs will usually suffice (key definitions given in Box 1). These are usually full or fractional factorial designs (Figures 1a,b). Fractional factorial designs are often lower resolution than their full factorial counterparts because they require fewer experiments and, hence, provide fewer data. The most economical design, in terms of the number of experiments, is fractional factorial Resolution (Res) III, but this design does not allow determination of interaction effects. For interaction models, Res IV or V designs are required; however, some of the interaction terms in the model will be confounded with others and further experimentation might be required to decouple these terms at a later stage. Nevertheless, fractional factorial designs are useful for first sets of experiments, to determine which of a set of factors are significant.

The screening designs will only support linear responses, so if a non-linear response is detected or a more accurate picture of the response surface is required, a more complex

design-type is necessary. Designs such as Box-Benhken or Central Composite Face (CCF) will support non-linear responses and are generally used for response-surface modelling (RSM) and optimisation applications (Figures 1c,d). It is generally best to conduct an initial screening phase with a larger number of factors, using a low-resolution fractional factorial design. When the key factors have been identified, an optimisation phase can be performed, using an RSM design. In this way, the total number of experiments is kept to a minimum. A further design-type is the D-optimal design [5]. This is a computer-generated design that is suited to situations in which a large number of qualitative factors (solvents, reagents, buffers and so on) need to be included in the design.

Box 1. Definitions of Key Terms

Factor: Experimental variable, which can be quantitative (time, temperature etc.) or qualitative (solvent, buffer etc.).

Response: Property of the system that is being measured. For example, yield, purity.

Interaction: A state where two or more factors are dependent on each other.

Confounding: Effects that cannot be estimated separately (e.g. a main effect cannot be separated from an interaction effect in the statistical model).

Resolution: Measure of the degree of confounding. Low-resolution designs are highly confounded and can only give limited information about the system under investigation.

Applications of DoE in the drug discovery process

The use of DoE in all areas of drug discovery has steadily increased over the past few years but it is far from being adopted as standard practice; many more demonstrations of the value of this method must be published before this can happen. Here, a broad selection of examples have been used to demonstrate the wide applicability of DoE. Although, of course, there are always new applications awaiting demonstration.

Chemical process development

Early examples of the application of DoE to chemical problems derive from process development, where optimisation of reaction protocols is of key importance to the success of the drug manufacturing process [6]. Finding robust and reliable protocols in as short a time frame as possible can save considerable sums of money. The use of DoE in this area has been shown to be of considerable benefit in comparison to traditional methods. In many cases, the additional advantage of coupling DoE with the use of automated reaction systems has led to the identification of optimum reaction conditions in a short time-period (weeks, as opposed to months). Owen and colleagues have provided a detailed description of how to apply DoE in a development chemistry setting [7]. This publication is, essentially, a blueprint for any researcher who wishes to learn how to use DoE for the optimisation of a chemical process.

More recently, Larkin *et al.* have highlighted the need to decouple reactions from work-up and isolation steps when performing DoE methods [8]. In their synthesis of 17α-methyl-11β-arylestradiol, they needed to optimise an aromatisation step, using acetyl bromide and acetic anhydride. Initial results were confused by the application of DoE to the full reaction protocol, including a saponification and crystallization. Further experiments focusing on the reaction step led to the identification of the optimal reaction conditions. Several examples of the application of DoE to screening and optimisation of reaction conditions during early process development have demonstrated the value of this approach over more traditional methods [9–12]. The use of DoE in process development has also been extended to the optimisation of crystallization conditions for the isolation of products with high yield and purity [13]. Here, another advantage of DoE becomes apparent: it is possible to model both yield and purity of the product simultaneously. An increased understanding of the process is gained as a result, making it possible to determine a 'sweet spot' on the response-surface, which satisfies both criteria at once.

Discovery chemistry applications

Much more recently, the use of DoE has extended into smaller-scale chemistries that are conducted at the earlier, discovery phase of a drug discovery project. The synthesis of small arrays and larger libraries of compounds requires a validation phase in which the chemical steps are optimised to ensure that compounds are prepared in sufficient quantity and purity for biological testing. Until recently, traditional methods have been used for this validation process, but a few examples of the application of DoE to this type of problem have recently emerged.

An early example of the application of DoE to the validation of chemistry before synthesis of a compound array was demonstrated by Jamieson *et al.* [14]. In this case, a DoE approach was employed for the optimisation of an amide formation, using a solid supported carbodiimide reagent. The use of DoE in conjunction with an automated reaction system, employing IRORI® Kan™ technology, enabled the chemistry validation to be performed rapidly, and an array of 80 amides to be prepared as a result. A

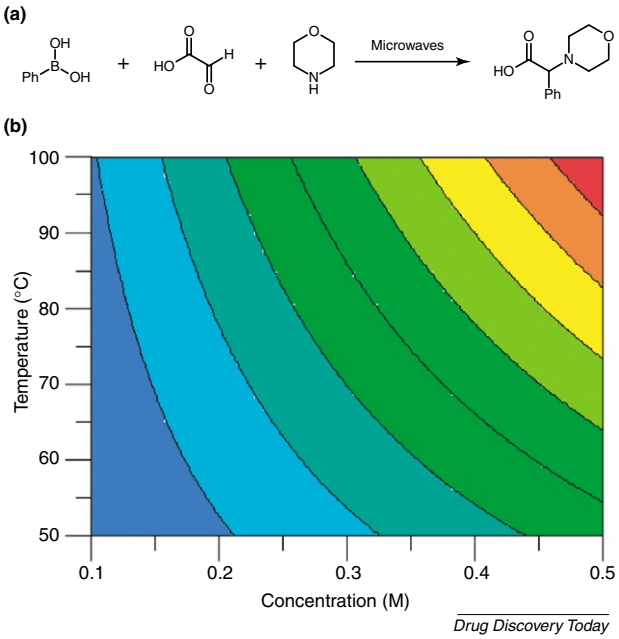

Figure 2. **(a)** Petasis reaction of phenyl boronic acid, glyoxylic acid and morpholine under microwave-assisted conditions. **(b)** Response contour plot for the microwave-assisted Petasis reaction. Contours show variation in the response (conversion) as the temperature and concentration of the reaction is varied. The red contour shows the region of highest conversion, which, in this case, corresponds to the highest temperature and concentration in combination.

further example of using DoE to optimise a reaction involving a solid supported reagent has been published by the same group [15]. Design Expert® software was used to generate a fractional factorial design that enabled the identification of the main factors affecting the yield in a S_NAr reaction. The optimal conditions that were identified in this initial screen were robust enough to be employed in the synthesis of an array of compounds.

Gooding and co-workers have reported an example in which a DoE approach was employed for screening and response-surface modelling of an amide formation reaction employing a polymer-supported HOBt (1-hydroxybenzotriazole) derivative [16]. The authors used a 2^{5-1} fractional factorial design to explore the main factors such as solvent composition, equivalents of reagents and reaction time. From the screening results, an RSM design was devised in which the main factors were varied and an additional factor of substrate type was added, generating a set of 25 reactions to be run. The results of this study identified a non-linear response to one of the reagents employed and enabled effective optimisation of the reaction conditions for both types of substrate studied. Subsequent to this, the optimal conditions were employed for the synthesis of a 3x16 library of amides, giving excellent results, except for the most chemically demanding cases. This is a good example of the way that DoE can simplify an otherwise complex optimisation process and reliably lead to a set of robust optimal conditions.

Another up-and-coming technology that is increasingly used for chemical synthesis is microwave irradiation [17]. Sophisticated apparatus are now available, allowing control of parameters such as power input, temperature and pressure for single and multi-mode microwave reactors. This enables reactions to be conducted on mili- to multigram scales, under reproducible conditions. One of the problems that are encountered when seeking to employ microwave-assisted heating in a synthetic reaction is knowing where the optimal conditions lie. This is particularly significant when working in pressurised systems that allow working conditions beyond those that are normally available in a synthetic laboratory. We and others have recently demonstrated the power of using DoE to identify optimal conditions for microwave-assisted reactions. Evans *et al.* employed a central composite CCF design to optimise the microwave-assisted synthesis of 1,2,4-oxadiazole derivatives [18]. This optimised procedure was subsequently used for the preparation of an array of 24 such derivatives. In our own work, we have used a DoE approach for the screening and optimisation of microwave reaction conditions and catalyst–solvent combinations [19,20].

The first example is of a microwave-assisted Petasis reaction (Figure 2a) [19]. The reaction between phenyl boronic acid, glyoxylic acid and morpholine, under microwave conditions, was screened using a two-level fractional factorial design. The factors that were varied were temperature, time, concentration and solvent. This screen determined that the solvent should be dichloromethane, temperature and concentration should be at high settings, and time was relatively irrelevant (Figure 2b). The screen was able to identify the key factors but did not meet our set objective because the conversion achieved was too low. Instead of running a further DoE design, we made use of a feature in the MODDE software called 'the optimiser', which allows extrapolation outside of the original reaction space, in the direction of the optimum. Using this feature, a set of reaction conditions was identified at higher temperature and concentration, which were predicted to give a better conversion. When these reaction conditions were applied, a significant improvement in the conversion was observed and the result that was obtained was considered to be optimal for this reaction. This method was then applied to the synthesis of an array of 19 compounds with good results in most cases. Thus, in just a few experiments (21), we were able to identify the key factors and optimal conditions for

the microwave-assisted reaction, using a DoE approach.

A second example is the screening of microwave reaction conditions and catalyst/solvents for a Biginelli reaction (H. Tye and M. Whittaker, unpublished). A microwave-assisted Biginelli reaction has previously been reported by Kappe *et al.* for the synthesis of an array of 48 dihydropyrimidines [21]. The conditions that were identified varied according to the substrates employed and the best Lewis acid catalyst identified was the expensive $Yb(OTf)_3$. The Biginelli reaction (condensation of a β-keto ester, urea and aldehyde to form dihydropyrimidines) is an ideal candidate for DoE optimisation and it was considered interesting to explore whether Kappe *et al.* had identified the best reaction conditions during their studies using conventional optimisation methods. To determine optimal microwave-assisted reaction conditions, a catalyst and solvent system (copper(II) triflate, acetronitrile) that worked well under thermal conditions was chosen initially (Figure 3a). A two-level fractional factorial screening design was generated in which mol% of Lewis acid, temperature, time, equivalents of urea and concentration were varied, as these were expected to be important factors. 19 reactions (including three centre points) were conducted and the data fitted using the MODDE software. The main factors were identified as concentration, mol% Lewis acid and temperature. A non-linear response was detected for the Lewis acid, and so a squared term in this factor had to be added to the model. The best conditions, giving more than 90% conversion, were identified to be 15 mol% Lewis acid at 80°C, for 10 min with 1 equivalent of urea and a reaction concentration of 0.5 M (Figure 3b). These results were consistent with those produced by Kappe and co-workers, who typically obtained a more than 90% yield when using 10 mol% catalyst, 120°C for 10 min at a reaction concentration of 3.3 M, albeit using a different catalyst and solvent. The next phase was to screen a range of Lewis acids and solvents under the optimal conditions as identified by the screen to see if copper(II) triflate was indeed the optimal catalyst. For this purpose, a D-optimal design was employed – this can cope well with multiple

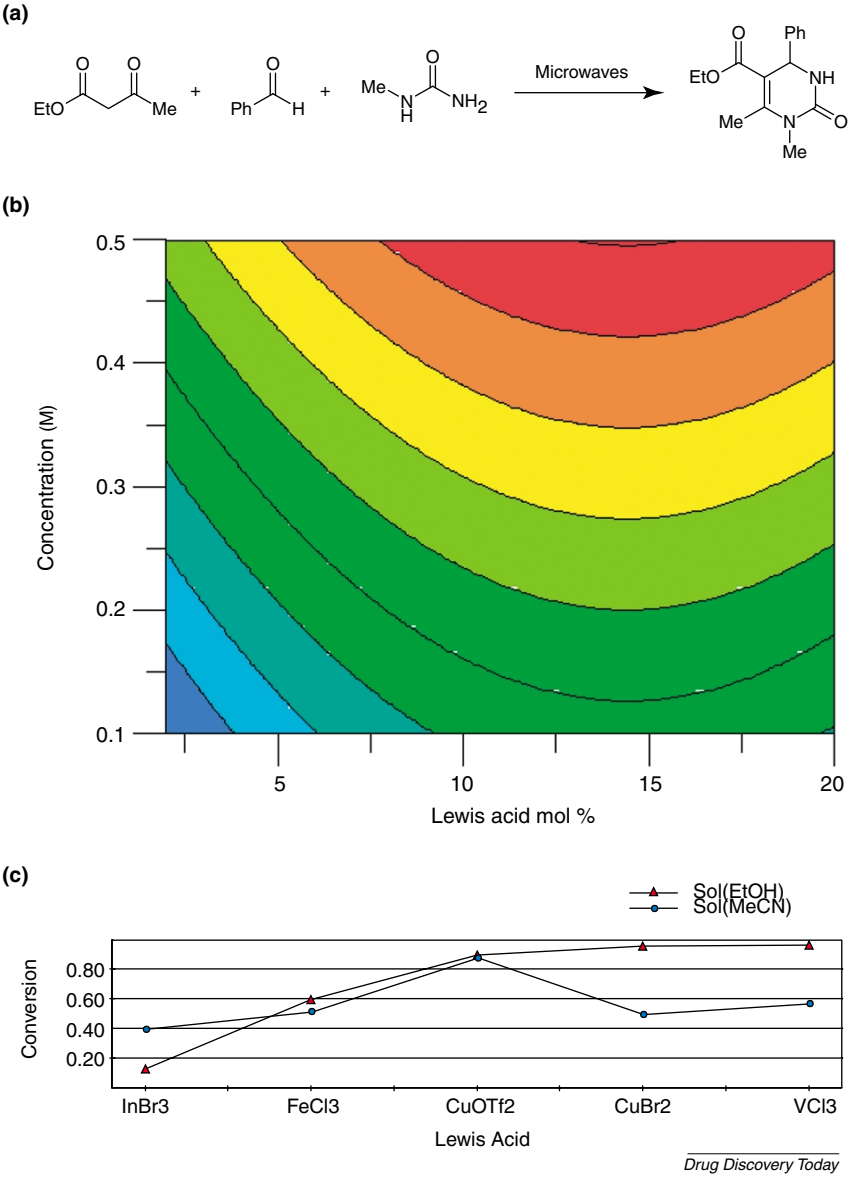

Figure 3. **(a)** Biginelli reaction of N-methyl urea, ethylacetoacetate and benzaldehyde under microwave-assisted conditions. **(b)** Response contour plot for the microwave-assisted Biginelli reaction. Contours show the variation in conversion across a range of reaction concentrations and mol% of Lewis acid. Curvature in the contours shows a non-linear response, with respect to mol% of Lewis acid. The darkest red contour shows the optimal region, corresponding to the highest concentration and 15 mol% of Lewis acid. **(c)** Effects interaction plot for the Biginelli reaction solvent/catalyst screen. Conversion (%) is plotted against several Lewis acids on the *x*-axis. The red triangles represent conversion achieved in EtOH solvent and the blue circles represent conversion achieved in MeCN solvent. The plot shows that there is a strong interaction between solvent and Lewis acid employed with EtOH solvent, giving a high conversion across a wider range of Lewis acids.

levels of qualitative factors. Five Lewis acids and two solvents were included in the design, giving 17 runs with three centre points. From the results, an interaction plot (Figure 3c) was plotted, showing that, when using acetonitrile as solvent, copper(II) triflate was the optimum catalyst. If, however, ethanol was employed as solvent, both copper(II) bromide and vanadium(III) chloride gave comparable results to copper(II) triflate. This result demonstrates the power of using the DoE approach, which can readily capture interaction effects between factors that more traditional approaches might well miss.

Analytical and purification applications

Use of a DoE approach can also be beneficial when attempting to optimise an analytical method or purification process using chromatographic techniques. Cole *et al.* have recently demonstrated the use of this approach in the optimisation of a high-throughput semipreparative LC method for the purification of compounds resulting from library synthesis [22]. In this case, the goal was to find conditions that gave a minimum run time, maximising through-put without compromising the separation achieved. The use of a DoE approach enabled the identification of the key factors that affected run time and separation. These were then optimised, resulting in conditions that gave a 50% reduction in the run time, a 25% reduction in solvent usage and a significantly improved resolution by comparison to the previous method used.

DoE has also been applied to the enantio-resolution of salbutamol by capillary electrophoresis and the development of an assay for an anticancer agent in pharmaceutical formulation studies [23,24]. A further recent application has been the optimisation of fusion protein purification, using metal affinity chromatography [25]. DoE enabled the better characterisation of fusion protein chromatography, in terms of yield and purity of the protein produced. Some of the key variables were found to interact significantly. This fact could only be determined via a DoE approach, again highlighting the value of this approach over the traditional approach of changing one variable at a time.

Biological applications

Like the synthesis of chemical libraries, biological assays can now be conducted on highly automated high-throughput platforms, thanks to recent advances in robotics and miniaturisation. However, the benefits of such high-throughput technologies can only be realized if high quality, robust and reliable assays can be performed with these systems [26]. In contrast to chemical synthesis systems where the size of the response signal (e.g. yield or purity) is of key importance, biological assays are best optimised by minimising signal variance and improving the signal:noise ratio. In a previous article in *Drug Discovery Today*, Michael Lutz gave a comprehensive account of how the use of a DoE approach is highly beneficial in assay development and optimisation [27]. One example in which signal variance was minimised was the optimisation of a scintillation proximity assay. The objective of the study was to minimise the variance of the K_i being measured and to reduce the amount of receptor used as far as possible. A full factorial design was employed and it was found that the main factors affecting variance in K_i were receptor and ligand concentrations. It was found that the best conditions, in terms of reducing variance and giving a good signal:noise ratio, required high concentrations of the receptor – a situation that was not desirable. However, the use of a DoE approach had yielded a good understanding of the system and it was possible to find a compromising set of conditions at the lowest ligand concentration that satisfied both of the objectives. A more recent example of combining DoE with automation was provided by Taylor *et al.* [28]. They demonstrated the use of a DoE approach for the optimisation of several biological assays, enabling them to be implemented on a high-throughput platform. Assay optimisation using their approach could be achieved in days, rather than months, and could be reliably used on an automated platform.

An alternative platform to microtitre plates for screening activity, particularly in the field of functional genomics, is the microarray (here, materials of interest are spotted as an array on a suitable substrate). In the field of functional genomics, this approach has enabled the measurement of the expression of thousands of genes simultaneously. One of the major problems that is associated with the use of such arrays is the complexity of the experimental protocols that need to be developed, such as probe amplification, array production, target labelling and hybridisation. A DoE approach is ideally suited to such complex systems, as shown by Wrobel *et al.*, who used it for the development of a buffer and spotting conditions for the production of a high-density microarray with increased reliability [29].

Conclusion

Although 'design of experiments' methods have been around since the mid-20th century, their application in the disciplines of drug discovery has only recently taken hold. The value of using such an approach for the screening of experimental parameters and optimisation applications cannot be disputed. The advent of sophisticated software packages that facilitate the implementation of DoE have

enabled non-specialists to employ DoE in their everyday work. There are an ever-increasing number of examples being published, a trend that is set to continue throughout the drug discovery business over the coming years. The full value of DoE will no doubt be demonstrated by such examples and it is possible that such an approach, in some form, will become routine in the future.

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