amc technical brief

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Experimental design and optimisation (1): an introduction to some basic concepts

Analytical scientists all too frequently think that the use of statistics and chemometrics is confined to the treatment of data obtained in completed experiments. In reality the proper planning of experiments using rigorous methods is equally important: without it the measurements may not produce results of the required quality, or may be unnecessarily elaborate. The linked areas of experimental design (ED) and optimisation are thus crucial areas of chemometrics. Some basic ideas that underpin these topics are introduced here.

Two Examples

In a classic mathematical puzzle, we are provided with 12 identical-looking balls, one of which is lighter or heavier than the other 11. Given a balance (but no weights) how can we identify the odd ball, and determine whether it is lighter or heavier than the rest, *in just three weighings*? This problem reflects the approach we should use in planning a chemical experiment: it demands that we use a minimal number of measurements to get the best outcome. And as in chemistry the solution to this problem – not simple! – requires careful thought and planning.

For a chemical example, we turn to the analysis of a drug and its metabolites in a urine extract using reversed-phase hplc (rp-hplc). The results of the analysis will depend on quite a large number of experimental variables, or **factors**. Some of the factors will relate to the mobile and stationary phases, some to the detector used (assumed to be optical), and some to other experimental conditions (see Table 1).

Table 1: Some factors affecting rp-hplc analysis

Mobile phase: Isocratic or gradient; organic modifier; pH, ionic strength, composition of the buffer component.
Stationary Phase: Chemical nature; particle size; manufacturer; batch number.
Chromatographic conditions: Temperature; flow rate.
Detector: Wavelength, spectral bandwidth.

Some terminology

While most of the factors in Table 1 are **quantitative**, others are **qualitative** in nature (e.g. MeOH or MeCN as the organic modifier). In either case the values they take are referred to as **levels**. An ED is thus characterised (in part) by the number of factors involved, and the number of levels of each that are studied. All the factors in the Table are **controlled** factors – their levels can be altered by the experimenter. In practice an analysis may also be affected by **uncontrolled** factors: for example reagent instability or instrumental drift might result in time-dependent trends in the results. Such uncontrolled effects are undesirable, and EDs which minimise them are available.

Crucial Steps

To get the best results, as efficiently as possible, out of an experiment three steps are involved:

• ***The aim of the analysis must be defined exactly***. In our example we might require the quantitative analysis of the parent drug only; or we might wish to resolve the drug and as many metabolites as possible; or we might seek an intermediate outcome, for example the analysis of the drug and its main metabolite. Each of these targets may require distinct experimental conditions, so the ED may be different in the three cases. It may seem obvious to state that the aim of an experiment must be very closely defined, but many ED and optimisation processes have failed because this first step was not properly considered.

• ***The factors affecting the outcome most significantly must be identified.*** This is the important ED stage.

• ***The levels of these crucial factors must be optimised.*** In principle it is sensible to apply optimisation methods only to the factors that really make a difference to the experimental outcome. But in practice the ED and optimisation steps are sometimes effectively combined.

Identifying the critical factors

In many experiments, although the number of factors is potentially large, the number of critical factors which have major effects on the outcome is gratifyingly smaller. How should we try to identify these? The most obvious method seems to be to take each factor in turn, and observe the effects of changing its level in two or more experiments while keeping the levels of the other factors fixed. This "one-at-a-time" approach is unacceptable for two reasons. First, in any analytical method in which a substantial number of factors are involved, the approach would be impossibly long-winded. The second problem with one-at-a-time studies is illustrated using our rp-hplc example again. Suppose we find in two experiments that changing the organic modifier from MeOH to MeCN at pH 7 improves the resolution of two chromatographic peaks, from 1.20 to 1.30. Suppose we also find in two further experiments that changing the pH of the aqueous solvent component from 7 to 8, the modifier being MeOH in each case, again improves the resolution from 1.20 to 1.30. What happens if we make both changes at once? The problem is simply summarised in the following Table:

Table 2: Effects of two factors on reversed phase-HPLC resolution

	MeOH	MeCN
pH 7	1.20	1.30
pH 8	1.30	*

If the effect of altering both factor levels is to give a resolution of 1.40 for the table entry marked *, then the two factors are said to be **additive**. But in practice the resolution obtained when both alterations are made simultaneously may be significantly greater or less than 1.40, in which case the two factors are described as being **interactive**. In this case the interaction means that the effect of changing the pH *depends upon* the choice of organic modifier, and *vice-versa*. Obviously such effects could not be detected by studying just one factor at a time. Table 2 also highlights a further problem encountered in practice. Suppose that when both the factor levels are changed the resolution is found to be 1.43. Has a (slight) positive interaction between the two factors occurred, or is the value 1.43 simply the result of random measurement errors, i.e. is it not significantly different from 1.40? It seems that to answer that question it would be necessary to make replicate measurements so that the effects of random errors can be separated from interaction effects, for example by using analysis of variance (ANOVA). In summary, efficient EDs involve varying two or more factors at once, to minimise the effort involved and to study interactions; but separating the interactions from random errors may require extra work. Compromises may thus be necessary, and the chemist must choose the design best suited to the identified aims of the overall experiment.

Factorial Designs

Table 2 provides the simplest example of what is known as a **complete factorial design**. We have two factors, each studied at two levels, so $2^2 = 4$ measurements are needed to study all possible combinations of the factors and levels. If each experiment is duplicated, to obtain an estimate of the random measurement error, $2^{2+1} = 8$ measurements are necessary. In general, if k factors are studied at 2 levels, the number of measurements in a complete factorial design is 2^k if the experiments are not duplicated, 2^{k+1} if they are. With 5 factors, therefore, either 32 or 64 measurements would be needed: in most cases such a protracted effort would be impossible. Complete factorial designs are evidently tedious in many cases, so **fractional factorial designs**, in which only some of the combinations of factors and levels are studied, are commonly used.

When there are 3 or more factors 3-fold or higher level interactions can occur. In our rp-hplc example if we studied the pH and the organic modifier as before, and also studied the buffer ionic strength, the three factors might all depend on each other simultaneously in their effects on the measured resolution. In practice it is often assumed that such higher order interactions are unlikely, so when their apparent values are calculated they can be used as a measure of the random errors occurring. This is one method by which the number of measurements in EDs might be kept within reasonable bounds. The use of only two levels for each factor (called **high** and **low** levels and given the symbols + and -, or 1 and 0, respectively in tables that summarise the measurements to be performed) also helps to keep the number of measurements down. But with quantitative factors it raises the question of how the high and low levels of each factor should be selected. If the two levels are too close together, the outcomes might not be distinguishable because of random errors. If they are too far apart they may give similar responses which actually lie on opposite sides of the best value. General knowledge of the analytical method may help the chemist to resolve this dilemma, but we could also use more than two levels for each factor. Such designs allow curved **response surfaces** for the experiment to be modelled. Again the number of measurements rises rapidly: a two-factor, three level design has $3^2 = 9$ measurements (Table 3). The three levels are designated 1, 0, and -1 here.

Table 3: Factorial Design: 2 Factors, 3 Levels

Factor	Levels
A	1 1 1 0 0 0 -1 -1 -1
B	1 0 -1 1 0 -1 1 0 -1

Summary

We have shown that, while good ED has much to offer, it may not be easy to combine the twin goals of identifying critical factors while performing a small number of measurements. Later papers will survey a range of practical ED and optimisation methods.

Available Software

Established suites of statistical software offer a wide range of ED facilities, and in many cases (*e.g.*, Minitab®) a good deal of tutorial guidance on their use.

This Technical Brief was prepared for the Analytical Methods Committee by the Statistical Subcommittee (Chairman M Thompson) and was drafted by J N Miller.

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