amc technical brief

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Experimental design and optimisation (2): handling uncontrolled factors

The first Technical Brief of this series showed that the results of even simple analytical methods are affected by a large number of experimental factors. To identify the most important factors with the minimum number of experiments, experimental design (ED) methods are used. There is an important additional need to be able to handle uncontrolled factors: this is normally done using randomization and blocking methods.

Trends and Uncontrolled Factors

In many experimental designs trends and/or **uncontrolled** factors may occur. We may be aware that the temperature in a laboratory increases during the day, and feel that this change should not affect our analytical results, but that may or may not be correct! Uncontrolled factors of which we are unaware may also occur: examples include the change in the pH of a solution due to absorption of atmospheric CO_2; drifts in the sensitivity of a detector; small differences in the impurity levels in a reagent used in consecutive experiments; and so on. Some uncontrolled factors are far from obvious, but it is still essential to take precautions against them. Failure to do so ignores one of the fundamental assumptions of the analysis of variance (ANOVA) methods described below which indicate the significant factors in EDs. ANOVA assumes that the observations in the trial experiments are independent of each other: this is clearly not true if there is a trend in the conditions, as successive observations will then be correlated. The most obvious way to avoid this problem is to use the process of **randomisation**, which can be illustrated with a simple example in which only one factor is studied.

Randomisation

Suppose we wish to study the effects of three different solvent compositions (A-C) on the resolution of a reversed phase hplc separation. The use of each solvent is often referred to as a **treatment**, a term derived from the early use of ED methods in agriculture. To estimate random measurement errors each solvent is used four times. If four experiments with one solvent are done first, then four with the second solvent and four with the third, we run the risk that any genuine effect of changing the solvent will be confused or **confounded** by a drift in the experimental conditions. The problem is avoided by assigning two-digit labels (01 – 12) to each experiment (Table 1), then using a random number table (or calculator or PC program) to randomise the order. Using statistical tables to provide the random numbers, we enter the table at an arbitrary point and read off the subsequent two-digit numbers, ignoring 00, 13 – 99 and duplicates. The initial numbers are then used to provide the order of the experiments; the table shows a possible outcome.

Table 1: Randomisation of Experiment Order

	Treatment		
	A	**B**	**C**
Initial number	01 02 03 04	05 06 07 08	09 10 11 12
Random order	09 04 02 10	06 05 07 03	08 01 12 11
Expt. order	C A A C	B B B A	B A C C

Although the 12 experiments are performed in a random order, the outcome evidently may still not be ideal. If, for example, the experiments were performed at the rate of 3 per day over 4 days, all the treatments using solvent B would be done on days 2 and 3, and most of those using solvents A and C would be done on days 1 and 4. Some time-dependent uncontrolled factors could then still affect the results. In other words complete randomisation may by chance leave some partial correlation.

Blocking

This difficulty can be overcome for known uncontrolled factors such as time by using the technique of **blocking**, i.e. *deliberately* dividing the experiments into groups that are (for example) performed on different days. This method is especially valuable in cases where there are known (or suspected) but uncontrolled factors, e.g. the use of a different hplc apparatus or new batches of the solvents on the separate days. In our hplc example the design could be blocked by using each of the treatments A-C once on each of the four days. To allow for uncontrolled variation within each day the order of the three treatments would be randomised, thus providing a **randomised block** design. A typical plan is shown in Table 2.

Table 2: A Randomised Block Design
Treatment Order

Day 1	A	C	B
Day 2	A	B	C
Day 3	C	B	A
Day 4	C	A	B

Data from such designs can be evaluated by using two-way ANOVA, the sources of variation then being the

between-treatment variation, the between-block (day) variation, and the random measurement error. Even if the between-block variation is (as we might hope) not significantly greater than the random error, these two sources of variation combined (i.e. in an unblocked experiment) might have been sufficient to prevent the detection of a significant between-treatment effect. In short, for any given number of measurements, blocked experiments are more sensitive than unblocked ones.

A Numerical Example
These principles are summarised by the following example, (Table 3) in which the data represent the resolution of two components separated by hplc:

Table 3: Hplc Resolution Experiment

	Solvent		
	A	**B**	**C**
Day 1	1.35	1.30	1.30
Day 2	1.32	1.32	1.33
Day 3	1.36	1.30	1.35
Day 4	1.38	1.32	1.36

Two-Way ANOVA

Source of Variation	**SS**	**df**	**MS**	**F**
Between-Block	0.00236	3	0.00079	2.34
Between-Treatment	0.00365	2	0.00183	5.43
Error	0.00202	6	0.00034	
Total	0.00803	11		

One-Way ANOVA

Source of Var^{n}.	**SS**	**df**	**MS**	**F**
Between-Treatment	0.00365	2	0.00183	3.75
Error	0.00438	9	0.00049	
Total	0.00803	11		

When the results are (correctly) evaluated using two-way ANOVA, the *F*-value for the between-treatment variation, 5.43, exceeds the critical value of 5.14 for $p = 0.05$, indicating that at this probability level the different solvents do affect the chromatographic resolution. The between-block variation is larger than the measurement error, but not significant at the $p = 0.05$ level ($F = 2.34$, $F_{crit} = 4.76$). (Note that this two-way ANOVA ignores the possibility of interactions between the blocks and the treatments, and that if this assumption is valid the measurement error can be estimated even though only one measurement for each treatment is made in each block). If blocking is neglected, and the data are studied using one-way ANOVA, the between-treatment *F*-value of 3.75 is less than the critical value of 4.26 ($p = 0.05$), suggesting that the different solvents do not have a significant effect. In both cases the between-treatment sum of squares is the same, but in one-way analysis the error sum of squares is the sum of the two-way ANOVA's between-block and error terms. The one-way error term has been inflated by the effect of run-to-run variation (the block effect) which makes the experiment less sensitive. This confirmation that a blocked experiment may be more sensitive than an unblocked one emphasises that even simple ED procedures deserve careful thought from the start.

Latin Squares
In one special type of design randomisation is not used at all. This possibility arises most obviously when the numbers of blocks and treatments are equal, as would be the case in our hplc example if each solvent had been tested three times instead of four. The **Latin Square** in Table 4 would then be possible for the solvent study:

Table 4: A Latin Square

	Treatment		
Day 1	A	B	C
Day 2	C	A	B
Day 3	B	C	A

Each treatment appears once in each column and each row of the Latin Square. ANOVA would then allow us to separate the between-block and between-treatment variations. Moreover if the treatments were applied in sequence during each day, the variation due to the time of day could also be extracted. The 3x3 Latin square has only one possible structure, but larger Latin squares where there is a choice of layouts can be used. Modified Latin Squares that do not demand equal numbers of blocks and treatments have also been described.

Conclusions
These simple examples show that uncontrolled factors must be taken seriously in EDs, and that it is possible to do this without a great increase in the number of trial experiments.

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

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