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Stat Note 10

In the tenth of a series of articles about statistics for biologists, **Anthony Hilton** and **Richard Armstrong** discuss:

The two-way analysis of variance

In a previous article in Microbiologist, Vol 8, No.2 July 2007 (Armstrong & Hilton, 2004), we described a one-way analysis of variance (1-way ANOVA) in a randomised design. In a 1-way ANOVA, an individual observation is classified according to which group or treatment it belongs and observations within each group are a random sample of the relevant population. The scenario to illustrate this analysis compared the degree of bacterial contamination on 2p coins collected from three types of premises, viz., a butcher's shop, a sandwich shop, and a newsagent. A sample of four coins was collected at random from each location and the number of bacterial colonies present on each coin was estimated. Such an experiment is often described as in a 'randomised design'. More complex experimental designs are possible, however, in which an observation may be classified in two or more ways (Snedecor & Cochran, 1980).

The scenario

We return to the scenario described in Statnote 7 (Hilton & Armstrong, 2006a). A hypothetical experiment was carried out to investigate the efficacy of two novel media supplements (S1 and S2) in promoting the development of cell biomass. Three 10-litre fermentation vessels were sterilised and filled with identical growth media with the exception that the media in two of the vessels was supplemented with 10 ml of either medium supplement S1 or S2. The vessels were allowed to equilibrate and were subject to identical environmental / incubation conditions. The vessels were then inoculated with a culture of Bacterium x at an equal culture density and the fermentation allowed to proceed until all the available nutrients had been exhausted and bacterial growth had ceased. The entire volume of culture media in each fermentation vessel was then removed and filtered to recover the bacterial biomass, which was subsequently dried and the dry weight of cells measured.

This experiment was originally carried out with 25 replicate vessels of each treatment and were analysed using a 1-way ANOVA in a randomised design; i.e., the treatments were allocated at random and without restriction to the original vessels (Hilton & Armstrong, 2006a). The present experiment, however, was carried out in a different way. First, 30 vessels were divided into 10 groups of three, each group representing 'a replication' with the intention of setting up and processing each replication (a control and each of the two treatments S1, S2) on each of 10 separate occasions and second, the treatments were allocated to the three vessels within a replication independently and at random.

The analysis appropriate to this design is analogous to that of the paired sample 't-test' described in Statnote 3 (Hilton & Armstrong, 2005) but extended to more than two treatments.

Terminology

In a two-way design, each treatment is allocated by randomization to one experimental unit within each group. The name given to each group varies with the type of experiment. Originally the terminology 'randomized blocks' was applied to this type of design because it was first used in agricultural experiments in which experimental treatments were given to units within 'blocks' of land, plots within a block tending to respond more similarly compared with plots in different blocks (Snedecor & Cochran, 1980). In the present example, the block is a single trial or replication of the comparison between treatments, the trial being carried out on 10 separate occasions. Furthermore, in experiments with human subjects, there is often considerable variation from one individual to another and hence a good strategy is to give all treatments successively to each 'subject' in a random order; the subject therefore comprising the 'block' or 'replication'.

Table 1. The effect of two novel media supplements (S1, S2) on bacterial biomass measured on 10 separate occasions

Occasion ('blocks')	Control	+S1	+S2
1	461	562	344
2	472	573	359
3	473	574	369
4	481	581	403
5	482	582	425
6	494	586	476
7	493	591	511
8	495	592	513
9	506	592	556
10	502	607	578

The statistical model

In the example given in Table 1, the fact that vessels are also grouped into replications, one complete replication for each of the 10 occasions, gives a more complex model. Using the commonly used notation to describe the basic model of an ANOVA described in Statnote 9 (Hilton & Armstrong, 2007a),

the two-way design includes a term for the replication effect 'b' in addition to the treatment effect 'a', *viz.*,

$$xij = \mu + a_i + b_j + e_{ij}$$

Hence, the ANOVA table (Table 2) includes an extra term for replications, i.e., the occasion on which the replication was sampled. In the terminology used in Statnote 9 (Hilton & Armstrong, 2007a), treatment (a_i) is a fixed-effect factor whereas blocks or occasions (b_j) are a 'random-effect' factor. In addition to the assumptions made in the randomized design, viz., homogeneity of variance, additive class effects, and normal distribution of errors (Armstrong *et al.*, 2000), this type of design makes the additional assumption that the difference between treatments is consistent across all replications (Snedecor & Cochran, 1980).

Table 2. Analysis of variance (two-way in randomised blocks) of the data in Table 1

ANOVA table:				
Variation	**SS**	**DF**	**MS**	**F**
Treatments	91373.4	2	45686.7	27.32*
Replications	35896.0	9	3988.45	2.39
Error	30097.3	18	1672.07	

* P < 0.001, + Not quite significant at P = 0.05; *Post-hoc* tests (Scheffé): Control v. S1 S = 14.39*, Control v. S2 S = 1.48 (P > 0.05), S1 v. S2 S = 25.11*

Interpretation of the results

The ANOVA appropriate to the two-way design is shown in Table 2. The design is often used to remove the effect of a particular source of variation from the analysis. For example, if there was significant variation due to replications and, if treatments had been allocated to vessels at random, then all of the 'between occasions' variation would have been included in the pooled error variance. The effect of this would be to increase the error variance and to reduce the 'power' of the experiment (Hilton & Armstrong, 2007b) thus making it more difficult to demonstrate a possible treatment effect. In a two-way design, however, variation between replications, attributable to occasions, is calculated as a separate effect and therefore, does not appear in the error variance. This may increase the 'power' of the experiment and make it more probable that a treatment effect would be demonstrated. In the example quoted (Table 2), there is a highly significant effect of treatment ($F = 27.32$, $P < 0.001$). In a two-way design, planned comparisons between the means or post-hoc tests can be performed as for the randomized design (Hilton & Armstrong, 2006b). Hence, Scheffé's *post-hoc* test (Hilton & Armstrong, 2006b) suggested that this result is largely due to the effect of supplement S1 increasing yield. In addition, in a two-way design, the variation due to 'replications' is calculated ($F = 2.39$) and this was not quite significant at $P = 0.05$. The borderline significance suggests there may have been some differences between replications and removing this source of variation from a comparison of the treatment effect may have increased the power of the experiment.

A comparison of the ANOVA table in Table 1 with that for a one-way ANOVA in a randomised design demonstrates that reducing the error variance by 'blocking' has a cost, *viz.*, a reduction in the degrees of freedom (DF) of the error variance which makes the estimate of the error variation less reliable. Hence, an experiment in a two-way design would only be effective if the 'blocking' by occasion or some other factor reduced the pooled error variance sufficiently to counter the reduction in DF (Cochran & Cox, 1957; Snedecor & Cochran, 1980).

Conclusion

The two-way design has been variously described as a matched-sample F-test, a simple within-subjects ANOVA, a one-way within-groups ANOVA, a simple correlated-groups ANOVA, and a one-factor repeated measures design! This confusion of terminology is likely to lead to problems in correctly identifying this analysis within commercially available software. The essential feature of the design is that each treatment is allocated by randomization to one experimental unit within each group or block. The block may be a plot of land, a single occasion in which the experiment was performed, or a human subject. The 'blocking' is designed to remove an aspect of the error variation and increase the 'power' of the experiment. If there is no significant source of variation associated with the 'blocking' then there is a disadvantage to the two-way design because there is a reduction in the DF of the error term compared with a fully randomised design thus reducing the 'power' of the analysis.

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