Statistical Analysis

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An experimental design and statistical technique are outlined for developing a decision rule for declaring residue or no residue in animal tissue studies. The procedure requires an operational definition of zero residue, which may be called relative zero or the limit of the sensitivity of the assay method.

THE problem in defining absolute zero and relative zero, as these terms apply to the limit of detection of an assay, is in an ambiguous state. The limit of detection as defined by Wilson (*6*) is based on controlling the risk of false positives and stresses that both the variation of the test sample and blank must be considered. It is further suggested by Roos (*5*) that the limit of detection should consider the risks of both false positives and false negatives. Kennedy (*3*) considers the problem of detection limit as it would apply to assays for tissue residues. He defines relative zero as the upper limit of the sensitivity range, and emphasizes that the inherent variation of the assay plus the statistical properties of the test procedure must be clearly stated.

This paper attempts to formulate these suggestions into a statistical technique for the specific problem of determining the sensitivity of tissue residue assays. The relationship between the model where compounds are added directly to control tissue and a model for animal tissue which has residue by the process of absorption and retention (hereafter referred to as unknown tissue) is not known. In fact, the variation measured in developmental studies may reflect solely assay difficulties. However, if this is true, the estimates of variation are probably smaller than would be obtained if more realistic developmental procedures were feasible.

Experimental Design

It is assumed that biological entities (animal or plant tissue), which are fed or treated with a compound, have the following model:

$$X_{ijk} = \mu + D_i + A_j + R_k + (DA)_{ij} + (DR)_{ik} + (AR)_{jk} + (DAR)_{ijk} \quad (1)$$

where μ = over-all mean of all assay results

D_i = time effect (usually called day effect). This term measures the influence of uncontrolled factors which influence all assay results from time period to time period. Some of the factors may be: incubation time, dilution or inoculum errors, aging effect on inoculum, agar differences, and growth and population concentration of test organisms.

A_j = animal or plant tissue effect. This effect may reflect differences in chemical composition of the tissue from animal to animal, thereby influencing solubility and recovery of compound. In unknown tissue this source of variation may reflect physiological differences in absorption and excretion (*1*).

R_k = residue effect of administered compound at level k. It is assumed that the ability to extract and measure the amount, k_i, from unknown tissue is the same for level k_i added directly to control tissue.

$(DA)_{ij}$ = interaction between assay time, D_i, and animal, A_j. This effect may be due to differences in blending consistency and the resulting effects of particle size and absorption on extractability. It may also reflect chemical composition heterogeneity with a tissue or pools of tissue.

$(DR)_{ik}$ = interaction between assay time, D_i, and dosage, R_k. This effect may be the result of dilution errors in the applied level, R_k.

$(AR)_{jk}$ = interaction of tissue, A_j, and dosage, R_k. This effect is probably the result of interaction of blending consistency and its associated effects, compound dilution errors, and within-tissue chemical heterogeneity.

$(DAR)_{ijk}$ = three-way interaction, considered to be all remaining sources of assay variation not expressed in the other terms.

These terms are explained for an assay system on a microbiological testing procedure. For this application the residue effect, R_k, is considered fixed; all other effects are random. In other test systems different sources of variation will be pertinent.

The expected value of Model 1 is $E(X_{ijk}) = R_k$ and $\sigma_{Xijk}^2 = \sigma_A^2 + \sigma_D^2 + \sigma_{DA}^2 + \sigma_{DR}^2 + \sigma_{AR}^2 + \sigma_{DAR}^2$. The basic problem is to determine the magnitude of R_k (the residue effect) within the realm of assay variation. An assay on control tissue (animal or plant that has not come in contact with the compound) is considered to have the following model:

$$X_{ij} = \mu + D_i + a_j + (Da)_{ij} \quad (2)$$

where a_j = the control animal or plant tissue effect. The expected value of Model 2 is $E(X_{ij}) = 0$ with variance $\sigma_{Xij}^2 = \sigma_a^2 + \sigma_D^2 + \sigma_{Da}^2$. The random sources of variation of Model 2 are estimated separately because the terms may be of a different magnitude than the same effects in Model 1. If extraction procedures are effective in eliminating all interfering substances, little or no variation in assay values should be obtained in control tissue. However, background substances cannot always be eliminated from control tissue; therefore, the sources of variation must be accounted for.

Amounts of compound in terms of micrograms per gram of standard are added directly to control tissue. The

Table I. Developmental Design for Each Tissue

(Microbiological assay)

Day	Animal	Amounts Added, μg./G. Std. 0	k	ak	bk
1	1	$\bar{x}_{110}$	$\bar{x}_{111}$	$\bar{x}_{112}$	$\bar{x}_{113}$
	2	$\bar{x}_{120}$	$\bar{x}_{121}$	.	.
	3	$\bar{x}_{130}$	.	.	.
2	1	$\bar{x}_{210}$	.	.	.
	2	$\bar{x}_{220}$	.	.	.
	3	$\bar{x}_{230}$	.	.	.
3	1	.	.	.	$\bar{x}_{313}$
	2	.	.	.	$\bar{x}_{323}$
	3	.	.	.	$\bar{x}_{333}$

Table II. Components of Variance for Developmental Study

Source of Variation	D. F.	EMS
Residue, R	2	. . .
Days, D	2	. . .
Animals, A	2	$\sigma_{DAR}^2 + 3\sigma_{DA}^2 + 9\sigma_A^2$
$D \times A$	4	$\sigma_{DAR}^2 + 3\sigma_{DA}^2$
$D \times R$	4	$\sigma_{DAR}^2 + 3\sigma_{DR}^2$
$A \times R$	4	$\sigma_{DAR}^2 + 3\sigma_{AR}^2$
$D \times A \times R$	8	σ_{DAR}^2

following 3 × 3 × 3 experiment (3 days, three animals, three levels of compound added, Table I) will be used to illustrate the statistical technique. It was necessary that the three levels bracket the point where the assay procedure can detect the ingredient added. Frequently preliminary testing was required before the correct levels were found. The experimental unit is an average of ten zones. Data are obtained on individual tissue whenever feasible, and on pooled tissue when necessary. For example, chicken kidneys must be pooled in order to obtain enough tissue to run an assay.

The components of variance of interest for a typical design are shown in Table II.

Statistical Procedure

The statistical procedure requires that one take the difference, $\bar{x}_u - \bar{x}_c = d$, between assay results on the same day between tissue from an animal fed the ingredient (unknown residue) and control tissue. A rule is developed that if d is less than a preassigned quantity, Δ (delta), a conclusion of no residue is reached, while a residue is claimed if d is greater than Δ. The expected value between unknown and control means is $E(\bar{x}_u - \bar{x}_c) = E(d) = \mu_d$. Therefore, the test procedure is of the form of a noncentral t statistic (*2, 4*). The t has d.f. = k and noncentrality parameter μ_d/σ_d.

The distribution is of this form:

$$t = \frac{(d - \mu_d)/\sigma_d + \mu_d/\sigma_d}{S_d/\sigma_d}$$

where

$$S_d^2 = S_A^2 + S_{DA}^2 + S_{DR}^2 + S_{AR}^2 + S_{DAR}^2 + S_a^2 + S_{Da}^2 \quad (3)$$

and S^2 denotes an unbiased estimate of the corresponding σ^2.

First a minimal difference $\mu_d = \mu_o$ is chosen to serve, in effect, as an operational definition of "no residue" material: one which will take into account the sensitivity of the assay method. This quantity is assigned the value $\mu_o = t_{0.95;k}\sigma_d$ where $t_{0.95;k}$ is the 95th percentile of Student's t distribution with k degrees of freedom. This choice corresponds to a true difference just large enough to be distinguished from control tissue approximately 50% of the time by a Student's t test.

Under the assumption that a sequence of differences (d) obtained from a given treatment generates a normal population, an X_k is chosen to satisfy

$$P\left(\frac{d}{S_d} < X_k \mid \mu_d = \mu_0\right) = 0.90 \quad (4)$$

The least significant difference, Δ, is approximated as

$$\Delta = X_k S_d \quad (5)$$

Results and Discussion

Examples of two replicated developmental studies are shown in Tables III and IV. These data were gathered at two laboratories with a time interval of approximately 6 months between studies. Each number within the tables is an average of ten zones. The smallest accurately measured zone is 9.00 mm. and this is considered no zone of inhibition. The data on individual zones are considered significant only to the first decimal point, but for purposes of calculation two decimal places are carried.

The Δ for test 1 was determined to be 2.27 mm. or approximately 2.3 mm. The sensitivity range is between 0.2 and 0.3 p.p.m.

The Δ for test 2 was determined as 2.86 mm. or approximately 2.9 mm. The sensitivity range is between 0.1 and 0.3 p.p.m.

Seventy studies, on four antibiotics and five tissues in cattle, chicken, and swine have been evaluated. The relative magnitude of each variance component to S_d^2 is found in Table V. It is noted that the relative magnitude of each average ranks the same within each animal—that is, the average $S_{DAR}^2/S_d^2 > S_{DR}^2/S_d^2 > S_{DA}^2/S_d^2 > S_A^2/S_d^2 > S_{RA}^2/S_d^2$ for each animal. In general, the relative magnitude of each component of variance was: S_{DAR}^2, 33 to 56%; S_{DR}^2, 20 to 28%; S_{DA}^2, 13 to 23%; S_A^2, 6 to 14%; S_{RA}^2, 3 to 8%. There is no

Table III. Results of Test 1 on Developmental Study of Antibiotic Z

Day	Animal	μg./G. Std. 0	0.2	0.3	0.5
1	1	9.00	9.00	9.00	13.46
	2	9.00	9.00	9.00	13.75
	3	9.00	9.00	9.00	13.56
2	1	9.00	11.19	13.99	16.03
	2	9.00	10.33	12.99	15.08
	3	9.00	9.46	11.72	14.61
3	1	9.00	9.00	11.84	13.38
	2	9.00	9.00	12.00	14.00
	3	9.00	9.00	10.00	14.13

Table IV. Results of Test 2 on Developmental Study of Antibiotic Z

Day	Animal	μg./G. Std. 0	0.1	0.3	0.5
1	1	9.00	9.00	14.11	16.17
	2	9.00	9.95	14.02	16.30
	3	9.00	9.00	13.57	15.61
2	1	9.00	9.00	11.85	15.98
	2	9.00	9.00	13.60	16.64
	3	9.00	9.00	13.16	16.52
3	1	9.00	9.00	15.16	17.88
	2	9.00	9.00	15.76	17.06
	3	9.00	9.00	14.82	17.17

Table V. Relative Magnitude of Variance Component

Tissue	Cattle	Swine	Chicken
	$(S_{DAR}^2/S_d^2)100$		
Fat	29.6	48.2	71.8
Lean	32.9	51.1	49.5
Liver	27.8	26.3	58.9
Kidney	46.4	57.0	44.0
Tripe	30.8	...	...
Av.	33.4	41.8	55.8
	$(S_{DR}^2/S_d^2)100$		
Fat	20.6	39.3	10.2
Lean	38.0	24.5	26.7
Liver	28.0	20.0	12.9
Kidney	30.0	25.0	31.6
Tripe	22.5	...	...
Av.	27.6	24.8	20.3
	$(S_{DA}^2/S_d^2)100$		
Fat	25.1	1.8	15.6
Lean	22.2	6.4	12.3
Liver	17.1	41.6	11.8
Kidney	14.0	14.3	12.9
Tripe	9.7	...	...
Av.	17.6	22.9	13.0
	$(S_A^2/S_d^2)100$		
Fat	13.2	10.7	9.8
Lean	4.0	3.2	8.8
Liver	17.1	10.1	7.3
Kidney	5.0	3.8	6.9
Tripe	29.0	...	...
Av.	13.7	7.4	6.2
	$(S_{AR}^2/S_d^2)100$		
Fat	11.3	0	1.5
Lean	3.6	14.9	2.7
Liver	10.0	3.7	9.0
Kidney	5.7	0	4.6
Tripe	8.1	...	...
Av.	7.7	3.2	4.6

apparent relationship between the variance components and the tissues studied on a within-animal basis.

Table VI. Results of 94 Independent Assays of Control Tissue plus Added Ingredient Indicated at Δ Level

Animal	No. of Independent Tests at Sensitivity Level	No. of Declared Residue	% Declared Residue
Cattle	33	26	78.7
Swine	4	3	75.0
Chicken	57	46	80.7
Total	94	75	79.8

The theoretical power of this procedure at the true Δ value is 50%. This means that there would be a β (beta) risk (failure to declare residue when truly there) of 50% at the Δ point if the sensitivity of the assay does not increase with increasing amounts of residue. It was of interest to check this empirically. As of this date, 94 independent studies were run at the parts per million estimated as the upper limit of the sensitivity ranges. These data are summarized in Table VI. These results indicate that the ability to detect residues at the Δ point is approximately 80%. This implies, therefore, that there is only a 20% β risk at the Δ criterion. It is realized that these data do not prove such power really exists for all tissues. However, studies will be continued and data will continue to be accumulated on the ability to detect residues at the approximate parts per million level indicated in the developmental studies.

A check on the reproducibility of the developmental system and method of analysis was attempted by replicating each of six 3 × 3 × 3 experimental systems twice. Table VII contains these results in terms of estimated parts per million of the test system. These data indicate that the approximate sensitivity of the test procedure is reproducible.

Table VII. Approximate Parts per Million Indicated by Replication of Development Assay Design

Test	Approximate P.P.M. Rep. 1	Rep. 2
1	0.03–0.05	0.05–0.08
2	0.20–0.40	0.30[a]
3	0.10–0.20	0.20[a]
4	0.05–0.10	0.05–0.10
5	0.20–0.30	0.10–0.30
6	0.03–0.05	0.01–0.03

[a] Dosage range failed to bracket Δ criterion.

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FORAGE ANALYSIS

Carbohydrate Content in Alfalfa Herbage as Influenced by Methods of Drying

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QUALITATIVE and quantitative determinations of carbohydrates are performed in various studies of agronomic crop plants. Drying and storage of plant tissue are often necessary because of the numbers of samples to be processed and the length of time involved in many chemical analyses. For carbohydrate analyses to be of maximum value, changes in plant composition during preservation and storage must be minimized. Investigators have observed the effects of various preservation procedures on a variety of plant materials (*2, 3, 6, 7, 9, 11*). Changes in organic constituents have been attributed to respiratory losses, metabolic interconversions, and deleterious effects of high drying temperatures. Studies of changes occurring during wilting (*10, 15*) also are pertinent, because conditions within a mass of plant tissue during early stages of drying may be conducive to metabolic changes and accelerated respiratory losses similar to those occurring during wilting (*12*).

Changes that may occur in the content of some carbohydrate constituents during the drying and preservation of alfalfa herbage are considered here.

Materials and Methods

Herbage from a stand derived from a single clone of Vernal alfalfa was harvested at or near bud stage on May 24, 1962, and again on October 11, 1962.

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