ANOVA & QUANTITATIVE ASSAYS

COMPARISON OF SEVERAL MEANS

- Suppose we want to know whether there are differences in the means of more than two independent groups.
- **Two questions here:** (1) How do we **measure** the "difference" among several means? and (2) How do we decide if the "difference" among several sample means is **large enough** to conclude that the population means are different?

ONE-WAY "ANOVA"

- What needed is **A**nalysis **o**f **V**ariance (ANOVA) or **One-way ANOVA**
- One-way ANOVA provides a "test" against the "**Global Alternative**": the ANOVA **F-test**; **if the F-test is significant, some pair or pairs of means are different.**
- We can start looking for that/those pairs- with "allowance" for multiple comparisons.

Methods for multiple comparisons:

Bonferroni

Benjamini & Hochberg

etc…

THE DATA

- We have continuous measurements X's from **k independent samples.**
- Data from the i^{th} sample can be summarized into **sample size n_i, sample mean $\bar{x}_i$, and sample variance s_i^2.**
- If we pool data together, the (grand) **mean of this combined sample** is:

$$\bar{x} = \frac{\sum n_i \bar{x}_i}{\sum n_i}$$

MEASURE OF TOTAL VARIATION

- In that combined sample of size $n=\Sigma n_i$, the variation. in X is measured in terms of the deviations $(x_{ij}-\bar{x})$, where x_{ij} is the jth measurement from the ith sample. The "total" variation, denoted by SST, is measured by the sum of squared deviations:

$$\mathbf{SST = \sum_{i,j} (x_{ij} - \bar{x})^2}$$

- For example, SST=0 when all observations x_{ij}'s are the same; **SST is the numerator of the sample variance of the combined sample**, the greater SST the greater the variation among all x-values.

COMPONENTS OF TOTAL VARIATION

- The total variation in the combined sample can be decomposed into two components as follows: $(x_{ij}-\bar{x}) = (x_{ij}-\bar{x}_i) + (\bar{x}_i-\bar{x})$:

(1) The first term reflects the **variation within the samples**; the following sum is called the "within sum of squares": $SSW = \sum_{i,j}(x_{ij} - \bar{x}_i)^2 = \sum_i (n_i - 1)s_i^2$

(2) The difference between the above two sums of squares, SSB=SST-SSW, is called the "between sum of squares" which **measures the differences between samples**: $SSB = \sum_{i,j}(\bar{x}_i - \bar{x})^2 = \sum_i n_i(\bar{x}_i - \bar{x})^2$

DECOMPOSITION OF SST

- SST measures the "total variation" in the combined sample with (n-1) degrees of freedom, $n=\Sigma n_i$ is the total size. It is decomposed into: SST=SSW+SSB
- (1) **SSW measures the variation within samples** with $\Sigma(n_i-1)=(n-k)$ degrees of freedom, and
- (2) **SSB measures the variation between samples** with (k-1) degrees of freedom; k=# of groups

Two questions here: (1) How do we **measure** the "difference" among several means? and (2) How do we decide if the "difference" among several sample means is **large enough** to conclude that the population means are different?

Answer to the first question:

Between-sample Sum of Squares

$$\mathbf{SSB} = \sum_{i,j} (\bar{x}_i - \bar{x})^2 = \sum_{i} n_i (\bar{x}_i - \bar{x})^2$$

(which is a concept similar to the "variance").

Next step is getting an "average difference":

MSB = SSB/(k-1)

“ANOVA” TABLE

The breakdowns of the total sum of squares and its associated degree of freedom are displayed in the form of an “analysis of variance table” (ANOVA table) as follows:

Source of Variation	SS	df	MS	F Statistic	p-value
Between samples	SSB	k-1	MSB	MSB/MSW	
Within samples	SSW	n-k	MSW		
Total	SST	n-1			

SSB is a concept similar to the “Sum of Squares”; SS – which is the numerator of the variance - applies to individual observations, SSB applies to sample means

$$SS = \sum_{i} (x_i - \bar{x})^2$$

MSB measure of the <u>average</u> variation within the k sample means.

MSW is a natural extension of the pooled estimate s_p^2 as used in the two-sample t-test; It is a **measure of the average variation (among observations) within the k samples.**

APPROACH TO QUESTION #2:

COMPARE the “average gap/difference” between sample means (MSB) to the average gap/difference between measurements in samples (MSW):

Use F=MSB/MSW

Why?

THE One-way ANOVA MODEL

Assume that the k samples come from k normal distributions with a common variance σ^2 and means μ_i. The model for the two-sample t-test is a special case of this "ANOVA model" with k = 2

$$X_{ij} = \mu_i + \varepsilon_{ij}$$

$$= \mu + \beta_i + \varepsilon_{ij}$$

$$\varepsilon_{ij} \in N(0, \sigma^2)$$

$$\sum_{i=1}^{k} \beta_i = 0$$

$$X_{ij} = \mu_i + \varepsilon_{ij}$$

$$= \mu + \beta_i + \varepsilon_{ij}$$

$$\varepsilon_{ij} \in N(0, \sigma^2)$$

$$\sum_{i=1}^{k} \beta_i = 0$$

The parameter β_i represents the "effect of the i^{th} group". This is often referred to as the **"Fixed Effects Model" or "Model I"**; it applies where **"the conclusion will pertain to just the k groups (say, k levels of a study factor) included in the study/analysis".**

HYPOTHESES

Model :

$$X_{ij} = \mu_i + \varepsilon_{ij}$$

$$= \mu + \beta_i + \varepsilon_{ij}$$

$$\varepsilon_{ij} \in N(0, \sigma^2)$$

$$\sum_{i=1}^{k} \beta_i = 0$$

Hypotheses :

$$H_0 : \beta_i = 0 \; for \; all \; i = 1,2..,k$$

$$H_A : \beta_i \neq 0 \; for \; some \; i$$

RESULTS

We have, under "Model I" (fixed effects):

$$\mathrm{E(MSW)} = \sigma^2$$

$$E(MSB) = \sigma^2 + \frac{\sum n_i \beta_i^2}{k-1}$$

Under H_0, F is distributed as:

$$F(df = k-1, df = n-k)$$

IMPLICATIONS

- MSW is an unbiased estimator of the variance of the error terms
- If all group (population) means are equal, MSW and MSB both estimate the same variance; but **if group means are not the same (under Alternative Hypothesis), "F statistic tends to be greater than 1 because MSB is larger.**

ANSWER #2:

We decide if the "difference" among several sample means is large enough to conclude that the population means are different by comparing:

F statistic vs. F distribution (df=k-1,df=n-k)

There are occasions when the groups included in the study are not of intrinsic interest in themselves, but **they constitute a sample from a larger population of levels** (say, three drug doses out of many possibilities). **If we want the conclusions apply to all levels**, we should consider "Model II", the "**Random Effects Model**".

That’s the case of Quantitative Bioassays; the doses uses are of no interest in themselves: we are looking for a global solution which applies to all doses (& all biological systems – if exists).

RANDOM EFFECTS MODEL

$$X_{ij} = \mu_i + \varepsilon_{ij}$$

$$= \mu + \beta_i + \varepsilon_{ij}$$

$$\boldsymbol{\beta \in N(0, \sigma_\beta^2)}$$

$$\varepsilon_{ij} \in N(0, \sigma^2)$$

$$Var(X) = \sigma_\beta^2 + \sigma^2$$

RESULTS

We have, under "Model II" (random effects):

$$\mathrm{E(MSW)} = \sigma^2$$

$$E(MSB) = \sigma^2 + n'\sigma_\beta^2$$

$$n' = \frac{1}{k-1}\left[n - \frac{\sum n_i^2}{\sum n_i}\right]$$

Under H_0, F is distributed as:

$$F(df = k-1, df = n-k)$$

Despite the fact that model II differs from Model I, the Analysis of Variance for one-way ANOVA is conducted in identical fashion.

In either model, one-way ANOVA F-test only provides a “test” against the “Global Alternative”; if the F-test is significant, some pairs of means are different. We can start looking for those pairs - with “allowance” for multiple comparisons. However, pairwise comparisons do not answer research questions.

CONTRASTS

A contrast is a comparison involving two or more group means; contrasts are formed to answer specific research questions. A contrast, denoted by "L" is defined as follows:

$$L = \sum_{i=1}^{k} \alpha_i \mu_i$$

$$\sum_{i=1}^{k} \alpha_i = 0$$

$$e.g.\ L = \mu_1 - \mu_2$$

RESULT

We can “test” and/or form confidence intervals using the variance:

$$L = \sum_{i=1}^{k} \alpha_i \bar{x}_i$$

$$Var(L) = \sigma^2 \sum_{i=1}^{k} \frac{\alpha_i^2}{n_i}$$

$$\cong (MSE) \sum_{i=1}^{k} \frac{\alpha_i^2}{n_i}$$

We can calculate “the sum of squares associated with a contrast”, SS(L), and form an “F-test” – L has 1 degree of freedom:

$$L = \sum_{i=1}^{k} \alpha_i \mu_i$$

$$SS(L) = MS(L)$$

$$= (L)^2 \Big/ \sum_{i=1}^{k} \frac{\alpha_i^2}{n_i}$$

$$= \frac{L^2}{n \sum \alpha_i^2} \quad if\ n_i = n$$

ORTHOGONAL CONTRASTS

Given two contrasts:

$$L_1 = \sum_{i=1}^{k} \alpha_{1i}\mu_i; \quad \sum_{i=1}^{k} \alpha_{1i} = 0$$

$$L_2 = \sum_{i=1}^{k} \alpha_{2i}\mu_i; \quad \sum_{i=1}^{k} \alpha_{2i} = 0$$

They are said to be "orthogonal" if :

$$\sum_{i=1}^{k} \frac{\alpha_{1i}\alpha_{2i}}{n_i} = 0, \text{ or}$$

$$\sum_{i=1}^{k} \alpha_{1i}\alpha_{2i} = 0 \; if \; n_i = n$$

An “orthogonal decomposition” is one where the component sums of squares add to the total sum of squares; for example: SST = SSB +SSW.

There exists a relationship between orthogonal contrasts and the orthogonal decomposition of SSB; **that is, there exists a set of (k-1) orthogonal contrasts so that SSB = ΣSS(L).** And the F-tests for individual contrasts are independent

PARALLEL-LINE ASSAYS

	Preparation					
	Standard Preparation			Test Preparation		
Dose (D; mmgcc)	0.25	0.50	1.00	0.25	0.50	1.00
X = log10(Dose)	-0.602	-0.301	0.000	-0.602	-0.301	0.000
Response (Y; mm)	4.9	8.2	11.0	6.0	9.4	12.8
	4.8	8.1	11.5	6.8	8.8	13.6
	4.9	8.1	11.4	6.2	9.4	13.4
	4.8	8.2	11.8	6.6	9.6	13.8
	5.3	7.6	11.8	6.4	9.8	12.8
	5.1	8.3	11.4	6.0	9.2	14.0
	4.9	8.2	11.7	6.9	10.8	13.2
	4.7	8.1	11.4	6.3	10.6	12.8

ANOVA ANALYSES

- ANOVA: can be used for
  (1) Checking assumptions: whether lines are parallel, linearity (for 6-point designs), and
  (2) Testing major hypotheses (difference between preparations, whether slopes are zero),
- ANOVA: can also be used for
  (1) Estimating the common slope and its standard error, and the difference between the two intercepts
  (2) Estimating Relative Potency too.

MAIN ANOVA RESULTS

Source of Variation	SS	df	MS	F	$F_{.95}$
Treatment	396.87	5	79.373	501.5	2.5
Block	1.28	7	.183		
Error	5.54	35	.158		
Total	403.69				

Results indicate that the 6 treatments are different and provides an estimate of the variance (MSE=.158); but to explain the various components/sources we need to **use the five (5) orthogonal linear contrasts.**

CONTRASTS FOR 6-POINT ASSAYS

The following table gives the coefficients α for five orthogonal linear contrasts associated with a 6-point assay

	Standard Prep			Test Prep			
Contrast	Trt#1	Trt#2	Trt#3	Trt#4	Trt#5	Trt#6	$\Sigma\alpha^2$
Lp	-1	-1	-1	1	1	1	6
Lr	-1	0	1	-1	0	1	4
Ld	1	0	-1	-1	0	1	4
Lqs	1	-2	1	0	0	0	6
Lqt	0	0	0	1	-2	1	6

- For L_p: No difference between preparations
- For L_r: Slopes are zero (conditional on L_d)
- For L_d: Lines are parallel
- For L_{qs}: Standard curve is not quadratic
- For L_{qt}: Test curve is not quadratic

Two important contrasts are:

For L_p: No difference between preparations

For L_r: Slopes are zero (conditional on L_d)

SIX-POINT ASSAYS

$L_p = (3)(\textit{Difference of Intercepts})$

$L_r = (4)(d)(\textit{Common Slope})$

d = Between consecutive doses on log scale

That's why we use L_p for "No difference between preparations"

& Use L_r to test if Common slope is zero

THE FOUR-POINT ASSAYS

- The 4-point parallel-line assays can be designed similarly; the test and standard preparations of the agent are tested at the same two dose levels. There and n replications at each dose of each preparation;
- It is designed with n dishes/plates, each contains 4 identical samples one in a “well”.
- The ANOVA analysis is the same; the main difference is the use of orthogonal contrasts. There are **3 contrasts L_p, L_r, and L_d (parallelism).**

CONTRASTS FOR 4-POINT ASSAYS

The following table gives the coefficients α (to be multiplied by column/treatment totals) for the three orthogonal linear contrasts associated with a 4-point assay

Contrast	Trt 1	Trt 2	Trt 3	Trt 4	$\Sigma\alpha^2$
Lp	-1	-1	1	1	4
Lr	-1	1	-1	1	4
Ld	1	-1	-1	1	4

Note: No tests for linearity; enough data to do it.

FOUR-POINT ASSAYS

$L_p = Difference\ of\ Intercepts$

$L_r = (2)(d)(Common\ Slope)$

$d =$ Between consecutive doses on log scale

That's why we use L_p for "No difference between preparations"
& Use L_r to test if Common slope is zero

LOG RELATIVE POTENCY

Four – point Assays:

$$m = \frac{dL_p}{L_r}$$

Six – point Assays:

$$m = \frac{4dL_p}{3L_r}$$

SIX-POINT ASSAYS

$$L_p = (3)(\textit{Difference of Intercepts})$$

$$L_r = (4)(d)(\textit{Common Slope})$$

$$d = \text{Between consecutive doses on log scale}$$

$$m = \frac{\textit{Difference of Intercepts}}{\textit{Common Slope}}$$

$$= \frac{4dL_p}{3L_r}$$

FOUR-POINT ASSAYS

$$L_p = (2)(\textit{Difference of Intercepts})$$

$$L_r = (2)(d)(\textit{Common Slope})$$

$$d = \text{Between consecutive doses on log scale}$$

$$m = \frac{\textit{Difference of Intercepts}}{\textit{Common Slope}}$$

$$= \frac{dL_p}{L_r}$$

SLOPE-RATIO ASSAYS

For balanced assays, ANOVA has been popular; two popular designs are balanced 5-point and 7-point designs which provide simple calculation of Relative Potency too.

FIVE-POINT DESIGN

For the most common assays follow a “five-point symmetrical” design. Doses for each preparation need not be identical; the design includes a “zero dose” - often called “blank”.

Preparation	Blank	Standard		Test	
Dose	0	XS/2	XS	XT/2	XT
Response Total	C	S1	S2	T1	T2

It usually follows a complete block randomized design with n responses to each of 5 doses/groups.

Now we can afford to have a ‘blank” (Dose = 0, because we do not have to take the log); the second dose is twice the first dose in a five-point assays.

CONTRASTS FOR SLOPE-RATIO ASSAYS

The following table gives the coefficients α (to be multiplied by column/treatment totals) for the two contrasts associated with "Intercepts" and "Blank".

Preparation	Blank	Standard		Test		sum of sq coefs
Dose	0	$x_S/2$	x_S	$x_T/2$	x_T	
Intercept Contrast, L_I	0	2	-1	-2	1	10
Blank contrast, L_B	2	-2	1	-2	1	14

VALIDITY & GOODNESS-OF-FIT

Relevant calculations required for ANOVA can be made in terms of the following contrasts for equality of intercepts (L_I) and validity of blank or control (L_B). The sum of squares for regressions, with 2 dfs, is obtained by subtraction. One carries out the relevance tests by comparing the F ratios $F_B = L_B^2/(14)(n)s^2$ and $F_I = L_I^2/(10)(n)s^2$ with the tabulated F value for 1 and 5(n-1) dfs.

RELATIVE POTENCY

A simple solution is to assume the model (after previous tests), ignore the blank and use the following “regression contrasts”:

Preparation	Blank	Standard		Test		sum of sq coefs
Dose	0	$x_S/2$	x_S	$x_T/2$	x_T	
Standard Prep, L_S	0	-1	1	0	0	2
Test Prep, L_T	0	0	0	-1	1	2

$$R = \frac{x_S L_T}{x_T L_S}$$

EXERCISES

1 In a slope ratio assay, how does the orthogonal contrast for intercept (LI) relate to the equality of the two intercepts.

2 Consider a 4-point parallel-line assay of exercise A2.1, calculate the log relative potency using ANOVA.

	Dose				
Block	0.015	0.045	0.015	0.045	
	(S1)	(S2)	(T1)	(T2)	Total
	45.07	60.2	49.75	66.35	221.4
	44.12	62.93	35.83	45.58	191.5
	39.64	48.44	44.94	54.26	187.3
	31.48	48.95	34.76	56.39	171.6
Total	160.31	220.52	165.28	225.6	771.7

3 Estimate the relative potency from the 4-point assay of Corticotrophin using ANOVA:

		Dose				
Preparation	Blank	Stadard		Test		
Dose	0	0.1	0.2	0.14	0.28	Total
	1.5	5	8	4.9	7.7	27.1
	1.4	4.7	7.7	4.8	7.7	26.4
	1.5	4.8	7.9	4.7	7.8	26.7
	1.6	5.1	7.8	4.8	7.9	27.2
Total	6	19.6	31.4	19.2	31.1	