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THE PROBLEM OF SELECTING UNIFORM SAMPLES OF LEAVES

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In order to study the effect of cultural or other treatments upon the chemical composition of excised leaves, it is necessary to collect a number of separate samples from the plants. One or more of these is at once dried or otherwise prepared for analysis to serve as a control; the others are subjected to the treatment and are then prepared for analysis in exactly the same manner. The necessary chemical determinations are subsequently carried out and the differences in composition between the treated samples and the controls are computed; these differences are assumed to furnish a measure of the effect of the treatment. The validity of this assumption is, clearly, dependent upon the precision with which the samples duplicated each other in composition at the time of collection.

Experience in this laboratory has shown that samples which duplicate each other more or less closely can readily be obtained. Nevertheless, this general method of investigation is subject to errors that occasionally interfere with the interpretation of the results. The chief source of these errors appears to be the failure of occasional individual samples to agree sufficiently closely with the others in initial composition. Inasmuch as the analytical data cannot be obtained until after the experiment has been completed, there is no opportunity to repeat an erratic observation, for the original plant material has meanwhile changed in composition.

To avoid improper interpretations arising from such possible sampling errors, it has been customary to disregard chemical changes of less than about 10 per cent. of the magnitude measured. Changes greater than this are assumed to be the result of the treatment, but smaller changes may be open to the suspicion of being affected by the sampling error.

The error in an analytical determination obtained upon plant material has two main components. One of these arises from variations in the analytical processes in the laboratory. This error can be estimated from the differences between the results of analytical determinations obtained in duplicate. The other component, arising from actual variations of the plant material itself, is open to study if different techniques of sampling are employed. Analysis of the data, after segregation of the analytical

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error, should then lead to judgments on the relative desirability of one or other of these sampling techniques.

In an effort to obtain objective evidence upon the magnitude of the sampling error in the selection of presumably similar samples of leaves, three methods of collection have accordingly been studied, *Bryophyllum calycinum* being used as the species investigated.

Methods of sampling

STATISTICAL METHOD.—The objective of the statistical method of sampling is to equalize the effects of the differences between the plants and the differences between leaf positions on the individual plants. The samples are so taken that each plant and each leaf position is represented to exactly the same extent in each sample. Because of the necessity of providing an exact balance of the several factors, there are restrictions on the relative numbers of samples, of plants, and of leaf positions that can be used.

The number of categories of leaf position that it is proposed to study is first established and a number of plants that is an integral multiple or submultiple of this number is selected. A number of samples that is also an integral multiple or submultiple of this number is then fixed upon. The selected plants are examined to make sure that each of the leaves or leaflets to be collected is normally developed and free from injury. The plants are assigned numbers at random, and an arbitrary plan of numbering the leaves or leaflets, *i.e.* the categories of leaf position, is set up. For example, with the Bryophyllum plants used in this study, each of the five leaflets on a pair of opposite leaves high on the plant was assigned the numbers 1 to 10 according to an easily remembered scheme. This system of numbering was then applied to the leaflets of the pair of opposite leaves in the same relative position on the stalks of each of the five plants used.

At the time of collection, leaflet 1 from plant 1 is placed in sample 1, leaflet 2 from plant 1 in sample 2, leaflet 3 from plant 1 in sample 3, and so on. If there are 5 samples, leaflet 6 will go into sample 1; if 10, the leaflets will go once around the set of samples. On plant 2, leaflet 1 is placed in sample 2, leaflet 2 in sample 3, and so on. On plant 3, leaflet 1 is placed in sample 3, etc. This progressive process is continued throughout the group of plants. To obtain larger samples, a second pair of leaves adjacent in position to the first pair is then treated in the same way and a third or fourth pair may also be included if desired.

The design employed is, in mathematical terms, a systematized Latin square and is selected for the present purpose to facilitate the picking of the samples. Alternatively, an ordinary Latin square in which the numbering of the leaflets is entirely randomized may be used but is more difficult to employ. A diagram of the system as it applies to the leaflets from one leaf on each of five plants is as follows.

Sample numbers to which designated leaflets are alloted according to a systematic Latin square designed to give 5 samples

Leaflet position	Plant number				
	1	2	3	4	5
	Sample number				
1	1	2	3	4	5
2	2	3	4	5	1
3	3	4	5	1	2
4	4	5	1	2	3
5	5	1	2	3	4

Leaflets 6 to 10 from the first pair of opposite leaves are collected according to a similar plan. In collecting the leaflets from the second pair of opposite leaves, the sampling plan may be identical, but, in the present case, was modified by setting up the Latin square beginning with sample number 2 in the upper left-hand corner and the third pair beginning with sample number 3 in the upper left-hand corner. Any other number from 1 to 5 may equally well be chosen. This is equivalent to extending the Latin square shown into a third dimension.

When the collection is finished, there is an equal number of leaflets in each sample, and each sample represents equally all of the plants, all of the leaflet positions on the leaves, and all of the leaf positions chosen. Accordingly, if the variability in the composition of leaves is assumed to be made up of a component that varies from plant to plant, one that varies with leaf position, one that varies with leaflet position, and an additional random component, each of these components is distributed evenly among the samples.

It is obvious that the 5-leaflet upper compound leaves of mature *Bryophyllum calycinum* plants restrict the number of samples that can be obtained by this method to 5 or a multiple of 5. If younger plants are used in which the upper compound leaves bear only 3 leaflets, a number of samples that is a multiple of 3 may be collected from this species. In this case, the number of plants used must also be a multiple of 3. In any case, although collection of the full mathematically determined number of samples is necessary, some of them may be omitted from the experiment, or the extra samples may be used for the duplication of controls or treatments.

OPPOSITE LEAF METHOD.—This method has long been used in physiological experiments (*e.g.* CHIBNALL (**2**)), on the assumption that the composition or behavior of opposite leaflets would, for morphological reasons, be so similar that one can properly be used as a control in studying the effect of treatment upon the other. In selecting the samples, there is no restriction upon the number of plants or the number of pairs of opposite leaflets employed. However, the number of samples must be evenly divisible by the number of categories of leaflets established. Differences between plants are balanced in that each sample contains an equal number

of leaflets from each plant. However, each sample consists of leaflets from the same position on the plant.

In selecting the samples, a suitable number of pairs of compound leaves in the same positions on a sufficient number of plants is selected and examined for freedom from abnormality and for similarity in appearance and development. In the present experiment with *B. calycinum,* the strict application of the method was slightly modified in that five categories of leaflets were set up: the first comprised the two leaflets on one side of one of a pair of similarly placed opposite leaves on each plant; the second, the leaflets opposite to these; the third, the two leaflets on one side of the opposite leaf; and the fourth, the two leaflets opposite these. A fifth category was made up of the terminal leaflets. In making the collection, all of the leaflets of the first category from each of the plants are placed in one sample, all of the second category in the next and so on, and a second and, if desired, third pair of leaves from all of the plants are treated in the same manner. At the end, each sample contains the same number of leaflets, and the plants are equally represented in each sample. Samples 1 and 2 represent mutually opposite leaflets as do samples 3 and 4. The fifth sample taken in the present case was, however, unique with respect to leaf position and would not be employed in an experiment designed to conform strictly to the opposite leaf method. However, its composition proved to be indistinguishable from the others and it was included to facilitate the mathematical analysis.

LEAF SIZE METHOD.—This method may be used with any species the leaves of which are of suitable size. It provides for randomization with respect to plant of origin and position on the plant. The plants are selected and a decision is made on the numbers and position of the leaves to be collected. There is no restriction on the number of plants nor upon the number of samples. However, randomization is best provided for in large samples. The leaves or leaflets are collected into a single receptacle, care being taken to collect a few more than will be required.

The correct total number of leaves for the set of samples is then counted out, discarding and replacing abnormal or damaged leaves, and the leaves are sorted according to size and degree of development, the number of grades set up depending on the variation in size. The leaves in each grade are then shuffled repeatedly so as to randomize them as much as is conveniently possible, and the number of leaves in each grade is noted. Where this number is not evenly divisible by the number of samples to be taken, adjustment is made by moving leaves of suitable size from one grade to another. After further shuffling, the leaves in each grade are distributed equally but at random to the samples.

The leaf size method has been used for many years in this laboratory. However, the sampling error problem that has occasionally arisen suggests that the degree of randomization that is attained in practice is not always adequate.

Experimental

Five samples of leaflets of *B. calycinum* were taken by each of the three methods described. There were 30 leaflets (five plants, three pairs of opposite leaves) in each sample taken by the statistical method, 36 (six plants, three pairs of opposite leaves) in each sample taken by the opposite leaf method, and 30 (150 leaflets from upper leaves of about 10 plants) in each sample taken by the leaf size method. The samples were collected by the statistical and opposite leaf methods in the early afternoon of a sunny day so that the organic acids would be at a low point and starch at a maximum (**7**). Those taken by the leaf size method were collected three weeks later also on the afternoon of a sunny day but after a prolonged period of cloudy weather.

Each set of samples was immediately dried at 80° C., equilibrated at

TABLE I

ANALYTICAL DATA GIVING DUPLICATE DETERMINATIONS IN GRAMS PER KILO OF INITIAL FRESH WEIGHT FOR EACH ALIQUOT OF POWDERED LEAF. SAMPLES IN SERIES ST. COLLECTED BY THE STATISTICAL METHOD, IN SERIES O.L. BY THE OPPOSITE LEAF METHOD, AND IN SERIES L.S. BY THE LEAF SIZE METHOD

Series	Sample No.	Total solids		Ash		Total nitrogen		Protein nitrogen		Starch	
St.	1	110.2	110.0	10.07	10.18	2.79	2.84	2.32	2.32	25.35	25.35
	2	105.9	105.8	9.85	9.87	2.78	2.78	2.23	2.24	24.35	24.35
	3	106.7	106.7	10.41	10.34	2.87	2.83	2.26	2.26	24.50	24.84
	4	105.9	106.2	9.96	10.28	2.73	2.75	2.23	2.23	25.47	25.02
	5	107.8	107.5	10.09	10.36	2.79	2.79	2.27	2.27	25.57	25.57
O.L.	1	110.2	110.1	10.77	10.40	3.05	3.05	2.43	2.42	24.05	24.05
	2	104.5	104.2	11.23	10.94	2.87	2.87	2.31	2.30	22.43	22.43
	3	106.2	105.9	11.20	11.27	2.91	2.91	2.31	2.30	23.64	23.25
	4	102.7	102.8	10.23	10.19	2.76	2.79	2.28	2.27	22.66	23.06
	5	108.6	108.6	10.24	10.10	2.81	2.81	2.31	2.30	23.99	23.99
L.S.	1	118.0	118.3	11.98	10.84	2.48	2.48	2.03	2.04	32.08	32.08
	2	121.6	121.7	11.69	11.79	2.59	2.63	2.06	2.05	33.50	33.50
	3	128.9	128.9	12.43	12.21	2.47	2.51	1.98	1.98	42.33	42.33
	4	119.4	119.4	11.30	11.23	2.37	2.41	1.92	1.92	35.83	35.83
	5	125.1	125.4	11.76	11.92	2.48	2.48	2.04	2.04	38.14	37.61

25° C. and 50 per cent. relative humidity in a constant temperature-constant humidity room until constant weight was attained and was then ground to powder in a Wiley mill for analysis.

The analytical determinations shown in table I were made, the data being expressed in grams per kilogram of fresh weight of the sample at the time of collection. The total solids were computed from the loss of weight of duplicate 500 mg. aliquots of the equilibrated powdered tissue when heated at 110° C. for exactly four hours. The same samples were used for the determination of the ash as described by VICKERY, PUCHER, WAKEMAN and LEAVENWORTH (**9**). Total nitrogen was determined on 100 mg. samples by the Kjeldahl method modified to deal with nitrate as described by VICKERY, PUCHER, WAKEMAN and LEAVENWORTH (**9**), except that a

loose plug of nonabsorbent cotton was placed in the neck of the Kjeldahl flask during the distillation as an additional precaution against entrainment of minute drops of alkali, and that the flask used to collect the distillate was cooled in a bath of cold water during the distillation.

Protein nitrogen was determined in duplicate 200 mg. samples by a recently developed procedure according to which the samples, securely wrapped in small squares of closely woven cotton fabric, are extracted with 70 per cent. alcohol in a continuous extraction apparatus for 16 hours (6). They are subsequently transferred to a centrifuge tube and extracted with hot water for 10 minutes and centrifuged. The extract is decanted through a filter paper and the residue is then washed quantitatively into a Kjeldahl flask and total nitrogen is determined. It is assumed that the nitrogen that remains insoluble under these conditions represents protein nitrogen.

Starch was determined by the method of PUCHER, LEAVENWORTH and VICKERY (8).

Statistical analysis of the data

A preliminary examination of the data for each constituent was made in terms of the coefficients of variation of the means of the duplicate determinations. In each case, the variation within the group of samples collected by the statistical method was less than within either of the other two groups, suggesting the superiority of this method, but no choice could be made between the other two methods. However, the results showed sufficient promise to warrant a more extended analysis.

The study concerned the relative variability of each component for each method of collection, so that the determinations in table I were transformed to logarithms for analysis. All of the computations reported here have been based upon these log values. Their means have been transformed back into the original units to obtain the "geometric mean content" of each constituent shown in table II.

The first step was to estimate the analytical error for each constituent from the differences in the five paired values in each of the three series of samples. The standard deviation in units of a single observation was computed initially for each series and the series compared. As would be expected, they did not differ significantly from one another. The variances for each constituent were then pooled, the square roots of these composite values representing standard deviations, each with 15 degrees of freedom. The percentage analytical errors in column 6 table II are the antilogarithms of each such standard deviation, diminished by 1 and multiplied by 100.

The differences in the paired determinations for percentage of ash were markedly more variable than those for any other constituent. Even with the most discrepant difference omitted, the percentage standard deviation from the remaining 14 values dropped only from 2.24 to 1.30 per cent. and the latter value was still nearly double that for the next most

variable constituent.[2] The other analytical errors varied considerably more from one another than would be expected by chance if, in fact, all were equally precise. The analytical error for the protein nitrogen was less than that for total nitrogen, but the differences in the analytical error could be attributed only in part to the larger aliquots used for protein nitrogen.

There is a possibility that the analytical error of these determinations is underestimated because of the custom of the analyst of weighing out the samples for duplicate determinations at the same time and carrying out the analyses in pairs. This practice carries with it the possibility of a subconscious bias when, for example, the titration of the second member of an identical pair is made. In point of fact, many of the duplicate determina-

TABLE II

COMPOSITION OF SAMPLES OF LEAVES OF *Bryophyllum calycinum* IN GRAMS PER KILO OF INITIAL FRESH WEIGHT. SAMPLES IN SERIES ST. COLLECTED BY STATISTICAL METHOD, IN SERIES O.L. BY OPPOSITE LEAF METHOD, AND IN SERIES L.S. BY LEAF SIZE METHOD. ERRORS REPRESENT STANDARD DEVIATIONS

CONSTITUENT	SAMPLE SIZE	GEOMETRIC MEAN CONTENT			ANALYTICAL ERROR	NET SAMPLING ERROR		
		ST.	O.L.	L.S.		ST.	O.L.	L.S.
	mg.	*gm. per kilo*	*gm. per kilo*	*gm. per kilo*	%	%	%	%
Total solids	500	107.26	106.34	122.60	0.14	1.61	2.89	3.64
Ash	500	10.14	10.65	11.71	2.24*	1.01	4.40	3.20
Total nitrogen	100	2.795	2.881	2.489	0.70	1.39	3.71	3.14
Protein nitrogen	200	2.263	2.322	2.005	0.23	1.59	2.51	2.85
Starch	100	25.03	23.35	36.15	0.66	2.00	3.04	11.48†

* Omitting the most discrepant pair in 15 reduces this value to 1.30 per cent.
† In subsequent statistical analysis, this value was replaced by 3.71 per cent. See text for explanation.

tions were exactly equal to the limits of observation. The data would have been improved for the present purpose if single determinations had been made throughout a given set of samples and then repeated. Furthermore, the statistical analysis would have been facilitated if an effort had been made to estimate an additional significant figure in the measurements.

Although separate aliquots were used for each constituent (except ash), some correlation might be expected among the five measured constituents of each sample. An analysis of variance was computed for each method of sampling, separating the variation among the totals of the five constituents in each sample (with 4 degrees of freedom) from the interaction of samples with constituents (with 16 degrees of freedom). In every case

[2] The difficulty of obtaining high precision in the determination of ash in plant tissues has long been recognized and much study has been given to the problem in this laboratory. It seems possible that some of the trouble arises from the instability of calcium carbonate at the temperature (590 ± 10° C.) of the muffle furnace.

the sample totals were the more variable. The variance ratios (F) for series St., O.L., and L.S. were 4.40, 3.56, and 2.52, respectively, the first two being statistically significant. These F ratios may also be expressed as intra-class correlations (**5**) with an average value of $r = 0.327$ on a scale of 0 to 1. Although the relative amounts of the constituents in each sample were correlated significantly, the correlation was small and has been disregarded in comparing the sampling variances of the different methods.

As has been mentioned, the variation in a constituent from sample to sample involves two components. The first is the analytical error which has already been measured; the second is the variation among samples within a series. In an analysis of variance, the mean square between duplicates within samples is an estimate of the first component, *i.e.*, the analytical error, but that among samples includes both components. In the present study, the net variance among samples could be isolated and

TABLE III

ANALYSIS OF VARIANCE OF NET SAMPLING ERRORS, COMPUTED IN UNITS OF THE LOGARITHM OF THE NET VARIANCE COMPONENT AMONG SAMPLES, DETERMINED FROM FIVE LOG-WEIGHTS OF EACH CONSTITUENT. DISCREPANT STARCH VARIANCE (SERIES L.S.) REPLACED BY MISSING PLOT TECHNIQUE

TERM	DEGREES OF FREEDOM	VARIANCE RATIO, F
Among constituents	4	0.36
Statistical samples vs. others	1	35.46
Opposite leaf vs. leaf size samples	1	0.01
Effect of discrepant starch variance on error	1	11.09
Residual error	7	1.00

was equal to one-half of the difference between two mean squares, that among samples and that between duplicates within samples (**3**). The net variances among samples were changed to square roots and the square roots to antilogarithms to obtain the percentage errors shown in the last three columns of table II, each with 4 degrees of freedom. It is evident by inspection that the statistical method of leaf sampling gave a smaller net sampling error than either the opposite leaf or the leaf size method. The results are generally consistent except that the starch determinations from the leaf size samples had an abnormally large sampling error. This set of samples was not taken concurrently with the other two sets, and intervening weather conditions may have been a factor in the greater variability in the starch content of these leaves.

To determine the significance of the variations in the net sampling error, an analysis of variance was computed from the logarithms of the net variances among samples, which, in turn, were computed from the log-weights of constituent. As is shown by BARTLETT and KENDALL (**1**), the variance in these terms conforms better to the hypotheses underlying the

analysis of variance. Two analyses were computed, one with the observed values and one in which the starch estimate for series L.S. was replaced by the missing plot technique (**4**). This substitution reduced the estimate of error so markedly that it has been used in table III, although substantially the same results were obtained with either the original or the substituted value.

From the variance ratios in table III, it is evident that the leaf samples varied about equally in each constituent. The samples collected by the statistical method were clearly more uniform ($P < .001$) than the samples collected by either of the other two techniques. The opposite leaf and leaf size samples, on the other hand, were equally variable. For measurements of leaf constituents of the same precision, from four to five times as many leaf samples would be needed if collected by either of these two methods instead of by the statistical technique.[3] The relatively large effect of the discrepant starch variance in the leaf size sample is evident from the variance ratio in the fourth row of table III.

Summary

The uniformity in composition of samples of leaves of *Bryophyllum calycinum* has been tested by statistical analysis of the data obtained in duplicate for solids, ash, nitrogen, protein, and starch. Three sets of five samples each were collected, one by a method, referred to as the statistical method, which balances equally among the samples the effects of differences among plants, leaf positions, and leaflet positions. A second set was collected by a modification of the opposite leaf method and a third by a method of randomization according to leaf size.

A comparison of the results of the duplicate determinations showed that the several analytical procedures were of unequal reliability, that for ash being the least precise. After segregation of the analytical error, the net variation among samples was determined for each method of collection and for each constituent. The five constituents showed no differences in variability and the statistical method gave the most uniform samples. To obtain by either of the other two methods the same precision as was secured by the statistical method, approximately four to five times as many samples would be needed.

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[3] This estimate is based upon the geometric means of the corresponding net sampling variances of the log-weights, which were 0.000041 for the statistical method and 0.000195 for the other two techniques. The number of samples required for equal precision is inversely proportional to these estimates.

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