ACCURACY OF QUANTITATIVE METHODS USED FOR THE BOTANICAL ANALYSIS OF OESOPHAGEAL FISTULA SAMPLES

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ABSTRACT

Diets containing known proportions of from three to six forage species were fed to sheep fitted with oesophageal fistulae. Extrusa was collected from the oesophagus of each sheep and the botanical composition determined by hand separation, and to a lesser extent, the microscopic point, microscope, and liquid maceration procedures. The hand separation method was the least accurate and the liquid maceration method the most accurate but with limited field application. The major sources of error in these methods were determined and their use discussed. It is concluded that the hand separation and microscopic methods are only sufficiently reliable to allow estimation of dietary components to within broad categories defined as minor (< 20%), *moderate* (21–50%), *and major* (> 50%) *proportions.*

INTRODUCTION

Ecological studies in semi-arid rangelands demand attention to the diets selected by grazing livestock. Several techniques have been used to assess dietary preference. Oesophageal fistulation is the most favoured method for the determination of diets (Anderson 1977). The reliability of the technique is greatly influenced by the method of analysing the extrusa. Manual separation (hand sorting) of fresh extrusa and the microscope point technique are the two most favoured methods. A number of reports are now available in which comparisons have been made between two or more methods but the conclusions drawn are conflicting (Hall and Hamilton 1975, Westoby, Rost and Weins 1976). Hall and Hamilton (1975) concluded that, after adjustments for losses of organic matter prior to separation and for unidentifiable particles that result in a variable portion of the samples being separated, the "estimates determined by manual separation were similar to the actual botanical composition of the diet". They also concluded that the microscope point method was more accurate than manual separation provided that microscope hits were adjusted for weight per unit area of each plant component. Westoby, Rost and Weins (1976) found that the microscopic analysis procedure gave poor results, mainly because there was tendency to underestimate or miss entirely taxa that were present in small amounts, and to over estimate those present in large amounts.

For studies on semi arid rangelands we need a technique which is accurate and dependable and which takes note of the minor components of a diet. The aim of the work presented here was to assess the accuracy of various quantitative methods of diet analysis on known mixtures of forage collected from the oesophageal fistulae of sheep.

METHODS

Methods of analysing extrusa

Four methods were investigated. The hand separation method (Stobbs 1969) was compared with two other published methods, the microscopic point method of Heady and Torrell (1959) and the modifications proposed by Sparks and Malechek (1968) (microscope method), and one method (liquid maceration) developed by one of us (JWM) during this investigation.

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Two methods (hand separation and microscopic point) examine only the larger plant fragments while the other two (microscope and liquid maceration) examine all fragments. The methods which use large fragments identify plant species by standard taxonomic features. The methods which examine all fragments identify, by light microscopy, those plant particles which have distinguishing epidermal structures. The large fragments have to be reduced to a size suitable for microscopy by grinding or blending.

Hand separation method

The extrusa samples were thoroughly mixed and washed in a sieve of 2 mm opening. Sieving had the effect of removing an unknown proportion of the smaller fragments. After draining, several small portions were taken and all recognisable items removed. The size of the fragment removed depended on the characters required to identify the species, or group of species. Further small portions were separated until approximately 2 g dry weight had been sorted. The material was allowed to dry for several hours and the identification of the fragments was checked during transfer to weighing bottles for oven drying to constant weight at 85°C. Triplicate subsamples, each of 2 g dry weight, were analysed.

Microscopic point method

This method was first used by Heady and Torell (1959) and has been refined by Harker *et al.* (1964). The oesophageal fistula sample was rinsed over several layers of butter-muslin, then spread on an oblong tray and allowed to dry partially. The fragments occurring under the crosshair of a stereoscopic microscope were then identified at a magnification of ×18. The 400 points were selected in an ordered 1 cm × 1 cm grid across the material and the frequency of counts converted to weight composition by prediction equations (Heady and Van Dyne 1965). These equations were developed from known mixtures of the species used in each experimental feed, prepared so that each species in turn was the major component of the mixture. The mixtures were macerated in a vitamizer. It was recommended by Heady and Van Dyne (1965) that the regression of dry weight on points should be adjusted to pass through the origin. This was not done in the present study because the material was identical to that used in the forage.

Microscope method

This method, developed by Sparks and Malechek (1968) as a modification of the microscopic point method, is less laborious since it relies on a statistical approach, recording only presence or absence of a particular species in the microscopic field. Oesophageal fistula samples were dried and ground in a hammer mill through a 1 mm screen. A sample of particles was mounted in a chloralhydrate solution to clear the tissues; identifications based on epidermal features were made at ×125 magnification. The presence or absence of each species from 20 microscope fields per slide was noted on 5 slides. The frequency percentages for each species were converted to particle density per field using a table provided by Fracker and Brischle (1944). The relative density of a species, expressed as a percentage of all species, had been shown for several forbs and grasses to be directly related to the percentage weight of that species.

Liquid maceration method

This method was developed during the present investigation. Masticated material was stored in formalin aceto-alcohol. It was washed over a 2 mm sieve in series with a 0.074 mm sieve and the 2 mm fraction was further macerated in a blender. Sieving and blending was repeated (sometimes a small amount of material had to be cut with a razor blade) until all the material would pass through the 2 mm sieve. It was then transferred to 70% alcohol and a few drops of Sudan IV added to stain cuticle. After 30 min. the material was centrifuged and the alcohol replaced by 30% glycerine.

After thorough stirring and before settling a small sample was withdrawn in a glass tube and mounted in glycerine jelly. Adjacent transects across the preparation were examined at a magnification of × 60 or higher, and the shapes of two samples each of 100 fragments of identifiable epidermis or cuticle were outlined on squared paper using a *camera lucida*. Folds in the fragments and the presence of both epidermes were also recorded. The area of the fragments was determined and converted to weight on the basis of leaf and stem areas (specific leaf area) per unit weight. This was done for each species by the method of counting squares. Shoot tips, leaf sheaths and inrolled laminae were carefully dissected; all leaves were laid flat and secured to a glass plate with transparent adhesive tape, and the outlines were obtained by contact photography. Duplicate or triplicate samples of 2–3 g fresh weight were estimated and oven-dried at 85°C to constant weight.

Feeding of diets and collection of extrusa

Ten diets of known botanical composition were fed to oesophageally fistulated sheep. Mixtures varied from simple 3-species mixes to a 6-species mix that resembled a diet eaten by sheep grazing a semi-arid rangeland (Leigh and Mulham 1966*b*).

Collection of forage samples

Forage plants were selected from the available rangeland and irrigated pastures to provide diets varying in their morphological structure and digestibility. All plant material was hand-collected daily and sorted to exclude dead portions. The stems were usually cut into 2.5 cm lengths to facilitate mixing. Three 10 g subsamples were oven dried at 85°C to constant weight to estimate the dry matter content. The ten mixtures are listed in Table 1.

Animals and management

Oesophageal fistulae were established in six 18-month-old Merino wethers by the method of Torell (1954). The sheep were maintained on irrigated pasture.

Before collection periods, the sheep were penned without food for several hours (usually overnight) and the experimental feed was offered at a time of day to correspond to the natural grazing time. In early experiments (1 to 5a), sheep were penned singly and fed approximately 150 g fresh weight of experimental feed, but in later experiments (5b–10) several sheep were penned together with a communal bin of feed and the oesophageal samples were bulked.

Collection of diet samples

The diets to be evaluated were fed separately over a period of weeks. Material extruded through the fistula was collected in a plastic bag suspended around the sheep's neck. The small amount of the test ration offered but not eaten was sorted, oven dried, and weighed so that the composition of the ingested material could be calculated.

RESULTS

Evaluation of the hand sorting technique

Table 1 lists the diets tested, the known composition, the estimated composition by hand sorting, the differences, and a measure of variability. The percentage in the known diet compared with the percentage estimated by hand separation is shown in Figure 1 (a–c).

Differences between known and estimated diets were frequently large (up to 22 percentage units, average 7.3) and in several instances the ranking of the species was altered. There was a tendency for grasses to be overestimated and the forbs and chenopods to be under-estimated, with considerable variation (Figure 1 (a–c)). Such regressions would be of little value for correcting estimated values. In 9 of the 10 diets the species with the highest dry matter content was over-estimated. In these nine diets, the over-estimated species was a wiry grass in six mixtures, a broad-leafed grass in one and a chenopod in two.

TABLE 1

The estimation of known diets by the hand separation method.

Diet No.	Species	Dry weight in diet (%)			S.D.[1]
		Known	Estimated	Difference	
1	*Danthonia caespitosa*	49	62	+13	4.1
	Maireana pentagona	36	22	—14	3.2
	Atriplex semibaccata	15	16	+ 1	3.2
2	*Stipa variabilis*	64	78	+14	4.4
	Lolium rigidum	21	17	— 4	3.3
	Hordeum leporinum	15	5	—10	1.2
3	*Maireana aphylla*	43	53	+10	2.9
	Atriplex vesicaria	39	27	—12	2.0
	Lolium rigidum	18	20	+ 2	1.3
4a	*Atriplex vesicaria*	44	39	— 5	4.1
	Erodium crinitum	29	26	— 3	3.0
	Lolium rigidum	27	35	+ 8	1.1
4b	*Atriplex vesicaria*	44	42	— 2	1.9
	Erodium crinitum	29	19	—10	2.8
	Lolium rigidum	27	39	+12	3.4
5a	*Stipa variabilis*	30	52	+22	6.2
	Maireana pentagona	35	15	—20	1.7
	Erodium crinitum	35	33	— 2	6.4
5b[2]	*Stipa variabilis*	29	43	+14	1.0
	Maireana pentagona	40	35	— 5	1.2
	Erodium crinitum	31	22	— 9	0.7
6	*Lolium rigidum*	28	50	+22	4.7
	Atriplex vesicaria	40	27	—13	6.2
	Erodium crinitum	32	23	— 9	2.1
7	*Stipa variabilis*	14	33	+19	5.4
	Maireana pentagona	17	17	0	1.2
	Lolium rigidum	11	10	— 1	1.0
	Medicago sativa	38	30	— 8	5.7
	Atriplex vesicaria	9	4	— 5	1.2
	Erodium crinitum	11	6	— 5	1.5
8	*Eragrostis* sp.	10	16	+ 6	2.9
	Maireana pentagona	20	19	— 1	0.7
	Atriplex vesicaria	15	15	0	2.0
	Medicago sativa	32	22	—10	1.4
	Phalaris tuberosa	13	14	+ 1	5.8
	Echium sp.	10	14	+ 4	1.4
9	*Eragrostis* sp.	10	17	+ 7	0.7
	Maireana pentagona	48	43	— 5	4.7
	Atriplex vesicaria	20	25	+ 5	1·7
	Phalaris tuberosa	22	15	— 7	3.0
10	*Maireana pentagona*	15	20	+ 5	2.6
	Atriplex vesicaria	67	62	— 5	3.0
	Medicago sativa	16	16	0	1.8
	Echium sp.	2	3	+ 1	2.0

[1]S.D.=standard deviation of 3 sub-samples.
[2] Diets 1-5a were fed to a single sheep fed individually in a pen.
Diets 5b-10 were fed to several sheep feeding from a comunal bin. The extrusa from these sheep were bulked.

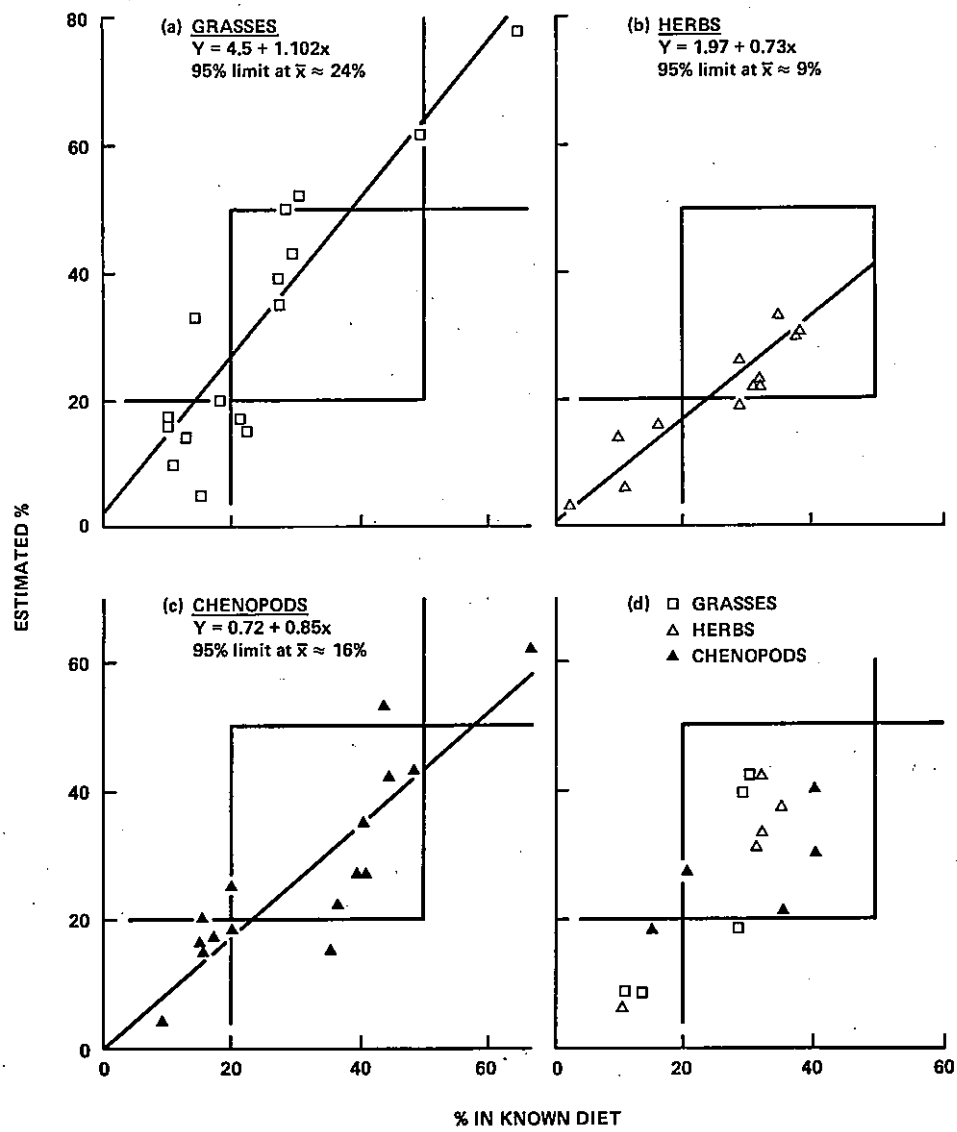

FIGURE 1

Known percentage of grasses (a) forbs (b) and chenopods (c) in diet plotted against percentage estimated by hand separation (a–c) and by microscopic point (d). Proportions of species in the diet have been grouped as minor (< 20%), moderate (21–50%) and major (> 50%).

In a recent study of hand sorting (Chacon, Stobbs and Haydock 1977) it was found that small particles (of the type that might well have passed through our 2 mm sieve) comprised 7–29% on a dry weight basis of the diet extruded by cattle. By identification of these particles on a ranking basis, the precision of their technique was improved. It is possible that if this modification had been applied by us that the variation in our study would have been less than the approximate 31% achieved (see Table 1).

Replication of samples for hand separation was extremely time-consuming. Since the differences between known and mean estimated amounts, when expressed as a percentage of the known, exceeded the coefficient of variation in 80% of the species it appears that replication is unjustified.

Two of the mixtures (diets 5b and 6) were analysed by all of the four procedures described earlier, two (5a and 8) by the hand separation and the microscopic point methods only (Table 2) and the remainder by hand separation only (Table 1). Because of the need to store samples while waiting analysis (see below) the procedure adopted was to take two 25 cc samples of the extrusa for either oven drying or preservation in formalin aceto-alcohol. Fresh extrusa were used for the hand sorting method and partly wilted material for the microscopic point procedure.

Storage of extrusa

All analytical methods are lengthy so there is inevitably an accumulation of oesophageal samples which must be stored. Samples for hand separation were washed, drained, and stored in polythene bags at 4°C with a few crystals of thymol to inhibit fungal growth. This material was readily identifiable for up to two weeks; the short storage time limits the number of samples that can be collected from an experiment. Deep freezing was found to be unsuitable because identification of plant parts was made more difficult. Deep freezing was used for the microscopic point method as the identification characters were not altered markedly when the material was thawed. For the microscope method, samples are dried, therefore storage places no limit on experimental design. In the liquid maceration method the samples were stored indefinitely in alcohol-based solutions. This is not acceptable for the other methods because of colour changes.

Comparison of other methods

Results obtained by the microscopic point, microscope, and liquid maceration methods are shown in Table 2 and Figure 1d. The differences between known and estimated diets in the microscopic point method averaged 6 per cent units and the largest difference was 12. The regression line in Figure 1d shows that a relationship exists between known and estimated values but that the variation is great. The association of over-estimation of the species with the highest dry matter content was not so marked as in the hand separation method. In the microscope method the largest difference was 34 per cent units and this may have arisen from insufficient training of operators or from an incorrect assumption that a direct relationship of per cent relative density to per cent weight held for this species. The specific leaf area of the grass was over twice that of the chenopod. In the liquid maceration method the largest difference was 6 per cent units.

A statistical comparison of the four methods by Tukey's test, calculated on an harmonic mean, shows that there was no significant difference between the species groups (grasses, forbs and chenopods) and the accuracy with which they were estimated, but there was a significant ($P < 0.05$) difference between the liquid maceration and the microscope methods. The errors in the microscope method were greater than the errors in the other three methods.

Major sources of error

In the hand separation method, the main error arose from the differential reaction of the various species to mastication. Trials with individual species showed that 92% (*Sporobolus caroli*) to 77% (*Hordeum leporinum*) of grasses eaten were retained on a 2 mm sieve, whereas the retention for other species was much lower (*Maireana pentagona* 51%, *Atriplex vesicaria* 67%). Analysis of fine fragments (those retained on a 1 mm sieve) did not improve accuracy.

The accuracy of the microscopic point method, using 400 points, when applied to macerated material was similar to that in the literature, as a mean point composition was estimated to within 20% at the 90% confidence level when 20–40% of a species was present (Galt *et al.* 1969, Grimes *et al.* 1965, Harker *et al.* 1964). However, when the method was applied to known diets collected through oesophageal fistulae, the percentage dry weight of the component species in the known diets were not estimated within these limits (Table 2).

In the microscope method a major source of error was found to be the differential loss of isolated epidermis on grinding. Examination of liquid macerated and ground preparations of the one sample showed that a sample of *Medicago sativa* changed from a mixture of 65:35 isolated epidermes : epidermes with mesophyll to a ratio of 50:50 after grinding. For *Atriplex semibaccata* the ratio changed from 23:77 to 0:100, whereas for *Lolium rigidum* the ratio changed from 15:85 to 35:65. Thus a variable error is introduced which depends on the differing extent to which epidermes are readily detached during mastication and subsequently destroyed by grinding.

The main source of error in the liquid maceration method is in sampling and measuring the areas of both the microscopic fragments and the intact leaves. The average error between the duplicate samples of 100 fragments was 14% and the average error between duplicate or triplicate estimations of specific leaf area was 12%.

DISCUSSION

Material collected from the oesophagus of a grazing animal contains the species in their chosen proportions and is a true reflection of the diet during the sampling time. However, the accuracy of the analytical methods used to estimate the botanical composition on a dry weight basis is mostly poor and decreases in the following order: liquid maceration, microscopic point, hand separation and microscope. The errors vary with the mixture of species in the forage. Although it would have been preferable to have tested all methods on all diet mixtures, the weaknesses of the liquid maceration and microscope methods were revealed without the need for extensive testing.

The accuracy of the two methods which examine mainly the larger fragments is influenced by the extent of destruction of the plants during mastication and this is determined by the plants and the animals used. The accuracy of the two methods which examine all fragments is affected by steps within the method. Destruction of dried extrusa during grinding is a feature of the microscope method so that this method is not recommended. The time needed to measure sufficient samples of fragments and to determine meaningful specific leaf areas is a major disadvantage of the liquid maceration method.

The liquid maceration method, while giving the best results with simple, known diets, would be difficult to apply in the field. It requires a knowledge of the epidermal pattern of all structures of a plant species, including seeds and fruits, and it is doubtful whether it can encompass thick, corky layers on secondarily thickened stems. The specific leaf areas of each species would have to be meaningful and if a higher proportion of fruit and seeds than of leaves and stems was ingested, then the same proportion would have to be represented in the sample taken for specific leaf area measurement.

The microscopic point and hand separation methods, in which only the larger fragments are identified, are less accurate but more practical for field application than liquid maceration. They cannot, however, be relied upon to give more than a broad description of the diet. When the proportions of species in the known diets were described as being minor ($< 20\%$), moderate (21–50%), or major ($> 50\%$) then fair agreement was found between the known and estimated diets (see Figure 1 (a–d)). The microscopic point method was 93% correct (15 points) and the hand

TABLE 2

The estimation of four known diets by two or four methods and difference from the known composition.

Diet No.	Species	Known composition (%)	Estimation of dry weight (%)							
			Hand separation	Difference	Microscopic point	Difference	Microscope	Difference	Liquid maceration	Difference
5a	*Stipa variabilis*	30	52	+22	42	+12				
	Maireana pentagona	35	15	—20	21	—14				
	Erodium crinitum	35	33	— 2	37	+ 2				
5b	*Stipa variabilis*	29	43	+14	39	+10	40	+11	24	—5
	Maireana pentagona	40	35	— 5	30	—10	35	— 5	39	—1
	Erodium crinitum	31	22	— 9	31	0	25	— 6	37	+6
6	*Lolium rigidum*	28	50	+22	18	—10	62	+34	29	+1
	Atriplex vesicaria	40	27	—13	40	0	19	—21	42	+2
	Erodium crinitum	32	23	— 9	42	+10	19	—13	29	—3
8	*Eragrostis* sp.	10	16	+ 6	8	— 2				
	Maireana pentagona	20	19	— 1	27	+ 7				
	Atriplex vesicaria	15	15	0.	18	+ 3				
	Medicago sativa	32	22	—10	33	+ 1				
	Phalaris tuberosa	13	14	+ 1	8	— 5				
	Echium sp.	10	14	+ 4	6	— 4				

separation method 82% (44 points). When the hand separation method was applied to the bulked oesophageal sample obtained from several sheep, the estimation of each species to within the correct category was improved. The bulking of samples in the field would therefore seem advisable, replication being obtained by sampling on successive days. This would also reduce the number of hand separations to be performed.

Although quantitative methods have been used to describe the diet of a population of grazing animals in terms of mean dry weight per cent of different species, the variability between animals has diminished the value of the results. Thus Leigh and Mulham (1966a, 1966b, 1967) who used the hand separation method, drew no more from their results than could be obtained had the figures been reported as falling in one of the three broad categories (minor, moderate, major) already described. Another observation they included was the frequency of occurrence of a particular species in the samples analysed. This value indicates whether a species is taken or ignored and when a high frequency for a minor species is recorded it may indicate a species which would become more important in the diet if more of it were available or if other species were less abundant.

It is concluded that the analysis of forage samples by the hand separation and microscopic point methods should not extend beyond the frequency of occurrence of species in those samples and an assessment of whether the species is of minor, moderate or major importance. These categories can be compared to the availability of herbage, similarly described, and a rough preference index established if relevant.

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