Factors Influencing Microhistological Analysis of Herbivore Diets

M. VAVRA AND J.L. HOLECHEK

Abstract

A study simulating herbivore diets was conducted to compare actual and estimated diet constituents as influenced by sample preparation technique and in vitro digestion. Nine plant species, three each representing grass, forb, and shrub forage classes were hand composited into three mixtures so that one forage class dominated each mixture. Samples of each mixture were then allotted to eight treatments involving combinations of grinding through a micro-Wiley mill, soaking in sodium hydroxide and in vitro digestion. Samples were then analyzed for botanical composition using the microhistological technique. In vitro digestion had the greatest impact on the difference between estimated and actual means. In digested samples grasses were overestimated while shrubs and forbs were underestimated. The preferred treatment involved grinding in a micro-Wiley mill and the sodium hydroxide soak.

Food habits studies using the microhistological technique of identifying diet constituents have appeared in the literature since Baumgartner and Martin (1939) first described the technique. Denham (1965) and Sparks and Malechek (1968) verified the technique by hand compounding mixtures of grasses and forbs.

Preparation of material for microscopic identification has varied considerably. Crocker (1959) simply diluted fecal material with water and spread the material between two microscope slides. Storr (1961) boiled, dried and ground samples in a mixture of nitric and chromic acids. The samples were then washed with water; stained with violet; and finally centrifuged and mounted on slides. Other techniques of sample preparation have been presented by Dusi (1949), Hercus (1960), Hegg (1961), Steward (1967), Zyznar and Urness (1969), Casebeer and Kess (1970), Ward (1970), Hansen et al. (1971), and Korfhage (1974).

In the laboratory we have found that grinding fecal and fistula material in a Wiley mill followed by soaking in a dilute solution of sodium hydroxide increased the number of identifiable particles.

This study was designed to investigate the effect of the sample preparation procedure just described on composited diets of different botanical composition. The primary objective was to determine whether particular procedures improved or hindered the identification of epidermal fragments. Sample preparation, in itself, particularly grinding in a Wiley mill as suggested by Sparks and Malecheck (1968) could destroy some epidermal fragments and invalidate the assumption that grinding the material to the same particle size is necessary to obtain the 1:1 ratio of relative density to percent weight. A second objective was to observe the influence of in vitro digestion on botanical composition determined by the microscope technique. Certain plant species may undergo greater destruction than others during digestion (Vavra et al. 1978). Digestion then, could make a diet sample more sensitive to destruction by sample preparation techniques. Results from fecal analysis would then be biased toward the more indigestible diet constituents.

Authors are associate professor and graduate research assistant, Oregon State University, Eastern Oregon Agricultural Research Center, Union and Burns, Ore. J.L. Holechek is currently assistant professor of range science, Department of Animal and Range Sciences, New Mexico State University, Las Cruces, New Mexico 88003.

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Materials and Methods

Common forage plants found in northeastern Oregon representing grass, forb, and shrub forage classes were hand compounded into mixtures. No attempt was made to use particularly digestion-sensitive plants. Orchardgrass (*Dactylis glomerata*), timothy (*Phleum pratense*), and a sedge (*Carex* spp.) were used to represent the grasses and grasslike species. At the time of clipping the reproductive structure of the sedge was not present and no attempt was made to key out the species. Orchardgrass and timothy were in the dough stage. The forbs were Canada milkvetch (*Astragalus canadensis*), velvet lupine (*Lupinus leucophyllus*), and Oregon checkermallow (*Sidalcea oregana*). All three forbs were in flower at the time of clipping. Snowbrush ceanothus (*Ceanothus velutinus*), mallow ninebark (*Physocarpus malvaceus*), and common snowberry (*Symphoricarpos albus*) were the selected shrub species. Only current year's growth of the shrubs was used.

After collection in mid-summer, the samples were dried in a forced air oven and then mascerated by hand so that stem lengths did not exceed 2 cm and leaves were crushed to allow even mixing of plant parts and more accurate weighing. All portions clipped of all plant species were included in the formulated diets. Three mixtures containing all nine plants were prepared. Each of the three mixtures was formulated so that one forage class dominated (Table 1).

Table 1. Percentages by weight of each plant species formulated into the three simulated diets.

Forage class level	High grass	High forb	High shrub
Grasses			
Orchardgrass	20	5	5
Timothy	20	10	10
Sedge	20	5	5
Shrubs			
Common snowberry	5	5	20
Snowbrush ceanothus	10	10	20
Mallow ninebark	5	5	20
Forbs			
Canada milkvetch	5	20	5
Oregon checkermallow	10	20	10
Velvet Lupine	5	20	5

The formulated diets were then subjected to eight treatment combinations that involved in vitro digestion (A), grinding through a micro-Wiley mill with a 1 mm screen (B), and soaking in a .043 M solution of sodium hydroxide for 30 minutes (C) (Table 2). The actual sequence of application was B,A,C. The in vitro digestion system employed was similar to that described by Tilley and Terry (1963); however, the pepsin stage was omitted as it should have had little effect on cellular destruction. Following the in vitro digestion, the material was dried prior to further treatment. Micro-Wiley mill grinding was omitted in some treatment combinations because some fragile epidermal structures could have been destroyed (Rogerson et al. 1976).

Table 2. Treatment combinations applied to formulated diets.

Treatment code	In vitro digestion	Grind	NAOH
$A_1B_1C_1$	Yes	Yes	Yes
$A_1B_1C_2$	Y	Yes	No
$A_1B_2C_1$	Yes	No	Yes
$A_1B_2C_2$	Yes	No	No
$A_2B_1C_1$	No	Yes	Yes
$A_2B_1C_2$	No	Yes	No
$A_2B_2C_1$	No	No	Yes
$A_2B_2C_2$	No	No	No

All materials, following the above treatments, were soaked in water for two hours and then blended for one and one-half minutes at high speed in a blender. After blending, the material was mounted on microscope slides following the procedure of Sparks and Malechek (1968).

Five replicate samples of three slides each were prepared per treatment combination and forage class level and 20 microscope fields per slide were observed. For the analysis of variance and for correlation analysis the number of observations was five. Frequency of occurrence of each species was calculated and converted to relative density, which was used as the percent weight estimate for each species in the diets as outlined by Sparks and Malecheck (1968).

A completely randomized nested factorial design was used to detect differences due to treatments (Anderson and McLean 1974). Duncan's new multiple range test was utilized to rank means where appropriate. Correlation procedures described by Neter and Wasserman (1974) were used to evaluate the degree of association between actual and estimated diets. Where linear regression was used to develop prediction equations for species subjected to different treatments, the procedures of Neter and Wasserman (1974) were used.

Results

Estimated and actual means for the different species used in the investigation are given in Table 3. These data indicate certain species undergo much greater destruction during sample preparation than others. The grasses and grass-like species used in this investigation were uniformly overestimated while forbs were uniformly underestimated. The analysis of variance revealed significant differences in forage class and species ($P<.01$) and the forage class-treatment interaction ($P<.05$). Browse species did not exhibit a uniform response. Common snowberry was greatly underestimated. In contrast snowbrush ceanothus was overestimated.

Forage classes at different levels did not respond uniformly to sample preparation (Table 4). The estimated and actual values were significantly, although not reliably, correlated ($r= .71$) ($P<.05$). The data presented show that the diet high in grasses and grass-likes was more accurately determined than the high forb or shrub diet.

Table 5. Interaction means for forage class and individual treatment combinations.

Treatment combinations	Grass (%)	Shrub (%)	Forb (%)
$A_1B_1C_1$	65^{a}	28^{b}	7^{b}
$A_1B_1C_2$	61^{a}	31^{ab}	8^{b}
$A_1B_2C_1$	65^{a}	27^{b}	8^{b}
$A_1B_2C_2$	61^{a}	29^{ab}	10^{b}
$A_2B_1C_1$	34^{b}	31^{ab}	35^{a}
$A_2B_1C_2$	33^{b}	30^{ab}	37^{a}
$A_2B_2C_1$	34^{b}	32^{a}	34^{a}
$A_2B_2C_2$	37^{b}	25^{b}	38^{a}

[a,b] Means within columns with different superscripts are significantly different at the .05 level.

Each forage class made up 33% of the prepared diet when the three forage class levels were pooled. Therefore values deviating greatly from 33% poorly represent the prepared diet. Those treatment combinations involving in vitro digestion were consistently different ($P<.05$) from those not digested (Table 5). Grasses and grass-likes were overestimated and forbs underestimated in those samples exposed to in vitro digestion.

Table 3. Estimated and actual means for plant species composition (%) over all treatment combinations.

	Grass and Grass-like			Shrubs			Forbs		
	Orchard-Orchardgrass	Timothy	Sedge	Snowbrush ceanothus	Common snowberry	Mallow Ninebark	Oregon checker-mallow	Canada Milk-vetch	Velvet lupine
Estimated means	14	19	16	19	2	8	8	7	7
Acutal means	10	13.3	10	13.3	10	10	13.3	10	10

Table 4. Means for the interaction of forage class and forage class level in the diet.

	Grasses (%)		Shrubs (%)		Forbs (%)	
Forage class level	Actual	Estimated	Actual	Estimated	Actual	Estimated
High grass	60	66	20	19	20	15
High browse	20	40	60	45	20	15
High forbs	20	40	20	23	60	37

Table 6. Correlation coefficients (*r*) between actual and estimated forage class levels for the eight treatments.

Treatment							
$A_1B_1C_1$	$A_1B_1C_2$	$A_1B_2C_2$	$A_1B_2C_2$	$A_2B_1C_1$	$A_2B_1C_2$	$A_2B_2C_1$	$A_2B_2C_2$
.47	.36	.33	.40	.99**	.94*	.96**	.88*

*Significant ($P<.05$)
**Significant ($P<.01$)

Correlation analysis was conducted to better evaluate the association between actual and estimated diets. Diets digested in vitro had low *r* values (Table 6). However, those not digested represented the formulated diets quite well. Treatments $A_2B_1C_1$ and $A_2B_2C_1$ had *r* values of .99 and .96, respectively. Both correlation coefficients were highly significant ($P<.01$). Further correlation analysis was conducted to evaluate the response of the two best treatment combinations with specific forage classes (Table 7). Treatment $A_2B_1C_1$ gave the best accuracy for grasses and grass-likes ($r = .96$) ($P<.01$). Shrub and forb determination was less accurate. Common snowberry was destroyed to a large degree by all treatment combinations. It specifically accounted for the reduced sensitivity of shrub determination. Treatment $A_2B_2C_1$ gave reduced sensitivity with grasses and grass-likes but did not improve analysis of shrubs and forbs.

Table 7. Correlation coefficients (*r*) between plant species in each actual and estimated forage class for treatments 5 and 7.

Treatment	Grass	Forb	Browse
$A_2B_1C_1$ (r)	.96**	.89*	.80
$A_2B_2C_1$ (r)	.77*	.90*	.83*

*Significant ($P<.05$)
**Significant ($P<.01$)

Discussion

In vitro digestibility greatly influenced the results of microhistological analysis particularly in the estimation of grass and forb content as indicated in Table 5. Shrub content showed less deviation; however, individual species were either over or underestimated. At the sampling intensity used, common snowberry was identified in only small amounts in some digested samples and was totally absent in others. Snowbrush ceanothus was uniformly overestimated in digested samples. This caused the total shrub content to approach the expected. Differential destruction of different species during digestion is substantiated in the literature (Vavra et al. 1978). However, Dearden et al. (1975) found all plant samples to be identifiable when collected from nylon bags placed in the rumens of cattle, bison, and reindeer and in fecal pellets from the same animals. It becomes obvious that there is tremendous variation in structural breakdown of plant species during digestion which influences identification of the particle in the feces.

Treatment $A_2B_1C_1$ is the combination of choice as indicated by the *r* values. This treatment involves grinding the sample through a Wiley mill to standardize particle size followed by soaking in sodium hydroxide. Even with this treatment some species can be expected to undergo some destruction. Rogerson et al. (1976) also indicated that certain plants contain very fragile cuticles. Grinding to reduce particle size as suggested by Sparks and Malechek (1968) could be destructive to some epidermal material. This results in deviation between percent weight in the diet and percent relative density. Dearden et al. (1975) also found that some plant species in their study did not have the 1:1 ratio of estimated percent weight and actual percent weight reported by Sparks and Malechek (1968). Dearden et al. (1975) mixed hand compounded diets and then estimated percent weight by microhistological technique. Regression equations were developed to correct estimated percent weight to the actual percent weight. A regression equation to correct common snowberry was developed in our study. The equation was $Y = 4.86 + 1.29x$ ($r^2 = .97$). This equation was highly significant ($P<.01$).

It seems possible that similar regression equations could be developed for use with fecal analysis. Hand compounded mixtures digested in vitro should be adequate to develop the equations. Pulliam (1978) developed a relative digestibility coefficient by feeding selected forages to elk. This was then used as a direct multiplier to correct fecal analysis. However, in vivo studies for baseline data would not be practical for wide spread use.

From the data presented, some guidelines for users of the microhistological technique can be suggested:

1) A sample preparation procedure that involved grinding through a Wiley mill with a 1-mm screen and soaking in sodium hydroxide should result in the best sample for analysis.

2) Some plant species, regardless of sample preparation, will be sensitive to epidermal destruction upon preparation. Therefore, in developing a reference collection, sufficient plant material should be collected so that hand compounded mixtures can be made. Regression equations can then be developed from estimated and actual values to correct for bias due to epidermal destruction.

3) Some plant species are sensitive to epidermal destruction during passage through the digestive tract. Because of this, some form of correction for the effects of digestion is recommended when fecal analysis is used to quantify animal diets.

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