TECHNIQUES FOR EVALUATING DIETS OF LIVESTOCK GRAZING NATIVE RANGES

James A. Pfister

and

John C. Malechek

1. Major field sampling techniques

A. Reliability of major sampling techniques

One may categorize the major field sampling techniques into six groups:

1) observation of free-ranging animals

2) hand plucking or "bite-count"

3) hand clipping plots before and after grazing

4) using esophageal fistualted animals

5) using ruminally fistulated animals

6) examination of feces or contents of the of digestive tract

Observational methods are generally inadequate for evaluating diets (Hardison et al., 1954; Lesperance at al., 1960; Galt et al., 1969). Errors of apprending, which are physical factors impeding the observation of the grazing animals, can be difficult to overcome in dense vegetation. Observer error or bias can influence observational results. It is often very difficult to determine the exact plant species being consumed at a given time. Observer effect, which may alter the feeding behavior of the animals, can be sutle and difficult to detect. See Lehner (1974) for a discussion about problems of observing animal behavior. Observational methods yield only qualitative data about animal diets (Theurer et al., 1976). The dry weight composition of diets can not be accurately determined, neither are samples available for later chemical analyses. However, the technique is inexpensive and easy to implement once, plant species are well known and animals accustomed to being observed.

Relatively few tests have been made of the bad plucking or "bite-count" technique (Reppert, 1960; Free et al., 1971). Under certain conditions, this technique appears to give reliable information (Theurer et al., 1976).

Because animals generally select forage higher in crude protein and lower in fiber than the average available in the vegetation complex, hand clipped samples (Coleman and Barth, 1973; Bredon, 1967) usually do not correspond well with the

chemical composition of animal diets. Hand clipped samples also do not give good estimates of botanical composition (Theurer et al., 1976).

Rumen fistulae have been used in domestic animals to allow total evacuation of rumen contents before actual collection of grazed diet samples (Lesperance et al., 1960a). Rumen fistula sampling provides reliable information, however sampling is laborious and time-consuming, thus it is difficult to sample adequate numbers of animals. Grab samples taken through rumen fistulae, are not representative of animal diets (Rice et al., 1971).

The use of esophageal fistulated animals is recognized as the procedure of choice for obtaining representative samples of animal diets (Theurer et al., 1976). Although the esophageal fistula technique may be used for a number of experimental purposes (McManus, 1981), the technique is most commonly applied to determine the botanical and nutritive content of animal diets.

Even though recovery of ingesta through esophageal fistulae is not 100%, such dietary samples are valid unless the fraction extruded through the fistula is not representative of the plants consumed. Hamilton and Hall (1975) demonstrated that incomplete sample recovery (54%) did not alter the botanical composition of the fistula samples of a known diet.

Certain chemical changes in extruded boli have been well documented (Lesperance at al., 1974). Considerable variation in results relative to changes in protein, soluble carbohydrates or nitrogen-free extract (NFE) and fiber have been reported (Lesperance et al., 1974). Generaly, ash contamination is significant (Grimes et al., 1965; Campbell et al., 1968; Berth and Kazzal, 1971; Scales et al., 1974). Therefore, chemical constituents should be expressed on an organic matter basis (Wallace et al., 1972). Changes in crude protein on nitrogen content have been variable, with some studies reporting no significant protein changes (Lesperance et al., 1960a; Grimes et al., 1965; Langlands, 1966; Barth and Kazzal, 1971; Little, 1972). Other studies, however, have noted significant changes in protein attributed to salivary contamination (Campbell et al., 1968;

Alder, 1969; Scales et al., 1974).

Variations in N content of extrusa due to salivary contamination have been questioned because of the large amount of saliva that must be added to ingesta to result in appreciable N increases (Galt et al., 1969; Hodgson, 1969). Losses of soluble protein and contamination by salivary protein may negate each other, although feeds low in protein may have a net addition of N because of lower leaching losses (Scales et al., 1974). Doyle (1967) found that salivary N contamination was positively related to the extent of mastication. Since the quantity of saliva secreted is related to mastication time (Batley, 1975), this may explain increased crude protein levels in low quality feeds (Scales et al., 1974). To minimize any possible effect of salivary N, fistulated animals should be maintained on pastures similar in N content to those pastures being sampled (Lesperance et al., 1974). In estimating the nutritive value of animal diets, either the total extrusa (Obioha, et al., 1970), or extrusa squeezed through cheesecloth and separated into liquid and solid fractions (Grimes et al., 1965) may be used. Generally there is only a small increase in accuracy gained from analyzing the solid and liquid fractions separatedly (Lesperance et al., 1960a, Hoehne et al., 1967; Marshall, Torell and Bredon, 1967). However, Cohen (1979) cautions that when the available forage has N values in excess of 2.7 N/100 g OM, it may be necessary to separate the two phases, because significant amounts of soluble N may be released on mastication and pass into the liquid phase. If one is conducting comparative research, where absolute values are not as important, little may be gained from using squeezed samples, assuming equal N losses across all treatments.

Soluble carbohydrate fractions are usually decreased in esophageal fistula samples (Grimes et al., 1965; Hoehne et al., 1967, Barth and Kazzal, 1971; Coleman and Barth, 1973). In many instances, this has been attributed to leaching of soluble carbohydrates by saliva when screen-bottomed collection bags are used.

Grimes et al. (1965) warned of possible losses of soluble carbohydrates if

the liquid phase of extrusa is not analyzed separately. This effect was particularly evident with sample material low in dry matter collected with a large amount of saliva.

Fiber has been shown to increase in extrusa in some studies, while others have reported no change in fiber values (Lesperance et al., 1974). Because fiber and soluble carbohydrates are calculated by subtraction, a decrease in soluble carbohydrates could result in a concomitant increase in fiber (Coleman and Barth, 1973). Scales et al. (1974) observed that significant differences in the fiber content of forages during one particular sampling year were a reflection of the loss of cell solubles rather than increases in fiber per se.

Fecal analysis to determine the botanical composition of animal diets has been widely used for a variety of herbivores includng kangaroos and wallabies in Australia (Storr, 1961), snowshoe hares (Adams et al., 1962), pocket gophers (Ward and Keith, 1962), reindeer (Deardon et al., 1975), bighorn sheep (Todd and Hansen, 1976), elk (Hansen and Clark, 1977), and deer (Zyznar and Urness, 1969). This technique has perhaps been best applied in quantifying wildlife diets because practically unlimited sampling is allowed, and disturbance of animals is minimal (Anthony and Smith, 1974). Because esophaeally-fistulated domestic animals are difficult to maintain, and sampling with these animals involves considerable effort, fecal analysis is sometimes used as an alternative. Analysis of digestive tract contents of killed animals will not be discussed in this paper. Ward (1970) reviewed digestive tract and fecal analysis methodology.

Disagreement is common in the literature about the reliability of fecal analysis (Johnson and Pearson, 1981; Vavra et al., 1978; Smith and Shandruk, 1979; Dearden et al., 1975; Anthony and Smith, 1974; Slater and Jones, 1971). Differential digestion of plant species may significantly influence results (Slater and Jones, 1971; Smith and Shandruk, 1979). Vavra et al. (1978) and Vavra and Holechek (1980) indicated that grasses are more resistant to digestion than are forbs. For this reason, use of fecal analysis should be preceeded by

preliminary trials conducted at several seasons of the year to determine correction factors for differential digestibility of major plant species. Because of the tedious nature of this type of digestion trial when repeated several times per year for each major diet component, strict and careful implementation of fecal analysis may be difficult.

A further source of potential error with fecal analysis is that digestion may fragment plant cuticle, reducing reliability of quantification procedures (Slater and Jones, 1971). In addition, digestion may influence identification of plant residues in feces (Vavara et al., 1978). Zyznar and Urness (1969) found that fecal samples seldom contained more than a low percentage of recognizable fragments even though deer in their trials were fed a single-species diet. Other workers maintaned that fecal analysis is reliable (Johnson and Pearson, 1981), but that perhaps the degree of accuracy expected from fecal analysis is less than that obtained from use of esophageal fistula extrusa (Vavra et al., 1978).

B. Application of esophageal fistula technique

Some basic considerations a researcher should carefully think through before beginning a collection scheme are discussed next. In establishing esophageal fistulae in experimental animals one invests considerable money and effort, therefore the animals should be used with care and forethought. If one simply begins collecting samples in a haphazard fashion, the results will likely be of limited value (see Platt, 1964, and Romesburg, 1981). An essential first step is to clearly define (in writing) the research problem, then review the literature pertinent to this problem. Before committing animals to the field, have a well-established treatment design written down. In determining the treatment design, a researcher brings to bear all his knowledge and experience about a problem. The treatment design specifies the structure of a set of treatments, and thus strongly influences the experimental design and statistical analysis of a data set.

An important question to be considered in using fistulated animals is the

number and seasonality of the collection periods. If one wishes to measure general dietary trends over a year, predetermined collection periods may be best. Statistically there are advantages to previously scheduled, regularly-spaced collection periods. However, biologically, it may make more sense to use either the grazing animals or the vegetation (for example, onset of growing season) as an indicator of when to sample. In this case, the research objectives govern the choice of sample periods. Diet studies are usually done over several years because dietary variations between years may be large, and are important to measure.

The number of animals used as sample collectors will need consideration before surgery is performed. One should consider reserve animals as well, as losses are inevitable. Sampling to determine the botanical composition of animal diets may require a large number of animals (Theurer et al., 1976). Even on ranges such as Utah winter range or dry caatinga forest where the number of plant species available is relatively small, at least eight fistulated goats or sheep should be used (Van Dyne and Heady, 1965; Harniss et al., 1975; Theurer et al., 1976). The minimum sampling number for cattle is probably four to six (Theurer et al., 1976).

One can compensate for fewer animal numbers by increasing the number of days sampled within one period. However, one must consider that the vegetation may be changing during the sample period, and rapid changes may greatly influence the botanical and nutritive content of the samples. In addition, the use of extra days to increase the sample number (n) will require the analysis of each individual animals sample for each day, so the animal and day sources of variation be partioned in the analysis of variance. One should evaluate the extra time required for botanical analysis of individual animals' samples collected over a series of days. Pooling of individual diet samples over days will not allow estimation of the variation associated with days, and n will equal the average daily number of fistulated animals used in a period. One must weigh the

increase in n against the analytical time required to determine the animal and day variation. If one has access to only a few fistulated animals, and the vegetation is not changing rapidly, increasing the number of days sampled, and keeping each animal's daily sample separate for later analysis, is probably wise. However, if one uses a large number of fistulated animals, perhaps pooling by weight for each individual animal across days would be better, if the variation in days is not of interest. Pooling samples over a several-day period should decrease variation in the major botanical components of the diet, and give more representative results.

The number of animals needed to determine chemcial composition of native ranges is less than for botanical composition (Harniss et al., 1975). Three or four fistulated animals for several days should adequately estimate the chemical composition of animal diets (Obioha et al., 1979; Harniss et al., 1975).

Because of diurnal variation in the botanical and nutritive content (especially N-content) of animal diets (Langlands 1965; 1967; Van Dyne and Heady, 1965), ideally one should sample twice daily, once in the morning and once in the afternoon (McManus, 1981). However, once-a-day sampling is probably more practical in most cases. Length of the collection period is variable. The only requirements are that animals have sufficient time to graze a representative diet, and that the sample is large enough to analyze. Sample periods of 20 to 45 minutes duration are established norms. Longer periods may result in rumen contamination of extrusa.

Because familiarity with pastures can affect animal diets, fistulated animals should be thoroughly acclimated to pastures from which collections are made (Langlands, 1967; Hodgson, 1969). An adpation period of 5 to 10 days has been widely used. However, Langlands (1967) has shown that a 10-day period may be the minimum allowable without bias entering into animal selectivity. Perhaps if pastures to be sampled are very small, one can justify a shorter adaptation period. Dean and Rice (1974) reported possible bias of results using esophageally

fistulated animals because of fence and corral location, even though the animals were familiar with the pasture design.

A precollection fasting period is often used to encourage grazing of an adequate sample (Obioha et al., 1970), and to prevent sample contamination from regurgitation of ruminal contents (Hodgson, 1969). The length of the fasting period may influence animal selectivity (Langlands, 1967; Grings and Morris, 1977; Sidahmed et al., 1977; Chacon and Stobbs, 1977; Arnold et al., 1964). If fasting is used, the fast should be of moderate duration (Sidahmed et al., 1977). Rumen contents can increase the N value of extrusa. The problem of regurgitation is often most pronounced with fistulaed goats, and fasting may not help. Occasionally a goat will apparently become nervous about the collection, and begin ruminating when the plug is removed. In this case, little can be done to prevent sample contamination except perhaps being extra gentle in handling that individual in future collections.

Supplementation of esophageally fistulated animals with a salt-mineral mix is essential. The ever-present leakage of saliva from many fistulae can lead to sodium depletion. Esophageally fistulated animals at pasture consume more salt than intact animals.

Experimental animals should be maintained in approximately the same body condition as the grazing animals in the population of interest. At times fistulated animals may need supplementation to maintan condition, but care should be used that the level of supplementation does not become excessive. Supplementation can affect animal grazing behavior (Box et al., 1965; Wagnon, 1963), and may influence the selection of a representative diet.

Preparation of fistula samples can have a marked effect on chemical composition (Harris et al., 1967). Sample preparation will affect botanical composition to the extent that plant diagnostic features are altered in the process, and to the degree that preparation affects assumptions inherent in a specific technique (Vavra and Holechek 1980; Havstad and Donert, 1978; Marshall

and Squires 1974).

The preferred method of preparation is immediate freezing of fresh extrusa, which can be followed by lyophilizing (freeze drying) (Harris et al., 1967). However, the cost of equipment of sufficient capacity to dry fistula samples oten precludes lyophilizing. Oven drying of extrusa must be used with care as oven drying increases the risk of nonenzymatic browning. This risk varies according to the dry matter content of the fistula sample, the drying temperaure, and drying time (Langlands, 1966). Drying temperatures in excess of about 50^{o} C greatly increase apparent fiber in wet forage samples (Van Soest 1969).

C. Application of fecal analysis

Even though the collection of fecal samples is simple, several precautions should be taken. As with esophageal fistula sampling, a predetermined sample collection aproach should be used. The choice between fixed collection dates and the use of animals or vegetation as biological indicators should be determined in advance. One should define what constitutes a subsample. This may vary from one to several pellets per pellet group, or from one cm^2 of cow dung to an entire defecation (Hansen and Lucich, 1980).

To obtain a representative sample, it is best to collect a very small part of many defications over several days (Hansen et al., no date given). These individual subsamples are composited to give a final, single sample. Hansen and Lucich (1980) suggested collecting one or two pellets per pellet group, or 2 g of material from 10 to 50 defications.

Care should be taken not to include extraneous soil or plant material with the sample. Fecal samples should be collected fresh to minimize the effect of weathering and destruction by insects, bacteria, and fungi (Ward, 1970). As samples are collected in the field, they need to be preserved to stop microbial action. Proper preservation facilitates later identification of fragments as soil bacterial and fungi can dissolve cutin, lignin, and cellulose (Hansen et al., no date given), which are the main components surviving digestion (Chatterton and

Powell 1974). Preservation methods include adding table salt, freezing, air or oven drying, and preservation in alcohol or formalin (Hansen et al., no date given). Williams (1969), in attempting to refine sample preservation, recommended that a 2 to 3 g sample of fresh feces be placed in a mixture of alcohol, formalin, and acetic acid (A.F.A) (for formulae see Appendix 1). This fluid is stable and most material can be stored for years (Berlyn and Miksche, 1976).

II. Microscopic analysis of samples

A. Reference slide preparation

Reference slides made from collected plant species indigenous to the study area are of critical importance for the microhistological technique. A simple reference collection of plants is sufficient for the microscope point method. Correct taxonomic identification of the plant species collected is basic to a successful research project. Reference slides may be made from fresh or dried plant material. Some plant species may change major diagnostic features as they mature (Davies, 1959), so reference material should be collected from young, as well as mature plants. Because differentiation with maturation will vary from species to species, only personal experience can help one decide which species change markedly during maturation.

Slides should initially be made from individual plant parts such as stems, leaves, flowers, seeds (Hansen, no date given). However, after one becomes comfortable with the major diagnostic characteristics of a plant, it may be helpful to grind together several entire plants of a species with all plant parts included. Using this type of reference material can greatly aid in training one's eye for actual diet determinations.

Metcalfe (1960) in his classic work on diagnostic characteristics of Poaceae, advocates learning monocot leaf features by a scraping technique first described by Prat (1948). In a modification by Pfister (1979), strips of grass leaf epidermis were soaked in a high density alcohol for several days. Then under low power magnification, a razor blade was used to scrape away the upper

epidermis, with vascular bundles, aclerenchyma and the lower epidermis being exposed intact. Household bleach was used to aid in the scraping process by softening the tissue. After the scraped epidermis was carefully turned over (the material was very thin and fragile), it was washed with distilled water and dessicated with 95% alcohol. Brusven and Mulkern (1960) used a similar method, only these workers used potassium hydroxide to soften tissues. Although this scraping technique is frustrating to learn, one can, with practice, expect to produce extremely clear reference samples of intact monocot leaves. Intact reference material will help the novice to learn monocot diagnostic features.

After leaf diagnostic characteristics are learned with relatively intact epidermal surfaces, leaves may be ground or agitated in a Waring blender (using just enough water to cover the blades) to provide more challenging and realitic material for study. For initial reference slides, monocot parts other than leaves may be placed in a Waring blender and agitated for several minutes to simulate mastication (Hansen 1971).

Reference slides for dicots are made according to the techniques of Hansen (1971), Storr (1961), or Steward (1967). These techniques are discussed below. Hansen's (1971) technique can be used with either ground material or material chopped in a blender. Reference material prepared following Storr's (1961) or Stewart's (1967) sample preparation methods should be satisfactory for dicots (Williams 1969).

A successful technique for clearing plant pigments from reference material is that of Shobe and Lersten (1967) (see Appendix 2). This clearing technique is not suitable for specimens fixed in A.F.A. because the denatured proteins will not dissolve in NaOH (Berlyn and Miksche 1976).

Seeds may pose special identification problems. Ward (1970) states that a valuable guide for seed identification is the Seed Identification Manual (Martin and Barkley 1961).

Line drawings and detailed notes on observed features are essential to

successfully mastering the study of plant diagnostic characteristics. In addition, one should have the budgetary capacity to take numerous microphotographs of the reference material. A carefully organized reference collection is a key step in a successful diet study, and its importance cannot be overemphasized.

B Microscope point method

Theurer et al., (1976) have reviewed the history, precision and reliability of the microscope point method. Our purpose in the discussion that follows will be to detail sample preparation procedures immediately prior to placing a sample in the tray, to list key diagnostic features of plants under low magnification (15 x), and to briefly discuss correction factors.

Several simple options are available to prepare frozen or dried samples for tray analysis. Van Dyne and Heady (1965) reported that after thawing the fistula samples, the samples were wrapped in cheesecloth, washed to remove saliva, then partially dried and spread evenly on trays. Malechek and Leinweber (1972) used frozen samples which were handchopped until free of clumps, then the samples were agitated in a water suspension. The water was removed by aspiration, and the saturated material was spread evenly in a tray. Hamilton and Hall (1975) stored extrusa for 5 days at 5 C, agitated the material in a beaker with water, then removed the water by vacuum filtration through large filter papers.

Diagnostic features under low magnification will be gross morophological plant characteristics. Animal diets will usually be composed of mostly leaf and stem material, with occasional fruits or flowers. Because of the low magnification, at times it will be diffcult to separate closely related genera or species using the microscope point method. The following morphological divisions are suggested as being useful for separating leafy material:

A. leaf margins

Hairs present (specify type and number)

Hairs not present

B. leaf venation

venation parallel

venation other (ex: pinnate, palmate, etc.)

C. Apices

Apex distinguished (if so, how?)

Apex undistinguished

D. leaf base

base distinguished (if so, how?)

base undistinguished

E. leaf surface

surface of leaf distinguished (i.e., unusual cell pattern, or surface feature such as alveolate or papillae cells).

surface of leaf undistinguished.

F. surface coverings (hairs)

hairs simple, unbranched

hairs branced or forked

hairs hooked or barbed

hairs without hooks or barbs

hairs scattered over surface, not restricted to margins

hairs restricted to margin, not superficial

hairs curled, interwoven or entangled

hairs shot or truncted to long or delicate, but not curled or interwoven

When one is attempting to classify hairs (ex: hirsute or stellate), referring to botanical texts is a necessity. Such a text can be of great assistance in classifying leaf surfaces. Hairs and surfaces are the most valuable leaf diagnostic features at low magnification. For stems, line drawings and notes

of the shape, and the number and type of hairs are valuable. For flowers, the floral parts (corolla and calyx) can be identified by relative insertion of the floral parts and by the venation, and hairs. Fruits (defined as a ripened ovary and contents, plus any other part attached to it, after Smith (1977) can be separated according to morphological characteristics.

Each investigator will need to determine a separation scheme based on detailed study of the reference material. A general dicotymous key may be useful during analysis for eliminating some genera or species. However, the degree of variability between and within plant species makes it doubtful if a key can be constructed which, when followed, can be used as the sole determinant of a species (Brusven and Mulkern, 1960). Using a broad key as a tool in analysis, in addition to notes, line drawings, and microphotographs, will probably yield the best returns.

The number of points necessary for precise analysis is variable (Theurer et al., 1976). Usually 100 to 400 points per tray have been read by investigators. The number of points will vary in each study depending on the degree of accuracy and precision desired by the researcher. In most diet studies, it is unrealistic to expect to reach a level of precision higher than $\pm$ 10% of the population mean at the 90% level of confidence.

A number of workers have increased the precision of the microscope point method by developing correction factors for biases in the technique. These correction factors are of three general types:

1) Seasonally relate the percent points to percent weight using masticated of fresh (clipped) or chopped (to stimulate mastication) herbage of known weight either singly or in preprepared mixtures (Harker et al., 1964; Van Dyne and Heady, 1965; Heady and Van Dyne, 1965; Galt et al., 1968).

2) Seasonally relate percent points to volume using mixtures with known weight/volume relationships (Chamrad and Box, 1964; Galt, 1972, as cited by Theurer et al., 1976).

3) Seasonally relate weight per surface area constants (WAC in mg/cm^2) for each species and adjust points by equation 1:

$$c_i = \frac{P_i \; a_i}{P_1 \; a_1 + P_2 \; a_2 \ldots . + P_n a_n} \qquad (1)$$

where p_1 is the total number of hits of component i, a_i is the WAC of component i and n is the number of components (Hamilton and Hal, 1975).

Types one and two are highly dependent on species specific mixtures and serious errors could result if the mixtures used differed greatly from the actual animal diets (Theurer et al., 1976). Regression equations become necessary when one cannot accept the assumption that the percent of points (i.e., hits) per species is equal to the percent weight of a species in the diet. In an Australian study, Hamilton and Hall (1975) determined weight per area constants for a legume and a grass species. Adjustments using WAC improved the method considerably. Van Dyne and Heady (1965) recommended reporting data as percent points rather than percent weight if adjustments are not made to relate percent points to weight.

C. Microhistological analysis

1. Fistula samples

Grinding of dried fistula samples through a 1 mm screen is standard procedure. Grinding reduces fragments to equal size, which is necessary to relate percent frequency to percent relative density (Sparks and Malechek, 1968).

Unequal sized fragments can introduce errors into the analysis (Havstad and Donart, 1978). Marshall and Squires (1979) and Vavra and Holochek (1980) warned of potentially serious destruction of epidermal tissue on grinding. Regression equations developed from hand-compounded mixtures can correct for epidermal fragmentation (Vavra and Holochek, 1980).

After grinding, microslides may be prepared following the procedures of Sparks and Malechek (1968) and Hansen (1971). In this procedure small amounts of ground material are placed on a microslide, using caution not to overlap the particles. A few drops of Hertwig's solution (Appendix 1) are added to the slide, then boiled off over an alcohol (or Bunsen) burner to clear plant pigments. The most numerous diet component should not have a frequency higher than 86% in actual diet determinations for quantification to be accurate. Through experience, adjustments are made in the number of fragments so that three to six recognizable fragments are visible per field at 100 x, resulting in satisfactory slides (Hansen, 1971). Hoyer's mounting medium (Appendix 1) is used to affix the coverslip. Air bubbles are removed by heating the slide until the Hoyer's solution boils, then rapidly wiping the bottom of the slide with a damp cloth or sponge. Microslides are then oven-dried. Vavra and Holochek (1980) found that soaking the material in a dilute NaOH solution helped to clear plant pigments and assisted in identification. Several minutes of soaking in full strength household chlorine bleach can also aid in clearing plant tissue, however, prolonged soaking may disintegrate tissues.

To the uninitiated researcher, the microhistological technique appears easy to implement. In fact the technique is initially difficult to implement, largely because of a lack of standardized terminology, and variations in microhistological characteristics (Havstad, unpubl. manuscript).

Even though the microscope field of plant fragments at one location is established at 100 to 125 x for the technique, magnification of a fragment up to 300 x can be useful if identification proves difficult. Usually one can readily

separate fragments into monocots or dicots. Monocots display parallel venation, elongated cell wall patterns, parallel rows of epidermal cells, sort cells (cork, silica, or silica-suberose couples) with thick walls found over the vein, stomata elongate with long axes parallel with epidermal cells surrounding them, the ends of the guard cells which are thin-walled and enlarged (dumb-bell shaped), and stomata alternating with rows of epidermal cells (Eames and MacDaniels, 1977). Dicots are noted for reticulate venation, and regular and irregular shaped epidermal cells which reflect non-linear venation (Metcalfe and Chalk, 1950).

Metcalfe and Chalk (1950) distinguished four types of stomatal complexes: 1) anomocytic- with no subsidiary cells, only ordinary epidermal cells around the stoma, 2) anisocytic- with three subsidiary cells, one much smaller than others, 3) paracytic- one or more subsidiary cells flank the stoma parallel to the longitudinal axis of the guard cells, 4) diacytic- with a pair of subsidiary cells with their common walls at right angles with the longitudinal axis of the guard cells. Trichomes (hairs, glands or scales) of dictos are often distinct in appearance, and provide indispensable diagnostic features. Detailed line drawings of trichomes will be invaluable aids in diet examinations.

Quantification of diets using the microhistological technique follows Sparks and Malechek (1968) and Hansen (1971). Five slides are made of each sample. At 125-X magnification, twenty fields of view are systematically located on each slide. Plant fragments are tabulated on a frequency basis (total fields per sample equal 100). Dry weight percentages of each species are figured using a conversion outlined by Sparks and Malechek (1968).

In this conversion, after percent frequency is calculated, particle density is determined using a table developed by Fracker and Brischle (1944). The table of Fracker and Brischle (1944) was generated to calculate the number of plants (n) per 100 quadrats likely to be present under strict mathematical probability when any given percentage (i) of quadrats containing one or more plants each. Sparks and Malechek (1968) adopted this table for use in diet determinations.

Once relative density expressed as a percent is calculated, it is used as a direct estimate of the percent dry weight of a species in the diet.

2. Fecal samples .

Endogenous solubles can be removed by washing over a 0.1 mm (200 mesh) screen with hot tap water for one of two minutes (Hansen, 1971).

Several clearing procedures to remove plant pigments from fecal material are described in the literaure. Hansen et al. (no date given) cleared material in chlorine bleach, then washed the material over a 200 mesh screen. If further clearing was desired, Hertwig's solution was used. Hoyer's medium was used to fix the coverslip.

Williams (1969) developed what he felt was an improved bleaching procedure for fecal samples. A 2 g preserved sample was diluted in 2 to 3 times its original volume with 70% alcohol, with the fragments dispersed but not subdivided. This slurry was placed in a 500 ml flask and allowed to stand for several hours to extract the chlorophyll. After filling with boiling water, the flask was left to stand overnight. Following decantation of the supernatant, the boiling water treatment was repeated. Three to four hours later the decanting was repeated and 10 to 15 ml of household bleach was added. Several hours later the bleach was removed through a Buchner funnel or by centrifugation.

A technique described by Storr (1961) was found useful in diet studies of kangaroos and wallabies. After drying and grinding, the sample was placed in a 150 ml flask containing 5 ml each of 10% nitric and chromic acid, fitted to a reflux condenser and boiled for one to three minutes until the mesophyll began to disintegrate. Storr (1961) indicated that ten minutes may be required for more fibrous species. When the mesophyll had disintegrated, the two integuments of each leaf drifted apart. The liquid was then poured off and water was added which contained a few drops of ammonia.

Stewart (1967) used a similar technique in determining the grass preference of East African herbivores. Seventy grams of fresh feces were fixed in A.F.A.

solution. A 1 g sample was placed in 4 ml of concentrated nitric acid and heated for two to three minutes over a waterbath. The sample was made up to 100 to 300 ml and boiled. With grasses, some mesophyll survived the process, indicating the treatment had not destroyed any cutinized epidermis.

Zyznar and Urness (1969) boiled deer fecal samples for 15 minutes in 10% sodium hydroxide to dissolve the external mucus coat. Anthony and Smith (1974) pointed out that discrepancies in the results of Zyznar and Urness (1969) and those of other studies may be due to technique. The boiling time is critical; if too short, inadequate epidermal clearing results; if too long, epidermal fragments disintegrate, leaving low quantities of recognizable fragments (Anthony and Smith, 1974).

Free et al. (1970) used the sample preparation technique of Sparks and Malechek (1968), in which a single clearing step consisted of boiling with a few drops of Hertwig's solution. Small, hard lumps of epidermal tissue were found in sheep feces which could not be easily dispersed on a microslide. Free et al. (1970) felt that these lumps were from the outside surface of sheep pellets where mucus forms a hard layer with the outer particles of feces.

Shandruk (1975) placed a 10 to 20 g subsample in a 150 ml beaker, and added enough 10% chromic and nitric acid (1:1 ratio) to completely cover the sample. The sample was digested for 12 hours at 22 C, then heated to a boil. After washing over a 200 mesh screen, the sample was deacidified with a weak ammonia chloride solution, and rinsed in distilled water.

Vavra and Holochek (1980), as noted above, simply soaked samples in a weak HaOH solution for 30 minutes. Scotcher (1979) advocated using a technique similar to that of Stewart (1967) to determine the diets of African herbivores.

Diagnostic features of residual fecal material, microslide preparation, and quantification are exactly the same as detailed above for fistula samples.

D. Summary of the relative merits of each technique

The microscope point technique is often recommeended because of practicality. The method requires few laboratory facilities, less intensive experience with plant histology, is easy to learn and implement, and is probably the least time consuming of the methods available.

However, the microscope point method is useful only in identifying relatively large particles under low power magnificaton. At times workers have difficulty properly separating closely related genera or species. In addition, there is a seasonal need to relate surface area (or points) to weight by regression equations or by weight per unit area constants, if one wishes to express plant species on a weight basis.

Even though the microhistological technique is difficult to learn and tedious to apply, the degree of accuracy achieved by the technique when used with fistula samples is perhaps higher than other methods, if the basic assumptions are fully met. Also, once the diagnostic features of plants are learned, the high magnificaton of properly cleared fragments enables one to confidently identify very small particles.

In using the microhistological technique, one cannot separate green (live) from dry (dead) material. Upon grinding, epidermal tissue may be destroyed and lost, hence correction factors may be needed to eliminate this source of error. One assumption of the technique is that the relationships between frequency and particle density, and also between relative density and weight, are about 1:1. Havstad and Donart (1978) have shown the former to be valid if all particles are equal sized. In their material, one forb species had large trichomes which survived grinding, and resulted in an over-estimation of this plant. To test the latter relationship, hand compounded mixtures are necessary. If one is working with material similar to that of Sparks and Malechek (1968), this assumption is probably not greatly violated.

Another assumption of the microhistological method is that the ratio of

identifiable to unidentifiable particles is 1:1. This assumption was not met in the tests of Havstad and Donart (1978).

Fecal analysis using the microhistological technique has proven to be accurate with perennial grasses due to the degree of cutinization, and appaently similar fragmentation on digestion, rate of digestion, and ratios of epidermis to volume of plant material (Smith and Shandruk, 1979; Scotcher, 1969). One must use the fecal method with caution, however. Digestion often reduces the identification of diagnostic characteristics (Dearden et al. 1972; as cited by Vavra et al., 1978). With dissimilar species such as forbs and browse, poor results have been demonstrated (Smith and Shandruk, 1979). Differential digestion and fragmentation of these types of plants make it imperative that workers develop correction factors to obtain reliable results. In Africa, investigators do not attempt to use fecal analysis to identify dicots in herbivore diets because of the limitations of the technique (Scotcher, 1979).

Often investigators ignore the necessity to develop correction factors for the biases of the various techniques, because that phase of diet examinations is tedious and time-consuming. Marshall and Squires (1979) have shown with known diets the large potential errors inherent in all microscope methods if correction factors are not applied. They concluded that diet studies done without correction for inherent errors should report results only in broad categories, with plant species described as being minor (20%), moderate (21-50%), or major (50%) diet components. Vavra et al. (1978) stated that actual percentages of a diet component are less important than the relative values (importance values) of the species. They concluded that by taking the most important diet components, the needs of most diet studies would be fulfilled.

In summary, we emphasize that there is no substitute for experience in diet examinations, and there are no shortcuts. For example, the lure of a technique such as fecal analysis may be strong because of the ease of collecting samples, and the desire to avoid caring for esophageally fistulated animals. However, time

saved at the sampling stage may be lost later later if one considers the time required to develop seasonal species-by-species correction factors, and to complete the difficult analytical task. Before choosing an analysis technique, investigators must consider the objectives of the study, the animal(s) being studied, the time, labor, and money avalable, and the experience and preferences of those involved in the study.

APPENDIX 1

A. F. A.

ethyl alcohol (95%)	50 cc
glacial acetic acid	5 cc
formaldehyde (40%)	10 cc
water	35 cc

Glacial acetic acid -1% acetic acid made by adding 10cc of glacial acetic acid to 990 cc water.

Formalin- the trade name used for an aqueous solution of formaldehyde containing 40% formaldehyde gas by weight.

(Berlyn and Miksche, 1976)

Hertwigs° solution

270 g chloral hydrate

19 ml 1 N HCL

60 ml glycerine

Hoyers° solution

200 g chloral hydrate

20 ml glycerine

50 ml water

30 g photopurified gum arabic

(Hansen, 1971)

APPENDIX 2

1. Fresh leaves are immersed in 70-95% ethyl alcohol until chlorophyll is removed. If the material is dry or has been preserved, omit this step and begin with step 2. In some cases acetone may be required to fully remove the chlorophyll.

2. Leach out cell content by placing the material in 5-10% solution of NaOH. The leaching process can be hastened at 60° C. Replace the colored NaOH until clear. If after several days of alkali treatment dark spots remain in the leaves, transfer to full strength chlorine bleach for 2-5 min. Use the least amount of time possible in the bleach solution because tissue breakdown may occur.

3. Rinse the material with three changes of distilled water (5 min. each).

4. Immerse in an aqueous solution of chloral hydrate (250 g/100 ml distilled water) for several hours. Except for the most lignified areas, the tissues should become quite transparent. Storage can be indefinite in this solution.

5. Repeat step 3. Fragile material should be passed through a dilution series prior to immersing in pure water to avoid interface turbulence or currents.

6. Dehydrate fragile tissues through a graded alcohol series to 95%; mature material may be placed directly in 95% ethyl alcohol without harm (three changes in 95%, 5 min. each).

7. Stain at this point.

(Shobe and Lersten, 1967).

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