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TECHNICAL NOTE

Collection, Handling and Analysis of Forages for Concentrate Selectors

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Abstract

Forage collection practices must be consistent among studies for accurate and comparable results. Forage samples should be collected in the context of the feeding habits of the focal species to accurately represent available nutrition, but inconsistent handling and analysis of forages also could bias nutrient reports. Previously described methods of forage collection based on agricultural protocols are adequate for studying diets of intermediate browsers and grazers, but likely are inaccurate for application to concentrate selectors. More specifically, the agricultural protocols generally underestimate nutritional quality for concentrate selectors because leaf collections avoid the physiologically young plant parts that concentrate selectors seek. Furthermore, agricultural drying practices are designed for forage samples lower in water content than the young plant parts selected by concentrate selectors, which may create inaccuracies in subsequent nutrient assays. Also, laboratory methods and accuracy may affect nutrient reports in addition to collection and handling procedures. As a whole, improper collection, handling, or analysis of forages leads to improper conclusions and invalid comparisons across studies. Herein, we review protocols reported in empirical studies from agricultural and wildlife research and provide guidelines for standardizing collection, handling, and analyses of forages with the goal of providing a framework for researchers studying diets of concentrate selectors and related nutritional indices. These protocols will ensure valid conclusions are drawn and allow valid comparisons among related studies in future research.

Introduction

More than 40% of ruminants worldwide are classified as concentrate selectors (see Hofmann [1] for list of common species in each foraging classification). Concentrate selectors are equipped with a digestive tract that is perfectly adapted to process highly digestible forages rich in soluble cell contents [1]. The other 60% of ruminants,

intermediate browsers and grazers, are better adapted to digest lignified material, requiring a less selective diet but greater intake rates and longer retention times to extract nutrients. Intermediate browsers fall between the dietary extremes, avoiding ligneous material when possible, but to a lesser extent than concentrate selectors; grazers frequent grass-dominated rangelands with little dietary selectivity and consume relatively lignified forages [1]. Understanding the fundamental differences between the concentrate selectors and other types of feeders are of extreme importance when studying dietary indices.

Researchers often collect forage samples with the intent of measuring forage quality and to compare habitat management regimes in terms of nutritional carrying capacity (NCC) [2-8]. Nutritional carrying capacity estimates for concentrate selectors have been approximated in various ways but generally are based on nutrient availability (most notably crude protein) coupled with an approximation of diet selection and intake rates [1-6, 8, 9]. Consistent estimation of nutrients is important because NCC estimates are affected by nutrient concentrations, even when diet selection, intake rates, and forage availability are similar [6]. Also, researchers may use nutritional indices to examine mechanisms guiding ungulate selectivity [10, 11]. For example, Tixier *et al*. [12] modeled diet selection of roe deer (*Capreolus capreolus*) and used plant nutrient levels as covariates to explain the variation in diet selection. Understanding the foraging strategy and nutritional quality of ungulates may be important in guiding feeding regimes in captive animals [13]. And, more recently, researchers have used plant constituents to shift diet selection to less desirable and problematic range plants [14].

Factors influencing nutrient concentrations in agricultural plant parts have been evaluated extensively [15], but the methods used are not appropriate for quantification of concentrate selector diets. Nutrient content varies among plant species and between physiologically young and mature parts in the same plant [15]. Hence, sampling protocols developed for most agricultural crops inform soil amendment recommendations to maximize crop yield [16] but are less suitable for sampling wildlife forages, particularly when studying a concentrate selector. Because concentrate selectors select specific plant species and normally focus on physiologically young growth [1], agricultural protocols are flawed for application to their dietary nutrition because the plant parts selected for assay are chosen based on their ability to estimate nutrient deficiency in the soil. Therefore, physiologically young plant parts are avoided in collections because young plant tissue is undergoing relatively rapid change in elemental content and may not correlate with soil mineral deficiency [15]. However, agricultural protocols likely are appropriate for intermediate feeders and grazers because of their lower relative selectivity of plant parts [1, 17]. Therefore, use of agricultural methods that measure only mature growth or a combination of mature and young plant parts provide an underestimate in diet quality for concentrate selectors.

A standardized protocol for collecting, handling, and analysis of forages for concentrate selectors is needed given the potential insufficiencies of agricultural crop sampling protocols. Accordingly, we used information from agricultural- and wildlife-based studies to propose a standardized protocol for collecting, handling, and analyzing forages with the fundamental goal of measuring nutrient availability for concentrate selectors.

Forage Collection

Numerous factors may affect the nutritional quality of plants. For example, secondary plant compounds such as tannins significantly reduce forage quality by reducing digestibility or preventing nutrient absorption [18, 19]. However, the relative maturity of a plant part is perhaps the most important consideration, particularly when measuring forage quality for concentrate selectors [Table 1; 15, 20].

Table 1: Concentration of nutrients in mature leaves and immature leaves from the same plant dried at 45°C for 36 hours, Fort Bragg Military Installation, North Carolina, June 2012.

Species		CP[a]	P	K	Ca	ADF[a]	NDF[a]
Flowering Dogwood[b]	Mature	9.8	0.11	0.83	2.15	15.5	17.5
	Immature	12.9	0.19	0.92	1.84	12.3	13.5
Mockernut Hickory[c]	Mature	13.6	0.16	0.83	0.84	29.2	31.3
	Immature	16.1	0.31	1.36	0.56	21.3	18.8
Turkey Oak[d]	Mature	8.2	0.10	0.64	0.24	30.1	37.2
	Immature	12.5	0.31	1.00	0.15	12.3	19.4

[a]CP, ADF, and NDF stand for Crude Protein, Acid Detergent Fiber, and Neutral Detergent Fiber, respectively.

[b]*Cornus florida*

[c]*Carya tomentosa*

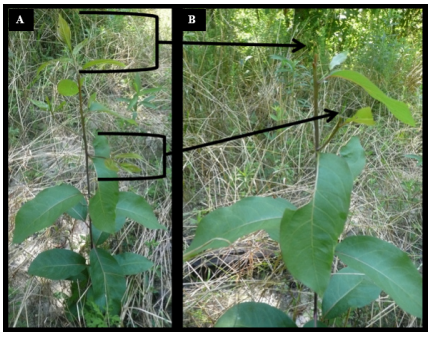
[d]*Quercus falcata*

Because concentrate selectors select specific plants and plant parts, consideration of which plant parts to collect for subsequent analysis is important given the fluctuations in nutritional quality among plant parts. [Fig. 1; 1, 21].

We suggest selecting parts of plants that mimic herbivory of the target species (i.e., physiologically young growth for concentrate selectors; Fig. 2) when studying diet quality [6, 22].

Figure 1 (*left*): Physiologically young plant parts before (A) and after (B) observed white-tailed deer herbivory. Notice only the physiologically young growth was consumed.

Figure 2 (*right*): Physiologically young (tender growth) and mature growth on a blackgum (*Nyssa sylvatica*). Notice the color change in leaves from red to green as growth matures.



For example, if the objective of the study is to quantify nutritional carrying capacity from total forage production, then all plant material within reach (Fig. 3) should be collected because some concentrate selectors consume more or all of the plant parts available under some conditions [e.g., high density, drought; 22, 23].

Subsequent nutrient assays may be performed on a composite sample of the plant parts collected to estimate NCC. However, if the objective is to measure diet quality

Figure 3: White-tailed deer browse line in a forest with a deer density that is exceeding nutritional carrying capacity. Notice plant material above 1.2m is still present.

when resources are abundant, we suggest collecting only physiologically young growth for concentrate selectors. Physiologically young plant parts are generally on or near twig tips and may be red to purple in coloration (presumably because of high concentrations of some nutrients; Fig. 4).

Also, discolored or otherwise damaged or abnormal plants and plant parts should be avoided for collections [15]. Researchers may be able to distinguish herbivory among wildlife species and between new and old bites, which in turn may provide a reference to plant part and plant species selection [Fig. 5; 24]. Physiologically young plant parts typically contain less lignin and break away from the plant much more easily than mature parts, making collection of young parts relatively consistent [22].

Figure 4 (*left*): Examples of color variation in physiologically young growth of common persimmon (*Diospyros virginiana*; left) and common greenbrier (*Smilax rotundifolia*; right). Notice growth changes from purple to red to green as it matures.

Figure 5 (*right*): Comparison of white-tailed deer and lagomorph herbivory on common greenbrier. Notice the differing bite morphology and the necrotic black tissue surrounding the older lagomorph bite.

Additionally, we suggest plant parts be collected during the time period of interest, and animal physiology and plant phenology should be considered. For example, in the southeastern U.S., plant samples collected in the late-summer stress period (August) would likely underestimate diet quality during early lactation (June) for white-tailed deer (*Odocoileus virginianus*) because plants from this region generally decrease

in quality as plant parts mature and senesce [21]. If the objective of the study was to determine if dietary nutrition of lactating females is sufficient to support young neonates, the plants collected late in lactation may reflect inadequate nutrition because of poorly timed forage collections. In this case, the consideration of animal physiology and plant phenology are important to accurately measure the dietary nutrition of females.

Forage Handling

Forage handling is equally as important as collecting representative plant parts in maintaining accuracy in subsequent assays. Samples may degrade quickly after collection, particularly in warm and humid climates. For example, samples from the southeastern United States inserted into a dryer without allowing proper ventilation may begin molding in less than 24 hours. Deterioration of samples may affect dry mass in the short term and subsequent assays may be affected substantially because nutrient levels are reported as a percentage of dry matter [15]. We suggest samples collected for nutritional assays should be inserted into paper bags and immediately transported to a dryer when testing forages for nutrient content [15]. However, analyses of non-nutrient plant constituents, such as secondary plant compounds, may require freeze-drying samples [25]. If short-term storage (<8 hours) is necessary, we suggest keeping samples in a well-ventilated area (e.g., shaded truck bed if in the field, refrigerated if in the lab) until processed in a dryer. Samples should not be stored in sealed plastic bags because they will retain moisture and expedite enzymatic deterioration. If samples cannot begin drying within 8 hours of collection, they may be stored in a refrigerator for 24 hours [15]. Samples should not be frozen because freezing water in the plant tissues may form ice crystals that can rupture cell walls, spilling the contents and compromising the subsequent assay [15]. Also, wet weight should be recorded when the samples are collected as a precautionary measure to ensure heat does not catalyze the Maillard reaction (non-enzymatic browning) when drying the forages [26]. Similar to caramelization, this process could artificially inflate lignin content in the subsequent assay, resulting in inaccurate results [22].

Heat drying, freeze drying, and vacuum drying are the 3 most commonly used methods of drying samples. Freeze drying or vacuum drying may better preserve plant tissues that are high in soluble sugars [27], though this is of greater concern in seeds than vegetation. Heat drying is used more commonly to dry other plant parts because ovens generally are more accessible and less expensive. Also, previous studies have demonstrated drying method has little effect on subsequent analyses of nitrogen [28, 29] and other mineral contents [30], unless exceeding 65°C [31]. Samples must be dried to constant mass and then may be removed from the dryer. A walk-in airflow dryer (designed for agricultural crops) is a commonly used dryer because moisture is quickly removed from the chamber mechanically with fans. Also, there are stand-alone cabinet (i.e., not walk-in) drying ovens with continuous venting that provide a more affordable alternative. However, a conventional oven (oven that applies heat without mechanized air circulation) may be used under the correct circumstances if the door is cracked to allow moisture to escape and samples are not overstocked. Samples should be dried at temperatures ranging from 45-65°C [15, 31]. A compromise must be met between drying time commitment and temperature. For example, Steyn [31]

reported citrus leaves dried at 50, 65, and 105°C were affected differently by thermal decomposition. Thermal decomposition was not present at 50°C, began at 65°C, and samples were severely damaged at 105°C. However, drying time is extended to remove sufficient moisture at lower temperatures. Also, samples that are not well vented (oven is overstocked or door not open) may deteriorate enzymatically while drying at lower temperatures [31]. For example, we dried a sample at 45°C in a conventional oven without proper ventilation and it began enzymatic decomposition (in this case molding) after 48 hours in the dryer. This is a common problem with samples high in moisture content but can be avoided easily by removing them at least once per 24 hours (e.g., when weighing for constant mass) and mixing them. Stirring samples exposes new surface area allowing evenly distributed evaporation. Plant species may become discolored after drying but generally should retain the natural vegetation color. Samples that have turned brown to black or have a burnt odor probably have begun thermal deterioration and should be discarded. Also, most forage labs provide an option to test for bound protein, which provides the opportunity to identify if heat damage has occurred and compromised the subsequent assay results.

The most likely culprit of thermal deterioration is a drying temperature that is too hot [15]. We suggest samples are devoid of moisture and safe for removal after 3 consecutive similar dry mass observations (6 to 24 hrs apart). Dried samples should be assayed as soon as possible but may be stored for longer durations (up to 6 months) because samples with $\leqslant$10% moisture are relatively stable when kept in the absence of sunlight and high temperatures [32]. Also, storage duration after drying may be extended by refrigeration (an additional 6-12 months), and freezing may further increase storage life following moisture removal because cell rupture is no longer possible [15].

Lab Analyses

Using laboratories (labs) certified by the National Forage Testing Association (NFTA) in the United States or the Bureau InterProfessionnel d'Etude Analytique (Bipea) in European countries will ensure accurate assays. These organizations ensure that laboratories meet a standard of accuracy by intermittent testing. For example, the NFTA is a volunteer group established by crop growers that tests the labs by submitting blind samples. The lab must match the true mean of individual nutrients within an acceptable range of variation to gain and maintain certification (see NFTA certification process and a list of NFTA certified labs at www.foragetesting.org). Therefore, NFTA certified labs have demonstrated accuracy and are more likely to provide accurate results [32]. Labs may be equipped to only report certain nutrients and may vary significantly in cost. Because most labs are in operation to test agricultural crops, the lab manager should be contacted to ensure the desired test can be performed, the lab is equipped to test non-crop forages, and the lab is equipped to use wet chemistry (separation of nutrients with chemical reactions) rather than near infrared reflectance (NIR). NIR is a procedure where a beam of infrared radiation is focused on a finely ground dried plant tissue sample and the reflected radiation is measured to estimate nutrient levels (primarily N) based on a previously developed regression equation [33]. Regression models are species specific and developed

primarily for agricultural crops. Therefore, wild forage nutrients estimations are unlikely to be accurate and in some cases cannot be performed [15]. However, NIR procedures may provide an accurate and economical alternative to wet chemistry if regression models have been developed for the submitted forages. We recommend recording the specific processes used to determine each nutrient when consulting the laboratory manager. For example, Lashley and Harper [22] reported crude protein (CP) using the combustion analyzer method [34] and acid detergent fiber (ADF) and neutral detergent fiber (NDF) using ankom fiber determination [27]. Both are accepted methods, and this information is important because lab results may vary depending on the method of nutrient determination [27]. For example, the Kjeldahl digestion procedure, one of the most widely used N determination methods, tends to give a 1-4% less N result relative to other methods [15]. The lower estimation is because N in the plant tissue as either NO3 or NO2 is not completely recovered during Kjeldahl digestion unless converted to NH4 by other pretreatments [35, 36]. Therefore, assays used must be reported to ensure comparability among studies.

Most labs require samples to be dried and ground before arrival. However, some labs will provide these services for an additional cost. If samples are to be ground before submission, particle size must be reduced after drying to <2mm [32]. This can be achieved using a Wiley mill with a 1-mm mesh strainer. One gram of dry matter is needed per nutrient assay (i.e., CP, ADF, and Ca requires ~3g) [32]. Physiologically young plant parts are generally >50% in water content but may be more than 90% water (Table 2). Therefore, we suggest collecting at least 10g (wet weight) for each nutrient to be assayed.

Table 2: Percent water weight of physiologically young plant growth after drying plant materials for 36 hours at 45°C until material reached constant mass, Fort Bragg Military Installation, North Carolina, June 2012.

Classification	Species	Common name	% Water
Forb	*Toxicodendron pubescens*	poison oak	61
Forb	*Lespedeza hirta*	hairy lespedeza	62
Forb	*Chamaecrista fasiculata*	showy partridge pea	74
Forb	*Phytolacca americana*	pokeweed	85
Forb	*Ambrosia artemisiifolia*	common ragweed	81
Grass	*Andropogon virginicus*	broomsedge	59
Grass	*Aristida stricta*	wiregrass	48
Grass	*Sorghum halepense*	johnsongrass	73
Grass	*Dichanthelium spp.*	low panicgrass	67
Sedge	*Carex spp.*	sedge	64
Shrub	*Gaylussacia dumosa*	dwarf huckleberry	66
Shrub	*Crataegus crus-galli*	hawthorn	64
Shrub	*Clethra alnifolia*	pepperbush	77
Shrub	*Vaccinium formosum*	high-bush blueberry	65
Shrub	*Callicarpa americana*	American beautyberry	77
Tree	*Nyssa sylvatica*	blackgum	70
Tree	*Cornus florida*	flowering dogwood	57
Tree	*Acer rubrum*	red maple	61
Tree	*Sassafras albidum*	sassafras	73
Tree	*Oxydendrum arboreum*	sourwood	76
Vine	*Campsis radicans*	trumpet creeper	70
Vine	*Rubus argutus*	sawtooth blackberry	73
Vine	*Smilax rotundifolia*	common greenbrier	88
Vine	*Vitis rotundifolia*	muscadine	69
Vine	*Parthenocissus quinquefolia*	Virginia creeper	78

Summary

Because nutrient levels are variable within plant parts, researchers should select plant parts representative of the diets selected by the target species. When selecting physiologically young growth for analysis of concentrate selector diets, care should be taken to dry plant parts at the appropriate temperature and store them in a manner that reduces sample degradation before transport to a laboratory. When choosing a laboratory, researchers should ensure they are certified by the appropriate association and that appropriate procedures can be performed. Taking these simple steps will ensure forage analysis results are accurate and comparable across studies.

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