14 Characterization of Forages by Chemical Analysis

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What is Worth Measuring?

The best evaluator of forage quality is animal performance. Intake, digestibility and efficiency of utilization are characteristics of forages that determine animal performance, with variation in intake accounting for 60–90% of the variation in digestible energy (Mertens, 1994). It would therefore be desirable to measure those forage characteristics that relate most closely to intake and digestibility. Chemical fractions that have been associated with intake and digestibility include fibre, lignin and protein (Cherney and Mertens, 1998). A routine system of chemical characterization of forages for ruminants, then, must include determination of fibre, lignin and protein and critical to these is an accurate dry matter (DM) determination. Some characterizations, such as the determination of water-soluble carbohydrates (WSC), may be of particular importance in silage research. Others, such as tannin assays, starch and soluble fibres, may be of particular importance for some types of forages and some feeding situations. The worth of any particular assay, other than for fibre, lignin, and protein, is thus dependent on the researcher's objectives and the likely economic impact of not performing the assay. If an anti-quality factor limits production, it may become the most important factor to analyse for.

What can it Tell us about Forages?

Chemical analyses can provide valuable information about the actual chemical constituents influencing digestion, unlike *in vitro* methods (Van Soest, 1994). Component chemical analyses, often expensive, can provide important biochemical information, leading to a better understanding of the factors that may limit animal performance. Chemical characterization methods cannot give a direct estimate of nutritive value, but rather rely on statistical association to measure digestibility

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and intake. Using these statistical associations, characterization of forage fibre, lignin, protein and other chemical components are used increasingly to predict animal performance.

Chemical analyses, along with the use of models, are increasingly used to predict performance. Forage chemical analyses can be used to identify factors in forages that may be limiting animal performance (Minson, 1981).

Chemical Characterization Methods

Dry-matter concentration

While not considered a chemical characterization *per se* by many, DM determination is included here because accurate determination of forage DM is essential for successful characterization of any other chemical constituent. Analytical results must be reported on a total DM basis for meaningful comparisons among feeds. Additionally, the accurate determination of DM is critical for balancing the diets of ruminants. Clancy *et al.* (1977) demonstrated that small errors in DM determination were amplified in the calculation of quality parameters, such that significant differences in quality resulted solely from the method of DM determination. Fresh forage or samples preserved as hay have several options for DM determination, while samples collected after ensiling must account for volatile compounds in ensiled forages. Options for determination of DM directly or by chemical means will be briefly discussed here (Table 14.1).

Oven-drying is commonly used for DM determination. At a drying temperature of 100°C, mechanically trapped water is evaporated, leaving chemically bound water (Brusewitz *et al.*, 1993). The amount of water removed from the sample is directly related to drying temperature, although a small amount of chemically bound water remains in samples dried at temperatures as high as 135°C. Officially approved oven-drying methods, according to the Association of Official Analytical Chemists (AOAC), include drying at 100°C under a vacuum of 1.3×10^4 Pa for 5 h (AOAC-934.01, 1990), drying samples in a forced-air oven at 135°C for 2 h (AOAC-930.15, 1990), or drying at 105°C for 16 h (AOAC-967.03, 1990). An officially approved method in the UK involves drying in a forced-air oven at 100°C for 18 h (MAFF-1, 1986). Further analyses should not be attempted on the dried sample, as drying at 100°C will alter some forage compounds making them less available (Van Soest, 1994).

A two-step procedure is generally used in the USA. Wet samples are dried in a forced-air oven at 55 or 60°C overnight to determine a partial DM value. Samples are then ground and can be used for the second step in total DM determination or for other analyses. The second step of the DM procedure usually involves one of the AOAC procedures.

Fermented feeds contain volatile components, which may be lost during the drying process. Direct laboratory measurement of water content of an as-received silage sample can reduce or eliminate DM determination errors due to loss of volatile components. Toluene distillation is one method for the direct measurement of water in a forage sample. Its use is warranted only for fermented samples,

Table 14.1. Comparison of laboratory methods for determining forage moisture contents (adapted from Brusewitz *et al.*, 1993).

Method	Operating principle	Moisture range	Accuracy*
Hot-air oven	Drying	0–1000	Good for dried forage, poor for silage
Hot-air oven	Drying with correction for volatiles	0–1000	Good
Toluene distillation	Boiling	0–900	Good
Toluene distillation	Boiling with correction	0–900	Very good for silages
Saponification	Chemical binding of water	0–1000	Very good
Karl Fischer	Titration	0–1000	Excellent
Gas chromatography	Physical separation of water from other extracted chemical	0–1000	Excellent
Near-infrared Reflectance Spectroscopy	Electromagnetic properties	0–400	Very good

* Precision for all listed procedures is ±10 g kg^{-1}.

due to the hazards associated with toluene (NFTA-2.3.1, 1993). The small amount of water not removed from the sample has been assumed to offset the volume of volatile components in the distilled-water phase (NFTA-2.3.1, 1993). A more accurate method is to measure the volatile components in the water phase in order to adjust the volume of water for these components (Dewar and McDonald, 1961; AOAC-925.04, 1990). Most studies have concluded that uncorrected toluene distillation results in higher DM content than oven-drying at a range of temperatures (Larsen and Jones, 1973).

The Karl Fischer titration method (Fischer, 1935) is based on the reaction of water with iodine in the presence of pyridine and methanol. This method also involves hazardous chemicals, and the relatively small sample size analysed will increase potential sampling error. Karl Fischer titration for DM determination has been considered too time-consuming for routine analyses (Hood *et al.*, 1971; Robertson and Windham, 1983), although others considered it a simple, rapid procedure for silage samples (Galletti and Piccaglia, 1988; Kaiser *et al.*, 1995).

Gas-chromatography procedures have been developed to measure the water content of forage (Fenton *et al.*, 1981; Burdick and McHan, 1982). Water and alcohol are separated using a gas chromatograph and the ratio of water to alcohol in unknown samples compared with the standards allows determination of sample water content. Accuracy and repeatability are excellent.

Because they can contain significant quantities of volatile compounds, fermented feeds present special problems. Several methods of correcting silage DM

content for loss of volatile components are used. Corrections to oven-dried silage DM have been made by analysing samples for fermentation products (Fatianoff and Gouet, 1969; Dulphy *et al.*, 1975). Galletti and Piccaglia (1988) did not find significant differences between Karl Fischer titration values and oven-dried values, after oven-dried silage values were corrected for fermentation products. Equations have been proposed for estimating a toluene DM determination from oven-dried grass-silage samples (Haigh and Hopkins, 1977; Barber *et al.*, 1984; Haigh 1995a, b, c). Such equations will work only if the exact oven-drying procedure is used. Loss of DM during drying is a function of the type and concentration of volatiles present, as well as oven temperature (Minson and Lancaster, 1963).

Kaiser *et al.* (1995) compared eight DM determination methods for silages. In general, silage × method interaction was highly significant, due to greater differences among methods at low silage DM compared with high silage DM. Kaiser *et al.* (1995) concluded that Karl Fischer DM values can be estimated in feed testing laboratories with sufficient accuracy from oven DM values and prediction equations.

Fibre determination

The proximate analysis system (Weende method) has been used for almost 150 years. Components of proximate analysis include crude protein, ether extract, crude fibre, ash and, by difference, nitrogen (N)-free extract (Fisher *et al.*, 1995). While this method is simple, repeatable and relatively inexpensive, there are several problems associated with the method that caution against its use. Ether removes waxes and some other compounds not considered digestible. The crude-protein determination assumes that all N is contained in protein with an N content of 16%, which is incorrect. Crude fibre does not provide an accurate representation of the least digestible fibrous part of the feed in many cases (Sollenberger and Cherney, 1995). Inversely, nitrogen-free extracts are not always a reasonable estimate of the highly digestible carbohydrate fraction. Crude-fibre digestibility exceeds nitrogen-free extract digestibility in about 30% of feedstuffs (Van Soest, 1994), with the largest error occurring in tropical grasses and straws. Perhaps the most serious error is the assumption that crude fibre contains all dietary cellulose, hemicellulose and lignin (Fig. 14.1). For these reasons, crude fibre and nitrogen-free extract are not and should not be routinely used. Crude protein has long been the standard for evaluating the protein value of forages and is still acceptable for many conditions (Vérité, 1980).

The primary standard for fibre evaluation of forages in the USA is the Van Soest system of fibre analysis (Marten, 1981). The Van Soest system separates carbohydrates into fractions based on nutritional availability (Sollenberger and Cherney, 1995). This separation results in a more reasonable estimate of the structural carbohydrates than does crude fibre (Fig. 14.1) and allows for prediction of other indices of forage quality, such as digestibility (Van Soest, 1994). The Van Soest system does not fractionate feeds into chemically pure fractions. It must be remembered, however, that what is important is the nutritional uniformity of the fraction in question.

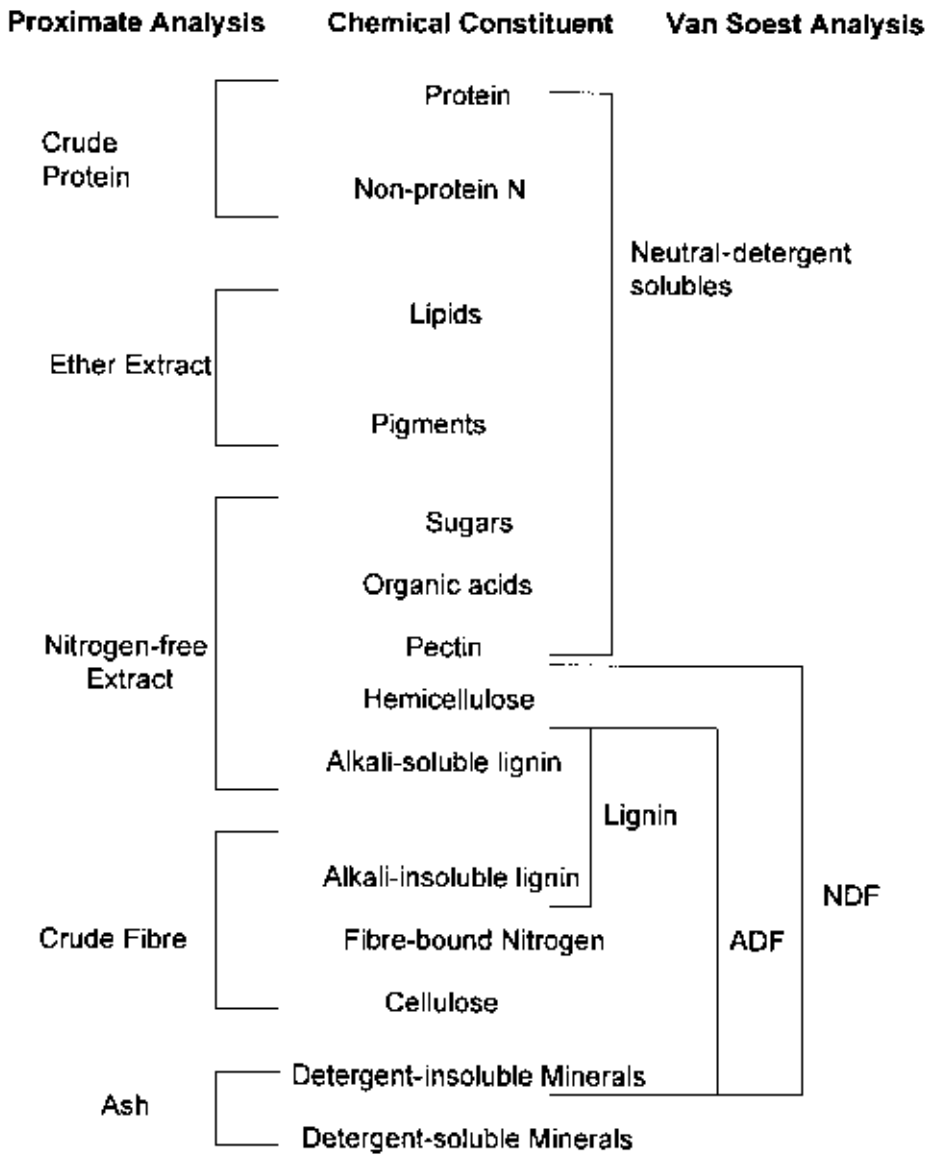

Fig. 14.1. Contrast of Weende system and Van Soest system of carbohydrate analysis (adapted from Fisher *et al.*, 1995). ADF, acid-detergent fibre; NDF, neutral-detergent fibre.

The Van Soest system of fibre analysis has remained relatively unchanged over the years, although a number of modifications have been proposed for special samples (Mascarenhas-Ferreira *et al.*, 1983). Current recommended procedures for neutral-detergent fibre (NDF) (Van Soest *et al.*, 1991) recommend elimination of sodium sulphite, discussed further below, and decalin from the original procedure

(Goering and Van Soest, 1970) and replacement of 2-ethoxyethanol with triethylene glycol. Other research has suggested that 2-ethoxyethanol is not necessary for the determination of NDF in forages and should be eliminated rather than replaced (Cherney *et al.*, 1989). Triethylene glycol may be used where excessive foaming is a problem during filtration. The use of amylase to aid in the removal of starch is also recommended (Van Soest *et al.*, 1991).

Starch contamination of NDF, particularly in those forages containing grain, can lead to overestimates of the NDF value (Robertson and Van Soest, 1977). Insufficient removal of starch from a sample often leads to difficulties with filtration, resulting in elevated NDF values (Cherney *et al.*, 1989). Generally, the use of heat-stable α-amylase is recommended (Van Soest *et al.*, 1991). Pretreatment with urea to remove starch may be necessary in forages with large concentrations of starch (Van Soest *et al.*, 1991).

Sodium sulphite, originally used to reduce nitrogenous contamination in fibre analyses, was removed from the system because it was thought to solubilize some lignin (Van Soest *et al.*, 1991). Recently, the inclusion of sodium sulphite in the NDF solution of high-protein forage samples was re-evaluated (Hintz *et al.*, 1996). Addition of 0.5 g sodium sulphite decreased nitrogen concentration in fibre and lignin values and reduced within-sample variance. Use of sodium sulphite in high-protein forages is therefore recommended.

Recently, a new modification, using the ANKOM fibre analyzer (ANKOM Technology, Fairport, New York), has been developed, which uses preweighed fibre filter bags (Komarek *et al.*, 1994). Fibre values obtained using this method compared to conventional systems were similar (Komarek *et al.*, 1994). This system may be advantageous for silage samples or samples with high soil contamination, which are routinely difficult to filter, as the ANKOM system does not require filtration. This system also offers advantages of reduced initial cost and reduced labour over the conventional system.

Another modification of the NDF system worthy of mention is the micro-NDF method (Pell and Schofield, 1993). There are numerous instances when the amount of sample available may be too small for conventional analysis. In the micro-NDF method, samples in the range of 10–50 mg are placed in 50 ml serum bottles with neutral-detergent solution and autoclaved for 60 min at 105°C. Results compare favourably with standard Van Soest fibre methodology (Van Soest *et al.*, 1991; Pell and Schofield, 1993).

Of the fibre fractions, NDF relates best to feed intake, representing the total insoluble fibre matrix (Van Soest, 1994). This makes NDF the most important fibre fraction to consider. The intended use of acid-detergent fibre (ADF) is as a preparative residue for the determination of cellulose, lignin, Maillard products, silica, acid-insoluble ash and acid-detergent-insoluble N (ADIN). Despite the statistical association in some cases, there is no valid theoretical basis to link ADF to digestibility (Van Soest *et al.*, 1991).

Modified ADF, slightly higher in acid concentration (0.5 mol l^{-1}) and longer in boiling time (MAFF-30, 1986) than Van Soest ADF, is common for fibre analysis in the European Union (EU). The original study for the method (Clancy and Wilson, 1966) reported that prolonged boiling with acid of higher concentration reduced bound nitrogen and improved the relationship between fibre and

digestibility. The use of modified ADF as a means of assaying for heat damage and unavailable protein is precluded, because this system includes oven-drying at 95°C as a preliminary step (Van Soest, 1994).

Lignin

Lignin and associated phenolics are most often identified as the chemical constituents in cell walls most limiting digestion (Van Soest, 1994; Buxton *et al.*, 1996). There are a number of procedures used to determine lignin in forage samples (Table 14.2). Insolubility of lignin in 72% sulphuric acid is the basis of most procedures (Barnes, 1973). Measurement of lignin in forage species is complicated by the presence of proteins, chemically bonded cinnamic acids (Iiyama and Wallis, 1990), Maillard products and other non-lignin substances insoluble in 72% sulphuric acid (Van Soest and Robertson, 1985). Klason lignin (72% sulphuric acid method), suitable for most woody species, leads to an overestimation of lignin, because protein coprecipitates with lignin (Norman and Jenkins, 1934). Most methods to determine lignin in forages, therefore, involve preliminary removal of protein, either by enzymes (Crampton and Maynard, 1938) or by pretreatment with acid-detergent solution (Van Soest, 1973).

Barnes (1973) suggested that problems associated with the use of lignin concentration and digestibility could be summarized as: (i) complex and unknown structure of lignin; (ii) lack of a standardized lignin procedure; and (iii) the inherent variation in lignin content among forages. Some 25 years later, the definition or concept of what constitutes lignin is still subject to debate (Van Soest, 1993) and there is, as yet, no universally accepted lignin standard. As to the third point, Van Soest (1993) suggested that a very large part of the diversity between species in the relationship between lignin content and DM digestibility is due to interaction with NDF content. If lignin is expressed on a fibre basis, lignin's relationship to digestibility appears to be reasonably uniform across legumes and grasses (Van Soest, 1993). It would thus appear logical to report lignin on a fibre basis, or to include fibre values when reporting lignin.

Giger (1985) performed a thorough review of current lignin methodology and concluded that a pure analytical lignin fraction, without any lignin loss or contamination by proteins, carbohydrates, cutins or tannins, is not possible with current methods. Methodological insufficiencies may be due to the complexity and diversity of the 'lignin fraction' (Barnes, 1973; Giger, 1985; Van Soest, 1994). Van Soest (1994) suggested that lignin assays be evaluated with the following criteria: (i) lignin recovery in digestion balances; (ii) degree of correlation between lignin and digestibility; (iii) low nitrogen content of lignin preparation; and (iv) recovery of phenolic matter. While this may not be a 'true definition' of lignin (Van Soest, 1994), it is certainly an operational definition. Lignin's value is in its relationship to digestibility or indigestibility. None of the methods listed in Table 14.2 or the more extensive list of Giger (1985) is clearly better based on the four criteria listed above. Some of the newer procedures, such as pyrolysis mass spectrometry, may be beneficial in determining the true structure of lignin. At this time, however, the equipment may be too expensive for use in a routine evaluation scheme. For any

Table 14.2. Methods for the determination of lignin.

Method	Comment	Reference
Quantitative		
Gravimetric		
Klason lignin	Most severe limitation is protein contamination	Giger, 1985
Acid-detergent lignin (ADL)	May contain cutin and other non-lignin carbohydrates	Van Soest, 1973
Difference after lignin removal		
Permanganate lignin	Yields higher values than ADL; cutin excluded, but may contain polyphenolic substances	Van Soest and Wine, 1968
Chlorite	Values may be higher than permanganate	Collings *et al.*, 1978
Triethylene glycol–HCl	Yields values similar to permanganate lignin; lignin preserved	Edwards, 1973
Absorbance	Most of these methods require calibration with gravimetric method	
Acetyl bromide	Protein and non-lignin fractions may contribute to absorbance	Morrison, 1972
Modified acetyl bromide	Non-lignin fractions cause little interference	Iiyama and Wallis, 1990
Bjorkman's ball-milling	Low lignin yields, carbohydrate contamination; used for structural studies	Lam *et al.*, 1990
Saponification	Only applicable to monocotyledonous plants containing ester bridges	Lau and Van Soest, 1981
Calorimetric	Heats of combustion vary due to lignin structure, cannot be used across genera	Schramm and Bergner, 1969
Pyrolysis mass spectrometry	Equipment expensive, must have definition of lignin; can distinguish differences between different lignin chemical methods	Morrison and Mulder, 1995
Non-quantitative	Used for anatomical studies	
Phloroglucinol–HCl		Akin *et al.*, 1990
Chlorine–sulphite		Akin *et al.*, 1990
Coupling with diazonium compounds		Akin *et al.*, 1990
UV fluorescence		Hartley *et al.*, 1990

HCl, hydrochloric acid; UV, ultraviolet.

new lignin method to be routinely used in evaluation, it would have to improve on the relationship with indigestibility, as well as be competitive with respect to time and effort *vis-à-vis* currently used methods (Van Soest, 1994).

Starch

In situations where forages containing high starch levels are fed, specific analysis for starch may be useful. Assays for the reliable extraction and hydrolysis of starch have been developed (Faichney and White, 1983). Accuracy of starch analysis, however, is dependent on the specificity of enzymes used in the analysis and on complete hydrolysis of starch to glucose (Hall, 1997). Starch digestibility is probably as critical as starch quantity in determining its value in a forage sample, and can have a large impact on animal performance (Allen *et al.*, 1997). Methods that evaluate starch digestibility quickly and reliably in the laboratory have not yet been developed, so starch digestibility is not currently routinely determined (Allen *et al.*, 1997).

Water-soluble carbohydrates

WSC concentration in pre-ensiled forage allows assessment of the available supply of energy for lactic acid production during ensiling, and therefore is an important measurement for studies involving these forages. Insufficient WSC will inhibit fermentation, while excess WSC provides substrate for undesirable organisms during storage and feed-out. Forage monosaccharides, glucose and fructose, the oligosaccharides, sucrose, melibiose, raffinose and stachyose, and fructosans can be extracted with cold water (McDonald and Henderson, 1964). Cold-water extractions of legumes and subtropical and tropical grasses do not yield starch, because amylose is partially soluble in hot water, while amylopectin is not soluble in water (Smith, 1981). Procedures that solubilize starch by enzyme or dilute-acid methods, as well as the sugars, are referred to as total non-structural carbohydrate methods (Smith, 1981).

If not inactivated, forage enzyme systems will quickly modify WSC concentration. Rapid freezing, followed by freeze-drying, can minimize modifications to WSC concentration, although freeze-drying does not inactivate enzymes. Rapid oven-drying will also minimize changes in WSC. Enzymes have been inactivated with hot water or ethanol (Kerepesi *et al.*, 1996), but cold-water extraction is recommended. Hot water may solubilize fibre components (McDonald and Henderson, 1964). Protein solubility and other interferences can be minimized by extracting sugars with alcohol solutions (Wiseman *et al.*, 1960).

Spectrophotometric methods used in the UK, involving the use of anthrone, are described elsewhere (Yemm and Willis, 1954; Thomas, 1977; MAFF-14, 1986). Total WSC concentration may also be determined using a spectrophotometric method based on the reaction of phenol–sulphuric acid with carbohydrate (Dubois *et al.*, 1956).

Other carbohydrates

Analysis for sugar components may yield valuable basic information. Methods used will depend on the carbohydrates present and the degree of fractionation required (Moore and Hatfield, 1994). Traditionally, schemes for fractionating carbohydrates were long and laborious (Van Soest, 1994). The development of automated high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC) equipment has eliminated this problem, but other problems remain. General schemes cannot be applied without knowing the peculiarities of the forage involved (Van Soest, 1994).

Soluble carbohydrates are approximately 98% digestible (Van Soest, 1994), but the diversity of carbohydrate fractions included prevents their treatment as a uniform nutritional entity. One fraction, soluble fibre, which includes non-strarch polysaccharides, such as pectic polysaccharides, is of particular interest. Unlike sugars and starches, with which they may be coextracted, soluble fibre is not digested by mammalian enzymes. Compared with starch and sugars, which tend to ferment to lactic acid and result in decreases in ruminal pH, soluble-fibre fermentation tends not to produce lactic acid (Hall *et al.*, 1997). The practical implications of this for ruminant nutrition, particularly high-producing dairy cows, justifies the separation of soluble carbohydrate into a fibre and non-fibre fraction. Colorimetric or chromatographic methods to extract fibre have problems with incomplete extractions, partial degradation of carbohydrates or interference by other substrates (Hall, 1997). Recently, a gravimetric method, using the neutral-detergent solution of the Van Soest method, has been proposed to estimate the neutral-detergent-soluble fibre fraction in forages. This method is relatively simple and inexpensive, suggesting its possible use in routine evaluation schemes for forages containing significant quantities of pectic substances.

Nitrogen fractions

Total N content is usually determined by macro- or micro-Kjeldahl methods. Samples are digested in sulphuric acid, with a copper sulphate or copper–selenium (MAFF-48, 1986; AOAC-984.13, 1990) catalyst. Nitrogen is converted to ammonia, distilled and titrated. A variety of other macro- and micro-Kjeldahl methods are available, based on similar principles. Total N content can also be determined effectively by combustion in elemental analyser instruments (Emteryd *et al.*, 1990). These instruments have become more affordable, and there are fewer environmental concerns related to chemical use and disposal than with traditional Kjeldahl methods. Because the N content of forage crude protein is not always 16%, it is suggested that N content be used with the correction factor for crude protein.

Total N may be only 60–80% true protein in fresh and ensiled forages, with the remainder being non-protein N and unavailable N (Van Soest, 1994). Simple crude-protein concentration determination is inadequate to describe protein quality, particularly in high-protein fresh or ensiled forages. Ideally, any method of assessing protein quality should be able to describe the degree to which a protein contributes to bacterial crude protein and undegraded intake protein (Broderick,

1994). Models such as the Cornell net carbohydrate and protein system (CNCPS) model (Fox *et al.*, 1992) have developed the need for even more comprehensive fractionation of forage proteins (Licitra *et al.*, 1996).

Protein solubility has long been used as a technique for determining degradation characteristics of proteins (Broderick, 1994). An ideal method for determining soluble protein would be a single laboratory analysis, and there has been much research into this methodology. Solubility and extraction methods for protein fractions are subject to many of the limitations of carbohydrate solubility methods (Van Soest, 1994). Unlike carbohydrate fractionation, however, in which the Van Soest method is standard in the USA for evaluating carbohydrate fractions in forages, there is no such standardization with protein fractions. This sometimes leads to difficulties in interpreting reported values for nitrogen fractions.

Sniffen *et al.* (1992) recently proposed the use of a series of chemical fractionations to identify five N components in crude protein. It provides a rational basis for the fractionation of protein, based on nutritional availability, much like the carbohydrate fractionation system of Van Soest (1994). A suggested methodology for these N components is reported by Licitra *et al.* (1996). This suggested methodology is outlined in Table 14.3.

Non-protein nitrogen is traditionally the protein passing into the filtrate after precipitation with a protein-specific reagent (Licitra *et al.*, 1996). Trichloroacetic acid (Krishnamoorthy *et al.*, 1982) and tungstic acid (Pichard and Van Soest, 1977) are two commonly referenced methods. These methods differ in the size of the amino acids included in the precipitate. Other methods are also available (Licitra *et al.*, 1996). The choice of the method will depend on the objective of the experiment.

Table 14.3. Suggested partitioning of nitrogen and protein fractions in forages (adapted from Licitra *et al.,* 1996).

Fraction	Estimation or definition	Enzymatic degradation	Classification*
Non-protein N	Not precipitable	Not applicable	A
True protein	Precipitate with tungstic acid		
True soluble protein	Buffer soluble but precipitable (TP-IP)	Fast	B_1
Insoluble protein	Buffer insoluble	Variable	B_2
Neutral-detergent soluble protein	IP-NDIP		
ND-insoluble protein, but soluble in AD	Protein insoluble in ND, but soluble in AD	Variable to slow	B_3
Insoluble in acid detergent	Includes heat-damaged protein and N associated with lignin	Indigestible	C

* From Pichard and Van Soest, 1977; Van Soest, 1994.
TP, true protein; IP, insoluble protein; ND, neutral detergent; AD, acid detergent; NDIP, neutral-detergent-insoluble protein.

A borate phosphate buffer (Krishnamoorthy *et al.*, 1982), which ensures pH stabilization, is recommended to estimate soluble true protein as the difference between nitrogen and non-protein nitrogen (Licitra *et al.*, 1996). Other fractions (Table 14.3) are based on Kjeldahl analysis of acid-detergent and neutral-detergent residues. The ADIN fraction is assumed to be indigestible (Licitra *et al.*, 1996). This fraction is also an indication of heat damage in silages (Van Soest, 1994). The neutral-detergent-insoluble protein fraction is that protein fraction which is not soluble in neutral-detergent protein (Licitra *et al.*, 1996).

Tannins

Tannins may modify the digestibility of dietary protein and structural carbohydrates in forages used for ruminants (Mueller-Harvey and McAllan, 1992; see also Chapter 20, this volume). This may be considered to be an anti-quality component or a desirable attribute, depending on the type of tannin and its concentration in forage (Broderick, 1995; Reed, 1995). High concentrations (50–100 g kg^{-1} DM) are generally considered toxic to ruminal microorganisms, while lower concentrations reduce the soluble non-protein nitrogen content of silage and improve efficiency of protein utilization (Albrecht and Muck, 1991).

Numerous tannin extraction and quantification procedures have been developed (Table 14.4). Most procedures are specific for condensed tannins. The majority have some difficulty associated with their use, either because the procedure is not specific or because the procedure is long and laborious. Additionally, toxic effects of tannins are strongly influenced by an interaction between animal species, animal adaptation and type of tannin, so that it may be difficult to predict (Robbins *et al.*, 1991). Because of these factors, no one procedure has emerged as the prevalent method in tannin analysis. An improved method, based on precipitation with trivalent ytterbium (Giner-Chavez *et al.*, 1997), exhibits promise for more routine quantification of tannins in forages.

Tannins are not prevalent in grasses or most temperate legumes. Routine evaluation for tannins for these forages is not necessary. Tannins are prevalent, however, among dicotyledonous forbs, shrubs and tree leaves (Haslam, 1979). For animals consuming diets with these types of forages, performance may be adversely affected. In these situations, energy or nitrogen content may not limit performance, but rather this anti-quality aspect of tannins. In these cases, it becomes important to routinely analyse for tannins. Other anti-quality forage components should be handled similarly (see Chapter 20).

Vitamins and minerals

Minson (1981) stated that a complete analysis of the elements would be his first priority where animal production was low. Forages clearly provide an important source of vitamin A and E and the minerals essential for growth (Fisher *et al.*, 1995). There are no simple procedures that can be routinely used for vitamin analysis in forages (Fisher *et al.*, 1995). Forages can be prepared for mineral analysis

Table 14.4. Methods for isolating and quantifying tannins.

Method	Comment	Reference
Extraction		
Absolute methanol		Terrill *et al.*, 1990
Aqueous methanol		Terrill *et al.*, 1990
Aqueous acetone		Terrill *et al.*, 1990
Aqueous acetone with ascorbic acid and diethyl ether	Ascorbic acid minimizes oxidation of tannins, but can interfere with subsequent analysis Some high molecular weight condensed tannins not soluble	Terrill *et al.*, 1992; Reed, 1995; Giner-Chavez *et al.*, 1997
Hot methanol		Reed, 1995
Isolation methods		
Sephadex LH-20	Commonly used method	Makkar and Becker, 1994
Trivalent ytterbium	Newer method yields results similar to Sephadex LH-20	Reed *et al.*, 1985; Giner-Chavez *et al.*, 1997
Binding procedures	Non-specific isolation	
Protein		Makkar *et al.*, 1988
Polyvinylpyrrolidine (PVP)		Makkar *et al.*, 1993
Polyethylene glycol (PEG)		Silanikove *et al.*, 1996
Quantification methods		
Gravimetric	Crude estimate of tannins	
Trivalent ytterbium	Does not work well for low levels of tannins	Reed, 1995
PVP	Not specific for condensed tannins	Makkar *et al.*, 1993
Colorimetric	All require standards; internal standards required	
Redox	Subject to numerous interferences	Hagerman and Butler, 1989
Vanillin–HCl	Commonly used, condensed tannins and flavan-3-ols specific	Broadhurst and Jones, 1978; Reed, 1995
Acid butonol	Best assay for condensed tannins; very specific	Hagerman and Butler, 1989
Protein binding	Less precise than colorimetric, may relate to biological effects	
Radial diffusion	Depends on protein structure and reaction conditions	Hagerman and Butler, 1989
Radioactivity		
^{14}C PEG	Pre-extraction of tannins is not required	Silanikove *et al.*, 1996

HCl, hydrochloric acid; ^{14}C, carbon-14.

by dry ashing at 550°C or wet digestion with nitric acid (AOAC-968.08, 1990). Mineral solutions are then analysed by atomic absorption spectrophotometry or flame photometry for individual elements or by inductively coupled plasma emission spectrophotometry for multiple elements. These procedures are labour-intensive and, unless a mineral deficiency or toxicity is expected, would not warrant routine analysis except for the macrominerals.

Chemical Analyses versus *In Vitro* Techniques

Many of the chemical characterization assays are designed to estimate *in vivo* digestibility. Numerous studies have concluded, however, that chemical methods are usually less highly correlated with *in vivo* methods of measuring forage quality than microbial and enzymatic methods (Barnes, 1973; Marten, 1981; Van Soest, 1994). The inability of a single chemical analysis or solubility index to accurately predict an *in vivo* parameter is due to the complexity of biological systems (Barnes, 1973). Legumes and grasses may have the same *in vivo* digestibility but be considerably different in chemical composition. Conversely, forages may be similar in chemical composition (e.g. NDF) but considerably different in digestibility (Cherney *et al.*, 1990). It is likely, therefore, that *in vitro* and/or *in situ* assays will continue to be used (Van Soest, 1994).

In vitro assays are not without difficulty. Standardization of techniques can be difficult and many factors will affect results (Marten, 1981; Cherney *et al.*, 1993). Soluble protein is often determined by direct *in vivo* measurement, although results obtained using this methodology have not always been successful and are difficult for commercial application (Broderick, 1994). Chemical characterization of forages is easier, quicker and less expensive than conducting *in vitro*, enzymatic or *in situ* assays (Weiss, 1994). Intensifying environmental and welfare concerns will make maintaining animals for research or commercial analyses increasingly difficult.

Microbial methods are sensitive to most intrinsic factors that limit *in vivo* digestion of a forage and can yield information concerning rates of digestion (Van Soest, 1994). Microbial methods, however, provide little information regarding the actual chemical constituents influencing digestion. Information from both of these types of analyses is likely to continue to be needed.

Future

The use of modelling to predict animal performance will continue to increase. Many of these models will rely heavily on chemical characterization, because of the speed, repeatability and generally low cost of chemical characterization. Fibre, lignin and protein assays will continue to be important, due to their strong association with factors affecting animal performance. Other assays, such as soluble-fibre and starch analyses, will become increasingly important as our level of understanding of the factors affecting animal performance increases. Automation and refinement of equipment will make chemical characterization faster and more repeatable.

There are numerous methods available for fibre, lignin, protein and other

chemical components. It is likely that new methods will continue to be developed. As with lignin, it is imperative that any new method be judged on its ability to improve on the relationship with intake or indigestibility, as well as be competitive with respect to time and effort *vis-à-vis* currently used methods. In addition, because there may be numerous assays to determine the same component, such as lignin, it is the responsibility of the researcher to know the limitations of their methods and what they actually are analysing (e.g. permanganate lignin versus acid-detergent lignin). Researchers should also take special care to report the exact methodology that they are using. With the advent of new methodologies and a better understanding of factors limiting animal performance, the chemical characterization of forages will continue to be a worthwhile field of endeavour.

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