The Detergent System for Analysis of Food Stuffs

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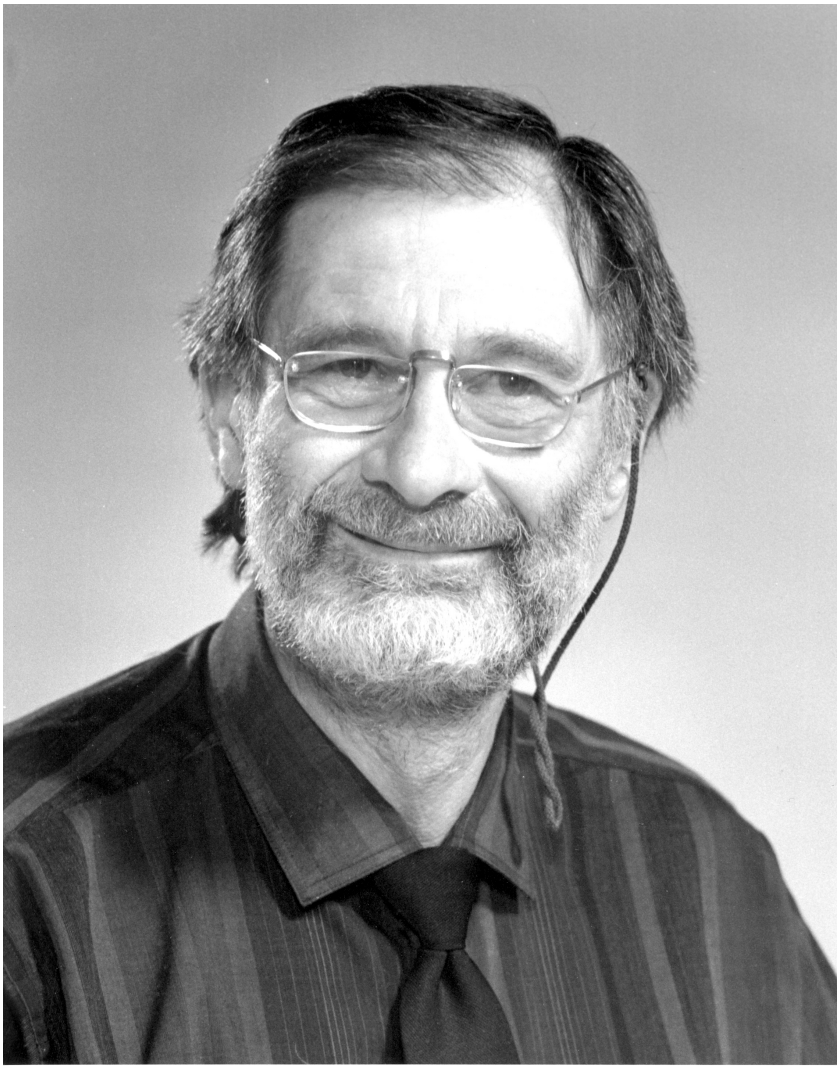


Abbreviations

A.R.: Analytical reagent

AACC: American Association of Cereal Chemists

ACS: American Chemical Society Standard

AD: Acid detergent

ADDL: Acid detergent dispersible lignin

ADF: Acid detergent fibre

ADL: Acid detergent lignin

ADR: Acid detergent fibre residue

AIA: Acid insoluble ash

ALS: American Chemical Society

aNDF: Amylase treated neutral detergent fibre

Anhy: Anhydrous

AOAC: Association of Official Agricultural Chemists

AR: Analytical reagent

BMR: Brown mid-rib

Coeff: Coefficient

CP: Chemically pure

CS: Corn silage

CTA: Cetyltrimethylammonium

CTAB: Cetyltrimethylammonium Bromide

CTAC: Cetyltrimethylammonium dichromate

DM: Dry matter

DNDF: Digestible neutral detergent fibre

EDTA: Ethylenediaminetetraacetic acid

ELS: monoethanolammonium lauryl sulphate

EtOAc: Ethylacetate

GC: Gas chromatograph

GLC: Gas liquid chromatography

HPLC: High pressure liquid chromatography

I.D.: Internal diameter

I: Insoluble

KL: Klason Lignin

KLS: Potassium lauryl sulphate

Lignin(pm): Permanganate lignin

Lignin(sa): Lignin sulphuric acid

MADF: Modified acid detergent fibre

M_i: Metabolic

NaOAc: Sodium acetate

NaOEt: Sodium ethoxide

NBS: Neutral buffer solution

ND: Neutral detergent

NDF: Neutral detergent fibre

NDR: Neutral detergent fibre residue

NF: National formulary (pharmaceutical)

NFE: Nitrogen free extract

NPN: Non-protein nitrogen

O.D.: Optical density

PCB: Polychlorinated biphenyl

peNDF: Physically effective fibre

pKa: Dissociation constant of an acid

Prep: Practical grade

R: Correlation

S: Soluble

SLS: Sodium lauryl sulphate

Tech: Technical grade

TELS: Triethanolammonium lauryl sulphate

UDR: Urea detergent reagent

USDA: United States Department of Agriculture

USP: US Pharmacopoeia

VFA: Volatile fatty acids

Wt.: Weight

Contents and pages to read

1. Fundamentals and background to the analysis of fibrous foods

1.1. Nutritional classification

Adequate dietary analyses of any sort require that the methods employed are relevant to a nutritional classification of the dietary chemical components. Such classifications have often depended upon availability of energy and the dietary components associated with it. On the other hand, dietary fibre and forage also have a major role in determining the gastrointestinal environment, which does not necessarily relate to a classification based on availability. For many animal studies, the problem of nutrient availability is dominant, whereas in the case of human nutrition and some other instances, the ecological question prevails. These two perspectives do not necessarily reflect the same criteria, leading to the solution that alternative approaches are needed.

Nutrients in foods and feeds have been traditionally classified according to chemical composition and presumed structure, such as carbohydrates and protein as in the proximate system; or according to relative energy content, such as fats *versus* proteins and carbohydrates, or minerals of no energy content at all. Other bases involve that of presumed availability, such as fibrous *versus* non-fibrous carbohydrates. Solubility tests for protein as quality parameters for ruminants also follow this logic. The underlying principle of all of these classifications is the desire to categorise component substances into groups possessing similar nutritional effects and properties. The most common classification is based on nutritive availability or expected digestibility. In this context fibre has a unique position nutritionally since it tends to measure less available components than those possessing relatively complete availability.

1.1.1. Nutritional availability

Systems of food and feed analyses are procedures that attempt to classify substances into groups related to their nutritional character and availability. They provide analytical values of composition of dietary ingredients upon which balanced dietary formulations are based. Alternatively, animal responses are predicted from dietary composition. In particular, the fibrous fractions are associated with unavailability of energy and nutrients and are, therefore, indices of quality for such calculations.

The association between fibre content and unavailability presumes a cause and effect relationship whereby the fibrous components either contain the unavailable fractions or promote loss or expenditure of nutrients and energy. These two aspects may be divided into those effects that regulate availability, and therefore digestibility, and those that influence the efficiency by which available energy and nutrients are used by the animal. Another component of nutritive value, in addition to digestibility and efficiency, is voluntary intake.

The unavailability of nutrients can be associated with a fibre fraction in two ways: either the component is contained as an unavailable fraction in the fibrous residue, or the fraction is sequestered by a fibrous component that affects other components. There also may be indigestible components which are not associated with fibre and may include soluble aromatics, phenolics and essential oils. These are the conditions of physicochemical association from which criteria for method evaluation may be derived. Feed fractions which do not appear to affect digestibility through these mechanisms, yet appear correlated through secondary association, usually environmental, have poor predictive value because the association is not direct and is

influenced by external factors that are not controlled in the model. This is the great limitation of empirical predictive statistics based on statistical correlations with digestibility as an evaluation of a particular analytical method.

Adequate prediction of digestibility would be achieved if chemical entities could be separated and sorted according to their nutritional uniformity. This concept was developed by H.L. Lucas in the form of a mathematical model (Lucas, 1964; Van Soest, 1994) which has been applied to digestion balances in various ruminant and non-ruminant species. The results of analysis by the Lucas model (Table 1.1) show that only the non-cell wall portion of feeds behaves in a nutritionally uniform fashion and that the cell wall associated carbohydrates are non-uniform sub-fractions (Van Soest, 1994). The non-uniformity of fibre carbohydrates is associated with lignification and secondary factors which are extrinsic cell wall factors influencing availability of other cell wall substances.

A disastrous consequence of this external effect is that the most elegant analysis for sugar components and carbohydrate fractions is unable to reliably evaluate forages (Chesson, 1983). It was assumed that detailed analysis of cell wall for structural components would account for indigestibility. However, this non-conformity of carbohydrates can be stated another way as: the physicochemical nature of the plant cell wall carbohydrates is of greater nutritional significance than is its chemical constitution and intrinsic composition. The probable chemical basis for this effect is that physicochemical associations of the respective constituent macropolymers are more important in determining availability than the intrinsic chemical structure and its

linkages. The most important indigestible cell wall component is lignin, which in itself is not a uniform fraction (see Section 5).

1.1.2. Neutral detergent solubles

The neutral detergent soluble fraction is completely available to digestion by ruminant microorganisms and is free of the effects of lignification (Van Soest, 1967; 1994). The neutral detergent soluble fraction is composed of mainly non-cell wall components, but -also some soluble cell wall, that are completely fermentable. These include pectins, beta glucans, galactans and some other polysaccharides (Hall, 2003).

The detergent fractionation has been criticised for its failure to definitively separate cell wall from non-cell wall (Pelletier et al., 2010). However, detergent fractionation was based on a Lucas analysis (Van Soest, 1994. Ch. 22.3) that distinguishes insoluble lignified cell wall from soluble non-lignified fractions and non-cell wall components that are completely fermentable. Soluble cell wall components may be assayed by the methods of Hall et al. (1999).

Since lignification is restricted to the insoluble plant cell wall, the content of cell wall moderates the effect of lignification in ruminants. As an example, alfalfa has a lower cell wall and a higher lignin(sa) content compared to a grass at the same digestibility. The higher content of cell wall is offset by a lower lignification in the case of a grass, allowing it to have an equivalent dry matter digestibility at a higher fibre content compared to the legume. The complete availability of cellular contents in ruminants does not signify that actual true digestibility will be complete. The principal factor limiting extent of digestion is the gastrointestinal retention and digestion rate. Starch is the

principal fraction exhibiting sufficiently slow digestion rates to exhibit a faecal loss of about 0.02 at maintenance, but that loss increases at high intakes, and at faster passage rates, and with crystalline structures. The uniformity observed in the Lucas analysis was based on digestion balances in which intake was restricted to near maintenance energy requirements.

Several other deviations from ideality occur in the case of some dietary materials. Soluble polyphenolics (*e.g.* tanniniferous forages, soluble lignins in the rumen and from alkali-treated straws, and soluble Maillard products in heated feeds) are excreted largely in faeces leading to a dilution of cellular contents and lowering of the apparent digestibility. Low molecular weight terpenoids and phenols are absorbed to a large extent and excreted in urine, producing no apparent effect on the true digestibility but in fact lowering its contribution to the metabolisable energy value of the feed.

An absolute requirement for the cell solubles and cell walls, as applied to digestion balances, is that the fibre analysis distinguishes the metabolic products, bacteria and their degradation products, from the truly undigested matter. Presently neutral detergent is the only reagent available for this purpose since enzymatic procedures fail because of the indegradability of microbial cell wall components in the faecal metabolic matter (Mason, 1978). However, this criticism does not apply as long as enzymatic analyses are restricted to foods and diets.

1.1.3. Classification of nutrients

Groupings of dietary fractions according to their availability and nutritional character are shown in Table 1.1. These categories are recognised as substances available to mammalian enzymes, substances unavailable to mammalian enzymes and

partially or wholly unavailable to gastrointestinal microorganisms. The grouping of all substances unavailable to mammalian enzymes has been defined as dietary fibre (Trowell, 1976; Bach Knudsen, 2001; Prosky, 1999; McCleary et al., 2010) and is relevant to non-ruminant and human nutrition, particularly to those animals lacking pregastric fermentation. The insoluble fibre as represented by neutral detergent fibre (NDF) is composed of fractions lacking complete availability to digestive enzymes. This fraction is important to ruminant nutrition as a factor limiting intake and energy efficiency, and is of significance for digestibility estimates for non-ruminants.

Table 1.1.
Classification of some dietary components

Component	Availability
Sugars (simple and disaccharides) Starches Lipids (fats)	Entirely available: potential limitation by rapid passage and digestive escape
Beta glucans Fructans Galactans Pectins	Available: only via gastrointestinal fermentation
Neutral detergent fibre) Cellulose Hemicellulose	Partially available: regulated by lignification and fermentability
Maillard products Retrograded/damaged starch Lignin Tannins Leather Cutin	Unavailable: not susceptible to gastric and fermentative digestion

Substances available to mammalian enzymes form the bulk of the plant cellular contents and include lipids, sugars, starches, true protein and non-protein nitrogen (NPN) compounds, including nucleic acids. The main limitation to utilisation of these substances is competition between passage and digestion rates, relevant in particular to the less soluble crystalline starches.

Substances that are completely fermentable, but unavailable to mammalian enzymes include pectins, gums, galactans, and fructans, and carbohydrates that are generally soluble in neutral detergent. Although pectins form a part of the cell wall, they are soluble because they have no covalent linkage to the cell wall matrix. Fructans are plant reserves, but the relation of beta glucan gums in oats, rye, and barley grains and galactans in legume seeds to the cell wall is less clear, since these carbohydrates may serve as plant reserves. The consequence of their inclusion in dietary fibre is that the comprehensive definition of nutritive fibre does not coincide with the botanical definition of cell wall. This is also the case when the NDF is used in the context of fibre for ruminants, since the degradable pectins are not included.

Other gums also occur in various plants, microorganisms and algae. These include glucomannans, (fermentable) alginic acids, and agar (soluble but highly unfermentable). Heat damaged starch is soluble but unfermentable and indigestible.

Neutral detergent fibre is comprised of partially fermentable and insoluble carbohydrates, a small amount of very insoluble proteins, undegradable lignin, cutin, Maillard products, and tannin condensates. No chemical method exists to distinguish available cellulose and hemicellulose from unavailable, which seems to be determined

by their associations with each other and extrinsic linkage of these carbohydrates with lignin. The limit of digestibility is largely set by the lignin to structural carbohydrate ratio. Accurate laboratory estimates of digestibility of cellulose and hemicellulose, therefore, depend on digestion with rumen organisms or cellulases. Estimation of cellulose and hemicellulose availability from the lignin content depends on statistical regressions with digestibility. The actual extent of *digestion in vivo* is determined by the competition of ruminal digestion with passage, as well as by lignification.

The obligately unavailable fraction is comprised of natural substances (*i.e.,* lignin, tannin, cutin, and keratin) and of synthetic components induced by, for example, treatment, heating, storage. These include heat damaged starch; Maillard products (polymers of aromatised sugars and amines), polymerised unsaturated lipids. Many of these substances are collectively isolated in crude lignin by the lignin(sa) procedure. However, the damaged starches and some tannins may also be soluble. Condensed tannins and tannins complexed with protein (also rendered indigestible) tend to isolate with the detergent fibres.

1.2. Criteria for non-ruminants

Animals without pregastric fermentation utilise fibrous components with lower efficiency than do ruminants (Van Soest, 1994), and compensatorily make more efficient use of non-fibre components since these are not subjected to fermentation before gastric digestion. In addition to the insoluble fibre components, there are a variety of polysaccharides in plants, such as pectins, gums, beta glucans, galactans and fructans, which are soluble and completely fermentable but not susceptible to

mammalian carbohydrases, leading to their collective classification as unavailable carbohydrates (Southgate, 1969). Unavailable carbohydrates have the definition that, although they are digestible through fermentation, their digestion in non-ruminants provides no direct sugar for mammalian metabolism, but instead provide energy in the form of volatile fatty acids (VFA) that are efficiently absorbed from the lower digestive tract (Argenzio and Southworth, 1975; McNeil et al., 1978).

The mechanism for utilisation of fermentable unavailable carbohydrates is of major importance to non-ruminant herbivores. They promote microbial fermentation in the hind gut which is the mechanism delivering an important source of dietary energy to the host animal. The quantities of these carbohydrates along with that of the celluloses and hemicelluloses that collectively ferment are responsible for the net effect. Increased intake of the unavailable sources leads to a higher portion of the energy being derived from VFA leading to higher gluconeogenesis from propionate and increased metabolism of butyrate by the colonic mucosa (von Engelhardt, 1983). The gluconeogenic effect is important in human diabetes and is part of the beneficial effect of high fibre diets for diabetics (Anderson, 1985). In non-ruminants, the effects of soluble unavailable carbohydrates in the diet are more aligned with the effects of cellulose and hemicellulose. The definition of dietary fibre has been extended to include these soluble components (Trowell, 1976), and enzymatic methods have been proposed for the determination of the entire dietary fibre complex (Asp, 1981).

The attractiveness of enzymatic methods to determine unavailable carbohydrates and fibre is that they follow the sequence and limitations of gastric digestion. However, they have several disadvantages. Enzymes may be variable reagents, and the

spectrum used by the dietary fibre assay (*e.g.,* proteases and amylases) do not remove microbial components and animal mucopolysaccharides. The consequence is that enzymatic procedures for fibre are unsuitable for dietary balances and the analysis of faecal matter. The requirement for an adequate analysis of a digestion balance is that the same substances fed must be recovered from faeces (if indigestible) without contamination from metabolic and endogenous products. No exact procedure exists for this purpose.

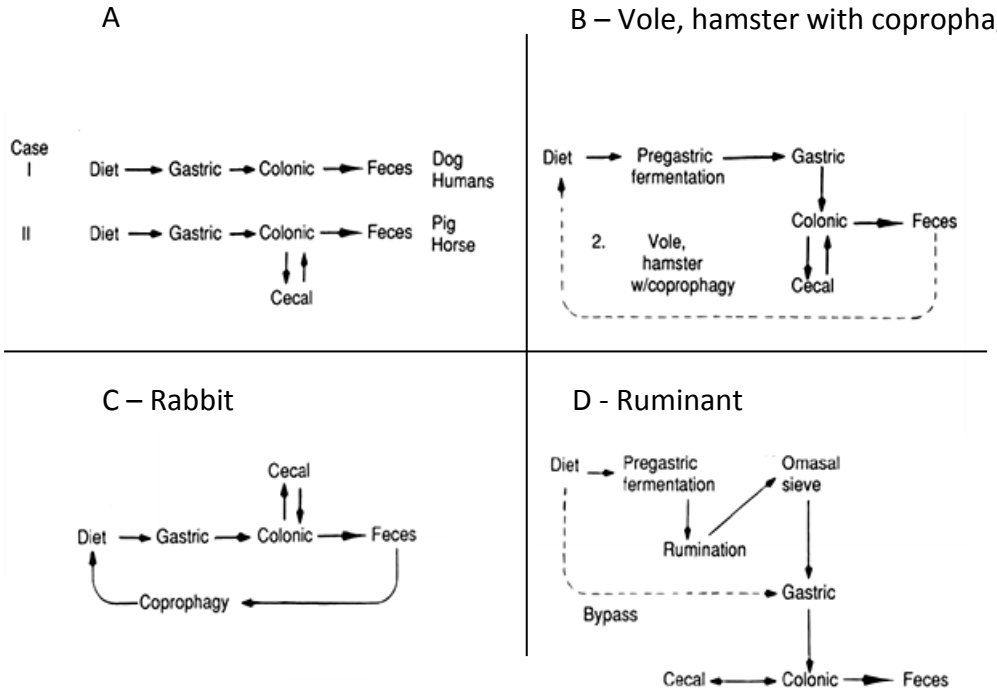
The special problem of separating faecal soluble polysaccharides from endogenous and microbial peptidoglycans is more difficult and requires special procedures (Horvath, 1984). In some cases, this separation may be unnecessary because of the relatively complete fermentability of most soluble unavailable carbohydrates. Agar, alginic acid and escaped starch are exceptions and will require special analyses.

1.2.1. Diversity in non-ruminants

The argument for the definition of dietary fibre was largely developed in the context of human nutrition, where it was assumed that pregastric fermentation is insignificant. However, a survey of the variations in non-ruminant mammals indicates that this is not a safe generalisation to be applied broadly to non-ruminants. Various members of this group possess pregastric fermentation or, alternatively practice coprophagy, allowing more complete utilisation of fermentation products. Relative to anatomical sequence, there are at least five general strategies of digestion (Table 1.2).

Table 1.2.

Sequence of digestion in order of relative complexity



The simplest sequence is that exemplified by man, dog and carnivores in which a cecum as a separate compartment is essentially lacking. However, it should be noted that the human colon is sacculated, in contrast to carnivores, which probably represents herbivorous evolutionary ancestry. Non-ruminants such as the pig and horse possess a significant cecum that pulsatively empties every day or two. In these animals the cecum is secondary in capacity to the colon, and they seem to possess higher capacity for fibre digestion than in man or the small rodents (Koller et al., 1978; Ehle et al., 1982a).

A third category, represented by many rodents, is the situation where the main site of digestion lies in the cecum, which is dominant over the colon, and many of these animals practice coprophagy. Within this group, there are more specialised species

such as rabbits and lemmings where the cecum selectively admits only fine matter, with coarse fibre being excluded and excreted in ‘day’ faeces. ‘Night’ faeces are reingested allowing utilisation of microbial protein and vitamins derived from the most fermentable carbohydrates (Hoernicke and Björnhag, 1980). Because the coarse insoluble fibre is rejected, fibre utilisation is very low. This aspect may be regarded as an evolutionary adaptation of small herbivores to the problem of the low energy density of fibrous plant matter.

The fourth category includes those animals which possess pregastric fermentation without rumination. These include a wide spectrum of mammals including kangaroos, hamsters, voles, colobine and langur monkeys, hippopotamus and, probably, other species that remain to be described. The argument for the definition of dietary fibre for these animals would follow that of the ruminant rather than the non-ruminant, since the sense of classification of fermentable carbohydrates as unavailable for absorption would apply in this case to soluble sugars and starches that are pregastrically fermentable.

1.2.2. Comparative digestion

The digestive capacity of herbivores varies according to their respective ability to fermentively digest NDF, represented by unavailable and slowly digesting carbohydrates. All animals are limited in this ability to digest insoluble carbohydrates (Foose, 1982; Van Soest, 1994), and fibre residues in faeces are largely comprised of unavailable fractions and variable amounts of cellulose and hemicellulose that have escaped digestion. The amount of these carbohydrates depends on fibre quality, fermentation rate, the inherent retention time in the fermentive compartment, and the

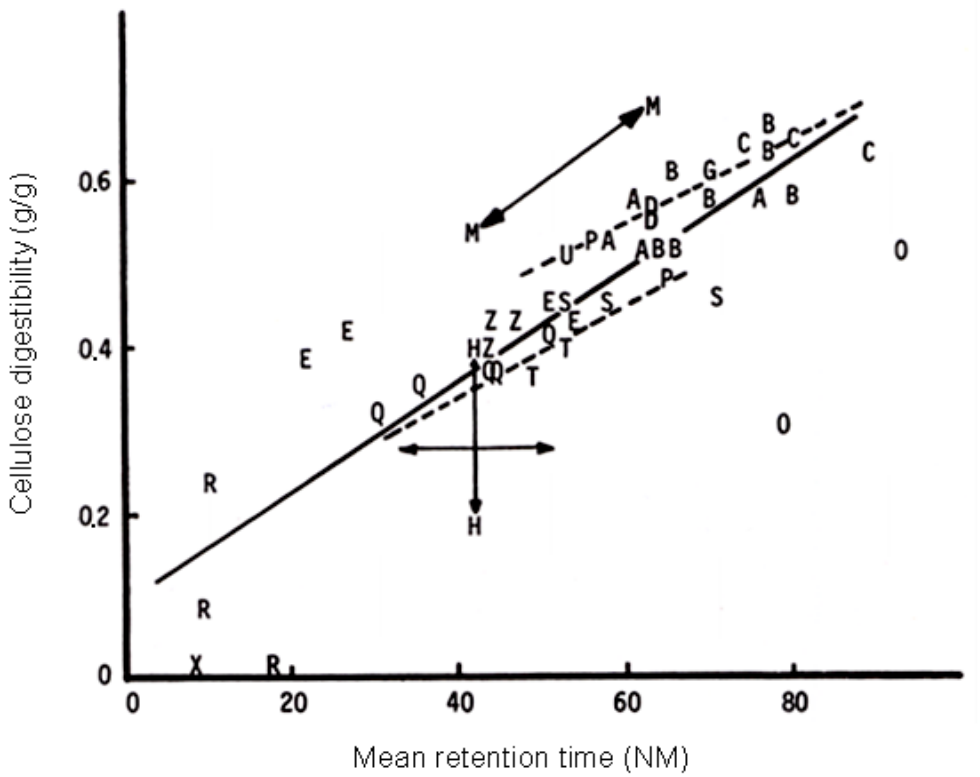


Fig. 1.1. The relationship between cellulose digestion and mean retention in 46 species of herbivorous mammals fed grass-type fibre. Ruminants such as antelopes (A), grazing bovids (B), camelids (C), deer (D), giraffes (G), sheep and goats (S), are better able to digest fibre than non-ruminants, but smaller species of both groups are at a disadvantage. The ranges of values for humans (H) and baboons (M) (indicated by arrows) are somewhat less than the figure for pigs (U) but higher than for rodents (R). Large capacity animals such as hippos (O) and rhinos (P) may approach the capacity of ruminants, but elephants (E) and smaller equids (Q) zebra (Z) and tapirs (T) are less efficient. Very small animals (rodents and lagomorphs [R]) have very low digestion capabilities. The panda (X) has the lowest of all (Dierenfield et al, 1982). The overall correlation between retention and digestion was 0.86; the individual correlation for ruminants was 0.69 that for non-ruminants was 0.78 (Van Soest, 1994).

level of intake. When controlled diets are fed, gastrointestinal retention is the dominant variable (Fig. 1.1). Smaller animals are less able to digest fibre because they have high metabolic requirements and, hence, high intakes relative to their gastrointestinal capacity, leading to short retention times (Demment and Van Soest, 1985).

Two problems arise from this interspecies variation in digestive capacity. One is in use of animal models as exemplified by modelling the human condition with rats, pigs, monkeys, etc., or application of domestic ruminant requirements to wild ruminants. The second is the estimation of digestibility of particular diets for a specific animal species from data derived from domesticated animals.

1.2.3. Composition and digestibility of forages

Lignification is the primary factor limiting the ultimate extent of fibre digestion since all herbivores are limited in digestive capacity by finite retention times. The limits of cell wall digestion are approached only in those animals with very long retention times (generally the larger ruminant) and in the case of the lowest intakes. Since lignification is only weakly correlated with cell wall content of forages and fibrous foods, the result is that fibre content is not highly related to extent of digestion. Fractions associated more closely to the lignified core are better estimates of digestibility in large grazing animals.

However, in those species with shorter retention times, and also in domestic ruminants fed at high levels of intake with concentrates, the cell wall (NDF) is more closely associated with digestibility (Henry, 1976; Keys and Van Soest 1970; Keys et al., 1969) and lignin may form a zero, or even positive, relationship. Since animal species form a continuous spectrum of retentions and digestive capacities (Fig. 1.1), the

estimation of digestibility in respect to any animal species is not simple, and more specific information is needed for many herbivorous animals.

1.3. Criteria for ruminants

Ruminants (Category V in Table 1.2) are characterised by selective pregastric digestion accompanied by rumination and the devotion of the greater part of the digestive tract to fermentative digestion. This allows ruminants to utilise available fibrous carbohydrates more completely. Because virtually all ingested carbohydrates are potentially available to fermentation in adult ruminants, the concept of unavailable carbohydrate, as applied in human nutrition (Southgate, 1969) has a new and special significance, namely that all otherwise available sugar and starches are thereby unavailable as sugar sources as a consequence of their fermentation, making the ruminant animal largely dependent on gluconeogenesis for maintaining blood glucose. Consequently, the division of dietary fibre into soluble and insoluble components has no meaning, since available pectins and beta-glucans have the same fate as sucrose and starch. Only if the respective carbohydrates escape the rumen to the lower tract, will the availability distinction apply. Even then, substantial amounts of starch tend to escape digestion and pass on to be fermented in the cecum and colon.

The problem of extent of digestion centres on the insoluble plant cell wall matrix, its lignification, rate of potential fermentive digestion, and retention time. Since the nutritional quality of forage cell walls varies independently from its amount; (non-uniformity in the Lucas analysis) measures of cell wall such as NDF, do not correlate well with apparent digestibility and relate better to voluntary intake (Van Soest et al., 1978). The special problem for ruminants is to sort the cell wall carbohydrates into the

potentially digestible and the obligately undegradable. Unfortunately, no chemical procedures are available for this purpose and resort must be made to biological assays with cellulolytic rumen organisms or fungal cellulases.

Division of analytical approaches into chemical and biological (enzymes and *in vitro* fermentation) allows a more complete description of respective forages and feeds. Enzymatic and fermentive systems assay availability to digestion and fermentation, but are insufficiently developed to provide meaningful description of chemical entities, which must be obtained from chemical fractionation and analyses. Adequate knowledge of the chemical composition will account for the behaviour of the feed material in *in vitro* digestion. Hence, the two approaches in the analysis of ruminant feeds and forages are complementary.

1.3.1. Agronomic factors

Most of the variation in forage quality is accounted for by plant maturity and responses of plants to environmental factors which determine the rate of plant development and distribution of synthetic resources in the plant. Another factor, relevant to practical animal nutrition, is the variation in quality expressed by individual forage species that may respond differently to environmental stimuli. Environmental effects on forage composition are complex but, temperature, light and moisture (in decreasing order) are the dominant factors affecting composition (Van Soest, 1994, Section 6; Wilson, 1982).

It is generally assumed that cell wall (total fibre) and lignin content of plants advance with age and both are negatively correlated with digestibility. This generalisation is quite unsafe and is true only in specific circumstances. Lignification is

primarily dependent upon environmental temperature (Wilson, 1982) and plant maturity, with temperature overriding the effect of maturity, while cellulose and total cell wall are probably influenced more by light patterns. Light and photoperiod promote photosynthesis and the production of sugars and metabolites that dilute the structural matter; hence a negative association between light and cell wall components occurs. The consequence of the interactions of temperature and light are shown in Fig. 1.2. Generally, stress factors promote digestibility through retardation of plant development (Van Soest et al., 1978).

Light-temperature interactions over the growing season have major effects on plant composition. The growing season is divided into three periods in the northern temperate zone: 1) In the spring, temperatures and day length are positively associated; 2) During midsummer, where day length decreases after June 21 and temperature tends to still increase; and 3) In the late summer and fall during which both temperature and day length decrease with time. In spring, plant development and lignification tend to be strongly associated, causing fibre to be negatively associated with digestibility. In midsummer, the relationships are much weaker, since cell wall content is more constant and varies less with lignification. In the fall cellulose and lignin can be negatively associated leading to possible inversion in the primary factors determining digestibility, and therefore the sign and degree of correlation of fibre with digestibility will depend on the particular fibre's secondary association with lignin. This is the ultimate consequence of the non-uniform character of the structural carbohydrates.

The practical problem of forage evaluation is thus made all the more difficult because of the complexity of the environmental interactions. To that of light and

temperature must be added the effects of soil, moisture and plant nutrients which promote regional and micro-environmental differences. Analyses of data, based on date-of-cutting, have been biased by first cutting (spring) values and have largely ignored interregional and late-season effects (Van Soest et al., 1978).

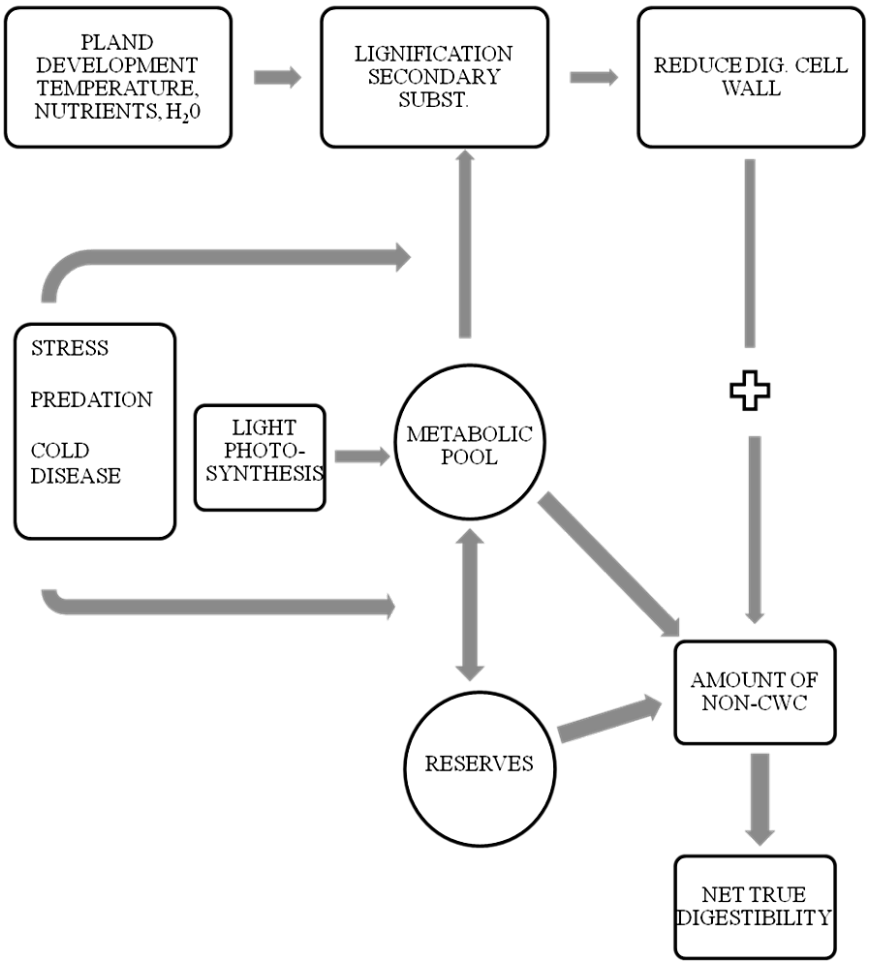


Fig. 1.2. Diagram showing the interaction of environmental factors upon metabolic processes and consequent composition and digestibility (Van Soest, 1994).

It is obvious from the foregoing that variation in environment will not allow any single criterion of forage quality to adequately estimate nutritive value. For this reason, and from the concern for adequate chemical analysis, the detergent system of forage

fractionation has emphasised: 1) Practical separation into chemical entities; 2) Separation, as far as is possible, into nutritive entities, where the major limitation of proximate analysis with the division of carbohydrates into crude fibre and nitrogen-free extract (NFE) which the detergent system was designed to overcome. Crude fibre does not represent a recovery of any particular cell wall component. It is mostly cellulose, although not all of that is recovered and most of the lignin and hemicellulose are lost to the NFE (Chapter 6 in Van Soest, 1994). Statistically, crude fibre behaves mostly like cellulose and suffers from the same limitations.

Analyses for evaluation of forages and feeds are only as good as their ability to account for the various limiting factors that exist in the particular forage or feed. The physicochemical associations of the macropolymers constituting the dietary fibre are of greater nutritional significance than the chemical structure of sugars and linkages.

The primary limiting factors to digestibility are the amount of plant cell wall and its intrinsic components, of which lignin is the principle. Unfortunately, the amount and composition of forage cell walls is greatly perturbed by environmental factors that determine the association between cell wall content and its lignification in forage. This effect contributes to the problem that fibre values, which are secondarily associated with quality through the primary ones such as lignification, are not reliable estimators of digestibility. It is not possible to estimate digestibility reliably with any fibre value unless the environmental variables are assayed. A single fibre analysis is, therefore, an unreliable means of evaluation since too many variables affect quality which cannot be evaluated in a single determination.

1.4. Fibre quality

The quality of fibre is an important factor influencing and maintaining the normal rumen environment. Apart from its quantity and concentration in the diet, particle size, surface exchange properties, fermentation rate and degree of lignification are relevant parameters of quality. Because of the variation in these quality factors, equivalent amounts of dietary fibre will not elicit equal effects in the animal.

Finely ground fibre lacks the ability to stimulate rumination despite the fact that its chemical composition and dietary quantity is not being altered. Grinding and pelleting of fibrous feeds decreases their potential water holding capacity and increases feed density, thereby allowing the animal to consume more feed at the expense of enhanced passage rate since particle size is one of the factors affecting rumen retention. In non-ruminants, the effects are opposite in that the increased density promotes longer retention time (Heller et al. 1980; Ehle et al., 1982b) since the non-ruminant lacks the screening system that retains larger particles which, in turn, stimulate rumination.

Further factors that affect hydration capacity and hence the effective bulk contributed by the fibre, is its cation exchange. Surface groups such as carboxyls, phenolics, amines and hydroxyls provide hydrophilic character as well as binding capacity for metal ions. This latter capacity is apparently associated with shorter lag times (delay of fermentation) and faster rates of microbial fermentation of the respective cell wall. Cation exchange can also serve as an important buffering capacity to the rumen. Legume cell walls possess more buffering capacity than do graminaceous cell walls and also are generally more lignified. The combination of lignification and exchange capacity allows the fibre particle to survive digestion and to continue to serve

as a bank of buffering capacity throughout the digestive tract; a property not possessed by the carbonate or bicarbonate salts that are commonly used to supplement and correct the acidity of low fibre diets (McBurney et al., 1986).

Vegetable cell walls are normally unlignified and generally have the highest exchange and hydration capacities while also exhibiting the fastest fermentation rates. The buffering capacity of calcium pectate is considerable since the metal ion is unfermentable and the calcium in pectate is a fairly available mineral source.

Unlignified cell walls tend to be completely fermentable and disintegrate into fine material during fermentation, since the cementing effect of the lignification matrix is lacking. Natural examples include vegetables and seed hulls such as soybeans. Chemically delignified wood and straw also exhibit the tendency to disintegrate in fermentive digestion, causing the disappearance of the coarse fibre effect required for stimulation of rumination. Lignification can be seen, in a sense, to be a positive factor since it is necessary for normal rumen function and contributes to exchange capacity.

Delignified straw is of higher *in vitro* digestibility than its untreated form, but often exhibit slower rates of fermentation and longer lag times (Van Soest, 1994). Because of the faster passage elicited by the fermentive disintegration, the lag and slower rate of digestion, an increase in *in vivo* digestive efficiency is not always obtained (Berger et al., 1979). Delignified materials usually have low cation exchange and hydration capacities, but it is not known whether these characteristics contribute to the fermentation lag.

1.4.1. The fibre requirement

Ruminants (dairy cattle in particular) generally require adequate dietary fibre for normal rumen function and maintenance of normal milk fat synthesis. Rumen function in dairy cattle is associated with adequate rumination and cellulose digestion to maintain cellulolytic microorganisms which characteristically produce the higher acetate and butyrate needed for normal lipid metabolism in the cow. Feedlot cattle are not fed much fibre for economic reasons. Nevertheless acidotic conditions and rumen parakeratosis are recognised pathologies that disappear if adequate fibre is fed. Low forage quality as affected by plant maturity and forage species, limits milk production in a way which cannot be overcome by other supplementation (Van Soest, 1994).

The level of fibre in the diet which provides optimum rumen conditions has been examined in several experiments (Lofgren and Warner, 1970; Briceno et al., 1987; Grant et al., 1990). Although different forages do not elicit equal efficiencies, maximisation appears to occur at 300 to 350 g/kg NDF in the diet as course forage (Van Soest, 1994). Particle size is an important modifier of the effectiveness of fibre (Briceno et al., 1987; Grant et al., 1990) with adequate particle size promoting rumination and control of rumen pH.

The concept of effective fibre has been further developed by Mertens (1997), and Grant and Cotanch (2005), and defined as physically effective fibre (peNDF). Physically effective fibre is the fraction of fibre that stimulates rumination and maintains the floating mat of large particles in the rumen (Mertens, 1997). Too fine a particle size fails to stimulate adequate rumination, and a limiting size appears to be about 1.18 mm for a sheep and somewhat larger for cattle, up to 4 mm (Grant and Cotanch, 2005).

Determination of peNDF requires the measurement of the amount of NDF greater than the limiting particle size done on a sieve or screen.

The NDF represents the sum of lignin, cellulose and hemicellulose which have diverse effects upon nutritive value. Cell wall is more highly correlated with voluntary intake than any other feed fraction, but is less correlated with digestibility than is ADF, of which lignin forms a larger proportion. Nevertheless, NDF recovers the truly indigestible portion of the diet to a higher degree than any other fibre fraction, since it represents the truly lignified cell wall residue. The ADF serves primarily as a preparation for cellulose or lignin determinations, and is not a valid replacement for crude fibre.

There are also studies indicating beneficial effects of fibre in other animal species such as pigs (Kornegay, 1981), guinea pigs (Fahey et al., 1979) and man (Burkitt, 1973). The feeding of alfalfa to growing pigs affects gross feed efficiency and body composition. Levels of 60 to 120 g NDF/kg DM in total diets to swine does not alter use of digested energy and may improve it (Kornegay, 1981). However, one of the significant effects is the alteration in body composition. Fibre-fed pigs are leaner with less fat and have a larger gut fill and a heavier gut mucosa (Kass et al., 1980). Fibre also stimulates caecal and colonic growth in the rat (Jacobs and Schneeman, 1981). Caecal and colonic mucosal growth occurs through VFA stimulation as in the ruminant (Stevens, 1978). The VFA's are absorbed in non-ruminants, including man, by the same mechanisms as from the rumen (McNeil et al., 1978; Fleming et al., 1983). Butyrate is metabolised by the colonic and caecal walls and is the most stimulatory of mucosal growth (Argenzio and Southworth, 1975).

The most extensive development regarding the nutritional role of fibre has been in human nutrition where Western diets, typically low in fibre, have become associated with diverticulitis, colon cancer, heart disease, diabetes and probably other diseases as well (Burkitt, 1973). The fibre hypothesis is an alternative to the high fat, cholesterol hypothesis for heart disease. These alternatives are not mutually exclusive and some information (Morris et al., 1977) indicates that adequate dietary fibre moderates the effects of fat and cholesterol because some kinds of fibres promote faecal loss of these lipids.

Fibre has important upper tract effects and, in man particularly, the gummy and hydrated types delay gastric emptying. This causes soluble substances, such as glucose, vitamins, and some minerals to be absorbed more slowly and efficiently (Roe et al., 1978; Anderson, 1985). In the case of glucose, the spike of absorption after eating is obliterated, requiring less insulin response. This effect, along with propionate production (a glucose precursor) from fibre fermentation in the colon, has permitted dramatic treatment of diabetics by feeding higher fibre diets allowing reduction, or even elimination, of insulin therapy (Anderson, 1985).

These effects may account for the apparent efficiency in pigs and health in man, elicited by moderate increases in dietary fibre. Benign fibre-digesting bacteria may compete against pathogens in the gut. The level of about 140 to 180 g NDF/kg DM in the total diet of pigs appears to be near optimal (Kornegay, 1981), with a relative level in human diets of about 80 to 100 g/kg DM, corresponding to about 40 g/d, roughly doubling the average current consumption in Western diets (Wrick et al, 1983).

Pigs can digest more fibre than man (Ehle et al., 1982a) and the apparent fibre requirement and/or tolerance observed by Kornegay (1981) is higher than that for humans who have less digestive capacity. A broader classification of non-ruminants might be divided into species of limited tolerance but substantial capacity for fibre digestion (*e.g.,* man, pig), intolerant with little or no capacity for fibre digestion (*e.g.,* carnivores, pandas, poultry, rats, mice) and high tolerance with little or no fibre digestion. Equids, horses, and zebras have more digestive capacity.

There does not seem to be any evidence for positive effects of fibre in poultry, which have little or no fibre digestive capacity. It can be suspected that a fibre requirement may be limited to herbivorous species. Nevertheless, one cannot be sure since, for example, dogs benefit from bran and can digest about 0.20 parts of its fibre (Visek and Robertson, 1973) and, while poultry may not seem to benefit, one cannot safely extrapolate to herbivorous birds. Their crops should be examined for rumen-like bacteria.

2. Theory of quantitative analytical procedures

2.1. Principles of assay

Any analytical procedure is a descriptive sequence of defined operations leading to compositional information on the sample offered for assay. Qualitative procedures have the objective of identifying chemical components, while quantitative procedures are designed to assay the content of known or presumed components. Definition of qualitative procedures sets the specificity for the chemical components, avoiding interferences from other components from that being assayed. Definition of quantitative procedures incorporates this aspect and procedural definitions which guarantee reproducibility and agreement in the values of concentration, or amounts, of the assayed component.

The sequential parts of any adequate analysis can be divided into three main parts: sample preparation, steps to eliminate interference or to isolate the desired components from interferences, and final measurement or endpoint (Table 2.1). Sample preparation (Section 3) seeks to convert the sample into a stable (storable) uniform state, so that repeated analyses do not vary because of intrinsic differences in composition. The steps in the second part of a procedure tend to vary inversely to the specificity and uniqueness of the final assay. Very specific endpoints, for example atomic absorption analysis for mineral elements depending on spectral lines, may require little more than preparation of solutions of ashed matter. In contrast, gravimetric methods place the whole business of isolating the assayed component from

interferences at the second stage. Thus, the specificity of most fibre and lignin procedures depends on the adequacy of the preparatory steps.

Table 2.1.
Steps in quantitative analysis

Obtaining the sample		
Sample preparation	Drying Milling	
Preliminary	Subsampling Separation of desired component from interference	Distillation Extraction Precipitation Chromatography, *etc*.
Endpoint	Weighing Titration Sensor → recorded output	

2.2. Analytical variation

Adequate chemical analysis should provide compositional information of sufficient reliability to distinguish true differences among samples. Practically, in biology and nutrition, accuracy should exceed biological significance to the extent that the method contributes minimally to variation among samples. Most statistical analysis of analytical data in biology and nutrition does not consider variation among replicate analyses, the analyst reporting the mean value of the best determinations. It is common practice to discard aberrant values and to choose the repeatable ones. This judgment is often based on the suspicion or knowledge of circumstances (*e.g.*, spillage, difficult filtering) that would account for aberrant values. This leads to the important statistical concept regarding analytical values, which is that unusual analytical values exist and can be discarded. A statistical test has been devised (Youden, 1963) based on the

departure of the odd value from the standard mean and standard deviation of the population of analytical values.

Another concept is that of the true or "right" value for a given sample, which is the average that repeated analyses should approach. However, from a chemical point of view the true value can only be established by use of a standard of known or theoretical composition and, where appropriate, in conformation to the laws of stoichiometry. This leads to the distinction between precision and accuracy. A precise method has good repeatability, but an accurate one produces values in conformity with established standards. For example, the procedure for crude fibre is a precise method (repeatable) but also a wholly inaccurate one, since produced values grossly underestimate the true fibre values of most foods and feeds. Good methods should be both precise and accurate.

Traditionally, it is common practice to calculate analytical error by dividing the difference between duplicates (or the average difference among replicate analyses) by the mean of the analysis. From a statistical point of view this approach is simplistic and ignores the kinds of variation that occur. However, it is a simple way for an analyst to keep in practice and increase skill in obtaining precision.

Statistically, the standard deviation of a group of replicate analyses allows the rational exclusion of deviant values that may lay several standard deviations from the mean. Partition of the variance occurring among analyses on a given day and sequential days can disclose environmental effects, reagent uniformity, and technician errors that are likely to be factors associated with the variation among analyses conducted at different times, and further deal with the variation that can exist among

various laboratories utilising the same procedures. Standardised collaborative studies have the objective of establishing reproducible methods (Youden, 1963).

Often the standard deviation is divided by the sample mean to calculate the coefficient of variation. This procedure assumes that variation is proportional to the content of the analysed substance in the sample. Actually, the variation is usually influenced by other factors unrelated to content, such as sample size and method sensitivity. Smaller samples lead to relatively larger errors and the sensitivity (*i.e.*, limit of the method) cause increase in relative error in samples of low content. The smallest difference or observation which can be distinguished above background error provided by blanks is referred to as the sensitivity of the method.

Systematic errors involved with the minimum difference which can be observed may be relatively constant, and become proportionally larger, when smaller amounts of the component are analysed. Examples of operations which give these include almost any apparatus with a fixed limiting error of observation. In the case of an ordinary burette, the limit is about 0.05 to 0.1 ml, or with weighing of samples, 0.5 mg on a 4 place balance.

2.3. Factors influencing the reproducibility of methods

The sources and distribution of analytical variation in any laboratory procedure varies according to the nature of the material being analysed and the number and kinds of steps involved in the assay. Influence of the sample involves sampling and the content of potential interferences. Usually sampling error is not considered a part of the variation inherent in an analytical procedure and, in collaborative studies, is controlled in

an attempt to gain information on the variation contributed by the analytical procedure. However, it must be emphasised that sampling error can be the major error under practical conditions of feed and food analysis. The sampling problem divides into several aspects which transcend the analytical problems: obtaining the sample, sub-sampling into portions suitable for laboratory preparation, and sampling of the prepared sample for analysis. The problems of sampling are covered in detail in Section 3. The rest of this discussion is devoted to the problems of analytical variation inherent in methods.

Analytical procedures are constructed of sequences often referred to as a sequence of numbered steps which are followed by the analyst much as a cook follows a recipe. Generally, the possibility of errors through sample alteration, contamination or loss is increased by handling so that variation is proportional to the number of steps or operations. Thus, it may be anticipated that long, lengthy, and tedious procedures are less likely to provide the precision of shorter procedures involving few steps. The same principle applies to the number of individual laboratory personnel involved in handling the material during the analysis. The consequence is akin to the so-called Murphy's Law which, given enough opportunities, everything bad will happen, but is fundamentally a consequence of statistical entropy.

Further sources of variation arise from the reliability of the reagents and equipment used in the procedure. These factors provide opportunity for design in methods to utilize reagents and systems, which will provide for greatest stability and reproducibility.

2.4. Manual versus instrumental methods

This commentary is generally applicable to manual procedures as instrumental procedures tend to remove the contribution of the analyst and place the burden of precision and accuracy upon the instrument. Since adequate instrumentation minimises measurement error the relative sources of error fall on sample preparation before it is placed in the instrument. In this respect, calibration by means of adequate standards becomes paramount.

In the case of other methodologies, such as those for fibre, cellulose, lignin or biological methods in general, primary standards are not possible because no stoichiometric reference standards are possible. Standardisation of such methods is highly dependent upon the definition applied to such materials as cellulose or fibre. The same problem applies to protein, even when it is determined by the precise Kjeldahl N procedure. In this case, the assumption is that determined nitrogen is from proteins, and the factor 6.25 is applicable. Such assumptions are relevant to the definition of the determined substance.

In the case of fibre, where the product is in fact defined by the method of preparation, the biological definition of the fibre is as plant cell wall or plant residues resistant to animal digestive enzymes. Such definitions which are based on reasonable biological and physiological principles, while not necessarily compatible with each other, provide grounds for severe criticism of inaccurate methods like crude fibre which can have no claims to represent any definite biological fraction of plants.

Some may wish for pragmatic reasons to establish a standard for fibre or lignin which relates to the maximum correlation with ruminant digestibility. Such a definition is

likely to conflict with chemical or biological criteria as they become more refined. The only practical standard has been to provide stocks of carefully prepared material made available for reference analysis. Collaborative work establishes a mean value that laboratories entering this type of analysis strive to reproduce. Such materials are not true standards, but reference materials. For example, a standard reference alfalfa cannot be reproduced when the stock is exhausted since the environmental conditions of growth that affect composition will not allow it. Thus, a new source will have to be obtained which, despite all attempts at similarity, will provide a new range of analytical values due to the unique conditions of its growth.

2.5. Standards

A common method of checking the accuracy and precision of any analytical method is use of standard samples (Kolthoff and Sandell, 1947). Such samples have known composition and allow testing as to whether correct values are being achieved. This allows, as previously mentioned, the distinguishing of two kinds of analytical error. The first as to whether the method tends to produce a mean value near the theoretically correct one (accuracy), and the second kind is the variation in replication within and among laboratories (precision).

An important difference in the nature of standards depends on whether or not they can be referred to stoichiometric elemental composition. In the case of the conventional Kjeldahl method, as a determination of ammonia or nitrogen, reference can be made to any number of crystalline nitrogen containing substances of known constant composition. Such materials are known as primary standards.

Requirements for suitable standards are that they are stable substances which can be readily obtained in reproducible stoichiometric composition. Stability of composition under laboratory conditions requires that they are not susceptible to humidity, air oxidation, and are otherwise stable to specified conditions of drying (Kolthoff and Sandell, 1947).

Commercial chemicals used as reagents have composition and a standard of purity on their labels. Such labels will state the analytical standard as set up by chemistry associations and societies. Some of the more relevant labels are shown in Table 2.2.

Many reagents that can be obtained, bearing labels of high purity and composition (Table 2.2), are not suitable because of their sensitivity to moisture, gain or loss as the case might be, such that once the bottle is opened composition may depart from that described on the label. As a result, some compounds are preferred as standards for given chemical entities. A list is presented in Table 2.3.

Table 2.2
Analytical standards of purity

Abbreviation	Meaning	Relative purity
AR	Analytical reagent	High grade
CP	Chemically pure	High grade
ACS	American Chemical Society Standard	High grade
USP	US Pharmacopoeia	High grade
NF	National formulary (pharmaceutical)	Middle grade
Tech	Technical grade	Lowest purity
Prac	Practical grade	Suitable for chemical manufacture

Table 2.3
Some primary standards

Analysed component	Standard
Calcium	$CaCO_3$
Iron	$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$
Chlorine	NaCl (dried)
	$AgNO_3$
Sodium	NaCl (dried)
Potassium	KCl (dried)
Magnesium	$MgNH_4PO_4$
Phosphorus	$MgNH_4PO_4$
Oxidation-reduction	$K_2Cr_2O_7$
	$Na_2S_2O_3 \cdot 5H_2O$
	$Fe(NH_4)_2SO_4 \cdot 5H_2O$
Acid-base	$KHC_8H_4O_4$ (potassium biphthalate)
	Na_2CO_3
	Succinic acid (dried)
Chromium	$K_2Cr_2O_7$

2.6. Reagents

An adequate reagent should provide the conditions of reaction such that the desired process or product is maximised and that slight variations in conditions will induce minimal variation in the result. This aspect involves the quality of the reagent ingredients, the net amount used and the time of reaction.

Reagent quality involves stability, the purity of the ingredients, and the freedom from interferences that might be caused by impurities. Unstable reagents degenerate with time and may need to be prepared anew for each batch of samples. Variation among batches of reagents can be a source of error, particularly if concentration and standardisation are not optimum. Volume and quantity of reagent solutions are set by

the limits of solubility, and the practical volumes required to contain optimum amounts. Concentration itself may be a critical variable.

The optimum amount of reagent required for any process is in a range of concentration and/or amount that variation in quantity will minimally affect the result. Too little reagent limits the process, and excess reagent introduces error because of difficulties in handling and obscuring of results. Inadequate quantities of reagent tend to cause the result to be proportional to the amount of reagent, and is a system for assay of the reagent. In a sense the assayed component becomes the reagent and the reagent the unknown. This situation is non-ideal relative to the criteria for an adequate method (Youden, 1963). This last described system is however the basis for enzymatic assays using excess substrate. The enzyme which can be considered a reagent must be inadequate for it to be capable of being assayed.

2.6.1. Effect of concentration of reagents

When fibre is prepared by acid hydrolysis as in the case of ‘normal acid fibre’ or acid detergent fibre, the concentration of acid is important relative to the range which will provide best recovery of the product, namely lignocellulose. A concentration is chosen which offers convenient handling with a reasonable time of reflux to give a practical result. Also, it is chosen such that variation among batches of reagent will cause minimal effect (Fig. 2.1 and 2.2). Fibre preparations of these sorts do not have any definite criteria of standardisation which becomes an arbitrary choice. Longer boiling and stronger acid concentrations tend to reduce the pentosan and Maillard product content of the residue, an objective that may be desirable from a purely chemical point of view, but undesirable from the criteria of the fibre residue containing

the substances most refractory to animal and microbial digestion. Fig. 2.1 and 2.2 show the effect of concentration of acid and detergent on the yield and nitrogen composition of ADF.

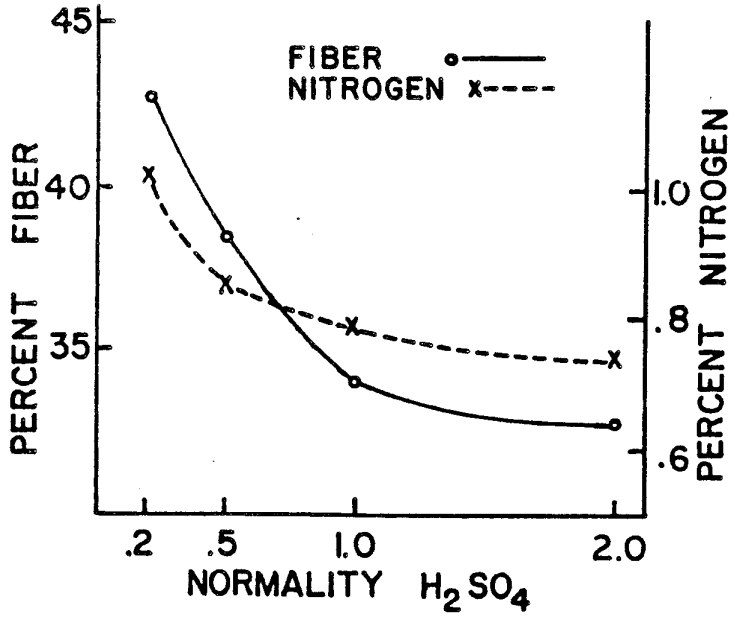


Fig. 2.1. The influence of normality of acid upon the yield and nitrogen content of acid detergent fibre from alfalfa (lucerne) (Van Soest, 1963). The standardisation of normal acid, used in normal acid fibre was subsequently adopted for ADF. Samples are boiled for one hour. Normality refers to Hydrogen ion concentration, and is twice the molarity of H_2SO_4 as defined by older literature.

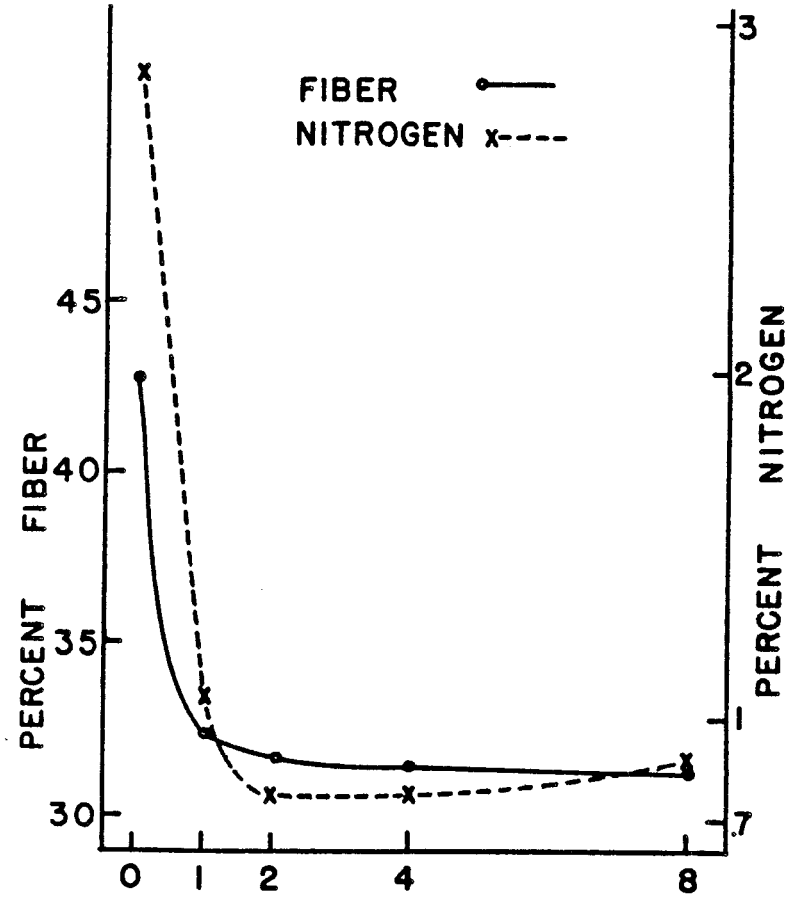


Fig 2.2. The effect of concentration of CTAB upon yield and nitrogen content of the fibre obtained from alfalfa (lucerne) refluxed 1 hour in 0.5 M H_2SO_4. This case offers a similar situation to that in Fig. 2.1 with the additional problem that the detergent complexes protein to prevent its precipitating with the fibre. Therefore, an amount of detergent must be used that is in excess of that required for anticipated high protein samples (Van Soest, 1963).

2.6.2. Time

The influence of time involves the rate of reaction. Most reaction rates tend to be first order under conditions where the reagent is not limiting and the rate is dependent upon substrate concentration. Such conditions cause the rate to be logarithmically related to time, reaching complete conversion at infinite time. As no analyst waits for infinity, a practical time is chosen at which most substrate will have reacted and at which time the variation due to changes of a few minutes will cause little effect. Long times of reaction may be undesirable from the effect of side reactions that alter or destroy the product. An example is the influence of time of boiling upon acid hydrolysis of polysaccharides wherein sufficient time produces a maximum yield, but not complete hydrolysis of the cellulosic residue. More boiling may hydrolyse the remainder, but results in some loss of product through decomposition of hydrolysed sugars.

When ADF is boiled for longer times than one hour, residues recovered decline at a slowing rate. There is a tendency to deplete nitrogenous components, particularly Maillard products and the pentosan content. This is the basis of the modified acid detergent fibre (MADF) of Clancy and Wilson (1966).

2.7. The concept of ruggedness

Methods used for general standardisation and control of food and feed composition must reproduce analytical results whether in the hands of different analysts or in different laboratories. Reproduction of values within specified limits of error are more easily possible with some procedures than with others. This characteristic of ease

of handling and achievement of good results is termed ruggedness (Youden, 1963) and includes a number of factors (Table 2.4).

Table 2.4
Factors affecting the ruggedness of an analytical procedure

Item	Comment
Clarity of description	Unambiguous
Minimum steps	Error proportional to number of steps
Stable reagents	Used at concentration and times that induces minimum variation
Optimum concentrations	Reagent is not limiting
Time of action	Reaction approaches completeness

Examples of some methods commonly used in nutrition that do not meet these criteria are listed in Table 2.5. These are kinds of methods that will tend to vary as they are applied in different laboratories although some degree of uniformity may be obtained within. Often the situation of non-ideality results from the conflicting desire to design reagents that reflect reported physiological conditions, although such concentrations and conditions are not optimal to give the most reproducible results.

Table 2.5
Examples of non-ideal methods lacking ruggedness

Method	Problems	Reagent characteristic
Tilley & Terry *in vitro* digestion	Many steps	Rumen fluid a variable reagent
Protein solubility[a]	Confoundation with NPN	Unstable buffer reagent (CO_2)
Protein degradability	Enzyme reagent is limiting	Protease

[a]Krishnamoorthy et al. (1983).

Inter-laboratory variation should not exceed that occurring among laboratories. Published methods often tend to give the best results in the laboratory where the procedure or technique was developed. This occurs because of the greater familiarity of the personnel with the intricacies of procedure, perhaps not sufficiently described in the

published method. Study of a method among collaborating laboratories is needed to overcome such limitations and the parent laboratory must be willing to concede and modify their method to overcome obscurities.

One example is the collaborative study on the Tilley and Terry method for *in vitro* rumen digestion (Barnes, 1967) which was conducted to obtain uniform adherences to a published procedure which was in itself not without problems (Chapter 7). However, the organising committee could not agree on concessions that would likely simplify and increase the precision of the method.

Another problem is the case where a new method is intended to replace an old familiar one. Most laboratories will be familiar with the old method and unacquainted with the new one, with the consequence that upon initial collaborative comparison analysis of variation has a systematic bias in favour or the older procedure, even though the new method may have many merits and be potentially superior.

2.8. Collaborative studies

The objective of proper collaborative study of a method is to obtain a rectified procedure that gives repeatable and reproducible results in all laboratories. There are three main objectives: to trace problems in a given laboratory, make unfamiliar personnel familiar with the critical details, and to purge the procedure itself of problematic steps and conditions. The collaborative experiment has been standardised by the AOAC (1989, 1995) and its statistical treatment is available in a publication (Youden and Steiner, 1975).

3. The conservation of composition

The objective of the proper collection and handling of samples is to obtain material in a form that represents the composition of the substance sampled, and also is suitable for chemical analysis and general laboratory handling. The preparation must not alter the composition in any way other than that which can be determined in quantitative terms. Moisture, organic matter and energy content are the components most easily altered in handling. Because moisture, in particular, is the most important factor affecting the repeatability of nutritional composition, its determination and the expression of organic composition relative to a defined basis of dry matter and moisture is necessary.

The definition of dry matter is arbitrary in that methods for water analysis are not exact. Similarly, the definition of organic matter, or that part of the dry matter that contains combustible energy, also is arbitrary, since it depends on determination of ash. Both moisture and ash depend on volatile loss measurements conducted at different temperatures. Standardisation has the objective of reducing variation and controlling the values in the range of biologically meaningful results. Thus, convenient procedures are needed even though more complex and accurate ones have been devised. The physicochemical problems associated with volatile losses present the most difficult aspects of the analysis of food and feeds, yet no other problems are so basic to the expression of reliable nutritional values.

3.1. Sampling

The acquisition of the original sample for the purpose of chemical analysis often involves more uncertainty or error than any other process in evaluating feedstuffs. Uncertainty is mentioned because sampling is not often done in replicate (or even duplicate) to ascertain some idea of the error involved. The error of sampling generally increases with the mass being sampled and with the ratio of the sampled mass to the quantity of sample taken.

Large masses (*i.e.,* large tonnages of baled hay, ensiled forages, piles of cereal grain, carloads *etc*.) are apt to be non-uniform because of the agronomic variation in their sources and the fact that they have been deposited non-uniformly in a historical sequence: the oldest at the bottom or deepest in and the most recent at the top or otherwise most accessible.

The adequate sampling of such masses requires sampling of the less accessible parts of the stack or pile, as well as sampling of sufficient regions of the stack or pile, to be able to approach a sample representing the average of that in the stack or pile. In large masses, 10 or more subsamples may need to be combined for this purpose. Some formula should be employed in selecting bales for coring, such as every fifth or tenth.

The manner of obtaining cores should be managed so as to sample the cross section (Fig. 3.1). This allows the sampling of the sequential variation established in the formation of the bale or stack. It is effective in stacks or silos only to the extent that a representative core be obtained, and this is difficult or impossible in deep piles. An alternative to this problem could be sampling as the structure or silo is being filled, and

would include adequate sampling of each load as it was being emptied into the structure. This procedure has no advantage in the case of moist forages and feeds which ferment and can heat; the consequences of which can only be assayed in the stored product.

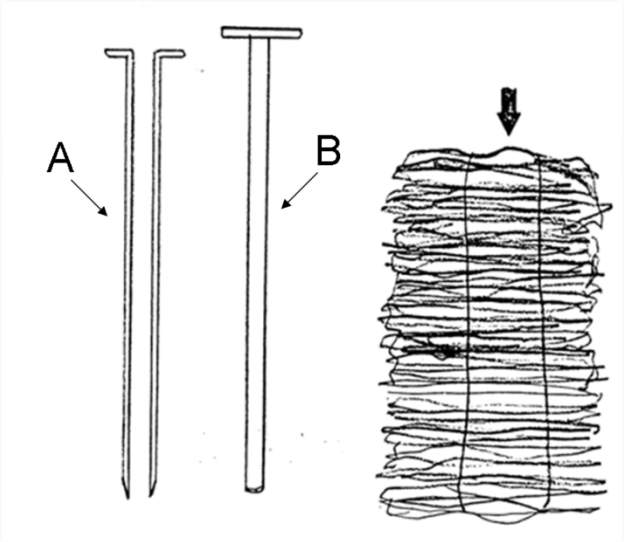


Fig. 3.1. Diagram of a coring device and the proper dimension of sampling. Core is obtained by pushing device A through the bale. Plunger B removes core from A.

Regardless of the amount of effort expended in primary sampling, it should be appreciated that even the best efforts can be inadequate. As an example, the author recalls the chopping and bagging of three hays for experiments at the energy laboratory at Beltsville (USA). Sufficient forage was pre-chopped and bagged to feed cows for a series of digestion experiments. Although attempts were made to randomize bags, subsequent digestibility varied about 10 units for each forage. Subsamples of the three lots of forage collected in individual animal balances showed correlations of 0.83, 0.91 and 0.94 between lignin content and digestibility across the range of the experiments,

(individual animal periods for the three forages respectively) indicating that true randomization of such large tonnages had not been achieved.

3.1.1. Procedure A (for dry feeds)

Use a coring device if available. Core bales or piles in the described manner (Fig. 3.1). Obtain sufficient corings (20 or more) to represent all parts of the stack, combine corings and follow procedures for sample preparation and subsampling. Combined weight of corings should not be less than 2 kg.

3.1.2. Procedure B (for moist feeds)

Collect the sample following protocol in procedure A. If the material is fresh forage, the combined material is placed in a porous paper sack, weighed and dried for 1 to 2 h at 100^{o}C, after which the temperature of the drying oven is reduced to 65^{o}C and the material is dried to constant weight.

If the material is silage or faecal matter, preliminary heating at 100^{o}C is unnecessary. However, drying will cause volatile loss. See special procedures (3.3), if this problem is to be avoided.

3.2. Sample handling and subsampling

The reduction of the primary sample into a form suitable for chemical analysis requires the maintenance of quantitative standards, such that the composition of the subsample represents that originally collected. Grinding and the preparatory steps must not alter the composition of the material.

Alteration of composition can occur in several ways: through disproportionation (selective loss of material of deviant composition); through contamination of extraneous

matter; through chemical or biological reactions involved in storing, drying, grinding, *etc*. The object of preparation and subsampling is to convert the sample into a stable storable form, and in a state of uniformity that small representative samples can be collected for analysis.

3.2.1. Grinding

Bulk samples are not easily resampled to obtain uniform representative material. Therefore, grinding of the entire bulk sample should precede subdivision. Many forages are difficult to grind in one step to the desired fineness for chemical analysis, usually to pass a one mm screen, making sequential steps required. Slightly moist materials can be difficult to grind, causing caking, jamming and heating of the mill and the sample. Consequently, samples must be dry and preliminary drying should be conducted at 65^{o}C even for apparently dry feeds, since ambient humidity can cause problems. Wet samples and feeds containing a high content of oil or fat will require special treatment (3.3).

Sufficiently dry feeds can be easily ground through a 2 to 4 mm screen, which is a sufficient degree of fineness for adequate subsampling. The subsample once collected can be ground to pass a 1 mm screen.

3.2.2. Moisture equilibration

Oven drying and even grinding cause loss of absorbed moisture that is contained in the material in equilibrium with ambient humidity. The effect is to notice a rise in net weight of dried or ground material when it is exposed to open air. The uptake of water can be to some degree prevented by using tight, moisture-proof containers.

The usual procedure is to allow the material to equilibrate with air and to determine dry matter on that equilibrated sample, which is henceforth kept in a tightly closed container to maintain the determined moisture content. The total sample may be weighed after drying and grinding and later after equilibration to provide correction factors. An important point to remember is that the laboratory determined dry matter may not be the same as that existent in the feed as fed and a direct value on the original material in the barn or field may be required.

Preparatory and drying procedures cannot be standardised to one method that would be satisfactory for all conditions; the experimenter must choose intelligently those that suit his/her purpose. A variety of preparatory and drying methods are presented. Incompatibilities are generally noted.

3.2.3. Procedure for dry feeds

Collect the entire sample obtained (3.1) and dry if necessary at a temperature not higher than 65^{o}C. If a dry matter (3.7.1) is desired on this material, a subsample should be obtained for this purpose. Any material dried at 100^{o}C is unsuitable for chemical analysis and should be discarded. The entire dried material is ground through a 2 to 3 mm screen. Collect the ground material in a large plastic bag fitted to the bottom of the mill. Combine any material remaining in the mill with the ground sample. Be sure to carefully clean the mill before grinding the next sample. Contents of the closed plastic bag can be mixed by rolling it around.

Spread the material on a clean smooth surface, preferably metal, and divide the pile into quarters. Sample equal portions from the quartered piles taking care to obtain

material from all portions of the respective piles. A total of at least 500 g should be collected.

Grind the subsample through a 1 mm screen, again taking care to collect the sample in as quantitative a manner as possible. Avoid as much as possible the loss of dust by keeping the top of the mill closed. Combine any remaining material in the mill with the ground sample.

Allow the ground material to equilibrate for one day by remaining open to the air. This may be done by storing in a shallow pan.

Subsample the material in the pan using the previously described quartering process. Place the collected material in a wide mouth sample bottle that can be closed tightly. Plastic bottles and polyethylene bags, provided they are closed well are satisfactory. Do not fill bottles more than two-thirds full, because this will make mixing of the material in the bottle difficult.

3.3. Handling wet samples

Problems with wet samples arise from their unstable condition and the desire to assay components that would be lost, destroyed or altered by ordinary drying procedures. Substances of interest include volatile acids, ethanol, ammonia, volatile essential oils including terpenes, and non-volatile substances that can be altered by drying at temperatures above 65^{o}C. Oven drying can change the analysis of other unmentioned components, since volatile loss of energy-containing compounds will reduce the net combustible energy content, and also underestimate dry matter. Loss of ammonia is apt to occur in the drying of silages and faeces with resulting

underestimation of Kjeldahl nitrogen in the respective samples. Tannins tend to undergo oxidative polymerisation with heat, air and moisture which decreases their solubility, resulting in an apparent reduction in extractable tannins and the appearance of anomalously high lignin, ADF and NDF contents. Crude protein is apt to accompany the tannin with consequent high nitrogen contents of the refractory residues.

Not every laboratory will have the facilities or the convenience of handling wet samples by sophisticated procedures and alteration of material in preparation may have to be tolerated. However, the order of decreasing preference is milling and handling of the frozen material, freeze drying followed by milling of the dried material or drying at 65^{o}C as described in Section 3.2.1.

Freeze drying has been proposed in handling silages. However, ethanol, some volatile acids amines and ammonia are likely to be lost under these conditions.

3.3.1. Procedure for wet materials

3.3.1.1. Grinding

Some kinds of materials such as faeces, may be in a sufficiently fine state to not require grinding. However, species such as sheep that void dry pelleted faeces will require grinding to disrupt the pellets. Alternative techniques are grinding frozen material in a mill with liquid nitrogen or dry ice (CO_2), or grinding with a meat grinder or salad chopper.

3.3.1.2. Grinding wet (frozen) materials with a Wiley (cutting) mill

This procedure is equivalent to grinding in a meat grinder, but it is not amenable to handling of large samples. Grind frozen material through an intermediate Wiley mill with a 2-mm (10-mesh) screen. Add charges of dry ice to keep sample frozen. Collect the

ground material in a plastic bag closed around the bottom of the mill. After grinding, refrigerate but allow the bag to be vented to allow escape of remaining CO_2. Do not overly expose the contents to outside air as this can cause moisture condensation and alter the composition.

3.3.1.3. Cattle faeces

Mix by hand or with a mechanical mixer daily collection thoroughly on a clean surface, quarter, and subsample 1 kg or 100 g/kg of wet weight. Store in a plastic container in a freezer. Daily samples may be thawed, composited, mixed, and subsampled at a later date.

3.3.1.4. Sheep faeces

Collect cumulative faeces from at least 5 days collection, and pass through a meat grinder with a 6 mm plate. Clean grinder, add all contents to the ground mass, mix thoroughly, and subsample 1 kg. Store frozen in a plastic container.

3.3.2. Silages and fresh forages

Pass not less than 2 kg frozen silage through a meat grinder with 6 mm plate. Tie a large plastic bag over the end of the grinder to collect ground material. Clean grinder and add all contents to the ground mass, and mix thoroughly. A salad chopper can be used in place of a meat grinder. Store bag and contents in a freezer.

Alternatively, chop the frozen silage in a salad chopper device of the type used in large cafeterias. Another alternative is to hand chop cold silage with a paper cutter. Attempt to keep length of fibre on the order of 1 cm or less. If this procedure is used, take care to adjust for dry matter increase due to moisture evaporation during this somewhat lengthy operation. This procedure can also be applied to fresh-cut forage.

3.3.3. Freeze drying (lyophilisation)

Wet samples can be freeze dried and ground through a Wiley mill. Heat damage is avoided, but loss of nitrogen in the form of ammonia occurs in faeces and silages. VFA are lost in freeze drying. See 3.7.3 for a comparison with other dry matter methods.

3.3.4. Drying with acetone

This method is suitable for lignin and other components of fibre and cell wall. Weigh 100 g of wet ingesta into a 500-ml wide mouth Erlenmeyer flask and add 400 ml of reagent grade acetone. Shake thoroughly and allow to stand with occasional shaking for 1 h. Acetone extracted material can be prepared with a Waring blender. Shake and pour mixed contents into a 10 cm fritted glass, coarse porosity, Buchner funnel previously tared to 0.1 g. Allow to settle before applying suction. Suck off excess acetone with vacuum. Remove vacuum and add 400 ml fresh acetone, while stirring to wash any remaining fibre from Erlenmeyer flask. One washing is sufficient. The preparations need not be washed free of pigment and the preparation is sucked dry on the filter. Allow to air dry for 24 h at room temperature. If humidity is high, funnels containing fibre may be dried at 40^{o}C for 4 h in a forced draft oven. Weigh funnel plus contents and calculate yield of acetone dried powder. Dry matter determinations should be made on original wet ingesta. Dried acetone powders are ground in an intermediate Wiley mill. Store in a tightly stoppered container. To convert acetone dry sample weight to the equivalent oven dry sample weight, multiply by the following correction factor:

Oven dry sample factor = dry matter of wet ingesta / acetone powder yield

3.3.5. Oven drying

Weigh 500 g sample into an 18 x 30 cm tared pan and dry at 65^{o}C in a forced draft oven. Remove pan and allow to equilibrate with air at room temperature for 24 h. Weigh and calculate yield. Grind dried material through 1 mm (about 20 mesh) screen in a Wiley mill. Grinding through finer screens (30 to 40 mesh) may tend to induce filtering problems. Caution - in faeces and silages, loss of nitrogen as ammonia results from oven drying. In silages there is also a serious loss of volatile organic acids and energy value. Damage to lignin, protein and carbohydrates can occur in all oven dried materials such that true values of individual components may not be obtained.

3.3.6. Sub-sampling of wet samples

Thaw and empty contents of forage subsamples from the gross sampling onto a clean surface and cover with a large sheet of plastic. Mix material by hand under the plastic. Quarter and subsample forage and fill a 250 ml wide mouth plastic polyethylene bottle not more than two-thirds full. For faecal material, mix in a plastic beaker with a food mixer (electric eggbeater) and subsample material to fill bottle two-thirds full. To weigh samples for analyses, close bottle with a plastic cap (Fig. 3.2) that has been punctured with a spatula allowing the spatula to remain in place primarily within the bottle. Weigh closed container to 1 mg and remove an amount of material with spatula equivalent to 0.5 to 2 g of dry matter to a requisite sample container. Replace cap and spatula assembly and re-weigh bottle. Take difference between first and second weights as weight of sample taken. Weigh samples for dry matter determination in conjunction with matter weighed for other determinations. Mix sample contents of bottle between weighing by stirring with spatula.

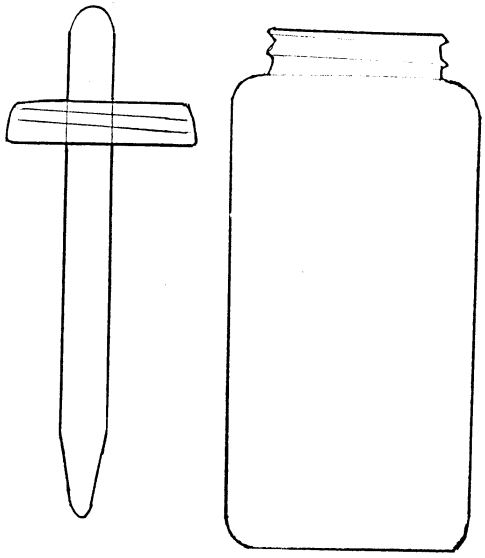


Fig. 3.2. Arrangement of cap assembly and 250 ml plastic bottle.

Total weight of bottle and contents should not exceed 160 to 200 g, the capacity of most analytical balances. Also, it is important that the height of the closed assembly fit within the housing of the balance pan. The advantage of this arrangement is that wet matter is minimally exposed to air and moisture loss and particles sticking to the spatula are returned to the bottle. The technique is devised so that the loss of weight in wet sampled material and material adhering to the spatula does not affect determination of net sample weight. Weighing the cap spatula assembly as well as the sample bottle and contents before and after removing the sample eliminates this source of error. The cap spatula assembly is removed after weighing is completed and replaced by an ordinary cap. Bottles are refrozen; they may be thawed, mixed and subsampled for further analyses at a later time.

3.4. Digestion experiments

Balance experiments involve somewhat special conditions. Stall devices and the means of separating urine and faeces are well described in the literature. The objective in

this discussion is to describe the conditions of quantitative recovery and maintenance of the compositional integrity. Digestion experiments can be conducted under rather simple conditions such as a pen with a concrete floor, provided quantitative measures are employed and great care and labour are not limiting to the sorting of faeces, spilled feed *etc*. The accuracy of a balance increases with the duration of the collection. Since refused feed and faeces must be collected over days, moist material must be preserved, preferably by freezing, in order to avoid loss of ammonia if faeces are dried. Collections from small animals (< 80 to 100 kg) may be composited whole in large bags or cans, while in the case of large animals voiding excessive amounts of faeces, collections will need to be mixed and an aliquot (*ca*. 100 g/kg) collected and composited.

An important feature of balance experiments conducted on preserved feed is to prepare the meals in bags ahead of the experiment. The bags are randomised for reduction in sampling error in preparing the bags. Refusals and faeces may be treated as single composites representing the total balance for each animal.

3.4.1. Reproducibility of feeding experiments

Collaborative studies and consequent recommendations for the management of feeding in digestion experiments have been done (Swift and Bratzler, 1959; Donefer, 1966; Barnes, 1968). Factors promoting the reproducibility of digestion experiments include the foregoing comments, but also the regulation of feed intake, which affects feed selection and passage rate. Generally, selective feeding is the overriding factor, so that an offering of feed or forage above the level that will be completely consumed (conventionally 100 to 150 g/kg refusal is allowed in intake experiments) to limit orts. Animals are able to

reject less digestible parts. If correction for this kind of selection is not made through careful chemical analyses of orts (refused feed) an overestimation of digestion results.

For these reasons, the conventional experiment is conducted at a level of offered feed at which no orts results. Such levels are near maintenance or below in the case of poor quality feeds. Conventionally, this level is 900 g/kg of the actual feed intake sustained by the animals on the same feed (Donefer, 1966). Such are the conditions under which the bulk of the temperate world's digestion coefficients have been obtained. Higher intakes of the same feed without selection reduces digestibility through loss via increased passage (Van Soest, 1994).

Tropical forages are generally not amenable to the above management, since the differential quality between stems and leaves is large and selection is easier. Practical levels of feeding tropical forages to small ruminants ordinarily involves up to 600 g/kg refusals (Olubajo et al., 1974; Zemmelink and 't Mannetje, 2002).

Conditions of feeding in digestion experiments have been compared collaboratively (Swift and Bratzler, 1959; Donefer, 1966; and Barnes, 1968). Results show that while variation within experimentation may be 1 to 2 units of digestibility, variation among sites is considerably larger, which must reflect, in part, the difficulty of sampling and obtaining large amounts of equivalent feed at the respective collaborative sites. It also indicates variable procedures among the sites and their ability to follow directions.

3.4.2. Feed sample from a digestion experiment

Chop hay to approximately 3.5 cm through a 2.5 cm screen. Weigh and bag hay for individual feedings before the digestion experiment but after the intake level has been established. Composite samplings from the bags to comprise not less than 5 kg. Dry

sampled forage if necessary at less than 65°C. Grind in a large Wiley mill through a 2 mm (10 mesh) screen. Return all contents of mill to the sample. Collect in a large plastic bag and mix, by rolling partially filled bag on floor. Subsample from all parts of the bag to an amount of at least 2 kg and grind through a 1 mm (about 20 mesh) screen. Allow ground material to equilibrate with air overnight before placing in closed containers.

3.4.3. Handling of faeces and orts

Handle composited moist materials as described in Section 3.3.6. Follow the precautions for dry matter determinations described in Section 3.5.

3.5. Volatile losses and the problem of moisture

Water and incinerable organic matter are commonly assayed as volatiles by applying appropriate heat, non-volatile matter remaining after incineration being denoted as ash. The separation of water from organic matter depends on choice of a temperature that removes only volatile water and does not degrade organic matter.

While water is one of the simplest of substances, it is nevertheless one of the most difficult to determine. No method of water determination is entirely free from errors and artefacts. This situation exists because of the great affinity of water for organic materials and the fact that water is one of the easiest degradation products to produce from organic substances. There is also the problem of organic volatiles having vapour pressures in the range of that of water. Such volatiles are characteristic of silages and other fermented products (3.5.2).

The standard time for drying is defined as the drying interval that results in constant weight. That is, no further change in weight upon further drying at that temperature (Fig.

3.3). This concept assumes that water is in equilibrium with the absorbing substance and leads to the expectation that apparent loss will be related to temperature, higher estimates of moisture appearing at higher temperatures that provide more thermal energy for desorption. When cooled, the substance will regain moisture at the equilibrium set by the new temperature. Humidity may well have some effect on the amount of equilibrated moisture. Ideal substances, such as Pyrex® glass, that have no other interaction with water than absorption will obey these rules. Most biological substances will not.

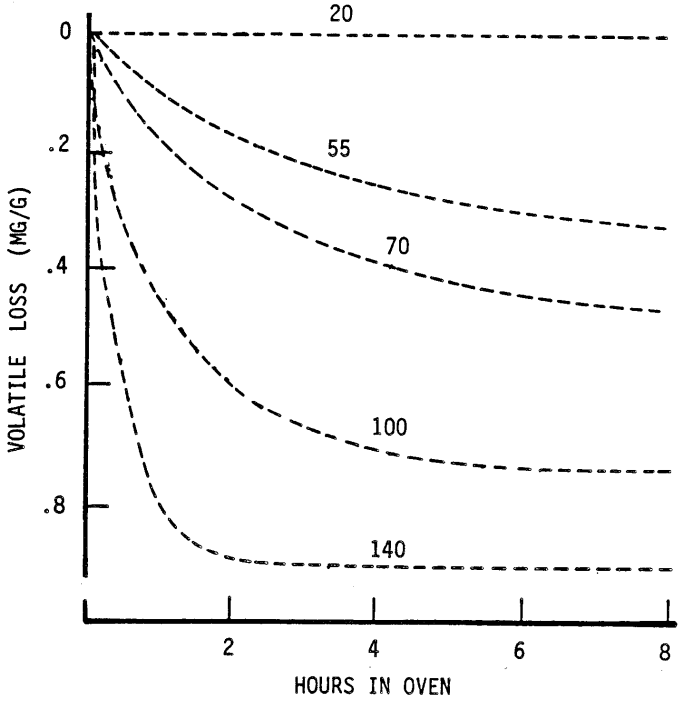


Fig. 3.3. Moisture desorption curves for an ideal substance (Pyrex®) (based on Willits, 1951). Time required to reach constant weight decreases with increased temperature, but with a greater volatile loss. Substances that lose hydrated or chemical water may not stabilise, particularly if the temperature range is in the steep phase of their decomposition (Fig. 3.4).

In sum, there is no drying protocol that will satisfy all types of samples. For example, Maillard reactions begin at 60°C and generate chemical water. Organic volatiles may be lost even in freeze-drying. If preservation of sugars in fresh cut forages is needed brief microwave treatment followed by drying at 55°C may largely conserve composition (Pelletier et al., 2010). Tanniniferous plants offer different challenges and freeze-drying might be preferred (Pagan et al., 2009).

Generally, substances that contain hydrates or lose chemical water irreversibly may not come to a constant weight in any reasonable time because the supplied thermal energy is too small to drive the reaction to completion. Raising the temperature may accomplish this. Such materials, in most cases, will not regain equal moisture upon cooling because the dehydrated product behaves as a new chemical individual.

Water usually exists in several forms in feedstuffs: free water in moist feeds, adsorbed water and crystalline water held as hydrates. Dry feeds contain little or no free water and most is apparently adsorbed. Crystalline hydrates are familiar in the form of many salts, carbohydrates, lactose hydrate, *etc*. These are compounds in which, although no bond exists between water and the substance, there is a crystalline space or a polar attraction between one of the ions and water to form a stable crystalline substance. Pure hydrates have the unique character of having discrete decomposition energies leading in each case to a particular temperature of decomposition with release of water. The more stable the molecular complex the higher the temperature of decomposition. The more stable hydrates decompose well above the boiling point of

water, and above the temperature at which water can also be formed from thermal decomposition of carbohydrates and other fragile organic substances. This is defined as water of chemical origin, as opposed to water from crystalline hydrates or clathrates which are molecular complexes.

Water of chemical origin can be formed from a variety of reactions, the most important including dehydration of alcohols (particularly beta hydroxy aldehydes and acids) to form alkenes, condensation of acids and alcohols to form esters, Maillard reactions [which involves the loss of water from sugars, and the dehydration of silicic acids to form anhydrides (ultimately SiO_2)]. Certain reactions can occur spontaneously at rather low temperatures below the boiling point of water as for example the dehydration of beta hydroxy acids and aldehydes, or the Maillard reaction under optimal conditions. Esterification reactions likely occur in the extraction of water by dry alcohol, such as is used in titrimetric measurements of water. The situation is such that undesired chemical water is formed at temperatures below that which adsorbed water is entirely removed.

Chemical reactions producing water tend to exhibit threshold temperatures like that of the hydrates. Carbohydrates, on the other hand, because of their multihydroxyl structure, do not show sharp decomposition temperatures. Amino acids and the presence of water reduce the temperature at which carbohydrates will degrade. Consequently, the weight loss exhibited by feeds and feed ingredients are comparatively smooth curves (Fig. 3.4). The lack of inflection indicates the purely arbitrary nature of the $100^{o}C$ used for standardised moisture determinations.

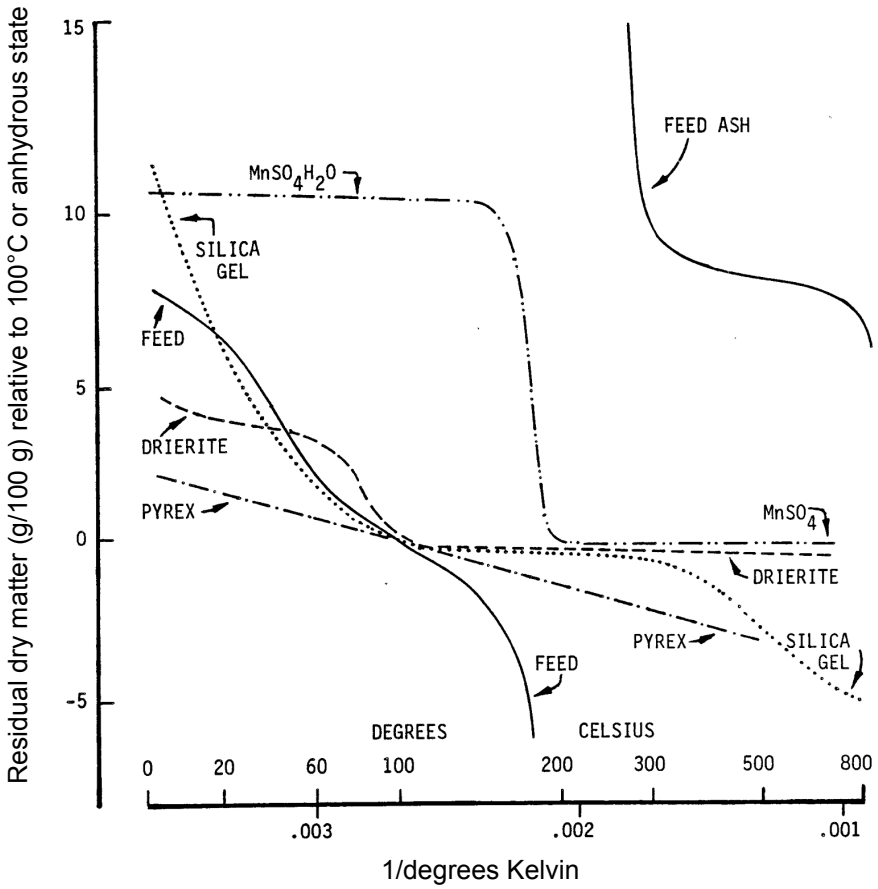


Fig. 3.4. The relative volatile loss of various substances when heated at different temperatures expressed as residual dry matter (Y axis) against the reciprocal of the absolute temperature (X axis). Each material at each temperature has been heated until equilibrium is reached. The line for Pyrex® is presented as the effect of the weight change of a 30 g beaker or crucible. Other materials are shown as weight loss of their own mass.

Pyrex® behaves as an ideal substance showing a constant desorption with the reciprocal of the absolute temperature (degrees Kelvin). Theoretically, the energy required for dissociation of any complex (compound) is balanced by the available thermal energy. Therefore, the degree of dissociation is inversely proportional to the absolute temperature; the slope of the relationship being a function of the stability, *i.e.*,

the greater the energy required for dissociation (activation) the steeper the slope. Each molecular combination is unique: an example is $MnSO_4 \cdot H_2O$ which is stable at 100°C and shows little desorption up to 140°C but above this temperature the curve is very steep as the salt decomposes into the anhydrous salt which is stable at temperatures above 200°C. Cooling $MnSO_4$ will not cause resorbtion since the salt is metastable at lower temperatures and can coexist with the hydrated form. Other substances shown are more complex. Silica gel and drierite ($CaSO_4$) desorb relatively steeply up to 100°C and show comparatively little change above 100°C. Silica loses more water above 300°C. In contrast to $MnSO_4$, silica gel and drierite dried at 100 to 120°C will readily resorb moisture from air when cooled. These materials are commonly used as desiccants. Another material is calcium chloride (not shown in Fig. 3.4), which will absorb so much water at lower temperatures that it will dissolve itself in it. Feeds show a complex curve and reveal no clear point of inflection that indicates a fixed relationship with adsorbed moisture. Maillard reactions that involve loss of chemical water and CO_2 can occur at temperatures well below 100°C. Charring becomes dominant above 150°C.

3.5.1. Losses at higher temperatures-ashing

Organic matter chars rapidly at 200°C and higher but does not completely oxidise to produce a clean ash until temperatures above 400°C are reached (Fig. 3.4). Even at this temperature the process is slow and inefficient. Total loss of organic material will occur at temperatures above 450°C. Old ashing procedures often use 600°C as the standard temperature, and require porcelain or other suitable dishes. However, Pyrex® glass melts above 550°C and practical work with sintered glass crucibles cannot exceed this temperature. For this reason ashing procedures with these crucibles are

standardised at 500^{o}C. Temperature for ashing is purely relative and has no theoretical value. It must be high enough so as to destroy all organic matter. Although the destruction of organic matter does not mean the removal of all carbon since carbon will persist in the form of alkali carbonates and possibly silicon carbides.

The evidence for organic matter at ashing temperatures is the presence of black carbon. A false positive is the presence of ferrous iron which in the form of oxide is also black. This substance will remain no matter how long the ashing is prolonged. Silicon carbides can be grey to black and also may persist.

The apparent ash value obtained at any temperature above 400^{o}C is not likely to come to a constant weight, because of complex slow-acting reactions. Chlorides of potassium and sodium are slowly volatile. These problems are discussed further in this section. It is not really possible to eliminate these effects, although they are related to time and temperature. Carbonates are the remains of organic matter while KCl is true mineral. The unfortunate situation is that desirable and undesirable reactions overlap in the temperature range used for ashing. The loss of volatile chlorides (and some other elements as well *e.g.*, Hg, S, I, Zn, K *etc*.) render ashing as an unsuitable means of preparing samples for mineral analysis of most volatile minerals.

Reactions involving silica and decomposition of hydrated silicates above 400^{o}C are also significant. There are several kinds of reactions which are potentially complex. These consist of the decomposition of silicic acids (silica gel), the formation of silicon carbides, and the reaction of silica as an acid with bases to displace CO_2 and form silicates that may potentially melt or sinter to form glass.

Hydrated silica and silicate minerals occur ubiquitously in feeds either as contaminants or deposited cell wall components. The loss of water from silicic acids proceeds with the dehydration of silanol groups to form siloxane bridges. These reactions occur in two ranges: at room temperature (as in the case of saturated silicic acid) and at high temperatures above 300^{o}C. Because of the wide range of temperatures over which weight loss occurs, the analysis of mineral matter is also confounded with that of water and organic matter. Ordinarily this error is small but can be appreciable in high silica materials *such as* rice bran, rice hulls, straws and other siliceous forages. It is also a potential error when siliceous filter aids (glass wool, celite, asbestos *etc*.) are used in procedures that involve drying and incineration. These materials lose water as shown in Fig. 3.4 at high temperatures and thus affect the estimate of organic matter.

When siliceous forages are ashed, the opaline biogenic silica is reactive toward the decomposing organic carbon compounds and organosilicon compounds result (Dymicky and Stedman, 1967). These substances contain silicone (Si-C) linkages which are stable. The result is a grey or dark coloured ash containing carbon that will not disappear upon prolonged ashing. Little can be done about this error within the limits of the ashing procedure. Carbides can be destroyed by ashing with nitrates or by evaporation with HF and reashing. However, these applications used in silicon determinations will not preserve the true ash value since nitrites are formed in one case and SiF_4 is volatised with total SiO_2 loss in the other.

The interaction of silica with carbonates occurs even though silicic acid (pk_1 = 9, pk_2 = 12) is a much weaker acid than is carbonic (pK_1 = 6.4, pk_2 = 10). The reaction is

driven by the fact that CO_2 is volatile and is removed while driving the equilibrium which allows for an intermediate pKa of 6.3 which approximates the acidity constant of CO_2. Many carbonates are unstable to temperature as well. All bicarbonates decompose into normal carbonates at relatively low temperatures. The only carbonates stable to ashing temperatures are those of the alkali and alkaline earth metals including sodium, potassium, and calcium, and these are unstable toward silica, decomposing in the following reaction:

$$K_2CO_3 + SiO_2 \text{ —› } K_2SiO_3 + CO_2$$

The carbonates of Na, K and Ca are stable at ashing temperatures if silica is absent. Most other carbonates will spontaneously decompose below ashing temperatures. However, any of the basic oxides can react with silica to form silicates. This reaction is accelerated if the ash mixture melts, in which it forms a glass (this reaction is used in making glass) which is a solution of the silicate mixtures in each other.

The melting of the mineral ash has serious consequences if the ash is to be used for analysis of mineral elements. While it may be inconsequential for the measurement of the gross ash value, the formation of a glass renders most of the mineral insoluble in water and acids and, therefore, can lead to low values for Na, K, Ca, PO_4, *etc*. where extraction of solution is a step in the analytical preparation. On the other hand, empirical methods such as acid insoluble ash (AIA) can also be seriously affected since the alkaline and normally soluble components will not extract leading to anomalously high values of insoluble ash. The problem with glass formation is that it represents the solution of one silicate mineral into another. This kind of reaction is capricious in relation

to temperature since melting points are relative to the eutectics formed between the respective dissolving compounds. Thus variation in K, Ca or SiO_2 content can widely vary the potential chance of melting. Generally, this problem can be avoided if lower temperatures are used (400 to 500^{o}C) although the conversion of some carbonate to silicate cannot be prevented.

3.5.2. Silages and volatile products

Most feeds and foods that have undergone fermentation contain volatile products from the anaerobic metabolism of microorganisms. These substances may contain considerable metabolisable energy (Table 3.1). Ammonia is the principle inorganic product that contains no metabolisable energy, but most of the other organic products contain more energy per gram than the carbohydrate from which they were derived. This increase in energy content arises from the loss of carbon dioxide and water in microbial metabolism. Since water and CO_2 have no combustible energy, the energy from the original carbohydrate is retained in the decarboxylated products minus a small loss in heat (provided it is anaerobic). However, the total energy of the fermentive system is decreased by the amount of heat and organic matter losses through effluent or volatilisation during fermentation. The heat loss is small in a purely anaerobic system (about 0.05) but becomes much larger if any aerobic respiration occurs.

Table 3.1.
Volatile substances and their energy values[1] compared with carbohydrates.

Substance	Boiling point (at 760 mm Hg)	Combustible energy
	°C	cal/g
Acetic acid	118	3490
Propionic acid	141	4962
Butyric acid	162	5958
Ethanol	80	7122
Lactic acid	dec[2]	3622
Cellulose (starch)		4179
Glucose	-	3739

[1]A miscellany of minor volatiles has been identified that individually are inconsequent but could collectively represent a significant portion of the loss (Morgan and Pereira, 1962); [2]can be distilled in a vacuum but decomposes at high temperatures to other volatile products.

Generally, the quantity of volatile products and the loss of heat (and feed value) are proportional to the extent of fermentation, and is also associated with the loss of sugar, protein and other potentially degradable components of the initial material (McDonald, 1981). The problems of silage fermentation and quality are further discussed in Chapter 14 of the Nutritional Ecology of the Ruminant (Van Soest, 1994).

Most silages with reasonable moisture contain about 100 g/kg volatiles (Coppock and Van Soest, 1959; McDonald, 1981), but poor quality ones substantially more, while haylages may contain less, particularly, since more of the fermented product is in lactic acid due to a more restricted fermentation and is less volatile than other products. Another point is that the ammonia and amine salts of VFA, particularly butyrate, are very volatile substances, so that pH has little effect on recovery of VFA or volatile nitrogen compounds.

The problem here is to comprehend the effects of volatile loss on the accuracy of silage analyses. If the volatiles contain more energy per gram than the unfermented feed components, a large volatile loss will cause the energy value of the non-volatile

residue to appear lower than the real value of the silage. Probably this mechanism biased early evaluations of silage with proximate analysis to make it appear that energy in silages was utilised more efficiently than that in hays. This is because the energy in the metabolisable volatiles had not been accounted for, although attempts were made to explain on the basis of acetate propionate ratios (Ekern and Reid, 1963).

Various systems have been suggested for correction or improved analyses of silages. One way is to base analysis on wet material, or to apply systems of correction which on the whole are less satisfactory. Drying at lower temperatures has no real benefit since many of the volatiles are quite volatile at low temperatures, although the losses are probably somewhat reduced. Actually, a higher dry matter is obtained because the lower temperature allows a greater residue water adsorption that may mask the volatile loss if a compensating error is allowed to counter balance the losses.

Systems for correction of dried material include collection of the volatiles in water from toluene distillation, or in a freeze trap attached to the drying system, or a systematic correction based on acetic acid. Measurement of acids ignores non-acid components, particularly ethanol, and thus underestimates the magnitude of the loss. The best work identifies ethanol, lactic acid, VFA, volatile nitrogen including ammonia and amines, and the best way for dry matter calculation is to measure water directly (3.7.2). Energy content of wet materials can be determined by bomb calorimetry using primers (Coppock and Van Soest, 1959; Fenner and Archibald, 1959). All of these methods require the use of wet sampling techniques (3.1.2 and 3.3).

Faeces also represent in a sense a fermented product and also contain volatiles. Unlike silage, faeces contain little organic energy-containing compounds so that volatile

losses for energy and net organic matter are comparably negligible (Fenner and Archibald, 1959; Flatt, 1957). However, ammonia is a prominent component and careful handling of wet samples are required for nitrogen balances. This condition is dependent on careful collection and prevention of fermentation in voided faeces. Large energy losses from faeces have been observed (Colovos et al., 1957).

3.6. Weighing

The precision of gravimetric analyses is dependent upon the ability to produce repeatable weights, accurate to at least a factor of 0.0005 to 0.001 of the respective components being determined. The measurement of a sample or dry matter, ash, *etc*. from that sample is similarly affected. Since weights of Pyrex® containers and of most materials are affected by temperature through adsorbed or desorbed water, standardisation of the influence of adsorbed moisture is imperative. Traditionally this has been accomplished by the use of desiccators and desiccants. Because of the particular capacity for cellulosic matter and Pyrex® containers to compete with desiccants for moisture, a hot weighing procedure has been developed. This method is presented as first choice, while the alternative choice of desiccators is offered optionally.

3.6.1. Hot weighing

In fibre analysis, the residues from the various extractions can be very hygroscopic. The glass of the crucibles can absorb up to 25 mg of water when cooled to room temperature. Even at 100^{o}C, all absorbed water in materials is not removed. Since most residues or extracts to be gravimetrically assayed are usually 500 mg or less,

absorbed water can result in substantial error. An alternative to the use of desiccators is to weigh all crucibles and residues directly from the drying oven. It is a more rapid weighing procedure and subject to less error.

3.6.1.1.Apparatus

3.6.1.1.1. Balance

A single-pan automatic macro-analytical balance with direct electronic readout to 0.1 mg; alternatively a balance with a full gram swing can be used and preferably have a digital rather than vernier readout for the last two digits. A counterweight (negative weight) can be balanced with an optical tare of at least 10 mg.

3.6.1.1.2. Oven

A forced draught oven with adequate heating capacity to maintain the temperature despite repeated opening should be used. A safety-type oven is best since there may be traces of flammable solvent on drying samples. Organic solvent explosions can be very unpleasant. The door should have only one handle such that it can be opened and closed by single-handed action. If the oven has two handles, remove one. The oven and balance should be set up conveniently to the left and right of the operator respectively (Fig. 3.5) so that the technician can open the oven, reach in for a crucible and weigh it with a minimum of movement.

Since the crucibles are weighed straight from a 100^{o}C oven, heat will be transferred from the crucible to the balance mechanism. This will cause distortion which, in the majority of balances, will result in a weight reading less than the true weight. Thus, with the mechanical balance, a positive tare is put on the balance, usually about 8 to 10 mg, with the optical tare mechanism. A Teflon plate made to fit over the balance

pan may be used. It will insulate and reduce the amount of heat transferred to the balance mechanism. This is especially useful in the hot weighing of filter paper. The initial hot weighings produce the most distortion in the balance while subsequent weighings tend to result in an equilibrium being reached between the heat input of weighing and the cooling which occurs between weighings. Thus, to heat up the balance, at least 2 weighing sequences should be completed, using blank crucibles, before the analytical weighing commences. For best results, the crucibles should be weighed in a set sequence. In cases where there are significant fluctuations in the laboratory temperature and humidity, standardising crucibles of known weight should be included in the weighing sequence to improve the accuracy.

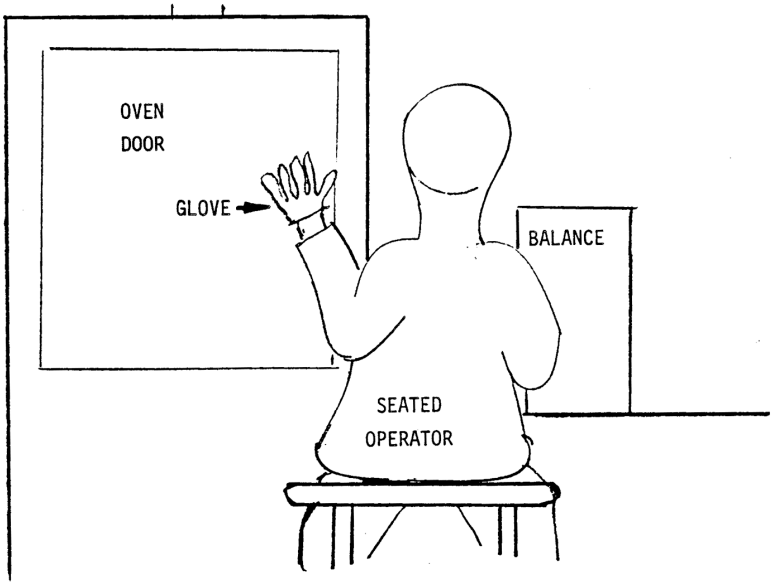


Fig 3.5. Arrangement of balance and oven showing how the operator has oven and balance within reach as they sit. Operator uses a glove on the left hand to manoeuvre hot crucibles and operates the balance with the right.

On a single pan automatic balance, the distortion is realised by a significant drop in the readout weight after several seconds on the scale. Typically, after placement of the crucible on the pan the reading will appear to stabilise, then drop an additional 2 to 3 mg and remain at this low point for several seconds (Fig. 3.6). This drop typically occurs within 10 sec after the crucible is placed on the scale. The weight will then appear to climb by 0.1 to 0.2 mg at 1 second intervals. This distortion is more noticeable in the first 2 to 3 measurements on a cold scale. Thus, at least 4 weighing sequences should be completed using blank crucibles, before the analytical weighing commences.

3.6.1.2.Procedures for hot weighing using a mechanical counterweight balance

(Goering and Van Soest, 1970; old procedure)

(1) Taring: Put an optical tare of 8 to 10 mg on the balance.

(2) Weighing sequence:

a. Dial in 39 g on the balance.

b. Remove crucible from the oven and place on the pan. Put the balance in 1/2 arrest and reduce the dialled weight until the scale image drops.

c. Put the balance in full release and read the gram fraction weight. The balance stabilises for a short period of time while the hot crucible is on the pan.

d. Put the balance in full arrest. Remove the crucible from the pan and the dialled weights from the balance.

e. Put the balance in full release and read the optical tare.

f. Repeat above sequence.

(3) Calculation of "true" weight: A series of crucible weights and optical tares will have been recorded. The "true" weight of the crucible will be its recorded weight minus the subsequent optical tare.

A timed sequence of weighing should be developed such that crucibles are weighed at the rate of one per minute. The optical tare is recorded about 30 sec after the crucible is weighed (Fig. 3.7). The crucibles used in the analysis of fibre normally weigh between 34 and 37 grams.

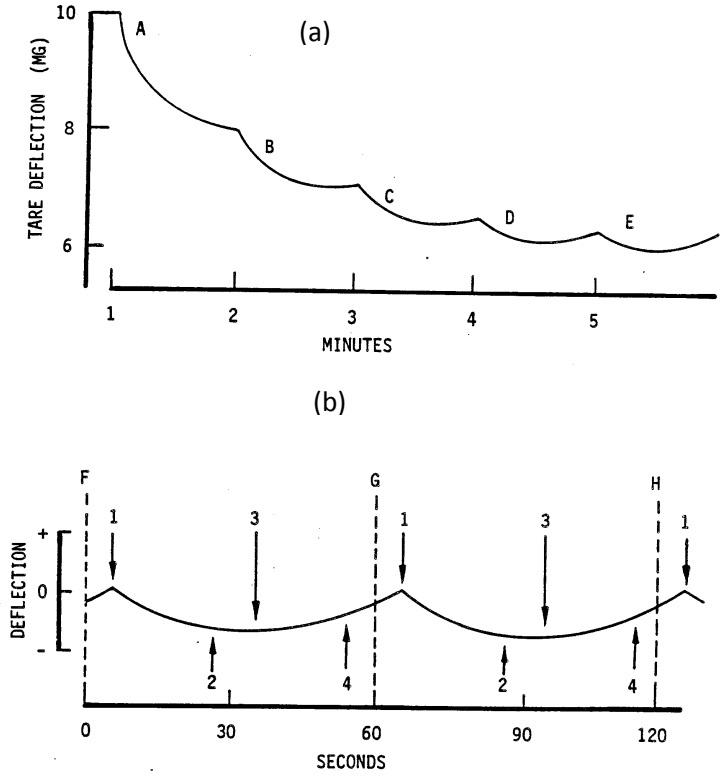


Fig. 3.6. Influence of the hot weighing sequence upon the apparent zero point of a balance (a). The apparent weight without a load on the pan will cycle between weighings, decline over the first 8 to 10 weighings then become stable, provided that the timed weighing sequence is maintained. Lower panel (b) shows steps in

the weighing operation: (1) place crucible on pan; (2) record weight; (3) remove crucible; (4) record optical tare.

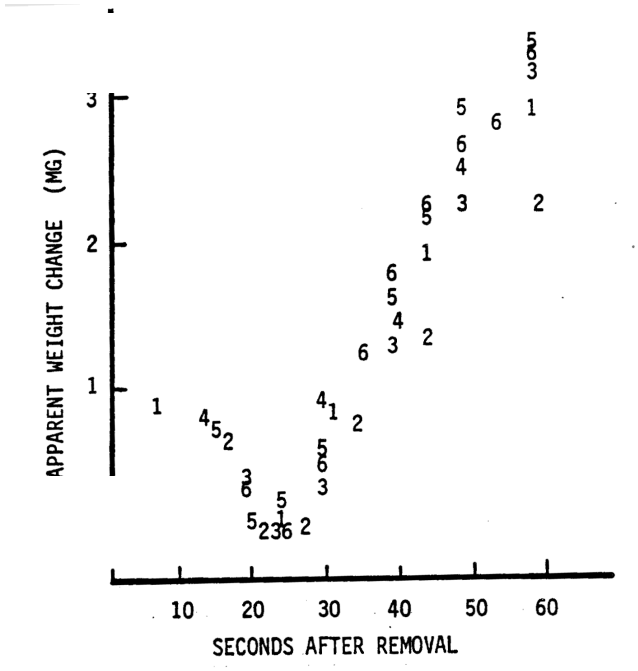


Fig. 3.7. Range in apparent weight of hot crucibles while on balance pan. Numbers indicate different crucibles. Note the minimum weight obtained at 20 to 30 seconds after removal of the crucible from the oven.

3.6.1.3. Procedure for hot weighing using an automatic single pan balance with electronic readout.

(1) Warming of the scale:

a. Weigh 4 pre-warmed crucibles in sequence. Allow each crucible to remain on balance for 10 sec.

(2) Weighing sequence:

b. Place hot crucible on balance.

c. Wait for reading to reach lowest point, and then take gross measurement.

d. Remove crucible and take tare measurement 10 sec after removal.

e. Repeat above sequence for each crucible.

A timed sequence of weighing should be developed such that crucibles are weighed at the rate of 3 to 4 per minute.

3.6.2. Procedure using desiccators

Desiccators have their own microenvironment in which the contents and desiccant come to equilibrium. Even when fresh, the common desiccants do not absorb all the water from the atmosphere of the desiccator. Each time the desiccator is opened, the conditions are altered since some air is exchanged, and the moisture introduced becomes distributed among sample crucible and desiccant. In using a desiccator, the items to be weighed should be removed in an ordered sequence and a strict time interval in weighing must be maintained.

The sequencing of crucibles will adjust for the greater exposure of the last crucibles to be weighed. Thus the same order of weighing in the taring operation must be kept when the crucibles are weighed containing sample.

3.6.2.1.Apparatus

A desiccator large enough to accommodate 24 crucibles should be used. The bottom of the desiccator under the porcelain supporting plate that holds the crucibles should be filled with phosphorus pentoxide P_2O_5 to a depth of 2 cm. Care should be taken that dry powder is always exposed, since P_2O_5 tends to form a gum-like pellicle that will greatly decrease its dehydrating potential. When most of the dry P_2O_5 powder is used up it will be necessary to renew it. Care must be taken in disposing of P_2O_5. If

much powder remains it should be added slowly to a large amount of water in a sink, with which it will react vigorously. Tongs and/or gloves Are needed for handling crucibles.

3.6.2.2.Weighing

Remove crucibles from oven with tongs or glove placing one by one in numerical order (this requires some preliminary planning). Replace the lid leaving a crack to vent the expansion of air. Allow 15 to 20 min for crucibles to cool to room temperature as needed. Close lid. However, keep this interval exact and keep it equal in later weighings of the crucible set. Weigh the crucibles one by one taking care to close the desiccator after each removal.

3.6.3. Comparison of hot weighing and the use of desiccators

A comparison of the hot weighing technique with the conventional system using desiccators was made at Beltsville (Goering and Van Soest, 1970). Desiccators were prepared with two desiccants: P_2O_5 and silica gel.

The average tare weight of a crucible (50 ml, high form) when weighed cold from a P_2O_5-containing desiccator was 20.3 mg greater than its respective hot weight. In the case of silica gel this value was 21.8 mg. The net increments for crucible plus fibre (about 400 mg fibre) was 26.3 mg for P_2O_5 and 35.9 mg for silica gel. Thus, in the case of P_2O_5, the average moisture accrued to a crucible by the sample was 26.3 - to 20.3 = 6.0 mg. For silica gel this increment was 14.1 mg.

Silica gel holds water reversibly over the range of 0 to 100^{o}C whereas P_2O_5-reacts irreversibly with water to form HPO_3 (metaphosphoric acid) and with more water to form H_3PO_4 (orthophosphoric acid). Thus, it can be seen that P_2O_5 is a much more

effective desiccant than silica gel. Also, it would appear that silica gel is superior to drierite ($CaSO_4$) in net capacity for water, but not necessarily in water absorption strength. Pyrex® is highly competitive with P_2O_5, but has a low capacity for absorption.

Estimation of water indirectly by volatile loss is a defined procedure since there are no exact conditions that are generally applicable (3.5). The minimum length of time for oven drying at 100°C is about 2 h. This time interval will apply provided (1) the oven is not overfilled, so as to reduce efficiency of drying; (2) there is no disturbance of the oven during the period *i.e.*, no fresh samples are put in, no samples taken out for weighing; and (3) the protocol applied is generally parallel to the manner of handling and weighing (3.6).

3.7. Methods for water

Water may be estimated through direct or indirect procedures. The most common method, that of measuring volatile losses at 100°C, is an indirect procedure in that it is the dry matter that is actually weighed and the volatile loss, assumed to be water, calculated by difference. Alternative procedures that directly measure water have been devised to render the measurement more specifically then that of water. It should be kept in mind that the purpose of a moisture measurement is to obtain a dry matter that when corrected for ash will reflect a recovery of metabolisable energy and that the choices are relative to this problem. An alternative is the direct measurement of combustible energy (3.9). However, this application is not meaningful unless a representative faecal sample is available for reference. This arises because lignin is a non-volatile component that is indigestible but with a very high energy of combustion.

The volatile losses of non-water origin are various. For most dry feeds they are unimportant. However, in the case of silages and other fermented plants, fermentation products in the form of volatile organic acids, ethanol, other volatile organics, ammonia, amines *etc*. are of consequence. Browses and certain other plants may contain volatile oils, usually of high energy content but of no value to the animal. This is in contrast to silages where the volatiles usually have a higher metabolisable energy than the average of the feed. Volatile oils may be inhibitors of digestion and metabolism. This leads to different purposes for the assay of the volatiles-not water and the accuracy of true water measurements.

When volatile metabolisable organics are lost as in the drying of silages, then the energy value is underestimated, and the water overestimated, whereas in browses and spices containing volatile oils, the available energy is not underestimated although the water value is off. Thus, silages are the feeds that have received most attention relative to this problem.

3.7.1. Moisture by volatile loss at 100°C (dry matter)

3.7.1.1. Equipment

Forced drying ovens and four place balance as described under weighing procedures (3.6.1.1), or desiccators with P_2O_5 if this method of weighing is used. Left hand glove if hot weighing is used. Dishes or beakers for holding samples.

3.7.1.2. Procedures

Weigh sample (1 to 3 g) into pretared containers which may be Pyrex® beakers or crucibles, porcelain dishes, or aluminium dishes. If ash (3.8) is to be done sequentially only Pyrex® or porcelain are satisfactory. Place samples in the oven in a

tray which retains the order in which they will be weighed. This is determined by the order in which the tare weights were obtained. Allow the samples to remain a minimum of 2 h or preferably overnight. Remove and weigh, following the appropriate procedures (3.6.2, 3.6.3).

3.7.2. Direct procedures for measuring water

3.7.2.1. Toluene distillation

The oldest method for direct measurement of water is the toluene distillation. While it is no longer a recommended procedure, there is still sufficient use and questions concerning it to present its principles and the problems associated with the method.

The principle of toluene distillation is that mutually insoluble liquids will distill in the presence of each other following the laws of the phase rule and partial vapour pressures. The ratio of toluene and water that distill will be in proportion to their partial vapour pressures at the effective distillation temperature. The sum of these vapour pressures will equal ambient barometric pressure. Thus, both liquids distill at a temperature somewhat below their respective boiling points. When water is exhausted, the boiling point rises to that of the toluene that remains. Thus, it is required that the sample be distilled in the presence of a large excess of solvent that is water insoluble. The maximum temperature of boiling is set by the boiling point of this solvent (Toluene, 110^{o}C, 760 STP). This boiling point will be decreased by the presence of water and increased by non-volatile components soluble in toluene (*e.g.* lipids).

The distillation is conducted in a glass apparatus in which toluene is allowed to drain back into the distillation while water is trapped in a calibrated receiver (Fig.

3.8).Distillation is continued until the water volume stabilises and becomes constant with time. The sample size required depends on the accuracy of volumetric measurement of water, the lower practical limit being about 3 ml. Thus, the lower the moisture the larger the sample that must be taken. A minimum sample weight at 900 g/kg DM to obtain 3 ml of water will be about 30 g. Samples of lower water content will require larger samples. This in turn will determine the volume of the distillation and the amount of toluene.

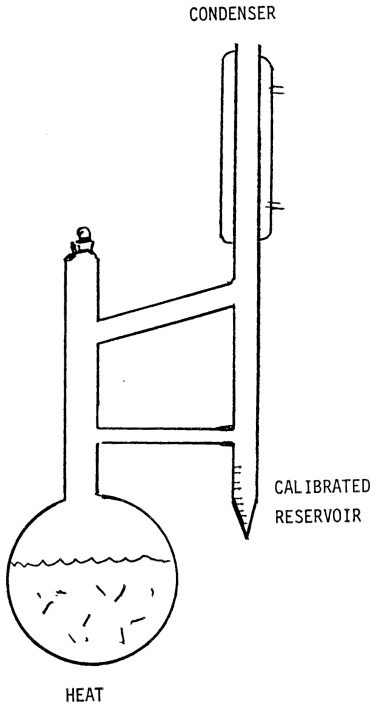


Fig. 3.8**.** Distillation apparatus for the determination of water using toluene.

Apparatus for other liquids than toluene to distill water are also described. Volatile oils, insoluble in water, can also be measured where water serves as the internal phase. The design of the apparatus varies relative to amounts of distillates expected, and whether the measured compound is lighter or heavier than the distilling

medium (Fetzer, 1951). Water is heavier than toluene, and essential oils are lighter than water.

The object of direct measurement of water is to reduce the influence of volatile organic substances other than water. Terpenoid oils, esters and other comparatively non-polar liquids are soluble in toluene, insoluble in water, and do not interfere with the water estimation. However, other polar liquids do offer a problem. The substances for consideration are VFA, their ammonium salts, ethanol and volatile amines. All of these acids and amine bases, ammonium or amine salts are volatile at the boiling point of toluene. The volatile acids are also miscible in all proportions with both toluene and water while the salts are soluble in water only. When presented with a toluene-water interphase, acids and ethanol strongly prefer the water phase at a ratio of 4:1 or greater. Thus, most VFA and ethanol plus their ammonium or amine salts will be present in the water distillate. The distortion upon volume will vary, since salts often tend to increase density of solution more than volume, while free liquids as VFA or ethanol will tend to add to the volume of the water.

The result is that the toluene distillation applied to silages tends to overestimate true water content though less than oven drying. Procedures have evolved for the correction of the contribution of VFA to the aqueous distillate, none of them entirely satisfactory because of the complex and variable composition of the contaminants. If the water is titrated for acidity, only free acids are measured, salts do not measure, nor do amines or alcohols. If VFA are measured by gas liquid chromatography (GLC), the amine and alcohol fractions are ignored. Some workers tend to estimate acidity as acetate either by titration or by GLC. Whatever the procedure, underestimation by as

much as 50% of the contamination is inevitable. Among the errors, if titration is applied, are that not only untitratable fractions are ignored, but the assumption that all acids are acetic acid leads to an underestimation of energy content since most other VFA (particularly butyric) will have a higher energy content per gram than acetic acid.

The final reason for not recommending the toluene distillation lies in the health hazard of continued exposure to toluene vapour. This can be minimised if the operations with open solvents are conducted under a safe and efficient hood.

3.7.2.2.Saponification Method (Hood et al., 1971)

The basis of this method is the saponification of an ester (ethyl acetate) that requires water as an obligate reactant. The reaction cannot proceed without water and will proceed to the extent that water is available in amount. Hence, assay of the extent of saponification becomes a measure of water. The reaction is shown in the following equation:

$$H_2O + EtOAc + NaOEt \rightarrow NaOAc + EtOH$$

where water is consumed in the reaction. If water is limiting, unused ethylacetate (EtOAc) and sodium ethoxide (NaOEt) will remain. Since NaOEt is a stronger base than sodium hydroxide and sodium acetate (NaOAc) is a comparatively neutral compound, assay of water is related inversely to the amount of NaOEt remaining which can be easily assayed by titration with standard acid with a suitable pH indicator (phenolphthalein).

Application of the technique requires extraction of water from the sample into a "dry" solvent. Since silages are usually acidic, a blank value for the net acid must be

titrated on this solution. Another aliquot of the solution is then treated with ethylacetate and sodium ethoxide and the unused NaOEt determined by titration with standard acid.

A number of problems exist relative to possible side reactions. Equilibration with alcohol can synthesise esters (catalysed by acid) liberating chemical water. This is apt to proceed with time of standing, such as a sample of silage soaking in alcohol. Acids converted to esters will no longer measure in the blank titration. The equilibration of wet silage with alcohol is subject to the same desorption problems as oven drying. The concentration of water in the alcohol (hence the ratio of sample and its moisture to the volume of alcohol applied) sets the effective "humidity" of the solution. Higher concentrations of water allow more equilibration toward adsorption upon the sample. Since sample volume ratios are constrained by laboratory glassware, a systematic error arises, because water concentration is then correlated with water content of the forage, and the adsorption is proportional to the water content. This leads to systematic underestimation of water in samples of low dry matter, and thus there is an overestimation of the dry matter in these samples.

This problem of equilibrium of adsorption also affects the Karl Fischer system. Another related matter is that the extraction is done at room temperature (or lower if refrigerated). The absorption therefore reflects ambient temperature, whereas oven drying reflects adsorption at a higher temperature which must be a smaller quantity.

Sample preparation of. wet forages and silages include freezing and grinding with dry ice in a Wiley mill fitted with a 1 mm screen. The ground silages are stored at -20^{o}C in plastic bottles to insure complete loss of CO_2, at which time the bottles are sealed. Samples are handled as described in Section 3.3.

Chemical determination of water is conducted in triplicate on 2 to 3 g (depending on moisture content) silage samples. Each sample is weighed to a 2.5 cm x 9.0 cm glass vial and 40 ml of absolute ethanol added. After sealing, each tube is shaken briefly and allowed to equilibrate for 24 h.

The reagents include:

Water-sensitive reagent. This stable reagent is prepared by mixing 650 ml of anhydrous ethyl acetate, 0.150 g of phenolphthalein and 350 ml of approximately 1.0 M sodium methoxide. If a precipitate develops, add methanol drop-wise until the reagent clarifies. The reagent has a capacity of 6 mg of water/ml of solution. Sodium methoxide is prepared by dissolving 23 g of freshly trimmed sodium metal in 1000 ml of cold anhydrous methanol. The reagent is conveniently stored in the reservoir of a 10 ml dispensing pipette fitted with an atmospheric moisture trap.

Ethanolic HCl. A stock solution is prepared by bubbling anhydrous HCl gas into cold absolute ethanol until a 25% increase in weight has been obtained. The stock solution is stored under refrigeration and diluted to approximately 0.15 M HCl with absolute ethanol (1 ml of stock solution per 45 ml of absolute ethanol) as required. The dilute acid is conveniently stored in a reservoir fitted with a 25 ml self-levelling burette.

Standardisation of the ethanolic HCl. Standardisation is achieved using a solution of 1.0 M water in absolute ethanol (18 g of water diluted to 1000 ml with absolute ethanol) as a primary standard and the same absolute ethanol serving as a blank. Using the following assay procedure to determine unreacted sodium methoxide, the titration of 1 ml of the 1M water standard (assay titre) and 1 ml of absolute ethanol (blank titre), the molarity of

the HCl is given by: 1/(B to A) with B = blank titre (0 mg water) and A = assay titre (18 mg water).

Water determination requires adding to each 50 ml glass stoppered Erlenmeyer flask containing 10 ml of the water-sensitive reagent, 1 ml of the ethanolic silage extract. The flasks are then incubated in a water bath at 50^{o}C for 15 min after which water is determined by titration with standard ethanolic HCl. To eliminate errors due to endogenous acids and bases, a blank titration must be performed on each silage. This is conveniently performed in a 50 ml Erlenmeyer flask by mixing 10 ml of water-sensitive reagent and 0.8 units of the standard ethanolic HCl required for the blank titre used in the standardisation of the HCL. The flask and contents are chilled in an ice bath for 15 min, at which time 1 ml of ethanolic silage extract is added and the titration continued until an endpoint is reached. The quantity of water in each flask is calculated using Equation I:

I. Water (g/ml) = X = 0.018•M•(D - C)

where: X = g water/ml silage extract, A = assay titre, B = blank titre and M = the molarity of ethanolic HCl. Silage water content, if calculated from g water/ml in equation I, is based on a dilution factor of 40. Actually, the dilution factor is 40 plus the volume contributed by extractable silage components. A correct water content can be calculated from Equation II:

II. Water content (g/kg) = (V/ 1-X) – V)•1000/W

where: X = g water/ml or ml water/ml silage extract from Equation I. V = volume of ethanol used to extract the silage (40 ml) and W = weight of silage (g). This equation is

derived by a series expansion: $\sum_{k=1}^{\infty}(V) * X^k$ and is expressed as (V/(1-X) –V when X <1.

3.7.2.3.The Karl Fischer titration

The Karl Fischer reaction is based on the oxidation of sulphite to sulphate with iodine. Formation of sulphite from sulphur dioxide (SO_2) uses water. This reaction requires a sufficiently high pH to convert SO_2 into sulphite when water is present. The water is divided: the hydrogens to protonate two pyridines to pyridinium ions and the oxygen to SO_2 to form sulphite ion (SO_3^{2-}) as shown in the equation:

$2\ Pyr + SO_2 + H_2O \rightarrow (Pyr^+)_2\ SO_3^{2-}$

The oxidation with iodine then proceeds as:

$(Pyr^+)_2\ SO_3^{2-} + I_2 \rightarrow (Pyr^+)_2\ SO_4^{2-} + 2I^-$

It is important to note that the oxidation is reversed in acidic conditions (*i.e.*, iodine reduces sulphate to produce SO_2 or bisulphite), while under alkaline conditions at pH 8 to 9, sulphite is oxidised to sulphate. This operation of the system requires an anhydrous alkaline solvent. Those characteristics are offered by pyridine. Water can be assayed by measurement of unused iodine which is inversely related to water content.

Historically, the method was developed and presented as a manual titration of unused iodine with starch as the colour indicator, the solution being titrated with thiosulphate until the brown colour of iodine has faded. Then a few drops of starch was added and the titration continued until the blue disappeared.

The procedure as such was almost unusable in the determination of water in forages and silages, because the natural brown yellow alcoholic extracts obtained from these feeds made the titration colours very difficult or impossible to see. This caused

forage analysts to favour the toluene procedure and ultimately the procedure of Hood et al. (1971) when it appeared. More recently the availability of an automatic titration apparatus in which the endpoint is measured electrochemically has caused the Karl Fischer procedure to become the favoured method. The automatic apparatus electrolytically generates halogen (actually bromine instead of iodine) thus eliminating the need for prepared iodine containing reagent. The amount of current required for the generated halogen is given in a digital readout. The current Karl Fischer titration is conducted in methanol with the following reagent mixture: imidazole (5 to 10%), 2-methylimidazole (5 to 10%), sulphur dioxide (5 to 10%), iodine (less than 10%) and 2-(2-ethoxyethoxy)ethanol (60.3 to 85%).

3.7.3. Comparison of methods for moisture

An evaluation of four methods of determining dry matter is shown in Table 3.2. Silage contains substantial non-water volatiles, whereas fresh forage and sheep faeces contain negligible amounts. Nevertheless, substantial differences exist among the four methods. Oven drying uniformly gives the lowest values, followed by freeze drying. The lower temperature of freeze drying as compared with oven drying causes more water to remain adsorbed at the lower temperature (3.5). On the other hand, volatile losses still occur, leading to an intermediate value. Freeze drying is obviously not a satisfactory substitute for oven drying as it gives lower values than the two direct methods.

While the values for the direct methods do not differ, the superiority of the Karl Fischer is evident in the lower standard deviation between duplicates. The Hood method is consistently higher in analytical variation than any of the other methods. The

higher values of apparent dry matter for alfalfa forage and sheep faeces that contain little volatile organic components (Flatt, 1957) are likely the result of moisture adsorption from the alcoholic solutions used in the two methods.

Table 3.2.
Comparison of four methods of determination of water (Olafson and Warner,1977)

Method	Alfalfa silage	Fresh alfalfa	Sheep faeces
Oven 100°C	$25.7^{a} \pm 0.18$	$25.7^{a} \pm 0.32$	$38.5^{a} \pm 0.40$
Freeze drying	$27.2^{b} \pm 0.11$	$26.4^{b} \pm 0.23$	$39.2^{a} \pm 0.13$
Saponification[1]	$28.8^{c} \pm 0.56$	$26.7^{bc} \pm 0.88$	$39.8^{b} \pm 0.61$
Karl Fischer	$29.0^{c} \pm 0.31$	$27.1^{c} \pm 0.48$	$39.9^{b} \pm 0.36$

[1]Hood et al. (1971); [a,b,c] values with different superscripts in each column are significantly different ($P<0.05$); ±: standard deviations of triplicate determinations.

3.8. Ashing

Procedures for ashing are parallel to the measurement of dry matter with the exception that a higher temperature is used. The older procedures used 600°C which requires porcelain or other suitably resistant dishes. Since Pyrex® softens at this temperature, the use of 500°C (maximum 550°C) is used in procedures utilising Pyrex® crucibles. At this lower temperature a somewhat higher ash content results (Fig. 3.4) probably through the lesser dehydration of silica, decomposition of carbonates, and losses of volatile salts.

Ashing procedure is integral to the determination of acid insoluble ash and silica (3.8.2). Procedures for these entities are components of the detergent system (4). Included here are the conditions for ashing and a discussion of other methods in the literature.

Data in Fig. 3.4 indicate that the apparent weight of Pyrex® is affected by temperature. The weights in that experiment were obtained by hot weighing at the

respective temperature of treatment. Since hot weighing out of the muffle furnace is impractical (although it can be done) the samples are equilibrated in the 100°C drying oven. Pyrex® regains its weight to the equilibrium at any temperature, but this is not so with many mineral substances of the type surviving in ash. Thus, the definition of ash is again a standardisation of the weight at 100°C like that of dry matter. This allows crucible tare weights to be obtained in the same manner as those for dry matter, which may be a part of the same sequential analysis.

3.8.1.1. Apparatus

Muffle furnace with temperature controller set at 550°C maximum. (this value should be checked using Tempilstiks® since many controllers are not accurate). Tongs for manoeuvring hot crucibles and a pan with asbestos sheet or porous ceramic tile lining the bottom.

3.8.1.2. Procedure for ashing

Weigh 2 to 3 g of sample into pretared Pyrex®-crucibles or 50 ml beakers. Numbering should be etched into glass with a diamond pencil. Any other kind of mark may not survive ashing and sample identity could be lost. Dry matter may be measured following procedure 3.7.1. Place crucibles or beakers in muffle furnace arranging toward the rear and away from the door which tends to be cooler. Ash overnight. Turn off furnace, and let temperature drop to below 250°C. Remove crucibles (use tongs) into a pan with an asbestos sheet or porous ceramic of low heat conductivity to prevent shock to glass and potential cracking of crucibles. Place pan in 100°C oven, and hot weigh after 30 min. Alternatively, place hot crucibles directly in a desiccator and follow procedures. Ash content is calculated as:

Amount of ash = (wt. after ashing – crucible tare)/(air dry sample•DM coeff.)

If dry matter was determined initially using the same crucibles, the denominator becomes (oven dry sample wt. - crucible tare).

3.8.2. Acid insoluble ash (AIA)

The significance of ash that is resistant to acid solution is that it represents (in theory) the nutritionally inert or unavailable minerals. Conventionally, it includes soil minerals, which are largely silicates, and opaline silica of plant origin. Properly conducted, it becomes a separation of silica from most of the other ash, and is thus preliminary to the determination of silica or silicon. Several versions of acid insoluble ash have been proposed as an internal marker, an alternative to lignin in digestion balances (Van Keulen and Young, 1977; Thonney et al., 1979).

Two generic procedures exist based on the order of sequential treatments. One is to ash the sample and to treat the prepared ash with acid and subsequent weighing of the insoluble matter. The Indian method (Shivastra and Talapatra, 1962), and the method of Van Keulen and Young (1977) are of this type (Table 3.3). The alternative is to digest the sample first in acid, then filter and ash the residue. The acid detergent insoluble ash and silica methods and the 4 N acid method of Vogtman et al. (1974) are of this latter type.

Methods for AIA evolved from the preparatory steps in the gravimetric determination of silica. The difficulty in evaluating the procedures for AIA reported by Van Keulen and Young (1977) is that of the problems associated with recovery of silica. These methods do not appear to have been tested for silica recovery, but by

comparison with proper gravimetric silica methodologies, it would seem that the Indian and Vogtman methods cited by them are adequate, while their 2 M HCl method may lose solubilised silica.

Table 3.3
Sequence of treatments in methods for AIA and silica

Method	Acid pretreatment	Ashing conditions	Post to treatment
Shrivastava and Talapatra (1962)	None	350 to 650^{o}C 5 h stepwise	Conc. HCL Evap. 2x Redissolve Filter and wash Re-ash 650^{o}C
Vogtman et al. (1974)	4 M HCL 0.5 h Filter	650^{o}C	None
Van Keulen and Young (1977)	None	450^{o}C	2 M HCL 5 min Filter, final ash 450 ^{o}C
Van Soest and Wine (1968)	Acid detergent 1 h in H_2SO_4 Filter	500 to 550^{o}C	8 M HBr for SiO_2
Van Soest (2006)	None	Ash in Pt dish	6 M HCL
Kolthoff and Sandell (1947)		Bunsen flame	Evap. 2x, Redissolve Filter, reash in Pt dish

Loss of soluble silica may occur as a result of reaction of alkali carbonates with silica to form soluble silicates. This is promoted by high temperature or long times of ashing and a high content of sodium or potassium. Conversion to silicate and loss of CO_2 with pure Na_2CO_3 and excess SiO_2 is 0.03 parts at 450^{o}C, 0.18 parts at 550^{o}C and

0.32 parts at 650^{o}C when ashed overnight. The acidification step attempts to precipitate silica. However, this is a difficult reaction to drive to completion since monosilicic acid is fairly soluble and dehydration and polymerisation is required. Dilution may cause some reversal of the polymerisation, through mass action. Prolonged refluxing or repeated evaporations with acid are needed and even then small amounts escape which render the method useless for determination of low levels (Kolthoff and Sandell, 1947). The 2 N method of Van Keulen and Young treats only 5 min with acid which is too weak to rapidly drive the reaction, and the time is hopelessly short.

Other problems include the formation of carbides and glasses that are resistant to acid digestion and lead to high results. Formation of carbides (resistant black ash) is favoured by the lower temperatures, while soluble silicates and glasses are favoured by higher temperatures of ashing. Solubility is favoured by high ratios of sodium and potassium. Calcium and magnesium silicates, though not soluble, are decomposable by acid, provided they have not fused. Iron and aluminium silicates are much more resistant to acid. Glass, if formed, will dissolve phosphates, nitrites, nitrates, probably sulphides (from protein) and also Pyrex® glass crucibles are etched. Thus, the result becomes an unpredictable consequence of interaction between sample composition and procedure and does not represent any physical or chemical definition of what the biological insoluble ash is supposed to be.

Only procedures adequate for the retention of silica are recommended. Included here are the acid detergent insoluble ash method and the platinum dish method with hydrofluoric acid (HF) (Kolthoff and Sandell, 1947). The extraction with neutral

detergent can be used as a tentative separation of plant from soil silica, provided the limits of solubility of opaline silica in neutral detergent are not exceeded.

3.8.3. Acid insoluble ash and silica (HBr method) (Van Soest and Wine, 1968)

These fractions may be determined in acid detergent fibre at the end of any of the lignin-cutin-cellulose sequences (Section 5) or directly after ashing the acid detergent fibre. See Section 4.8 for a discussion of the problems and interactions of sequential analysis. Pre-extraction with neutral detergent will tend to remove biogenic silica and leave soil minerals in the residue. Sodium hydroxide or ethylenediamine extraction of the insoluble residue will tend to accomplish the same purpose (3.8.5).

Gravimetric analysis for silica is suitable for most siliceous forages containing enough silica to be of biological importance in digestion balance. The practical minimum level is 0.5 g/100 g. Below this level, the colorimetric measurement as silicomolyblate may become more practical.

3.8.3.1.Procedure

Prepare ADF by the usual method using a 1 g sample. Filter on a previously tared 50 ml coarse porosity Pyrex® crucible. Lignin determinations may optionally follow Van Soest and Wine (1968). Ash the ADF (or the residue from the sequential treatment) at 500°C until free from carbon. Cool and weigh. Add drops of concentrated 8 M HBr, moistening all particles. Use no more than 4 ml acid. Allow to stand 1 to 2 h. Add more drops of HBr if much red colour forms. Suck off excess acid on vacuum and wash once with acetone. Use no water. Dry and ash briefly at 500°C. Cool and weigh. Report silica as the difference between this weight and the original tare.

3.8.4. Silica by the hydrofluoric acid (HF) method

This is an absolute method for silicon, since it depends upon the volatilisation of silicon as the tetrafluoride, the gravimetric measurement being based on weight loss. Use of sulphuric acid in the ashing and platinum dishes eliminates interferences from titanium, glass, etc. For a thorough discussion of the problems of silicon determination and variations in this method see Kolthoff and Sandell (1947). Values by this procedure compare quite closely with those from method 3.8.2, provided silicate mineral contamination is not too large. The relationship for a range of forages is shown in Fig. 3.9.

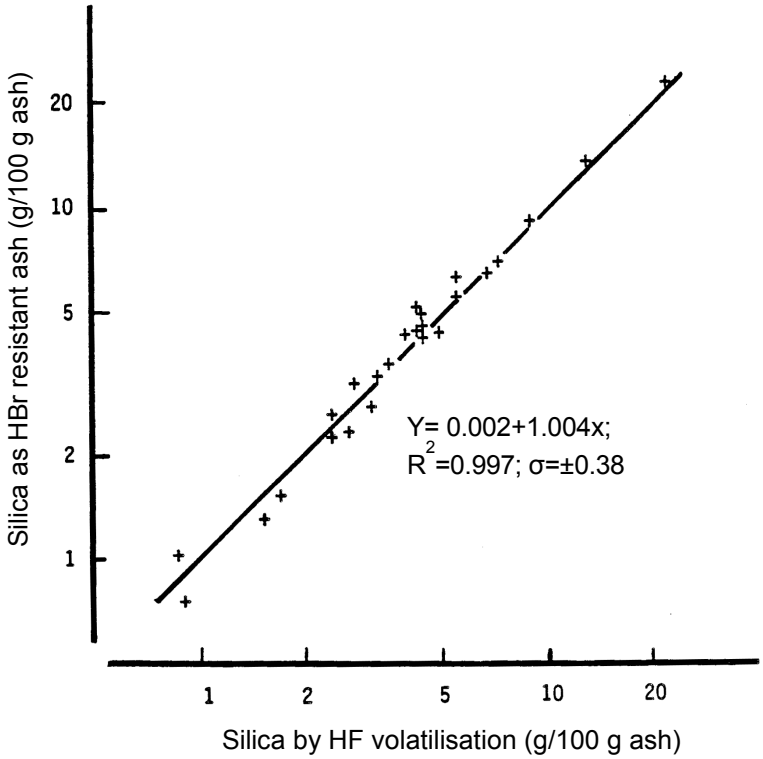


Fig. 3.9. Relationship between silica determined as the HBr-resistant portion of acid detergent insoluble ash and by HF volatilization (Van Soest and Lovelace, unpublished).

3.8.4.1. Procedure

Weigh 1 to 2 g of sample into a platinum dish and ash over a Bunsen burner (*ca.* 800^{o}C). Total ash can be weighed optionally. Platinum dishes do not need to be hot weighed since Pt does not adsorb moisture, but are weighed as soon as they have cooled to room temperature. Care must be taken in ashing that the Pt is always in the oxidising flame. Dishes can be damaged if heated in the bright reducing flame.

Add 20 to 30 ml 6 M HCl to the ash and evaporate to dryness on a steam bath to dehydrate silica (until odour of HCl can no longer be detected). Add again 20 to 30 ml 6 M HCl to dissolve metals other than silicon. Filter hot through Whatman No. 54 paper and wash precipitate free from chlorides (*ca*. 200 ml H_2O). Return paper and filtered ash to the Pt dish. Ignite paper holding precipitate to a white ash at 500^{o}C. Cool and weigh. The removal of soluble bases can be eliminated in samples that have been previously extracted or are otherwise free from soluble bases. Moisten ash completely with drops of concentrated H_2SO_4 (1 to 2 ml). Add cautiously drops of concentrated 8 M HF (up to 1 ml) under an efficient hood. Vigorous reaction ensues. Carefully evaporate over a Meker (multiple open flame) burner, avoiding contact of platinum with the reducing part of the gas flame. After dense fumes of SO_3 are eliminated, heat dish to redness. Cool and weigh. Silica is reported as the loss from the previous weight.

3.8.5. Soil contamination

Dust and mud can collect upon forages, particularly on the lower parts of the plants near the soil. This contamination is difficult or impossible to remove by washing without concomitant loss and disproportionation of organic composition. Most soil minerals are comparatively insoluble in contrast to opaline silica that dissolves 430 mg/l

(as SiO_2) in boiling neutral solutions. A separation based on solubility is imperfect in that opaline silica returned to soil through decayed dead plants would retain its chemical nature.

Apart from this limitation, three possible methods are available for separation of soil silica based on its insolubility. These are the silica remaining in ash from neutral detergent fibre, the mineral matter resistant to dilute hot sodium hydroxide, and the silica insoluble in hot diamineethylene. These three methods are offered here, subject to their respective limitations.

High amounts of silica render the ND method impractical, while the NaOH treatment cannot be done in sintered glass crucibles. The ethylenediamine is less destructive than NaOH to silicate minerals but requires a safe hood because of the fume hazard.

3.8.5.1.Soil silica by sodium hydroxide

Transfer ash to a Pyrex® beaker. Add 20 ml 1 M NaOH. Cover and heat on the steam bath 30 min. Filter immediately on Whatman No. 54. Wash thoroughly with water until paper and residue are free of NaOH (*ca*. 200 ml). Proceed with either of the above methods for determination of residual silica. Do not leach sintered glass crucibles with sodium hydroxide since their tare weight will decrease.

3.8.5.2.Soil silica by ethylenediamine

Alternatively, ash in sintered glass crucibles may be treated with 4 M ethylenediamine, covered on the steam bath for 1 h. Caution: keep under an efficient hood. Filter and wash with a small amount of water. Ash at 500^{o}C, cool and weigh.

Report residue as soil contamination. There may be important amounts of other elements in this residue, particularly aluminium and iron.

3.8.5.3.Silica by ash from neutral detergent

Extract a 0.5 g sample with 100 ml of neutral detergent in the usual procedure. Filter on sintered glass and after using, apply the HBr procedure. Soil silica is the residue provided the saturation limits of silica are not exceeded (43 mg/l). Alternatively, the fibre may be filtered on Whatman No. 54 paper, washed and ashed in a Pt dish. Follow with the HF procedure for silica (3.8.3).

4. The analysis of fibre and the detergent system

4.1. History of fibre methodology

The earliest attempts to isolate and determine the fibre content of food plants (*ca*. 1800) consisted of maceration in water followed by filtering on cloth. This produced values close to modern-day NDF values (Einhof, 1806; Van Soest, 1994). The advent of acid-alkaline extractions is not known (Tyler, 1975) although early results were published by Gorham, (1820); Horsford, (1846); and Wolff, (1856). The triumph of acid-alkaline extraction (crude fibre) which produced much lower values when compared to the maceration techniques of Pearson and Einhof (Tyler, 1975; Van Soest, 1994), seems to be founded on the criteria of low ash and nitrogen content and elemental ratios of carbon, hydrogen and oxygen as in pure carbohydrate (Horsford, 1846).

However, the conduct of digestion experiments and the discovery of lignin by Schultz in 1855 (Henneberg and Stohmann, 1860) indicated the defects of crude fibre. Henneberg and Stohmann, in applying crude fibre, standardised acid and base concentrations and time of boiling, hence the procedure acquired the name of Weende, the experiment station where Henneberg did his studies. Ignorance of the earlier literature on fibre led to the erroneous supposition that Henneberg and Stohmann invented crude fibre. Actually, Henneberg with the help of Stohmann (1864) demonstrated the loss of lignin in the alkaline extraction that partly accounted for the problem of the low digestibility of the nitrogen free extract (NFE), which was supposed to represent available carbohydrate (Paloheimo, 1953). However, this information did

not prevent the acceptance of crude fibre and its further standardisation by the AOAC, and the confusion has persisted to the present day.

Crude fibre cannot be regarded as a serious procedure for the analysis of fibre and is, therefore, excluded from the compendium of procedures described in this section. Technically and scientifically, crude fibre fails to isolate any definable fibre fraction and cannot be related in any meaningful way to the total fibre content of foods and feeds. Its further use is discouraged and should be discontinued.

Since the adoption of crude fibre the main contributions to fibre research have consisted of improvements in analytical precision and apparatus which have been generally useful in applying newer fibre procedures. The continuing search for alternatives to crude fibre is dependent on the evolution of nutritional concepts and the consequent definition and role of fibre in nutritional evaluation. It should be remembered, however, that throughout the history of fibre studies, fibre has tended to be defined by the procedures employed.

In the hundred years or so since the standardisation by Henneberg and Stohmann (1860, 1864), efforts toward basic understanding have centred on definitions and methods for the structural components such as cellulose, lignin, *etc*. Hemicellulose, poorly defined, has remained more problematic, but even here the old chemists instituted the measurement of furfural, a byproduct of the reduction of the pentose sugars comprising hemicellulose. A list of relevant publications is given in Table 4.1. No attempt is made here for an inclusive list of all attempts to determine fibre. Emphasis is on the more significant contributions.

If achievement of a total fibre procedure has been elusive, methods for the components could provide some idea of the total fibre by summation of the analysis for seven components (Salo, 1961; Paloheimo and Vainio, 1965). This approach became quite tedious, and in the interest of providing a single value for fibre, by methods economically competitive with crude fibre, led to enzymatic approaches and other preparations of isolated total fibre. The latter came into vogue about 1950 at a time when most dietary fibre research was dominated by ruminant studies.

Realisation of the importance of fibre in monogastric and human nutrition is a recent phenomenon (since 1970) and is largely due to observations by Trowell (1973), Cleave (1974) and Burkitt et al. (1974), associating human disease and dietary fibre deficiency. However, this recognition has induced a whole wave of analytical effort applying modern tools of chemistry to fibre methodology.

Modern approaches have to a large degree recapitulated earlier sequences (Table 4.1). They consist of a few main types: macerations and extractions with reagents to give residues that appear to represent the less digestible fractions, or enzymatic digestions to more positively isolate the resistant matter. The enzymatic approach to fibre methodology has arisen from the new definition of dietary fibre as polymeric substances of plant origin that are resistant to mammalian digestive enzymes (Trowell, 1976). This has led to AOAC approval of a wholly enzymatic system, (Prosky et al., 1984) of which the analytical problems have not been entirely resolved. The problems of general application of this definition to animal species including man have been discussed in Section 1. Here, the focus is upon analytical integrity. Residual isolates that are measured gravimetrically have no real defined composition and the

inference as to the composition is based on the assumption that the fraction behaved in the expected manner. Enzymatically resistant residues may be particularly heterogeneous and suffer also from the problems of using enzymes as reagents and the occurrence of enzymatic inhibitors such as tannins. The application of component analysis will define the composition of obtained residues; however, this usually (as applied) does not resolve the problem of definition as determined by digestive or enzymatic studies and model analysis by Lucas methods.

Table 4.1.
Fibre methodologies according to approach

Approach	Method	Reference
Mechanical	Maceration	Pearson (1803)[a] Einhof (1806)
Solubility	Crude fibre	Gorham (1820)[b] Horsford (1846)[b] Henneberg and Stohmann (1860)[ab]
	Detergent fibres	Van Soest (1963)
	Modified ADF	Clancy and Wilson (1966)
Defined entities	Cellulose	Crampton and Maynard (1938)
	Enzymatic fibre	Williams and Olmstead (1936)
	Unavailable carbohydrates	Southgate (1969) Hellendoorn et al. (1975) Prosky et al. (1984)
	Component analyses	Englyst et al. (1982) Theander and Westerlund (1993)

[a]Cited by Tyler 1975; [b]cited by Van Soest 1994.

However, sophisticated modern approaches have not resolved the problem of definition of methods. Approaches involving maceration, solubility or the residue after enzymatic treatment become essentially gravimetric measurements. Isolation or determination of sugar components by hydrolysis followed by instrumental chromatography (GC or HPLC) effectively defines the fibre fraction by the method of isolation used to prepare the residue upon which this component analysis is conducted. For example, if one were to determine cellulose by component analysis, then glucose becomes the criterion for standardisation, provided that all non-fibre glucose-containing substances have been eliminated in the preliminary extraction. Definition based on glucose will effectively exclude the pentosan component that is contained in all gravimetric residues. Use of acids, bases or enzymes leaves the assumption that all insoluble glucose is in cellulose. Only further determination of glycosidic linkages can define this problem. Here enzymes have been useful.

Component analysis is thus dependent upon adequate preparation of residues and is subject to the limitations of the extraction and isolation procedures that are the weakest links of the chain in defining dietary fibre fractions. The validation of the detergent procedures has been based on the comparison of feed and corresponding faeces, with the application of the Lucas analysis to ascertain ideal entities. No true fibre fraction appears to be ideal (other than lignin) and ideality appears to be a feature of non-fibre components (Van Soest, 1994). Nevertheless, this analysis has provided a definition of fibre.

4.2. Maceration methods

Isolation and determination of fibre by mechanical maceration followed by washing and filtering the pulp was the oldest form of fibre analysis. This approach is still relevant today when the interest is in obtaining chemically or biochemically unaltered preparations particularly for botanical study (Harris and Lowry, 1979). Maceration procedures are not rapid and are usually applied for special purposes. The principle of isolation depends upon resistance to comminution, thus overgrinding or trituration can cause loss of very fine matter. Soluble components are lost, but could be recovered by alcoholic precipitation. Fine matter can be recovered by use of settling tanks. The residues obtained by mechanical treatment are apt to contain considerable protein and other non-fibre polymers. Therefore, use of any of the published procedures should be followed up with analysis for nitrogen (protein), non-structural carbohydrates and ash.

4.2.1. Procedure A (Wrick, 1979): applicable to fresh vegetables, fruits, and forages not containing starch

A unit weight of fresh plant material is combined with sufficient ethanol to form approximately a 1:4 water-ethanol mixture. The mass is blended for about 2 min. Filter on cloth. Resuspend in aqueous ethanol solution (950 ml/L). Allow to soak for an hour. Repeat ethanol extraction until no more colour is removed. Suspend in acetone, filter and dry. The residue will contain most of the protein, but soluble gums and pectin are recovered. If protein must be removed, follow one of the enzymatic procedures. Neutral protease is preferred as acid-pepsin treatments are likely to degrade hemicelluloses and pectins.

4.2.2. Procedure B (Einhof, 1806 (modified)): suitable for starchy vegetables and cereals

Cereal grains are soaked for 24 h to allow swelling and then macerated in a mortar. Alternatively, vegetables like potatoes are grated or blended in water. The macerated material is suspended in a tall glass cylinder and allowed to settle. Starch, being heavy, will fall to the bottom, fibrous matter settling more slowly. Alternatively, the slurry may be filtered on coarse cloth allowing starch (and some fibre) to pass through. Separation processes will need to be repeated until most starch is removed. The final pulp is washed twice with ethanol and finally with acetone and dried.

4.3. The detergent system

This method for estimating dietary fibre and fractioning it into its major components is based on the use of anionic and cationic detergents. It was developed at the USDA Beltsville by Van Soest (Van Soest, 1963; Van Soest and Wine, 1967; Goering and Van Soest, 1970). These determinations were primarily intended for evaluation of roughage feeds for ruminants but also have been subsequently applied to the estimation of the fibre content of non-ruminant feeds and human foods.

The adaptations required to accommodate a wider variety of feeds and foods has led to the proliferation of modifications of the original procedures (Southgate, 1969; Southgate, 1981; Robertson and Van Soest, 1981). Further, definition of the dietary fibre complex to include soluble gums and pectins not digested by mammalian enzymes necessitated development of a detergent procedure for these components. Other modifications include attempts to overcome the interferences of starch (McQueen and

Nicholson, 1979; Mongeau and Brassard, 1982). Micro methods have been developed for small samples (Waldern, 1971).

4.3.1. Theory

The detergent procedures are divided into the neutral systems seeking to measure total or insoluble fibres and the acid detergent fibre (ADF) that isolates the insoluble cellulose and lignin and some other components.

The methods depend upon the capacity of detergents to render proteins soluble so as to prevent their interference and isolation with the fibre (Van Soest, 1963). All detergents form strong complexes with proteins; however, only ionic types form complexes with convenient solubilities. Ionic detergents form complexes with proteins and lipids in which the lipophilic tail of the molecule is attracted to the core of the protein or into the surface of the oil droplet, and the ionic group to the outer solvent phase (Fig. 4.1), the consequence being that the complex behaves as a giant polyvalent ion. Positive charges are distributed over the surface in the case of anionic detergents leading to attraction of cations; while with quaternary detergents, cationic charge is distributed over the surface leading to attraction of anions.

The attractions are such to produce electrical neutrality. Solubility is influenced by the type of detergent and the ionic species in the solution. Some salts of detergent protein complexes are insoluble, and such interferences must be avoided (Table 4.2). The problem of precipitation is particularly evident in the case of neutral detergent where polyvalent cations generally precipitate the anionic complex. Therefore, a chelating agent (EDTA) must be present to eliminate this effect. Most of the common anions occurring in foods form soluble salts with cetyltrimethylammonium cation, with

the exception of long chain fatty acids, which, however, can be removed by prior extraction. The only important insoluble anionic components involving quaternary detergent and its complexes are the polyuronides. In the case of sodium lauryl sulphate, the potassium salt or the more water-soluble ethanolamine salt have high alcohol solubility and are useful for soluble dietary fibre recovery. The insoluble pale violet cuprammonium salt is a sensitive test for lauryl sulphate ion. Some of the insoluble salts of cetyltrimethylammonium are useful in the recycling of the acid detergent reagent. Procedures also exist for recycling the sodium lauryl sulphate and the EDTA used in the neutral detergent and acid detergent (Section 6).

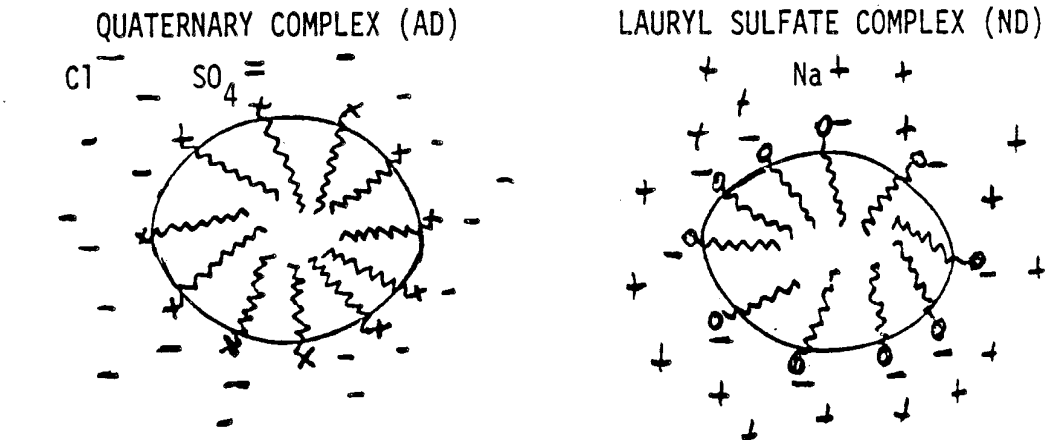


Fig. 4.1. Diagram of molecular models of detergent-protein complexes. The charge of the detergent ion imparts the major charge to the surface of the complex such that it behaves as a very large polyvalent ion.

Table 4.2.
Solubilities of ionic detergent salts and their protein complexes

		Solubility[b]	
Detergent	Ion	Detergent salt	Protein complex
Na lauryl sulphate	K^+	Sol hot	Sol hot
	Ca^{2+}	I	I
	Mg^{2+}	S	Slightly sol
	Cu^{2+}	S (I in NH_4OH)	I
CTAB[a]	Cl^-	S	S
	SO_4^{2-}	S	S
	PO_4^{3-}	S	S (in acid)
	CO_3^{2-}	S	I
	$Acetate^-$	S	S
	ClO_4^-	I	I
	MnO_4^-	I	I
	Dichromate $^{2-}$	I	I
	Dithionate $^{2-}$	I	I
	Long chain fatty acids	I	I

[a]Cetyltrimethylammonium bromide; [b]I=insoluble, S=soluble.

4.4. Apparatus for detergent fibres

Equipment required for NDF and ADF determinations is generally the same as those for crude fibre, although certain types of apparatus have more utility than others. The essential requirements are that the boiling rate of beakers can be individually regulated and the volume and concentration of reagents must be controlled with an adequate condenser. Adequate refluxing requires convection of feed particles (failure to achieve this will lead to high results) but at a sufficiently slow rate of boiling that excess foaming does not occur (this also leads to high and erratic results because of lodging of feed particles high on the walls of the beaker out of contact with the reagent). Apparatus

which uses air condensers with Fleaker® beakers is inadequate because concentration of reagents cannot be controlled due to volatile loss of water. Low recoveries are obtained for ADF.

Several types of adequate refluxing apparatus are available from various laboratory supply companies. An example is the FOSS Fibertec® instrument from Denmark, along with the ability to create a system for boiling and filtering. All recommended systems use straight-sided beakers or refluxing tubes; Erlenmeyer (conical) are generally not recommended because of the problem of foam regulation. An exception is in the case of macro-fibre preparations (4.5A) that utilise large round bottom flasks. When these are used, it is important that they are not more than half-filled.

Of the commercial units the FOSS is preferred for the sake of utility and convenience in conducting sequential operations since the filtering function is an integral part of the unit. The Labconco has the disadvantage in the maintenance of the enclosed electrical system; if acid is spilled onto the plates and wiring becomes burned out, replacement is not easy. However, repairs to the FOSS instrument can be complicated and the Labconco unit does have a price advantage. The Labconco and home constructed apparatus require the adjunct of a filtering manifold.

A home-constructed refluxing apparatus is by far the most economical, while it may not match commercial units in convenience. The essential requirements of an efficient apparatus is individual control of heating plates, and a filter manifold with as many filters (usually 6) as there are heating plates.

4.4.1. List of apparatus

Glassware (suitable for the specific unit).

Berzelius beakers without pouring spout, 600 ml capacity (Corning® 1040 or equivalent). At least 36 beakers, and preferably 72, should be available so that enough samples may be weighed out for a day's refluxing.

Gooch-type crucibles, high form, fritted disc, coarse porosity of maximum pore size 40 µm (Corning® 32940 or equivalent). An adequate supply would be about 100 for each technician. The crucibles should be permanently engraved with identification numbers. These crucibles will melt or become misshapen if they are subjected to temperatures much above 550°C. They should not be removed from the muffle furnace after ashing until the temperature has dropped to 250°C, and when removed, should be placed on an asbestos sheet or porous ceramic tile or brick, rather than directly on the metal surface of a tray.

Coarse-fritted glass crucibles of the large size are preferred (40 to 60 µm porosity). Crucibles are fitted with glass microfiber filter disks (discussed in section 4.4.4.) as an alternative or enhanced filtration aid in the event small particles are present.

Photographic trays (shallow pans, constructed of acid- resistant material). Suitable sizes are 42 x 26 x 6 cm and 31 x 20 x 5 cm. Enough trays are required to hold the crucibles for weighing, transport between laboratory locations, lignin determinations and other analytical procedures. The larger trays hold 36 and the smaller hold 18 crucibles.

Asbestos sheets or other fire-proof heat-insulating material sheets, about 3 mm thick, and cut to fit the bottom of the enamel trays. If no longer be available because of the health hazard of asbestos, porous ceramic tile or brick may be satisfactory.

4.4.2. Reflux unit

For optimum analytical throughput, the reflux unit should have heating plates in banks of six. Each plate should have its own heat regulation with enough capacity to bring 100 ml room temperature detergent solution to boiling in 5 to 6 min and maintain a vigorous boil. The unit should be adapted to straight sided containers and the condensers should allow for adequate cooling to keep the volume of the boiling solution constant without excessive foaming.

If the laboratory has a reflux apparatus used for crude fibre determinations it can be used without modification for the detergent procedures. If no reflux unit is available, one may be constructed or adapted from available equipment.

4.4.3. Materials required to construct a reflux unit

Depending on the sophistication desired, not all of the materials suggested may be needed.

Hotplate 400 watt, flat steel top, 9.5 cm diameter or equivalent. If individual hotplates are to be used, a multiple outlet electric box with adequate amperage capacity will be needed.

Condensers to fit 600 ml beakers. Boiling flasks of 250 to 300 ml capacity may be used. The boiling flasks are simple and an adequate series with flowing water are optional. A half-filled 300 ml round bottom or Florence flask is adequate, when the water becomes heated (this will be indicated by foaming), it can easily be replaced with a

fresh flask containing cold water. If the reflux rate is sufficiently regulated at a low level, this change will not need to be made very often. Suitable reflux condensers are manufactured.

Support stand for six individual hot plates needed: 1) Flexaframe® rods: 5 x 30 cm, 3 x 60 cm, 2 x 90 cm; 2) Flexaframe® hook connectors, approximately 36; 3) 2 Flexaframe® feet; 4) support rings, cast iron, 10 cm O.D., 6; 5) Clamps, extension 7.

Rubber tubing (thick wall), 8.0 mm I.D., 3.5 mm wall. The Flexaframe® unit supports the condensers and the cast iron rings which are positioned above the hot plates to prevent the beakers being bumped off.

The final reflux apparatus used may be as simple as one hot plate with a boiling flask acting as the condenser through various combinations of heating and condensing units to commercially designed units such as the Labconco crude fibre extractor or FOSS Fibertec®.

4.4.4. Filtration

Samples filtered onto sintered glass crucibles result in significant loss of fines. This is overcome by the use of filter discs (Udén, 2006; Raffrenato and Van Amburgh, 2011).

Glass microfiber filter discs (934-AH ™ by Whatman® ; Whatman Limited- G.E. Healthcare, Maidstone, U.K.) are inserted in 50 ml Pyrex® Gooch crucibles with 40 to 60 µm porosity discs. Losses from conventional filtration on sintered glass without proper filter are serious for lignin (Table 4.3). All other filters systems using bags or other conveniences are inadequate.

Table 4.3.
Percent difference in recovery of acid detergent fibre (ADF) and sulphuric acid lignin (lignin(sa)) when using a glass microfiber filter with 1.5 µm pore size for the ADF and lignin(sa) procedures (Raffrenato, 2011)

Forage Group	ADF	Lignin(sa)
Grasses	1.3 (-0.5 – 5.0)	10.1 (-2.2 – 19.3)
Wheat straws	0.1 (-0.4 – 0.7)	12.3 (-1.16 – 29.3)
Immature grasses	19.1 (1.9 – 11.1)	38.3 (19.25 – 90.5)
Conventional CS	3.3 (-0.5 – 9.9)	23.2 (2.7 – 41.0)
BMR CS[a]	7.0 (-1.8 – 15.1)	27.5 (-1.5 – 67.2)
Alfalfas	2.7 (-1.0 – 11.2)	3.2 (-1.6 – 10.1)
Faeces	5.9 (2.3 – 10.3)	18.9 (8.4 – 32.0)

[a]Brown midrib corn stover

4.4.5. Filter manifold

The filter manifold should have receivers for at least six crucibles to match the number of refluxing units (Fig. 4.2) and vacuum to each crucible must be individually controlled. This will allow slow-filtering samples to be handled without disruption of the timed sequence of others in the batch.

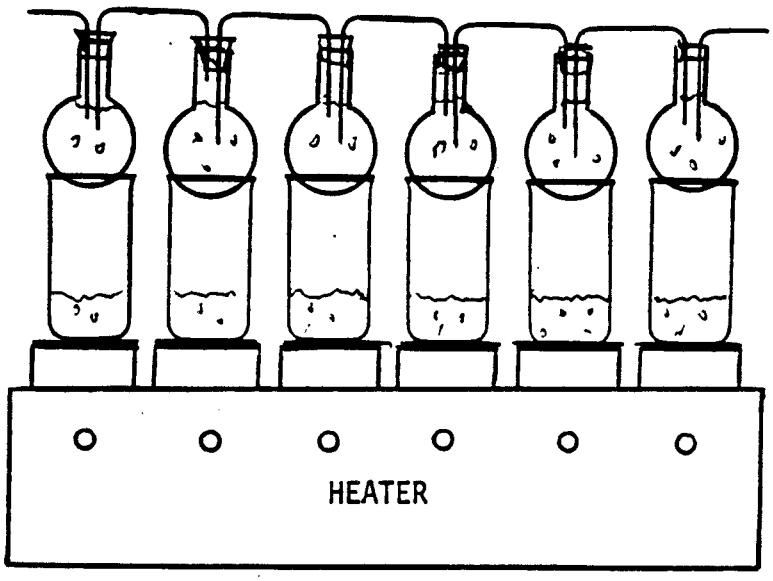


Fig. 4.2. Diagram showing arrangement of reflux units in a home-made construction.

Alternatives to the home constructed variety is the FOSS device (which only fits FOSS crucibles) and the Holst apparatus, constructed of glass in such a form that the crucible can be kept hot, to improve filtering of samples that are prone to form gels on cooling.

Since strong acids and organic solvents are used in the detergent procedures, the manifold is best constructed from acid-resistant material. Plastic pipe has this quality and polyethylene is used here. The pipe and tubing are held in position by a wood frame (Fig. 4.3.). Materials required to construct a filter manifold for crucibles are:

a) Wooden support frame.

b) Polyethylene pipe, 60 cm long, 2.5 cm I.D., 3 mm wall.

c) Polyethylene tubing, 4.8 mm I.D., 1.5 mm wall.

d) Glass tubing, 3 mm I.D., 1 mm wall.

e) Rubber tubing, 6 mm I.D., 3 mm wall.

f) Rubber stoppers, No. 6 1/2, 1 hole.

g) Clamps, pinchcock, Flatjaw, 70 mm long.

h) Clamp, pinchcock, Hoffman, small.

i) Funnels, Buchner, porcelain, size 0.

j) Rubber adapters, size B.

k) Gooch tubing, 32 mm flat diam.

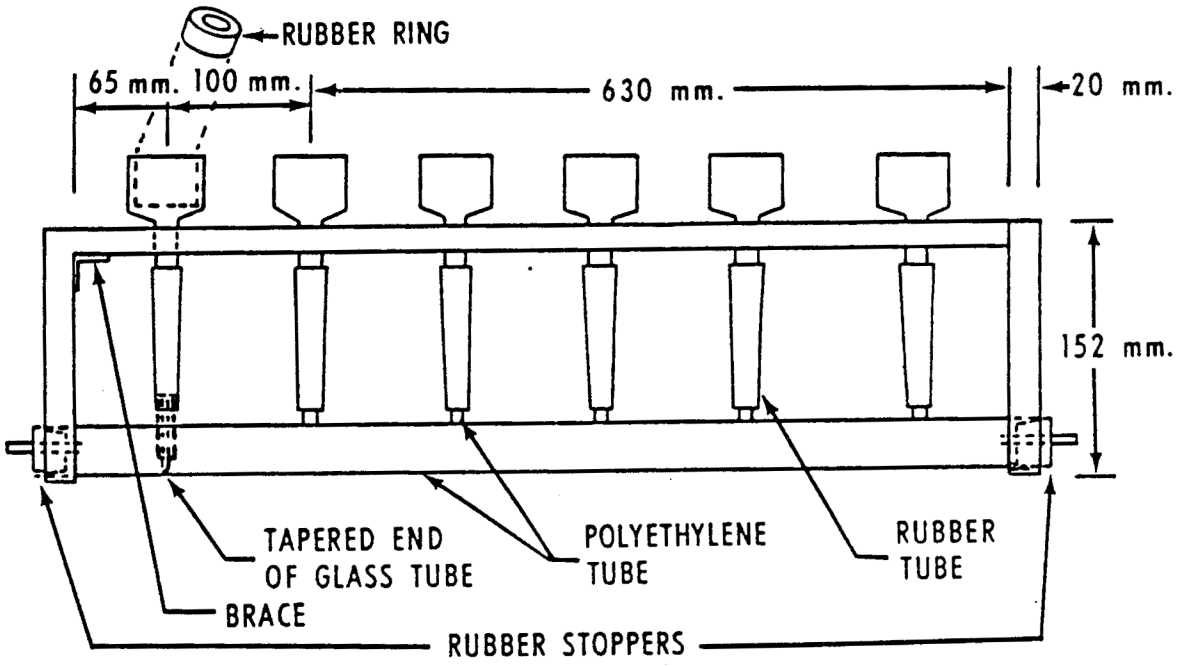


Fig. 4.3. Diagram of a filtering apparatus: home construction.

The manifold is made by welding, with polypropylene six tubes (3.8 cm long x 3.5 mm I.D.) into drilled 3.5 mm holes in a 3 cm ID pipe. These are spaced 100 mm apart (Fig. 4.3). If polyethylene is used a glass lining (5 mm O.D.) is inserted into each

polyethylene tube to prevent collapse due to heat. Thick walled rubber tubing connects the polyethylene tubes to the Buchner funnels supported on the wooden frame. This tubing should not be so stiff that it cannot be closed by a pinch clamp. Flatjaw pinchcock clamps on the rubber tubing control the vacuum. Gooch tubing is placed over the funnels containing the adapters to give added support to the crucibles. The polyethylene pipe is closed by the rubber stoppers through which 10 cm lengths of polyethylene tube are inserted, and the manifold is attached to the wooden frame. To one end of the manifold is attached rubber tubing and a Hoffman pinchcock clamp to control the amount of vacuum by allowing bleeding from the atmosphere. The other end of the manifold is connected through a filter trap to the vacuum source by thick walled rubber tubing. (See Fig. 4.3.) The manifold is constructed so that vacuum space beneath the filter is minimal. This is to allow for rapid elimination of residual vacuum when the unit is closed with a clamp. This feature is very necessary to avoid difficult filtering of finely divided samples.

Holst (1973) has designed an alternative manifold for detergent analyses in which the vacuum to each crucible can be carefully controlled and regulated. This apparatus is constructed of glass and is quite expensive. Circulating hot water surrounds the crucibles and their holders to prevent the solutions from cooling down while being filtered.

A water aspirator supplies insufficient vacuum for filtering detergent solutions due to the excessive foaming which occurs in the filtrate. Vacuum pumps are better suited but have to be protected from the filtrate, thus a filter trap has to be inserted between the manifold and vacuum source. It should consist of at least two 4 L filtering flasks

which will handle the filtrate from about 12 detergent analyses. Anti-foamants should be put on the filtering flasks, especially in the neutral detergent procedure. Normal butanol controls foam but silicone based ones are more economical.

4.4.6. Hot water source

An adequate supply of hot (90 to 100^{o}C) distilled water, directly above the filtering manifold, is needed to wash the various residues. Water heaters such as large coffee percolators, electric units or those designed for crude fibre (Holst, 1973) may be used. Alternatively, a glass unit may be constructed.

Materials:

a) Flask, distilling, 3 L, 3 neck. It must be modified by adding a well with a water inlet to the bottom and water outlet on the side.

b) Immersion heater, quartz, 1000 watts.

c) Support ring, cast iron, 15 cm O.D. To allow for expansion and contraction, remove 5 cm from ring and cover with rubber tubing.

d) Condensers.

e) Rubber holder. Supports the immersion heater in the flask.

f) Transformer, variable, 10 amp.

g) Bottle, solution, 18 litre capacity, (Corning® 1595 or equivalent) to store distilled water.

h) Crucible holder, filter tube. 42 mm I.D. x 160 mm long. Needed for the automatic filling device.

i) Clamps, extension.

j) Flexaframe® rods or support stand.

k) Clamp, flat jaw.

l) Polyethylene tubing 9.5 mm I.D. x 1.5 mm wall; 6 mm I.D. x 1.5 mm wall.

m) Rubber tubing, 6 mm I.D. x 2 mm wall.

n) Rubber stoppers.

Distilled water is stored in an elevated 18 L reservoir above and to the side of the water heater. A closed siphon connects the reservoir to the automatic levelling device with the water inlet tube (6 mm I.D. polypropylene) set about 2.5 mm lower into the automatic levelling device (the crucible holder) than the air return tube (9.5 mm I.D. polypropylene). An air vent tube, set above the inlet and air return tubes in the automatic levelling device allows for air pressure compensation between the atmosphere and the reservoir contents. A 9.5 mm I.D. polypropylene tube connects the reservoir to the work bench area to facilitate filling of the reservoir. The rubber stoppers closing the reservoir and automatic levelling device are sealed with bee's wax. The automatic levelling device is clamped to the support stand and connected to the well of the 3 L flask with rubber tubing. It is adjusted vertically to give the desired level of water in the 3 L flask which rests on the 15 mm O.D. cast iron ring attached to the support stand. The quartz immersion heater is connected to a variable transformer so that the rate of boiling can be controlled. A thick wall rubber tube feeds hot water from the flask to the manifold and is closed with a flat jaw clamp. The feed hose terminates with a polypropylene tube (about 3 mm I.D.) to allow a controlled stream of hot water for

washing. The end of the hot water feed tube should be wrapped in insulation for ease of handling.

To fill the reservoir, the tube between the automatic levelling device and the flask is closed off. Then vacuum is applied through the air vent tube to lift distilled water from the level of the work bench area up into the reservoir. When the system is not in use, the tube between the automatic levelling device and the flask should be closed. The inlet tube between the reservoir and the automatic levelling device should not be one piece but split and connected with rubber tubing. Thus, if the siphon is broken, vacuum can be applied to the water inlet tube to restart the flow.

Cotton gloves should be available for handling the equipment during reflux and hot weighing. Rubber gloves should also be available.

Two macro-analytical balances should be available. For specifications, see section on hot weighing (3.6.1). Top loading balances should be available to weigh out reagents.

For oven specifications, see Section 3.6.1 on hot weighing.

The muffle furnace should be large enough to accommodate 36 crucibles for ashing. A floor area of about 36 cm x 24 cm will accomplish this. An energy requirement of about 6000 watts is needed to rapidly heat up the furnace and maintain ashing temperatures. Since the crucibles should not be subjected to temperatures much above 500^{o}C, control of the furnace temperature is very important. Most manually controlled rheostats are not precise enough, and it is advisable to use an electronic control unit.

4.5. Reagents

The mixing of reagents used in the detergent system requires the measurement of fairly large volumes of water. Suitable containers for handling these volumes are, at best, only accurate to ±0.05. Thus, it is convenient to have a series of 2, 4 and 6 L Erlenmeyer flasks calibrated to contain exactly their amounts. The calibration may be done by weighing into the flask the amount of water equivalent to the desired volume and etching the flask at the meniscus.

4.5.1. Reagents for neutral detergent fibre

Reagents used for making a neutral detergent solution are shown in Table 4.4.

Table 4.4.
Reagents for neutral detergent solutions

Desired volume:	1 L	18 L
Distilled water (L)	1	18
Sodium lauryl sulphate, lab. grade (g)	30	540
Ethylenediaminetetraacetic acid·4 H2O, A.R. (g)	14.61	263
Sodium hydroxide, A.R. (g)	4	72
Sodium borate·10 H2O, A.R. (g)	6.81	122.6
Disodium hydrogen phosphate, anhy., A.R. (g)	4.56	82.1
Triethylene glycol (purified grade; ml)	10	180

The EDTA and NaOH may be replaced by the molar equivalent (335 g) of the disodium salt of EDTA (Na_2 EDTA·$2H_2O$).

Dissolve the NaOH in about 3 L H_2O and add the EDTA and $Na_2B_4O_7 \cdot 10H_2O$. Separately dissolve the Na_2HPO_4 in about 400 ml H_2O over heat. Add the dissolved components to a 20 L container. Mix the solution while still warm. Dissolve the sodium lauryl sulphate (270 g in about 4 L H_2O) and add to the container using a funnel (note device to minimise foam, Fig. 4.4). The triethylene glycol is added as necessary to control the foam. Add the remaining water and triethylene glycol to the container and

mix well. The following day, check the pH of the solution (see Section 6.7.8. for further explanation). It should be between 6.9 and 7.1 and can be adjusted to that range, if necessary, with NaOH or HCl. If the solution is not stored at temperatures above 20^{o}C, the detergent will precipitate out but can be re-dissolved on heating. Note that the ingredients are added to 18 L water and that the resultant volume of the finished solution will be about 18.5 L.

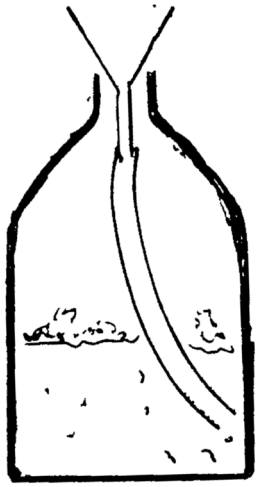


Fig. 4.4. Arrangement of funnel and hose allowing the filling of a carboy without induction of foam.

4.5.2. Enzyme solutions for NDF in starch-bearing foods and feeds

Termamyl, a heat stable solution of amylase is used for the analysis of starch-bearing foods and feeds (Sections 4.6.3, 4.6.4).

4.5.3. Reagents for acid detergent fibre

Prepare acid detergent solution by first standardising 0.5 M sulphuric acid between 0.49 to 0.51 M). Add cetyl trimethylammonium bromide according to recipe (Table 4.5). Dissolve and mix. Solution is indefinitely stable. Note that the detergent is added to the standardised volume. Final volume is somewhat increased. Therefore, acid standardisation must precede addition of detergent.

Table 4.5.
Reagents for acid detergent solutions

Desired volume:	1 L	18 L
Sulphuric acid, A.R. 0.5 M (L)	1	18
Cetyltrimethylammonium bromide, tech grade (Bromat®; g)	20	360

4.5.4. Cleaning solution for crucibles

Prepare the cleaning solution according to Table 4.6. Solution is very caustic. Wear rubber gloves and goggles for eye protection.

Table 4.6.
Reagents for a 2 L glass crucible cleaning solution

Reagent	
Distilled water (L)	2
EDTA (disodium salt; g)	10
Trisodium phosphate (anhydrous; g)	100
Potassium hydroxide (850 g/kg; g)	400

4.5.5. Cleaning of Crucibles

Between each determination, the crucibles should be washed and distilled water sucked backwards through the plate. After repeated use, however, the sinter glass plate of the crucible becomes clogged with mineral matter. When this occurs will depend on the amount of acid insoluble ash that the samples contain; however, it is rare to have to

demineralise the sintered glass plate more than once per year. Many of the deposited minerals are insoluble in strong acids, such as chromic or sulphuric, thus a strong alkaline solution containing a chelating agent (EDTA) is used which, in addition to dissolving the mineral matter, can also attack the sintered glass plate.

Crucibles should not be cleaned with this procedure any more than is necessary, because the process erodes, wears out the plate and shortens the life of the treated crucible. Routine recycling of crucibles may be as simple as tapping out the ash after the final step in the muffle furnace, back flushing with distilled water to remove lodged particles, followed by 100^{o}C drying before taring for the next run.

Make a suction device (Fig. 4.5) by boring a hole in a #12 rubber stopper and inserting one end of a 50 ml pipette into the hole. Connect the other end of the pipette through a trap (1 or 2 L filtering flask) to a vacuum source.

Place the ashed, water-washed crucibles in an enamelled pan, add about 50 ml of the cleaning solution to each crucible and heat over a steam bath for about 15 min. Using the suction device, suck the cleaning solution back into the crucible until 2/3 full. Allow the solution to filter through. Repeat wash, and suck off all cleaning solution. The sinter glass plate should be in contact with the cleaning solution for 1 h at most. The cleaning solution is saved for future use. Wash the crucibles under running hot tap water for at least 30 min to remove all traces of alkali, and then wash the crucibles in distilled water.

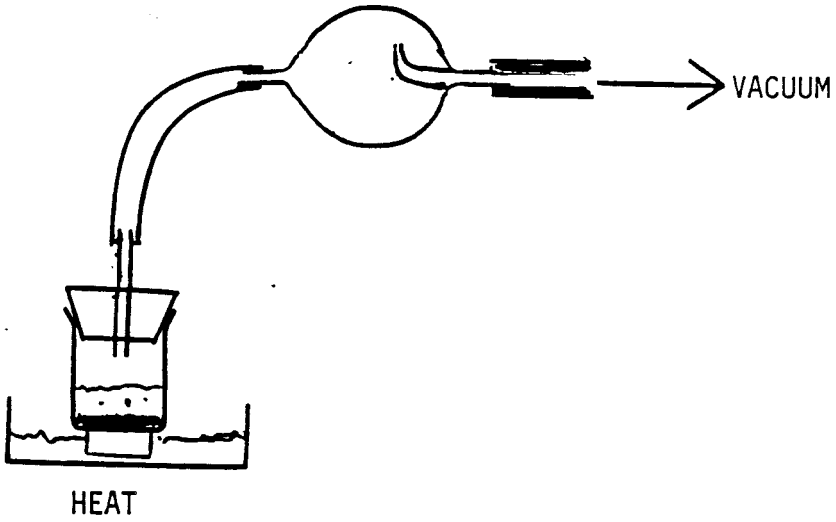


Fig. 4.5. Suction device for cleaning crucibles. Bulb in the line prevents the caustic solution from advancing up the vacuum lines. The bulb may be the trap from a macro Kjeldahl distillation unit.

4.6. Neutral detergent fibre (insoluble dietary fibre)

The NDF represents the insoluble matrix of the plant cell wall, substances covalently linked, or so intimately associated through hydrogen bonding, crystallinity, or other intramolecular association so that they are resistant to solutions within the range of physiological concentrations. Since the neutral detergent reagent is non-hydrolytic most of these interbondings should be preserved in the NDF residue. This is reflected in the compared *in vitro* digestibility of isolated NDF (without sulphite) as compared to digestibility of NDF in the intact forage. Washing of NDF before *in vitro* digestion may remove natural inhibitors and is to be avoided. However, there are a few associated components which are removed by ND resulting in higher digestibility of isolated NDF from the forages containing them. These include silica (Van Soest, 1981) and some kinds of tannins (Robbins et al., 1975).

Not all cell wall components are bound in the matrix. Approximately 0.9 of the pectin, for example, is removed by ND (Bailey and Ulyatt, 1970) and is also completely fermentable, showing no effect of lignification, which exerts major reduction in digestibility if the bound components in the NDF. Thus, NDF cannot be claimed to represent total plant cell wall, but represents a residue (insoluble fibre) of nutritional significance, in that it retains the unavailable lignified matrix, and the physically insoluble structure that has special effects in the rumen, and in the gastro intestinal tract of non-ruminants as well.

Fibre is commonly used as a negative index of dietary quality, since it generally represents the less digestible portions of feedstuffs. Although NDF recovers the indigestible components, unlike ADF (loses hemicellulose) or crude fibre (lignin and hemicellulose), its correlation with digestibility for ruminants is generally inferior to ADF. This has caused some to use ADF as a standard for forages although NDF is better related to rumination, efficiency and feed intake (Van Soest, 1994). Fibre requirements for ruminants can only be quoted in terms of NDF, since both hemicellulose and cellulose contribute. The value of NDF is that of all fibre analyses, it is the only one that will equate different forages and concentrate by-products according to their fibre potential, provided particle size is considered. Thus, NDF is the only analysis that ranks all feedstuffs in a continuum from feeds containing no fibre, low fibre concentrates, to high fibre straws and cellulose.

Considerable attention is now being made to the soluble components of dietary fibre that include the cell wall components such as pectin removed by neutral detergents, but also galactans and gums that are probably storage compounds and not

cell wall substances as far as the plant is concerned, but fall into the dietary fibre classification, because they are resistant to mammalian digestive enzymes. Current effort to include these and all insoluble fibre in a method for total dietary fibre is claimed by the new AOAC procedure of Asp (Prosky et al., 1984).

It is necessary to defend NDF to the observations that the soluble components have different physiological effects compared with the insoluble matrix. For ruminants, the soluble complex is completely fermentable, and thus justifiably included in the ND solubles along with starch and sugars that largely suffer the same fate in the rumen. For non-ruminants, the gummy components exert effects upon gastric emptying in the abomasum, upon glucose absorption and tolerance than the matrix substances, which in their turn exert much larger effects upon transit and forage and regulation of lower tract fermentation. The philosophy here is that dietary components of differing character ought to be distinguished in the analytical scheme.

The NDF is recognised as an important food fraction in human diets (Baker 1981), and has been modified by Schaller (1976) for use in cereals. This procedure has been approved by the American Association of Cereal Chemists (AACC, 1978). The NDF has also been collaborated by the AOAC (Mertens, 2002).

4.6.1. Problems and interferences with NDF

One of the great problems of general procedures for foods and feeds is the great diversity of the kinds of samples, their composition and likely analytical problems. This diversity is such that it is difficult to write a universal method. Many modifications have been published for one purpose or another. The main problems are outlined in the next section.

The most common difficulty with NDF analysis is filtering, which can arise from several causes: faulty apparatus or technique (See 4.4.4), high viscosity due to starch as well as other matter, too finely ground samples, crucibles with clogged or defective plates. Many of the starch containing feeds or foods will be greatly helped and the values much improved if starch is removed enzymatically (4.6.2). One of the enrichment procedures may be resorted to (4.6.6).

Protein and sodium sulphite. Sodium sulphite is incorporated because of its ability to aid in the solution of protein by attacking disulphide bridges. The use of sulphite has a tendency to lose lignin (Hartley, 1972; Robertson, 1978). However, in the case of hair contamination (keratin proteins that are protein resistant) sulphite offers the only avenue of removing that interference. Hair contamination of faecal samples is a common problem, but not usually in the case of diets, unless animal products are included. The content of insoluble nitrogen in acid detergent fibre will generally recover unavailable nitrogen including that in keratins. Sulphite is used in the AOAC procedure by Mertens (2002), but not in the simpler procedure described in Section 4.6.3. Use of sulphite reduced analytical variation in the AOAC study, but is incompatible with the use of neutral detergent to distinguish slow digestion of cell wall protein (Krishnamoorthy et al., 1982, 1983). Addition of sulphite precludes the use of ND residues for further sequential analysis because sulphite has altered the lignin cell wall matrix (Section 4.8; Van Soest et al.,1991).

Inclusion of sodium sulphite in the NDF procedure can result in some loss of lignin which could compensate for the reduction in residual nitrogen (Table 4.7). Small

but significant loss in lignin is reflected in increased digestibility of sulphite treated NDF on the order of 10 to 15%.

Table 4.7.
Effect of sodium sulphite and α-amylase (*B. subtilis*) on neutral detergent fibre values (g/kg DM)

Feedstuff	No enzyme	Enzyme	No enzyme	Enzyme
	With sulphite		No sulphite	
Orchard grass	649	624	682	662
Timothy	735	722	762	743
Alfalfa	298	292	335	313
Light bran	391	354	412	388
Corn bran	588	550	627	576
Lima beans	527	101	556	119
Black eyed peas	244	82	147	90
Chicken feathers	90	90	844	844
Average	440	352	546	467
Lignin recovery	0.847	0.89	1.05	1.06
Residual N (g/kg of total N)	159	123	312	265

High levels of protein can also cause filtering problems. It may help to use an Oklahoma State filter screen (Labconco®) to remove protein detergent complexes prior to filtering the neutral detergent residues through the crucible. Increasing the reflux time to 2 h has been suggested (King and Taverner, 1975), but excessive boiling could result in formation of Maillard products and increasing the time beyond 1 h is not recommended for this reason. Proteases, which are compatible with detergents (*e.g.* ICN Pharmaceuticals Cat. No. 101027), have been used in analysis of vegetables rich in protein.

The neutral detergents will accommodate lipids as long as a second phase does not form, wherin excess lipids result in separate oil-water phases. Since the detergent is oil-soluble its action in water to complex protein is inhibited and high values are obtained. The ratio of 100 ml detergent to 0.5 g sample will tolerate about 100 g/kg lipid

in the dry matter. Materials with higher levels will need to be pre-extracted (see 4.6.6). It is important that the pre-drying steps (AOAC) for ether extract determination be avoided as this will produce Maillard products and generate its own interference.

Samples may be difficult to filter for various reasons some of which could be: the nature of the sample that is too finely ground or high in starch or gums, mismanagement of vacuum and the filtering process or dirty crucibles. Specifications on crucibles and their cleaning is described in the following section. Some kinds of samples are difficult to filter, but application of proper technique will minimise problems.

Proper management of filtering involves protection of the filter plate from clogging by fine particles and avoidance of excess vacuum. The construction of the filtering apparatus to allow easy release of vacuum and regulation of low pressure is essential. High pressure tends to clog filters more quickly, and thus, no more vacuum should be used than is necessary. Slurries once added to crucibles should be allowed to settle 10 to 15 sec before turning on vacuum. Many samples will filter easily by gravity if crucibles are clean. Samples and water should be kept hot to avoid gelation. The Holst apparatus automatically does this. However, return slurry to the beaker on the hotplate if necessary in the case of slow filtering samples. Amylases remove starch and reduce gelation.

In most instances, it cannot be assumed, unless proven, that a filtering aid is inert. Blank determinations need to be made on all new batches of such materials, since their affinity for water may vary. Loss through acid extraction should also be watched.

Some materials contain high levels of pectins, mucilages and gums. When these materials are refluxed in neutral detergent solution, unfiltrable gels are formed. One

attempt to overcome high pectin levels (as calcium pectinates) is to increase the concentration of EDTA in the neutral detergent solution. This is best done by adding 2 ml of a solution of 2 M EDTA in monoethanolamine (pH 7) to the sample and beaker prior to refluxing. This modification works well for vegetables containing high levels of pectin. Filtering at 100^{o}C through crucibles held in a special manifold that can maintain the crucible and its contents at a high temperature often works well (Holst, 1973).

If increasing the EDTA concentration or using high temperature filtration fails, the best estimate of cell wall may be that obtained from doing an ammonium oxalate or ethyl alcohol (800 ml/L) extraction of the air dry fat-free sample and correcting for the residual protein (Sullivan, 1964).

4.6.2. Modifications of NDF

There have been many alterations to the original NDF procedure, some of them from the original authors and some from others. Some of these other methods are doubtful, some real improvements. Modifications can be classified into categories: (1) that alter the basis of NDF such as Fonnesbeck (1976) or Moir (1971) who wished to exclude as much protein as possible and yet retain pectin. Fonnesbeck thus reduced the pH of the solution, (2) the methods of Moir (1971), which devised special equipment for handling and filtering. While Moir uses an enzymatic system for cell wall, his equipment has considerable utility and could be used with the preferred modifications indicated below. (3) Alterations with amylases have the aim of either removing starch or improving filtration. Amylase treatments may precede extraction or incorporate amylase into the detergent reagent.

Not all of these systems have been adequately compared as to their accuracy and precision, although Mascarenhas-Ferreira et al. (1983) compared eight procedures in a collaborative study. Criteria for judgment are not simple, since there is no primary standard for precision. However, some procedures giving lowest results, regarded as a criterion by some, are clearly too low. The judgment of these authors was that if an amylase treatment reduced the value of non-starch containing forage, the enzymatic treatment was clearly degrading non-starch components. Thus, the criterion is that the amylase treatment on non-starch containing forages gives equal values to the procedure of Van Soest and Wine (1968).

Low results are obtained with long pre-treatments with enzyme, and hemicellulose is likely lost either through fermentation or impure enzyme. Higher temperatures of enzyme treatment tend to eliminate the problem. The shorter procedures are also favoured because they are more economical and more likely to be reproducible. One of the Mascarenhas-Ferreira procedures is semi-micro and is included in Section 4.6.7.

Mascarenhas-Ferreira et al. (1983) compared amylases from *B. subtilis* and pancreas in all of the eight combinations in Table 4.8 and these did not alter results. Either enzyme can be used. However, the advent of the very thermostable Termamyl has led to an even simpler method where the enzyme is confined in the ND reagent in a simple one hour reflux.

Table 4.8.
Comparison of eight modifications of NDF (g NDF/kg DM; Mascarenhas Ferreira et al., 1983)*

Method	Non-starch containing forage (	Starch containing faeces
Van Soest and Wine (1968)	535[a]	-
Robertson and Van Soest (1977) (also Mongeau and Brassard, 1982)	539[a]	213[ab]
Giger et al. (1981)	540[a]	221[a]
Wainman et al. (1981)	467[e]	167[d]
McQueen and Nicholson (1979)	487[d]	173[de]
Wainman et al. (1981)A**	472[e]	204[b]
Wainman et al. (1981)A**	512[c]	183[e]
Mascarenhas-Ferreira et al. (1983)	516[bc]	218[a]
Mascarenhas-Ferreira et al. (1983)	520[b]	218[a]

*B. Subtilis amylase used in all procedures except Wainman et al. (1981)A; ** modified by Mascarenhas-Ferreira; [a,b,c,d,e]values with same subscript are not significantly different (P<0.05).

4.6.3. Simple procedure (Van Soest and Wine, 1967; Van Soest et al., 1991)

Weigh up to 0.5 g of the air-dried sample, ground to pass a 1 mm screen, into a 600 ml Berzelius beaker; add 100 ml of neutral detergent solution. Sodium sulphite and decalin are not used. Place on a hot plate and heat to boiling in 5 to 6 min. Adjust heat to an even boil, timed from the onset of boiling. When several samples are being measured, allow about 3 min between each determination.

Place tared Gooch crucibles on the filter manifold. Heat the crucibles by adding hot (90 to 100^{o}C) water and allowing it to filter through. After the 60 min reflux, remove the beaker, swirl to suspend the particles, and fill the crucible 3/4 full. Allow to settle 10 to 15 sec. Use minimal vacuum and start the filtering process. At no time should the beaker contents be added to an empty crucible under vacuum, but they can be poured slowly into the crucible containing liquid. Increase the vacuum as needed to achieve

filtration. Rinse the sample from the beaker with a minimum of hot water. Wash the sample twice with hot water, twice with acetone and suck dry. If filtration seems to be impeded, the filter plate can be cleared by back flushing: the vacuum to the crucible is closed, the crucible removed and then pressed back into the holder creating a back pressure of air which will lift and break the fibre mat.

Dry the crucibles for 8 h or overnight at 105^{o}C in a forced air oven and weigh to obtain the yield of NDF. Ash the crucibles at 525^{o} for 3 h, remove to the oven and weigh. The loss in weight is the ash-free NDF (NDFom). The use of tared crucibles may be eliminated if only NDFom is determined.

4.6.3.1. Expression of results: ash correction

Some laboratories correct for ash expressing results on an organic basis (NDFom), while others follow the original procedure of Van Soest and Wine (1967), which includes ash (NDF). Forage samples, particularly grasses, may contain ash of biogenic origin and from soil contamination. The biogenic silica is largely dissolved by neutral detergent while ash of soil origin is largely retained in ND insoluble residue. The NDF ash contamination is still a part of the indigestible dry matter, and the biogenic silica a part of the cell wall structure limiting degradability (Van Soest, 1981, 2006). Methods are presented in Section 3.8 to separate biogenic silica from soil minerals. Significant amounts of residual ash should be reported (over 20 g/kg of the fibre residue).

4.6.4. Alternative procedures

4.6.4.1.Procedure A

This procedure is identical to 4.6.3 and represents the least effort to remove starch as the thermostable enzyme is added directly to the reagent. Add 0.25 ml of Termamyl 120 to each 100 ml of ND reagent. Proceed as in 4.6.3.

4.6.4.2.Procedure B

An amylase digestion is incorporated into the reflux step of the neutral detergent procedure. This procedure does not increase the time to analyse NDF.

Reagents

Neutral detergent solution (4.5.1).

Prepare the enzyme solution on the day of use by mixing 20 g/L of amylase. (Sigma® A1278) in water. Filter through Whatman 54.

Procedure

Weigh 0.5 to 1.0 g sample ground to pass a 1 mm screen into a 600 ml Berzelius beaker. Add 50 ml room temperature neutral detergent solution, place on hot plate and heat to boiling. Adjust heat to an even boil, keeping the feed particles suspended.

Forty minutes from the onset of boiling, remove beaker and add 50 ml room temperature neutral detergent solution and 2 ml of enzyme solution. Return beaker to hot plate without adjusting the thermostat.

Place tared Gooch crucibles with filter disc (4.4.4) on the filter manifold and warm with hot water. One hour after the initial onset of boiling, filter the residue onto the crucible. Wash twice with hot water. If filtering problems occur, add 1 to 2 ml enzyme solution to the crucible and contents. Add about 30 ml of hot water to the crucible and 2

ml enzyme solution. Let stand 5 to 10 min, then filter, wash twice with hot water, twice with acetone and suck dry. See Mertens (2002) for a detailed description of filtration management.

Dry the crucibles for 8 h or overnight in a forced air oven at 105^{o}C and weigh to obtain yield of cell wall (aNDF). Ash crucibles at 525^{o}C for 3 h; remove to oven and weigh. The loss in weight is the ash free NDF (aNDFom). Values for ash should be reported.

4.6.4.3. AOAC official method 2002.04. Amylase treated NDF (aNDF).

The aNDF method of Mertens (2002) has been collaborated and approved by the Methods Committee on Feeds, Fertilizers and Agricultural Related Products as First Action. The method is outlined here. However, details of the method are in the publication “Official Methods Program Actions” (2002) Inside Laboratory Management [September/October issue].

The Mertens method has been developed for a minimal inter-laboratory variation, and focussed on feeds important to the ruminant feeding industry. The inclusion of amylase treatment is justified for these reasons. The use of amylase to remove starch has been critically evaluated in the Mertens study. The procedure provides evaluation of amylases and their activities which vary with source. Results are expressed on an ash-free basis. Samples are filtered with the use of a special acid-wash sand. Crucibles are conducted with blanks. Also the pH of the neutral detergent reagent is measured and controlled within the range 6.95 to 7.05, and adjusted if necessary with HCl or NaOH.

There is no mention of the loss of fines in the filtration system.

4.6.5. Method using 8 M urea (for starchy feeds)

Urea detergent reagent (UDR). Dissolve 480 g urea and 100 g sodium lauryl sulphate in 630 ml distilled water. Use a 2 L flask with heat and stirring. Total volume is 1100 ml and is essentially saturated relative to urea and detergent.

Reagents.

Amylase-urea detergent solution (for use with UDR). Suspend 2.2 g amylase (B. subtilis) in 50 ml of distilled water. Let stand 20 min and filter into a clean 100 ml graduated cylinder. Dilute to 63 ml. Transfer to a 250 Erlenmeyer and add 48 g urea and 10 g sodium lauryl sulphate. Heat gently to dissolve.

Concentrated neutral buffer solution (NBS).

Prepare buffer the same as for ordinary neutral detergent, except the sodium lauryl sulphate is omitted and the concentrations are increased by a factor of 1.4.

Procedure.

Weigh 500 mg of feed (ground 1 mm) into a 600 ml Berzelius beaker. Add 30 ml of urea-detergent reagent and 2 ml of amylase solution. Swirl gently to mix contents. Cover beaker with watch glass. Let stand at least 3 h or overnight in a warm place, or a sufficient time to allow solution of starch.

Add 70 ml NBS solution and 2 ml of amylase solution. Heat to boiling. Filter on a pre-tared sintered glass crucible (size 50 C). Wash twice with hot water and twice with acetone. Suck dry. Dry overnight at 105^{o}C and weigh. Ash at 525^{o}C for 3 h. Weigh. Loss in weight is neutral detergent fibre.

4.6.5.1. Macro-procedures for NDF

Larger amounts of prepared NDF are needed for various purposes, such as particle size measurement (Smith and Waldo, 1969) or for preparing marked material for passage studies. Prepared NDF is also a useful means of studying microbial inhibitors that may be in the feedstuffs. Macro preparations can also be a means of enrichment (4.6.6) to serve in analysis of foods and feeds that are very low in fibre.

4.6.5.2. A preparation of macro amounts of NDF (Smith and Waldo, 1969)

Macro amounts of prepared fibre (10 g or more) are needed for many studies where sequential treatments and analysis require large amounts of uniform material, such as for determination of particle size of fibre, mordanting for digesta passage studies, or for replicated *in vitro* fermentation studies. These procedures are alternative to the maceration methods that can also yield less well extracted material for these purposes. Extraction of macro quantities can also serve as a means of isolating small amounts of fibre from sources of low content, and is an alternative to the micro procedures listed in 4.6.8.

Reflux 20 to 50 g of forage for 1 h in 1 L of neutral detergent, use a 2 L round bottom flask and a glass cool heater. Attach a wide mouth Allihn condenser fitted with a rubber stopper. Adjust heating such that foaming is controlled. Butanol may be dripped down the condenser if further control of foaming is desired.

Filter the fibre onto a large piece of 40 µm mesh cloth 40 x 40 cm, held in a large Buchner funnel. Cloth should be large enough so that it can be tied off as an enclosed bag. Wash the fibre on the cloth three times with litre portions of hot water. Tie off the fibre cloth into a bag and suspend in a large container (*i.e.*, bucket and soak in tap

water overnight). Finally, suspend the bag in a sufficient amount of aqueous ethanol (950 g/ kg H_20) and with manual sloshing, remove as much pigment as possible. Wash twice with acetone. Air dry the closed bag at 24^oC. If the bag is tare weighed, a rough estimate of yield can be obtained by recording the final weight of the bag and its air dry contents.

4.6.5.3.Procedure

The handling of very large samples on the order of a kg may be necessary for coarse materials. Large quantities present special problems. Prepare a large sack of stout filter cloth *ca*. 200 µm pore size. Sew the bag shut with forage inside. Sew the bag so there is plenty of extra surface. Use a sewing machine and double sew such that there is no danger of loosening and spilling of contents. The bag is then laundered in an automatic washer first with warm water. Do not put through the spin cycle. Remove, drain water from bag and machine and repeat washing cycle with 2 L neutral detergent plus water to fill the machine. Repeat 2 more cycles with hot water each time avoiding the spin cycle. Finally, immerse in a large bath of aqueous ethanol (950 g/ kg H_20), 20 L or more. Allow to soak overnight with the bath covered. Agitate from time to time. Ethanol treatment may be repeated if much pigment remains. Dry contents in air at a temperature <40^oC.

4.6.6. Enrichment procedures

Enrichment may remove interfering matter which may be lipid, protein, gums etc., or concentrate the fibre in samples of low fibre content. Macro-preparation may be satisfactory in the case of low content, but unsatisfactory in case of interference. Suggested procedures follow for specific kinds of problems.

4.6.6.1. High-lipid samples

Preliminary extraction of samples high in fat or oil is needed in cases of content above about 100 g/kg DM where the lipid forms a separate phase. The ethanol dehydration from Section 3 is a suitable procedure. It is not necessary to completely remove lipid, only reduce it to a tolerable level. It may be necessary to pre-extract lipid to be able to grind foods very high in fat. Sohxlet extraction or other methods using much heat should be avoided, as they will alter lignin, insoluble protein and hemicellulose.

4.6.6.2. High-protein samples

Protein is conveniently removed by using neutral protease that is compatible with detergent (Megazyme Int. catalogue reference: E-BSPRT).

Dissolve 1 g enzyme in 100 ml distilled water. Let stand 15 min. Filter on Whatman No. 54 paper. Add 1 ml of this solution to 100 ml of ND reagent.

Weigh samples into fibre beakers, or larger container if more sample is needed. Add 100 ml reagent/g of sample. Let stand overnight. Proceed with the ND procedure (4.6.3).

4.6.6.3. Gums

Several kinds of samples, such as psyllium and guar are so viscous that no normal handling in a fibre procedure can be done. For enrichment on high gum and low insoluble fibre the following is recommended.

Warm tertiary butyl alcohol above 25^{o}C to melt crystals.

Weigh 10 g ground material into a 4 L beaker. Add 200 ml tertiary butyl alcohol. Place beaker on a hot plate with a magnetic mixing device. Place magnetic stir bar into

the beaker and set speed to vigorous mixing. Add 3 L of cold distilled water. Heat to boiling. Cover and let stand overnight. Insoluble fibre will settle out. Decant gelled supernatant. Reserve insoluble matter for regular ND extraction (4.6.3).

4.6.7. Micro procedures

The chief reason for resorting to micro procedures is in the economy of reagents, and availability of small sample quantities at hand, which may be very expensive in developing countries or because of limiting amounts of sample. This situation is apt to arise in the case of agronomic plant part separations or in the case of digestion balances with small animals such as mice, voles, hamsters etc.

Reduction in sample size will increase the analytical error however. Acceptable results should be obtainable with care in sampling, etc. down to a sample size of 100 mg. It is important to keep the ratio of sample to reagent volume within the limits of the regular procedure. Under these conditions, no significant effects of reduction in sample and regent should occur. Mascarenhas-Ferreira et al. (1983) reduced volume to 50 ml and used a sample size of 250 to 500 mg with no significant effects. A reduction of one half may be done with regular equipment. However, greater reduction will require smaller beakers. The procedure of Waldern (1971) uses tubes. Care should be taken that reflux and agitation of sample in the reagent are maintained. The Reed modification (4.6.7.1) reduces reagents and a sample size by a factor of four.

4.6.7.1. Procedure of Reed (personal communication) reduction 4X

Equipment needed include 200 ml Berzelius beakers, suitable hotplates, 100 ml round- bottom flasks for condensers.

The procedure Is the same as 4.6.3 or modifications in 4.6.4 except that 25 ml reagent and a 250 mg sample is used.

4.6.7.2. Procedure of Mascarenhas-Ferreira et al. (1983)

The reduction 2X method is conducted on ordinary equipment. The Tecator fibre unit was used in the original work.

in this procedure, weigh 0.5 g ground sample into a fibre beaker or Tecator crucible and use 50 ml ND reagent.

4.6.7.3. Micro method of Waldern (1971)

Waldern specifies a 0.35 g of sample, but this could be reduced to 0.10 g or less if care is taken in sampling.

The same reagents as described in 4.5.1 and 4.5.3 are used, but decalin and sodium sulphite are eliminated from the procedure as described by Waldern (1971).

The digestion units consist of 50 ml universal lifetime red line Taylor tubes, 25 x 200 mm (see Figure 4.2). These tubes are placed in two aluminium blocks which are situated on top of a 2600 watt Lindberg heavy-duty heater, 50.8 x 30.5 cm. Each aluminium block is 25.4 cm wide x 30.5 cm long x 7.6 cm deep, and has 56 holes which are 26.0 mm in diameter, 58.5 mm deep and 61.1 mm from outside to outside of any two holes. A large marble, approximately 25.4 mm in diameter, serves as a condenser.

The sintered glass crucibles and filter discs are the same as described in 4.4.4.

Weigh 0.35 g of air dry sample, ground to pass a 1 mm screen, into a 50 ml Taylor tube. Add 35 ml of room temperature acid- or neutral detergent solution. Place a large marble on top of each tube. Slowly bring the sample to a boil (10 to 15 min) to avoid foaming. Boiling should be gentle to avoid pushing feed particles up the sides of

the digestion tubes. Feed particles collecting above the digestion fluid level should be returned to the boiling mass by gentle agitation of the tube while in the block, or by washing down the sides of the tube with a small amount of warm acid- or neutral detergent solution. Excessive cooling during the wash down will cause bumping and loss of sample. Continue the digestion for approximately 1 h with the temperature of the aluminium blocks maintained at approximately 124^{o}C. Maintain a constant volume of digesta during this period through the addition of appropriate volumes of warm acid- or neutral detergent solution. This step is very important in order to keep variation between duplicates at a minimum. In the subsequent steps of the acid detergent lignin procedure (Van Soest and Wine, 1968), permanganate was reduced to 15 ml.

4.7. Acid detergent fibre (ADF)

Extraction of plant residues with acid detergent was developed for the purpose of isolating the lignocellulose fraction and as a preparation step for lignin analysis on a residue freed from the interferences of protein (Van Soest, 1963). Further work disclosed that ADF also isolated biogenic silica and the obligately unavailable nitrogen produced in the Maillard reaction through heating of feeds.

The ADF residue is also correlated better than other fibre fractions with digestibility (Van Soest, 1994). While the association is empirical and subject to substantial errors, this feature, nevertheless, has led to its frequent use as a predictor of digestibility, although the association of ADF with other parameters of nutritive value (intake and efficiency) is not as good as NDF.

Another use of ADF is a rough partition of the insoluble cell wall into acid detergent soluble hemicellulose and the insoluble lignin and cellulose. While the crude arithmetic difference between NDF and ADF may be satisfactory in some cases, accuracy focuses on the interferences involved in the difference. Sequential extraction (4.8) will remove most of these (precipitated pectins, silica and soluble tannins). However, a more precise definition of hemicellulose requires component analysis for sugars to distinguish the pentose fraction, some of which obstinately remain in the ADF residue.

Some reports have promoted exclusively sequential extractions in obtaining ADF. This desire, based on the isolation of a purer lignocellulose, sacrifices recovery of biogenic silica that is soluble in neutral detergent but is recovered in ADF and some of the Maillard products which are partially soluble in neutral detergent. There is no universal sequence that is applicable to all samples and all needs. Sequential and parallel analyses are discussed in Section 4.8.

The residual pentose in ADF from grasses consists of xylose and a small amount of arabinose (Bailey and Ulyatt, 1970). These polysaccharides appear to be linear, less soluble and closely associated with cellulose and have a digestibility considerably lower than either the associated cellulose or the acid soluble pentosans (Gaillard, 1962; Lyford et al., 1963). This behaviour no doubt contributes to the association of ADF with indigestibility. Nevertheless, zealous definition of cellulose has led some to attempt their minimisation by increasing acid strength and prolonging the reflux in the modified acid detergent fibre (MADF) of Clancy and Wilson (1966). This modification which is not recommended, sacrifices the capability of assaying heat damage (through the nitrogen

content of ADF, which is also largely lost through the extra acid strength and time of boiling. The problem has been discussed in greater detail elsewhere (Van Soest, 1994).

Another modification of ADF is that of Baker (1977) where the strength of acid is reduced and buffered to recover more of the non-cellulosic residue. This procedure was intended more as a competitor of NDF, but it has not found favour.

On the other hand, ADF has been criticised for not recovering some of uncrystalline celluloses *i.e.*, glucose in cell wall, leading to a modification with reduced acid strength. These residues are inevitably higher in non-cellulosic polysaccharides mentioned above.

The ADF procedure is standardised on the normal acid extraction and is a compromise, since there is no one set of conditions that will satisfy all criteria. The dissolved "celluloses" are very highly digestible and more digestible than the insoluble carbohydrate. Criticisms for the failure of ADF to recover lignin is discussed in Section 5. Cellulose residue in ADF is virtually identical in content and yield with the other common gravimetric cellulose analyses (Crampton and Maynard, 1938).

Only one general methodology for acid detergent fibre is presented here and follows the recommendations of the AOAC Collaborative Study (Van Soest, 1973). Sequential analysis (preceded by neutral detergent extraction) does not alter the basic procedure. Decalin has been omitted (Van Soest, 1973). Corrections for filtering, drying and weighing should be observed.

4.7.1. Procedure

Weigh 1.0 g air dry sample ground to pass a 1 mm screen into a 600 ml Berzelius beaker. Add 100 ml acid detergent solution, place on a hot plate and bring to

boiling in 5 to 6 min. Adjust heat to an even boil, keeping the feed particles in suspension and reflux for 60 min from the onset of boiling.

Put pre-tared Gooch crucibles with filter discs on the filter manifold. After the 60 min reflux, remove the beaker; swirl to suspend the particles and filter, starting with minimum vacuum. Wash the residue at least 3 times with hot water, making sure all the acid has been removed (taste test is possible), twice with acetone and suck dry.

Dry the crucibles for 8 h or overnight at 105^{o}C in a forced air oven and weigh to obtain the yield of acid detergent residue. Residues should be ashed to determine residual silica (3.8).

The ADF yield is calculated in the same manner as the NDF (4.6).

Care should be taken in washing with hot water and acetone that lumping does not occur. Lumping (hard-to-breakup) will occur when the moist fibre is allowed to dry on the filter. Prompt application of acetone to dehydrate the residue will eliminate the problem and the acetone damp material will dry to a friable mat. This precaution is very important in the case of sequential lignin determinations, since lumping will cause inadequate extraction of the fibre by the lignin reagents.

Concern about apparently aberrant ADF values arises when the ADF is as high as, or greater than, the NDF. Such occurrence may be indicative of problems with a particular sample, but not necessarily an error. Where interferences are involved, sequential analysis is recommended (4.8). However, samples that are very badly heat damaged may be depleted in hemicellulose. Malliard products may be partially soluble in neutral detergent. This lack of recovery is likely related to incompleteness of polymer-isation of the Maillard intermediates.

Another case of higher ADF than NDF arises in plants with very high biogenic silica and low hemicelluloses, *viz*. Equisetum. Since biogenic silica is recovered in ADF but largely dissolved in neutral detergent, the anomaly of high ADF also arises. In both these examples, the high figure must be regarded as a true value. However, because of the large contents of ash and other non-carbohydrate components, the results cannot be directly used in the usual assessment of hemicellulose, lignin and cellulose. Parallel sequential analyses would be needed for this purpose.

Another cause of anomalous ADF values is the precipitation in acid solutions of organic compounds. Soluble at neutral pH, tannins, alginic acid, etc. are quantitatively recovered in the ADF. Pectic substances are variable in their solubility in acid detergent solution (Bailey and Ulyatt, 1970). These interfering substances may be removed by refluxing the ADF residue in neutral detergent solution for 60 min or by doing a sequential analysis (see below). No sodium sulphite should be used since it affects lignin recovery (Hartley, 1972).

The precipitation of tannins in the acid detergent procedure will result in elevated lignin estimates but, because they precipitate protein which is probably unavailable nitrogen, their inclusion in the ADF is justified when acid detergent insoluble nitrogen (see below) is determined.

Any errors in estimating the neutral detergent and/or the acid detergent residue could result in errors in the estimates of hemicelluloses, lignin and cellulose. There is some evidence in heat damaged feeds and in some pelleted products that Maillard complexes may be solubilised by ND due to the action of sodium sulphite, but recovered in the ADF residue.

4.8. Sequential and parallel analyses

Sequential analysis should be considered if there is a limited amount of sample available for analysis, or if it contains interfering substances, or if enrichment of the fibre content is required for the sequential ADF. The usual sequence would involve sequentially, refluxing in neutral detergent, refluxing in acid detergent, lignin analysis (permanganate or sulphuric acid) and ashing at 525^{o}C. Other sequences have been devised for special purposes such as biogenic (Van Soest, 1981) and soil silica (3.8) and removal of unknown insoluble inhibitors (Robbins et al., 1975). The alternative combination of sequences for lignin and cutin using combined sequential order of permanganate and 12 M H_2SO_4 is discussed in Section 5. The principle purposes of this section are to delineate sequences for the purpose of attaining estimates of carbohydrate fractions. These sequences are inevitably associated with component analysis and for understanding high values of ADF relative to NDF. This latter question is discussed in Section 4.8.1.

A summary of the types of analyses and their yield and results are shown in Table 4.9. The sequences by which they are most accurately obtained are shown in Fig. 4.6. Procedure for conducting sequential analysis are given in Section 4.8.2.

Table 4.9.
Summary of analysis using detergents

Fraction	Main components	How obtained
NDR	Hemicellulose, cellulose, lignin, bound protein	SLS, EDTA Borate pH 7.0 Boil 1h
ND solubles	Sugars, organic acids, starch, soluble protein, nucleic acids, pectin	Calculate by difference
ADR	Cellulose, lignin, silica	CTAB in 0.5 M H_2SO_4 Boil 1 h
Crude lignin	Lignin, cutin, Maillard products, (silica)	12 M H_2SO_4 of ADR for 3 h
Cutin	Cutin + some Maillard products	$KMnO_4$ treatment of 12 M acid residue
Cellulose	Pectin-free if sequential ND-AD sequence contains about 150 g/kg pentosan	Organic residue after $KMnO_4$ corr. for cutin
Hemicellulose	-	Difference between NDF and sequential ADF. May need to correct for ash and CP

ADR: acid detergent fibre residue; NDR: neutral detergent fibre residue; CTAB: cetyltrimethylammonium bromide; SLS: sodium lauryl sulphate; EDTA: ethylenediaminetetraacetic acid.

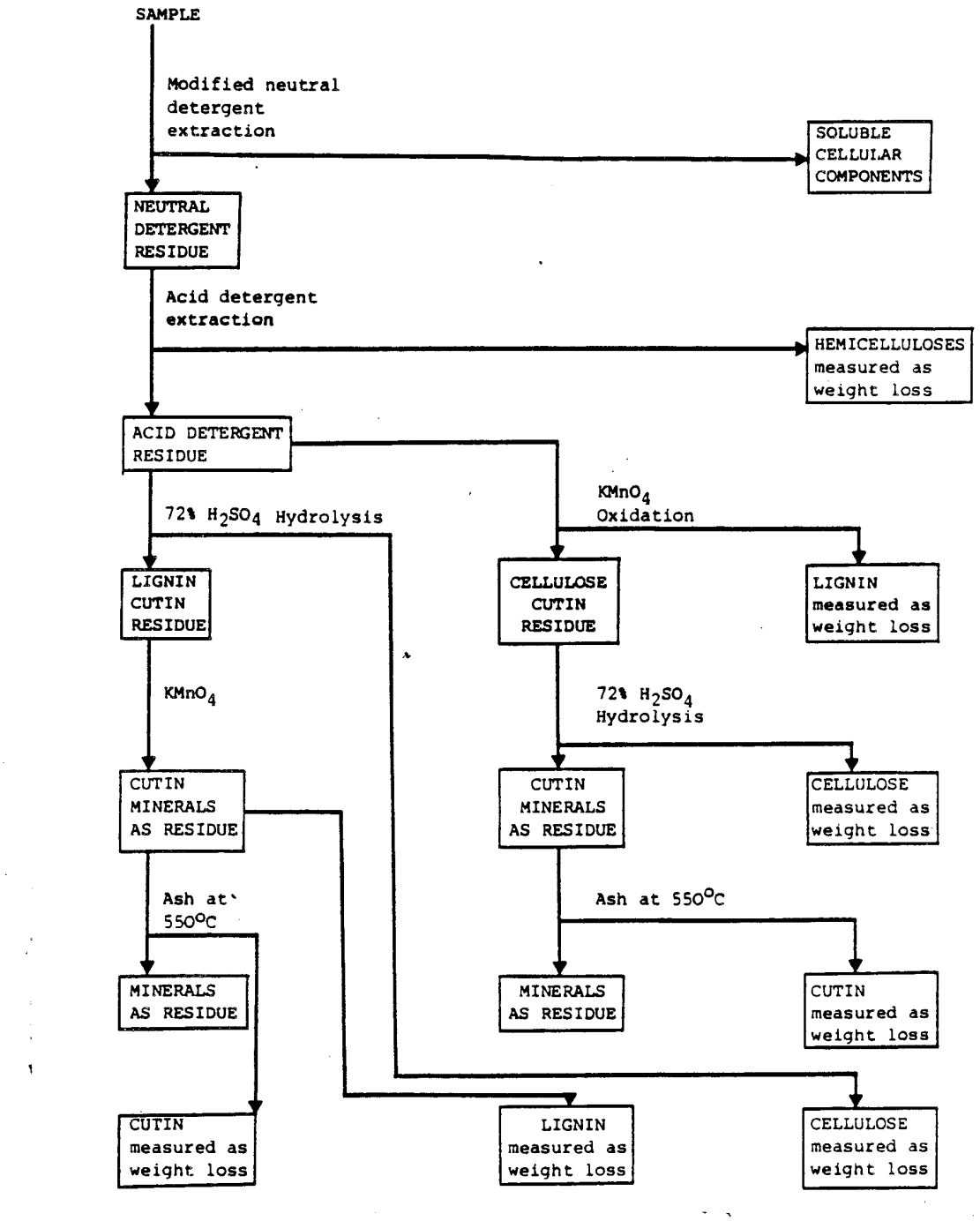


Fig. 4.6. Flow diagram for sequential analysis (Robertson and Van Soest, 1981).

The principal problems to be resolved by sequential analysis are the interfering components in ADF that affect the value of hemicellulose as a difference between NDF and ADF. These can be pectin (polygalacturonic acid) silica, protein and tannins.

Protein can be adjusted in subtracting nitrogen times 6.25. The remaining problem is the pectin and biogenic silica which are least soluble and recovered in ADF but not in NDF. These lead to diminishing the difference between NDF and ADF and the crude estimation of hemicellulose. Protein, in contrast to silica and pectin, tends to be higher in NDF and thus widens the NDF-ADF difference. For statistical purposes, the difference may be an adequate estimate of hemicellulose because of the compensatory effects of protein with silica or pectin.

The cellulose value from direct ADF is affected by pectin which elevates the value. Sequential analyses will correct for this. If cellulose is estimated by ADF- lignin, the cutin will be eliminated if the lignin(sa) method is used, but left in the residue if $KMnO_4$ is used. Sequential lignin analysis with 12 M sulphuric acid and $KMnO_4$ eliminates this effect.

Sequential analysis is an alternative to direct analyses, but there is no one adequate sequence. For example, total biogenic silica is recovered in ADF but largely dissolved in ND. The ND treatment tends to recover silica from soil contamination. On the other hand, the sequential procedure yields a cleaner value for hemicellulose and cellulose that is uncontaminated with poly-galacturonic acid from pectin. Generally, grasses are siliceous and contain very little pectin, such that the direct analysis is preferred. Dicotyledonous plants including legumes are seldom silicified, although there are some rare species of this type. Hence, sequential analysis may be a better routine for most of these kinds of samples.

4.8.1. The significance of higher values of ADF over NDF

Various factors contribute to the elevation of ADF relative to NDF in a feed or forage sample. Among these already discussed are pectin and silica. Some samples

such as citrus pulp have nearly identical NDF and ADF values. This occurs because hemicellulose in citrus pulp is very low while pectin content is very high. Another factor is heat damage in which the Maillard reaction consumes hemicellulose, converting it along with protein into artefact lignin. Severely heat damaged silages, particularly legumes which are low in hemicellulose anyway, may have ADF even higher than NDF because some Maillard products soluble in NDF have acidic tanning properties and are thus less soluble in AD.

Discrepant NDF and ADF values can occur, but it should always be checked that the result is not due to analytical errors, for in most feedstuffs and foods, it is an unexpected result. Sequential analyses are the means of ascertaining the true state of the sample.

4.8.2. Procedures for conducting sequential analysis for NDF and ADF

The diagram for sequential analysis is provided in Fig. 4.6. There are several systems for handling sequential extractions. The simplest requires no change is the FOSS Fibertec™ as samples are refluxed in their respective crucible and no transfer is required. This system is a development from the earlier Tecator Fibertec™.

The second preferred system is to place the filter crucible containing NDF on its side in a large enough Berzelius beaker where it is refluxed with AD. At the end of the reflux, the crucible is fished out, rinsed for any fibre clinging to outside or bottom, and the suspension is filtered through the crucible and disc on a filter manifold in the usual way. All other sequences (Table 4.9) are treated similarly.

5. Indigestible Components

Most of the analyses conducted for the purpose of assaying nutrient availability in fact measure the unavailable: a paradoxical endeavour which nevertheless recognises the true situation relative to cause-effect, since dietary organic matter would be generally available to digestion except for the existence of inhibitory and limiting factors.

Under the general heading of unavailable substances that affect the availability of other components (insoluble plant cell wall) is lignin, which has received the largest share of attention in this matter. However, lignin is not the sole factor affecting the extent of digestion nor is crude lignin itself a uniform single substance in a nutritional sense (discussed further in this section). Lignin mainly affects the maximal extent of digestion by setting a limit, while other aspects of digestion may involve losses of potentially available substances through competition between passage and slow rates of digestion. In addition to lignin, the cuticle similarly sets a barrier to digestion and affects perhaps both rate and extent. Tannins are easily confounded with crude lignin measurements and certainly are inhibitors thus having an influence on rate, but also they form leather which is undegradable and thus represents a further limitation upon extent. Biogenic silica is also a limiting factor in silicious grasses (Van Soest, 2006).

Since insoluble lignin protects a definite fraction of the plant cell wall matrix from digestion, this obligately indigestible residue is of interest both as a predictor of indigestibility and its use as a marker. This fraction can only be estimated by *in vitro* rumen digestion. These *in vitro* rumen methodologies are discussed in Section 7. Other indigestible fractions include acid-insoluble ash and silica are discussed in Section 3.

Defining lignin has been an elusive endeavour. As more sophisticated chemical methods have been employed, what is known about the complexity of lignins has evolved. This literature is not coherent and contains confusion (5.4). Conceptually, lignin is the phenolic polymer that gives rigidity to plant cell walls and offers resistance to rumen digestion. Because it is a condensed non-hydrolysable polymer, structural detail is difficult to ascertain. Three phenylpropanoid alcohols (p-hydroxyphenyl, guiaiacyl and syringyl) units are primary building blocks of wood lignins (Grabber, 2005). Forages are more complex, particularly grasses that contain cross-linked phenylpropanoid acid esters, particularly of p-coumaric and ferulic acids. This requires an extension of the conventional definition of lignin (Ralph et al., 2004). Forage lignins also contain nitrogen not present in any important amounts in wood lignins. Another problem is where to place the monomers that are obviously the materials for the biogenic polymer. Ralph and Helm (1993) do not consider them as lignin, which for them is a polymer. On the other hand, Klason lignin may very well include them, although accurate information is lacking.

5.1. Klason lignin (KL)

The KL is the oldest of the existing lignin methods and was developed for wood. The basic procedure, as currently conducted, is removal of extractives with boiling alcohol and chloroform followed by treatment of the dry residue with 12 M sulphuric acid. The sulphuric acid paste is diluted to 0.5 M and boiled for another time, then filtered, dried and weighed. The KL residue is corrected for ash. The purpose of the secondary boiling is to condense and precipitate otherwise soluble phenolic

components (Theander and Westerlund, 1986). Various versions for KL are described by Whitehead and Quicke (1964) with differing times of the secondary boiling. More recently, autoclaving has been substituted for boiling (Jung et al., 1997).

The lignin product is altered from that in the native cell wall, and any non-lignin substances that are insoluble in 12 M H_2SO_4 will automatically contaminate the residue and lead to high values, which is the most common problem with lignin determinations in forages and other green plants that contain a wider variety of substances than mere wood. In short, wood-based methods cannot be directly applied to green plants, and a long history of pre-treatment methodologies exist with the object of reducing the interferences, the most serious of which are protein and Maillard products.

Since insoluble minerals (particularly silica) are also likely to isolate with lignin, the value is determined as the organic matter lost upon ashing and, therefore, all of the problems and precautions applying to ash determinations (3.5) must be considered. The principal practical problem of the use of silicious filter aids (not recommended) such as asbestos, celite and glass wool, is the irreversible loss of water upon ashing that will contribute to an apparent lignin value (Section 3).

The Klason method is based on the insoluble organic matter in 12 M sulphuric acid, which defines the product obtained, like fibre and other similar residues, and no proper standards exists. Since this is a defined procedure, standardisation of conditions must be rigorous: the concentration of H_2SO_4, and the time and temperature of treatment must be controlled. However, the Klason procedure varies in the literature and has so far not been collaborated by the AOAC.

The ideal acid strength is that which will remove cellulose without alteration of the lignin. This optimum is likely a compromise, since lower acid strength yields high values and high acid strength low ones. The traditional concentration generally adopted is 12 M (24 N) which corresponds to a 720 g sulphuric acid/kg with a specific gravity of 1.634 at 20°C.

The exposure of lignocellulosic residues to strong sulphuric acid, causes slow degradation of lignin and carbohydrate even at room temperature. Hence, the time of treatment needs to be regulated. The optimum time at 20°C appears to be near 3 h (Fig. 5.1).

The rate of any chemical reaction is a function of the absolute temperature. Some reactions will not proceed unless a threshold temperature is reached (3.5). For solution of cellulose by sulphuric acid, the threshold is on the range of 0 to 15°C. While reaction of lignin with the acid is slow at these temperatures, it rises slowly above 25 to 30°C. Thus, the range of temperatures suitable for separation of cellulose from lignin lies between 15 to 25°C (Fig. 5.1), a temperature range that yields minimum values that are the least perturbed by interferences.

The lignin prepared by 12 M H_2SO_4 at 20°C is nevertheless chemically altered from that in the native plant. Absorption in the ultraviolet is significantly altered and the prepared lignin is sulphonated as evidenced by a significant sulphur content not present in mature lignin. Some part of the lignin seems to be lost since yield is lower than that obtained by the permanganate ($KMnO_4$) oxidation procedure. This recovery may well be high relative to the real situation since it may be partly compensated by the sulphonation reactions.

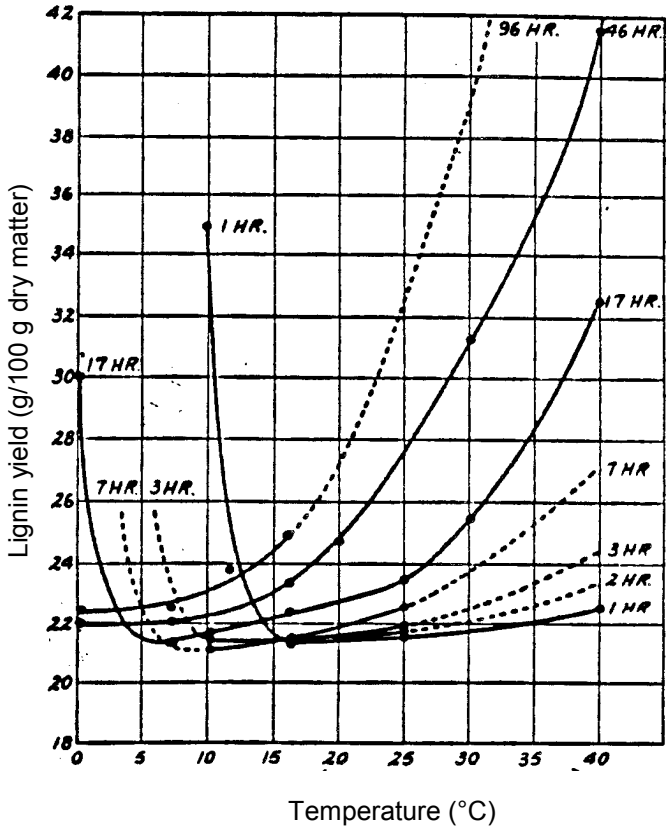


Fig. 5.1. The relationship between yield of Klason lignin and temperature of the 12 M sulphuric acid (Ritter et al., 1932). Low temperatures give high values due to inefficient removal of carbohydrate. Higher temperatures also give high results because of the reaction of carbohydrate degradation products with lignin and sulphuric acid (Norman and Jenkins, 1934a

5.2 Development of acid-detergent lignin - lignin(sa)

The lignin(sa) assay was developed in the early 1960's to provide a more rapid method for fibre residues low in nitrogen and was then named acid detergent lignin (ADL) (Van Soest, 1963) and accounted for NDF digestibility (Van Soest et al., 1963, 1973). There is also a modification using permanganate that also measures cellulose and silica (Van Soest and Wine, 1968) (see Section 5.7). Earlier antecedents are

procedures of Moon and Abou-Raya (1952) and Sullivan (1959) who abandoned boiling of the dilute sulphuric acid in the second stage of the KL. The lower values obtained by lignin(sa) as compared to KL was regarded as a loss of lignin and a serious fault by current lignin chemists, nevertheless, lignin(sa) remains the mainly used method in the animal nutrition literature.

5.2.1. Procedure for lignin(sa)

This treatment must be applied to a prepared fibre residue that is as low in nitrogen content as possible and filtered to recover fines (Section 4). The original procedures utilised asbestos as a filter aid. This has been abandoned with the discovery of the health hazard of asbestos. No filter aids are used, since they have been associated with interference in the lignin value (Van Soest, 1973). The lignin(sa) procedure is approved first action and is in the Methods of Analysis (AOAC, 1975).

5.2.2. Equipment

MCA volumetric flask(s) [2000 ml] are volumetric flasks made of Pyrex® with a bulb in the neck above the calibration line. The tare weight of the flask which is needed at various points in the preparation of 12 M sulphuric acid is conveniently etched onto the surface of the flask.

Trays, photographic or beakers [100 ml] are needed to hold crucibles during treatments and collect the effluent acid as it drains. Trays are convenient for numbers of samples; beakers are used with individual crucibles or samples.

Stirring rods, short and fat in design of about 0.8 to 1.0 cm in diameter and about 7 to 8 cm long are needed. They can be made of glass, but are better made from Teflon as this material does not erode the bottom plates of crucibles as glass rods do.

An air conditioner and a laboratory cart to hold trays near each other. Air conditioning device is for maintaining a temperature near 20 to 22°C. Temperature regulation is most conveniently managed by placing trays holding samples on a cart near a window air conditioner. Cart is moved closer for lower temperature, further away to increase it.

A thermometer: with a range of 0 to 50°C.

A op-loading balance of 5 kg capacity with a 0.1 g sensitivity.

5.2.3. Reagents

Sulphuric acid 720 g/kg, 12.000 molar, specific gravity 1.634. This solution is prepared by careful dilution of concentrated acid in the following manner: For 2000 ml the total wt = (2000 x 1.634) 3268 g. Reagent grade sulphuric acid is labelled with the manufacturers' assay on label (usually 950 to 980 g/kg). The remainder is water. For a 2000 ml 12 M solution, 2354 g of anhydrous H_2SO_4 are needed (2 x 12 x 98.08). The required amount of reagent grade is obtained by dividing 2354 by the assay. This weight is subtracted from 3268 g to obtain the required weight of water (about 800 to 850 g). The calculated quantity of water is weighed into the pre-weighed flask. Place the flask in a sink made of stone or other acid resistant material. Add concentrated H_2SO_4 in 50 to 100 ml portions, mixing carefully by gently swirling after each addition. Take care that the solution does not boil. This may happen if too much acid is added at once. After 4 or 5 additions, the reaction becomes less violent and larger amounts can be added. Fill flask up into the bulb above the meniscus. Cover with a 250 ml beaker. Cool under tap water to room temperature (ca. 30 min). Remove from the sink. Dry outside of flask with paper towels. Place on balance and add sulphuric acid to a total weight of 3268 g

plus tare. Stopper and mix contents by inversion. Measure temperature. Bring temperature of contents to 20±0.5C° by using either warm or cold tap water. If cold water is above 20°C, place flask in a refrigerator. (Always mix before re-measuring temperature.) When flask is near 20°C, note position of meniscus relative to the 2000 ml calibration mark.

Standardisation of the acid is satisfactory and requires no adjustment if the meniscus is within ±5 mm of the calibration mark. If the concentration is too high (meniscus is below the calibration), estimate the volume required to fill to the mark. For each 3 ml needed remove 5 ml of contents[1]. Replace flask on balance and add water back to the required weight (3268 g + tare). Remix, cool if necessary and compare meniscus and calibration mark at 20°C.

If the concentration of the flask is too low (meniscus is above the calibration mark), note the volume in excess above the calibration mark. For each ml in excess remove 8 ml of contents. Replace on balance and add concentrated H_2SO_4 to the correct total weight (3268 g + tare). Mix, cool if necessary, remix and compare meniscus and calibration mark at 20 ± 0.5°C.

5.2.4. Procedure

Place crucibles containing prepared fibre in a tray. Place a stirring rod in each crucible. Place the thermometer in the tray. Add enough chilled 12 M acid from a 400 ml

[1]The specific gravity of the flask contents are very near 1.634, while water is 1.000 and concentrated H_2SO_4 is 1.84. Thus, the volume ratio of 12 M sulphuric acid to water is about 8:5 (1.000 divided by 1.634) and for 12 M acid to concentrated acid 8:9 (1.634 divided by 1.84). Thus if the concentration is too high, 8 ml of water must replace each 5 ml of contents to achieve a 3 ml increase in volume. The net weight must be kept constant. When the concentration is too low, 8 ml of concentrated acid must replace each 9 ml of contents for each ml that the solution is above the meniscus.

beaker to moisten the fibre. Acid is pre-chilled to 15°C to avoid slight overheating at the first step.

Stir the acid and fibre into a paste. Add a little more acid. Stir more to break up all lumps making sure acid is in contact with any fibre particles high on the walls of the crucible. Add acid until the crucible is about half full, mix and allow to stand at 20°C. After 1 h, fill crucibles to the half level again. Repeat at the second hour. After 3 h, place crucibles on the vacuum manifold (4.4.5) and suck off excess acid. Wash with portions of boiling water rinsing sides of crucible; shut off vacuum to allow lignin to soak. Restore vacuum after 20 sec. Repeat until crucibles are free from all acid. Dry crucible at 105°C. Weigh[2] and place in muffle furnace and ash for 3 or more hours at 525°C.

Remove crucibles when the temperature of the furnace has dropped below 250°C and place in 100°C oven and weigh[2] after 30 min. Weight of crude lignin is represented by the loss in weight upon ashing. Weight of mineral matter remaining in the crucible is acid-insoluble ash (3.8.2).

5.3. Faecal recovery

The lignin(sa) is the only method that so far has been tested for nutritional uniformity (Lucas test) and faecal recovery (Van Soest, 1994). An early test (Van Soest, 1967) indicated near zero true digestibility, and a more extensive examination with 279 digestion balances, based on the data of Giger (1985) indicated an average recovery of 0.86 (Fig. 5.2). Lack of recovery may not mean digestion, because sintered glass filters

[2]Alternatively, at this point the hybrid permanganate procedure (5.7.1) may be followed.

with a 40 µm aperture do not accomplish a complete recovery of the fine particles (Udén, 2006; Raffrenato, 2011). Problems also occur in polyester bags.

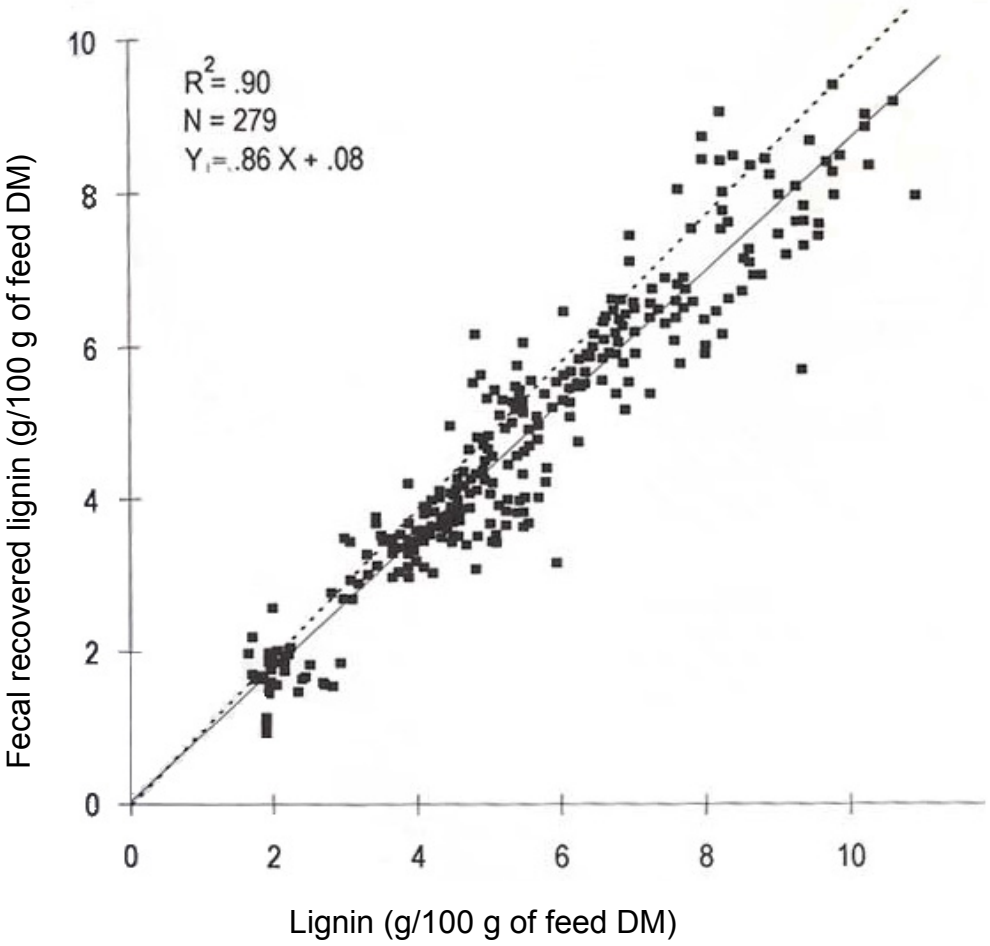


Fig. 5.2. Lignin balance data calculated from Giger (1985) presented in the form of a negative Lucas test (p 360 to 363 in Van Soest 1994). Recovery (indigestibility) of lignin(sa) is 860 g/kg (solid regression line). The dashed line represents complete recovery. See p. 191 in Van Soest (1994).

These problems arise because lignin in feed and forages is selectively distributed in larger particles, whereas, in faeces lignin is contained in the finer particles (p 242 in Van Soest, 1994). Muntifering (1982) found variable faecal recoveries with lignin(sa).

Permanganate lignin is also poorly recovered (Chestnut et al., 1986). The limitation of permanganate to oxidise samples of higher lignin content may be due to a requirement of longer reaction time (Rowland and Roberts, 1994).

Large variation is seen in apparent digestibility of lignin(sa) at low concentrations in immature forages. This occurs because the average standard error in a lignin(sa) determination was about 3 g/kg in a collaborative study (Van Soest, 1973). However, variation is likely larger, (*e.g.,* 5 g/kg when no standard samples are compared). Thus, in a forage with 20 g/kg of lignin(sa), the error in its apparent digestibility will be on the order of a least a factor of 0.25 with bias toward lower recoveries. Practical use of lignin(sa) as a marker in feeds requires at least 50 mg/kg.

5.3.1. Ultimate extent

The lignin(sa) has been related to the ultimate extent in the studies of Chandler et al. (1980) who fermented waste residues for 60 or 90 days in methane fermenters. A mean ratio of undegraded NDF to initial lignin(sa) content was 2.36 ± 0.41. However, inclusion of the glass microfiber filter (4.4.4) has produced higher ratios (Huhtanen et al., 2006; Raffrenato, 2011).

5.4. Comparison of lignin(sa) *and KL*

The KL value is assumed by many lignin chemists to represent true total lignin (Hatfield et al., 1994; Lowry et al., 1994; Hindrichsen et al., 2006). It gives higher values than the lignin(sa) (about 2 to 5 times higher), particularly in grasses (Huhtanen et al., 2006; Gomes et al., 2011; Raffernato, 2011). The material in the difference between KL and lignin(sa) (Δ L) is polyphenolic (Lowry et al., 1994). It may represent less

polymerised fractions, such as the soluble lignin complexes in rumen fluid (Galliard and Richards, 1975; Neilson and Richards, 1978). The KL has not been examined to the same degree as lignin(sa) for faecal recovery, although considering the conditions, its recovery is probably high (Lowry et al., 1994). However, exactly what is recovered in the KL has not been examined (recovery of monomers, for example). On the other hand, KL and ADL will certainly contain tannins and cutins (Van Soest, 1994).

Gomes et al. (2011) compared tropical grasses and legumes. Permanganate lignin and lignin(sa) ranked high. However, KL showed separation of legumes and grasses with different regressions. Correlations are heavily influenced by interactions between plant families. The literature on lignin methods is confused by the different purposes for which the procedures are intended. Recovery of phenolics is the intent of KL, practical application to forage quality in the case of lignin(sa). Reproducibility and convenience of procedure are other issues. Lignin(sa) has been collaborated by the AOAC (Van Soest, 1973), whereas KL has not been collaborated. The comments of Rowland and Roberts (1994) are pertinent.

> " – the procedure in operation in this laboratory is a method based on Klason [*sic*] lignin which is rather difficult to reproduce without favourable laboratory conditions and considerable experiences. It could not therefore be recommended."

On the other hand, these authors consider lignin(sa) to be reliable and free from operator bias and easy to implement.

Gaillard and Richards (1975) and Neilson and Richards (1978) found soluble carbohydrate-lignin complexes in rumen fluid of cattle fed speargrass. They estimated that the complex represented on the order of half of the KL in this tropical grass, a value roughly of the same magnitude as recovery of ΔL in the lignin(sa) and acid pepsin-insoluble matter in the Tilley-Terry *in vitro* procedure (Jung et al., 1997; Table 5.1). These complexes appear to be polymers containing some carbohydrate and protein (Conchie et al., 1988) and are thought to pass undigested into the faecal neutral detergent solubles (Lowry et al., 1994). Other papers reporting enzyme-soluble lignin include Akin and Benner (1988), Grabber and Jung (1991), Grabber et al. (2002), and solubilisation by rumen fungi (McSweeney et al., 1994). Lowry et al. (2002) have also found neutral detergent soluble lignin complexes which appear to limit gas production during *in vitro* fermentation. Lignin-related phenolics released by enzymes decline with forage maturity (Hartley et al., 1974), as shown in Fig. 5.3.

This low weight fraction is likely to be positively associated with digestibility in contrast with the more insoluble lignin (Hartley, 1972). The soluble fraction is also elevated in the brown midrib mutants of corn that have a higher digestibility (Hartley and Jones, 1978). The suggestion has been made that this low weight fraction comprises unpolymerised lignin that has not been converted to a mature high molecular weight product, polymerisation being required for the antiquality effect.

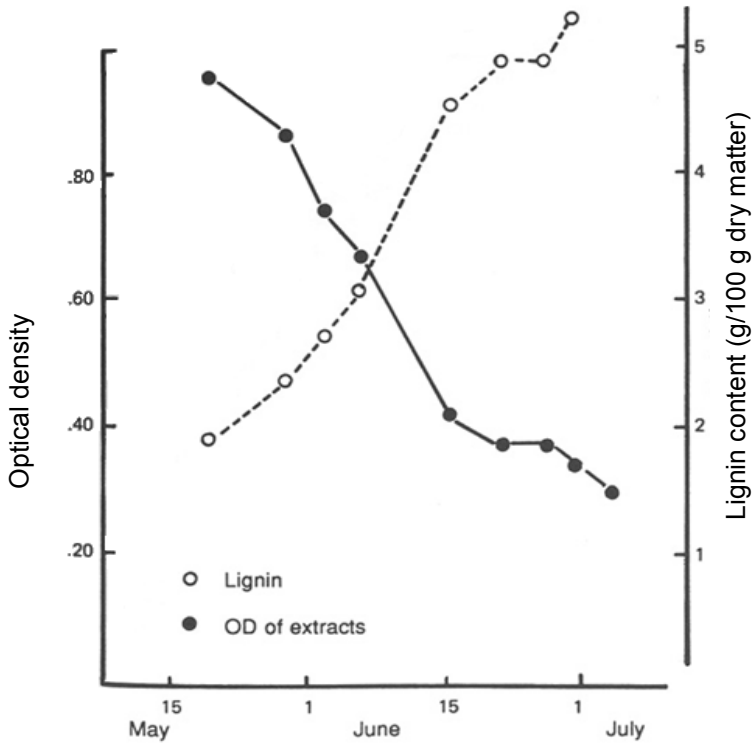


Fig. 5.3. Relation of ferulic acid and p-coumaric esters released by cellulase as measured by UV absorption of extracts (●—●) in data from Hartley et al. (1974). Other curve (○- -○) shows lignin(sa) content changing with date of cutting of ryegrass (p.185 in Van Soest, 1994).

Jung et al. (1997) presented a comparison of 36 forages with 48 h *in vitro* digestion, and for 20 of these forages, sheep *in vivo* digestion values were available. Values of KL and lignin(sa) on a dry matter basis were correlated with the digestibility of NDF on an NDF basis. Correlation coefficients in their paper did not indicate any advantage of either method. However, recalculation of their decoded data to a common denominator (NDF basis) gave a different result (Tables 5.1 and 5.2).

Regression of NDF digestibility on lignin on a dry matter basis resulted in an interaction between NDF and dry matter that confounded the slope of lignin on NDF matter. Further, the use of lignin in practical feed models (CNCPS) utilises lignin on an

NDF basis. For these reasons, the figures in Jung et al. (1997) were enlarged, placed on a grid, and values of dry matter and NDF digestibilities were decoded to the nearest part per hundred. Thirty-four of the *in vitro* values and 19 of the *in vivo* values could be identified. These were correlated with lignin contents of NDF for KL and lignin(sa), as well as the differences between KL and lignin(sa) (ΔL), all divided by NDF calculated from Table 1 in their paper, respectively. The correlations between these recalculated values of lignin contents and the decoded digestibilities of NDF are shown in Table 5.1. Correlations on an NDF basis are higher for lignin(sa) compared with KL. In the case of KL, only the total population is significant. Apparently, the interaction between NDF and dry matter improved correlations for KL in the original work of Jung et al. (1997). Correlations with the differences (ΔL) tend to be positive. The total population for *in vitro* is highly significant if the two outliers are omitted.

Table 5.1.
Correlations among lignins as proportion of NDF and digestibilities of NDF *in vitro* and *in vivo*[a]

		Lignin		
Class	N	Lignin(sa)	KL	ΔL[b]
In vitro data				
C3 grasses	15	-0.84**	-0.22	0.27
C4 grasses	8	-0.94**	-0.17	0.42
All grasses	23	-0.82**	-0.16	0.29
Legumes	11	-0.77**	0.19	0.72*
All forages	34	-0.75**	-0.53**	0.50**[c]
In vivo data				
Grasses	13	-0.53*	-0.63*	-0.27
Legumes	6	-0.72	-0.27	0.45
All forages	19	-0.73**	-0.72**	-0.13

[a]Calculated from the digestibility of NDF reported in Jung et al. (1997) and reconstructed from the figures in the paper; [b]ΔL, the arithmetic difference between KL and lignin(sa) divided by NDF, calculated from the Table 1 in Jung et al. (1997); [c] two

outliers omitted (>two standard deviations from the regression line), which if included, yielded a correlation of 0.42*; *0.05; **0.01.

Further calculations on the data of Jung et al. (1997) are shown in Table 5.2. Correlations of ΔL (dry matter basis) with *in vitro* dry matter digestibility were not significant, legumes giving positive values. However, correlations with M_i, the difference between true digestibility (based on NDF) and apparent digestibility (Van Soest, et al., 1966; Van Soest, 1994) are significantly positive for the *in vitro* data. The M_i values represent the material that is soluble in neutral detergent, but insoluble in acid pepsin in the second stage of Tilley-Terry *in vitro* procedure. Values for the *in vivo* data are also insignificant. Since lignin(sa) is contained within KL, co-association is responsible for some KL correlations.

Table 5.2.
Correlations of ΔL (Klason lignin - lignin(sa)) with apparent dry matter digestibility (DDM) and with metabolic matter (M_i) (dry matter basis) using data from Jung et al. (1997)

Class	DDM	M_i[a]
In vitro data		
C3 grasses	-0.37	0.71**
C4 grasses	-0.23	0.51
All grasses	-0.3	0.60**
Legumes	0.12	0.78**
All forages	-0.03	0.69**
In vivo data		
Grasses	-0.42	-0.3
Legumes	0.09	-0.74
All forages	-0.35	0.21

[a]Three outliers were omitted, two C3 grasses and one C4 grass. One C3 grass and one legume were missing from the figures; *0.05; **0.01.

The positive correlations between ΔL and M_i are further examined in Fig. 5.4. The regression shows a positive slope of 0.91, not different from unity, which suggests that the ΔL passes into the neutral detergent solubles, but is precipitated in the acid pepsin stage of the Tilley-Terry procedure along with undigested microbial cell walls. This observation is in agreement with Lowry et al. (1994) and Gaillard and Richards (1975) who found soluble carbohydrate-lignin complexes in rumen fluid. Lowry et al. (1994) suggest this as the fate of the acid detergent dispersible lignin (see Section 5.5), to pass into the neutral detergent soluble part of the faeces. Thus, it is likely that ΔL does not limit the extent of NDF digestibility, but instead becomes soluble, depressing the apparent digestibility of neutral detergent solubles. The substances soluble in AD, but likely occurring in KL, include phenolics, small amounts of protein and carbohydrate (Gaillard and Richards, 1975).These observations suggest that KL is not nutritionally uniform, and consists of at least two fractions differing in the manner in which digestibility is reduced.

All publications by authors that promote KL assume lignin to be a uniform inhibitory substance. Yet evidence here is otherwise. The soluble lignin that is released by cellulolytic enzymes and which occurs in the rumen is an overlooked problem. Release of soluble lignin might affect rate of cell wall digestion, but to account for effect on extent of insoluble fibre is more problematic. From physicochemical principles, degradation of an insoluble substrate would require formation of a complex with the substrate for which there is no evidence. The positive correlations of digestibility with ΔL (Table 5.1) suggest that soluble lignin has no effect upon extent. The ultimate indigestibility of NDF is limited by insoluble lignin.

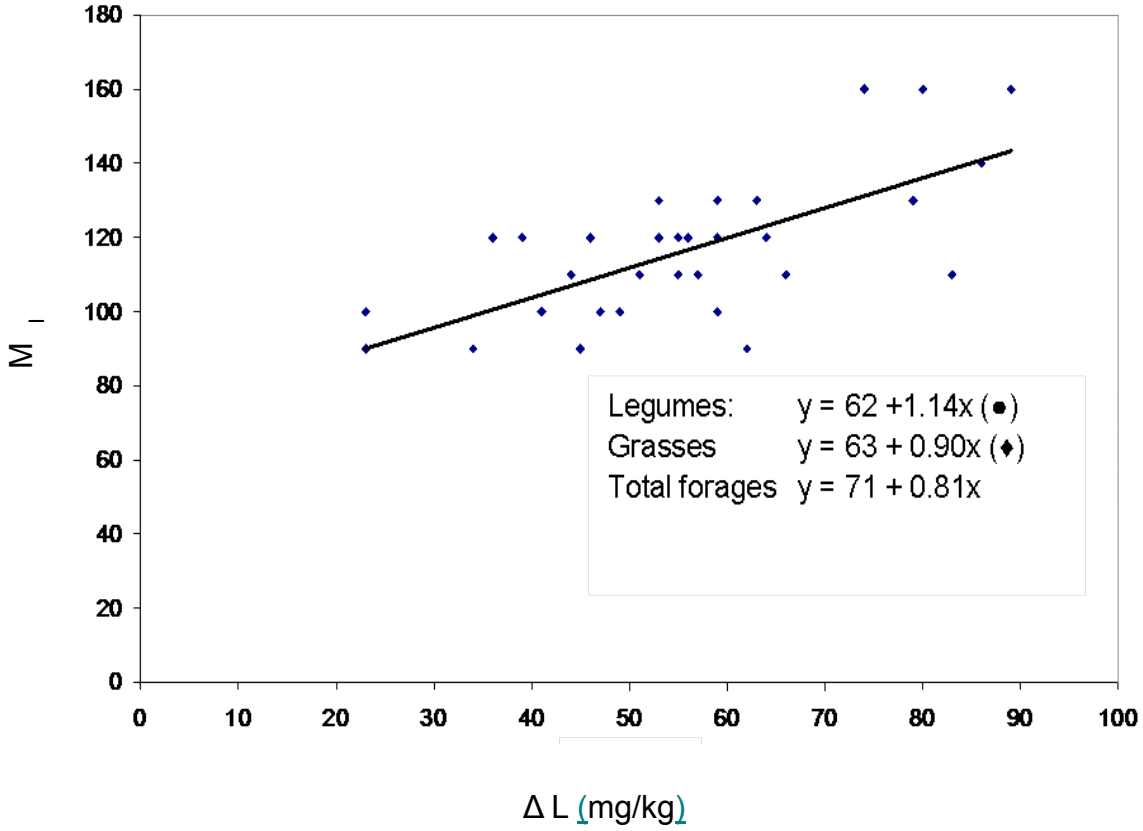


Fig. 5.4. Relationships between ΔL (Klason lignin - lignin(sa)) and Mi for grasses and legumes from *in vitro* digestibilities, recalculated from data of Jung et al. (1997). See Table 5.2 for correlations. Slopes are not different from unity. Three outliers were omitted. Mi is the arithmetic difference between apparent digestibility of dry matter and the digestible amount of NDF (NDF x DNDF) and represents metabolic matter.

5.5. Acid detergent dispersible lignin (ADDL)

Lowry et al. (1994) have published a procedure for ADDL that measures the absorbance at 280 nm of the phenolics dissolved by acid detergent. These authors determined that ADDL is indeed largely polyphenolic including lignin-like polymers. Presumably this soluble polyphenolic fraction is related to ΔL but no compositional data

were presented. There are no other studies in the literature that have used this procedure.

Lowry et al. (1994) suggest that the ADDL represents soluble fractions not digested in the rumen and recovered in undigested neutral detergent solubles, in agreement with our analyses of the data of Jung et al. (1997). This is also the case of alkali-treated straws where cleaved lignin becomes soluble in neutral detergent and likewise reduces digestibility of neutral detergent solubles in treated straws (Lau and Van Soest, 1981; McBurney, 1985).

5.6. The problem of nitrogen

Classical lignin structures do not allow for the presence of nitrogen. However, all forage lignins contain some nitrogen. Early forage chemists, as well as current lignin chemists, regard the source of nitrogen as protein contamination (Reeves, 1993). Much effort has been expended to remove it (Norman and Jenkins, 1934b; Ellis et al., 1946; Whitehead and Quicke, 1964; Gomes et al., 2011), either by subtraction of nitrogen times 6.25, or by pre-treatment with proteolytic enzymes such as acid pepsin (Reviewed by Whitehead and Quicke, 1964). None of the procedures using enzymes, or indeed the acid detergent extraction, remove all of the protein from isolated lignins. It is suggested that amino acids isolated from lignin(sa) are somehow covalently bonded to lignin (Brinkmann et al., 2002). Lignins also have an apparent ability to adsorb heterocyclic amines in the gut (Funk et al., 2006). Several papers have examined the nitrogen contents of lignin(sa) (Van Soest, 1965; Reeves, 1993; Brinkmann et al., 2002; Ross, 2004) and amino acids have been recovered (Brinkmann et al., 2002; Ross, 2004).

Legume lignins have high nitrogen contents. The bonding in legumes is unclear, but might be peptide linkages involving possibly tyrosine, which has the same functional condensative sites on the phenyl ring as p-coumaric acid. This could explain how peptides could be incorporated into lignin and explain the higher nitrogen content of lignin(sa) in alfalfa and other legumes. The study of Conchie et al. (1988) indicates that peptides are very tightly attached to the soluble lignins and cannot be removed by trypsin or pepsin. Other studies (Van Soest, 1965; Chaudhry and Webster, 1993) indicate low digestibility of nitrogen in acid detergent fibre and is largely recovered in lignin(sa).

However, Hatfield et al. (1994) prepared freeze-dried alfalfa and grass forages to which various nitrogenous substances, such as bovine serum albumin, were added in the KL procedure without much effect of nitrogen content of lignin. Calculation of their data indicates up to a third of the ΔL in alfalfa may be Maillard polymers. The elevation of nitrogen content in lignin is linked to pre-treatments involving heat (Van Soest, 1965).

Higher nitrogen contents were due to pre-treatments, not the lignin procedure itself (Van Soest, 1965). The increase in nitrogen and artefact lignin is in part due to the Maillard reaction and includes phenolic matter created from the destruction of sugar and amino acids in the Maillard reaction (Popoff and Theander, 1972, 1976; Lau and Van Soest, 1981).

5.7. Acid detergent permanganate lignin and cellulose

Phenolic and unsaturated compounds are generally oxidised by permanganate at room temperature, while saturated substances including carbohydrate are attacked only

at higher temperatures or in strongly acidic solutions. Thus, buffered permanganate has been used as a lignin-specific stain in the microscopy of plant cell wall tissues and is a titrimetric method for lignin in wood pulp. This latter method has the disadvantage that the oxidation equivalent of lignin is obscure and the endpoint is arbitrary, such that the lignin value can only be quoted in terms of the amount of permanganate used per gram of wood pulp. In the procedure presented here, permanganate is used in excess to oxidise and destroy lignin, which is then measured as the weight loss in the ligno-cellulosic preparation. This characteristic is in common with several other methods that remove lignin which is then measured as a loss. These include oxidation with chlorine dioxide and dissolution of lignin in acidic triethylene glycol (Edwards, 1973).

The permanganate ion is reduced by phenolics in weakly acidic and neutral solutions to manganese dioxide in which manganese has the valence of four, thus gaining three electrons in the drop from valence seven in permanganate in the reaction:

$$2\ KMnO_4 + H_2O \rightarrow 2\ MnO_2 + 3[O] + 2\ KOH$$

Destruction of the strongly acidic permanganate ion results in a substantial rise in alkalinity, due to liberated potassium hydroxide. The manganese dioxide is very insoluble and is deposited at the site of lignin in the oxidised fibre. These conditions place two requirements on a gravimetric method: the oxidising solution must be strongly buffered, and all manganese (deposited mineral) must be removed from the delignified preparation.

The buffering is accomplished with the use of very strong acetic acid (3 M) in the oxidising solution. Since deposition of MnO_2 is necessary and cannot be prevented, the manganese is removed in a sequential demineralisation step. Thus, the treatment of the fibre residue divides into two stages.

The first stage oxidation step requires further control to prevent side reactions. Permanganate is unstable toward light and can autocatalytically decompose, especially in the presence of traces of halogen (chloride, for example). Silver ion is a good complexer and precipitant for halogens, and it also forms a complex with permanganate and appears to catalytically improve its oxidative capability. Deletion of silver from the permanganate reagent results in instability of permanganate, and in many cases manganese gels that are impossible to filter. Silver is also deposited in the fibre, and thus its level is limited to that below gravimetric detection. A small amount of silver ion also has the remarkable ability of stabilising the permanganate solution over time (Van Soest and Wine, 1968).

Further problems occur in the second stage where the fibre is demineralised with a mixture of oxalic acid and hydrochloric acid in 800 ml/L ethanol. The mixture of oxalic and hydrochloric acids were chosen for maximum speed in dissolution of manganese dioxide. The reactions of these acids are complex and involve the reduction of MnO_2.

This proceeds stepwise from tetravalent oxide to trivalent and to divalent manganese chloride:

1. $MnO_2 + 4HCl \rightarrow MnCl_3 + [Cl] + 2H_2O$

2. $MnCl_3 \rightarrow MnCl_2 + [Cl]$

3. $4H_2C_2O_4 + 2MnO_2\ (H+) \rightarrow Mn_2(C_2O_4)_3 + 2CO_2 + 4H_2O$

4. $Mn_2(C_2O_4)_3$ (H+) → $2MnC_2O_4 + 2CO_2$

Oxalic acid is the more powerful reductant but requires exogenous acid, since acid is consumed in the reactions. Production of too much manganous (II) ions can cause the precipitation of manganous oxalate (MnC_2O_4), which is very difficult to remove. Manganous oxalate is nearly white when formed, but decomposes into black Mn_3O_4 upon ashing. Hence, the interference is represented by unusually high residual ash of black colour. The oxalate precipitates when it occurs and usually exceeds the loss in oxidised lignin leading to apparently negative values.

Prevention of this problem depends on having sufficient ferric (Fe (III)) iron in the system which forms soluble complex oxalates with manganese (II). Thus, this interference can be avoided. Ferric iron co-deposits with MnO_2 if present in the first stage and is thus more conveniently included in the oxidation step, rather than at the second stage where it is operative. The objective at the second stage is to demineralise quantitatively, such that inclusion of a mineral ion in the reagent at this point is counterproductive and reduces efficiency of demineralisation. Thus, ferric nitrate is added to the Stage 1 buffer along with enough potassium acetate to prevent formation of free nitric acid through hydrolysis of the nitrate salt.

Demineralisation destroys all metal complexes with the cellulosic carbohydrates, and allows possible solution of small amounts of pectic acid and hemicelluloses that are associated with the cellulose. Such an occurrence would give inflated lignin values. To prevent this, the oxalic-hydrochoric acid mixture is made in ethanolic solution that will precipitate all polysaccharides. Too high an alcohol concentration must not be used since some water is required for the reduction of manganese and also for the desorption

of HCl from the cellulose. If too little water is in the reagent (as is the case for 950 ml/l ethanol), sufficient acid remains adsorbed that the cellulose and will char upon drying in the oven. To prevent this, about 200 ml/L of water is added to both the demineralising solution and the ethanolic wash that follows.

5.7.1 Method for permanganate lignin (lignin(pm); Van Soest and Wine, 1968)

The ordinary sequence of analysis is to oxidise acid detergent fibre with permanganate in crucibles in the same manner as in the lignin(sa) assay. After demineralisation and washing, the crucibles are dried, weighed and the loss in weight taken to represent lignin. Cellulose, cutin and acid-insoluble ash remain in the crucible. For many purposes, loss on ashing is taken to be cellulose although cutin is included. These can be separated by treating the residue with 12 M sulphuric acid (5.2).

5.7.2. Equipment

Same as for lignin(sa) (5.2) including cart, trays, air conditioner, thermometer and short fat stirring rods.

5.7.3. Reagents

Saturated potassium permanganate is prepared by dissolving 50 g reagent grade $KMnO_4$ and 0.05 g reagent grade Ag_2SO_4 in 1 L water. Keep solution out of direct sunlight.

To create the buffer solution: dissolve 6.0 g $[Fe(NO_3)_3]•9H_2O$ and 0.15 g $AgNO_3$ in 100 ml distilled water. Combine with 500 ml glacial acetic acid and 5.0 g potassium acetate. Add 400 ml tertiary butyl alcohol and mix. Use grades of acid and solvent passing the dichromate test.

Combine the two solutions by mixing 2 parts saturated potassium permanganate and 1 part buffer solution, v/v, before use. Prepare amounts as needed. Do not store. The mixed solution should not contain any precipitate and be purple in colour.

Prepare the demineralising solution by dissolving 50 g oxalic acid dihydrate in 700 ml 950 ml/L ethanol. Add 50 ml *ca*. 12 M HCl and 250 ml distilled water; mix.

Ethanol (about 800 ml/L) is prepared by mixing 155 ml distilled water and 845 ml 950 ml/L ethanol.

Use an acetone grade that is colourless and leaves no residue upon evaporation.

5.7.4. Procedure

Dry forage or food samples at <65°C and grind through 20 to 30 mesh. Prepare and determine acid detergent fibre on 1 g sample according to the standard procedure (4.7.1). Prepare a shallow enamel pan or photographic tray with 2 to 3 cm cold water. Combine and mix saturated potassium permanganate and buffer solution, 2:1, v/v, and add 25 ml to crucibles. Note that crucibles containing fibre of high lignin contents will require more permanganate solution. Immerse each crucible after addition in the pan of water. Adjust level of water in the pan immediately to prevent excessive flow of solution out of crucibles[3]. Place short glass or Teflon rod in each crucible to stir contents, to break lumps, and to draw permanganate solution up on sides of crucibles to wet all particles. Let crucibles stand at 20 to 25°C for 90 ± 10 min, adding more mixed permanganate solution as necessary. If crucible is full, filter solution by vacuum and add more reagent. Mixture must be purple at all times. Note that a brown hue without any

[3]Flow of permanganate solution through the crucibles must be carefully standardised, particularly in the case of immature grasses where a single addition of permanganate solution suffices. Fibre from immature grasses is very rapidly delignified and there is danger of loss of cellulosic carbohydrates if there is too much flow.

red or purple coloration indicates that permanganate is exhausted. However, avoid addition of more solution than is necessary. Remove crucibles to filtering apparatus and suck dry; do not wash. Place in clean enamel pan, and fill not more than half-full with demineralising solution. After 5 min, suck dry on filter apparatus and refill halfway with demineralising solution. Repeat after second treatment if solution is very yellow. Incomplete removal of lignin is indicated by a yellow colour in the centres of larger fibre particles. Rinse sides of crucibles with solution from wash bottle with fine stream. Let stand until fibre is white (total time required, 20 to 30 min). Fill and thoroughly wash crucible and contents with 800 ml/L ethanol. Suck dry and repeat wash twice. Wash twice in similar manner with acetone. Suck dry. Dry at 105°C overnight and weigh. Calculate lignin(pm) content as loss in weight from acid detergent fibre.

5.8. Cellulose and acid insoluble ash

The residue remaining after permanganate delignification is mainly cellulose although cuticular component remains in it. The values obtained upon ashing this residue are essentially identical to Crampton cellulose (Van Soest and Wine, 1968).

5.8.1. Procedure

The residue remaining after permanganate oxidation and demineralisation is ashed at 525°C as specified in procedure for total ash. The ash residue is acid insoluble ash and can be used for total silica determination (Sections 3.8.2).

5.9. Hybrid procedures

The utility of 12 M sulphuric acid and the permanganate reagents that remove cellulose and phenolic lignin, respectively, allow combining of procedures in a sequential way. If the cellulose residue from the lignin(pm) procedure (5.7.1) is treated with 12 M sulphuric acid, cutin remains as the non-oxidisable and non-hydrolysable portion of crude lignin. This is the original cutin procedure of Van Soest and Wine (1968). Alternatively, as suggested by Southgate (1981), the lignin(sa) residue from Section 5.1 can be treated with permanganate leading to a loss of phenolic lignin and a residue of cutin. In either case, the cellulosic fraction is obtained as a value corrected for cutin. The hybrid lignin procedure offers the advantages of both the lignin(sa) and lignin(pm) procedures as the lignin value is corrected for some of the inherent errors in the individual procedures.

Filter aids must not be used in either sequence, following the prohibition of asbestos in the Klason procedure. Most siliceous filter aids lose weight upon ashing leading to artificially high values (3.5.1).

5.9.1. Original procedure for cutin

The cellulose residue from 5.7.1 after drying and weighing is treated with 12 M sulphuric acid in the Klason procedure. The organic matter loss upon ashing is taken to be cutin. Residue in the crucible after ashing is acid insoluble ash and can be followed with a silica determination using the HBr procedure (3.8.3).

5.9.2. Acid detergent sulphuric – permanganate lignin

The crude lignin residue from 5.1 is dried and weighed and then treated according to the permanganate lignin procedure in 5.7.1. The loss after drying and

weighing is taken to be lignin and the residue consists of cutin and acid-insoluble ash. This residue is ashed at 525°C, and reweighed. Loss is taken to be cutin, and only acid-insoluble ash remains. Silica can also be determined on this residue with the HBr procedure (3.8.3).

5.10. Comparison of values: lignin(sa) *and lignin(pm)*

Permanganate lignin (lignin(pm) values are higher than those obtained by the 12 M sulphuric acid (lignin(sa) method. The ratio between values obtained by the two methods depends upon the material analysed. Nevertheless, for 75 comparisons, the correlation was 0.997 and the coefficient of variation was 0.072. The regression and scatter are shown in a proportional projection (Fig. 5.5). The slope of the regression of lignin(sa) on lignin(pm) was 0.81 with an insignificant zero intercept.

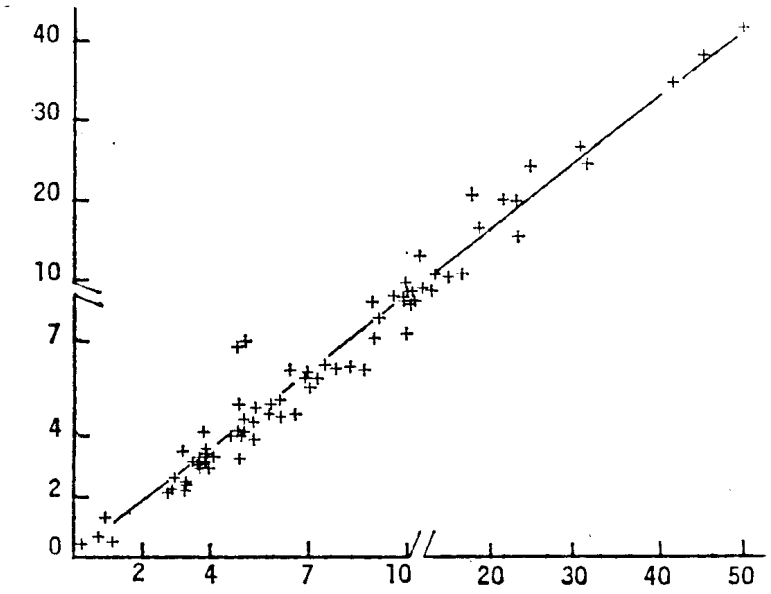


Fig. 5.5. Relation of permanganate lignin (lignin (pm)) with 12 M sulphuric acid (lignin (sa)) for 75 samples. Values by the two methods are very similar with a correlation of 0.992 and a coefficient of variation of 0.036. Certain materials yielded lower values by the permanganate method. Both celluloses contained a significant sulphuric acid-insoluble fraction.

Important differences between the methods arise from the fate of cutin, which is largely retained by the lignin(sa) method and is excluded by the lignin(pm) method. This leads to higher values of Klason lignin as compared with the permanganate lignin in seed hulls, barks, *etc*. that are high in cutin. Higher values for lignin(pm) are expected in forages and lignified tissues that are low in cutin.

Another comparison is that of the cellulose values which are obtained by difference. The values of cellulose from lignin(sa) will exclude cutin that remains with the lignin while permanganate cellulose will include it. The permanganate cellulose, being the residue after delignification, will contain it. This inclusion is also a characteristic of all celluloses prepared by oxidative delignification as, for example, Crampton cellulose which uses nitric acid as the oxidant.

The resolution to the cutin problem is to subject the residual cellulose to the lignin(sa) procedure. Thus, sequential treatment will give a cutin estimate.

Lignin(pm) may yield a value closer to a true theoretical lignin value, that is, it excludes some non-lignin components. It will also be affected by factors which also affect other lignin methods. Polyphenolic and other unsaturated substances such as tannins, pigments or proteins that may not be completely removed in the acid detergent fibre, will react with permanganate and appear as lignin(pm). Lignin(pm) is subject to increases by heating of the assayed material, as is lignin(sa). However, some observations show that this method may be somewhat less susceptible to that artefact (Southgate, 1981).

The lignin residue from the lignin(sa) method has a higher carbon content than carefully prepared lignins (Van Soest, 1963) and is undoubtedly degraded. The

ultraviolet absorption spectra of this residue are also greatly altered, unlike spectra of forage lignins not treated with sulphuric acid. Also, there are lignin fractions that may be soluble in 12 M sulphuric acid. Thus, there is evidence to support a higher true lignin figure than that which is obtained by the lignin(sa) method.

5.10. Lignin as a marker

Another use of lignin methods is as internal digestibility marker. Here, the requirement is for faecal recovery. While there is no good evidence that any metabolisable energy derives from lignin, faecal recovery is often incomplete due to a failure in filtration. This failure is more often due to methodological problems than to any real disappearances of lignin.

Lignin in feed is selectively distributed in larger particles, while in faeces it is contained in the finer ones (Van Soest, 1994). Particle size affects results from both the lignin(sa) and lignin(pm) methods, since larger particle size decreases efficiency of the reagent by the rate at which the lignified matrix is penetrated. In the case of the lignin(sa) procedure, lignin is the residue, and a reduced rate of penetration will lead to higher values. In contrast, the permanganate that removes lignin and leaves a cellulosic residue may give lower results in some cases. A further effect is the quantity of lignin presented to the reagent. Larger quantities may reduce reagent efficiency leading again to higher results with the KL and lower values with the lignin(pm) method.

Faecal particles are often finer but also contain much more lignin. Thus, the effects of fineness and amount are partly compensatory, and the balance of effects is responsible for differences in recovery by the respective methods. Generally, the lignin(sa) method gives better recovery than the lignin(pm) method and has thus been

recommended over the permanganate for balance studies. The poorer recovery with the permanganate procedure can be overcome by increasing the time of oxidation of faecal samples from 90 to 180 min (Enzman et al., 1969). This is consistent with the observation that the permanganate oxidation is rate-limited.

An alternative is to use the hybrid procedure which eliminates many of the problems with both the Klason and permanganate procedures. Excess non-oxidisable and non-hydrolysable material, isolated by the KL method, is eliminated by the permanganate by inclusion in the cuticular residue, while the penetration problem for permanganate is improved by the prior removal of the bulk of the sample in the form of cellulose. Samples of low lignin content tend to give high values by the permanganate method, possibly through excessive oxidation. This is eliminated in the hybrid method since all carbohydrate has been removed by the prior lignin(sa) treatment.

5.12. Other Lignin Methods

Various methods other than KL and lignin(sa) exist but have been less used. Some of these depend on removal of lignin by oxidation with chlorite (Collings et al., 1978) or permanganate (Gomes et al., 2011, reviewed by Hatfield and Fukushima, 2005), or solubilisation with triethylene glycol (Edwards, 1973), solution with acetyl bromide (Gomes et al. 2011) or thioglycollic acid (Hatfield and Fukushima, 2005). None of these methods have found practical use in forage evaluation.