Challenges in measuring insoluble dietary fiber

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ABSTRACT: Objectives of this review are to define criteria for evaluating insoluble dietary fiber (IDF) methods, discuss their relevance in meeting the nutritional needs of ruminants and herbivores, describe problems with empirical IDF methods, and assess their relative merits. The challenge for the researcher, nutritionist, and analyst is to select fiber methods that are relevant and reproducible. Without relevance, there is no reason to measure IDF, and without reproducibility, there is no value in doing so. Insoluble dietary fiber is a complex matrix of chemical components, and there are no primary standards that can be used to establish the validity of methods. Thus, the definition of fiber is crucial in determining method relevance. For ruminants and nonruminant herbivores, the appropriate physiological definition for selecting IDF methods may be as follows: the organic fraction of the diet that is indigestible or slowly digesting and occupies space in the gastrointestinal tract. Crude fiber does not match this definition, and its use should be abandoned. Acid detergent fiber does not measure all IDF but is useful when included with other dietary fiber methods to describe some feeds. Several current methods, including amylase-treated neutral detergent fiber (aNDF) and enzymatic-gravimetric methods, are relevant for measuring IDF. In a collaborative study, aNDF obtained a standard deviation of reproducibility (SD_R) of 1.3%. Enzymatic-gravimetric methods of measuring IDF have been evaluated using too few feed materials to make statistically valid conclusions, but the SDR of IDF, for the few feeds evaluated, were similar to aNDF (0.9 to 2.4%). The enzymatic-chemical method of measuring IDF as the sum of insoluble nonstarch polysaccharides and lignin agrees with NDF, but the SD_R of neutral sugar analysis using acid hydrolysis and chromatography is greater (3.2%) than other dietary fiber methods. Empirical methods—such as those used to measure IDF, although based on nutritional concepts—actually define the fraction being measured and must be followed exactly, without modification. The selection of a suitable method for IDF depends on the purpose of analysis. Analysis of sugars in insoluble polysaccharides provides more information but is less reproducible and more expensive to obtain. For routine nutritive evaluation of feeds and formulation of rations, aNDF seems to be a reasonable choice for measuring IDF based on relevance and reproducibility.

Key Words: Analytical Methods, Detergent Fiber, Dietary Fiber, Fiber Analysis, Repeatability, Reproducibility

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Introduction

Fiber concentration has been a useful measure for describing feeds and estimating energy value for nearly 150 yr (Dougall, 1956; Sullivan, 1964; Van Soest, 1964; Tyler, 1975). Hipsley (1953) was the first to use the term *dietary fiber* to describe a nutritional property of diets. This generic term will be used to indicate the general concept of fiber as it relates to nutrition. The main challenges facing insoluble dietary fiber (**IDF**) methods are relevance and reproducibility. Without relevance, there is no reason for measuring dietary fiber; without reproducibility among laboratories, there is no value in reporting dietary fiber results. To be relevant, IDF methods must provide useful nutritional information and must have utility as either a quantitative description of a feed or a means of evaluating feeds or formulating rations. Reproducibility of IDF methods is crucial to have confidence that results are accurate and comparable among research, regulatory, and feed-testing laboratories.

Dietary fiber is unique among feed constituents because it is defined only on a nutritional basis (that is, in terms of the digestive and physiological effects that it elicits) but must be measured chemically. Thus, the nutritional definition for dietary fiber is key to method relevance. The usefulness of dietary fiber results vary from its value as an indicator of physiological health benefits to its value as a predictor of digestibility and energy value of feeds. Furthermore, the relevance of

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dietary fiber data may be different between research and practical use, and vary within each use.

Numerous methods have been proposed for measuring dietary fiber, and some have become routine analyses for research and practical use. The scope of this review will be limited to the official methods of fiber analysis as described by the Association of Official Analytical Chemists (**AOAC**) International. These methods can be used in situations in which accuracy and precision are required and often are the ones routinely used in research and practical applications to describe feed characteristics. Objectives of this review are to define the criteria needed to evaluate IDF methods, discuss the relevance of each method in meeting nutritional needs, describe analytical problems in reproducing empirical dietary fiber results, and assess the relative merits of IDF methods.

Methods

Throughout this discussion, the term *materials* will be used to describe individual feeds or lots of feed, *sample* will be defined as the portion of a material that is prepared for analysis, and *test sample* will be used to describe the part of the sample that is actually analyzed.

AOAC Official Methods for Dietary Fiber

With a few exceptions, dietary fiber is determined gravimetrically as the difference in weights of a test sample before and after extraction in a solution(s). There are two AOAC official methods for crude fiber (**CF**) in animal feeds: 962.09—Crude fiber in animal feed and pet foods, ceramic fiber filter method, or 978.10—Crude fiber in animal feed and pet foods, fritted glass crucible method (AOAC, 2002). In the most recent versions of Method 962.09, the precoating of the Oklahoma filter screen or California Buchner funnel with ceramic fiber when analyzing extremely fine samples was clarified. Acid detergent fiber and acid detergent lignin using sulfuric acid (**ADSL**) can be determined using AOAC Official Method 973.18—Fiber (acid detergent) and lignin in animal feed. Several clarifications have been included in the more recent version of Method 973.18, such as 1) description for cleaning crucibles, 2) specification of particle size for preparing samples, 3) preextraction of test samples containing >10% fat with acetone or similar solvent, 4) time of soaking of residues after acid detergent extraction to remove acid, and 5) addition of formula to report results on a as-is or as-received basis (AOAC, 2002). Amylase-treated neutral detergent fiber (**aNDF**) can be measured by AOAC Official Method 2002.04—Amylase-treated neutral detergent fiber in feeds using refluxing in beakers or crucibles.

There are several AOAC official methods for measuring total dietary fiber (**TDF**), IDF, and soluble dietary fiber (**SDF**). The first AOAC official method for TDF was 985.29—Total dietary fiber in food, enzymatic-gravimetric method, which did not allow separation of dietary fiber into soluble and insoluble fractions. Insoluble dietary fiber can be determined using AOAC Official Method 991.42—Insoluble dietary fiber in foods and food products, enzymatic-gravimetric method (phosphate buffer) and SDF by Method 993.16—Soluble dietary fiber in food and food products, enzymatic-gravimetric method (phosphate buffer). These methods for measuring TDF, IDF, and SDF have been superseded by Official Method 991.43—Total, soluble, and insoluble dietary fiber in foods, enzymatic-gravimetric method (MES-Tris buffer). Official Method 992.16—Total dietary fiber, enzymatic-gravimetric method, uses neutral detergent extraction with amylase treatment and measurement of SDF to determine TDF. More detailed analysis of TDF can be determined using Official Method 994.13—Total dietary fiber (determined as neutral sugar residues, uronic acid residues, and Klason lignin), gas chromatographic, colorimetric, gravimetric method, which is based on acid hydrolysis and chromatographic analysis of sugar residues.

Collaborative Studies

One of the primary purposes of the AOAC is to sponsor collaborative studies for evaluating analytical methods under actual laboratory conditions with a diversity of materials, personnel, environments, equipment, and so on (AOAC, 1993). Under these conditions, the total precision of a method (reproducibility) can be determined (Steiner, 1975), which the AOAC uses to make an informed decision about the acceptability of the method as official. The total precision of an analytical result is the sum of variability among laboratories and within laboratories. Reproducibility of a method is defined as the variation among single results for the same material when determined by different laboratories (different analyst, apparatus, environment, time, etc.). Repeatability of a method is defined as the variation among results for the same material determined in similar conditions within a laboratory (typically successive analyses within the same run: same analyst, apparatus, reagents, etc.).

To assess the reproducibility and repeatability of a method requires replicated analyses of multiple materials within multiple laboratories. Youden (1975) suggested that the absolute minimum design for a collaborative study would be five laboratories analyzing three pairs of materials (low, medium, and high concentrations of the analyte). He also suggested that matched pairs of materials (Youden pairs) provide more statistical information than blind duplicates for the same number of analyses. The minimum design provides 30 observations, which is the minimum number needed to obtain an acceptable estimate of standard errors (Wernimont and Spendley, 1985). Typically, the AOAC requests at least eight laboratories and five materials (duplicate analyses) for most collaborative studies

(AOAC, 1993). The materials should represent the full range of composition matrices to which the method will be applied. A study with more than one material would result in the following statistical model to obtain estimates of variation pooled across materials (Wernimont and Spendley, 1985):

$$Y_n = \mu + L_i + M_{ij} + L_i \cdot M_{ij} + r_{ijk}$$

where

Y_n = result for a single analysis of the nth material,
μ = mean observation,
L_i = ith laboratory,
M_{ij} = jth material analyzed in the ith laboratory,
$L_i \cdot M_{ij}$ = ith laboratory by jth material interaction, and
r_{ijk} = kth replicate of the jth material within the ith laboratory.

An acceptable method must have a nonsignificant $L_i \cdot M_{ij}$ interaction and variations within and among laboratories that are small relative to the variation among materials. Assuming the sources of variation are independent, the variances can be partitioned so that the variation for reproducibility of the method can be calculated:

$$\sigma_R^2 = \sigma_L^2 + \sigma_r^2$$

where

σ_R^2 = variance of reproducibility of an individual measurement from any laboratory,
σ_L^2 = variance among laboratories, and
σ_r^2 = variance of replicated analysis within laboratories and the standard deviation of reproducibility (**SD_R**) and of repeatability (**SD_r**) are the square roots of the respective variances.

This model applies to study designs in which replicates are run successively or within the same run or day. Because replicates are analyzed successively, this model assumes that repeatability within a laboratory is primarily a function of random variation among replicate test samples and that techniques, equipment, reagents, and so on, within laboratories do not vary among runs or days.

However, it is reasonable to assume that within-laboratory repeatability has two sources of variation: replication within a run in which conditions are relatively uniform with respect to environments, apparatus, reagents, and so on, and variation among runs, which is associated with uncontrollable laboratory conditions over longer periods of time. These sources of repeatability within laboratories can be assessed by designing a collaborative study in which laboratories replicate analyses in different days or runs.

Horwitz (1982) compared the results of numerous AOAC collaborative studies and observed that the relative standard deviation of reproducibility (**RSD_R** = SD_R/mean) was related to the concentration of the analyte across a wide variety of methods and generated a formula for the Horwitz relative standard deviation of reproducibility (**$HRSD_R$**):

$$HRSD_R \text{ (\% of the mean)} = 2e^{(1-0.5\cdot\log_{10}C)}$$

where C is the fractional concentration of the analyte. Thompson and Lowthian (1997) confirmed that the $HRSD_R$ provides an expected standard deviation of reproducibility for any method, which varies from 4% of the mean at 1% concentration to 2% of the mean at >90% concentration. The $HRSD_R$ indicates that the expected standard deviation of most feed analysis methods, which have means of 10 to 60% of DM, would be 2 to 3% of the mean. Dividing the RSD_R observed for a specific method by the $HRSD_R$ generates the Horwitz ratio (**HORRAT**), which permits the comparison of reproducibility among diverse methods (Horwitz et al., 1990). A HORRAT of 1.0 or less indicates that a method has reproducibility similar to other methods approved by AOAC. With some exceptions, a HORRAT of >2.0 suggests that a method is unacceptable with respect to the reproducibility of other official methods.

Proficiency Testing of Laboratories

The ongoing validity of each individual laboratory's ability to generate reliable results is accomplished by proficiency testing or performance check programs. In these programs, carefully prepared, homogeneous samples are analyzed by participating laboratories to compare results. The Association of American Feed Quality Control Officials operates a check sample program in which results of participating laboratories are summarized and reported back to the participants for use in monitoring their results. The National Forage Testing Association (**NFTA**) was established under the auspices of the American Forage and Grassland Council, the National Hay Association, and commercial forage analysis laboratories to certify the proficiency of participating laboratories. The results of each laboratory are compared to a consensus reference value for each material and, if they fall within a specified range, the proficiency of the laboratory is certified by the NFTA (Mertens, 1998b). Results for other analyses are monitored by NFTA, but only DM, CP, ADF, and aNDF are used for certification of proficiency.

Ideally, materials used for proficiency testing would have a known composition for each analyte. However, there are no primary standards for dietary fiber or dry matter, and it is necessary to establish reference values for each material used in a proficiency-testing program. The NFTA program allows each laboratory to select the method used to measure each of the analytes, and requires only that laboratories analyze the proficiency-testing samples with the same method they use in routine practice. The results of each laboratory are com-

pared to a consensus value, the reference method average (**RMA**) for each analyte (Mertens et al., 1994). The reference method for each analyte is either an AOAC official method or a method accepted by the NFTA (Undersander et al., 1993). The results used to calculate the RMA are selected based on each laboratory's answers to a questionnaire about the specific details of their routine methods to determine whether they followed the reference method. Results of laboratories using reference methods are censured by selecting only those within one standard deviation of the median; that is, results are ranked and the top and bottom 15.8% are discarded. Typically, 10 to 30 laboratory results are used to generate the RMA. Censuring ensures that anomalous results are not used to determine the RMA to which all laboratories are compared for certification of proficiency. Six materials are analyzed each year, and laboratories are certified as proficient if their results fall within $\pm 3 \cdot HRSD_R$ of the RMA for CP, ADF, and aNDF and within a modified HRSD for DM.

Discussion

Relevance of Dietary Fiber Methods

Dietary fiber is a nutritional entity that can be truly measured only by the digestive process of the animal. In the laboratory, chemical or enzymatic methods are devised to measure dietary fiber, but accuracy and relevance of a method is based on how well the analytically measured fiber matches its nutritional definition. Thus, the development of fiber methods must be based on an acceptable definition of fiber. The concept of dietary fiber for humans was developed initially by Burkitt et al. (1972) and Trowell (1974) to describe plant cell wall components in the diet that were resistant to hydrolysis by mammalian digestive enzymes. Later, Trowell et al. (1976) broadened the definition of dietary fiber to include all indigestible polysaccharides, such as gums and mucilages, whether or not they originate from plant cell walls. This physiological-chemical definition of dietary fiber as "polysaccharides and remnants of plant materials that are resistant to hydrolysis (digestion) by human alimentary enzymes" is the basis for AOAC official methods for TDF that have been accepted for human food labeling (Cho et al., 1997). Because TDF is based solely on resistance to digestion, it contains both SDF and IDF. Partitioning TDF into SDF and IDF may provide important nutritional information for nonruminants because their recovery in the feces and impact on the physiological processes of digestion (fermentability, viscosity, water-holding capacity, distension, etc.) may be quite different.

The definition of TDF for humans, which limits dietary fiber to components that cannot be digested by mammalian enzymes, may be unduly restrictive for ruminants and herbivores, which have a symbiotic relationship with microorganisms and other adaptations of digestive physiology that enable significant digestion of dietary fiber. In the most general terms, dietary fiber is the coarse-textured portion of edible materials that is difficult to digest and adds bulk to digesta and feces. Mertens (1985) proposed that dietary fiber for herbivores be defined as the "indigestible or slowly digesting portion of feeds that occupies space in the gastrointestinal tract." Perhaps to distinguish dietary fiber from indigestible ash, this definition should be modified to include only "indigestible or slowly digesting organic matter of feeds that occupies space in the gastrointestinal tract." These definitions of dietary fiber exclude rapidly fermenting polysaccharides of plant cell walls (such as pectin) and soluble polysaccharides that do not occupy space in a liquid environment (such as fructans and gums), but would include slowly fermented, complex polysaccharides that are digested by fermentation in the alimentary tract of herbivores (such as cellulose and hemicellulose). Essentially, this more restricted definition of dietary fiber describes IDF, which is the feed component that is variable in digestibility and affects the total DM or OM digestibility of feeds or diets by ruminants. It excludes the rapidly fermentable SDF because they have true digestibilities similar to plant cell contents. Although SDF may alter ruminal fermentation, its effect on the health and performance of ruminants are unknown. Insoluble dietary fiber affects the digestibility and passage rate of feeds and diets in all animals. Due to their high intakes of dietary fiber, the space-occupying characteristics of IDF and its requirement for chewing to reduce particle size for passage through the alimentary tract may be factors that make IDF more important to herbivorous animals than SDF.

For a dietary fiber method to be practicable, it must apply to all potential feed ingredients and compound mixtures of feeds. Therefore, the restriction that dietary fiber comes only from plant sources is practically inappropriate and nutritionally inconsistent with the definition of dietary fiber. The strictly physiological definition does not require that dietary fiber originates from plants or their cell walls, and even TDF as defined for humans contains compounds that do not occur naturally in plants. Although fiber has been linked to plant cell walls because they contain similar chemical components in forages, fiber and cell walls are not synonymous terms. Insoluble dietary fiber is not cell walls because analytical methods often isolate insoluble components in feeds other than plant cell walls, and cell walls are not IDF because some plant cell wall components, such as pectin, are rapidly fermented and are solubilized by many fiber methods.

The goal of dietary fiber methodology is to accurately evaluate nutritive value and ultimately be useful in improving the nutritional quality of animal diets. Theoretically, dietary fiber methods should be developed to fit a nutritional definition and not vice versa. However, it is unlikely that any chemical or enzymatic measurement will mimic all of the nutritional effects of fiber in the animal. Although dietary fiber should be defined by nutritional concepts and not analytical methodology,

practically, dietary fiber is an empirical measurement that is defined by the method per se. In this situation, consensus dietary fiber values can be generated for reference materials by averaging the results of several laboratories that follow exactly a prescribed method. To ensure the accuracy of results, efforts are needed to generate reference materials that can be used to document the accuracy of dietary fiber analyses among research, regulatory, and feed-testing laboratories.

A practical method should not require exotic instruments, reagents, equipment, laboratory environment, and the like so that it is suitable for routine analyses by feed-testing and regulatory laboratories. In addition, the method should be rapid, convenient, and economical to allow multiple samples of materials to be analyzed in a timely manner. Given the variability in materials and their sampling before being sent to the laboratory, routine methods should be designed to allow multiple determinations, rather than be so expensive or time consuming that nutritional decisions must be based on a single sample. Specificity is required to ensure that the method measures fiber accurately without interference or artifacts that alter the fractions being measured. Finally, a method must have a clearly defined limit of reliability that determines the method's ability to discriminate among analyte levels in materials and detect concentrations different from zero.

For research, specific methods that require sophisticated equipment and techniques may be needed to provide detailed analyses for comparisons among treatments or to assess molecular or structural characteristics of dietary fiber components. However, if their conclusions are to be relevant to field applications, researchers have a burden to provide routine analytical information as well as their detailed analyses to establish a connection between research findings and field applications. In addition, researchers have a responsibility to ensure that routine methods used in their laboratory correspond to those used in the field. Therefore, research laboratories should participate in proficiency-testing programs with regulatory and feed-testing laboratories, and provide their performance statistics in reports and manuscripts.

Reproducibility of Dietary Fiber Methods

After it has been established that a dietary fiber method is relevant because it matches the definition of fiber, it must be established that it is reproducible. Reproducibility is related to how well analytical results are measured (Steiner, 1975). It is a deceptively simple term that represents the sum of variation associated with accuracy and precision. Accuracy is the ability to measure the "true" value of a primary standard with known composition or of the consensus concentration of a reference material that is determined by a group of analysts exactly following a defined method. Precision is the ability to repeat a measurement, or more quantitatively, the variation among repeated results.

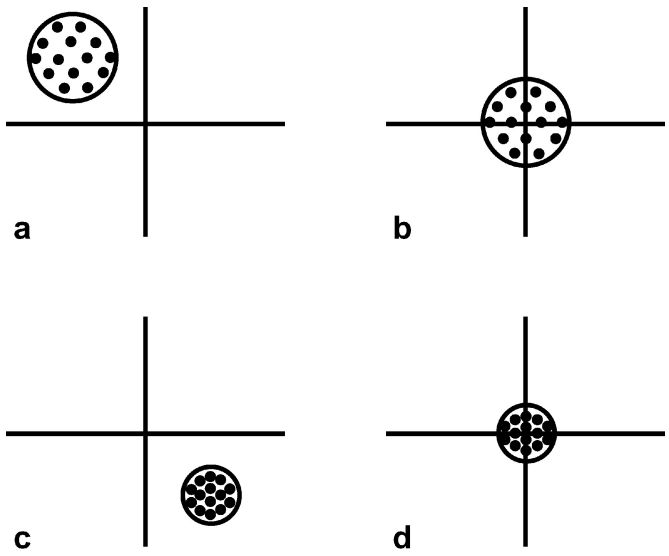

Figure 1. Illustration of the concepts of accuracy and precision: a) inaccurate and imprecise, b) accurate and imprecise, c) inaccurate and precise, and d) accurate and precise.

As illustrated in Figure 1, accuracy and precision are independent characteristics of a method's reproducibility. The error associated with precision (scatter about the average value) tells us nothing about the error associated with accuracy (how close the average result matches the true value). Yet we often hear that the results of a method used in a laboratory must be correct because duplicate variation (only a measure of precision) was small. Precision does not guarantee accuracy because it is possible to precisely determine the wrong result (Figure 1c). No one would argue that an inaccurate and imprecise method (Figure 1a) is useful; however, it could be argued that precision is not particularly relevant because the true value can be detected using an imprecise method simply by averaging a large number of replications (Figure 1b). Conversely, researchers sometimes argue that precision is more important than accuracy because the true value is unattainable or irrelevant when we are only concerned about detecting differences among treatments or feeds. However, if data are compared among institutions or laboratories, if new knowledge is built on one another's results, or if research conclusions are to be applicable in the field, then research analytical results must be both accurate and precise (Figure 1d) to allow others to reproduce and use the information that is generated.

Statistical reproducibility is the total variation of an individual dietary fiber measurement from any laboratory. It is the sum of the variance of results among and within laboratories. Variances among laboratories or among days within laboratories are systematic because they represent a consistent pattern of deviations in mean results. These systematic biases are related to the ways methods are performed, equipment is adjusted

or calibrated, or reagents are prepared that cause consistent differences. For an ideal method, the variance among laboratories, among days within laboratories, and among material × laboratory interactions would be zero, and the variance of individual results from any laboratory would be equal to the variance among replicate analyses. This replication variance is due primarily to random differences in test samples that are taken from prepared samples that are not completely homogeneous. To truly measure within-laboratory repeatability, analyses must be performed in different runs or days. This ensures that the results would be repeatable in that laboratory if measured at some future time; therefore, they are valid for making comparisons with other results generated within that laboratory. Replication within a run, particularly if measured consecutively, does not provide such assurance.

Unlike research laboratories, commercial feed analysis laboratories typically analyze only one test sample for each material received. Thus, the reproducibility (approximate 95% confidence interval) among single analyses performed by two laboratories on representative samples of the same material is $2.8 \cdot SD_R$. The reproducibility of a method is a quantitative measure of its robustness, or its power and sturdiness in measuring the analyte in all types of materials using generally accepted practices within laboratories. Several performance characteristics of a method determine its reproducibility or robustness: ruggedness, practicality, specificity, and limit of reliability (Wernimont and Spendley, 1985). Ruggedness refers to a method's ability to generate acceptable results when small, uncontrolled changes in operating conditions occur. Ruggedness testing of a method (Youden, 1975) involves evaluating the impact of making small perturbations in the reagents (concentrations, sources, etc.), conditions (temperature, time, etc.), equipment (settings, models, etc.), and steps (skipping or modifying). Ruggedness testing can be a daunting task when methods are complex and involve sophisticated equipment, and typically these methods are less thoroughly tested. Thus, complex methods that are less rugged are more demanding in expertise and in exactness of following procedures than are simple solubility methods.

Types of Dietary Fiber Methods and Sources of Variation

Fiber methods are typically categorized into three types (chemical-gravimetric, enzymatic-gravimetric, or enzymatic-chemical) based on the ways fibrous residues are isolated and measured. Isolation of dietary fiber residues is done by extraction in chemical solutions, enzymatic hydrolysis of nonfibrous constituents, or a combination of the two. After the fibrous residue is isolated, it is measured either gravimetrically (weighing the residue) or chemically (hydrolyzing the residue and measuring individual components, such as sugars and lignin).

A primary factor affecting gravimetric reproducibility is the accuracy and precision of the balance. Accuracy of a balance depends on its ability to report the true value when tested with a known weight and its smallest weight of detection. It is clear that balances should be routinely maintained and standardized, and should be calibrated or checked for accuracy at each use. Even with daily calibration of balances, we have observed an unexplained systematic bias that is consistent for all weights taken within weighing sessions. Correcting for blanks accounts for this systematic bias in weighing and has greatly improved the precision of replicates and the accuracy of results for test samples that have small residue weights (such as lignin or dietary fibers <10% of DM) in our laboratory. The improvement in the accuracy of results occurs because the systematic bias is often a significant proportion of the residue weight (Mertens, 2002). The problem of the lowest weight of detection may be less obvious because laboratories occasionally weigh test samples or residues only to the nearest 0.01 g without recognizing that this negatively affects results. If test samples of 0.50 g are used, results can be reported legitimately to only two significant digits (nearest 1 percentage unit) because this is the limit of information in the original weight regardless of the number of digits generated during calculation.

The reproducibility of results is also determined by the precision of weighing the test sample, such as, if the precision of this balance is ±0.01, then the 95% confidence interval for the test sample weight is 0.48 to 0.52 and the potential variation in weighing overwhelms the remaining factors associated with method variability. However, the converse of this situation is not true. If the balance used weighs to the nearest 0.0001 g, this does not guarantee four significant digits of precision because other characteristics of the method can affect the precision of results. Sokal and Rohlf (1981) suggest that, in general, the number of decimal places for reporting results should be based on the standard error of the mean using the following guideline: divide the standard error by 3 and use the decimal place of the first nonzero digit to determine the significant digits to report. Because the standard error of most dietary fiber methods is less than ±2.5, results should typically be reported to the nearest 0.1%.

Cherney et al. (1985) demonstrated that the variation in fiber results is also affected by the amount of test sample. The effects of weighing error increased as the test sample amount decreased, especially when <0.3 g (for alfalfa containing about 26% NDF and 18% ADF). Goering and Van Soest (1970) reported that weighing materials hot directly from the oven instead of transferring oven-dried materials to a desiccator before weighing is not only faster and more labor efficient, but also more accurate. The accuracy of the hot-weighing technique is better than when using desiccators because any change in the zero value of the balance is recorded and subtracted from the hot weight to arrive

at the true weight of the material. In effect, this corrects each weight for slight changes in the balance between weighings. In addition, hot-weighing eliminates the variation due to the use or inadequate maintenance of desiccators. Both Horwitz et al. (1990) and Mertens (2002) reported that the minimum SD_R for gravimetric methods is about 0.3 to 0.4 percentage units when the concentration of the analyte approaches zero. Thus, the HORRAT may be an inadequate estimate of the expected reproducibility of gravimetric methods when factional concentrations are <0.1. In gravimetric methods, the error associated with weighing does not approach zero as the analyte concentration approaches zero as predicted by the $HRSD_R$ equation, which is based on all methods including instrumental methods that have small limits of detection (approximately parts per billion or less).

Crude Fiber

Crude fiber was supposed to measure the indigestible ballast of feeds. The method was based on chemical extraction with alkali and acid solutions, which were the known characteristics of the digestion process before enzymes were discovered. The organic matter lost during ashing is calculated as CF, which was initially called *wood fiber* or *crude woody fiber* (Dougall, 1956). It was discovered that CF was digested as it passed through the alimentary tract of ruminants (Henneberg and Stohman, 1860), but their method became a standard part of the proximate analysis scheme. The CF method was first approved as an official method for the AOAC in 1890 (Wiley, 1890), and it became the de facto definition of dietary fiber for over 100 yr. The current AOAC Official Method 920.86 was adopted for flour in 1920 and Official Method 962.09 was adopted for animal feeds in 1962. The CF method is extremely robust in that it can be easily measured in all types of feeds and foods and can be reproduced within and among laboratories. Although the method is empirical (defined solely by the method used to measure it), CF has been useful, historically, in estimating digestibility or energy value within feed types.

The major deficiency of CF is its lack of relationship to any acceptable nutritional definition of dietary fiber and its inability to advance understanding about physiological responses to dietary fiber or its impact on digestibility. The proximate system partitions carbohydrates and allied compounds into two fractions: CF, which is measured analytically, and nitrogen-free extract (**NFE**), which is calculated by difference (NFE = 100 – CP – EE – CF – ash). Neither of these fractions meets criteria for uniform nutritional availability or for the definition of dietary fiber (Van Soest, 1967). Depending on the feed material, CF may contain only 40 to 100% of the cellulose, 15 to 20% of the pentosans from hemicellulose, and 5 to 90% of the lignin. Lignin is dissolved, especially in grasses, by the alkali extraction step in the CF method that is used to remove protein. Because much of the hemicellulose and lignin is included in the NFE fraction, the digestibility of NFE, which is supposed to contain the easily digested carbohydrates, is less than the digestibility of CF for 25% of the feeds listed by Morrison (1956). Currently, CF is used only for quality control and specification of feeds (minimum CF) by regulatory agencies. Its lack of accuracy in measuring dietary fiber and abandonment by researchers and practicing nutritionists suggests that its use for feed regulation should be abolished.

Acid Detergent Fiber and Acid Detergent Sulfuric Lignin

Like CF, ADF (AOAC Official Method 973.18) is an empirical method that was designed to be a preparatory step for the determination of ADSL. The reason for specifying that lignin was determined using sulfuric acid is that ADF can also be the preparatory step for determining lignin using permanganate (**ADPL**); therefore, the term *acid detergent lignin* is not adequate to differentiate between the two correlated but different measures of lignin (ADPL typically has higher values than ADSL). In the ADF method, protein is removed from the fibrous residue by the cationic detergent cetyl trimethylammonium bromide to minimize the nitrogen contamination of lignin. Acid extraction is used to remove nonfibrous compounds while minimizing the losses of alkali-labile lignin. Acid detergent fiber does not meet the nutritional definition of dietary fiber or IDF for ruminants because acid-soluble hemicelluloses are removed and some rapidly fermented pectin is not. The precipitation of pectins in strong acid may be the reason that some feeds containing high proportions of pectin (e.g., immature alfalfa, citrus pulp) may have ADF results that are higher than NDF.

The reproducibility of the ADF determined during an AOAC collaborative study (Van Soest, 1973) was good, with a SD_R of 1.13 and a HORRAT of 1.2 (Table 1). The SD_r was approximately one-third of the total SD_R of individual analyses among laboratories. This indicates that the variation among laboratories is twice the variation within laboratories, which falls within the typical range for most methods (Horwitz, 1982). The SD_R for ADSL was 0.62, but the HORRAT was 3.1, primarily because the small mean of ADSL results in an unrealistically small $HRSD_R$ for a gravimetric method (Mertens, 2002).

The keys to measuring ADF are to standardize the acid to 1 *N*, properly prepare samples for analysis, and adequately soak fiber residues in hot water after extraction to remove acid and acid detergent solubles. To prepare samples for ADF analyses, they must be dried at <60°C and ground through a cutter mill with a 1-mm screen. Cyclone mills tend to produce a finer grind with the same apertures in the screen, so a 2-mm screen in a cyclone mill results in a particle size similar to that of a 1-mm screen in a cutter mill. It has recently been discovered that samples with fat >10% can result

Table 1. Repeatability and reproducibility of the acid detergent fiber (ADF) and acid detergent sulfuric lignin (ADSL) AOAC Official Method 973.18 (Van Soest, 1973)

Item	ADF	ADSL
Number of materials	6	6
Number of laboratories	10	10
Mean, % of DM	39.47	6.66
SD_r[a]	0.38	0.29
SD_R[b]	1.13	0.62
Repeatability within laboratories[c]	1.06	0.81
Reproducibility among laboratories[d]	3.16	1.74
HORRAT[e]	1.24	3.10

[a]Standard deviation of repeatability within laboratories.
[b]Standard deviation of reproducibility among laboratories.
[c]$2.8 \cdot SD_r$, which is the approximate 95% confidence interval for duplicate analyses within a laboratory.
[d]$2.8 \cdot SD_R$, which is the approximate 95% confidence interval for single analyses between two laboratories.
[e]Horwitz ratio, which is the SD_R divided by the expected SD_R based on the equation of Horwitz (1982).

in ADF values that are artificially high. Therefore, the most recent version of the AOAC Official Method 973.18 was modified to require preextraction of the test sample with acetone or other suitable solvents to remove fat when the material contained >10% fat. The method was also modified to indicate that extracted fiber residues must be soaked three times in 90 to 100°C water for at least 3 min to equilibrate acid from within particle pores. It is essential that all residual acid be removed from the fiber before it is dried. During drying of ADF, any residual acid is wicked to the surface of particles and concentrated as water evaporates. Residual concentrated sulfuric acid will char the edges of particles, especially when heated in a >100°C oven. Blackened or charred ADF residues indicate that acid was not completely removed during the residue-washing steps and that ADF results will be low.

Although it is not an AOAC official method, there are circumstances when it may be desirable to measure ADF sequentially (**sADF**) after neutral detergent extraction. Sequentially determined ADF is almost always less than ADF determined by the official method because neutral detergent removes some components that are not removed as well by acid detergent, such as pectins and tannin or phenolic acid complexes. Hintz et al. (1996) determined ADF sequentially on NDF residues that were isolated using heat-stable α-amylase with (aNDF) or without sulfite (neutral detergent residue, **NDR**). When sADF was determined on NDR, values were 1 to 3 percentage units lower than ADF measured using the official method and this difference increased to 2 to 4 percentage units when sADF was determined on aNDF (Table 2).

Neutral Detergent Fiber

The NDF method was designed initially to isolate the insoluble dietary fiber components in plant cell walls: cellulose, hemicellulose, and lignin (Van Soest and Wine, 1967). The significant nutritional attribute of neutral detergent extraction is that it separates feeds into two major fractions that are distinctly different in their digestibility and intake by ruminants and herbivores, and in many cases by nonruminants as well (Mertens, 1993). Whereas NDF has variable digestibility, occupies space in the alimentary tract that can be a physical constraint on intake, and requires significant chewing to reduce particle size, neutral detergent solubles (**NDS**), which represent the inverse of NDF (NDS = 100 – NDF), have nearly constant true digestibilities near 100%, occupy little space because they are rapidly solubilized, and require minimal chewing. Van Soest (1967) reported that NDS have a true digestibility of about 98% and a relatively constant endogenous loss of 12.9% when consumed by ruminants at maintenance levels of intake. The level of intake is important because, at maintenance levels of intake, herbivores, especially sheep, chew feeds adequately, which allows complete digestion of NDS.

Neutral detergent fiber is measured using a chemical solubility-gravimetric method. Proteins are extracted using anionic detergent and sodium sulfite. Fats are removed using hot detergent and acetone. Soluble dietary fiber is removed primarily by hot detergent extraction, and the solubility of easily fermented pectin is enhanced by chelating calcium bound in pectin complexes using EDTA. In the original NDF method (Van Soest and Wine, 1967), soluble carbohydrates and starch were extracted by hot solutions. It was discovered that the original NDF method inadequately removed starch from some feeds and foods. Numerous modifications of the NDF method have been proposed since the original publication of the method (McQueen and Nicholson, 1979; Robertson and Van Soest, 1980; Mascarenhas Ferreira et al., 1983; Van Soest et al., 1991). Of these modifications, the NDR method of Robertson and Van Soest (1980), which uses a heat-stable α-amylase to remove starch during detergent extraction and eliminated the use of sodium sulfite, became the de facto method for measuring NDF.

The original NDF method of Van Soest and Wine (1967) was never evaluated by a collaborative study. However, a method for measuring IDF based on the NDR modification of Roberson and Van Soest (1980) was evaluated as a method for measuring the IDF portion of TDF (Mongeau and Brassard, 1990). Although most of the materials used in the collaborative study were human foods, the comparison for wheat bran may represent results for high-fiber by-product feeds (Table 3). The SD_R for wheat brans (±1.92) was slightly higher than that observed for ADF (±1.13), and the RSD_R was also slightly higher (4.7 vs. 2.9%). The SD_R was higher for TDF compared to IDF in wheat brans and foods, suggesting that the SDF contained in TDF may be less reproducible than IDF.

The aNDF method was developed as an IDF method that could be used on all feeds, including forages,

Table 2. Comparison of acid detergent fiber (ADF) determined by AOAC Official Method (973.18) to ADF determined sequentially after neutral detergent extraction using heat-stable α-amylase with ($sADF_{aNDF}$) or without sodium sulfite ($sADF_{NDR}$)

Feed type	ADF	$sADF_{NDR}$ (–sulfite)	$sADF_{aNDF}$ (+sulfite)	Difference between ADF and:	
				$sADF_{NDR}$	$sADF_{aNDF}$
Heated by-products	14.1	11.5	10.2	2.6	3.9
Oilseed meals	17.6	16.5	15.2	1.1	2.4
Grass forages	45.2	42.4	41.1	2.8	4.1
Legume forages	32.9	29.7	28.7	3.2	4.2

grains, oilseeds, and plant and animal by-products used for animal feeds (Mertens, 2002). The method is a modification of the original NDF method, which included the use of sodium sulfite, but added the use of a heat-stable α-amylase standardized to remove starch during neutral detergent extraction and with specific modifications (sand and other filter aids) that solve filtering difficulties for all types of materials. The aNDF method allows results to be reported as either aNDF with fiber-associated ash or as ash-free aNDF organic matter (**aNDFom**), and each of these results can be reported with or without blank correction. It is anticipated that aNDF will be reported for routine feed analyses because it does not require an ashing step before reporting results. Although crucibles are routinely cleaned by ashing, that step represents an additional time delay in reporting results and most, if not all, commercial feed testing laboratories currently report aNDF.

For the most accurate estimate of insoluble dietary fiber, blank-corrected aNDFom is recommended. Blank correction is especially important when fiber results are <25% aNDF because systematic weighing variation can have substantial impact on these small residue weights (Mertens, 2002). Reporting results as aNDFom more accurately matches the definition of insoluble dietary fiber as organic matter and improves the accuracy of calculating nonfibrous carbohydrates because the ash in fiber is not subtracted twice. It is often unclear whether NDF results reported in the literature are ash-free organic matter, but neither the original method of Van Soest and Wine (1967) or the handbook of Goering and Van Soest (1970), which are often cited as sources of methods, indicate that NDF should be determined as ash-free organic matter.

Determination of aNDF has SD_R among laboratories (Table 4) similar to that reported for ADF (Table 1). It is surprising that the SD_r within laboratories, which is due primarily to random variation in test samples, was a much larger proportion of SD_R for aNDF (79%) than for ADF (34%). This suggests that either the variability in aNDF among test samples is much larger than for ADF or that other sources of variation within laboratories contributed to repeatability differences for aNDF. The repeatability value in Table 4 indicates that analyses should be rerun if duplicates differ by more than about 2.9 percentage units. The reproducibility value indicates that results for 19 out of 20 laboratories performing a single analysis on a well-mixed material should be within 3.7 percentage units of each other.

The original NDR method (Robertson and Van Soest, 1980) differs from the aNDF method (Mertens, 2002) in the use of sodium sulfite and type of amylase. However, current implementations of the NDR method use amylases similar to the aNDF method because the original

Table 3. Results of a collaborative study (Mongeau and Brassard, 1990) to evaluate AOAC Official Method 992.16 for total dietary fiber (TDF) or insoluble dietary fiber (IDF), which was determined using neutral detergent extraction with α-amylase (NDR)

Item	IDF(NDR)	IDF(NDR)	TDF	TDF
Material used	Wheat bran	Foods	Wheat bran	Foods
Number of materials	2	6	2	6
Number of laboratories	9/10	7/10	9/10	8/10
Mean, % of DM	40.48	6.95	46.80	10.38
SD_r[a]	0.71	0.45	0.86	0.70
SD_R[b]	1.92	0.70	2.80	1.02
Repeatability within laboratories[c]	1.99	1.26	2.39	1.96
Reproducibility among laboratories[d]	5.38	1.96	7.86	2.86
HORRAT[e]	1.57	2.55	2.02	2.67

[a]Standard deviation of repeatability within laboratories.
[b]Standard deviation of reproducibility among laboratories.
[c]$2.8 \cdot SD_r$, which is the approximate 95% confidence interval for duplicate analyses within a laboratory.
[d]$2.8 \cdot SD_R$, which is the approximate 95% confidence interval for single analyses between two laboratories.
[e]Horwitz ratio, which is the SD_R divided by the expected SD_R based on the equation of Horwitz (1982).

Table 4. Results of a collaborative study (Mertens, 2002) for amylase-treated neutral detergent fiber (aNDF) when blank-corrected (aNDFbc) or when expressed as ash-free fiber organic matter (aNDFom and aNDFombc)

Item	aNDF	aNDFbc	aNDFom	aNDFombc
Material used	Feeds	Feeds	Feeds	Feeds
Number of materials	11	11	11	11
Number of laboratories	11	11	11	11
Mean, % of DM	38.7	38.6	37.7	37.4
SD_r[a]	1.05	1.02	1.02	1.00
SD_R[b]	1.33	1.28	1.28	1.24
Repeatability within laboratories[c]	2.94	2.86	2.85	2.80
Reproducibility among laboratories[d]	3.72	3.59	3.58	3.48
HORRAT[e]	1.49	1.44	1.47	1.43

[a]Standard deviation of repeatability within laboratories.
[b]Standard deviation of reproducibility among laboratories.
[c]$2.8 \cdot SD_r$, which is the approximate 95% confidence interval for duplicate analyses within a laboratory.
[d]$2.8 \cdot SD_R$, which is the approximate 95% confidence interval for single analyses between two laboratories.
[e]Horwitz ratio, which is the SD_R divided by the expected SD_R based on the equation of Horwitz (1982).

amylase is no longer available. Sulfite was eliminated when the NDR modification was developed because it may extract lignin and phenolic complexes. Removing sodium sulfite from the NDF procedure increases the protein contamination of fibrous residues, which has been used to define a slowly degrading protein fraction in feeds (Sniffen et al., 1992). However, protein contamination can greatly inflate the IDF values of feeds, especially heated feeds (Table 5). Using sodium sulfite reduces aNDF values for forages and oilseed meals by only 1 to 4 percentage units; however, it reduces the fiber values of heated by-product feeds, such as brewer's and distiller's grains, by about 11 percentage units. Thus, the use of sodium sulfite in the aNDF method is a compromise between losing a small amount of phenolic compounds in some feeds or having IDF contaminated by a large amount of protein that is apparently digested in other feeds. With the exception of grass forages, which have the smallest difference in fiber due to the use of sodium sulfite, most of the material extracted from feeds by sulfite is crude protein equivalent (Table 5). Although NDF, NDR, and aNDF can be corrected for protein contamination by measuring the nitrogen content of the respective fiber residues, this additional step requires time and expense, which are large disadvantages for a routine method and have little impact on results in comparison to variation within and among laboratories for IDF results. The aNDF method was designed to improve the routine determination of IDF in all feeds; therefore, sulfite was retained in the method to remove most protein contamination. Sodium sulfite has the additional benefit that it improves filtration (and precision) during the analysis of some problem materials.

The NDF method has a reputation of being variable and difficult to accomplish. Most of the variability in NDF results among laboratories is related to differences in the specific modification of the method that was used. Much controversy about differences in NDF results among laboratories would be eliminated if laboratories would state exactly how they measured it and used specific nomenclature to identify it. Unfortu-

Table 5. Composition of the material extracted when sodium sulfite is used during neutral detergent extraction

				Composition of the difference		
Feed type	NDR[a]	aNDF[b]	Difference[c]	CPE[d]	sADSL[e]	Other[f]
	(% of DM)			(% of difference)		
Heated by-products	45.5	34.4	11.1	67	8	25
Oilseed meals	26.9	22.8	4.1	54	6	40
Grass forages	73.1	71.1	2.0	24	76	0
Legume forages	40.3	38.9	1.4	75	25	0

[a]Neutral detergent residue (Robertson and Van Soest, 1980) determined with heat-stable α-amylase and without sodium sulfite.
[b]Amylase-treated neutral detergent fiber (Mertens, 2002) determined with heat-stable α-amylase and sodium sulfite.
[c]NDR – aNDF.
[d]Crude protein equivalent (nitrogen × 6.25).
[e]Acid detergent lignin using sulfuric acid determined sequentially after neutral detergent extraction.
[f]Undefined composition determined by difference.

Table 6. The effect of different dry matter adjustments among laboratories on the concentration of nutrients with small magnitudes (crude protein and ether extract) or large magnitudes (neutral detergent fiber) when expressed on a dry matter basis

Laboratory and nutrient	As-is, %	DM fraction[a]	DM-adjusted, %
Laboratory 1			
Ether extract	2.2	0.88	2.5
Crude protein	10.0	0.88	11.4
Neutral detergent fiber	65.0	0.88	73.9
Laboratory 2			
Ether extract	2.2	0.92	2.4
Crude protein	10.0	0.92	10.9
Neutral detergent fiber	65.0	0.92	70.7

[a]The difference in DM between laboratories, which alters DM-adjusted results, is not real, but due to differences in technique or method for DM determination.

nately, it is not recognized that because NDF (like any IDF) is not a homogeneous chemical entity, its magnitude and properties are defined by the method used to measure it. Thus, every modification measures something slightly different from the original NDF method, and results should not be indiscriminately called NDF to avoid confusion when comparing results among experiments or laboratories. Mertens (1998a) and Hintz et al. (1996) reported the variation in dietary fiber concentration among the three major modifications of the NDF method. In general, the NDR modification (Robertson and Van Soest, 1980), which uses heat-stable α-amylase, but not sulfite, results in slightly higher values (approximately 0 to 1 percentage unit) for feeds with little starch and moderate protein and much lower values for starch-containing feeds (approximately 1 to 5 percentage units) compared to the original NDF method (Van Soest and Wine, 1967). The aNDF modification (Mertens, 2002), which uses both sodium sulfite and heat-stable α-amylase, generates lower results than either NDF or NDR.

One of the unappreciated sources of variation in NDF results is the effect of DM adjustment. Most chemical entities are measured on a test sample that is not completely dry; thus, the analysis is determined on an as-is, or as-received, basis. Although feeds are bought, sold, and regulated (feed tag specifications) on an as-is basis, nutrients are also reported on a DM basis to allow a more direct comparison of nutrient densities without the confounding factor of moisture in the material. However, small apparent differences in DM determination, which may be caused by poor technique or differences in methodology, can result in large artifact differences in nutrient concentration on a DM-adjusted basis when the magnitude of analyte concentration is large (Table 6). The small discrepancy between these two laboratories for EE or CP is inconsequential, but the larger difference in NDF on a DM basis between these laboratories would be disconcerting. This illustrates that nutrient concentrations should not be compared among laboratories on a DM basis because these results combine the potential errors in both DM and nutrient determinations, making it impossible to determine which is the culprit when discrepancies occur. Unfortunately, there is no AOAC official method for routine measurement of DM that is acceptable for all feeds and this situation needs to be rectified. The determination of DM is empirical and methods vary significantly among laboratories. The 100 laboratories that responded to a 1993 NFTA questionnaire about drying methods indicated that they used 47 different combinations of time and temperature (Mertens, 1994). Twenty-one temperatures (ranging from 57 to 140°C) and 16 drying times (ranging from 2 to 48 h) were used for DM determinations.

Total, Insoluble, and Soluble Dietary Fiber

Total dietary fiber in human foods has been measured by two major types of methods: enzymatic-gravimetric or enzymatic-chemical. Enzymatic-gravimetric methods can be used to measure TDF directly by AOAC Official Method 985.29 or by summing IDF (Official Method 991.42) and SDF (Official Method 993.19). These three methods use essentially the same procedure. Official Method 991.43, which supersedes these earlier methods, is based on the same principle as Official Method 985.29 but uses an organic buffer instead of the phosphate buffer. The organic buffer used in Official Method 991.43 results in a method that is simpler, uses fewer reagents, and generates lower and more reproducible blanks; this method can be used to measure TDF and its components IDF and SDF. All of these methods involve the isolation of fiber residues by using α-amylase and amyloglucosidase to hydrolyze starch and proteases to hydrolyze proteins. Insoluble dietary fiber is the residue remaining after enzymatic hydrolysis. Soluble dietary fiber is precipitated from the hydrolyzed solution with ethyl alcohol at a final concentration of 78%. Ash and protein is determined on one of the duplicate IDF and SDF residues and used to correct IDF and SDF for protein and ash.

Although few materials were analyzed that are typical animal feeds, the results in Tables 7 and 8 suggest that enzymatic-gravimetric methods measure IDF with reproducibility and repeatability similar to those of the aNDF method (Prosky et al., 1985, 1992, 1994; Lee et al., 1992). The values for IDF using these methods are within the range of aNDF values expected for these feed sources. However, these methods have not been used on as wide a variety of feeds as the aNDF method and it is difficult to draw firm conclusions about comparisons between aNDF and enzymatic-gravimetric methods. Enzymatic-gravimetric methods are more complex and require more intermediate analyses than aNDF; therefore, it is unlikely that reproducibility would be better. The complexity and time required to conduct enzymatic fiber assays makes it difficult to envision

Table 7. Results of collaborative studies for enzymatic-gravimetric methods that use phosphate buffers to measure total dietary fiber (TDF), insoluble dietary fiber (IDF) and soluble dietary fiber (SDF)

Item / AOAC Official Method:	985.29[a]	991.42[b]	993.19[c]	993.19	993.19
Dietary fiber:	TDF	IDF	TDF	IDF	SDF
Material used	Wheat bran	Soy bran	SB fiber[d]	SB fiber	SB fiber
Number of materials	1	1	1	1	1
Number of laboratories	9	13	10	10	10
Mean, % of DM	42.65	65.24	66.07	45.57	20.65
SD_r[e]	0.99	0.91	1.15	0.67	0.80
SD_R[f]	1.21	2.40	1.59	0.98	1.35
Repeatability within laboratories[g]	2.78	2.55	3.22	1.88	2.24
Reproducibility among laboratories[h]	3.38	6.72	4.45	2.74	3.78
HORRAT[i]	1.25	1.72	1.13	0.96	2.58

[a]Prosky et al. (1985).
[b]Prosky et al. (1992).
[c]Prosky et al. (1994).
[d]Sugar beet fiber.
[e]Standard deviation of repeatability within laboratories.
[f]Standard deviation of reproducibility among laboratories.
[g]2.8·SD_r, which is the approximate 95% confidence interval for duplicate analyses within a laboratory.
[h]2.8·SD_R, which is the approximate 95% confidence interval for single analyses between two laboratories.
[i]Horwitz ratio, which is the SD_R divided by the expected SD_R based on the equation by Horwitz (1982).

that they will be adopted as routine methods for feed analysis. Their appeal is based on the concept that the measurement of dietary fiber by enzymes mimics the process of digestion; however, it is clear that neither the conditions nor the enzymes used for measurement approach the complexity of hydrolysis in the gastrointestinal tract. Nonetheless, SDF measured by enzymatic methods appear to affect physiological processes in humans.

An alternative to the enzymatic-gravimetric approach is to measure the monomeric composition of structural components of plants (cell walls) or of nonstarch polysaccharides. The former is directly related to the physiological definition of dietary fiber as the dietary components resistant to hydrolysis by mammalian enzymes, whereas the latter is based on a chemical definition of dietary fiber as nonstarch carbohydrates and lignin (Theander et al., 1994; Englyst et al., 1994). The Uppsala method (Theander et al., 1995) is the only enzymatic-chemical method for measuring TDF that is an official method. Official Method 994.13 of the AOAC uses α-amylase and amyloglucosidase to hydrolyze and remove starch, but no proteases are used to remove protein. After starch hydrolysis, ethyl alcohol is used to precipitate soluble polysaccharides. The combined soluble and insoluble fiber residue is hydrolyzed to neutral sugars and uronic acids using 12 *M* sulfuric acid at 30°C for 1 h followed by diluting the sulfuric acid to 0.4 *M* and autoclaving at 125°C for 1 h. Klason lignin is determined as the loss of acid insoluble residue after

Table 8. Collaborative study results of AOAC Official Method 991.43, which used MES-Tris buffer (Lee et al., 1992) to measure total dietary fiber (TDF), insoluble dietary fiber (IDF), and soluble dietary fiber (SDF)

Dietary fiber	TDF_d[a]	TDF_s[b]	IDF	SDF
Material used	Brans[c]	Brans	Brans	Brans
Number of materials	2	2	2	2
Number of laboratories	11	11	11	11
Mean, % of DM	42.03	42.23	35.13	7.04
SD_r[d]	1.04	0.78	0.78	0.51
SD_R[e]	1.56	1.22	0.94	0.87
Repeatability within laboratories[f]	2.90	2.17	2.17	1.43
Reproducibility among laboratories[g]	4.37	3.40	2.62	2.44
HORRAT[h]	1.23	1.62	1.48	4.48

[a]Total dietary fiber measured directly.
[b]Total dietary fiber measured as the sum of IDF and SDF.
[c]Soy and oat brans.
[d]Standard deviation of repeatability within laboratories.
[e]Standard deviation of reproducibility among laboratories.
[f]2.8·SD_r, which is the approximate 95% confidence interval for duplicate analyses within a laboratory.
[g]2.8·SD_R, which is the approximate 95% confidence interval for single analyses between two laboratories.
[h]Horwitz ratio, which is the SD_R divided by the expected SD_R based on the equation by Horwitz (1982).

Table 9. Collaborative study results of Uppsala method (Theander et al., 1995) for measuring total dietary fiber (TDF) as the sum of acid hydrolyzed uronic acids and neutral sugars and Klason lignin (AOAC Official Method 994.13)

Dietary fiber	TDF	Uronic acids	Neutral sugars	Klason lignin
Material used	Brans[a]	Brans	Brans	Brans
Number of materials	2	2	2	2
Number of laboratories	9	9	9	9
Mean, % of DM	51.35	2.70	39.75	8.70
SD_r[b]	1.28	0.24	1.31	0.44
SD_R[c]	3.16	0.37	3.15	0.80
Repeatability within laboratories[d]	3.58	0.66	3.67	1.22
Reproducibility among laboratories[e]	8.85	1.04	8.82	2.24
HORRAT[f]	2.78	3.98	3.45	3.18

[a]Wheat and oat brans.
[b]Standard deviation of repeatability within laboratories.
[c]Standard deviation of reproducibility among laboratories.
[d]$2.8 \cdot SD_r$, which is the approximate 95% confidence interval for duplicate analyses within a laboratory.
[e]$2.8 \cdot SD_R$, which is the approximate 95% confidence interval for single analyses between two laboratories.
[f]Horwitz ratio, which is the SD_R divided by the expected SD_R based on the equation by Horwitz (1982).

ashing and the acid-hydrolyzed filtrate is analyzed for neutral sugars and uronic acids. The neutral sugars are measured as alditol acetates by gas chromatography and uronic acids are measured colorimetrically.

The enzymatic-chemical chromatographic method is time consuming and expensive in terms of both labor and equipment. It requires highly skilled and trained personnel to manage sensitive chemical reactions and to operate and maintain chromatographic instruments. The method is currently used mostly in research laboratories and is not used extensively by commercial or regulatory laboratories. Although the method is chemically sophisticated, the reproducibility of both TDF and total nonstarch polysaccharides (Table 9) indicates that variability among laboratories is about twice that of gravimetric assays for dietary fiber (aNDF or enzymatic).

Combining quantification of sugar monomers with complementary chemical information can be used to describe secondary and tertiary structures that affect the physicochemical properties of fiber that influence digestive physiology and digestibility. This is a valid research endeavor, but until the nutritional value of extensive monomer information is demonstrated and the reproducibility of these methods is improved, it seems premature to recommend these methods for the routine description of feeds. The relatively large SD_R for TDF and neutral sugars (Table 9) suggests that laboratories have difficulty reproducing monomeric information, which appears to be related to the empirical conditions used to isolate enzymatic residues and compromises between maximum solubilization and minimum degradation of sugars during acid hydrolysis. The benefits of extensive carbohydrate analysis will be realized in the short term only if routine analyses are reported concomitantly to provide a bridge between current and future fiber analyses and to determine when alternative methods of analysis provide similar or complementary information.

Knudsen (1997) reported detailed analysis of nonstarch polysaccharides and Klason lignin in feeds using modifications of the Uppsala method, that were reported as soluble, insoluble, and total nonstarch polysaccharide and lignin, which were summed to obtain TDF (Table 10). Although NDF was not measured by Knudsen (1997), several samples of each feed were analyzed, which can be compared to typical NDF values reported by NRC (2001). The IDF of feeds based on insoluble nonstarch polysaccharides plus lignin are generally similar to NDF.

Problems with Empirical Methods

The Codex Alimentarius Commission (1986) describes empirical methods as Type I Defining Methods because the values obtained can be generated only in terms of the specific method—that is, defined by the method. This classification is not a negative reflection on the quality of a method or its repeatability within or reproducibility among laboratories. Because there are no primary reference standards for these methods, they cannot be validated for accuracy in determining the "true" value for the constituent. To minimize systematic errors (bias) among laboratories, empirical methods must be followed exactly because even slight variations in methodology can result in the measurement of a different constituent. Systematic bias among laboratories for empirical methods can be determined only using consensus values, which are the average results of laboratories that follow the method exactly.

It might seem that dietary fiber can be defined more accurately and precisely by specifying and quantifying the chemical monomers in dietary fiber carbohydrates. However, this assumes that sugar analysis is exact and that detailed knowledge of polysaccharide analysis will lead to nutritional insights. Although the accuracy of chromatographic measurement of sugars can be determined using primary standards, the preparation of en-

Table 10. Enzymatic-chemical analysis of insoluble dietary fiber (IDF) determined as the sum of insoluble nonstarch polysaccharides (NSP) and Klason lignin in feeds as reported by Knudsen (1997) compared with neutral detergent fiber (NDF)

Feed	Soluble NSP	Insoluble NSP	Total NSP	Klason lignin	IDF[a]	NDF[b]
Corn grain	0.9	8.8	9.7	1.1	9.9	9.5
Soybean meal	6.3	15.4	21.7	1.6	17.0	14.9
Wheat bran	1.5	35.9	37.4	7.5	43.4	42.5
Beet pulp	40.7	37.2	77.9	3.5	40.7	45.8
Grass meal	3.1	36.6	39.7	16.1	52.7	57.7
Alfalfa meal	7.7	25.2	32.9	12.8	38.0	41.6

[a]Insoluble dietary fiber calculated as the sum of insoluble NSP and Klason lignin.
[b]Reported by NRC (2001).

zymatic residues is relatively empirical and can affect the quantities of sugars and lignin recovered (Marlett et al., 1989). In addition, polysaccharides are converted to monosaccharides, and monosaccharides are degraded at different rates depending on acid hydrolysis conditions and characteristics of the enzymatic residues. Because there is incomplete recovery of sugars, correction factors (based on typical analyses) are needed to correct results and generate adequate quantification.

The nonuniform digestibility of fibrous carbohydrates suggests that knowing their monomeric composition, no matter how accurately, provides little information about their availability to the animal. The physicochemical nature of fiber and its relationship to noncarbohydrate components, such as lignin, may have greater nutritional significance than its intrinsic monosaccharide composition. The consequence of this speculation is that even the most elegant analysis of carbohydrate composition may add little to our ability to evaluate feeds nutritionally. The rationale for dietary fiber analysis is derived from its nutritional consequences. Caution should be exercised to ensure that research on dietary fiber is not shifted from an approach that uses the nutritional definition of dietary fiber to develop methods for measuring it to an approach that develops a method that measures chemical constituents and attempts to define fiber on the basis of its composition.

Dietary fiber methods are now, and may always be, empirical because the result is dependent on the reagents and conditions used in each method to isolate fibrous residues. Horwitz et al. (1990) reevaluated the collaborative studies of all methods used for nutritional labeling and concluded that all fiber methods had poor reproducibility among laboratories when compared to the measurement of crude protein. They suggested that the lack of reproducibility was related to the empirical nature of these methods. The relatively poor reproducibility of dietary fiber methods compared with other methods may be related to several factors.

1. The physical and chemical properties of dietary fiber are distinctly different from other components in feeds, which makes high-fiber feeds more heterogeneous. Thus, it is difficult to prepare a homogenous sample of most feeds because fiber components tend to segregate. Assuming that technique is relatively stable within a laboratory, most of the variation in repeatability within laboratories is related to random differences in the test samples.
2. Many dietary fiber methods are multistep processes that often require corrections for ash or protein contamination and for component recoveries. Each of these steps or supplemental assays has associated random and systematic errors that contribute to the total variation of dietary fiber methods.
3. Random and systematic errors in weighing constitute a significant source of error in gravimetric methods when fiber residues are small. If 1.0000-g test samples are analyzed that contain <5% fiber, the final fiber residue that is weighed is <50 mg. A balance precision of ±0.0001 would result in a weighing error alone of 4% (±2·SD). This error is greatly magnified when the test sample or fiber residue is smaller. In addition, small test sample amounts may make it difficult to obtain a representative test sample of heterogeneous materials.
4. Among-laboratory variation is large for empirical methods because analysts often perform methods in nonstandard ways that do not follow the official method. In addition, quality assurance programs instituted to verify results in laboratories often are inadequate or even nonexistent. Many dietary fiber methods have not been optimized to ensure adequate suitability for all types of material or to identify those steps that must be executed in detail. Often the limitations of methods and rationale for specific steps in a method have not been published or have not been properly relayed to the analyst. But perhaps most of the among-laboratories variation is associated with the desire of analysts to improve efficiency by shortening times, eliminating steps, or failing to follow the details of a method and assuming that these deviations should or would not affect results. These sometimes well-intentioned deviations ignore the fundamental property of empirical methods, such as dietary fiber, which re-

Table 11. Performance statistics of certified laboratories participating in the proficiency-testing program of the National Forage Testing Association[a]

Year and parameter	Dry matter	Crude protein	ADF	NDF
2000				
RMA[b]	91.6	17.2	27.0	37.8
HSD_R[c]	0.24	0.44	0.66	0.87
N[d]	136	139	138	128
SD_L[e]	0.65	0.57	1.14	1.48
2001				
RMA	92.2	14.5	29.4	39.8
HSD_R	0.23	0.38	0.71	0.91
n	133	131	134	129
SD_L	0.65	0.48	1.02	1.40

[a]Published with permission of the Board of Directors of the National Forage Testing Association.
[b]Reference method average of six materials to which all participating laboratories were compared.
[c]Horwitz standard deviation of reproducibility (Horwitz, 1982) calculated from the RMA and used to determine certification of proficiency (HSD_R for dry matter was calculated from moisture concentration). Laboratories must have been within 4·HSD of the RMA for certification in 2000 and 3·HSD of the RMA for certification in 2001.
[d]Number of laboratories eligible for certification for each method.
[e]Standard deviation from the RMA among all certified laboratories for the mean of triplicate analyses.

quires that they be followed to the utmost detail, including calibration of equipment.

Proficiency of Laboratories in Using Empirical Methods

Although the standard deviation from the RMA among laboratories (**SD_L**) eligible for NFTA certification for each method is not the same statistical parameter as the SD_R determined in AOAC collaborative studies, it provides a similar indication of variation among laboratories. The SD_L differs from the SD_R because it does not include variation among replicates within laboratories and is based on the average of triplicates rather than single analyses; however, it includes systematic bias because it is determined in relation to the RMA. Observed SD_L (Table 11) of ADF and NDF are larger than the HSD_R (ranging from 144 to 173%), which may indicate that laboratories participating in the NFTA proficiency-testing program are not required to follow any specific method for their analyses (although they are encouraged to do so). It may also indicate the variability of empirical fiber methods because the HORRAT for all of the dietary fiber methods are >1.0. Surprisingly, the relative SD_L for CP, ADF, and NDF were similar (3.3 to 4.2% of the mean), which disagrees with the conclusion of Horwitz et al. (1990) that fiber results are less reproducible than protein results. The small relative SD_L (<5%) for the routine methods of feed analysis suggests that analyses among laboratories certified as proficient by the NFTA are comparable when replicated results are reported.

Table 12. Average regression parameters for the relationship between digestibility and dietary fibers adapted from Giger-Reverdin (1995)

Dietary fiber	Intercept	Slope	R^2	SE_{reg}[a]
CF	89.7	–0.77	0.63	5.31
	(7.2)[b]	(0.27)	(0.16)	(2.53)
NDF	97.7	–0.54	0.67	5.92
	(7.8)	(0.17)	(0.12)	(3.28)
ADF	95.4	–0.71	0.68	4.95
	(9.1)	(0.43)	(0.16)	(2.53)
Lignin	83.1	–2.98	0.79	4.94
	(3.7)	(0.68)	(0.08)	(2.86)

[a]Standard error of regression.
[b]Values in parentheses are standard deviations of the parameter from 15 experiments.

Practical Utility of Empirical Methods

One criterion for the usefulness of dietary fiber methods is their ability to predict DM digestibility. Giger-Reverdin (1995) summarized a large number of published reports that provided independent regression equations for predicting digestibility based on various fiber fractions, which were averaged to obtain the mean and standard deviation of intercepts, slopes, R^2, and standard deviations of regression (Table 12). The highest average and smallest SD of R^2 indicates that ADSL had the best average relationship with digestibility. However, the large SD for the slope of the regression for ADSL suggests that its quantitative relationship with digestibility is variable among populations of materials. Although it has the highest and most variable average standard error of regression, NDF obtained the most robust relationship with digestibility as evidenced by the smallest variation in the slope. The average regression parameters for NDF suggest that about half of NDF is typically digested and that the digestibility of NDS is about 97.7% (i.e., the digestibility of a feed containing no NDF and therefore 100% NDS).

Choice of Insoluble Dietary Fiber Methods

There is no perfect method for measuring IDF because the needs of each user of analytical information are unique. All dietary fiber methods are compromises between nutritional relevance and analytical convenience and reproducibility. Dietary fiber methods are of variable and unequal value in measuring nutritional utility. Assuming that it is nutritionally relevant, the selection of an IDF method depends on several factors: reproducibility, repeatability, labor efficiency, timeliness, personnel requirements, cost, and use of the results. Occasionally, nutritionists may benefit from fractionation of TDF into SDF and IDF when attempting to explain physiological responses of animals, and researchers may want to expend the time and effort to obtain a detailed description of monomeric composition. Even in these situations, reproducibility and ruggedness of the IDF method are requisite so that results

can be interpreted reliably and applied universally. However, complete separation and quantification of IDF polymers is impractical, expensive, and unnecessary for routine descriptions of feeds and formulation of diets. Until the nutritional implications of detailed dietary fiber analysis are understood and adapted to ration formulation, it appears that simple empirical methods of IDF analysis will provide the nutritional information of interest.

Conclusions

Methods for measuring insoluble dietary fiber are inherently more variable than methods for other nutrients. Acceptability of a dietary fiber method is based on its ability to match the definition of fiber and its reproducibility among laboratories. Several current methods, including aNDF and the enzymatic-gravimetric methods for measuring TDF, seem to be relevant for measuring IDF because they match the appropriate definition of dietary fiber for herbivores. These methods have acceptable reproducibility among laboratories when followed exactly. Variability in NDF among laboratories is related primarily to differences in modifications of the method that are used, and laboratories need to be more exact in describing their methods and naming their results. Official methods for measuring TDF using enzymatic hydrolysis and measurement of monomers and Klason lignin after acid hydrolysis do not report an IDF fraction but could be modified to do so. The sum of insoluble nonstarch polysaccharides and Klason lignin agrees with IDF measured by NDF, but the reproducibility of TDF when using acid hydrolysis is less than that of other dietary fiber methods. The selection of a suitable method for IDF depends on the purpose of analysis. Analysis of insoluble polysaccharides provides more chemical information but is less reproducible among laboratories and more expensive to obtain. For routine nutritive evaluation of feeds and formulation of diets, aNDF seems a reasonable choice for measuring IDF. To verify that their dietary fiber results are comparable, research, regulatory, and commercial feed-testing laboratories should participate in check-sample or proficiency-testing programs. Alternative methods of dietary fiber analysis may provide supplemental or complementary information, but routine analyses should be reported for these experiments to provide a bridge between current and future dietary fiber analyses.

Implications

All dietary fiber methods are empirical to some degree. This places additional burden on the analyst to avoid shortcuts and follow the details of the method exactly, during every analysis. Although IDF analysis requires attention to detail and good laboratory techniques, its practical utility in predicting digestibility, intake, and nutritive value makes it a necessary part of routine and research analysis of feeds. More research is needed to evaluate IDF methods for their reproducibility when analyzing typical feeds and forages, and to directly compare their ability to provide both similar and complementary nutritional information.

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