Symposium review: Fiber and in vitro methods, analytical variation, and contributions to feed analysis*

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ABSTRACT

At least 2 basic inputs are needed to formulate rations: the nutritional requirements of the animals to be fed and the nutritional composition of the feeds. David R. Mertens not only defined fiber requirements for dairy cattle but became a leading expert in the laboratory measurement of fiber in feeds, digesta, and feces. Fiber is a heterogeneous nutritional entity composed mainly of polysaccharides and polyphenolics. Because the method defines the fiber that is measured, methods must be described thoroughly and followed exactly to obtain results that are repeatable within a laboratory and reproducible among others. Filtration of neutral detergent fiber (NDF) can be difficult, and those who have worked in his laboratory can attest that Mertens rigorously studied this, along with other method details to improve NDF analysis from sample preparation to blank corrections. Mertens’s procedure for amylase-treated NDF (aNDF), using α-amylase and sodium sulfite with crucibles, culminated in the Association of Official Analytical Chemists Official Method 2002.04 for aNDF, which was also accepted as International Standard ISO 16472:2006 and is used worldwide as a reference method for feed evaluation. Because aNDF digestibility is variable and a key factor in overall digestibility, Mertens also worked to improve in vitro ruminal digestibility and gas production procedures within and among laboratories, including procedures using flasks or filter bags. His in vitro gas production method is currently used by commercial laboratories that generate a significant share of the aNDF digestibility results reported worldwide. Outside of the laboratory, his extensive outreach to commercial and research laboratories has had a huge impact on fiber analysis, in vitro digestibility, and other laboratory procedures. While advising the National Forage Testing Association, Mertens provided program infrastructure that improved laboratory proficiency in more than 120 laboratories in the United States and around the world. Most importantly, thanks to his advances in fiber analysis and in vitro digestibility techniques, Mertens has enhanced the evaluation of feeds and the nutrition and health of dairy cows. These contributions have helped thousands of dairy farmers and nutritionists around the globe and continue to have a substantial impact on the industry.

Key words: Mertens, neutral detergent fiber, aNDF, in vitro fiber digestibility

INTRODUCTION

With the overarching premise that DMI is the main determinant of animal performance, David R. Mertens worked exhaustively to relate the nutritional composition of forages and forage digestibility to rumen fill and DMI (Mertens, 1973). Mertens strove to improve 2 laboratory methods of analysis: the measurement of amylase-treated neutral detergent fiber (aNDF) and the determination of in vitro ruminal aNDF digestibility in feeds commonly fed to ruminants.

In the practice of ruminant nutrition, knowing the nutritional composition of feeds is critical for ration formulation. In this regard, Mertens’s work has had a substantial impact on livestock production systems through the refinement of laboratory methods that have become standard methods of analysis (Undersander et al., 1993; Mertens, 2002; ISO, 2006). Implementation of these methods by commercial laboratories around the world to characterize ruminant feeds has directly or indirectly influenced thousands of livestock farming operations and continues to do so.

The objectives of this review are to describe Mertens’s major accomplishments in fiber and in vitro ruminal digestibility assays and to describe the influence that his work has had on the dairy industry at national and international levels.
EMPIRICAL METHODS OF ANALYSIS

Analytical methods are designed to measure an analyte of interest in a complex feed matrix. Generally, the analyte is an ion or molecule (e.g., calcium, phosphorus, lysine, nitrate). The Codex Alimentarius, or food code, defines type II methods as those in which an identifiable analyte, such as an ion or a molecule, is quantified (Zielinski et al., 2013). These methods are also called rational or theoretical methods. The Codex Alimentarius also defines type I methods as non-analyte-specific methods in which the measurand is defined by the method and is not a specific ion or molecule (Zielinski et al., 2013). These methods are also called empirical methods.

Type I methods are frequently used in feed testing and include fiber methods, fat methods, loss on ignition (ash), loss on drying (DM), in vitro ruminal digestibility, and lignin, to mention a few. In the true spirit of empirical methods, only a single method can be associated with a specific analyte name. Also, great care must be taken when performing empirical methods so that they are executed exactly as validated and published, given that even the slightest modification can cause the test results to be biased from the published method. Generally, empirical methods are not calibrated with reference standards, since such standards do not exist.

Using lignin as an example, the concentration of lignin in animal feeds is frequently determined through the sequential extraction of a feed sample in acid detergent and then 72% sulfuric acid (Van Soest, 1963; AOAC, 1990; Hintz et al., 1996). However, the concentration of lignin in animal feeds can also be determined using other empirical methods, such as the extraction of lignin using acetyl bromide (Ferreira et al., 2021) or the Klason method (Theander et al., 1995; Van Soest et al., 2020). Given that the analytes measured by these 3 methods are different, the analyte cannot be called “lignin” indistinctly. Rather, it should be specifically called ADL, acetyl bromide lignin (ABL), or Klason lignin, respectively, according to the method of analysis. To highlight the importance of thoroughly following a described methodology, obtaining lignin concentrations after extracting fiber in neutral detergent will yield different results than after extracting fiber in acid detergent.

The existence of several empirical methods is not unique to lignin and includes determining the concentration of fiber in animal feeds. For example, Hintz et al. (1996) reported that the concentration of amylase-treated NDF in brewers’ grains was 52.3% when sodium sulfite was omitted but 40.9% when sodium sulfite was used as a reagent in the method. Similarly, when using filter bags, Ferreira and Mertens (2007) reported that NDF in corn silage was 47.3% when amylase was omitted but 43.3% when amylase was used in the method (with sodium sulfite used in both treatments). These examples demonstrate that empirical methods of analysis should be clearly described and that, when a reference method exists, care must be taken that the method is executed exactly as published, because any modifications will bias test results (Mertens, 2002).

AMYLASE-TREATED NDF

The detergent system of forage analysis was developed by Peter Van Soest and collaborators in the early 1960s (Van Soest, 1963; Van Soest and Marcus, 1964). Originally, cell wall constituents (i.e., lignin, cellulose, hemicellulose, and some wall-bound proteins) in non-starchy forages were extracted by boiling 1 g of sample for 1 h in 100 mL of pH 7 detergent solution containing 3% sodium lauryl sulfate and 1.86% disodium EDTA (Van Soest and Marcus, 1964), 0.68% sodium borate decahydrate, and 0.46% disodium hydrogen phosphate (Van Soest and Wine, 1967). The resulting residue, insoluble in the mentioned neutral detergent (ND) solution, was termed neutral detergent fiber (NDF). The original NDF method included the addition of sodium sulfite to increase the solubilization of proteins (Van Soest and Wine, 1967) and reduce protein contamination in the ND-insoluble residue through the cleavage of disulfide bonds (Van Soest et al., 1991). The original NDF method was modified to add heat-stable amylase and triethylene glycol to remove interference from starch (Van Soest et al., 1991). Finally, because it solubilizes some of the phenolic compounds from lignin (Hintz et al., 1996), the addition of sodium sulfite was considered optional (Van Soest et al., 1991). All these variants to the NDF method are not trivial, as each modification will result in different test results. Consequently, it is paramount that these empirical methods are properly distinguished, test results from each method are labeled accordingly, and test results are interpreted taking method conditions or modifications into consideration. The variation in methodology is especially crucial when interpreting meta-analysis of animal responses to fiber and feed evaluation. Often the method is not described adequately to allow for comparisons among similar studies.

Although an Association of Official Analytical Chemists (AOAC) International official method for determining the concentration of acid detergent fiber (ADF) in animal feeds (AOAC Method 973.18; AOAC, 1990) existed, no official method had been approved for determining the concentration of NDF in animal feeds by 1993 (Undersander et al., 1993). The lack of an official or reference method for determining the con-
Mertens began a project at the USDA-ARS, US Dairy Forage Research Center in Madison, Wisconsin, to investigate and address the problems in the analysis of fiber. Mertens's early development of the method “Amylase Neutral Detergent Fiber (aNDF) by Refluxing in Beakers or Crucibles,” which included sodium sulfite and added heat-stable amylase during both refluxing in ND and filtering the residue, was proposed as a reference method (Undersander et al., 1993) for commercial feed testing laboratories seeking certification by the National Forage Testing Association (NFTA). This method was proposed for any type of forages and feeds (Undersander et al., 1993).

Among many factors, filtration technique and residue contamination with starch or protein were 2 major factors causing variation in NDF analysis (Mertens, 1992). Improvements to filtration techniques have been addressed in multiple papers (Van Soest et al., 1991; Mertens, 1992; Mertens, 2002), and laboratory technicians should be aware of them. To avoid contamination of NDF residue with starch, Van Soest et al. (1991) added heat-stable amylase during refluxing in ND, but Mertens observed that amylase was more effective and assisted filtration when it was added as a split dose during both refluxing in ND and also during filtering the residues. Although Van Soest et al. (1991) considered the use of sodium sulfite optional and were concerned about its effect on removing lignin components, Mertens and his collaborators (Hintz et al., 1996) observed that adding sodium sulfite avoided protein contaminants in NDF residues, especially when feeds were heated. Also, Mertens wanted the official method for aNDF to be able to analyze all types of feeds, and not just forages. Therefore, as no optional reagents can be in an official method, both heat-stable α amylase and sodium sulfite are critical reagents in aNDF analysis (Mertens, 2002).

Mertens’s work on fiber analysis culminated in a collaborative study proposal to AOAC for the analytical determination of aNDF. The collaborative study, involving 12 research, feed company, regulatory, and commercial feed testing laboratories (Mertens, 2002), met repeatability and reproducibility standards and resulted in adoption of the method as AOAC Official Method 2002.04 (Mertens, 2002; AOAC International, 2016). Mertens received the 2003 Collaborative Study of the Year Award from the AOAC, with the citation that

This study is deserving of this award for its complexity and impact on the international agricultural community ... The method truly deserves to become the much-awaited international standard for the determination of neutral detergent fiber.

Mertens’s method filled many needs, including NFTA’s need of a reference method to assess the proficiency of commercial feed testing laboratories seeking certification. Through this study, Mertens encouraged numerous laboratories to improve aNDF methodology. Ultimately, the repeatability and reproducibility of aNDF in difficult feed matrices was substantially improved by laboratories implementing the aNDF method.

IN VITRO RUMINAL FIBER DIGESTIBILITY

The determination of in vitro ruminal digestibility of fiber is also an empirical method of analysis with substantial variation within and among laboratories. In addition to the lack of a reference method, the dependence of the system on animal donors to provide one of the “reagents,” the ruminal microorganisms, makes in vitro ruminal digestion systems extremely variable. Also, great variation in the methodology of in vitro digestion systems exits, including the incubation of samples in water baths within flasks or tubes in which the substrates are fermented (Mertens, 1973; Grant and Mertens, 1992a), the incubation of samples in bottles from which gas production is measured (Mertens and Weimer, 1998), or the incubation of samples in filter bags within a rotating-jar system in which the undigested residue is measured (Ferreira and Mertens, 2005).

Mertens’s work on in vitro ruminal digestion was focused on reducing analytical variation and maximizing fiber digestion using many of the existing in vitro systems. For example, to minimize analytical variation, Mertens (1973) highlighted the importance of minimizing the time from rumen fluid collection to inoculation of the in vitro system to minimize variation, especially for short intervals of fermentation (e.g., 24 h or less). Under Mertens’s mentorship, Ferreira (2002) evaluated different sources of variation that affect in vitro ruminal digestion when using a rotating-jar system and found that the position of the jar within the incubator can have a substantial effect on in vitro fiber digestibility of corn silage. Given the design of the heating source and the air-circulating fan within the incubator, Ferreira (2002) observed that in vitro ruminal digestibility was positively correlated with the temperature of the inoculum within the jar. Because in vitro ruminal digestibility is affected by the position of the jar within the incubator,
Mertens's work on in vitro ruminal digestion also aimed to maximize the possibility of detecting differences in substrates based on their intrinsic properties. In this regard, Grant and Mertens (1992a) recommended the use of continuous CO₂ gassing and the addition of reducing agents to promote earliest initiation and fastest digestion of the substrates when using in vitro digestion systems. To minimize variation among in vitro ruminal digestibility runs, Mertens also evaluated the concept of apparent donor specificity of the ruminal bacteria (Weimer et al., 2010). Using an in vitro gas production system, Mertens et al. (1998) reported that cow donors of rumen fluid, more importantly than diets, affected gas production kinetics and concluded that differences in gas production kinetics are associated with ruminal pH. The use of a composite inoculum prepared from ruminal solids blended with anaerobic media and rumen fluid obtained from different cows became Mertens's recommendation and common standard procedure (Ferreira and Mertens, 2005; Grabber et al., 2009; Schlau et al., 2021).

Another of Mertens's major contributions has been the interpretation of in vitro fiber digestibility through mathematical models. Using semi-log plots, Smith et al. (1971) reported a high linear correlation between in vitro undigested fiber and time of fermentation and concluded that fiber digestion follows first-order digestion kinetics with an intercept equal to 100 at time 0. However, Mertens (1973) observed that in most cases the intercept of in vitro undigested fiber was larger than 100 and proposed that fiber digestion follows first-order digestion kinetics with a discrete lag time. According to Mertens (1973), the lag time could be attributed to the growth and adaptation of the bacteria in the in vitro system or to the nature of the substrate to be fermented. To test the nature of the substrate, Mertens and Loften (1980) determined the in vitro ruminal fiber digestibility of forages with the addition of different amounts of starch and observed that the lag time associated with fiber digestion increased with the addition of starch in the substrate. Grant and Mertens (1992b,c) later reported that reducing the pH of the in vitro system increases the lag time and reduces the 96-h digestion of the fiber, and that starch accentuates this effect for some substrates.

**MERTENS'S IMPACT AND LEGACY**

Throughout his career, Mertens has worked within his laboratory and collaborated with others to improve methods of analysis. Because most of this work was conducted in his laboratory, Mertens's contributions might be interpreted as having limited impact. Quite to the contrary, his contributions have had a tremendous impact on the livestock production industry at both national and international levels.

Mertens's validation of his aNDF method through the process to Final Action as an AOAC Official Method (Mertens, 2002; AOAC International, 2016) was adopted by the International Organization for Standardization as a standard method for the determination of aNDF in all types of animal feed (ISO, 2006) and established its value as a reference method for NFTA certification. Mertens's method is used confidently for aNDF determinations across government, university research, regulatory, and private commercial laboratories worldwide.

Outside the laboratory, Mertens's impact is also remarkable. At least 2 things are needed to formulate a ration: knowing the nutritional requirements of the animals to be fed and knowing the nutritional composition of the feeds. Therefore, thousands of farmers and millions of cattle have been directly affected by Mertens through forage testing for feed evaluation and as part of a ration formulation process. For example, 2 commercial laboratories certified by NFTA estimate that aNDF or in vitro rumen digestibility test results have reached 8,683 stakeholders within the United States and more than 1,820 stakeholders throughout 49 countries since 2015 (D. Taysom, Dairyland Laboratories, Arcadia, WI, and R. Ward, Cumberland Valley Analytical Services, Waynesboro, PA, personal communications). Also, through his work with NFTA, Mertens has contributed to the certification process of 99 laboratories in the United States and 38 laboratories around the world (NFTA, 2022).

Mertens's contributions through NFTA and the Near Infrared Reflectance Spectroscopy (NIRS) consortium have also been significant. The NFTA was established in 1984 to reduce variation among participating analytical laboratories, and Mertens played a significant role in this research priority. In 1992, Mertens was elected to the Board of NFTA. In this new role, he was asked to develop a new protocol for certification, which is still implemented today. Unfortunately, very few university and research laboratories are aware of such a certification program. Participation in this rigorous proficiency testing program could ensure that analytical results of university and research laboratories are reproducible and comparable to the analyses used in the field to implement research conclusions. To train laboratory personnel in proper methods for fiber analysis, Mertens additionally conducted several hands-on workshops, made numerous presentations at...
NFTA annual meetings, and helped many laboratories solve specific problems with their analytical methods. From 1984 to 1994, Mertens served as the US Dairy Forage Research Center’s representative to the NIRS consortium. In a collaborative effort, the consortium developed the technology associated with the use of NIRS for rapid forage analysis (Windham et al., 1989). The use of NIRS for feed analysis likely represents more than 75% of all reported results, given that it is both rapid and inexpensive. That being said, Mertens has urged researchers and feed analysis laboratories to continuously validate NIRS results for research use (Buxton and Mertens, 1991; Jung et al., 1998). Mertens’s work with aNDF method evaluation, NFTA, and NIRS culminated with his efforts to identify and quantify sources of variation in fiber analyses, and the factors that could be controlled to minimize the analytical results used to formulate dairy rations (Mertens, 2007).

Another significant contribution emanates from Mertens’s mentorship legacy, which includes scientists such as Mike Allen and Rick Grant and his close working relationships with colleagues in Denmark, Finland, France, Italy, and New Zealand, all of whom have made substantial contributions in the dairy industry nationally and internationally. Those who had the privilege of working under Mertens’s mentorship obtained invaluable laboratory skills for using, developing, troubleshooting, evaluating, and improving laboratory methods. These laboratory skills might be increasingly scarce in the future, as important parts of the analyses performed on forage research are delegated to commercial laboratories.

After retiring from the USDA-ARS in 2010, Mertens founded Mertens Innovation & Research LLC and continues to work with laboratories and companies developing products for fiber digestion, designing experiments, developing appropriate laboratory methods, improving the accuracy of measurements, and interpreting results.

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