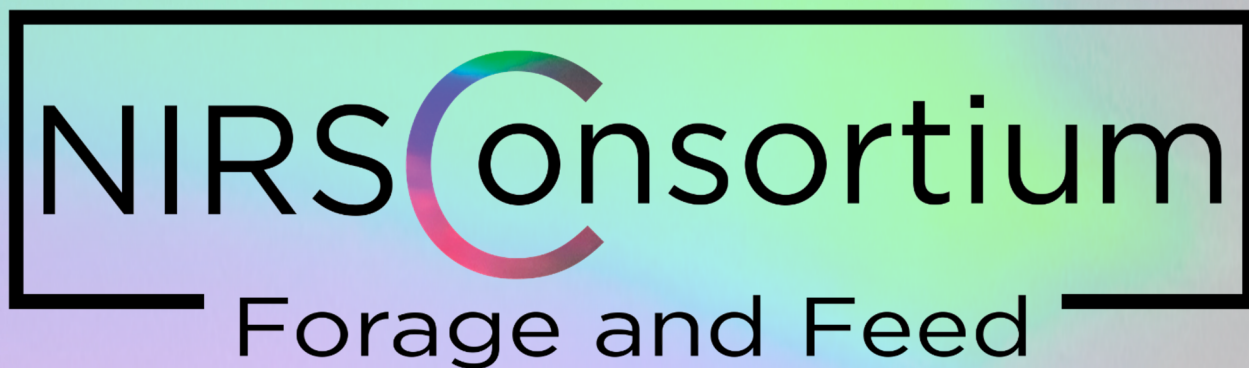




GUIDELINES FOR OPTIMAL USE OF NIRSC FORAGE AND FEED CALIBRATIONS IN MEMBERSHIP LABORATORIES

SECOND EDITION

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1.1 Brief History of Near Infrared Spectroscopy

Spectroscopy is the branch of science concerned with the investigation and measurement of spectra produced when matter interacts with or emits electromagnetic radiation. The electromagnetic spectrum is the range of all the types of electromagnetic radiation, or energy, that exists within the near infrared region defined as 780-2500 nm (Figure 1.1).

Near infrared radiation (NIR) was first identified in 1800 by William Herschel, who was curious as to whether a certain color was associated with the heat of sunlight (Hindle, 2008). In 1938, near infrared spectroscopy (NIRS) was described in scientific literature and neglected because it was believed that other types of spectroscopies were more useful (Ellis and Bath, 1938). Then, in 1968, NIRS was first used to describe the characteristics of agricultural products by Karl Norris and associates at the USDA Instrumentation Research Laboratory in Beltsville, MD (Workman and Shenk, 2004).

This was an important breakthrough since Norris was the first to combine the right spectrometer, computer, and advanced chemometrics to develop prediction models for animal feeds. By the 1970s, an array of chemical analysis methods was commonly being used by labs to describe the nutritional characteristics of forages. In 1976, Karl Norris and his group took chemical analysis reference data, NIR spectra, and used chemometrics to predict crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF) (Norris et al., 1976). Concurrently, John S. Shenk and colleagues at Penn State University were developing advanced calibration and prediction software for performing NIRS analysis (Shenk et al., 1978).

By the 1980s, both hardware and software were available for forage and feed testing. Some university and commercial agricultural testing labs had adopted the technology, however, there were variations in techniques and early adoption of this secondary analysis was slow to be accepted.

1.2 The NIRS Forage and Feed Consortium

Scientists at six United States Department of Agriculture Agricultural Research Service (USDA-ARS) locations formed the National NIRS Forage Research Project Network in 1978; and, worked for nearly a decade to summarize their findings in a report by Marten and co-workers (Marten et al., 1989). In 1990, a group led by John S. Shenk was formed to evaluate the variation among testing labs and determine ways to standardize instrument and software methodology.

That group later became the NIRS Forage and Feed Testing Consortium (NIRSC) in 1992. The organization created specific goals of standardizing instrumentation and software which were developed into standard operating procedures in the industry and for NIRSC members. The NIRSC was first incorporated in 1998, then in 2002 elected a Board of Directors, created bylaws, and established the 501(c) non-profit organization.

These goals remain intact today within a much larger scope of testing strategies and animal nutrition constituents within forages and other feedstocks. The NIRSC is a 501(c)(6) non-profit organization composed of Instrument Partners, Collaborators, Sponsors, Commercial Laboratories, Research Laboratories, University Laboratories, Plant Breeders, and Producers. The NIRSC strives to unify knowledge, accuracy, and application of NIR technology to help members in the use of calibrations, educational efforts, and support with instrumentation.

The membership shares in the effort and costs to produce calibrations for use with continued work to strengthen forage and feed testing and to encompass new technology as it comes available. The NIRSC works toward a vision to lead collaboration and applications for agriculture through educational efforts, promotion of best sample handling and preparation practices, and proper NIR instrument and calibration use. The goal of the NIRSC is to create uniformity in all aspects of NIRS analysis of feeds and forages for the membership and beyond.

Our mission is to promote and standardize the use of NIRS through the development of robust, accurate prediction calibrations.

1.3 Goals of this Guide

This guide consolidates methods and resources specifically for NIRS analysis for NIRSC calibrations used on instrument partner machines. Specific recommendations for sample preprocessing and scanning required to ensure compatibility with NIRSC calibrations are highlighted. Additional materials are included on the use and reporting of NIRSC predictions.

Goals of this guide are to:

1. Develop an understanding of basic NIRS technology and calibration use.
2. Provide guidance on how to monitor internal instrumentation and calibration performance.
3. Create a resource for correct sample preparation, handling, and scanning.

2.1 Instrumentation

A source of light is filtered to produce near-infrared light. It is then directed onto the sample. Some of the light interacts with the sample and is absorbed, while some interacts and is reflected. Hydrogen, oxygen, and carbon bonds are especially important. These molecular bonds vibrate, bend, and stretch. Some vibrations are more active in the NIR region than others, explaining why certain constituents are more successfully predicted by NIRS analysis than others. Because of active bond vibrations, NIR light interacts with a sample in a specific way. The information from that light/sample interaction is collected (Workman and Shenk, 2004). Depending on the NIR instrument, transmittance and/or reflectance can be measured. Both use conversions to absorbance. The spectrum is made up of the absorbance values at a set of wavelengths. The spectrum obtained from a sample is therefore a composite of all chemical and physical external information. Every sample has a unique NIR composite spectrum.

2.2 Calibrations

An NIRS calibration is developed to predict unknown values of a sample based on spectral characteristics using principles of chemometrics. This is done by acquiring spectra and known reference chemistry values on a group of samples that should cover the complete range of possible reference values and spectral variability for a given product type. This means that a good representation of the low, medium, and high-quality samples should be included in the calibration dataset. A broad range of growing conditions, species, cultivars, as well as multiple cuttings, and years of growing seasons should be considered to acquire ranges for each quality constituent.

When selecting samples for calibration development, care must be taken to cover not only the full range of reference values but also the complete variation in multi-nutrient relationships. These relationships occur due to species composition in a sample that may be similar in the spectra; but have a difference

in a unique composition identifier, therefore should be included in a dataset. Once a robust sample set is obtained, population structuring is used to eliminate redundant samples and outliers. A quality set of samples with spectra and reference values allows for a calibration to be built (Workman, 2007). The individual calibration is created for each constituent of interest. Generally, multiple calibrations are put into one file specific to a desired grouping specific to a product of similar characterization.

Although NIRS analysis of feed and forage is a rapid and effective method for determining sample composition there are limits to its application and these limits must be observed. Failure to observe these limits, can lead to inaccurate or misinterpreted results. The NIRSC offers several different calibrations for a wide variety of feedstuffs. Each calibration was developed to analyze a specific group of materials such as legume hay, grass hay, mixed hay, haylage, and both fermented and un-fermented corn silage. Each product calibration includes multiple constituents, or parameters. For NIRS results to be reliable, it is imperative to limit calibration application to the range of sample types known to be included in the calibration set. The membership submits samples from across the United States, Canada, and several international locations. These samples are used to updated calibrations annually. This continued expansion results in a database that encompasses species, cultivars, growing conditions, harvests, weed content, and mixtures.

2.3 Calibration Performance

Calibration performance is best evaluated with a validation set. The group of samples selected for validation should be independent of the calibration dataset and have accompanying wet chemistry which was done with methods matching those used in the original calibration development. It is important to first evaluate the reference laboratory error. NIRS is a secondary method that works within the error limits of the reference method. Data from the laboratory on blind replicates can be used to calculate the standard error of the laboratory reference method (SEL), also known as the standard error of a single test, which is the standard deviation of analytical results of replicates of one or more samples (Williams et al., 2017).

While equations for SEL vary, Workman (2007) and ASTM (2012) define it as:

$$SEL = \sqrt{\frac{\sum_{i=1}^n \sum_{j=1}^r (y_{ij} - \bar{y}_j)^2}{n(r-1)}}$$

The i index represents different samples and the j index different measurements on the same sample, y_{ij} is an individual replicate of a sample, $\bar{y}_j$ is the mean of replicates of a sample, n is the number of samples, and r is the number of replicate analyses for each sample. Simpler equations for the case of

duplicate analyses of each reference sample are given by Workman (2007) and Murray and Cowe (2004). Next, the NIRS predicted output values are compared to the wet chemistry reference values and the Standard Error of Prediction (SEP) is calculated. As a rule of thumb, SEP should be 1.5-2 times the lab error for most constituents.

The SEP, Workman (2007) and ASTM (2012) define it as:

$$SEP = \sqrt{\frac{\sum_{i=1}^n (Lab - NIRS)^2}{n}}$$

While reproducibility of NIRS predictions is very good, there is added performance error due to the repeatability of the reference method. Calibration performance accuracy is the sum of the reference error and the error of the NIRS system.

2.4 Calibration Statistics

Separate from independent validation, each calibration contains a group of statistics calculated by software during calibration development. It is important to note that calibration statistics are directly related to the range of samples used in the calibration dataset. Please note that transferred database, from a master instrument to clones, calibration statistics are specific to each calibration set and instrument model. Please consult with your manufacturer and the NIRSC to determine this.

Number of samples in the calibration dataset (N) is influenced by the natural variation in the trait of interest. The number of samples required to create a quality calibration is dependent upon the analytical and spectral diversity within the dataset selected for developing the calibration. There are varying opinions and methods that determine the number of samples required to develop a calibration. For example, some calibrations have vast amounts of samples included to predict well; then, others have less to produce good results as well. There is no specific number of samples needed to develop a calibration. Sample data sets should be focused on over-all goal of the calibration outcome with appropriate range, treatments, species, cultivars, harvests, growing conditions, and regional representation.

The Standard Error of Calibration (SEC) is the standard error between reference values and NIR values when the calibration sample set is predicted with that calibration. These values should be low in relation to the range of the dataset population. The SEC values will be higher for those constituents with more range. This uses the calibration dataset predicted back on itself and is therefore the most optimistic validation with highest correlation; often used for feasibility studies and comparisons.

An R-squared (RSQ) or coefficient of determination, describes the correlation between reference and NIR predicted values for the calibration set. The RSQ should be between 0.70 and 1.0, however is heavily dependent on the range of samples of the dataset. A calibration set of similar samples will produce exceptional R-squared values and may be over-fit, while robust datasets with range that are more ideal for forage analysis may have lower RSQ.

The Standard Error of Cross Validation (SECV) describes the standard error between reference values and NIR values for the calibration set when samples are systematically removed from the calibration process using cross validation. This is the best estimate of prediction accuracy available from the calibration samples. SECV values close to the SEC values are ideal.

2.5 Utilizing Global and Neighborhood Distances

NIRSC calibrations are supplied with, or contain, appropriate data for NIRS software to calculate and report Global H (GH) and Neighborhood H (NH) distance values. These are statistical guidelines for how well a sample fits within the calibration dataset. These values are based on the principles of *Mahalanobis Distance* that is used to determine if the spectrum collected has a prediction is suitable for analysis (Whitfield et al., 1987). It is important to monitor these values closely as they can be used to provide guidance to the user in the following situations. However, please consult with the NIRSC technical staff to determine how to use these values as they can be reported differently depending on instrument manufacturer software with the same principles outlined below.

The GH is global spectral distance, or how close that sample is to the mean spectrum of the calibration set (Shenk, 1989). And, the NH is neighborhood spectral distance, or how close that sample is to its nearest neighbor in the calibration set (Shenk, 1989). Typically, an NIRS predicted result is considered acceptable when the GH value is less than 3.0, and NH is less than 1.0 (Shenk et al., 2008; Williams et al., 2017). However, it should be noted that the accuracy of the predicted value is not always poor with high H values. GH and NH values are statistical guidelines for how well a sample fit within the calibration set but should not be interpreted as a relative measure of accuracy. Therefore, normal quality measures and validation must still be carried out. After first confirming that all sample preparation and handling procedures were done properly, samples with high global and neighborhood values are often good candidates for future calibration database additions. Please note, some instrument software packages have renamed the fit statistics to another form, however, the same principle is met.

Examples of the causes for GH and NH issues:

1. *A sample may be misclassified.* For example, a legume hay sample was analyzed with a corn silage calibration.
2. *A sample is not dried appropriately.* NIR is very sensitive to moisture. Under and overdried samples that fall outside the acceptable moisture range (93.7-97% DM), depending on forage type) will often have high GH and NH values.
3. *A sample is not ground correctly.* Second to moisture, NIR is most sensitive to particle size. Samples that are ground to a particle size that is different than those in the calibration set (1 mm, cyclone mill) will have higher GH and NH values. Screens should be replaced periodically when any sign of wear is visible. Worn screens produce samples with varying particle sizes.
4. *A sample is high in ash.* High GH and NH values can indicate levels of minimal absorbance, therefore high ash levels in a sample. There are situations where a calibration is developed with a spectral range including the visible spectrum that this principle of absorbance will not be the case.

Any sample exceeding suspect H value limits (GH >3.0) should be investigated for identification and classification accuracy, proper drying, two-step grinding, and ash content. An error in sample preparation should prompt re-prepping and re-analyzing the sample. Errors in any case should prompt rescanning the sample. If upon re-analyzing the sample the H values remain high, it should be concluded that the sample is not close to any of the samples in the calibration database. **Please note that NH and GH values may be higher on transferred databases.*

Reporting results for samples that yield high Global and/or Neighbor H values without discussing the situation with the client or confirming predicted values with wet chemistry analysis is a disservice to the client. This practice may also lower the quality and reputation of the laboratory, and by association, the NIRSC. Such samples should be saved and sent to NIRSC for selection and possible inclusion in calibration updates. However, this sample may have contamination of some sort as well. If sample contamination and preparation/scanning errors ruled out; then, the sample should be sent to the NIRSC for further investigation and possible inclusion into the next calibration update.

2.6 Instrument Performance and Environment Considerations

Maintaining consistent conditions and cleanliness for NIR instruments is important in generating reproducible results. Instrument and environment are important as variation in temperature, humidity, vibration, and power supply affect instrument performance and production of spectra. Analysis of forage samples by NIRS requires additional attention be paid to environmental conditions for sample presentation and proper use of instrumentation.

Environmental conditions to monitor in the lab environment:

1. *Run instrument diagnostics.* Do this with instrument manufacturer requirements, and any other routine internal quality measures your lab has established.
2. *Equalize temperature and humidity of unknown test sample.* Samples should be acclimated to the room environment of the instrument.
3. *Maintain a room temperature between 68-72°F.* This will provide instrumentation ambient cooling.
4. *Clean instrument.* Follow instrument manual for tips on keeping instrument and parts clean and free of dust contaminants.
5. *Control humidity levels in the lab.* High humidity levels in the laboratory affect your sample moisture content and can damage instrument components over time as well.

Best practices for instrument performance:

1. *Ensure instrument has a stable consistent power supply.* We recommend a backup battery to protect against power outages and surges.
2. *Always leave lamp turned on.* This is the most important thing to do even if scanning once a week. This allows for stable scanning and temperature consistency of the instrument. Some software packages might need to be left on as well. The instrument computer may be “locked” when not in use and can be restarted when necessary. Bulbs tend to burn out at the same rate no matter if lamp is left on continuously or turned off and on over time. Keep spare lamps on hand for issues such as a bad lamp, power surge that blows one prematurely, and to change at least once a year even if you have no issues to continue quality scanning with your instrument. Please remember that a bulb does not last forever and should be closely monitored.
3. *Scanning internal monitoring samples.* This is a good way to catch this issue and several manufacturers have software functions that report the bulb intensity and reliability. However, this is not always the best determination of a bulb's performance due to the range of “acceptable” limits.

2.7 Instrument Diagnostics, Check Cells, and Internal Standards

In accordance with the instrument manufacturer guidelines, NIRSC recommends running instrument diagnostics on a weekly basis, and analyzing a sealed check cell daily or at the beginning of each shift. Diagnostics and check cell results should be recorded along with lab conditions, including temperature and humidity. Failure of check cell or diagnostics should be brought to the attention of lab managers and, if necessary, directed toward the instrument manufacturer. A control chart should be maintained to plot check cell scan results over time can be used to identify unacceptable results, bulb variation, and instrument drift.

Check cell protocols may vary between instrument models. Permanent check cells may be available from instrument manufacturers. Laboratories often have access to a variety of samples that could be used as bulk standards for internal monitoring. Bulk samples of 200 grams or more may be produced from a large amount of an individual sample. When using a bulk sample, wet chemistry reference data in at least triplicate must be available for comparison. Bulk standards/samples must be kept in sealed, dark containers in cool conditions. Samples must be preprocessed according to the sample preparation recommendations of the NIRSC. Failure to do this will result in internal standards that do not perform well. It is important that the wet chemistry methods used for reference match those of which were used in development of the NIRS calibration being monitored. Multiple NIRS analyses over time will develop a baseline of acceptable prediction output.

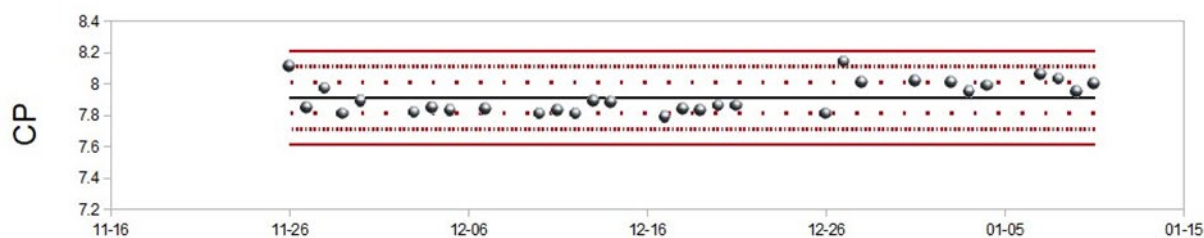
If creating an independent check cell, it is important to follow the recommended steps to prepare a sealable cup:

1. Use properly ground and dried material that is well mixed with multiple subsamples from the sample.
2. The sample must be packed tightly enough to avoid particle shifting and sealed well enough to prevent moisture alterations.
3. The sealed cup must be able to hold up to extensive handling and temperature changes.
4. It should be stored in a small, airtight, and light occluding box.
5. Once the cup is scanned, the predicted data is used to develop a control range.
6. Instrument drift and other problems can be detected by using a permanent check cup daily.
7. Each time the check cup is scanned, compare the results to the mean and if within two standard deviations from the mean. It is recommended the sample be scanned daily to detect drift. Weekly scanning may suffice if daily scanning is not achievable.

An efficient way of monitoring acceptable output over time is to use an S Control Chart. This is a control chart that uses the standard deviation to establish control limits of acceptability, using the average over time as a center line from which an individual analysis is compared.

An example chart for Crude Protein is below (Figure 2.1). This is an S Control Chart with an upper control limit of 2 standard deviations above the mean and a lower control limit of 2 standard deviations below the mean. The mean of the values forms the center line (shown in black). For constituents less reliably measured by NIR, 3 standard deviations may be acceptable. Further in-depth discussion relating to confidence limits and statistical evaluation has been described by ASTM (2012).

Figure 2.1. Control Chart for crude protein (CP) showing upper and lower control limits of ± 2 SD.



2.8 Bulk Samples as an Internal Quality Control

Labs that participate in the National Forage Testing Association (NFTA) Proficiency Program have a supplemental quality control method available to them. The use of NFTA bulk samples as outside quality control standards can help monitor NIRS performance. NFTA will send duplicate samples bags of specifically requested certification samples for a fee. When utilizing these samples over time, it is important be aware of storage conditions. Changes may occur in DM as well as sample degradation, therefore altering values.

When using NFTA, or any other source for bulk samples, as quality control standards, it is prudent to find a sample that is representative of samples typically received by your lab. The NFTA samples all have Reference Method Averages (RMA) for dry matter (DM), crude protein (CP), Acid Detergent Fiber (ADF), and amylase treated Neutral Detergent Fiber (aNDF), against which labs participating in the NFTA program are statistically graded.

The RMAs are calculated from the results of all participating labs' test sample submissions and reported with each lab's Certification Report. These samples can also be used as permanent sealed check cells, however, please note each time the sample is scanned, compare the results to the corresponding RMA values from NFTA with changes in the samples moisture and contents in mind. Please make sure to always prepare and load the dried and ground NFTA bulk sample as recommended for your instrument's sample presentation before scanning. Reference methods and other information about the NFTA's Proficiency Certification Program can be found at their website: <https://www.foragetesting.org/>.

2.9 Instrument Realignment

Depending on the instrument manufacturer and model, the manufacturer may have a synthetic cell available that realigns the instrument back to factory standards. Periodic use of these standard cells is important in keeping the instrument stable. The NIRSC recommends running a realignment on a regular monthly basis, however, follow manufacturer recommendation procedures as timing this procedure will vary.

Please note, if you are realigning the instrument more frequently due to check cell failures, usually a new bulb is recommended even if it is still working or testing close to manufacturers specs. However, The bulb may not have the energy required to produce optimal spectra for forage analysis. Contact the NIRSC if you have questions about this.

2.10 Instrument Maintenance and Troubleshooting

Please contact your instrument manufacturer if you have questions about maintenance or any other issues you may have. The NIRSC is not responsible for or able to perform maintenance on any instrument. The technical staff can help you troubleshoot to a point where, if recommended, you should contact the manufacturer or a trusted company for further help. The NIRSC is responsible for only standardization, calibrations, and use thereof.

2.11 Instrument Standardization

Calibrations developed by the NIRSC can be transferred to the members only if their instruments spectrally match the NIRSC Master. An instrument is defined as a standardized clone when it produces a similar prediction value as the master instrument after scanning the same sample. Use of sealed cell samples (natural or synthetic) drastically improves instrument standardization of clone to master instruments (Osborne et al., 1999; Tillmann et al., 2000).

Most instruments require standardization, however contact the NIRSC for standardization protocols specific to your instrument. Currently, NIRSC utilizes a primary set of sealed samples of diverse products for characterization. An additional set of forage samples is used to evaluate the standardization performance. If the evaluation is acceptable, the residual error of the host standardized predictions is small compared to the master predictions. Detailed instructions for scanning of these samples are

supplied by the NIRSC. Standardization evaluation of each NIRSC member instrument is typically required upon initial set up and is scheduled on a continuous rotation. In the event of certain instrument repairs an instrument must be re-standardized.

SAMPLE COLLECTION AND HANDLING

3.1 Sample Collection

The usefulness and applicability of any analytical results are limited first and foremost by the quality of the sample submitted to the laboratory. The best practices in any lab cannot compensate for an improperly collected sample that ultimately does not scan well in the laboratory. Though we have no control over the sampling procedures employed by a lab's clientele, we must make every effort to emphasize the importance of proper sampling techniques to customers. In addition, many NIRSC members are researchers who must be proficient in collecting primary samples for analysis.

Laboratories and researchers must be proactive in their approach to proper sampling. The NIRSC cannot stress the importance enough about proper sampling and the techniques that facilitate representative sample collection. Protocols must be observed before the sample meant for analysis reaches the laboratory. Laboratories cannot fix an unrepresentative sample; the laboratories can only analyze it. For ways producers misuse forage analysis refer to Common Abuses of Hay Testing Results (Putnam, 2019). When improperly collected samples arrive to the lab (e.g., extremely small samples, grab samples, or cores from a single bale) potential discrepancies among laboratories can be minimized using these proactive measures rather than dealing with sampling issues in a reactive manner.

3.2 Sample Handling

Sample handling is easily the most significant contributor to the accuracy and precision of analytical results. Errors can be introduced to the system in the laboratory at any stage from splitting and sub-sampling to contamination and induced physical alterations such as using grinders and equipment not for forage and feed preprocessing. These errors add to the errors introduced in obtaining the primary

sample. We cannot overemphasize the importance of proper sample handling technique on the performance of NIRS analysis.

3.3 Sample Composition

Heterogeneity of forage material makes the processes of splitting and sub-sampling prone to errors. Not all particles within a sample exhibit the same composition (e.g., a sample may contain particles of leaf and particles of stem), thus it is vital that a sample and associated sub-sample accurately reflect the makeup of the entire lot. Achieving this accurate reflection is further complicated by the non-random distribution of particles within the given sample (at any stage of collection or analysis). Sufficient sample size and proper mixing are the best mechanisms to mitigate these effects. Samples are also vulnerable to physical or chemical alterations induced by improper sample handling, chemical treatments, mold, and moisture. Contamination (from either foreign sources or separate samples), electrostatic separation, or ‘loss of fines’, and the effects from grinding or excessive heat exposure (e.g., *Maillard Reaction*) are known to alter the chemical makeup of forage material and can alter analytical results.

3.4 Theory of Sampling

Multiple resources are available that discuss the Theory of Sampling (TOS). It is important to remember that even though the laboratory is not responsible for collecting the sample, there is a shared responsibility in the sampling, chemical analysis, and data analysis (Undersander et al., 2005). The laboratory must be aware of the accumulation of errors in these steps to reduce or prevent errors by understanding TOS. In addition, the laboratory must understand TOS because proper sampling theory will be required in selecting a test portion from the laboratory sample.

4.1 Preprocessing Theory

When a sample is received by the laboratory the NIRSC recommends that the entire laboratory sample submitted be dried and ground for analysis. Any laboratory sample must be handled and documented for evidentiary integrity so that the test result(s) can be traced back to the lot. This includes controlling error during processes that select a portion of the laboratory sample and during processes that prepare the sample and includes reception of the laboratory sample. As follows, any handling or manipulation of a sample in any stage must preserve the primary sample integrity (AAFCO, 2018; Thiex and Ramsey, 2016). Therefore, sample preparation that converts the sample received at the laboratory into a homogeneous material suitable for analysis (Undersander et al., 1993) must attempt to keep sampling and analytical errors as low as possible.

During all laboratory steps that handle the sample, AAFCO (2018) describes two major processes: the non-selection processes and the selection processes. The non-selection processes include any steps of sample handling or preparation in which there is no mass reduction or selection of mass (AAFCO, 2018). During any of these steps, multiple errors will be combined to determine the Global Estimation Error (GEE), which is the Total Sampling Error plus the Total Analytical Error (AAFCO, 2018). During any of these steps, beginning with receipt of the laboratory sample, efforts must be made to understand and reduce errors that contribute to GEE (AAFCO, 2018). For example, these errors include introduction of extraneous material, loss of sample material, contaminants, not selecting enough mass, etc.

4.2 Receiving Samples

It is the premise of NIRSC that all samples that are received will be analyzed by NIR. Following that premise, for the portion of sample going to an NIR or other chemical analysis (other than any portion going to Dry Matter determination), all samples will be dried enough (93.5-97% DM) to be ground using

a Wiley Mill™ with a (2 mm) screen, or equivalent grinder; then, finely ground (1 mm) with a cyclonic type of grinder and presented, per instrument manufacturer, into an NIR sample vessel for analysis.

Recommended procedures for receiving a sample for NIRS analysis can be found below; these may vary depending on laboratory and type of sample received:

1. Label each sample with a unique identification number, barcode, or other form of ID.
2. Record sample information for reporting according to individual laboratory methods.
3. Remove roots and any soil from the sample.
4. If “as-received/as-is” moisture needs to be reported that process should begin here with a wet weight and can be a subsample of the entire sample received. This procedure should be determined by your lab with drying time and calculation.
5. Chop whole plants or long stems into one-half inch pieces using clippers or a laboratory forage chopper (Undersander et al., 1993).
6. Cored hay probe sampled material needs no further reduction in material size until grinding.
7. Place the entire sample into a labeled drying pan for subsequent drying.
8. If the entire laboratory sample cannot be dried because the sample is too large, a systematic mass reduction protocol should be implemented.
9. Reducing the size of the sample mass may be necessary at multiple points in sample preparation including upon receipt of the laboratory sample, after coarse grinding, and/or after final grinding.
10. If laboratory has other protocols to follow, please incorporate those where necessary to ensure sample tracking and integrity can be accomplished.

4.3 Mass Reduction

Mass reduction or splitting may be necessary to further process the sample, however whatever methods are used must preserve the sample integrity. When reducing the size of the laboratory sample or analytical sample, steps must control compositional and distributional heterogeneity (AAFCO, 2018; ISO, 2012). Splitting is a process that divides a mass or volume into two or more equal portions. It is highly recommended that forage labs use a stationary riffle splitter (preferably gated) for subsampling of cored hays, forages, and silages that have been dried and ground.

The laboratory may receive samples that consist of whole plants (either green or dry) or a large sample size of green chop, silage or haylage. While NIRSC strongly recommends that the entire laboratory sample received be dried before grinding, it may not be practical to do so with all types of samples. The following techniques can be used on samples that are not flowable or do not have small enough sample

size to pass through riffle splitters. Please note that large samples received in the lab will most likely be split manually before this point.

If a large mass of sample has been dried, a subsample can be split out before fine grinding. However, it is essential that the entire partially dried sample be coarse ground from 2-8 mm before being split. Coarse grinding reduces the particle size to ensure a more representative subsample is obtained by the riffle splitting process. Splitting of a sample may also occur after fine grinding.

Recommended procedures for mass reduction when grinding/chopping whole plants or fresh forage, as needed (Undersander, 1993):

1. Remove sample from shipping container and discard any roots from plants and brush off dirt. Note and report removed material and any other sample manipulation.
2. Chop samples of whole plants into about half-inch pieces using either hand clippers, a paper cutter, or a laboratory forage grinder/chopper.
3. Final quartering or grinding of the whole sample should take place at this time; then, further sample reduction with a riffle splitter or manual method can occur.

4.4 Sample Drying

Even though there are portable NIR devices and other NIR technologies that can analyze samples of standing forage, harvested piles, and stored feed; the standard presentation for laboratory forage NIR analysis is a dried and ground sample. Samples that are dried and ground remove variables including analysis interference from water and heterogeneity, and stabilizes the material (Murray and Cowe, 2004).

Drying samples in the laboratory has two purposes. First, the laboratory sample (AAFCO, 2018) must be dried enough to process it further for subsequent analysis; and a test portion must be used to determine the dry matter of the forage material submitted to the laboratory. All samples, regardless of the judged dryness of the laboratory sample, should be dried further before pre-processing or scanning.

Samples should be dried in a 55°C forced-air oven (Undersander et al., 1993). Oven drying is generally preferred over microwave protocols (Murray and Cowe, 2004) and is the only acceptable method for subsequent NIR analysis using NIRSC calibrations. Drying at higher temperatures (>55°C) may cause chemical changes in the sample that could alter the subsequent constituent analysis. All samples analyzed by NIRSC calibrations must be oven dried to obtain reliable results.

Please note that there are different types of drying for use in reporting lab analysis (as-received/as-is) and the dryness level needed to process a sample to scan it properly using NIRS instrumentation. Predicted dry matter or moisture in a sample is the presented moisture content of a sample to the NIR instrument at time of scanning.

4.5 Subsampling Techniques

There are numerous subsampling techniques that are implemented in laboratories, however, please note that subsampling often takes place at the sample submission even before it reaches the lab. Removing a portion from the top of a sample (whole, cored, chopped/ground) in any way, is not representative of the whole. This is apparent when multiple samples are submitted to/across laboratories for analysis.

Using a technique known as the Quadrant Subsampling Technique (QST) is a straightforward splitting technique with several advantages (Bohnert et al., 2011). This QST method also can be implemented in the laboratory or field, does not involve extra equipment, has minimal clean-up and decontamination requirements. Furthermore, the QST is quick to practically implement through training of laboratory personnel. The laboratory received sample is mixed well, spread out and divided into 9 tic-tac-toe style. Approximately $\frac{1}{3}$ or 3 of the 9 quadrants are reserved as the test portion of the sample. This sub-sampling technique provides unbiased results on cored hay samples compared with un-subsampled results (Bohnert, et al., 2011).

The 9-quadrant method has been shown as an acceptable way to reduce the mass of cored hay, chopped silage/haylage (Bohnert et al., 2011):

1. Pour the laboratory sample onto a large plastic or paper sheet.
2. Mix the sample by pulling the corners of the sheet diagonally over the sample from one corner to its opposite.
3. Turn the sheet to the next corner (one-quarter turn) and repeat for at least 8 corners.
4. Notice that the sample will have a more uniform color and texture as you mix it.
5. If the sample does not appear to have uniform color and texture to your eye by 8 rolls, mix it additional times.
6. Spread the sample over 9 “tic-tac-toe” like quadrants across the sheet.
7. Choose 3 quadrants to retain as the sub-sample to be tested in the laboratory.

Figure 5.1. Quadrant method example.



4.6 Mixing Techniques

Mixing a dried and ground sample is an important step before riffle splitting, NIR analysis, or wet chemical analysis (Williams, 2008). Grinding stratifies a sample and mixing is vital to ensure sample homogeneity. If the dried and ground sample is not mixed properly, the resulting analyses will not be representative of the sample.

Non-acceptable mixing techniques include putting a scoopula in the top of a sample container and stirring a few times or shaking the sample container (bags, jars, cups, etc.). These are especially bad techniques if the container is full or almost full. The material at the bottom is not adequately mixed in and the stirring may have promoted segregation making the mass reduction error even greater. Any type of mixing will not work with a container that is full or almost full. Even though a sample is mixed, it will still exhibit variation such as distributional and compositional heterogeneity. These can be controlled by taking multiple increments for analysis later (AAFCO, 2018). Whenever mixing is used as part of a procedure, its effectiveness should be evaluated with a performance test. The ISO (2012) section 8.4 provides a performance test for mixing.

The NIRSC recommends that labs choose an acceptable method, and a performance test should be used to determine effectiveness of the mixing technique used to account for sample heterogeneity (even after mixing) take multiple increments for the test portion.

4.7 Riffle Sample Splitting

The most common technique for splitting of dried and ground samples in forage laboratories is using a riffle device (gated and non-gated). Gated rifflers have a trap door between the hopper and the chutes. After the sample is poured into the hopper, the trap door must be opened to allow the sample to flow through the chutes into the receiving pans. A non-gated riffler does not have a trap door. The sample is poured over the chutes and immediately falls through the chutes into the receiving pans. Of the two types of stationary riffling, it is generally accepted that gated riffling provides more accurate splits. The reason for this is a function of operator error and condition of equipment. While both types of rifflers should theoretically give equivalent results and both must be used properly, the gated riffler is more forgiving of operator error. In addition, gated rifflers seem to be kept in better working order (perhaps due to design). Use of gated rifflers is recommended for these reasons, but non-gated rifflers are acceptable if used and maintained properly.

Proper Use of Stationary Riffle Splitters (ISO, 2012):

1. The riffle splitter should be on a firm, level surface.
2. Ground finished sample material should be used in a riffle splitter only. Samples larger than 2 mm will not drop correctly.
3. Do not feed the sample too fast (the chutes can fill up and overflow).
4. For non-gated rifflers, do not feed the sample into the riffle splitter with the receiving pan (it is not the correct width).
5. Material in the feeding scoop should be spread out evenly prior to pouring into the splitter.
6. Material must be fed slowly in the center of the chutes (to prevent overflow of material from the shallow chutes into the deeper ones).
7. For gated rifflers, slowly feed the material into the hopper in a back-and-forth motion. The material should be evenly distributed in the hopper after feeding.
8. If the first split portion is too large of a subsample, then transfer one of the split portions to the hopper and split a second time. Continue this splitting process until a split subsample of the correct size is obtained.
9. Fines may stick to splitter due to static electricity. If this occurs and measurement of the fines is important, you may try grounding the riffler or using an anti-static mat.

10. Riffle splitters are very operator dependent and must be used properly. Tests, such as performance tests, are performed with material like the material to be split to demonstrate their performance.

4.8 Grinding Procedures and Recommendations

Grinding is the most physically intensive process to which samples are exposed prior to analysis. Particle size is a very important factor in NIR analysis because finer milled particles are more reflective to NIR light, provide adequate surface area, and create a homogeneous sample (Murray and Cowe, 2004). Due to the complex processes occurring during grinding (e.g., leaves and stems may require different amounts of time to pass through the screen) it is vital that all material which enters the mill be collected into the final sample container. These processes may also introduce striations into the sample. Additionally, improper grinding protocols may lead to heating of the materials, which may alter DM or other analysis procedures for NIR or wet chemistry (Undersander et al., 1993; Williams, 2008; Williams et al., 2017). The publication ISO (2012) provides performance tests for particle size reduction (grinding) to determine grind quality and recovery.

Regardless of grinding equipment, all material for NIRS analysis must have a final pass through a 1 mm screen of a cyclonic mill. Abrams (1989) showed that the finer and more uniform particle size produced with 1 mm cyclonic milling reduced Standard Error of Calibration (SEC), and ISO (2012) reports that expected coefficient of variation goes down with smaller particle sizes. It is important to make sure the same grinding method which was used in developing the calibration is used for routine analysis. Always grind the entire sample that has been dried. After this step, if splitting is necessary, use of an approved splitting technique is recommended.

The NIRSC Instrumentation Hub uses a two-step grinding process in which the sample is first ground to 2 mm with a Wiley Mill™, then reground through a cyclonic mill, or equivalent, passing through a 1 mm screen as indicated by Hall (1990). Always remember that drying the samples is a step that provides easier grinding. It is good practice to remove humidity from the samples before starting to grind, thus making the entire process easier. Only some samples may need to be dried to more than 85% DM to grind properly in a cyclonic mill; followed by drying for NIR scanning after sample preparation is completed. Keep all equipment clean to avoid contamination across samples. Heating of the sample in excess will change the chemical analysis. To maintain mills, follow the operator's manual, paying attention to the condition of the screens, rotors, blades, and other grinding surfaces.

Two-step grinding procedures recommended by the NIRSC to match the material used to create calibrations:

Step One: Coarse grinding using a cutting/grinding mill

1. Make sure the mill is clean.
2. Insert screen into the mill (2-6 mm sizes acceptable). Please note some forage samples can easily pass a 1 mm screen if properly dried.
3. Following your mill's operating instructions, slowly feed the entire sample into the mill.
4. Once all material has passed through the mill, a higher pitched "empty" sound may be noted.
5. After grinding is complete, shut off the mill and remove the collection container.
6. Position the collection container beneath the grinding chamber as you open the mill.
7. Sweep and/or scrape any incompletely ground residue from the mill into the container.
8. Remove the screen and brush residue from it into the collection container.
9. If the collection container is different than the storage container, transfer all contents to the storage container.
10. Close storage container.
11. Clean the entire mill using compressed air, a brush, and/or vacuum.
12. Maintain the cutting mill by following the operator's manual, paying attention to the sharpness of the knives.
13. Knives must be properly aligned on re-installation for efficient grinding to achieve the required particle size.
14. The screen should be replaced if it becomes dented, corroded, or distorted.
15. Clean dust out of the motor housing as needed.
16. Record mill maintenance and repairs in a designated logbook.

Step Two: Fine grinding with a cyclonic mill

1. Make sure the mill is clean.
2. Insert a 1 mm screen.
3. Insert a clean sample collection bottle beneath the cyclonic and turn the mill on.
4. Following your mill's operating instructions, slowly feed sample material into the mill.
5. If the sample bottle becomes full, shut off the mill and empty the collection bottle into a larger container.
6. Replace the sample collection bottle and resume slowly feeding sample material into the mill.
7. Repeat as necessary until entire sample is ground; or enough material is collected representative of the whole sample.
8. After grinding is complete, shut off the mill and remove the lid.
9. Transfer any incompletely ground residue to the sample bottle.
10. Transfer all sample material to a labeled proper storage container.

11. Clean the entire mill using compressed air, a brush, and/or vacuum.
12. Transfer samples quantitatively. Do not leave any portion of sample in the mill.
13. Record mill maintenance and repairs in a designated logbook.

4.9 Sample Storage

Williams et al. (2008) explains that the stored sample must be the same as the sample presented for analysis. During sample handling to storage changes in temperature, moisture, and volatiles can all occur during any storage step. Store the dried, finely ground, mixed analytical samples in airtight containers, acceptable plastic bags (Whirl-Pak™, etc.). Light, heat, and humidity can also affect the samples viability and life span with the NIRSC recommending that samples older than 3-years should no longer be analyzed. The NIRSC does not recommend freezing undried samples as it causes cell wall bursting and loss of nutrients.

Additional information on storage methods and non-acceptable ways to store samples to include freezing after initial spectral collection is included in Williams et al. (2017). To store bulk samples over time, large air-tight containers/bags should be used to keep outside environmental elements from changing the samples.

PREPARING SAMPLES FOR SCANNING

5.1 Sample Presentation

Proper technique for loading dried and ground material into a sample cup for NIR scanning is vital to obtaining accurate spectral data. If the dried and ground sample is not handled properly while packing a sample cup, the resulting analysis will not be representative of the sample. For example, a non-homogeneous ground sample will result in a distorted sample prediction, while a dusty sample cup or instrument window can produce a faulty artifact in the sample spectra, therefore altering predicted value. Consistency and thoroughness in packing a sample cup for scanning will help produce NIR analyses representative of the original sample. In addition, consistency among NIRSC labs in preparing samples for NIR scanning will help reduce discrepancies between laboratories. Specific instructions for NIR analysis (including apparatus, instrument operation, and sample preparation) can be found in specific instrument operation manuals.

To maintain compatibility and predictive accuracy, all samples to be predicted using NIRSC calibrations must undergo matching drying protocols. NIRSC strongly recommends that all samples (regardless of their original dry matter content) must be dried, using the same method, prior to grinding. Drying of all samples (not only those determined to be “wet”) reduces variability in sample treatment as well as moisture range. Moisture in samples can cause issues in correctly predicting constituents and data fluctuation across a project or subsampled material. The optimal range of water in a ground sample for NIR analysis is roughly between 93.5-97% Dry Matter (DM) (McIntosh, 2019). McIntosh (2019) showed that samples scanned at between 84-93.5% DM underestimated, in most samples (unless noted below with a different scanning range) crude protein, overestimated fibers and ash, and underestimated sugars, carbohydrates, and digestibility.

Optimum Scanning Ranges (McIntosh et al., 2019):

- Grass Hay (GH) 95 to 97% DM
- Mixed Hay (MH) 94 to 97% DM
- Legume Hay (LH) 93.5 to 95% DM
- Haylage (HL) 93.5 to 95.5% DM
- Corn Silage (CS & UC) 94 to 95.5% DM

Determining if the sample(s) about to be scanned is within the recommended scanning range by product it is always good practice to randomly select a few samples and scan them for current dry matter (DM).

If the DM falls below the specific optimum scanning range, follow the procedure below:

1. Use a forced air oven set to 55°C. Do not use greater temperatures as it will damage contents of the sample.
2. Dry samples in food grade tin containers.
3. Do not over fill the oven with samples as that will lengthen drying time.
4. Start process by drying entire sample (Typically a 2-4 oz WhirlPak™ bag) for 15 min.
5. Remove one sample and let cool until sample is at room temperature (2-3 minutes).
6. Pack a sample cup, or ring cup, and scan. Noting the increase in DM.
7. Add time accordingly in 15 min increments until the sample(s) are at, or near, the above ranges.
8. If samples become dryer than the range, leave on bench top uncovered for 15 min and material should equilibrate across all samples depending on relative humidity levels. Scan for DM to test.
9. To hold sample(s) at the optimum range use a large airtight container or bag.
10. Do not prepare too many samples at one time that cannot be scanned within a 2-hour period. They will begin to gain moisture quickly after the first 15 minutes out of the oven.

*Please note certification and proficiency program samples are graded compared to wet chemistry DM reported across laboratories. Procedures on NFTA samples that are received by NIRSC laboratories can be found on the NFTA website: <https://www.foragetesting.org/>.

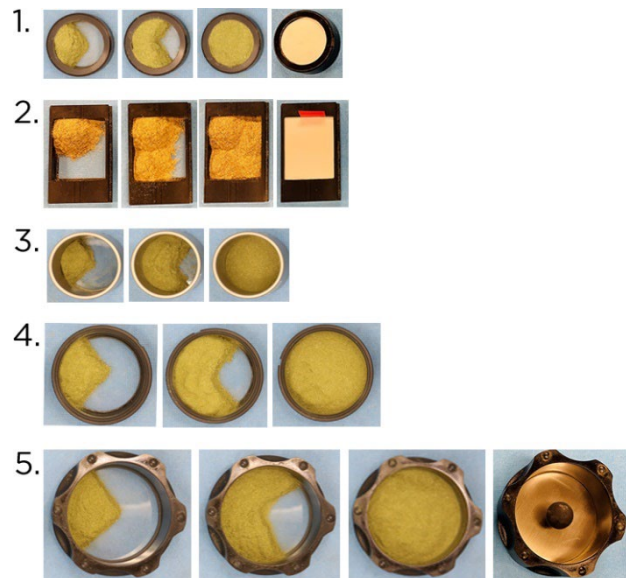
5.2 Packing and Scanning a Sample

This procedure is different depending on the sample cup size and type, however, the subsampling from the sample bag/container is the same. See Figure 5.1 to illustrate packing a sample cup with different cups available with instruments. It is important to clean your ring cups regularly as buildup of residues does occur. For cleaning use a mixture of distilled water and a 1% Ethyl alcohol at a 10:1 ratio where only a droplet is used with Kimwipes® disposable wipers. Do not press down too hard or scrape glass. Watch for the condition of the glass. If it is broken or loose do not use it for scanning and repair/replace that cup. Take note that often there are cups that do not scan like the others and care should be taken to remove that cup when doing certification samples. Not doing these maintenance steps will cause variation in the scans, and especially if scanning certification samples.

The NIRSC recommends the following steps to ensure the sample scanned is representative for optimum presentation to the instrument:

1. Mixing of dried and ground samples is important to alleviate any settling and stratification that might have occurred from grinding. Stratified samples produce varying size fractions in the ground sample and in turn produce different analytical results. Use of proper mixing protocols should be followed.
2. Subsample using a scoopula or pour from container taking multiple subsamples from separate areas of a sample bag or specimen cup taking from various locations vertically as well as horizontally. If samples are in cups, rotate cups as subsampling occurs. Three subsamples are recommended for a ring cup, and a similar protocol should be followed for other sample cup types. Finish filling the sample cup with additional subsamples (Figure 5.1).
3. Be sure that enough material is placed in the sample cup. The sample should be covering the bottom of the cup, packed in a way that avoids shifting. Please note: the NIR light travels into a sample at least 10 mm, however, make sure there is at least 4mm in the cup for good scanning. Too little sample will skew spectra collection and thus results will be incorrect as well.
4. Scrape off excess sample from the cup with a straight scoopula edge. If required, press the back onto the sample cup. Do not over fit or press down too much. Some instrument cups do not require any type of backing, see manufacturer recommendation for this step. Dust all cup surfaces with a soft brush or gently clean using air to avoid transfer to the instrument.
5. To collect spectra, load the sample cup into the instrument and scan in accordance with instrument manufacturer's instructions. Make sure to be scanning under the appropriate calibration that fits the samples contents.

Figure 5.1. Packing a sample cup: (1) small ring cup with foam backer [Universal], (2) quarter cup with foam backer [Foss], (3) sample cup [Bruker], (4) small cup [Foss], and (5) large open cup with plunger [Blue Sun Scientific]. Remember some of these require a backing for scanning, see manufacturers recommendation for that step specific to the NIR machine in use. *Cup sizes represented here are not to scale and note there are other cups available.*



6.1 Certification Programs and Bulk Sample Checks

NIRSC commercial laboratory membership requires participation in the NFTA Proficiency Certification Program. Not all NIRSC members are required to implement internal monitoring programs. However, any laboratory with a commitment to providing accurate and reliable results should also commit to tracking internal performance of their routine analysis.

Laboratories use the National Forage Testing Association (NFTA) Proficiency Certification Program to improve reproducibility and general quality of forage analysis by identifying the most accurate methods of analysis, and documenting evidence of satisfactory laboratory performance. To be designated as certified, labs will be required to achieve certification for all analysis (DM, CP, ADF, NDF) averaged across eight samples sent out by the NFTA each year.

The NIRSC advocates three methods for maintaining optimum internal monitoring of NIR results beyond those recommended by the instrument manufacturer:

1. Participation in the NFTA Proficiency Certification Program.
2. Use of bulk homogenous samples with wet chemistry reference as internal standards for quality. Past NFTA bulk samples with RMA values can be used when using NIRSC calibrations. Dry matter changes over time in the samples and your results will change if this happens. Learn to accommodate for this shift in data and the range it causes.
3. Use of other sourced material for sealed test samples with wet chemistry associated; again, dry matter changes affect the results with these samples.

Scanning NFTA samples:

1. At receipt of the samples, the NIRSC recommends that samples are scanned for DM immediately. Then for future scanning, place in desiccator or airtight container until time to scan replicates. Failure to do so will result in accumulation of moisture causing your results to not be in line with other labs.

Moisture does change even in the sealed sample bags. If you notice a problem with the bags, contact NFTA immediately for replacements or a recommended solution.

2. Take note that often there are cups that do not scan like the others and care should be taken to remove that cup when doing certification samples. Not doing these maintenance steps will cause variation in the scans, and especially if scanning certification samples.
3. Pre-scan one subsampled cup of material on the appropriate calibration to determine the DM and determine next steps. Remember that it is your responsibility to watch the GH and NH statistics to make sure sample is scanning properly on the appropriate calibration. When samples are received, they are labeled for NFTA use. Therefore, choosing the appropriate calibration for scanning is extremely important knowing that some hay samples could be a mixture of grass/legume species. Also, remember the (AH-Alfalfa) sample from NFTA is a legume hay (LH) sample.
4. If DM is below the NIRSC recommended scanning range, please scan 9 subsamples out of each sample bag under the appropriate calibration. This DM data will be used in the final steps before reporting to NFTA. Discard the rest of the data from these scans keeping DM only. Proceed to drying samples to optimum range.
5. If scans are within ranges for NIRSC optimal scanning, proceed with the 3-day, 3-rep regimen (ending in 9 scans per sample to select best scans from) recommended by the NFTA guidelines.
6. Data from samples dried after determination optimal scanning range was not sufficient on the received NFTA samples uses the DM mean value from the 9 subsamples out of each bag. You may report the mean across all 3 reps to NFTA or use the closest to the mean to report.
7. For reporting and wet chemistry comparison, use the scanned data from the dried subsamples that is at 100% DM with the mean or 3 closest reps of DM to do the conversions to “as-is” calculated as $(\text{Predicted NIR DM} \times \text{Constituent Value} / 100)$.
8. Please contact the NIRSC if you need help with this procedure or if you are having any other issue related to the calibrations and conversions.

Protocol for undesirable NFTA grades:

1. When member labs are utilizing NIRSC calibrations for NIRS analysis and are unable to receive passing grades based upon NFTA guidelines, they should first make sure they have reported as-is or as-received values to the NFTA. **NIRSC calibration values are reported at 100% DM**, therefore the predicted values must be converted to “as-is” or “as-received.”
2. To calculate “as-is” use: $(\text{Predicted NIR DM} \times \text{Constituent} / 100)$.
3. The member should determine if any instrument related issues were present at the time of analysis of the NFTA samples. The NIRSC is not responsible for improper maintenance, bad bulbs, packing and scanning mistakes.
4. If a source of error cannot be determined by the member, they may report the issue to the NIRSC, however, please note that most issues happen at the laboratory bench level and are not related to the calibration performance.

5. The NIRSC will investigate sources of error and provide guidance and/or action toward improvement of performance within reason. Please note that the NIRSC is not at liberty to discuss results from other labs and may not share any data concerning NFTA samples.
6. The NIRSC is committed to maintaining the highest accuracy and greatest transparency in NFTA performance, without violating the spirit or the letter of the NFTA's ethics statement. In accordance with this, NIRSC will refuse to discuss predictions or performance of NFTA standard samples until after results have been released by NFTA.

REPORTING, VALIDATION, AND RESOURCES

7.1 Reporting for Publication

Referencing NIRSC calibrations on lab reports, scientific publications, and any other form of documentation should follow a standard reporting format to encourage understanding among labs, nutrition professionals, as well as producers.

NIRSC members, or anyone that uses data herein, that use NIRSC calibrations should include the following in the methods section:

1. Calibration name and release year.
2. Constituents used.
3. NIRS Forage and Feed Consortium, Berea, KY.
4. Instrument type, model, and software(s) used.
5. Calibration performance statistics, available upon request from the NIRSC and is platform specific.

7.2 Required Validation for Publication

To publish in scientific literature, required validation of a lab's use of NIR and NIRSC calibrations is required and recommended. Validation is a process of testing a calibration with samples for accuracy by evaluating differences between calibration predictions and reference chemistry values. Estimates from the calibration and the reference method, and to test the agreement between estimates made with the model and the reference method (Workman and Shenk, 2004).

Additionally, certification programs do not offer validation on the sample sets being published in research. Care should be taken to randomly select a representative set of samples from the data set for wet chemistry validation. Please note, the reported constituents used in the project must match that of

the wet chemistry methods used in the calibration development. The number of wet chemistry samples should depend on the treatments, species, and factors involved in the outcome of the project. Publishing with NIR results is different than with what is reported for wet chemistry analyses, and you should consult with the publisher about what is required.

A general rule is to take 10% for wet chemistry validation to be compared to the NIRS scanned results. Use the splitting procedures and make sure NIRS sample data is from the same sample sent off to compare to the wet chemistry results. Also, convert values at either 100% DM, or “as-is” accordingly or results will never be comparable. Use validation calculations and statistics to report accordingly for publication purposes; and, as required in specific fields/scientific journals.

7.3 Wet Chemistry Reference Laboratories

The NIRSC utilizes two reference laboratories for wet chemistry analyses and all validation sets should be set to these for correct comparisons to the calibrations used:

Dairyland Laboratories, Inc.

217 E. Main Street

Arcadia, WI 54612

Phone: 608.323.2123

Website: <https://www.dairylandlabs.com>

Cumberland Valley Analytical Services

4999 Zane A. Miller Drive

Waynesboro, PA 17268

Phone: 1.800.CVASLAB

Website: <https://www.foragelab.com>

7.4 Calculated Parameters

The end user should refer to appropriate sources for calculated values. The NIRSC recommends the most current editions of *Nutrient Requirements of Beef Cattle*, *Nutrient Requirements of Dairy Cattle*, *Nutrient Requirements for Horses*, and *Nutrient Requirements of Small Ruminants*. Additionally, always consult

other trusted sources and land grant university resources. The NIRSC is not responsible for calculated parameters that are used by our membership and those used should be acquired independently outside the NIRSC. The calibrations provide the necessary data to perform almost all forage related calculated parameters currently in use.

7.5 Using the NIRSC Logo

An important goal for NIRSC membership is to maintain optimal performance, and any logo use should reflect this. The NIRSC logo may be used by any NIRSC member and placed on a lab report or literature, please contact NIRSC for the versions available to our membership. Using this logo does not imply that the NIRSC is liable for misuse of calibrations and data reported. Additionally, if reports are combined with other in-house made or purchased non-NIRSC calibrations, use of the NIRSC logo is not recommended unless specifically noted on the reports or publications.

7.6 Additional NIRS Companion Resources

The Handbook of Near Infrared Analysis, Fourth Edition (by Emil W. Ciurczak, Benoît Igne, Jerome Workman, Donald A. Burns) explores the factors necessary to perform accurate and time- and cost-effective analyses across a growing spectrum of disciplines. This updated and expanded edition incorporates the latest advances in instrumentation, computerization, chemometrics applied to NIR spectroscopy, and method development in NIR spectroscopy. It underscores current trends in sample preparation, calibration transfer, process control, data analysis, instrument performance testing, and commercial NIR instrumentation. The NIRSC recommends this offering as the most complete single-source guide of its kind.

7.7 Contact Information

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NIRS Applications Specialist:	Bobbi jo Anderson-Husmoen bobbijo@nirsconsortium.com
Executive Director:	Tina Bowling tina@nirsconsortium.com
Instrumentation Hub Manager:	David McIntosh dmcintos@utk.edu
Instrumentation Hub Address:	NIRSC Instrumentation Hub c/o David McIntosh 2505 EJ Chapman Drive, PBB 112 Knoxville, TN 37996

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Calibration and Constituents

Constituent	NIRSC Calibration and Constituents*					
	Grass Hay GH	Mixed Hay MH	Legume Hay LH	Haylage HL	Fermented Corn Silage CS	Unfermented Corn Silage UC
Dry Matter	DM	DM	DM	DM	DM	DM
Crude Protein	CP	CP	CP	CP	CP	CP
Acid Detergent Fiber	ADF	ADF	ADF	ADF	ADF	ADF
Neutral Detergent Fiber	aNDF	aNDF	aNDF	aNDF	aNDF	aNDF
Digestible Neutral Detergent Fiber 24 Hour						dNDF24
Digestible Neutral Detergent Fiber 30 Hour	dNDF30		dNDF30	dNDF30	dNDF30	dNDF30
Digestible Neutral Detergent Fiber 48 Hour	dNDF48	dNDF48	dNDF48	dNDF48	dNDF48	dNDF48
Ash	Ash	Ash	Ash	Ash	Ash	
Fat	Fat	Fat	Fat	Fat	Fat	
Lignin	Lignin	Lignin	Lignin	Lignin	Lignin	
Fructan	Fructan	Fructan	Fructan			
Starch	Starch	Starch	Starch	Starch	Starch	Starch
Sugars	ESC	ESC	ESC	ESC	ESC	ESC
Water Soluble Carbohydrates	WSC	WSC	WSC	WSC	WSC	WSC
<i>In-vitro</i> True Dry Matter Digestibility 24 Hour						IVTDM24
<i>In-vitro</i> True Dry Matter Digestibility 30 Hour	IVTDM30		IVTDM30	IVTDM30	IVTDM30	IVTDM30
<i>In-vitro</i> True Dry Matter Digestibility 48 Hour	IVTDM48	IVTDM48	IVTDM48	IVTDM48	IVTDM48	IVTDM48
Neutral Detergent Fiber Digestibility 30 Hour						NDFD24
Neutral Detergent Fiber Digestibility 30 Hour	NDFD30		NDFD30	NDFD30	NDFD30	NDFD30
Neutral Detergent Fiber Digestibility 48 Hour	NDFD48	NDFD48	NDFD48	NDFD48	NDFD48	NDFD48
Calcium	Ca	Ca	Ca	Ca	Ca	
Phosphorus	P	P	P	P	P	
Potassium	K	K	K	K	K	
Magnesium	Mg	Mg	Mg	Mg	Mg	
Acid Detergent Insoluble Crude Protein	ADICP	ADICP	ADICP	ADICP	ADICP	ADICP
Neutral Detergent Insoluble Crude Protein	NDICP	NDICP	NDICP	NDICP	NDICP	NDICP
Insoluble Crude Protein	Insoluble CP	Insoluble CP	Insoluble CP	Insoluble CP	Insoluble CP	Insoluble CP
*Please note that this constituent list is from the 2022 Calibration update. This list is subject to change annually. All are presented in percent (%) with results adjusted to 100% DM.						

Wet Chemistry Primary Methods

Constituent	Wet Chemistry Method*	Unit
DM	Oven-drying in forced air oven at less than 60C until sample contains less than 6% moisture. This is followed by NFTA Method 2.1.4 Dry Matter by Oven Drying for 3 hours at 105° C.	%
CP	Protein (Crude) in Animal Feed: Combustion Method. AOAC Official Method 990.03 16th Edition, Ch 4 pg 18.	%
ADF	Fiber (Acid Detergent) and Lignin in Animal Feed: AOAC Official Method 973.18 (1996) (Modification includes use of Sea Sand for filter aid as needed).	%
aNDF	Amylase-Treated Neutral Detergent Fiber in Feeds AOAC Official Method 2002.04 2005 (Modification includes use of Sea Sand for filter aid and Whatman GF/C filter paper for residue collection).	%
Ash	Ash of Animal Feed: AOAC Official Method 942.05(1996).	%
Fat	Fat (Crude) or Ether Extract in Animal Feeds, AOAC Official Method 920.39 16th Edition ch4 pg25 (Automated with Foss Soxtec 2047 instrument with the use of diethyl ether).	%
Lignin	Fiber (Acid Detergent) and Lignin in Animal Feed: AOAC Official Method 973.18 (1996) (Modification includes use of Sea Sand for filter aid as needed, use of Whatman GF/C filter paper to collect residue, and holding crucibles in beakers to cover fiber with 72% sulfuric acid for full time required).	%
ESC	Neutral Detergent-Soluble Carbohydrates Nutritional Relevance and Analysis, Hall, M.B., Lab Manual (University of Florida) Bulletin 339.	%
WSC	Extraction procedure adapted from Derias, R.E. 1961. J. Sci. Fd. Agric. 12:152 (Method for determination water soluble carbohydrates). Detection procedure from Neutral Detergent- Soluble Carbohydrates Nutritional Relevance and Analysis, Hall, M.B., Lab Manual(University of Florida) Bulletin 339	%
IVTDMD	Fermented in flasks using the method of Goering and Van Soest (1970) with the modification that a composite inoculum containing strained ruminal fluid and blended ruminal solids. Residue analyzed by NDF procedure.	% DM
Ca	50% Nitric Acid in High Energy CEM MarS Express microwave diluted to 10% then analyzed on ICP.	%
P	50% Nitric Acid in High Energy CEM MarS Express microwave diluted to 10% then analyzed on ICP.	%
K	50% Nitric Acid in High Energy CEM MarS Express microwave diluted to 10% then analyzed on ICP.	%
Mg	50% Nitric Acid in High Energy CEM MarS Express microwave diluted to 10% then analyzed on ICP.	%
Starch	Starch Analysis in Animal Feed: Method workshop from 30th Annual MW AOACI Meeting and Exposition. Method that is undergoing AOAC collaborative study. (Modification: Glucose analysis completed on YSI 2700 Select Biochemistry analyzer instead of using GOPOD).	%
ADICP	Residue from ADF method is collected by use of Whatman 541 (nitrogen-free) paper or Whatman GF/C filter paper before analysis for nitrogen by Crude Protein method.	%
NDICP	Residue from the NDF method (minus sodium sulfite) is collected by use of Whatman 541 (nitrogen-free) paper or Whatman GF/C filter paper before analysis for nitrogen by Crude Protein method.	%
Reporting Clarification*		
Insoluble CP	Insoluble Protein= protein value after extraction	%
Soluble Protein	CP-Insoluble Protein	%
Potein Solubility	(Soluble Protein/CP) *100	%
aNDF	NDF (amylase treated)	%
uNDF	100-IVTDMD	%
IVTDMD	100-uNDF	%
dNDF	aNDF-uNDF	%
NDFD	dNDF/aNDF * 100	%
*Please note that calculated parameters cannot be predicted and should be done separately from the data collection in NIRS		

Calibration Attributes, Species, and Qualities

Calibration Attributes			
All Hay Types (Square, Round, Furrow, Loose)		Molded	
Bunker		Regional	
Grinds Represented (1-mm Cyclone, 1-mm Wiley)		Scissor Cut	
Growing Conditions (Drought, Wet, Unknown, Field, Small Plots, Nursery, Irrigated, Not Irrigated)		Grab Sample	
Hand Clippings		Oven-dried	
Haylage, Silage, Baleage (all types of fermentation)		Microwave dried	
Heat Damaged		Multiple Cuts	
Trench		Silo Samples	
Species and Qualities by Calibration			
Grass Hay- GH	Mixed Hay- MH	Legume Hay- LH	Fermented Corn Silage- CS
Alfalfa/Grass	Alfalfa/Grass	Alfalfa (All stages and Combinations)	BMR Traits
Alfalfa (Lower Lignin, Lower Sugars)	Alfalfa (Lower Lignin, Lower Sugars)	Alfalfa (Lower Lignin, Lower Sugars)	Forage Cut to all Stages
Alfalfa/Oats	Alfalfa (Lower Lignin, Lower Sugars)	Alfalfa/Grass Mixtures	Lightly Fermented
Bahia	Alfalfa/Oats	Canola	Some grains by product
Bermudagrass	Bahia	Clovers (Red, White, Arrow, Misc)	Some Unfermented CS
Big Bluestem (Forage to Dormant)	Bermudagrass	Cowpea	Strong Fermented
Brome	Big Bluestem (Forage to Dormant)	Hemp	Sweet Corn Silage
Clover/Grass	Brassicas	Lespedeza	Unfermented Corn Silage- UC
Coastal Bermudagrass	Brome	Pea	BMR Traits
Common Bermudagrass	Canola	Peanut	Forage Cut to all Stages
Crabgrass	Clover/Grass	Percentages of Legume and Weeds in this group	Fresh Cut
Crested Wheatgrass/Brome	Coastal Bermudagrass	Sainfoin	Some grains by product
Dwarf Forage Sorghum BMR	Common Bermudagrass	Soybean	
Endophyte Free Fescue	Crested Wheatgrass/Brome	Haylage- HL (ensiled/fermented)	
Fescue (Every type and E+ with E-/Free)	Fescue (Every type and E+ with E-/Free)	Alfalfa	
Fescue/Alfalfa	Fescue/Alfalfa	Barley	
Fescue/Clovers	Fescue/Clovers	BMR Traits	
Fescue/Weeds	Fescue/Weeds	Forage Cut to all Stages	
Forage Sorghum	Hemp	Grass	
Indiangrass (Forage to Dormant)	Indiangrass (Forage to Dormant)	Grass and Legume Mixtures	
Little Bluestem	Little Bluestem	Legumes	
Millet	Millet	Mixed Species	
Mixed Grasses (Not specified)	Mixed Grasses (Not specified)	Oatlage	
Oats	Oats	Pea	
Orchardgrass	Orchardgrass	Pearl Millet	
Pea/Grass	Pea/Grass	Percentages of Legume and Weeds in this group	
Pearl Millet	Percentages of Legume and Weeds in this group	Red Clover	
Percentages of Legume and Weeds in this group	Rye	Ryegrass	
Quackgrass	Ryegrass (Per and Ann)	Small Grain Silage Mixtures	
Rye	Sorghum	Small Grain Silages	
Ryegrass (Per and Ann)	Sorghum Sudangrass	Some grains by product	
Sorghum	Straw	Sorghum	
Sorghum Sudangrass	Sudangrass	Sorghum	
Sorghum x Sudangrass BMR	Switchgrass (Forage to Dormant) (Low and High)	Sudangrass	
Straw	Teff	Sudex	
Sudangrass	Timothy	Triticale	
Switchgrass (Forage to Dormant) (Low and High)	Triticale	Wheatlage	
Teff	Wheat		
Timothy			
Triticale			
Wheat			

Instrument Partners



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Bruker Optics FT-NIR analyzers for Feed and Forage deliver the accuracy and repeatability necessary for complete animal nutrition management. For Dairy and Livestock producers, Bruker's measurement repeatability means lower feed costs by knowing the exact nutritional contribution between forages and TMRs that make up the animal's diet. Measurement accuracy for Research means you can be sure which aspects of nutrition changes result in measurable performance changes in the test animals. Instrument drift and calibration adjustment that increase operating cost for other NIR technologies are a thing of the past thanks to Bruker's Fourier Transform (FT) technology. If your operations involve managing formulations at multiple sites you will appreciate how Bruker's NIR analyzers will produce consistent results across your fleet of analyzers. <https://www.feed-analysis-nir.com/feed.html>

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