Sources of variation in feed analytical composition and minimizing their impact on ration formulation

David R. Mertens

USDA-ARS

US Dairy Forage Research Center

Introduction

- Rations on the farm
  - Ration formulated
  - Ration given to the feeder
  - Ration mixed and fed
  - Ration eaten by the cow
- Last ration is important to the cow
- First ration requires accurate analytical information

Objectives

- Describe methods for defining analytical variation
- Estimate expected variation for routine methods
- Discuss sources of analytical variation
- Propose approaches to minimize the effects of analytical variation on rations

Principles of Analytical Variation

1. All analytical results are ESTIMATES of the composition of the feed
2. Variation is natural and unavoidable
3. Variation can be partitioned into accuracy and precision
4. Replication and statistical inference are needed to detect differences and define confidence intervals

1. All Results are ESTIMATES

- Only a small portion of the feed is analyzed
  - 20 cores of .5 oz = 10 oz from 200 ton
  = 1/640,000th of the stack of hay
  - 1 g test portion of the 10 oz is analyzed
  = 1/181,600,000th of the stack
- Representative samples do estimate the analysis very accurately, but results will not be identical

1. All Results are ESTIMATES

- If a second sample was taken and analyzed by a second laboratory, should we expect the result to be the same?
  - No, the samples and the laboratories are not identical
- If they do not agree, is one correct and the other result wrong?
  - No, each result is an independent estimate of the composition of the stack
  - The best estimate is the average of the two
  - Must accept that more than one estimate exists

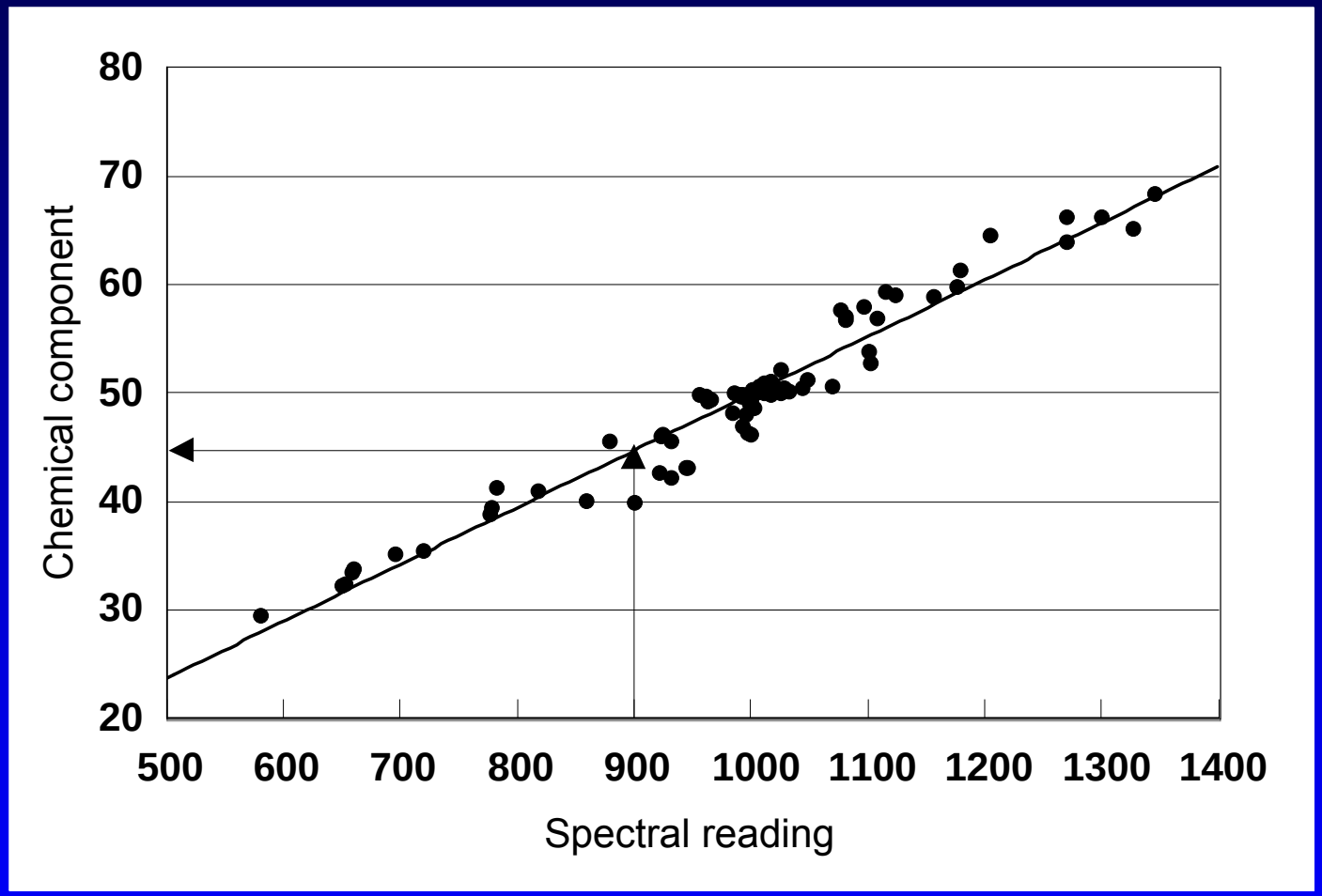
2. Variation is Natural and Unavoidable

- Prefer the terms “variation”, “deviation” or “bias” to the term “error” because error implies that a mistake was made
- Variation is related to the sensitivity of the measurement – there is more variation in sensitive measurements
  - e.g., measuring to the nearest inch or 1/16th inch
- Variation is due to differences in samples because feeds, especially forages, are heterogeneous
  - Source of most within-laboratory variation
  - NIR scans verify this

2. Variation is Natural and Unavoidable

- Variation is related to the analytical method
  - Complex methods are more variable
  - Predictive methods, such as NIRS, are less variable
    - This is due to statistical prediction that that estimates the mean expected value for the result

Prediction Is Less Variable



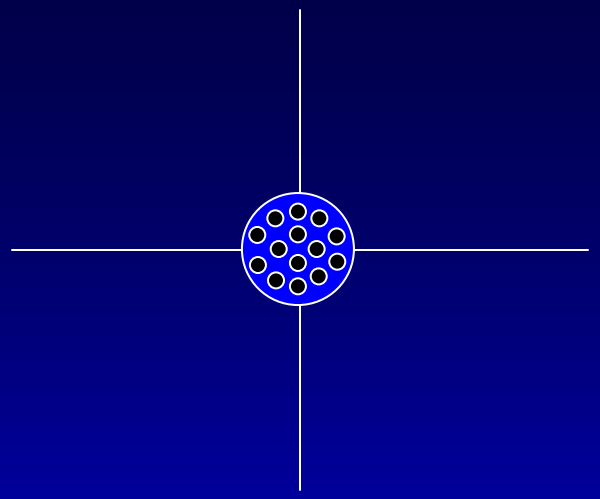
Regression predicts only one result for a spectral reading, whereas actual measurements have variation not predicted by regression

2. Variation is Natural and Unavoidable

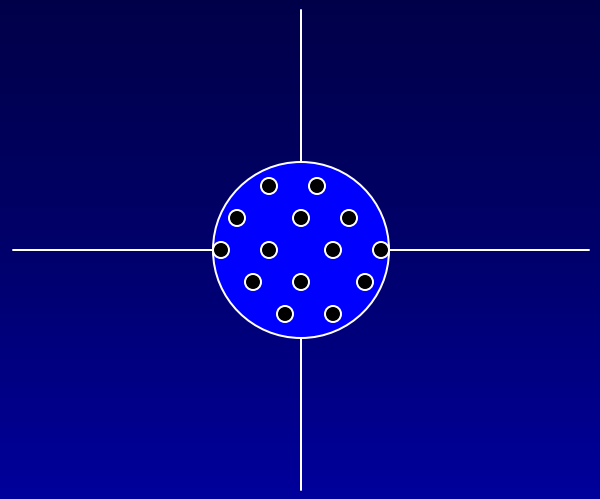
- Because it cannot be eliminated, variation can only be
  - Controlled (minimized) or
    - Representative samples
    - Excellent laboratory techniques
  - Reported (and accounted for)
    - Knowing the variation can be helpful in making decisions
    - Can be used to adjust rations to meet requirements with greater probability of success
    - Determines the usefulness of information

3. Variation = Accuracy + Precision

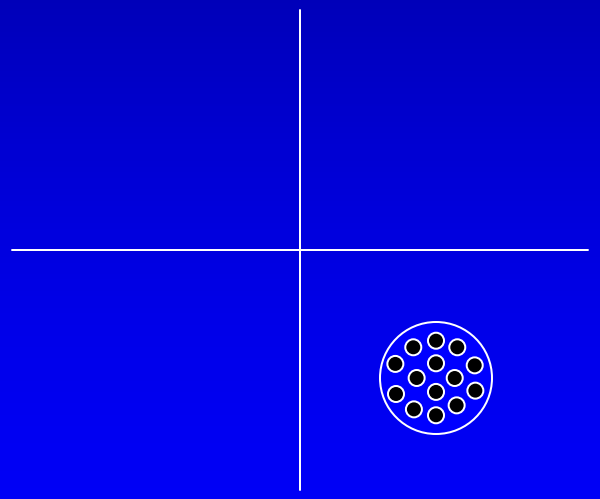
- Definition of crucial terms:
  - ACCURACY = closeness of the result to the known or consensus reference value
    - Related to SYSTEMATIC BIAS (error)
    - Controllable ‘error’; mistakes
  - PRECISION = consistency of results from repeated analyses
    - Related to RANDOM VARIATION (error)
    - Uncontrollable, natural random ‘error’
  - Accuracy and Precision are completely independent components of variation



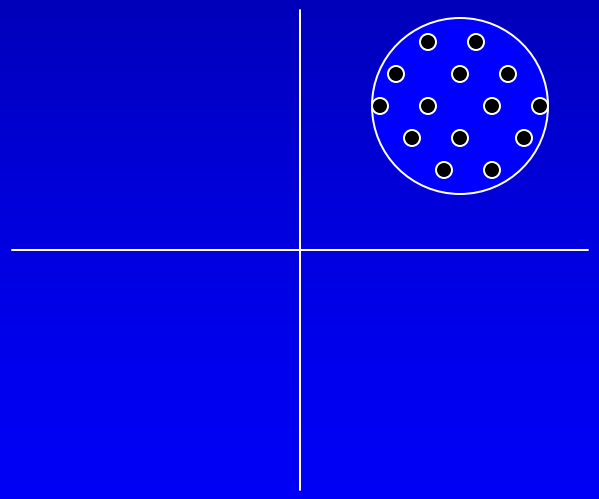
a. Accurate & Precise



b. Accurate, but Imprecise



c. Inaccurate, but Precise



d. Inaccurate & Imprecise

3. Variation = Accuracy + Precision

- Need both ACCURACY and PRECISION
  - Precision
    - insures confidence in results
    - minimizes costly replication of results
    - tells nothing about accuracy, it is possible to repeatedly get the same wrong result!
  - Accuracy
    - insures that results are comparable among laboratories
    - insures that analytical measurements are compatible with dietary requirements
      - Cow performance is the ultimate measure of accuracy
      - Inaccurate analyses require the “art of feeding”

4. Replication and Statistics Are Keys

- Replication is needed to:
  - Measure variation
  - Partition it into accuracy and precision
  - Solve the problem of imprecise measurements
  - Determine the probability that differences are real or true
  - Estimate confidence intervals around the average (the range in results in which we would expect additional measurements to fall)

4. Replication and Statistics Are Keys

- Replication is needed to:
  - Provide statistical information
    - One measurement (38.5% aNDF) gives no statistical information because there are 0 degrees of freedom (df = n – 1)
    - Two measurements (38.5 & 41.5% aNDF) give a mean or average (40.0%) and a ± standard deviation (SD = ± 1.5%), which can be used to calculate an expected range
  - Normal distributions
    - 68.3% of measurements should be within ± 1*SD
    - 95.4% of measurements should be within ± 2*SD
    - 99.7% of measurements should be within ± 3*SD

4. Replication and Statistics Are Keys

- Replication is needed:
  - For confidence intervals (large numbers of observations)
    - 90% of measurements should be within ± 1.64*SD
    - 95% of measurements should be within ± 1.96*SD
    - 99% of measurements should be within ± 2.58*SD
  - For a between-laboratory variation in aNDF analyses of 1.1% aNDF and a result of 40%, our 95% confidence interval would be
    - 40 – 1.96*SD to 40 + 1.96*SD
    - 40 – 1.96*1.1 to 40 + 1.96*1.1
    - The range 38.8 to 42.2% aNDF should include 95% of the analyses reported by laboratories

4. Replication and Statistics Are Keys

- Necessity and value of replication
  - Hay producer A sends a sample of a hay to Lab B and receives the result of 38.2% aNDF
  - Hay buyer C sends a sample of the hay to Lab D and receives the result of 42.6% aNDF
  - Both parties are upset and typically blame the laboratories for inaccurate results
  - But we have two potential sources of variation:
    - Samples from A and C (1 df for samples)
    - Analyses from Labs B and D (1 df for Labs)
    - With two analyses, there are no degrees of freedom to determine the true source of the variation (n = 2 –1 –1 = 0 df)
  - Best estimate of the aNDF in the hay is the average (40.4% aNDF)

4. Replication and Statistics Are Keys

- Necessity and value of replication
  - Solution is to have each lab analyze the sample from the other
  - We have four results (two for each sample and two for each laboratory
  - Three possible scenarios can occur

Necessity and value of replication

Scenario		Sample A	Sample C	Lab average
2	Laboratory B	38.2	42.4	40.3
2	Laboratory D	38.0	42.6	40.3
2	Sample average	38.1	42.5	
2	Overall average			40.3
3	Laboratory B	38.2	38.4	38.3
3	Laboratory D	42.8	42.6	42.7
3	Sample average	40.5	40.5	
3	Overall average			40.5
4	Laboratory B	38.2	40.8	39.5
4	Laboratory D	40.8	42.6	41.7
4	Sample average	39.5	41.7	
4	Overall average			40.6

4. Replication and Statistics Are Keys

- Average or mean results are consistent
  - Mean or average measurements are less variable because they represent more than one result
  - Mean standard errors decrease by 1/n, where n is the number of results averaged
  - Averaging repeated measurements has the effect of reducing the variability of the mean
    - Imprecise measurements can be overcome by repeating analyses and averaging results
    - Less precision = more replications

4. Replication and Statistics Are Keys

Number of	SD of individual replications			
Observations (n)	**1.00**	**2.00**	**3.00**	**4.00**
1	**1.00**	**2.00**	**3.00**	**4.00**
2	**0.71**	**1.41**	**2.12**	**2.83**
4	**0.50**	**1.00**	**1.50**	**2.00**
8	**0.35**	**0.71**	**1.06**	**1.41**
16	**0.25**	**0.50**	**0.75**	**1.00**
32	**0.18**	**0.35**	**0.53**	**0.71**
64	**0.13**	**0.25**	**0.38**	**0.50**
128	**0.09**	**0.18**	**0.27**	**0.35**

4. Replication and Statistics Are Keys

- Practical Confidence Intervals
  - .05 probability level, which is ± 1.96*SD, is very conservative (19 out of 20 times research results would agree)
  - Use “reference or expected” SD based on many observations to determine confidence intervals and evaluate results
    - Small replications (<30) can be erratic in determining SD
  - Samples and laboratories are not randomly selected
  - Practical multiplier is ± 2.0*SD

4. Replication and Statistics Are Keys

- Practical Confidence Intervals - Example
  - 3 samples of hay were analyzed by different labs – results were: 38.8, 42.6, 47.2% NDF
  - Reference SD for the sampling technique used is ± 1.4% NDF and reference SD for analysis of NDF is ± 1.5% NDF
  - The SD for a combination of variables is the square root of the sum of the squares of the variables
    - $\pm\sqrt{(1.4*1.4 + 1.5*1.5)} = \pm 2.05$
  - Practical confidence interval is the mean (42.9%) ± 2*2.05 = 38.8 to 47.0%
  - One result (47.2) is outside the confidence interval, (even when it was used to calculate the mean) and it is suspect
    - May be best to use the median or midpoint number to calculate the confidence interval

Variation in Analyses

- How do we assign the reference or expected SD for analytical results?
- Two well-established programs quantify analytical variation
  - Association of Official Analytical Chemists (AOAC, Int.) collaborative studies for evaluation methods
  - National Forage Testing Association (NFTA) proficiency certification program for analytical performance of laboratories

AOAC Official Methods Collaborative Studies

- Purpose is to evaluate the validity (Reproducibility) of a method among labs before conferring Official Method status
- Minimum of 8 laboratories should participate
- Minimum of 5 samples with blind duplicates or 5 Youden pairs of samples
- Only one analysis per sample

AOAC Official Methods Collaborative Studies

- Reproducibility SD(R)
  - Observed among-laboratory variation in results
    - Results compared among laboratories
  - More a function of accuracy than precision
    - Differences in methodology
- Repeatability SD(r)
  - Observed within-laboratory variation in results
    - Measurements of blind replicates compared within labs
  - More a function of precision than accuracy
    - Consistency in technique, environment, equipment and reagents
    - Random variation in the test sample

Expected Analytical Variation Standard SD of Analyses

Constituent	Labs	Material	No.	Avg.	SD(r)	SD(R)
Lignin	10	Forage	6	6.66	0.29	0.62
ADF	10	Forage	6	39.5	0.38	1.13
aNDF	11	Forage	5	52.2	0.84	1.10
aNDF	11	All feeds	11	38.7	1.05	1.33
IDF	10	Beet pulp	1	45.6	0.67	0.98
SDF	10	Beet pulp	1	20.6	0.8	1.35
TDF	10	Beet pulp	1	66.1	1.15	1.59
TDF	9	Brans	2	54.4	1.28	3.16
Uronics	9	Brans	2	2.70	0.24	0.37
N. Sugars	9	Brans	2	39.8	1.31	3.67
Klason lig.	9	Brans	2	8.70	0.44	0.80

AOAC Official Methods Collaborative Studies

- Horwitz (1982) summary of variation relationships in collaborative studies
  - Relative SD(R) or CV(R) was a function of the mean concentration across all types of methods
    - $RSD(R) = 2^{(1-.5*Conc*.01)}$
    - Probably occurs because methods and equipment are more sensitive when the concentration of the analyte is small
    - May not hold if concentration varies from 0 to 100% for a gravimetric method (based on weighing)
  - SD(r) was typically 1/3rd to ½ of the SD(R)
    - Random variation in test samples

National Forage Testing Association Proficiency Certification Program

- Purpose is to evaluate the proficiency of feed analysis laboratories to generate reproducible data for routine nutritional analyses
  - Voluntary, not regulatory evaluation
- Certification is based solely on the results
  - Reference Method Procedures are provided but not required
  - Focus is on results not process

NFTA Proficiency Certification Current Program 1995-present

- NDF was added to DM, CP, and ADF analyses
- 1 sample of corn silage and grassy hay were added to the 4 alfalfa hay samples
- Pass/Fail was replaced with grades of A to F
- Average results of three replicates are compared to a Reference Method Average (RMA) instead of the median value of all laboratories
- Horwitz standard deviation (HSD replaced the Median Absolute Deviation or MAD)
  - $HSD = RMA*.01*2^{(1-.5*RMA*.01)}$
  - A modified HSD is used for DM:
    - ModHSD = .381 +.0159*RMA

NFTA Proficiency Certification Current Program 1995-present

- Reference Method Average
  - A questionnaire is used to identify laboratories using the Reference Method for each analyte
  - Results are censored to remove outliers
    - Results for all potential Reference Method labs are ranked
    - Top and bottom 15.8% (greater than ± 1 SD) are removed and tested to eliminate outliers
- Every 20th sample is analyzed by NIR to verify homogeneity of samples before samples are submitted to the laboratories

NFTA Proficiency Certification Current Program 1995-present

- Certification criteria
  - Bias Total Accuracy (magnitude and precision of Bias from the RMA)
  - Cumulative Bias Total Accuracy across all six samples for each analyte
  - Grading scale
    - A = Bias ≤1.00*HSD
    - B = Bias 1.01 to 2.00*HSD
    - C = Bias 2.01 to 3.00*HSD
    - D = Bias 3.01 to 4.00*HSD
    - F = Bias ≥4.00*HSD
    - Penalty for missing data 5.5*HSD

NFTA Proficiency Certification Current Program 1995-present

- Certification criteria
  - Pass = Grade of C or above averaged over all 6 samples for each analyte (DM, CP, ADF & NDF)
- Additional information provided to labs
  - Precision and Total Accuracy of replicated analyses for each analyte
  - Replicated data is not used for certification because they can be easily manipulated (could replicate results and only report the closest three results)

Total Accuracy of Nutritional Analyses

- NFTA defines accuracy differently from the classical definition
- NFTA defines the difference between a measurement and the reference value as BIAS (instead of accuracy) and
- NFTA defines Total Accuracy as the sum of variation due to both bias and imprecision to indicate the overall (total) ability to measure and report the target value of the analyte

NFTA Proficiency Testing Report

Sample	RMA	HSD	Rep 1	Rep 2	Rep 3	Avg	SD	Bias	Grade
05AH06	32.14	0.763	31.87	34.27	30.27	32.14	2.013	-0.003	A
05GH05	43.53	0.987	42.92	44.32	42.32	43.19	1.026	-0.343	A
05CS04	47.32	1.059	47.88	45.48	49.48	47.61	2.013	0.293	A
05AH03	40.28	0.924	39.74	41.04	39.04	39.94	1.015	-0.340	A
05AH02	32.48	0.769	33.05	30.75	34.75	32.85	2.007	0.370	A
05AH01	42.97	0.976	42.68	44.68	41.68	43.01	1.528	0.043	A
Last six RMA						39.79			
Last six HSD						0.913			
Last six average within sample Total Accuracy								1.684	
Last six average within sample Bias Variation								0.276	
Last six average within sample Precision							1.661		
Last six among sample BIAS TOTAL ACCURACY								0.303	A
Last six among sample BIAS AVERAGE								0.003	A
Last six among sample BIAS TOTAL PRECISION								0.303	A

This lab has a very small BIAS with a large and consistent between-replicate variation.

NFTA Proficiency Testing Report

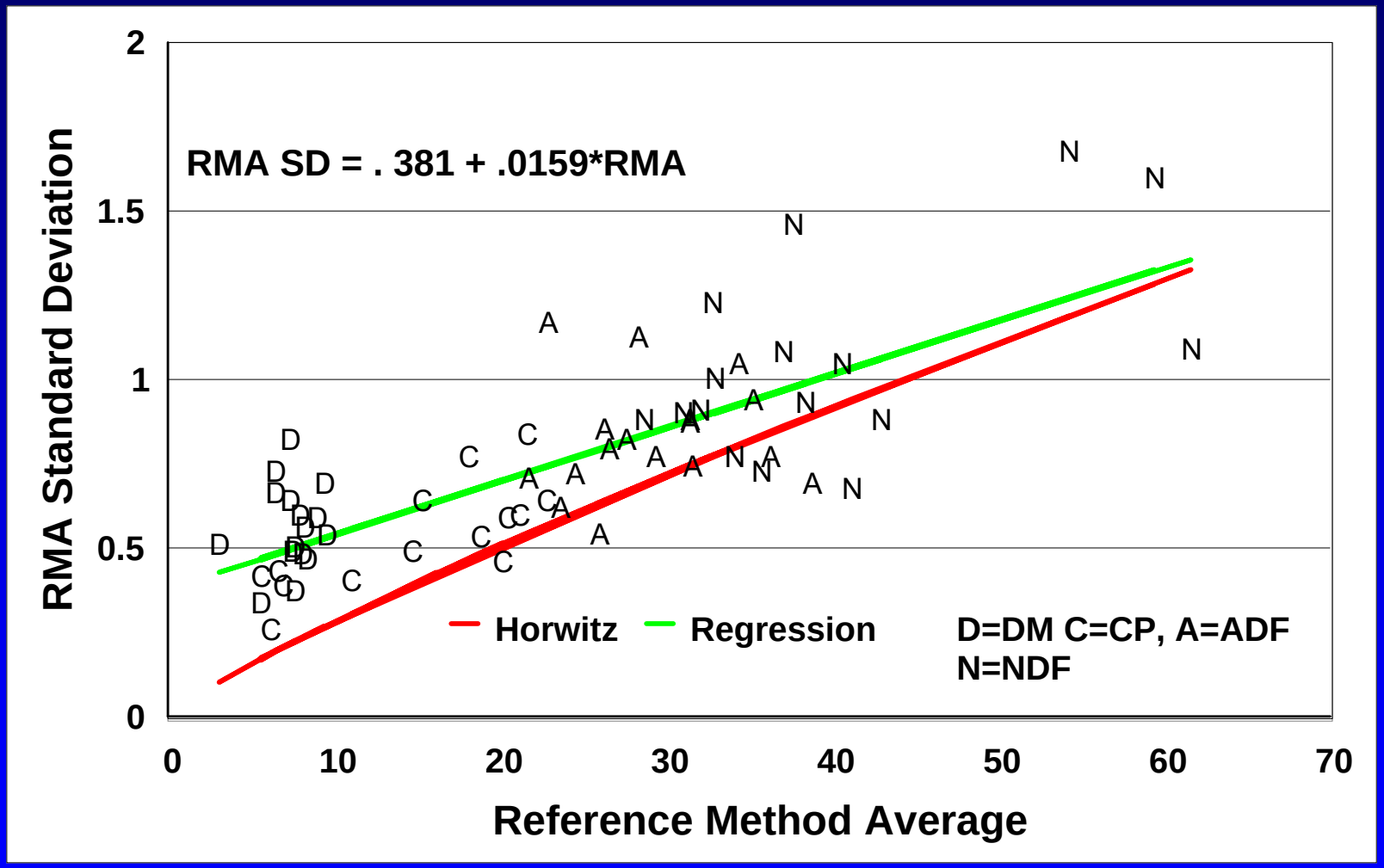
Sample	RMA	HSD	Rep 1	Rep 2	Rep 3	Avg	SD	Bias	Grade
05AH06	32.14	0.763	31.25	31.40	31.69	31.45	0.224	-0.693	A
05GH05	43.53	0.987	42.88	42.70	42.99	42.86	0.146	-0.673	A
05CS04	47.32	1.059	46.57	45.89	47.01	46.49	0.564	-0.830	A
05AH03	40.28	0.924	39.58	39.17	39.36	39.37	0.205	-0.910	A
05AH02	32.48	0.769	31.59	31.76	31.46	31.60	0.150	-0.877	B
05AH01	42.97	0.976	41.77	41.74	41.67	41.73	0.051	-1.243	B
Last six RMA						39.79			
Last six HSD						0.913			
Last six average within sample Total Accuracy								0.933	
Last six average within sample Bias Variation								0.891	
Last six average within sample Precision							0.276		
Last six among sample BIAS TOTAL ACCURACY								0.976	B
Last six among sample BIAS AVERAGE								-0.871	A
Last six among sample BIAS TOTAL PRECISION								0.441	A

This lab has a very small between-replicate variation with a very consistent BIAS.

Standard or Expected SD for Analyses AOAC Horwitz and NFTA Data

%Conc.	HCV(%)	HSD	Analyte/Source	Obs. SD	NFTA
5	3.14	0.16	Ash, Lignin	ADL = .62	0.46
10	2.83	0.28	Ash, Lignin	Klas.Lig.=.80	0.54
15	2.66	0.40	CP forages		0.62
20	2.55	0.51	CP forages		0.70
30	2.40	0.72	ADF forages		0.86
40	2.30	0.92	ADF for., NDF leg.	ADF for. = 1.13	1.02
50	2.22	1.11	NDF legumes	aNDF for. = 1.10	1.18
60	2.16	1.30	NDF grasses	TDF bp = 1.59	1.34
70	2.11	1.48	NDF grasses		1.49
80	2.07	1.65	NDF straws		1.65

NFTA Reference Method Average and SD



Sources of Analytical Variation

- Sampling of the material that is analyzed
  - Probably the major source of within lab variation
- Preparation of samples for analysis
- Methodological differences
- Technique differences among analysts
- Environment, reagent, equipment, and calibration differences among laboratories
- Errors (mistakes) in application or operation of methods
- Errors in calculating or adjusting results

Always compare analyses on an as-is or as-received basis

DM adjusted results contain variation in both nutrient and DM determinations

Nutrient	Lab A&B	Lab A	Lab B	Lab diff
	As-Is %	88%DM	92%DM	
		DM basis	DM basis	DM basis
CP	20	22.7	21.7	1.0
NDF	50	56.8	54.3	2.5

Putting Analytical Variation into Perspective

Comparison of Sources of Variation for NDF

Analytical	SE	Hay	SE	Silage	SE
Avg 'A' Lab single analysis	0.60	5 cores	2.15	5 loads	2.46
Avg 'A' Lab duplicate analysis	0.54	10 cores	1.52	10 loads	1.74
Two avg 'A' Labs single analysis	0.48	15 cores	1.24	15 loads	1.42
Avg 'B' Lab single analysis	1.50	20 cores	1.07	20 loads	1.23
Avg 'B' Lab duplicate analysis	1.35	5 grab	3.00	5 grab	2.37
Two avg 'B' Labs single analysis	1.21	10 grab	2.12	10 grab	1.68
Avg 'C' Lab single analysis	2.50	15 grab	1.73	15 grab	1.37
Avg 'C' Lab duplicate analysis	2.27	20 grab	1.50	20 grab	1.19
Two avg 'C' Labs single analysis	2.00				

Putting Analytical Variation into Perspective

- Sampling variation is almost always greater than analytical variation
  - Too often only a single sample is split to detect analytical variation
  - Splitting multiple samples would evaluate both sampling and analytical variation
  - Multiple samples should be the rule rather than the exception
  - Minimum analytical variation cannot compensate for a poorly collected and mixed sample

Putting Analytical Variation into Perspective

- Analytical variation is important in setting economic value and formulating rations, but is irrelevant to the cow
- Cows eat, digest and metabolize the nutrients actually in the feed and not the analysis reported
- Analytical variation is small relative to the relationships between nutrient content and animal responses

Relationship of Digestibility to Fiber

adapted from Giger-Reverdin, 1995

(average parameters and SD of 15 expts)

	Intercept	Slope	R^2	Reg.SE
CF	**89.7**	**-0.772**	**0.63**	**5.31**
	7.2	**0.259**	**0.16**	**2.53**
NDF	**97.7**	**-0.544**	**0.67**	**5.92**
	7.8	**0.174**	**0.12**	**3.28**
ADF	**95.4**	**-0.714**	**0.68**	**4.95**
	9.1	**0.429**	**0.16**	**2.53**
ADL	**83.1**	**-2.975**	**0.79**	**4.84**
	3.7	**0.683**	**0.08**	**2.86**

Minimizing Effects of Analytical Variation

- Analyze multiple representative samples using laboratories that have demonstrated ability to obtain accurate results (NFTA certified)
- The average of at least 2 samples submitted to 2 labs is a minimum estimate of the value of the feed
  - If the two values are outside standard confidence intervals, then a third analysis is needed to detect and eliminate suspect values
- The goal is not to obtain identical values, but obtain independent results that when averaged give the best estimate of the feed's nutritional value

Minimizing Effects of Analytical Variation

- Variation in total mixed rations (TMR) can be minimized by using multiple feed ingredients
  - It is unlikely that all the feeds will vary in the same direction at the same time
- Dry matter variation in feeds causes more variation in the TMR than analytical variation because it affects the proportions of feeds actually mixed

Implications – Take-Home Message

- All analytical results are estimates of the actual average nutritional value of a feed
- Although variation can be minimized, some random variation is natural and unavoidable
- Accuracy (closeness to the reference value) and precision are independent sources of variation – Precise (repeatable) results may not be accurate
- Standard deviations are a measure of scatter in results around a mean or average value
- Replication is needed to obtain more accurate averages and make statistical decisions

Implications – Take-Home Message

- Both AOAC International collaborative studies and the National Forage Testing Association Proficiency Certification Program provide reference estimates of analytical variation
- Analytical variation is highly correlated with the average analyte concentration
- Reference or expected standard deviations for obtaining a representative sample are often larger than those for analysis
- Variation due to sampling and analysis can be combined to determine the overall standard deviation for determining confidence intervals

Implications – Take-Home Message

- Sampling and analytical variation are important to the feed seller, buyer and nutritionist
- But not important to the cow because she eats, digests and metabolizes the nutrients actually in the feed, not what is reported
- When the cow's performance disagrees with analysis we must accept her assessment
- Analytical goals of replicated analyses are:
  - Estimate nutritional value accurately
  - Generate ration formulations that optimize animal performance

Sources of variation in feed analytical composition and minimizing their impact on ration formulation

Questions?

David R. Mertens

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