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| Title: Combustion Reference Method for Protein in Grains, Oilseeds, and Commodities | | |
| Lab: USDA,GIPSA,FGIS,TSD,ARTS | Program: Protein Reference Program | |
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**"CONTROLLED DOCUMENT"**

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## Changes From Previous Revision

1. In section 3. References:
   Added *SPC for the Rest of Us*.

2. In section 5. Equipment:
   (k) Added 8.75" x 17.75" x 6 mil polyethylene sample bags.

3. In section 6. Materials:
   (q) Moved to s. New q added for bulk soybean sample.
   (r) Added for bulk corn sample.

4. In section 7.2.a Sample Preparation:
   (4) Moved to 5. New 4 added to describe double bagging the samples.

5. In section 7.2.b Parameters for Use:
   Added definition of "batch."
   (1) Rewritten to better describe under what circumstances the LCS must be analyzed.
   (2) Added "…(at least one hour)…"
   (3) Replaced "…required per grinding session…" with "…required per batch…", added "…for no longer than two weeks…", and changed "…days' morning sample" to " …days' samples."
   (4) Added to emphasize the importance of getting a moisture result on the LCS as early as practical.

6. In section 7.2.c Determining the target Value:
   Moved to 10.2.b.1

7. In section 10.2 Acceptance Criteria
   Completely reworked to describe the development of a control chart and the rules for using it.
   (b) Moved to c.

## Table of Contents

## 1. Purpose and Scope of Application

The purpose of this Working Instruction (WI) is to establish the operational parameters, methodology, and requirements for the quality assurance and acceptability of data in the determination of protein in wheat, barley, corn, soybeans, and processed commodity samples.

## 2. Analyst Qualifications and Responsibilities

a) The analyst will receive on-the-job training in the conduct of this WI from a technician who has prior experience and training in using the WI, and/or from the Supervisory Chemist (or Project Leader).

b) The analyst will follow the WI as written. The Supervisory Chemist (or Project Leader) is responsible for ensuring this WI is followed, and modified as necessary or appropriate. All revisions to this WI must be approved by the Branch Chief of the Analytical, Reference and Testing Services Branch, prior to implementation.

c) The analyst is responsible for checking that the difference between replicate analyses of a sample, and the difference between the average of two replicates and the value determined by the Near Infrared Transmission (NIRT) instruments, meet the tolerances specified in this WI. The analyst is also responsible for repeating the analysis on any sample that exceeds these tolerances.

## 3. References


- American Association of Cereal Chemists (AACC) Method 46-30.
- Association of Official Analytical Chemists International (AOAC) Method 992.23.
- American Oil Chemists Society (AOCS) Ba 4e-93.
- American Society of Testing Materials (ASTM).
- National Institute of Standards and Technology (NIST).
- Kenyon, A.S., Black, J.C., & Layloff, T.P. (1995) *J. Assoc. Off. Anal. Chem.* **78**, 1109-1111.
- Pitt, H., 1993: *SPC for the Rest of Us*. Addison-Wesley, 419 pp.


## 4. Safety and Hazardous Waste

Refer to the Technical Services Division Chemical Hygiene Plan.

## 5. Equipment

a) Analytical balance accurate to 0.1 mg and equipped with RS-232 serial data interface.

b) Computer and software, IBM-compatible, equipped with a barcode wand, RS-232 serial interface, Microsoft Excel, and Beckman Laboratory Information Management System (LIMS).

c) Desiccator cabinet with shelves and desiccant tray.

d) Combustion Nitrogen Analyzer (CNA), FP-2000 (LECO Corporation, St. Joseph, MI).

e) Reference weight set, NIST Class S or ASTM Class 1, including 1.0g, 10g, and 50g weights.

f) Air compressor capable of providing pneumatic pressure of 40 ± 4 PSI, and equipped with a filter for trapping moisture and oil.

g) Gas regulators for oxygen, helium, and compressed air sources.

h) Udy Cyclone Mill, 3/4 horsepower, belt drive, using a 1 mm round-hole screen for grinding wheat and barley, and a 2 mm round-hole screen and automatic feeder for grinding soybeans and corn.

i) Stein M-2 or M3/B Mill.

j) Boerner grain divider.

k) Polyethylene sample bags, 6” x 13” x 6 mil, and 8.75” x 17.75” x 6 mil.

l) Poly bag impulse sealer.

## 6. Materials

a) Ethylenediaminetetraacetic Acid (EDTA), American Chemical Society (ACS) grade.

b) Nicotinic Acid p-Toluene sulfonate (NATS), #22781-24, HACH Company, Loveland, CO.

c) 2-Amino-2-(hydoxymethyl)-1,3-propanediol [tris(Hydroxymethyl)aminomethane; “THAM”; “Tris”; “TRIZMA”], NIST Standard Reference Material 723.

d) Magnesium perchlorate anhydrous, reagent grade (desiccant).

e) Lecosorb (sodium hydroxide on inert base).
f) Copper sticks.

g) Copper turnings.

h) N-catalyst, #502-049, LECO Corporation, St. Joseph, MI.

i) Alumina oxide pellets.

j) Glass wool.

k) Steel wool.

l) Ceramic boats.

m) Oxygen, at least 99.99% pure.

n) Helium, at least 99.99% pure.

o) Compressed Air, oil and water free.

p) Bulk sample of wheat, minimum size: 3000 grams.

q) Bulk sample of soybeans, minimum size: 1500 grams

r) Bulk sample of corn, minimum size: 1500 grams

Humidity indicating cards

## 7. Quality Control Procedures

### *7.1 Instrument Parameters*

#### 7.1.a FP-2000

1. Startup

   All steps in the "Installation," "Option Installation," and "System Setup" sections of the FP-2000 Instruction Manual should be completed before performing the following procedures.

   a. From the "Front Panel" screen confirm that the following settings are configured as shown:

      i. Method = instrument serial number (3355 or 3669)
      ii. Furnace Temperature = 1050 ± 2 °C
      iii. N TC Cell = 4.0 ± 0.5 Volts
      iv. Analysis Gas = On
      v. Sampler = Auto
      vi. Printer = On-Line

   b. Print the "Front Panel" screen.

   c. If the counter for the Catalyst Heater, Aliquot Absorption Tube, or Anhydrone Tube is over the limit, perform the required maintenance as described under the "Maintenance" section of the manual, then reset the counter(s) to zero. Document the maintenance performed in the Maintenance logbook.

   d. Check the pressure in the oxygen and helium tanks and replace if empty.

   e. If necessary, adjust the regulators on the oxygen, helium, and compressed air lines to supply 40 PSI each.

   f. Inspect the compressed air line for moisture by opening the drain valve that terminates the line. Drain the line if moisture is found.

2. System Check

   Refer to the "Diagnostics" section of the FP-2000 Instruction Manual for the following procedures.

a. Perform the helium and combustion leak checks. If either leak check fails, stop and refer to the appropriate leak troubleshooting procedure in the "Service" section of the manual. Print the leak check data.

b. File the print outs from the "Front Panel," leak checks, and counters in the "LECO Records" box. Store the box when full in the downstairs storage area (room 118).

3. Blank Calibration

Refer to the "Operation" section of the FP-2000 Instruction Manual for the following procedures.

a. At the "Analyze" menu, touch "Select ID Code" and highlight or type in the "Blank" ID Code. The sample weight should default to 0.2000 grams; if not, change it to read 0.2000 grams. Enter ten to fifteen blanks.

b. Analyze the blanks.

c. Check the last three or four blank results. If they are all $0 \pm 0.003$, proceed to the next step. If they are outside this range, refer to the "Blanks" chapter under the "Operation" section of the Instruction Manual and recalibrate the Nitrogen Blank.

i. If the blanks are running high, recalibrate the Nitrogen Blank again.

ii. If the blanks are still high, check the doser valve for dirt or rust, and check the oxygen tank pressure level to make sure it is not empty.

d. Print the data and file the print out in the "LECO Records" box.

4. EDTA Calibration

a. Place a ceramic sample boat on the analytical balance.

b. Tare the balance by pressing down the tare button.

c. Select the EDTA ID code from the FP-2000 "Analyze" menu.

d. Weigh 0.2000 to 0.2300 grams of EDTA into a ceramic sample boat.

e. Enter the weight into the FP-2000 by pressing the "print" key on the analytical balance.

f. Place the ceramic sample boat into the autoloader.

g. Repeat steps a through f for two more portions of EDTA.

h. Analyze the three portions of EDTA.

i. If the average result of the three EDTA's is within ± 0.02% of the value established during the last Trizma calibration, proceed to step 8.3, Calibration Checks.

ii. If the average result of the three EDTA's exceeds the tolerance, use at least two of the EDTA results and perform a drift correction (refer to "Drift Correction" under the "Operation" section of the Instruction Manual).

iii. Weigh and analyze two more portions of EDTA.

iv. If the average result of these two EDTA's is within ± 0.02% of the value established during the last Trizma calibration (see 8.2), proceed to step 8.3, Calibration Checks.

v. If the average result of these two EDTA's exceeds the tolerance, stop and investigate the process and/or the instrument to determine the cause and correct it. Repeat steps a-h.

### 7.1.b Analytical Balance

1. Refer to the balance operating manual for information regarding the operation and calibration of the balance.

2. Leave the power to the balance on at all times to ensure that the balance is at temperature equilibrium with the surroundings. If power is interrupted, the balance must be allowed one hour to return to equilibrium before a weighing is made (unless it is known that the interruption was brief, e.g., less than ten minutes, in which case the balance will reach equilibrium more quickly -- see Kenyon et al.).

3. Locate the balance in an area that is as free as possible of drafts and temperature fluctuations, preferably on a stone balance table with vibration-

damping pads separating the table top from the legs.

4. The balance must be cleaned, serviced, and calibrated with NIST-certified weights twice a year by a qualified service engineer.

5. Before using the balance, calibrate it at the beginning of the workweek, and anytime the response seems questionable (see balance operating manual for instructions on calibrating the balance).

6. Make sure all balance doors are closed before reading weights.

7. On the first work day of the week, weigh the 1, 10, and 50 gram weights from a set of NIST class S or ASTM Class 1 weights to check the balance for drift or other malfunction.

    a. Record the weights and the date in the FP-2000 maintenance logbook.

    b. Gross deviation from the known weights (i.e., a milligram or more) indicates a malfunction. Remove the balance from service and have it repaired by a qualified service engineer. Record the action taken in the logbook.

    c. A small deviation from the known weights that is continuously in the same direction on all three weights, and that grows larger over time, is indicative of a drift error. Remove the balance from service and have it repaired by a qualified service engineer. Record the action taken in the logbook.

### 7.1.c Reference Weight Set

Recertify the calibration of the reference weights once a year by sending them to a facility equipped to provide the service using NIST-traceable standards.

### 7.1.d Udy Grinder

1. Maintenance Required When Grinding Wheat or Barley

    The grinder must be disassembled and cleaned after grinding 20 samples and when switching from one class of wheat to another. The grinder may be cleaned when switching from Hard Red Winter to Hard Red Spring wheat by grinding approximately 10 grams of wheat of the next class to be ground, however, disassembly and cleaning is still required after every 20 samples.

The grinder may require more frequent cleaning when grinding high moisture or soft wheat samples.

a. To clean the grinder, unplug it, remove the lid, and vacuum or dust all parts, including the cover, impeller, grinding ring, screen, separator, cyclone, and filter assembly. Vacuum or dust all other accessible surfaces.
b. The grinding ring and the screen in the grinder must be replaced after not more than 8,000 samples have been ground. The grinding ring and the screen must be replaced at the same time.

c. Replace the grinding screen separately if it becomes damaged before the scheduled maintenance.

d. When a belt is replaced, always replace both belts. Maintain a record of all grinder service and maintenance.

e. Adjust the feed gate to allow a flow rate of approximately 1.5 grams per second. This may be checked by slowly pouring the sample into the feed hopper away from the feed gate allowing gravity and vibration to move the sample through the feed gate. Automatic feed hoppers may be used if they deliver approximately 1.5 grams of wheat per second to the grinding chamber.

f. The grinder motor should not be allowed to "drag" or run at a reduced rpm when the sample is introduced. If motor drag occurs and cannot be eliminated by adjusting the feed rate properly or replacing the belts, take the grinder out of service until it is repaired.

g. Some grinders have a switch for reversing the grinder motor. Always operate the grinder with the motor switch in the forward position.

2. Maintenance Required When Grinding Soybeans or Corn

Disassemble and clean the grinder after grinding every sample.

a. To clean the grinder, unplug it, remove the lid, and vacuum or dust all parts, including the cover, impeller, grinding ring, screen, separator, cyclone, and filter assembly. Vacuum or dust all other accessible surfaces.

b. The grinding ring and the screen in the grinder must be replaced after not more than 5,000 samples have been ground. The grinding ring and the

screen must be replaced at the same time.

c. Replace the grinding screen separately if it becomes damaged before the scheduled maintenance.

d. When a belt is replaced, always replace both belts. Maintain a record of all grinder service and maintenance.

e. Some grinders have a switch for reversing the grinder motor. Always operate the grinder with the motor switch in the forward position.

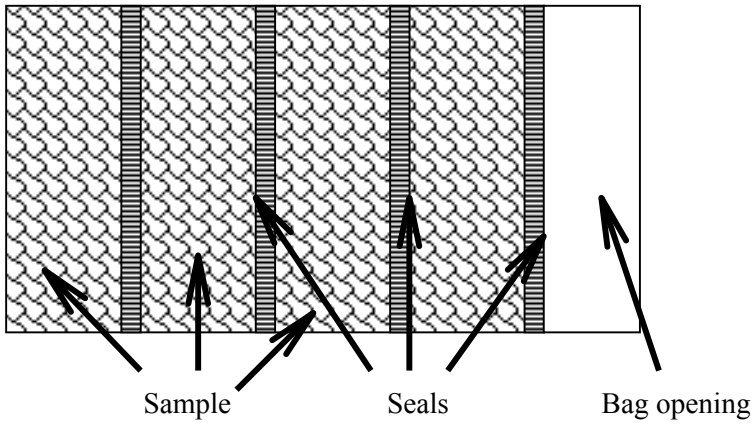
## *7.2 Wheat, Soybean, and Corn Lab Control Samples*

7.2.a Sample Preparation

1. Mix the bulk sample thoroughly.

2. Cut the bulk sample into portions of approximately 30 grams each using the Boerner divider.

3. Put the individual portions into plastic sample bags and seal. One bag can accommodate four portions as long as the bag is sealed after the addition of each successive portion as shown below:




4. Place the individually sealed portions inside a second plastic bag and seal. Reseal the outer bag each time a portion is removed for testing.

5. Keep the portions in refrigerated storage until needed.

7.2.b Parameters for Use

Note: a batch of samples is defined as all samples analyzed on the same workday.

1. One portion of an LCS must be ground and analyzed with every batch of like samples (use the wheat LCS when analyzing barley samples).

2. The portion(s) must be retrieved from the refrigerated storage and be allowed to come to room temperature before grinding (about one hour).

3. Although only one portion of an LCS is required per batch, up to four may be kept outside the refrigerated storage in order to prevent delays in processing the next batch of samples.

4. Because the disposition of the rest of the day's samples relies on the status of the LCS, it should be ground and analyzed as early as practical in the day.

## 8. Sample Analysis

### *8.1 Sample Preparation*

8.1.a Wheat and Barley

1. Check the Udy grinder to make sure it is clean and that a 1mm round-hole screen is installed in it. Turn the grinder on and allow it to reach maximum RPM.

2. Place a clean, empty Udy sample jar under the cyclone body and slowly pour the sample into the feed hopper away from the feed gate and allowing gravity and vibration to move the sample through the feed gate, or pour the entire sample into an automatic feed hopper if one is being used. A 20 gram portion should be completely ground in approximately 15 seconds.

3. After the sample is ground, press the plunger and tap above the clear plastic cyclone body top several times to clear the grinder of all loose flour.

4. Hold the plunger down, remove the sample jar, and cap it. Technicians should remove all jars from the grinder in a similar manner to obtain uniform drying of ground samples. Shake and rotate the sample jar to loosen caked flour.

5. Disassemble and clean the grinder according to information in section 7.1.d.1.

8.1.b Soybeans

1. Check the Udy grinder to make sure it is clean and that a 2mm round-hole screen is installed in it. Turn the grinder on and allow it to reach maximum RPM.
2. Place a clean, empty Udy sample jar under the cyclone body and slowly pour the sample into the feed hopper away from the feed gate and allowing gravity and vibration to move the sample through the feed gate, or pour the entire sample into an automatic feed hopper if one is being used. A 30 gram portion should be completely ground in approximately 90 seconds.

3. After the sample is ground, press the plunger and tap above the clear plastic cyclone body several times to clear the grinder of all loose meal.

4. Hold the plunger down, remove the sample jar, and cap it. Turn off grinder. Technicians should remove all jars from the grinder in a similar manner to obtain uniform drying of ground samples. Shake and rotate the sample jar to loosen caked meal.

5. Disassemble and clean the grinder according to information in section 7.1.d.2 after grinding each sample.

8.1.c Corn

1. Place the sample into a Stein Mill cup, raise the cup to the upper cup support and lock in place with the retaining knob.

2. Turn the mill on and crack the corn by grinding for five to ten seconds.

3. Lower the cup and brush the corn from the blade, blade arbor, and lower surface of the upper cup support into the cup.

4. Transfer the sample from the cup to a glass sample bottle.

5. Grind the cracked corn on the Udy following the procedure under 8.1.b.

8.1.d Processed Commodities

Most are already in ground condition. Check with CTL supervisor for any

necessary prep work.

### *8.2 Standardizing EDTA Against Trizma*

Note: This procedure is performed only when opening a new bottle of EDTA, approximately midway through the bottle, and towards the end of the bottle.

EDTA is used to calibrate the FP-2000. The theoretical nitrogen content of EDTA is 9.59%. Most suppliers of EDTA specify it's purity to be between 99.4% and 100.6%. Unless the purity is known more precisely, there can be little confidence in the accuracy of the protein determinations derived from an EDTA calibration.

By calibrating the FP-2000 against a very pure, NIST-certified standard (such as Trizma) and analyzing samples of the EDTA against that calibration, a more precise estimate of the purity of the EDTA is made. In turn, the calibration based on the improved estimate of the EDTA purity yields protein results that are more accurate.

a. Pour about 20 grams of Trizma and 50-100 grams of EDTA into separate dry, clean, glass petri dishes (approximately 140mm in diameter and 20mm high).

b. Place the petri dishes into a desiccator containing anhydrous magnesium perchlorate. Close the desiccator.

c. Use a humidity indicating card to check the humidity level in the desiccator. If the humidity is higher than 20%, replace the desiccant with fresh magnesium perchlorate.

d. Dry the chemicals at room temperature for at least 24 hours before using.

e. Upon removal from the desiccator, transfer the chemicals to tightly sealing bottles. Return the chemicals to the desiccator at the end of the work period.

f. Immediately after performing steps 1 through 3 under 7.1.a, calibrate the FP-2000 using ten 0.2000 to 0.2300 gram portions of Trizma (refer to the "Drift Correction" procedure under the "Operation" section of the Instruction Manual). The tolerance for Trizma nitrogen determinations is ± 0.02%. Trizma SRM 723 contains 11.55% nitrogen by weight, so the range of acceptable values for a Trizma analysis is 11.53-11.57% nitrogen.

g. Check the calibration with six more portions of Trizma. The calibration

should be stable (no widely fluctuating readings). If not, maintenance should be performed to improve the response of the instrument, and the calibration procedure should be repeated.

h. Once the calibration is stable, analyze six 0.2000 to 0.2300 gram portions of EDTA to determine the purity of the EDTA. With the actual percent of nitrogen in the EDTA known (for example, 9.56 ± 0.02%), the EDTA will be used as the working standard to calibrate the FP-2000 without using Trizma.

i. Approximately midway through a bottle of EDTA recheck the purity with Trizma following the eight steps above. As the EDTA gets close to being used up, perform this purity check once again.

j. The purity of EDTA is expected to differ from lot to lot (between 99.4% and 100.6%), but the purity determined for the chemical coming from the same bottle should not vary more than ± 0.2% from the previously determined average value for that container. If the change in calculated purity exceeds this tolerance, it is an indication that maintenance is required on the machine.

k. Whatever value is measured for EDTA (based on a Trizma calibration) will have a tolerance of ± 0.02% nitrogen. For example, if the average of the EDTA portions measured by the FP-2000 is 9.56% nitrogen, then the range of acceptable values for any one determination would be 9.54-9.58% nitrogen.

### *8.3 Calibration Checks*

Log in, weigh, and analyze two NATS samples. Sample weight should be between 0.2300 and 0.2400 grams.

1. If both NATS results are between 4.54 and 4.75% nitrogen, proceed to 8.4, Sample Analysis.

2. If either of the NATS results is outside the acceptable range, check the troubleshooting guide (Attachment 1) and the Instruction Manual for a cause and correct it. Repeat 8.3.

### *8.4 Sample Analysis*

Processed commodity samples are analyzed in single cuts. All other samples are analyzed in duplicate.

1. Enter the sample ID into the FP-2000 by scanning the barcode label, or manually enter the data if the sample has no barcode.

2. Weigh between 0.2300 and 1.0000 gram of sample into a ceramic sample boat.

3. Enter the weight into the FP-2000 by pressing the "print" key on the analytical balance.

4. Place the ceramic sample boat into the autoloader.

5. Repeat steps 1 through 4 for remaining samples.

## 9. Determination

The calculation for the percentage of protein in the sample is made by multiplying the amount of nitrogen detected by a protein factor (5.7 for wheat, wheat flour, and wheat-based processed commodities; 6.25 for barley, corn, corn-based processed commodities, soybeans, and soybean-based processed commodities). This is routinely done by the LIMS, but may be done by hand if necessary.

Protein results are normally corrected to a particular moisture basis using the following equation:

$$\%\, P_{MB} = \frac{P_{AI}\, x\, (100 - MB)}{100 - M}$$

Where $P_{MB}$ is the protein content of the sample on the desired moisture basis,
$P_{AI}$ is the "as is" protein content of the sample,
$MB$ is the desired moisture basis, and
$M$ is the moisture content of the sample.

## 10. Data Validation

### *10.1 Data Entry*

Protein results are either sent directly to a LIMS via an Excel spreadsheet interface, or are hand-entered into the spreadsheet on an "as is" basis. The protein is then moisture corrected by the spreadsheet using the calculation above.

### *10.2 Acceptance Criteria*

10.2.a Results on wheat samples supplied by the Wheat Protein Group for comparison to the NIRT instruments must agree with the NIRT by ± 0.50%.

Precision can be verified by comparing the differences between replicate analyses. Differences between replicates should be ≤ 0.50% protein for all soybean and soybean meal samples, ≤ 0.30% protein for barley and corn samples, and ≤ 0.15% protein for wheat and flour samples.

1. If the difference between the LCS's replicates falls within the cut tolerance for that grain or oilseed, the average of the two replicates is checked for accuracy as under 10.2.b below.

2. If the difference between the LCS's replicates exceeds the tolerance, the data is stored in the LIMS with the designation of "Fail" and the sample must be repeated (unless an assignable cause is known to have caused one of the two replicates to be in error, in which case the unaffected replicate may be considered "Valid" and used in the accuracy check below).

10.2.b Accuracy can be verified by comparing the LCS's replicate analyses to the target value.

1. Determining the Target Value

   a. In order to obtain a good estimate of the method's variability and have a control chart in place for a new LCS by the time the old LCS is expended, an action date should be estimated, and twenty portions of the new LCS should be analyzed in duplicate (one portion every couple of days over a period of eight-to-ten weeks). If for any reason this cannot be accomplished before the new LCS is needed, a less robust estimate can be developed by analyzing ten portions of the new LCS in duplicate on the same day.

b. Calculate the average value and standard deviation of the results.

c. The average is the target value for all future analyses of the LCS.

d. The standard deviation value is used to set the acceptable tolerance of all future analyses of the LCS.

2. Comparing the LCS to the Target Value

a. The LCS result is acceptable and data for all like samples within the batch will be validated and reported to the customer (pending agreement of the other samples' replicates with the tolerance specified under 10.2.a) if none of the following is true:

i. The result is outside the 3 standard deviation lines
ii. The result is the 8$^{th}$ point in a row on one side of the average line
iii. The result is the 6$^{th}$ point in a row all going up or down
iv. The result is outside the 2 standard deviation lines, along with more than 5% of the others

b. If any of the above scenarios are true, the data for all like samples within the batch are stored in the LIMS with the designation of "Fail" and the process, equipment, materials, etc. are investigated for an assignable cause.

i. If an assignable cause is found, document and correct the problem.

ii. Whether or not an assignable cause was found, grind and analyze another portion of the affected LCS, along with all of the samples from the batch.

1. If the LCS does not violate any of the four rules above the entire batch will be validated and reported to the customer.

2. If the LCS again violates one of the four rules above, the Project Leader (or, in his/her absence, the technician) must use discretion in deciding how to proceed.

10.2.c Commodity samples

Repeat the analysis on any sample for which a Quality Control (QC) request is issued through LIMS.