32.2.03A

AOAC Official Method 997.06
Protein (Crude) in Wheat
Whole Grain Analysis
Near-Infrared Spectroscopic Method
First Action 1997

[Applicable to wheat containing 9–16% protein (12% moisture basis).]

See Tables **997.06A–D** for results of the interlaboratory study supporting the acceptance of the method.

A. Principle

By means of linear chemometric algorithms, near-infrared (near-IR) transmittance or diffuse reflectance spectra are used as the basis for determining crude protein content in bulk wheat. Combination and overtone frequencies of NH, CH, and OH, which occur due to presence of proteins, carbohydrates, and water are of sufficiently high magnitude in near-IR region (850–2500 nm) to be measured and quantitatively related to protein content (compensated for moisture content). Procedure entails standardizing near-IR instrument, using a minimum of 20 wheats (termed standardization samples) of known protein content, before analysis of unknowns. Range in protein of standardization samples (preferably uniformly distributed) must be equivalent to or broader than that expected for unknowns.

B. Apparatus

(**a**) *Near-IR spectrophotometers.*—Use one of the following, or equivalent: (*1*) *Tecator Infratec 1221, 1225, 1226, or 1229.*—Available from Foss Tecator AB, Box 70, S-26321, Höganäs, Sweden. Instrument specifications: light source optics, 50 W tungsten halogen lamp; method of dispersion, ruled grating; mode of energy capture, transmittance; detector, silicon; dynamic response, 5 absorbance unit (AU); scan range, 850–1050 nm; wavelength resolution, 2 nm; bandpass [full width at half height (FWHH)], 6 nm. (*2*) *Foss Grainspec.*—Support and service available from Foss Decator AB, Box 70, S-26321, Höganas, Sweden. Instrument specifications: light source optics, 20 W tungsten halogen lamp; method of dispersion, bandpass filters and focusing wheel; mode of energy capture, transmittance; detector, silicon; number of interference filters, 11; number of focusing positions per filter, 3; wavelength range 808–1075 nm (provides uniformly spaced readings over wavelength range); wavelength resolution, 8.3 nm. (*3*) *Pertcon Inframatic 9100.*—Available from Perten Instruments, GMBH, Grossmootkehre 3, D-21079, Hamburg, Germany. Instrument specifications: light source optics, 8.5 W tungsten halogen lamp; method of dispersion, bandpass filters; mode of energy capture, reflectance; detector, lead sulfide; dynamic response; 2.3 AU; number of interference filters, 12; wavelength range 1077–1372 nm; bandpass (FWHH) for filters, 12 nm; wavelength accuracy, ±2 nm; and RMS noise, $<1 \times 10^{-5}$ OD. (*4*) *NIRSystems 6500/5000.*—Available from Foss NIRSystems, 12101 Tech Rd, Silver Spring, MD 20904 USA. Instrument specifications: light source optics, 75 W tungsten halogen lamp; method of dispersion, holographic grating; mode of energy capture, reflectance; detector, lead sulfide; dynamic response, 4 AU; scan range, 1100–2498 nm; wavelength resolution, 2 nm; bandpass (FWHH), 10 nm; wavelength accuracy, 0.3 nm; stray light, 0.1% at 2306 nm; and RMS noise, $<2 \times 10^{-5}$ AU.

(**b**) *Storage containers.*—For test samples; 500 and 1000 mL glass canning jars with rubber-lined metal caps and screw bands. Store laboratory and test samples in tightly sealed containers to minimize moisture transfer.

(**c**) *Software.*—Wheat protein content equation corrected to a fixed moisture basis supplied with each near-IR instrument. Each manufacturer's equation, in terms of chemometric technique employed and wavelengths used, is unique to particular instrument model. Following manufacturers' instructions, check and adjust

Table 997.06A Interlaboratory study results for determination of protein in hard red winter wheat (whole grain analysis) by near-infrared spectroscopy [in percent protein (N × 5.7) at 12% moisture basis]: Tecator Infratec

Sample	No. of labs	No. of labs excluded	Reference value[a], % w/w	Mean value, % w/w	s_r[b], % w/w	s_R, % w/w	RSD_r[b], %	RSD_R, %	r[c], % w/w	R[d], % w/w
1	10	0	8.98	9.32	—	0.086	—	0.92	—	0.242
2	10	0	11.42	11.50	—	0.071	—	0.61	—	0.198
3	10	0	11.78	12.02	0.087	0.089	0.74	0.74	0.242	0.250
4	10	0	12.37	12.18	—	0.069	—	0.57	—	0.194
5	10	0	12.25	12.57	—	0.079	—	0.63	—	0.220
6	10	0	12.56	12.85	0.049	0.055	0.38	0.43	0.138	0.154
7	10	0	13.24	13.53	—	0.068	—	0.50	—	0.191
8	9	1	13.58	13.75	0.045	0.056	0.33	0.41	0.125	0.157
9	10	0	13.87	14.14	—	0.064	—	0.45	—	0.179
10	10	0	13.93	14.01	—	0.099	—	0.71	—	0.277
11	10	0	14.57	14.52	0.088	0.112	0.61	0.77	0.245	0.313
12	10	0	16.17	16.27	—	0.092	—	0.57	—	0.259

[a] By combustion nitrogen analysis.

[b] By Youden pair analyses on sample pairs 2–3 and 10–11 (listed on lines for samples 3 and 11, respectively), blind duplicate analyses for samples 6 and 8.

[c] $r = 2.8 \times s_r$.

[d] $R = 2.8 \times s_R$.

Table 997.06B Interlaboratory study results for determination of protein in hard red winter wheat (whole grain analysis) by near-infrared spectroscopy (in percent protein [N × 5.7] at 12% moisture basis): Foss Grainspec

Sample	No. of labs	No. of labs excluded	Reference value[a], % w/w	Mean value, % w/w	s_r[b], % w/w	s_R, % w/w	RSD_r[b], %	RSD_R, %	r[c], % w/w	R[d], % w/w
1	10	0	8.98	8.79	—	0.163	—	1.85	—	0.456
2	10	0	11.42	11.34	—	0.201	—	1.77	—	0.563
3	10	0	11.78	11.87	0.096	0.187	0.83	1.58	0.268	0.524
4	10	0	12.37	12.29	—	0.248	—	2.02	—	0.694
5	10	0	12.25	12.45	—	0.172	—	1.38	—	0.480
6	10	0	12.56	12.85	0.053	0.174	0.41	1.35	0.148	0.486
7	10	0	13.24	13.18	—	0.222	—	1.69	—	0.622
8	10	0	13.58	13.58	0.058	0.201	0.42	1.48	0.162	0.562
9	10	0	13.87	13.99	—	0.182	—	1.30	—	0.511
10	10	0	13.93	13.92	—	0.256	—	1.84	—	0.718
11	10	0	14.57	14.81	0.138	0.216	0.96	1.46	0.388	0.604
12	10	0	16.17	16.46	—	0.120	—	0.73	—	0.335

[a] By combustion nitrogen analysis.

[b] By Youden pair analyses on sample pairs 2–3 and 10–11 (listed on lines for samples 3 and 11, respectively), blind duplicate analyses for samples 6 and 8.

[c] $r = 2.8 \times s_r$.

[d] $R = 2.8 \times s_R$.

near-IR equations periodically, using well-characterized standardization samples that are representative of range in constituent concentration, commodity class, and climate conditions of commodity analyzed.

(**d**) *Additional apparatus*.—Necessary if performing reference protein analysis on standardization samples: (*1*) *Reference protein analyzer*.—Any instrument or device designed to measure nitrogen by combustion (*see* **992.23** [*see* 32.2.02]) or Kjeldahl (*see* **979.09** [*see* 32.2.03]) method. *See* **976.05B** and **C** (*see* 4.2.05) for specific analyzer and reagents. (*2*) *Mill*.—Udy Cyclone (Udy Corp., 201 Rome Ct, Ft. Collins, CO 80524, USA) mill equipped with 1 mm screen, or equivalent mill, for preparing test samples for moisture analysis. Allow mill to run at least 30 min before grinding to ensure stable operating temperature. Amount and feedrate should be ca 15 g

Table 997.06C Interlaboratoy study results for determination of protein in hard red winter wheat (whole grain analysis) by near-infrared spectroscopy (in percent protein [N × 5.7] at 12% moisture basis): Perten Inframatic 9100

Sample	No. of labs	No. of labs excluded	Reference value[a], % w/w	Mean value, % w/w	s_r[b], % w/w	s_R, % w/w	RSD_r[b], %	RSD_R, %	r[c], % w/w	R[d], % w/w
1	8	0	8.98	9.03	—	0.088	—	0.98	—	0.247
2	8	0	11.42	11.12	—	0.126	—	1.13	—	0.352
3	8	0	11.78	12.09	0.150	0.153	1.29	1.27	0.420	0.428
4	8	0	12.37	12.58	—	0.288	—	2.29	—	0.807
5	8	0	12.25	12.10	—	0.145	—	1.20	—	0.407
6	8	0	12.56	12.96	0.124	0.212	0.96	1.64	0.348	0.594
7	8	0	13.24	13.23	—	0.204	—	1.54	—	0.572
8	8	0	13.58	13.45	0.063	0.181	0.47	1.35	0.177	0.507
9	8	0	13.87	14.10	—	0.139	—	0.98	—	0.388
10	8	0	13.93	13.89	—	0.157	—	1.13	—	0.439
11	8	0	14.57	14.74	0.143	0.210	1.00	1.42	0.401	0.587
12	8	0	16.17	16.18	—	0.170	—	1.05	—	0.477

[a] By combustion nitrogen analysis.

[b] By Youden pair analyses on sample pairs 2–3 and 10–11 (listed on lines for samples 3 and 11, respectively), blind duplicate analyses for samples 6 and 8.

[c] $r = 2.8 \times s_r$.

[d] $R = 2.8 \times s_R$.

Table 997.06D Interlaboratory study results for determination of protein in hard red winter wheat (whole grain analysis) by near-infrared spectroscopy (in percent protein [N × 5.7] at 12% moisture basis): NIRSystems 6500/5000

Sample	No. of labs	No. of labs excluded	Reference value[a], % w/w	Mean value, % w/w	s_r[b], % w/w	s_R, % w/w	RSD_r[b], %	RSD_R, %	r[c], % w/w	R[d], % w/w
1	9	0	8.98	8.87	—	0.149	—	1.68	—	0.418
2	9	0	11.42	11.46	—	0.154	—	1.35	—	0.432
3	9	0	11.78	11.98	0.189	0.144	1.61	1.20	0.528	0.403
4	9	0	12.37	12.19	—	0.078	—	0.64	—	0.219
5	9	0	12.25	12.54	—	0.141	—	1.13	—	0.396
6	9	0	12.56	12.67	0.128	0.136	1.01	1.07	0.357	0.381
7	9	0	13.24	13.48	—	0.160	—	1.19	—	0.449
8	9	0	13.58	13.82	0.115	0.143	0.83	1.03	0.321	0.400
9	9	0	13.87	13.90	—	0.141	—	1.02	—	0.401
10	9	0	13.93	13.97	—	0.172	—	1.23	—	0.481
11	9	0	14.57	14.57	0.112	0.126	0.79	0.86	0.314	0.352
12	9	0	16.17	15.91	—	0.172	—	1.08	—	0.481

[a] By combustion nitrogen analysis.

[b] By Youden pair analyses on sample pairs 2–3 and 10–11 (listed on lines for samples 3 and 11, respectively), blind duplicate analyses for samples 6 and 8.

[c] $r = 2.8 \times s_r$.

[d] $R = 2.8 \times s_R$.

in 5 s. Run mill additional 30 s after grinding each test sample to ensure that product is clear of chamber. (*3*) *Drying oven.*—Convection oven maintaining 130 ± 1°C. Used for determining moisture in standardization samples, for reporting protein content on a fixed moisture basis.

C. Reference Protein Content in Standardization Samples

This procedure is necessary when reference protein contents are not furnished with standardization samples. If protein contents are furnished, values must be accurate to ±0.2% (±1σ) protein to ensure an accuracy for standardization set average to ±0.1% (±2σ) protein.

(**a**) *Moisture determination.*—From 15 g ground test sample, weigh two 2–3 g test portions, place in ca 55 mm diameter by 15 mm height aluminum dishes, and dry 1 h at 130°C. Cover dishes and cool in desiccator containing activated alumina, molecular sieves (type 4A or 4A X W), or equivalent desiccant. Weigh cooled portions and calculate percent moisture gravimetrically. Repeat if duplicate determinations differ by more than 0.2% moisture, wet basis (*wb*). Report moisture as average of duplicate determinations.

Seal unanalyzed test portions in glass vials for determination of reference protein content.

(**b**) *Reference protein content.*—Determine by combustion (*see* **992.23** [*see* 32.2.02]) or Kjeldahl (*see* **979.09** [*see* 32.2.03]) method. Adjust protein content (*pc*) to protein content at fixed moisture basis ($pc_{x\%\ moisture}$), typically 12% moisture *wb*, using moisture content (*mc*) from **C**(**a**) and equation:

$$pc_{x\%\ moisture} = \frac{100 - x}{100 - mc} \times pc$$

For combustion method (*see* **992.23** [*see* 32.2.02]), additional procedural information is as follows: (*1*) *Calibration of analyzer*.—Following instrument manufacturer's instructions, calibrate analyzer using (preferably) U.S. National Institute of Standards and Technology Standard Reference Material (NIST-SRM) 723b, 2-amino-2-(hydroxymethyl)-1,3-propanediol (commonly known as "Tris"; theoretical content = 11.55% elemental N) or (acceptably) EDTA (ACS grade, theoretical content = 9.59% elemental N). Accuracy is demonstrated by making successive determinations of Tris or EDTA. Tolerance for determinations using either compound is ±0.02% (± 2σ) N. (*2*) *Analysis of wheat reference material*—Perform combustion N analysis on each reference material in duplicate determinations (230 mg each) and calculate protein content (N × 5.7). Report average protein content if determinations differ by <0.15% protein content, otherwise reanalyze. If reanalyzing, report average protein content when new determinations differ by <0.15% protein content. If new determinations differ by >0.15% protein content, report average of all 4 determinations. For Kjeldahl method (*see* **979.09** [*see* 32.2.03]), additional procedural information is as follows: (*3*) *Check of procedure*.—Blanks consist of 1.00 g pure sucrose, analyzed by Kjeldahl procedure. Value for blank is subtracted from analyte value for determination of N in reference material. Reference standards consist of (*1*) 0.10 g lysine, HCL (theoretical content = 15.34% elemental N) plus 0.90 g sucrose and (*2*) 0.2 g $NH_4H_2PO_4$ (11.08% elemental N) plus 0.80 g sucrose. Purities and recoveries from Kjeldahl (percent of initial N) should be as follows: lysine–HCL (purity ≥98.5%), N recovery 94.5 ± 1.4% (±1σ); $NH_4H_2PO_4$ (purity ≥99.5%), N recovery ≥99.5 ± 0.45% (±1σ). (*4*) *Analysis of wheat reference material.*—Perform Kjeldahl analysis on each reference material in duplicate (0.9900–1.0000 g each). Reanalyze if duplicates differ by >0.15% protein content.

D. Maintenance of Near-IR Instrument

(**a**) *Start-up.*—Follow manufacturer's recommendations for instrument warm-up. Generally, instrument, including lamp, should run at least 1 h before analysis.

(**b**) *Diagnostic tests.*—Perform set of tests provided by manufacturer to ensure photometric reliability. This may consist of scanning

ceramic material referenced to itself and expressing the root mean square (RMS) or peak-to-peak noise in log(1/Reflectance) or log(1/Transmittance) units; and/or scanning a test sample, predicting concentration of constituent, and comparing prediction to reference value and to historical predictions of the test sample for detection of instrument drift. Scanning instruments typically have procedure to evaluate wavelength accuracy by comparing measured locations of sharp absorption bands within rare earth oxide (e.g., didymium, dysprosium oxide) or stable polymer (e.g., polystyrene) to known values.

E. Near-IR Determination

(**a**) *Tecator Infratec*.—Configure instrument with 18 mm path length test cell from instrumental display menu. Select protein content equation for hard red winter wheat (U.S. Department of Agriculture–Grain Inspection Packers and Stockyard Administration [USDA-GIPSA] identification No. HW032593, distributed by Foss North America, 7682 Executive Dr, Eden Prairie, MN 55344, USA). Pour each reference material (ca 600 g) into upper hopper of instrument. Ensure that temperature of grain is 15–27°C. Upon initializing scan, grain is automatically metered in 10 discrete batches into transmittance chamber. Transmission spectrum is collected, transformed to log(1/*T*), and stored to internal memory for each batch. When final batch is completed, average spectrum is calculated and stored to magnetic disk. Instead of saving spectrum, protein content equation may be immediately applied to spectrum in computer memory, and protein content displayed on screen. After standardization (*see* **F**), repeat procedure with test samples. Protein content is reported on a fixed moisture basis set by the manufacturer's calibration equation. (Tolerances for U.S. Official Analyses reported in 7CFR801.7.)

(**b**) *Foss Grainspec*.—Grainspec is kept on continuously; otherwise, minimum 2 h warm-up period is required. Configure instrument with 18 mm path length test cell. Verify that operating software is version 6.02 or higher. Grainspec must have calibration "Protein AACC HRWW 339741390" installed, with 'slope' of one and 'bias' of zero. Instrument standardization map must be set to use standard zero for commodity zero. Standardization factors for standard zero must be those supplied by manufacturer.

Upon starting software program, pour each reference material (ca 400 g) into upper hopper of instrument. Grainspec scans reference material in discrete batches, with each batch spectrum normalized (to minimize batch-to-batch packing density variation), then corrected to manufacturer's master instrument before average spectrum is stored to disk. After standardization (*see* **F**), repeat procedure with test samples.

(**c**) *Pertcon Inframatic*.—Let system warm up at least 45 min from power-up. Configure product settings for wheat to Subsamples = 31 and Jogsize 300 (standard factory settings). Upon starting software program, pour each reference material (ca 400 g) into upper hopper of instrument. Instrument scans 5 discrete batches and averages them. After standardization (*see* **F**), repeat procedure with test samples. Protein content is reported on a fixed moisture basis set by the manufacturer's calibration equation. (Tolerances for U.S. Offical Analyses reported in 7CFR801.7.)

(**d**) *NIRSystems 6500/5000*.—Use natural products cell [NPC; available from Infrasoft International (ISI) Co., RD #1, 109 Sellers Ln, Port Matilda, PA 16870, USA] inside bulk transport module. Configured with NPC, instrument should have been corrected for whole grain analysis to master instrument located at ISI Headquarters, thus allowing spectra to be transportable between instruments.

Warm up instrument a minimum 1 h with lamp on. Set control options as follows: "Cup fullness" = Full, "Reflectance/Transmission" = Reflectance; "Number of reference scans to average before sample" = 10; "Number of sample scans" = 25; "Number of reference scans to average after sample" = 0; and "Number of complete scans to average" = 2. Adjust motor speed of transport mechanism so that 25 scans are completed with 1 downward pass of NPC. If needed, adjust speed by turning potentiometer screw (ccw for faster) located at the bottom of circuit board that is near the left side of the lower chamber of bulk transport module. Perform instrument diagnostics: "Instrument response" (for setting gain of detector amplifier), "Repeatability" (for examining instrument noise), and "Wavelength accuracy." Apply any needed corrections as described in ISI manual.

Evenly pour ca 150 g each reference material in NPC, seal, and insert into transport module. Remove NPC, empty contents, refill with same material, and reanalyze. Log(1/*R*) spectrum is corrected to ISI master instrument and average spectrum from 2 fills is stored to disk. After standardization (*see* **F**), repeat procedure with test samples. Protein content is reported on a fixed moisture basis set by the manufacturer's calibration equation. (Tolerances for U.S. Offical Analyses reported in 7CFR801.7.)

F. Equation Standardization

Perform standardization with each set of analyses. Standardization depends on instrument model.

(**a**) *Tecator Infratec*.—Near-IR protein content is determined on reference materials by partial least squares (PLS) equation developed by USDA–GIPSA. Standardization bias, i.e., mean difference between near-IR and reference predictions of reference materials is incorporated into PLS equation before predictions are made on test samples.

(**b**) *Foss Grainspec*.—Procedure similar to (**a**) is applied to PLS equation (339741390) supplied by manufacturer.

(**c**) *Perten Inframatic*.—Skewness (slope) and offset (intercept) of near-IR predictions of reference materials are corrected by simple linear regression of reference values on near-IR-predicted values. Slope correction is applied to equation when *t*-test determines that slope of regression equation is significantly different ($P = 0.01$) from unity.

(**d**) *NIRSystems 6500/5000*.—Instead of bias or slope and intercept correction, PLS equation (supplied by ISI) is redeveloped during process known as "expansion" by including standardization set spectra with company's calibration set spectra (mathematically reconstructed from calibration equation file ISI130SR.EQA), thus forming an enlarged pool of reference materials for recalibration. Principal component analysis is performed on reference pool, whereupon materials with uniquely different principal component scores are placed in new calibration set, regardless of their origin. Nonunique materials are left out of new calibration set. Recalibration by PLS is performed on this new set, and the resultant equation is applied to test samples.

Model accuracy is characterized by: (*1*) bias, i.e., mean difference between predicted and reference protein contents; (*2*) RMSD, i.e., root mean square of differences between predicted and reference values; and (*3*) SEP, i.e., standard deviation of these differences.

Reference: *J. AOAC Int.* **81**, 587(1998).

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