FOOD COMPOSITION AND ADDITIVES

Protein Content of Wheat by Near-Infrared Spectroscopy of Whole Grain: Collaborative Study

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A collaborative study was performed to assess accuracy, repeatability, and reproducibility of a near-infrared (near-IR) method for determining crude protein content (PC) of whole-grain wheat. Four types of commercially available near-IR instruments, representing various combinations of wavelength region, mode of energy capture, method of energy dispersion, and treatment of spectral data, were used. Eight, 9, 10, and 11 collaborators were involved, the exact number depending on instrument type. All collaborators received 22 samples of whole-grain hard red winter (HRW) wheat. They were furnished reference PCs (i.e., protein concentrations, w/w) corrected to a 12% moisture basis for instrument standardization. AOAC Method 990.03—combustion analysis—was the reference procedure. Standardization consisted of performing one of the following treatments to the instrument manufacturer's (or federal agency's) PC equation: (*1*) bias correction, (*2*) slope and intercept correction, or (*3*) recalibration with inclusion of standardization sample spectra. Standardized equations were then applied to a test set of 12 unknown HRW wheat sample spectra, with 2 samples blindly duplicated. The PCs of test samples ranged from 9 to 16%. Near-IR predictions were compared with reference measurements. Averaged within instrument type, root mean square of differences were 0.22, 0.24, 0.25, and 0.26% PC, depending on instrument. Corrected for bias within the test set, standard errors became 0.22, 0.18, 0.21, and 0.24% PC, respectively. These values were approximately twice the estimated lower limit for error (representing sample inhomogeneity). Overall repeatability relative standard deviation (RSD_r) values were 0.92, 0.36, 0.42, and 0.74%, respectively. Overall reproducibility relative standard deviation (RSD_R) values were 1.15, 0.61, 1.53, and 1.38%. Such values for within-laboratory and between-laboratory variations of the near-IR methods were equivalent to values reported for the combustion method (990.03) for wheat. An in-house study that examined all 6 U.S. wheat classes with one of the 4 instrument types produced repeatability and reproducibility values similar to those of the collaborative study, suggesting that the near-IR technique may be applied to red, white, hard, soft, and durum wheats. The near-IR method for determination of PC of whole-grain wheat has been adopted First Action (997.06) by AOAC INTERNATIONAL.

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The recommendation was approved by the Methods Committe on Commodity Foods and Commodity Products and was adopted by the Official Methods Board of AOAC. *See* "Official Methods Board Actions" (1997) *J. AOAC Int.* **80,** 84A, and "Official Methods Board Actions," (1997) *Inside Laboratory Management*, August issue.

Protein content (PC) is extremely important in defining the functional properties of wheat, determining its suitability in various products such as pan bread, crackers, cakes, noodles, flat bread, and biscuits. Aside from wheat class, PC is often the most important factor in defining the price of a wheat lot. Official methods for determining wheat PC include combustion (**990.03**) and Kjeldahl analysis (**979.09**) (1).

In the past 20 years, near-infrared (near-IR) spectrophotometry has gained widespread use for PC determination for cereals. A

Table 1. Standardization samples

Sample	Cultivar	NIR hardness	Protein (at 12% moisture), %
1	Colby	29.1	8.511
2[a]	Jules	59.4	9.423
3	TAM200	47.6	8.924
4	Yuma	56.6	9.081
5	Vista	48.9	9.236
6	KS92PO263-137Exp	74.8	10.819
7	TAM200	52.5	11.646
8	Cimarron	64.4	11.552
9	Karl92	61.5	12.854
10	2163	63.8	12.772
11	KS92PO263-137Exp	74.4	13.032
12	TAM107	72.5	13.271
13	Tomahawk	66.2	13.883
14	TAM200	57.1	13.294
15	XH1529Exp	73.4	13.336
16	Laredo	71.8	13.674
17	Newton	67.5	14.305
18	TAM300	81.6	14.039
19	Arapahoe	74.1	14.414
20	Karl	55.4	14.981
21	Karl	65.9	15.147
22	Karl	66.7	16.446
Average	(n = 21)	63.1	12.629
Standard deviation	(n = 21)	12.0	2.223

[a] The reference protein content for standardization sample 2 (listed as 9.423%) was suspected to be not representative of the portion distributed to each collaborator. Therefore, this sample was removed from standardization procedures.

secondary method by nature, near-IR spectrophotometry relies upon either of the 2 aforementioned official methods for calibration and instrument standardization. However, it is often preferred to the reference methods because it is rapid (typically <2 min per sample), accurate, and cost effective; it does not require skilled operators; and it does not generate hazardous waste. Although an Approved Method of the American Association of Cereal Chemists (AACC) for PC measurement by near-IR (Method 39-11 in reference 2) exists along with a collaborative study (3), the method requires that the wheat be ground before near-IR scanning. Only in the past 5 years have a variety of commercial near-IR instruments been released for whole-grain analysis. Previous single-laboratory studies on wheat and barley (4), rapeseed (5), and rice (6) have demonstrated the feasibility of PC determination by whole-grain near-IR analysis. Recent collaborative reports on the use of near-IR analysis for agricultural products have dealt with moisture concentration in forages (7, 8), demonstrating that near-IR methods can achieve lower within-laboratory and equivalent between-laboratory variabilities compared with conventional methodology. The present report is the first published collaborative study on near-IR analysis of whole-grain wheat.

Near-IR instruments are supplied by several manufacturers and vary widely in method of radiation dispersion, wavelength range, presentation of radiation to sample, and chemometric equation used to predict constituent concentration. Although the optical and chemical principles of how radiation interacts with a substrate are universal, the uniqueness of each instrument model precludes the use of a common equation for all instrument models. For an official near-IR technique to have the breadth representative of that existing in commercial instruments, the collaborative near-IR study was designed to encompass a multitude of instrument designs, each tested by a sufficient number of collaborators.

Collaborative Study

Four models—possessing different combinations of reflectance versus transmittance, short wavelength (850–1050 nm) versus long wavelength (1100–2500 nm), and scanning versus filter—were selected as representative of commercial instrumentation. Twenty-two standardization hard red winter (HRW) wheat samples (400–600 g each) were sent to 10 collaborating laboratories for each instrument model. Reference PC values obtained by combustion (**990.03**) were furnished with standardization samples. Air oven moisture contents (2; AACC Method 44-15A) were determined in duplicate for each sample so that reference PCs were reported on a 12% moisture (wet) basis. An additional 14 HRW wheat samples, with 2 samples blindly duplicated, were sent without reference values to each collaborator. Samples were sealed in one-pint or one-quart

Table 2. Test samples

Sample	Cultivar	NIR hardness	Protein (at 12% moisture), %
1	XH1756Exp	62.7	8.975
2	7846	63.1	11.417
3	Karl92	54.7	11.779
4	TAM107	65.8	12.374
5	Pecos	66.2	12.251
6[a]	Karl	57.8	12.564
7	Larned	74.7	13.245
8[a]	Tomahawk	74.1	13.579
9	Ponderosa	64.5	13.873
10	Arkan	63.6	13.934
11	Discovery	53.4	14.568
12	2180	95.0	16.166
Average	(n = 12)	66.3	12.894
Std. dev.	(n = 12)	11.1	1.802

[a] Test samples 6 and 8 were each blindly duplicated.

Table 3. Homogeneity within test samples based on protein by combustion[a]

Sample	Ground portion of Associate Referee's sample	Portion of same grind from which the reference analysis was performed	Difference
1	9.134	9.021	0.113
2	11.491	11.329	0.162
3	11.862	11.704	0.158
4	12.504	12.456	0.047
5	12.512	12.296	0.216
6	12.884	12.559	0.325
7	13.360	13.211	0.149
8	13.722	13.538	0.185
9	14.144	13.993	0.151
10	14.124	13.998	0.126
11	14.785	14.562	0.222
12	16.259	15.824	0.436
Average	13.065	12.874	0.191
Std. dev.	1.809	1.753	0.102

[a] Protein analyses was performed with a combustion analyzer at the Associate Referee's laboratory. Model of analyzer was the same as that used to perform reference analyses.

glass jars, with the jar size depending on the amount of material required by the instrument. Collaborators were instructed to keep the jars sealed at room temperature until the time of scanning. Although collaborators were advised to scan samples within 1 month of receipt (and most complied), samples were sufficiently stable to permit indefinite storage without degradation until the eventual time of scanning (no greater than 4 months for any collaborator).

PCs ranged from 8.511 to 16.446% for standardization samples and from 8.975 to 16.166% for test samples (Table 1). Within an instrument type, each collaborator's standardization samples were uniquely randomized and then sequentially numbered from *xx*01 to *xx*22, where *xx* is the collaborator number (11 to 20). Test sample numbers, *xx*23 to *xx*36, were similarly assigned. Collaborators were instructed to scan all standardization samples in order before scanning test samples. All 36 samples were to be scanned on the same day.

997.06, Protein (Crude) in Wheat, Whole Grain Analysis, Near-Infrared Spectroscopic Method

First Action 1997

[Applicable to wheat containing 9.0–16.2% protein (12% moisture basis).]

Method Performance:

[values are in percent protein (N × 5.7) at 12% moisture basis]:

Tecator Infratec: s_r = 0.047; s_R = 0.079; RSD_r = 0.36%; RSD_R = 0.61%

Bias = 0.162; RMSD = 0.236; SEP = 0.178

Foss Grainspec: s_r = 0.055; s_R = 0.198; RSD_r = 0.42%; RSD_R = 1.53%

Bias = 0.058; RMSD = 0.247; SEP = 0.206

Perten Inframatic: s_r = 0.098; s_R = 0.179; RSD_r = 0.74%; RSD_R = 1.38%

Bias = 0.058; RMSD = 0.260; SEP = 0.244

NIRSystems 6500/5000: s_r = 0.121; s_R = 0.149; RSD_r = 0.92%; RSD_R = 1.15%

Bias = 0.047; RMSD = 0.220; SEP = 0.218

Note: Repeatability and reproducibility values reflect near-infrared method alone, as accomplished by using 1 laboratory for reference (combustion) protein determination, thus avoiding the confounding of near-infrared (near-IR) and reference method variabilities. Hence, reported repeatability and reproducibility values are lower than those to be expected if each laboratory had performed both reference and near-IR determinations.

A. Principle

By means of linear chemometric algorithms, near-IR transmittance or diffuse reflectance spectra are used as the basis for determining the crude protein content (PC) of bulk wheat. Combination and overtone frequencies of NH, CH, and OH, which are due to proteins, carbohydrates, and water, are of sufficiently high magnitude in the 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 and using a minimum of 20 wheat samples (termed standardization samples) of known PC before analysis of unknown samples. Range in protein of standardization samples (preferably uniformly distributed) must be equivalent or broader than that expected for unknown samples.

B. Apparatus

(**a**) *Near-IR spectrophotometers.*—Use one of the following or equivalent: *(1) Tecator Infratec 1221, 1225, or 1226.*—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; dy-

Table 4. Standardization bias, slope, and intercept values used to correct raw protein readings from 3 instrument models[a]

Laboratory (grouped by instrument[b])	Standardization bias[c]	Slope[c]	Intercept[c]
	Tecator Infratec		
A	−0.597	—	—
B	−0.346	—	—
C	−0.403	—	—
D	−0.325	—	—
E	−0.359	—	—
F	−0.415	—	—
G	−0.365	—	—
H	−0.318	—	—
I	−0.297	—	—
J	−0.319	—	—
	Foss Grainspec		
A	−0.101	—	—
B	0.082	—	—
C	−0.226	—	—
D	0.243	—	—
E	0.018	—[d]	—
F	−0.455	—	—
G	1.254	—	—
H	−0.467	—[d]	—
I	−0.614	—	—
J	−0.611	—	—
K	0.257	—	—
	Perten Inframatic		
A	—	1.253	−1.552
B	—	1.200	−14.947
C	−0.012	0.999[e]	0.024
D	−0.127	1.025[e]	−0.186
E	—	1.173	−1.819
F	—	1.247	−5.728
G	—	1.247	−6.197
H	—	1.234	−0.722

[a] Values are based on analysis of 21 standardization samples. Equations subsequently applied to test samples are as follows: $protein_{corrected} = protein_{raw}$ − standardization bias, applied to Tecator Infratec and Foss Grainspec; $protein_{corrected}$ = intercept + slope × $protein_{raw}$, applied to Perten Inframatic. Values for intercept, slope, and standardization bias appear in table. Dashes indicate unneeded information.

[b] Only the instruments that were slope- and intercept- or bias-corrected are listed. The correction procedure for the NIRSystems instrument was a principal component expansion, as explained in text.

[c] Intercept and slope were determined from a simple linear regression of reference protein contents on raw near-IR protein contents of the standardization samples. Standardization bias was the average raw near-IR and reference protein contents minus the average reference protein content of the standardization samples.

[d] Slope was significantly different from unity, although a slope correction was not applied to test samples.

[e] Nonsignificant difference from unity slope, therefore, only a bias correction (i.e., first equation in footnote *a*) was applied.

namic response, 5 optical density (OD); scan range, 850–1050 nm; wavelength resolution, 2 nm; bandpass [full width at half height (FWHH)], 6 nm. *(2) Foss Grainspec.*—Available from Foss Electric Development, Millfield Lane Industrial Estates, Wheldrake, York YO46NA, United Kingdom. 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 and range 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) Perten Inframatic 9100.*—Available from Perten Instruments, 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 OD; number of interference filters, 12; wavelength range, 1077–1372 nm; bandpass (FWHH) for filters, 12 nm; wavelength accuracy, ± 2 nm; and root mean square (RMS) noise, $<1 \times 10^{-5}$ OD. *(4) NIRSystems 6500 or 5000.*—Available from Foss NIRSystems, Inc., 12101 Tech Rd, Silver Spring, MD 20904. 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 OD; 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, $<2 \times 10^{-5}$ OD.

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

(**c**) *Software.*—Wheat PC equation at fixed moisture basis supplied with each near-IR instrument. Each manufacturer's equation, in terms of chemometric technique and wavelengths used, is unique to each particular instrument model. Per manufacturers' instructions, check and adjust near-IR equations periodically, using well-characterized samples that are representative (i.e., range in constituent concentration, commodity class, 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* **990.03** [*see* 4.2.08]) 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 (Ft. Collins, CO) mill with 1 mm screen, or equivalent mill, for preparing samples for moisture analysis. Allow mill to run at least 30 min before grinding to ensure stable operating temperature. Amount and feed rate should be ca 15 g in 5 s. Run mill additional 30 s after grinding each sample to ensure that sample is clear of chamber. *(3) Drying oven.*—Convection oven capable of maintaining 130° ± 1°C. Used for determining moisture in standardization samples so that PC may be reported on a fixed-moisture basis.

Table 5. Tecator Infratec raw data: test set results in % protein [adjusted to 12% moisture (wet basis)]

	Sample													Model statistics		
Laboratory	1	2	3	4	5	6		7	8		9	10	11	12	Bias	RMSD	SEP
A	8.58[a]	10.95	11.48	11.54	12.00	12.23[a]	12.20[a]	13.00	13.20	13.19	13.65	13.41	14.01	15.90	−0.398	0.432	0.177
B	8.93	11.25	11.59	11.81	12.09	12.48	12.53	13.15	13.40	13.34	13.75	13.58	14.04	15.89	−0.230	0.282	0.170
C	8.84	11.02	11.58	11.89	12.06	12.39	12.36	13.15	13.33	13.31	13.84	13.41	14.07	15.92	−0.269	0.312	0.167
D	9.02	11.08	11.74	11.90	12.18	12.49	12.55	13.20	13.45	13.48	13.75	13.75	14.18	15.87	−0.176	0.234	0.161
E	8.98	11.12	11.56	11.81	12.26	12.48	12.55	13.00	13.38	13.41	13.77	13.67	14.13	15.93	−0.220	0.272	0.168
F	9.01	11.13	11.55	11.69	12.27	12.38	12.55	13.16	13.31	13.27	13.70	13.59	14.34	15.80	−0.233	0.296	0.191
G	8.97	11.14	11.80	11.82	12.27	12.52	12.57	13.19	13.28	13.39	13.74	13.78	14.28	15.87	−0.172	0.240	0.175
H	9.11	11.25	11.59	11.97	12.27	12.56	12.58	13.22	13.48	13.53	13.78	13.74	14.12	15.97	−0.139	0.212	0.168
I	8.94	11.24	11.83	11.86	12.25	12.55	12.54	13.28	13.54	13.43	13.88	13.83	14.22	15.95	−0.113	0.200	0.173
J	9.09	11.08	11.69	11.77	12.28	12.52	12.44	13.17	13.63[b]	13.32	13.76	13.63	14.12	15.85	−0.178	0.276	0.221
n[c]	9	10	10	10	10	9		10	9		10	10	10	10	10	10	10
Outliers[d]	1	0	0	0	0	1		0	1		0	0	0	0	0	0	0
Mean[e]	8.988	11.126	11.641	11.806	12.193	12.502		13.152	13.373		13.762	13.639	14.151	15.895	−0.213	0.283	0.178
Ref.[f]	8.975	11.417	11.779	12.374	12.251	12.564		13.245	13.579		13.873	13.934	14.568	16.166	—	—	—
s_r[g]	—	—	—	—	—	0.053		—	0.044		—	—	—	—	—	—	—
RSD_r[h]	—	—	—	—	—	0.42		—	0.33		—	—	—	—	—	—	—
r[i]	—	—	—	—	—	0.148		—	0.122		—	—	—	—	—	—	—
s_R[j]	0.083	0.100	0.117	0.121	0.105	0.069		0.089	0.106		0.065	0.146	0.105	0.050	—	—	—
RSD_R[k]	0.92	0.90	1.00	1.02	0.86	0.55		0.68	0.79		0.47	1.07	0.74	0.32	—	—	—
R[l]	0.232	0.281	0.327	0.338	0.293	0.193		0.249	0.296		0.181	0.408	0.294	0.141	—	—	—

[a] Reproducibility test outlier by single-value Grubb's test (2-tail, $p = 0.025$). With laboratory A, sample 1 included ($n = 10$), the statistics are as follows: mean = 8.947, $s_R = 0.151$, $RSD_R = 1.68$, R = 0.422. With laboratory A, blind duplicate sample 6 included ($n = 10$), the statistics are as follows: mean = 12.474, $s_r = 0.050$, $RSD_r = 0.41$, r = 0.142, $s_R = 0.112$, $RSD_R = 0.90$, R = 0.313.

[b] Repeatability test outlier by Cochran test (1-tail, $p = 0.025$). With laboratory J, blind duplicate sample 8 included ($n = 10$), the statistics are as follows: mean = 13.384, $s_r = 0.081$, $RSD_r = 0.60$, r = 0.226, $s_R = 0.116$, $RSD_R = 0.86$, R = 0.324.

[c] Number of laboratories retained after removal of outliers.

[d] Number of outlying laboratories removed.

[e] Arithmetic average of results of n laboratories.

[f] Reference protein value (12% moisture) by combustion (N × 5.7).

[g] Repeatability standard deviation.

[h] 100 × repeatability standard deviation/mean (dimensionless).

[i] Repeatability value ($r = 2.8 \times s_r$).

[j] Reproducibility standard deviation.

[k] 100 × reproducibility standard deviation/mean (dimensionless).

[l] Reproducibility value ($R = 2.8 \times s_R$).

C. Reference Protein Content in Standardization Samples

This procedure is necessary when reference PCs are not furnished with standardization samples. If PCs 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 sample, weigh two 2–3 g portions, place in ca 55 mm id × 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 portions in glass vials for determination of reference PC.

(**b**) *Reference protein content.*—Determine by combustion (*see* **990.03** [*see* 4.2.08]) or Kjeldahl (*see* **979.09** [*see* 32.2.03]) method. Adjust PC to PC at fixed moisture basis ($PC_{x\%\ moisture}$), typically x = 12% moisture WB, using moisture content (MC) from *C*(**a**) and the following equation:

$$PC_{x\%\ moisture} = [(100 - x) / (100 - MC)] \times PC$$

For combustion method (*see* **990.03** [*see* 4.2.08]), additional procedural information is as follows: *(1) Calibration of analyzer.*—Per 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 nitrogen) or (acceptably) EDTA (ACS-grade, theoretical content = 9.59% elemental nitrogen). Accuracy is demonstrated by making successive determinations of Tris or EDTA. Tolerance for determinations using either compound shall be ± 0.02% (± 2σ) nitrogen. *(2) Analysis of sample.*—Perform combustion nitrogen analysis on each standardization sample in duplicate successive determinations (230 mg each) and calculate PC (N × 5.7). Report average PC if determinations differ by <0.15% PC, otherwise reanalyze. If reanalyzing, report average PC when new determinations differ by <0.15% PC. If new determinations differ by >0.15% PC, 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 per Kjeldahl procedure. Value for blank is subtracted from sample value for determination of nitrogen in sample. Chemical reference standards consist of 0.10 g lysine-HCl (theoretical content = 15.34% elemental nitrogen) plus 0.90 g sucrose and 0.2 g ammonium dihydrogen phosphate (11.08% elemental nitrogen) plus 0.80 g sucrose. Purities and recoveries from Kjeldahl (% of initial N) should be as follows: lysine-HCl (purity ≥ 98.5%), N recovery ≥ 94.5 ± 1.4% (± 1σ); ammonium dihydrogen phosphate (purity ≥99.5%), N recovery ≥99.5 ± 0.45% (± 1σ). *(4) Analysis of sample.*—Perform Kjeldahl analysis on each standardization sample in duplicate (0.9900–1.0000 g each). Reanalyze if duplicates differ by >0.15% PC.

D. Maintenance of Near-IR Instrument

(**a**) *Startup.*—Follow manufacturer's recommendations for instrument warmup. Generally, it is recommended that instrument, including lamp, is running for 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 RMS or peak-to-peak noise in log(1/*R*) or log(1/*T*) units and/or scanning sample, predicting concentration of constituent, and comparing prediction to reference value and to historical predictions of sample for detection of instrument drift. Scanning instruments typically have a 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. Determination by Near-IR Analysis

(**a**) *Tecator Infratec.*—Configure instrument with 18 mm path length sample cell. Select PC equation for HRW wheat (U.S. Department of Agriculture, Grain Inspection Packers and Stockyard Administration [USDA-GIPSA], identification No. HW032593.) Pour each standardization sample (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 in internal memory for each batch. When final batch is completed, average spectrum is calculated and stored on magnetic disk. Instead of saving spectrum, PC equation may be immediately applied to spectrum in computer memory and the prediction reported on the computer screen. Upon standardization (*see F*), repeat procedure for test samples.

(**b**) *Foss Grainspec.*—It is recommended that Grainspec is turned on continuously. Otherwise a minimum 2 h warmup is required. Configure instrument with 18 mm path length sample cell. Verify that operating software is version 6.02 or higher. Grainspec must have calibration "Protein AACC HRWW 339741390" installed, with slope of 1 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 standardization sample (ca 400 g) into upper hopper of instrument. Grainspec scans sample in discrete batches, with each batch spectrum normalized (to minimize batch-to-batch packing density variation) and then corrected to manufacturer's master instrument before average spectrum is stored in disk. Upon standardization (*see F*), repeat procedure for test samples.

(**c**) *Perten Inframatic.*—Allow system to warm up at least 45 min from powerup. Configure product settings for wheat to Subsamples = 31 and Jogsize 300 (standard factory settings). Upon starting software program, pour each standardization sample (ca 400 g) into upper hopper of instrument. Instrument

Table 6. Tecator Infratec, with bias correction from standardization set: test set results in % protein [adjusted to 12% moisture (wet basis)]

	Sample													Model statistics		
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12		Bias	RMSD	SEP
A	9.18	11.55	12.08	12.14	12.60	12.83	12.80	13.60	13.80	13.79	14.25	14.01	14.61	16.50	0.199	0.261	0.177
B	9.28	11.60	11.94	12.16	12.44	12.83	12.88	13.50	13.75	13.69	14.10	13.93	14.39	16.24	0.116	0.199	0.170
C	9.24	11.42	11.98	12.29	12.46	12.79	12.76	13.55	13.73	13.71	14.24	13.81	14.47	16.32	0.134	0.208	0.167
D	9.34	11.40	12.06	12.22	12.50	12.82	12.88	13.52	13.78	13.80	14.08	14.08	14.50	16.20	0.149	0.214	0.161
E	9.34	11.48	11.92	12.17	12.62	12.84	12.91	13.36	13.74	13.77	14.13	14.03	14.49	16.29	0.139	0.213	0.168
F	9.42	11.54	11.96	12.10	12.68	12.80	12.96	13.58	13.72	13.68	14.12	14.00	14.76	16.22	0.182	0.258	0.191
G	9.34	11.50	12.16	12.18	12.64	12.88	12.94	13.56	13.64	13.76	14.10	14.14	14.64	16.24	0.193	0.255	0.175
H	9.43	11.57	11.91	12.29	12.59	12.88	12.90	13.54	13.80	13.85	14.10	14.06	14.44	16.29	0.179	0.240	0.168
I	9.24	11.54	12.13	12.16	12.55	12.85	12.84	13.58	13.84	13.73	14.18	14.13	14.52	16.25	0.184	0.247	0.173
J	9.41	11.40	12.01	12.09	12.60	12.84	12.76	13.49	13.95[a]	13.64	14.08	13.95	14.44	16.17	0.141	0.254	0.221
n[b]	10	10	10	10	10	10	10	9	10	10	10	10		10	10	10
Outliers[c]	0	0	0	0	0	0	0	1	0	0	0	0		0	0	0
Mean[d]	9.321	11.500	12.015	12.180	12.567	12.848	13.526	13.754	14.136	14.013	14.525	16.269		0.162	0.236	0.178
Ref.[e]	8.975	11.417	11.779	12.374	12.251	12.564	13.245	13.579	13.873	13.934	14.568	16.166		—	—	—
s_r[f]	—	—	—	—	—	0.049	—	0.045	—	—	—	—		—	—	—
RSD_r[g]	—	—	—	—	—	0.38	—	0.33	—	—	—	—		—	—	—
r[h]	—	—	—	—	—	0.138	—	0.125	—	—	—	—		—	—	—
s_R[i]	0.086	0.071	0.089	0.069	0.079	0.055	0.068	0.056	0.064	0.099	0.112	0.092		—	—	—
RSD_R[j]	0.92	0.61	0.74	0.57	0.63	0.43	0.50	0.41	0.45	0.71	0.77	0.57		—	—	—
R[k]	0.242	0.198	0.250	0.194	0.220	0.154	0.191	0.157	0.179	0.277	0.313	0.259		—	—	—

[a] Repeatability test outlier by Cochran test (1-tail, $p = 0.025$). With laboratory J, blind duplicate sample 8 included ($n = 10$), the statistics are as follows: mean = 13.758, $s_r = 0.081$, $RSD_r = 0.59$, r = 0.228, $s_R = 0.081$, $RSD_R = 0.59$, R = 0.228.
[b] Number of laboratories retained after removal of outliers.
[c] Number of outlying laboratories removed.
[d] Arithmetic average of results of n laboratories.
[e] Reference protein value (12% moisture) by combustion (N × 5.7).
[f] Repeatability standard deviation.
[g] 100 × repeatability standard deviation/mean (dimensionless).
[h] Repeatability value ($r = 2.8 \times s_r$).
[i] Reproducibility standard deviation.
[j] 100 × reproducibility standard deviation/mean (dimensionless).
[k] Reproducibility value ($R = 2.8 \times s_R$).

scans 5 discrete batches of sample and then performs averaging. Upon standardization (*see F*), repeat procedure for test samples.

(**d**) *NIRSystems 6500/5000.*—Use natural products cell (NPC; available from Infrasoft International (ISI) Co., Port Matilda, PA) 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 (counterclockwise for faster) located at the bottom of the 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 of each standardization sample 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 on disk. Upon standardization (*see F*), repeat procedure for test samples.

F. Equation Standardization

Perform standardization on each set of data. Standardization depends on instrument model.

(**a**) *Tecator Infratec.*—Near-IR PC is determined on standardization samples by partial least squares (PLS) equation developed by USDA-GIPSA. Standardization bias (i.e., mean difference between near-IR and reference predictions of standardization samples) 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 standardization samples 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 samples for recalibration. Principal component analysis is performed on sample pool, whereupon samples with uniquely different principal component scores are placed in new calibration set, regardless of their initial origin. Nonunique samples 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 bias (mean difference between predicted and reference protein contents) RMSD (root mean square of differences between predicted and reference values) and SEP (standard deviation of these differences).

G. Sample Homogeneity

This study was not performed by collaborators. To estimate homogeneity of each test sample, combustion analyses (*see* **990.03** [*see* 4.2.08]) were performed on material from 2 sources: (*1*) vials returned from reference protein analysis and (*2*) ground portions of Associate Referee's collaborative test samples. A combustion nitrogen analyzer (Leco Corp., St. Joseph, MI; model No. FP-428) determined PCs of samples from both sources within a 24 h period. Moisture content analyses, similar to that described in *C*, were simultaneously performed on the noncombusted portion of samples from the second source. Thus, PCs for each source were recorded on a 12% moisture basis. A disparity in a sample's PC between the 2 sources served as the best estimate of that sample's homogeneity. Large disparities would be indicative of subsampling errors stemming from inadequate mixing of the 12–49 kg/sample parent material prior to subdivision into 40 portions, one of which became the Associate Referee's set.

H. Extension to Other Wheat Classes

This study was not performed by collaborators. To demonstrate that the near-IR method applied to more than just HRW wheat (the largest of the 6 market classes in the United States), reproducibility and repeatability statistics were determined on the other U.S. wheat classes, based on data collected at USDA-GIPSA, Technical Services Division's laboratory in Kansas City, MO. For each class of U.S. wheat (HRW = hard red winter, HRS = hard red spring, SRW = soft red winter, DUR = durum, HWW = hard white, SWW = soft white), 5 check samples whose PCs span the typical range for the class, were analyzed on each of 2 Tecator Infratec instruments during a 2-month period. The number of analyses per wheat class depended on the frequency of need for analysis of unknown samples from the respective class. For each day that a sample from a particular class needed analysis, the 5 check samples from that respective class were analyzed in duplicate on each instrument. Daily readings were pooled, yielding the total number of replicate analyses per check sample and instrument: HRW = 42, HRS = 42, SRW = 12, DUR = 34, HWW = 8, SWW = 22.

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

Results

Cultivar, hardness, and reference PCs of standardization and test samples are listed in Tables 1 and 2, respectively. Each tabulated PC is the average of 6 determinations (230 mg/determination) from 2 combustion nitrogen analyzers (i.e., triplicate determinations from each of 2 Leco FP-428 analyzers housed at GIPSA, Kansas City, MO, except for standardization sample 12, for which 5 determinations were made). Samples 6 and

Table 7. Foss Grainspec, with bias correction from standardization set: test set results in % protein [adjusted to 12% moisture (wet basis)]

	Sample													Model statistics		
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12		Bias	RMSD	SEP
A	8.8	11.1	11.8	12.2	12.2	12.7	12.7	13.0	13.4	13.4	13.8	13.8	14.8	16.2	−0.051	0.162	0.160
B	8.69	11.42	11.98	12.30	12.54	12.92	13.00	13.36	13.84	13.82	14.20	14.06	14.98	16.56	0.179	0.274	0.217
C	9.03	11.24	11.82	12.12	12.34	12.80	12.66	13.00	13.24	13.30	13.78	13.72	14.58	16.19	−0.074	0.181	0.172
D	8.85	11.56	12.07	12.63	12.35	12.93	12.93	13.13	13.44	13.46	13.87	13.70	14.86	16.36	0.082	0.211	0.203
E	8.62	11.17	11.55	11.97	12.30	12.75	12.70	13.00	13.51	13.61	13.84	13.79	14.50	16.41	−0.109	0.222	0.202
F	8.95	11.78	12.21	12.78	12.86	13.29	13.22	13.54	13.87	13.85	14.34	14.36	15.26	16.44	0.413	0.457	0.204
G	8.78	11.43	11.71	12.32	12.39	12.85	12.88	13.27	13.64	13.71	13.92	14.18	14.83	16.48	0.091	0.179	0.161
H	8.49	11.18	11.78	12.12	12.58	12.80	12.78	13.22	13.84	13.74	14.02	14.18	14.86	16.54	0.074	0.272	0.273
I	8.92	11.20	11.92	12.12	12.47	12.71	12.63	13.35	13.53	13.36	13.84	13.84	14.64	16.54	0.028	0.177	0.182
J	8.77	11.21	11.90	12.33	12.29	12.81	12.72	13.12	13.51	13.47	13.95	13.74	14.79	16.47	0.017	0.176	0.182
K	8.79	11.25	11.78	12.21	12.40	12.76	12.84	12.76	13.50	13.42	14.13	13.60	14.81	16.58	−0.010	0.258	0.269
n^a	10	10	10	10	10	10	10	10	10	10	10	10	10	10	11	11	11
Outliers[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean[c]	8.787	11.345	11.873	12.291	12.452	12.850	13.175	13.584	13.991	13.918	14.811	16.457			0.058	0.247	0.206
Ref.[d]	8.975	11.417	11.779	12.374	12.251	12.564	13.245	13.579	13.873	13.934	14.568	16.166			—	—	—
s_r[e]	—	—	—	—	—	0.053	—	0.058	—	—	—	—			—	—	—
RSD_r[f]	—	—	—	—	—	0.41	—	0.42	—	—	—	—			—	—	—
r[g]	—	—	—	—	—	0.148	—	0.162	—	—	—	—			—	—	—
s_R[h]	0.163	0.201	0.187	0.248	0.172	0.174	0.222	0.201	0.182	0.256	0.216	0.120			—	—	—
RSD_R[i]	1.85	1.77	1.58	2.02	1.38	1.35	1.69	1.48	1.30	1.84	1.46	0.73			—	—	—
R[j]	0.456	0.563	0.524	0.694	0.480	0.486	0.622	0.562	0.511	0.718	0.604	0.335			—	—	—
n^k	11	11	11	11	11	11	11	11	11	11	11	11			—	—	—
Outliers	0	0	0	0	0	1[l]	0	0	0	0	0	0			—	—	—
Mean	8.788	11.326	11.869	12.286	12.432	12.797	13.162	13.570	13.976	13.910	14.813	16.434			—	—	—
s_r	—	—	—	—	—	0.050	—	0.055	—	—	—	—			—	—	—
RSD_r	—	—	—	—	—	0.39	—	0.41	—	—	—	—			—	—	—
r	—	—	—	—	—	0.141	—	0.154	—	—	—	—			—	—	—

Table 7. (*Continued*)

Laboratory	Sample												Model statistics		
	1	2	3	4	5	6	7	8	9	10	11	12	Bias	RMSD	SEP
s_R	0.155	0.201	0.178	0.236	0.176	0.104	0.215	0.198	0.180	0.245	0.205	0.137	—	—	—
RSD_R	1.76	1.78	1.50	1.92	1.42	0.81	1.64	1.46	1.28	1.76	1.38	0.84	—	—	—
R	0.433	0.564	0.499	0.660	0.493	0.290	0.603	0.556	0.503	0.685	0.574	0.384	—	—	—

[a] For the *n* line and the next 9 lines thereafter (but not pertaining to the 3 rightmost columns), values from laboratory A are not included in repeatability and reproducibility statistics because values were reported to only 1 decimal place. *n* = Number of laboratories retained after removal of outliers.
[b] Number of outlying laboratories removed.
[c] Arithmetic average of results of *n* laboratories.
[d] Reference protein value (12% moisture) by combustion (N × 5.7).
[e] Repeatability standard deviation.
[f] 100 × repeatability standard deviation/mean (dimensionless).
[g] Repeatability value ($r = 2.8 \times s_r$).
[h] Reproducibility standard deviation.
[i] 100 × reproducibility standard deviation/mean (dimensionless).
[j] Reproducibility value ($R = 2.8 \times s_R$).
[k] For the *n* and all following lines in the table, values from laboratory A are included in repeatability and reproducibility statistics.
[l] Laboratory F was a reproducibility test outlier by single-value Grubb's test (2-tail, $p = 0.025$). With laboratory F included ($n = 11$), the statistics are as follows: mean = 12.839, $s_r = 0.050$, $RSD_r = 0.39$, r = 0.141, $s_R = 0.171$, $RSD_R = 1.33$, R = 0.478.

8 of the test set (Table 2) were each blindly duplicated for calculation of repeatability. Hardness, an indicator of milling and end-use characteristics, was measured in accordance with AACC Method 39-70A (2). Because of a presumed sampling error in standardization of sample 2, this sample was removed from all analyses. Consequently, equation standardizations were redone by the Associate Referee for all instruments to reflect the smaller, but presumably more accurate, standardization set. The mean PC of the standardization set ($n = 21$) was similar to that of the test set mean ($n = 12$; 12.629 versus 12.894%), although the standard deviation (2.223 versus 1.802%) was greater for standardization samples. For measurement of repeatability of the combustion nitrogen analyzer readings, a 2-way analysis of variance (ANOVA) of PC was performed, with sample and combustion nitrogen analyzer as main effects. With PC from an analyzer determination as the random variable, overall repeatabilities (i.e., square root of the residual mean square) were 0.046 and 0.048% PC for standardization and test sets, respectively.

Homogeneity results are summarized in Table 3. Generally, each sample was fairly homogenous (range in differences = 0.047 to 0.436% PC), demonstrating an average difference of 0.191% PC between the portion on which reference values were based and the Associate Referee's collaborative portion. Therefore, the standard deviation of 0.102% PC represents the best estimate of the lowest possible SEP achievable on a collaborative test set, assuming a perfect near-IR model.

The collaborators were asked to write comments regarding the procedure on a separate sheet accompanying each set of samples. Of the comments received (from approximately half of the collaborators), most were remarks about loose jar lids or cracked jars. In the case of the later, replacement samples were sent immediately to the collaborators. One collaborator using Foss Grainspec (laboratory G) noted that test samples were scanned one day after standardization samples, with the second day at higher temperature and humidity than the first. A spot check of that collaborator's PC values and the subsequent Grubb's outlier tests indicated that the values were not affected by the 1 day delay. Two collaborators using Perten Inframatic instruments (laboratories D and E) indicated that some samples were not large enough for 1-pass analysis, thus requiring a small portion of the seed in the collection bin to be returned to the top hopper. Again, this departure from prescribed operations did not appear to affect PC readings.

Bias, slope, and intercept corrections to the equations for Tecator Infratec, Foss Grainspec, and Perten Inframatic are listed for each collaborator in Table 4. Correction for non-unity slope was not performed on the Infratec data; rather, only a bias correction was made. This was in accordance with GIPSA procedures, because all Infratec collaborators were GIPSA facilities, and as such, slope correction is periodically performed on these instruments with a separate set of internal samples. A *t*-test for non-unity slope of each of the Infratec collaborators' standardization samples confirmed that slope correction was not needed. In compliance with manufacturer's recommendation, the Grainspec equation was also bias- but not slope-corrected, despite a slope significantly ($p = 0.05$) different from

Table 8. Perten Inframatic 9100, with slope and bias correction from standardization set: test set results in % protein [adjusted to 12% moisture (wet basis)]

	Sample													Model statistics		
Laboratory	1	2	3	4	5	6		7	8		9	10	11	12	Bias	RMSD	SEP
A	9.05	11.06	11.87	12.39	11.97	12.90	12.96	13.17	13.37	13.25	14.05	13.65	14.67	16.14	−0.036	0.205	0.211
B	9.04	10.99	12.02	12.38	11.90	12.70	12.89	13.06	13.24	13.31	13.98	13.81	14.44	16.23	−0.078	0.222	0.217
C	9.12	11.17	11.96	12.26	12.16	12.63	12.81	13.30	13.39	13.32	14.09	13.97	14.54	16.21	0.008	0.139	0.144
D	9.00	11.16	12.13	12.44	12.18	12.86	13.12	13.44	13.45	13.47	14.06	13.86	14.57	16.09	0.041	0.179	0.182
E	8.84	10.94	12.25	12.88	11.95	12.96	12.66	12.91	13.25	13.36	13.91	13.80	14.89	15.98	−0.014	0.335	0.349
F	9.00	11.08	12.29	13.11	12.10	13.22	13.32	13.18	13.56	13.65	14.11	13.98	14.87	16.28	0.172	0.358	0.328
G	9.03	11.28	12.21	12.50	12.31	12.92	12.99	13.25	13.50	13.62	14.35	13.90	14.97	16.50	0.168	0.270	0.221
H	9.12	11.29	11.98	12.66	12.20	13.17	13.22	13.56	13.80	13.73	14.23	14.18	14.96	16.00	0.200	0.294	0.225
n[a]	8	8	8	8	8	8		8	8		8	8	8	8	8	8	8
Outliers[b]	0	0	0	0	0	0		0	0		0	0	0	0	0	0	0
Mean[c]	9.026	11.121	12.090	12.578	12.096	12.959		13.234	13.454		14.096	13.894	14.738	16.179	0.058	0.260	0.244
Ref.[d]	8.975	11.417	11.779	12.374	12.251	12.564		13.245	13.579		13.873	13.934	14.568	16.166	—	—	—
s_r[e]	—	—	—	—	—	0.124		—	0.063		—	—	—	—	—	—	—
RSD_r[f]	—	—	—	—	—	0.96		—	0.47		—	—	—	—	—	—	—
r[g]	—	—	—	—	—	0.348		—	0.177		—	—	—	—	—	—	—
s_R[h]	0.088	0.126	0.153	0.288	0.145	0.212		0.204	0.181		0.139	0.157	0.210	0.170	—	—	—
RSD_R[i]	0.98	1.13	1.27	2.29	1.20	1.64		1.54	1.35		0.98	1.13	1.42	1.05	—	—	—
R[j]	0.247	0.352	0.428	0.807	0.407	0.594		0.572	0.507		0.388	0.439	0.587	0.477	—	—	—

[a] Number of laboratories retained after removal of outliers.
[b] Number of outlying laboratories removed.
[c] Arithmetic average of results of n laboratories.
[d] Reference protein value (12% moisture) by combustion (N × 5.7).
[e] Repeatability standard deviation.
[f] 100 × repeatability standard deviation/mean (dimensionless).
[g] Repeatability value ($r = 2.8 \times s_r$).
[h] Reproducibility standard deviation.
[i] 100 × reproducibility standard deviation/mean (dimensionless).
[j] Reproducibility value ($R = 2.8 \times s_R$).

Table 9. NIRSystems 6500/5000, with equation expansion on standardization set: test set results in % protein [adjusted to 12% moisture (wet basis)]

	Sample													Model statistics		
Laboratory	1	2	3	4	5	6	7	8	9	10	11	12		Bias	RMSD	SEP
A	8.71	11.41	12.03	12.07	12.48	12.81	12.70	13.43	13.97	13.93	14.17	14.32	14.55	16.07	0.108	0.254	0.240
B	8.88	11.38	12.17	12.26	12.52	12.69	12.85	13.42	13.62	13.81	13.98	13.97	14.71	15.68	0.046	0.217	0.222
C	8.92	11.57	11.90	12.12	12.58	12.80	12.64	13.53	13.95	14.10	14.04	14.03	14.68	15.88	0.106	0.227	0.210
D	8.79	11.31	12.13	12.26	12.35	12.64	12.71	13.44	13.60	13.89	13.91	14.05	14.61	15.92	0.024	0.162	0.167
E	9.05	11.66	11.83	12.13	12.79	12.38	12.64	13.29	13.91	13.85	13.83	13.69	14.33	16.06	0.019	0.240	0.250
F	9.08	11.70	11.78	12.26	12.69	12.82	12.83	13.83	13.67	13.65	13.78	13.91	14.45	16.14	0.115	0.248	0.229
G	8.95	11.31	12.04	12.16	12.56	12.55	12.66	13.61	13.91	13.76	13.73	13.87	14.48	15.70	0.012	0.244	0.254
H	8.81	11.53	12.08	12.29	12.52	12.53	12.72	13.42	13.89	13.80	13.77	14.03	14.62	15.73	0.041	0.214	0.220
I	8.63	11.31	11.83	12.17	12.36	12.42	12.75	13.35	13.63	13.87	13.91	13.88	14.67	15.98	−0.049	0.150	0.148
n[a]	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Outliers[b]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean[c]	8.869	11.464	11.977	12.191	12.539	12.674	13.480	13.823	13.902	13.972	14.567	15.907		0.047	0.220	0.218
Ref.[d]	8.975	11.417	11.779	12.374	12.251	12.564	13.245	13.579	13.873	13.934	14.568	16.166		—	—	—
s_r[e]	—	—	—	—	—	0.128	—	0.115	—	—	—	—		—	—	—
RSD_r[f]	—	—	—	—	—	1.01	—	0.83	—	—	—	—		—	—	—
r[g]	—	—	—	—	—	0.357	—	0.321	—	—	—	—		—	—	—
s_R[h]	0.149	0.154	0.144	0.078	0.141	0.136	0.160	0.143	0.141	0.172	0.126	0.172		—	—	—
RSD_R[i]	1.68	1.35	1.20	0.64	1.13	1.07	1.19	1.03	1.02	1.23	0.86	1.08		—	—	—
R[j]	0.418	0.432	0.403	0.219	0.396	0.381	0.449	0.400	0.401	0.481	0.352	0.481		—	—	—

[a] Number of laboratories retained after removal of outliers.
[b] Number of outlying laboratories removed.
[c] Arithmetic average of results of n laboratories.
[d] Reference protein value (12% moisture) by combustion (N × 5.7).
[e] Repeatability standard deviation.
[f] 100 × repeatability standard deviation/mean (dimensionless).
[g] Repeatability value ($r = 2.8 \times s_r$).
[h] Reproducibility standard deviation.
[i] 100 × reproducibility standard deviation/mean (dimensionless).
[j] Reproducibility value ($R = 2.8 \times s_R$).

Table 10. Youden pair analyses for sample pair 2–3 [values in % protein (12% moisture basis)] (reference protein content = 11.417 and 11.779%, respectively)

	Instrument				
Statistic	Tecator, raw data	Tecator, bias corrected	Foss, bias corrected	Perten, slope and bias corrected	NIRSystems, expanded
n[a]	10	10	10[b]	8	9
Outliers[c]	0	0	0	0	0
Mean[d]	11.384	11.758	11.608	11.605	11.721
s_r[e]	0.087	0.087	0.096	0.150	0.189
RSD_r[f]	0.76	0.74	0.83	1.29	1.61
r[g]	0.242	0.242	0.268	0.420	0.528
s_R[h]	0.109	0.087	0.195	0.150	0.189
RSD_R[i]	0.96	0.74	1.68	1.29	1.61
R[j]	0.305	0.242	0.546	0.420	0.528

[a] Number of laboratories retained after removal of outliers.
[b] Although 11 laboratories possessing the Foss instrument participated, Youden pair analysis was performed on 10 laboratories. Data from laboratory A (*see* Table 7) was not used in analysis because values are reported to only 1 decimal place.
[c] Number of outlying laboratories removed.
[d] Arithmetic average of $2n$ values.
[e] Repeatability standard deviation.
[f] 100 × repeatability standard deviation/mean (dimensionless).
[g] Repeatability value ($r = 2.8 \times s_r$).
[h] Reproducibility standard deviation.
[i] 100 × reproducibility standard deviation/mean (dimensionless).
[j] Reproducibility value ($R = 2.8 \times s_R$).

unity for 2 (laboratories E and H) of the 11 collaborators. For the Inframatic instrument, a slope correction was applied on 6 of the 8 collaborators' equations, with the other 2 collaborators' equations demonstrating a slope not significantly different from unity.

Summaries of the collaborative test results for the 4 instruments are shown in Tables 5–9. For the Tecator Infratec, results are tabulated for both before and after equation standardization (Tables 5 and 6, respectively). The reason for reporting prestandardization predictions is that these values are the raw predictions from the GIPSA field office locations (recalling that all Infratec collaborators were GIPSA facilities). By internal agency procedures, field offices are required to check instruments daily with a standard set of samples. Less frequently (typically, once per year), instruments are checked for skewness (slope) with another set of standard samples. Therefore, of the 4 instruments examined, it is expected that the Infratec would require the least radical standardization procedure within the present collaborative study.

Three samples of the Infratec prestandardized values (Table 5) were designated as outliers (11; with values for the type I error level, as revised at a May 1994 IUPAC meeting in Delft, The Netherlands, noted in parentheses in table footnotes), of which 2 (samples 1 and 6) were single-value Grubb's reproducibility outliers (2-tail, $p = 0.025$) from laboratory A and the other (sample 8) was a Cochran repeatability outlier (1-tail, $p = 0.025$) from laboratory J. Repeatability and reproducibility values were determined with outliers removed. However, values determined with outliers are listed in the table footnotes. Outlier detection and reporting of statistical values were similarly performed on the Tecator standardized values as well as those from the other 3 instruments. Repeatabilities of the Tecator prestandardized values, expressed as a relative standard deviation, $RSD_r = 100 \times s_r/\text{mean}$, were 0.42 and 0.33% for the lower (sample 6) and higher (sample 8) protein duplicate samples, respectively. Also included in Table 5 (as well as in Tables 6–9) are prediction intervals for repeatabilities and reproducibilities, designated as r and R, respectively. These intervals refer to the upper limit for which duplicate measurements may differ 95% of the time, assuming differences to be normally distributed. Overall RSD_r was 0.37%. Reproducibilities, also expressed as a relative standard deviation, $RSD_R = 100 \times s_R/\text{mean}$, ranged from 0.32 to 1.07%. When all samples were considered, excluding second readings for each of the blind duplicate samples in the ANOVA (9, pp. 80–81), overall RSD_R was 0.78%.

Consistent with all instruments, model accuracy statistics for the Infratec prestandardized values were calculated with any previously identified outlier present. Without the standardization bias correction, RMSD values ranged from 0.200 to 0.432% PC, averaging 0.283% PC. Values of bias ranged from –0.398 to –0.113% PC (averaging –0.213% PC), indicating that the Infratec instruments had a tendency to underestimate PC. SEP ranged from 0.161 to 0.221% PC and averaged 0.178% PC. Laboratory A, which had the greatest RMSD, also had the largest absolute bias (|bias| = 0.398% PC), explaining why its standard error (SEP = 0.177% PC) was equivalent to the 10-laboratory mean.

With standardization (bias correction) of the Infratec equation (Table 6), repeatability (RSD_r = 0.38 and 0.33% for samples 6 and 8, respectively, and 0.36% overall) was similar to the

Table 11. Youden pair analyses for sample pair 10–11 [values in % protein (12% moisture basis)] (reference protein content = 13.934 and 14.568%, respectively)

Statistic	Instrument				
	Tecator, raw data	Tecator, bias corrected	Foss, bias corrected	Perten, slope and bias corrected	NIRSystems, expanded
n[a]	10	10	10[b]	8	9
Outliers[c]	0	0	0	0	0
Mean[d]	13.895	14.270	14.364	14.316	14.269
s_r[e]	0.085	0.088	0.138	0.143	0.112
RSD_r[f]	0.61	0.61	0.96	1.00	0.79
r[g]	0.239	0.245	0.388	0.401	0.314
s_R[h]	0.127	0.106	0.237	0.184	0.150
RSD_R[i]	0.91	0.74	1.65	1.29	1.05
R[j]	0.355	0.297	0.663	0.516	0.421

[a] Number of laboratories retained after removal of outliers.
[b] Although 11 laboratories possessing the Foss instrument participated, Youden pair analysis was performed on 10 laboratories. Data from laboratory A (*see* Table 7) were not used because values were reported to only 1 decimal place.
[c] Number of outlying laboratories removed.
[d] Arithmetic average of $2n$ values.
[e] Repeatability standard deviation.
[f] $100 \times s_r$/mean (dimensionless).
[g] Repeatability value ($r = 2.8 \times s_r$).
[h] Reproducibility standard deviation.
[i] $100 \times s_R$/mean (dimensionless).
[j] Reproducibility value ($R = 2.8 \times s_R$).

non-bias-corrected results. Reproducibility (RSD_R = 0.41 to 0.92%; 0.61% overall) improved after standardization. Standardization also eliminated the need to treat samples 1 and 6 from laboratory A as outliers, although the repeatability outlier (sample 8 from laboratory J) remained an outlier. Model accuracy also improved with standardization, which was particularly noticeable with RMSD values (range = 0.199 to 0.261% PC; average = 0.236% PC). The standardization bias correction resulted in a tendency to overestimate PC, as seen in the range for bias, being 0.116 to 0.199% PC (average = 0.162% PC). However, on an absolute basis, values for bias were smaller after standardization. Because of the manner in which SEP is defined, standardization by bias correction does not affect SEP.

For the Foss Grainspec (Table 7, above double line), laboratory A values were excluded from calculation of repeatability and reproducibility statistics because PCs were reported to 1 rather than 2 decimal places as specified in the protocol. Cochran and Grubb's tests confirmed the absence of repeatability or reproducibility outliers. On the basis of 10 laboratories, RSD_r was 0.41 and 0.42% for blind duplicate samples 6 and 8, respectively. RSD_R ranged from 0.73 to 2.02%, with an overall value of 1.53%. When values from laboratory A were included (Table 7, below double line), negligible changes in repeatabilities or reproducibilities occurred, except for the improvement in the reproducibility of sample 6 because of removal of laboratory F as a single-value Grubb's test (2-tail, $p = 0.025$) outlier. Further discussion on Grainspec repeatability or reproducibility is limited to the case in which laboratory A values are excluded. Ranges for accuracy statistics were as follows: bias = –1.09–0.413% PC, RMSD = 0.162–0.457% PC, and SEP = 0.160–0.273% PC. On the basis of all 11 laboratories, accuracies averaged 0.058, 0.247, and 0.206% PC for bias, RMSD, and SEP, respectively.

For the Perten Inframatic (Table 8), Cochran and Grubb's tests did not identify any outlying samples; therefore, all repeatability, reproducibility, and accuracy calculations were performed with data from all 8 laboratories. RSD_r of the lower protein duplicate sample (sample 6) was more than twice that of the higher protein sample (sample 8; 0.96 versus 0.47% PC). RSD_R values ranged from 0.98 to 2.29%, with an overall value of 1.38%. Bias, RMSD, and SEP had ranges of –0.078–0.200%, 0.139–0.358%, and 0.144–0.349% PC, respectively. Average values were bias = 0.058% PC, RMSD = 0.260% PC, and SEP = 0.244% PC.

No repeatability and reproducibility outliers were detected by Cochran and Grubb's tests applied to the NIRSystems 6500/5000 data (Table 9). RSD_r values of blind duplicate samples were 1.01% (sample 6) and 0.83% (sample 8), producing an overall RSD_r of 0.92%. RSD_R values ranged from 0.64 to 1.68%, with an overall value of 1.15%. Bias, RMSD, and SEP had respective ranges of –0.049–0.115%, 0.150–0.254%, and 0.148–0.254% PC. Average values for bias, RMSD, and SEP were 0.047, 0.220, and 0.218% PC, respectively.

Repeatability and reproducibility for each instrument model was also determined by treating samples 2 and 3 and samples 10 and 11 as Youden matched pairs (YMPs). The pairs were selected because of their relatively low and high protein contents, respectively, compared with the usual values for HRW wheat. Results of repeatability and reproducibility analyses are summarized in Table 10 for pair 2–3 and in Table 11 for

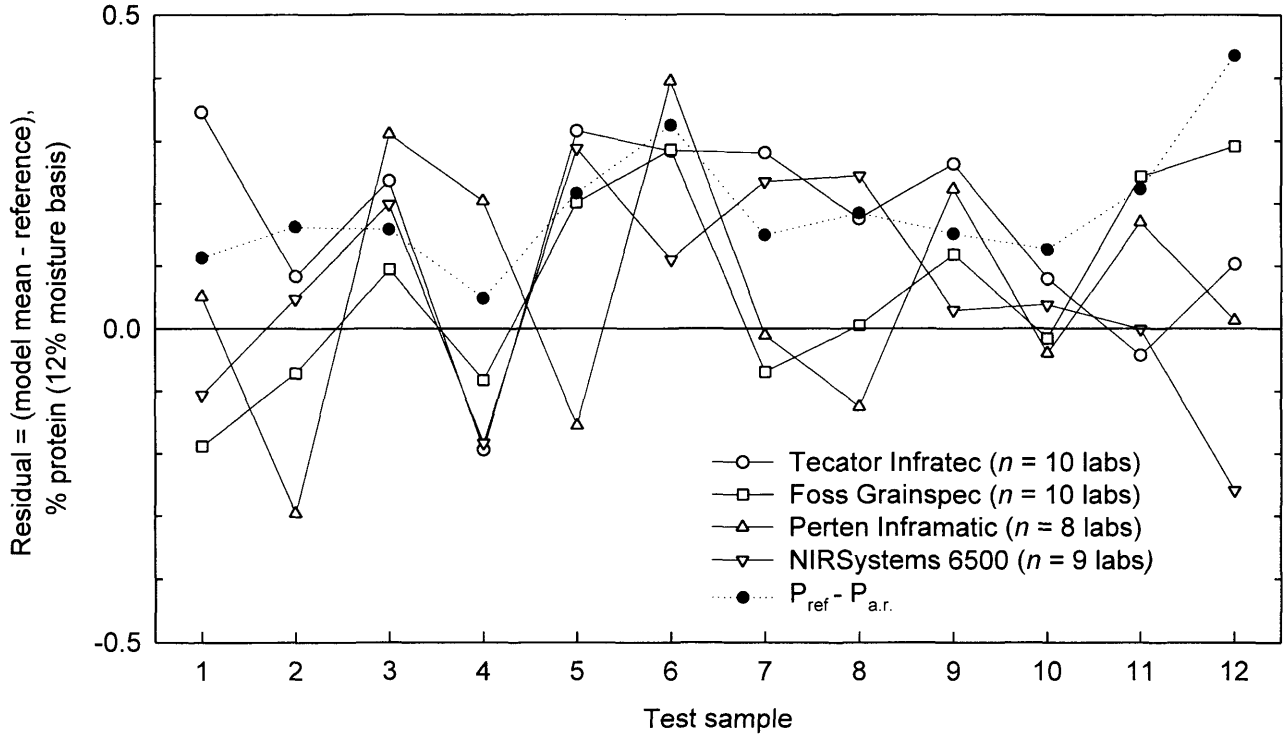

Figure 1. Residuals expressed as the difference between the standardized near-IR modeled value for protein content and the reference value for each sample of the test set. Near-IR modeled values are the means over all laboratories (outliers, as noted in Tables 6–9, excluded) within an instrument type. Also included is a plot of the differences between combustion protein values of the reference subsamples and the Associate Referee's collaborative subsamples (measured on the same combustion instrument), labeled as ($P_{ref} - P_{a.r.}$).

pair 10–11. For pair 2–3, RSD_r values ranged from 0.74% for the bias-corrected Infratec equation to 1.61% for the NIRSystems equation, while RSD_R values ranged from 0.74% (Infratec bias-corrected) to 1.68% (Grainspec). Repeatability values were lower for pair 10–11 (RSD_r = 0.61 to 1.00%) than for pair 2–3 for 4 of the 5 equations. Reproducibility for pair 10–11 (RSD_R = 0.74 to 1.65%) was similar to that for pair 2–3, with the exception of being lower for the NIRSystems equation (1.05 versus 1.61%). In general, repeatability values when determined by analysis of YMPs 2–3 and 10–11 (Tables 10 and 11, respectively) were approximately twice as large as those determined by analysis of blind duplicate samples 6 and 8 (Tables 5–9), with the exception of the NIRSystems equation on pair 10–11 (RSD_r = 0.79% versus 1.01% and 0.83% for samples 6 and 8, respectively). This trend of higher repeatability is most likely because the PCs of the YMP samples were more distant from the median PC of the standardization set than the PCs of the blind duplicate samples.

In all but 2 instances, reproducibility values from YMP analyses were bracketed by the corresponding reproducibilities of the individual samples that formed the pairs. For example, the following values were obtained by comparing the reproducibilities from the raw Infratec equation applied to samples 2 and 3 (Tables 5 and 10): (sample 2, RSD_R = 0.90%) < (YMP 2–3, RSD_R = 0.96%) < (sample 3, RSD_R = 1.00%). The 2 instances of exception were the Perten and NIRSystems equations applied to pair 2–3, in which YMP analyses for both equations produced higher reproducibilities than those from individual sample analyses.

Discussion

Each of the 4 instruments demonstrated repeatabilities and reproducibilities that were comparable with values reported for combustion of ground wheat (12). The largest overall RSD_r value (0.92%) in the present study was slightly lower than that reported for combustion (RSD_r = 0.99%; 12). Similarly, the largest overall RSD_R value (1.53%) in the present study was smaller than RSD_R = 1.74% from the same report. Previous collaborative studies on wheat protein content by Kjeldahl and near-IR reflectance of ground material have demonstrated values for repeatability [RSD_r = 0.36% (Kjeldahl) and 0.61% (near-IR) in reference 3; RSD_r = 0.71% (Kjeldahl) and 1.50% (near-IR) in reference 13] and reproducibility [RSD_R = 1.27% (Kjeldahl) and 1.48% (near-IR) in reference 3; RSD_R = 2.62% (Kjeldahl) and 2.14% (near-IR) in reference 13] that are similar to those of the present study. Hruschka (Table VI in reference 10) reported nearly identical repeatabilities (termed "reproducibility" in table) for PC of ground wheat by Kjeldahl (s_r = 0.154% PC) versus near-IR (s_r = 0.141% PC) procedures, where each sample's Kjeldahl reading was based on the average of 16 subsamples whereas the near-IR reading was the average of 4 subsamples. Overall reproducibility values of the present study [RSD_R = 0.61% (Infratec) to 1.53% (Grainspec)] are

Table 12. Comparison of AOAC collaborative study with in-house study of all U.S. wheat classes: repeatability and reproducibility statistics [values in % protein (12% moisture basis)]

Wheat class[a]	Protein content range, % w/w	s_r, % w/w[b]	s_R, % w/w[c]	RSD_r, %[d]	RSD_R, %[e]	r, % w/w[f]	R, % w/w[g]
HRW: AOAC Collaborative	9.0–16.2	0.048	0.099	0.37	0.78	0.136	0.277
HRW	10.8–14.9	0.065	0.068	0.49	0.52	0.181	0.191
HRS	12.4–16.5	0.057	0.062	0.39	0.43	0.159	0.174
SRW	9.0–11.7	0.067	0.068	0.65	0.66	0.188	0.191
DUR	11.4–14.2	0.071	0.075	0.52	0.55	0.198	0.210
HWW	11.9–13.7	0.064	0.081	0.50	0.63	0.181	0.226
SWW	7.8–11.4	0.067	0.073	0.71	0.77	0.188	0.203

[a] HRW = hard red winter, HRS = hard red spring, SRW = soft red winter, DUR = durum, HWW = hard white, SWW = soft white.
[b] Repeatability standard deviation.
[c] Reproducibility standard deviation.
[d] $100 \times s_r$/overall class mean.
[e] $100 \times s_R$/overall class mean.
[f] $2.8 \times s_r$.
[g] $2.8 \times s_R$.

actually less than those determined from best fit lines of historical data compiled by Margosis et al. (14) on collaborative studies of gravimetric and titrimetric methods for pharmaceutical preparations (e.g., at a concentration of 0.1, RSD_R = 1.62 and 1.65 for gravimetric and titrimetric methods, respectively).

The ranges in model accuracy for the current study (SEP = 0.16–0.22% PC, Infratec; 0.16–0.27% PC, Grainspec; 0.14–0.35% PC, Perten; 0.15–0.25% PC, NIRSystems) were comparable with the range reported by Osborne and Fearn (3; SEP = 0.15–0.33% PC) for near-IR analysis of wheat flour. A plot of residuals (near-IR PC – reference PC) is shown in Figure 1. In this case, the near-IR PC for each sample is an average for all laboratories (Cochran and Grubb's outliers removed). With the exception of 3 samples (samples 3, 6, and 9), the residuals for each sample were both positive and negative, although positive in most circumstances. Included in this plot are the differences between combustion analyses on reference subsamples and the Associate Referee's subsamples as reported in Table 3. The tendency toward a small positive value for bias may be due partially to slight changes in moisture content of the reference subsamples occurring between the oven moisture analysis and the combustion measurement 3 months later. Likewise, a delay of the same magnitude, although in the opposite direction, occurred for the Associate Referee's subsamples. Humidification of either set's subsamples during storage would result in a tendency toward a positive bias.

Generalization of Instrument Performance

Simple linear regressions were applied to establish the existence of any relationship between reproducibility [$\log(s_R)$ or $\log(RSD_R)$] and PC by a *t*-test on the slope of each instrument's regression line. Nonzero slope for the regression line $\log(s_R)$ on PC was not determined as significant (at $p = 0.05$) for any of the 4 standardized instruments. For the regression line $\log(RSD_R)$ on PC, a nonzero slope was significant ($p = 0.013$) for only the Grainspec. In this case, relative error declined slightly as PC increased. Because of the small number of repeatability values per instrument (2 blind duplicates plus 2 YMPs), regression analyses to establish statistical trends of repeatability error with PC were not performed.

A 1-way ANOVA of either $\log(s_r)$ or $\log(RSD_r)$, in which repeatability values from the blind duplicates were combined with those from the YMPs, showed no significant differences [at $p = 0.05$, for numerator degrees of freedom (df)/denominator df = 3/12) among the 4 standardized instruments. However, a similar ANOVA on the 12 values per instrument of either $\log(s_R)$ or $\log(RSD_R)$, excluding the YMP reproducibilities (to avoid redundant information from samples 2, 3, 10, and 11), indicated a significant ($p < 0.0001$, df/df = 3/44) instrument effect. When separate ANOVAs were conducted for accuracy terms, bias, log(RMSD), and log(SEP), only bias was significantly different ($p = 0.042$, df/df = 3/34) among the 4 standardized instruments.

Generalization to Other Wheat Classes

A 2-way ANOVA was performed on USDA-GIPSA check sample data for each wheat class, with instrument and sample as main effects. Repeatability and reproducibility values are summarized in Table 12. Overall RSD_r values ranged from 0.39% (HRS) to 0.71% (SWW), with 0.49% for HRW. These values are slightly higher than RSD_r = 0.37% obtained from non-bias-corrected Tecator Infratec collaborative study data (Table 5), most likely reflecting daily instrument variation associated with the check sample study that was not measured in the collaborative study. Conversely, overall RSD_R values of the check sample study (0.43% for HRS to 0.77% for SWW, and

0.52% for HRW) were lower than the corresponding value (0.78%) from the AOAC collaborative study, reflecting a difference between "reproducibility" as defined by physical location (collaborative study) and "reproducibility" as defined by instrument (check sample study). However, the fact that the repeatability and reproducibility values for HRW in the check sample study fell within the narrow ranges for all wheat classes suggests that the near-IR procedures have application to all U.S. wheat classes.

Conclusion

Near-IR procedures for determination of PC of whole-grain wheat have precisions that are equivalent to those of combustion or Kjeldahl procedures and have accuracies that are equivalent to those of near-IR procedures for ground grain.

Recommendation

On the basis of its simplicity, rapidity of operation, and ability to generate nonhazardous waste, it is recommended that the near-IR method for determination of PC of whole-grain wheat described herein be adopted official first action as an alternative to the combustion (**990.03**) or Kjeldahl (**979.09**) method.

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