FOOD COMPOSITION AND ADDITIVES

Preparation of Validation Materials for Estimating Gluten Recovery by ELISA According to SMPR 2017.021

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Background: **In September 2017, the AOAC INTERNATIONAL Stakeholder Panel for Alternative Methods adopted *Standard Method Performance Requirement* (SMPR®) 2017.021, "Quantitation of Wheat, Rye, and Barley Gluten in Oats," as guidance for the validation of methods for measuring gluten in oat products. The SMPR requires prospective methods to demonstrate adequate recovery (50–200%) based on the analysis of a set of reference samples.** ***Objective:*** **This document provides specific methods and data on the preparation of such validation materials and their analysis by an R5 ELISA kit to demonstrate the SMPR recovery estimation procedure.** ***Methods:*** **Seven reference samples were made by spiking wheat, rye, and barley into gluten-free oat flour at two levels, 10 and 20 mg/kg. The levels of gluten were determined by a wet chemical method based on the Codex Alimentarius definition of gluten.** ***Results:*** **The recoveries for wheat, rye, and barley were 122, 425, and 349%, respectively, for the R5 ELISA kit. The wet chemical method for estimating gluten in a sample of pure grain demonstrated repeatability relative SDs ranging from 1.40 to 2.75%.** ***Conclusions:*** **The reference materials are suitable to estimate ELISA kit responses to wheat, rye, and barley and calculate recoveries.** ***Highlights:*** **A series of oat flours spiked with wheat, rye, and barley flours were developed to be used as reference materials. A wet chemical method was established to estimate gluten contents based on the Codex definition. The reference materials are available for purchase to support further method development and validation.**

Celiac disease (CD) is a chronic small intestinal, immune-mediated enteropathy caused by ingestion of gluten from wheat, rye, and barley in genetically predisposed individuals, and it affects about 0.7 (biopsy-confirmed) to 1.4% (seropositive) of the population worldwide (1, 2). The only known therapy is a gluten-free diet, which means that CD patients must not ingest more than 20 mg gluten/day to avoid small mucosal damage, symptom recurrence, and long-term complications (3). Gluten-free products are, therefore, required to have gluten levels below 20 mg/kg gluten based on the product to provide adequate safety. Codex Alimentarius defines gluten as "a protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl" (4). ELISAs are most commonly used to assess regulatory compliance, and they need to fulfill specific requirements in terms of specificity, LOD and LOQ, accuracy, and recovery (4–6).

In the past, gluten methods were evaluated for accuracy based on quantitatively spiking wheat gluten, gliadin, or wheat flours into various gluten-free matrices and estimating recovery of the method by calculating the percentage of analyte recovered during a multilaboratory study. Recent quantitative methods, such as AOAC INTERNATIONAL *Official Methods of Analysis*℠ **2012.01** and **2014.03**, have used this validation process (7–9). In the case of validating an ELISA method for gluten in oats, it will be essential to evaluate the kit responses not only to wheat but also to barley and rye. *Standard Method Performance Requirement* (SMPR®) 2017.021 (10) has indicated that for this method project, the responses of wheat, rye, and barley must be estimated independently as part of single-laboratory validation. In order to facilitate the validation process, a series of reference samples were prepared, each spiked with a single grain type at specific levels. This paper describes the techniques used and data collected to substantiate the gluten levels in each sample.

The process we describe here involved obtaining gluten-free oat flour and quantitatively spiking in contaminant grain flours to produce quantitative reference materials. The contaminant grains were ground to the particle size of flour and then analyzed by different methods for gluten content prior to spiking. Table 1 shows the proposed set of seven samples and the target concentrations of each contaminant grain. The SMPR requires kit developers to analyze the series of samples (with replication) and estimate recovery for each grain independently.

The intention of this paper is to document the procedures used and data collected to validate the wet chemical methods used to establish the reference values of the materials. Additionally, we present here a proposed method for estimating the recovery of a prospective ELISA kit for wheat, rye, and barley proteins. As an example, we have analyzed the seven reference samples by a commercially available R5 ELISA kit in order to demonstrate the proposed method of estimation.

Received March 19, 2019. Accepted by SB May 31, 2019.
Appendixes are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
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DOI: https://doi.org/10.5740/jaoacint.19-0081

Table 1. Samples needed for studies on recovery of wheat, rye, and barley gluten in oat products

Sample name	Contaminant grain	Level (as gluten), mg/kg	Diluent grain
Blank	None	0	GF oats[a]
W10	Wheat	10	GF oats
W20	Wheat	20	GF oats
R10	Rye	10	GF oats
R20	Rye	20	GF oats
B10	Barley	10	GF oats
B20	Barley	20	GF oats

[a] GF = Gluten-free.

Materials and Methods

Gluten-Free Oat Flour

A large quantity of gluten-free oat flour was obtained by mechanically cleaning oats and dehulling the oats to groats (General Mills, Minneapolis, MN). The groats were then processed through an optical sorter to remove contaminant grains so that the resulting groats were less than 1 mg/kg gluten on average by the R5 ELISA method (at 18 replicates with 5 g test portion). Flour at this level is deemed low enough in gluten to be used in spiking experiments. The groats were milled through a Retsch mill (Model ZM 200 with 0.5 mm screen). Samples were made in a series of seven samples, all made from the same lot of "clean" oat flour as diluent.

Contaminant Grains

As we encountered in previous work, the decision on what contaminant grains to use in spiking studies was not straightforward, and it was difficult to reach consensus. The general question in such a context is whether to use a single cultivar or to use a blend of common grain varieties that are widely planted in the region (11). Single cultivars have been used in previous applications and have the advantage of being well-characterized genetically. However, even single cultivar samples can vary by crop year and growing region with respect to expressed protein levels and epitope expression rates (12). When choosing a single cultivar, researchers run the risk of selecting a cultivar that is abnormal with respect to these two critical parameters. For these reference materials, the Working Group consensus was to use blended cultivars as spiking materials.

Several common rye and barley cultivars currently planted in North American oat regions were obtained for study. Using data for planting acres from the Saskatchewan Ministry of Agriculture, the Manitoba Agricultural Services Corporation, and the U.S. Department of Agriculture National Agricultural Statistics Service, we selected barley and rye cultivars common to the oat growing regions of Canada and the United States. Samples were obtained from the breeding programs at North Dakota State University with the help of Richard Horsley, Department Head of the Department of Plant Sciences, and Steve Zwinger, Research Specialist at the Carrington Research Extension Center. We obtained six cultivars of two-row barley (Austenson, Conlon, Copeland, Hocket, Metcalf, and Synergy), three cultivars of six-row barley (Celebration, Lacey, and Tradition), and eight cultivars of rye (Hancock, Sponner, Rymin, ND Dylan, Dacold, Aroostok, Hazlet, and Wheeler).

For wheat spiking material, a sample of the single cultivar Carberry was obtained. Analysis of this sample showed high levels of protein and high response to the R5 antibody. We subsequently obtained several samples of wheat from General Mills wheat process streams. Obviously, these are not registered cultivars but unidentified commercial samples, as received at the wheat flour milling facilities. In addition, we obtained commercially available whole-wheat flours for study. Because of the lack of available pure cultivar samples, we decided to blend the available ground grains along with the commercially obtained whole-wheat blended flours to use as spiking material for wheat. Appendix A summarizes the samples of wheat and wheat flours that were characterized and studied for spiking and the masses of each sample used to make the spiking blend.

Estimation of Gluten in Contaminant Grains

In September 2017, the Working Group considered four options for characterizing the gluten level of the spiking materials. They were as follows: (*1*) the method of Wieser et al. (13), using reversed-phase HPLC (RP-HPLC) after modified Osborne fractionation. The method is well-established to characterize grain protein composition (11, 12, 14). (*2*) The wet chemical method, involving the extraction of nongluten proteins by water and 0.5 M NaCl to remove soluble proteins [which are nongluten by Codex definition (4)], followed by centrifugation and then Dumas nitrogen on the remaining pellet. (*3*) Extraction of nongluten proteins with 0.4 M NaCl in phosphate-buffered saline (PBS; pH = 7.5) followed by Dumas nitrogen analysis of the resulting centrifuged pellet. (*4*) Estimation of gluten by Dumas nitrogen for total nitrogen, using a factor for each grain based on empirical observations [e.g., for wheat, multiply protein by 0.8, and for rye and barley, multiply protein by 0.55 (14)].

The Working Group decided in November 2018 to use method (*2*) above, as it is linked to the gluten definition of Codex Alimentarius (4). The following procedure was developed.

Wet Chemical Method

The procedure for determining gluten concentration of pure grains was as follows. (*1*) Mill the grains through Retsch mill ZM 200 with 0.5 mm screen. (*2*) Weigh 150 mg sample grain into a 2 mL microcentrifuge tube. Record the weight to the nearest 0.1 mg. (*3*) Add 1.5 mL water to the tube. Cap and mix on a vortex mixer to completely disperse the sample. (*4*) Let the sample stand at ambient temperature for 15 min, mixing on a vortex mixer every 5 min. (*5*) Centrifuge in a microcentrifuge for 10 min at 3400 rpm. (*6*) Decant off the supernatant, making sure not to lose any solids. If solids are not completely at the bottom of the tube, recentrifuge for an additional 10 min. (*7*) Repeat steps (*3*)–(*6*) with water. (*8*) Repeat steps (*3*)–(*6*) two times with 0.5 M NaCl/100 µmol/L PBS (pH 7.5) solution. (*9*) Place the tube in a vacuum oven and dry overnight at 70°C under vacuum for 16 h. (*10*) Remove from vacuum oven, put pellet in Dumas foil, and drop in furnace to measure nitrogen content. Use original flour weight as mass for Dumas calculation. (*11*) Report nitrogen content per sample weight of original sample before washing. (*12*) Compare nitrogen content versus Dumas reading with no solvent treatment.

Gluten Analysis by R5 ELISA Kit

The seven test samples were analyzed for gluten by the R5 ELISA test kit (R7001, with Mendez Cocktail extraction buffer, R7016; R-Biopharm AG, Darmstadt, Germany). The samples were analyzed 18 times at 5 g test portion extraction.

Results

Appendixes B–D contain analytical raw data and results for the wet chemical gluten analysis of wheat, rye, and barley samples, respectively. Appendixes B–D also contain analytical data for the results of wet chemical tests for wheat, rye, and barley blends, respectively, made from equal-mass blends of all the cultivars. The rye and barley blends were made from seeds prior to grinding through the Retsch mill. The wheat blends were made up from flour and ground seeds, which were ground in the Retsch mill prior to blending. The blends were then analyzed for gluten by the wet chemical extraction method and also analyzed for total unextracted protein by Dumas. Total protein was estimated as nitrogen × 5.83 (15).

Appendix B shows the samples of wheat and wheat flours ranging from 9 to 14% protein, with the gluten proteins ranging from 70 to 77% of the total protein mass. Appendix C shows that for rye, the amount of residual protein left after aqueous wet chemical extraction was lower, ranging from 40 to 47% of the total protein mass remaining as gluten. Appendix D shows that the gluten results varied from 68 to 74% with a mean of 71% versus total protein. After blending, the wheat, barley, and rye blends were analyzed five times for total protein by Dumas nitrogen and five times by the wet chemical method. Results are given in Appendixes B–D. Table 2 summarizes the averages of all extractions for each set of samples.

The average gluten level in the wheat blend was estimated to be 9.21 g/100 g, which, when compared to the total protein content of the wheat, is 73.9% of the protein content as gluten. This is very consistent with the weighted mean level of gluten estimated in all of the individual samples at 72.7% of the total protein as gluten (weighted as proportional to the mass used in the blend).

For all three blend samples, the empirical values obtained by analyzing the finished blends were used to determine the amount of blended flour to use in the spiked production of reference materials.

Validation of the Wet Chemical Method

Samples of the wheat, rye, and barley spiking blends were analyzed by RP-HPLC by the method of Wieser et al. (13)

Table 2. Summary of gluten fractionation experiments

	Avg. gluten protein, g/100 g	Avg. total protein, g/100 g	Fraction gluten
Wheat samples	8.53	11.61	0.73
Wheat blend	9.21	12.46	0.74
Rye cultivars	3.34	7.63	0.44
Rye blend	4.04	7.82	0.52
Barley cultivars	7.20	10.18	0.71
Barley blend	7.93	10.17	0.78

Table 3. Comparison of results from the wet chemical method (wet chem) versus analysis by reversed-phase HPLC (RP-HPLC)

Blend	Dumas protein, g/100 g	Gluten by wet chem, g/100 g	Fraction by wet chem	Gluten by RP-HPLC, g/100 g	Fraction by RP-HPLC
Rye	7.82	4.04	0.52	3.2	0.41
Barley	10.17	7.93	0.78	5.7	0.56
Wheat	12.46	9.21	0.74	8.2	0.66

(Table 3). The AOAC Working Group was especially concerned with the barley fraction of 78% as the insoluble portion of protein retained as gluten. Previous experience suggested that more than 22% of the protein should be dissolved in the aqueous solvents. One hypothesis given to explain the observed result was that 15 min (water × 3, 0.5 M NaCl/PBS pH 7.5 × 3) was not long enough to completely solubilize all nongluten proteins. To test this hypothesis, we conducted an experiment comparing these results on the barley blend to a method with a 6 h soak in 0.5 M NaCl in PBS buffer. A different sample of barley blend was used for this test. Samples were performed in triplicate. Table 4 summarizes the results of this experiment. No significant difference was observed between the proposed wet chemical method and the 6 h extraction variation.

In all instances, we performed these wet chemical extractions with replication on all samples and calculated pooled SDs on the results in order to estimate method repeatability. Table 5 summarizes the pooled precision statistics from the replicate analyses. Pooled relative SDs (RSDs) ranged from 1.40% for barley to 2.75% for rye. The overall pooled RSD for the method was 1.93%. The precision for this method seems reasonable given the nature of the test and the amount of physical handling steps in the extraction.

Formula for Spiking

Given the estimated gluten levels required in each spiking blend, target levels for spiking amounts into oat flour for each of the spiked samples required in Table 1 have been calculated. Table 6 presents the levels of spiking materials required to produce reference materials at 10 and 20 mg/kg spike levels.

Production Scale Spiking

All materials, unspiked clean oat flour, and three spiking blends were shipped to Trilogy Analytical Laboratory (Washington, MO) for blending and packaging into 50 g pouches for distribution. Packaging took place in February and March of 2017. All weights of blank oat flour and spiked blends were recorded and carried through the calculation to estimate final gluten concentrations in the final blends. Table 7 summarizes the recorded spiking concentrations of preliminary master mixes and final blends in units of milligrams per kilogram gluten. The blends were successively diluted into Master Mix A, Mix B, and Mix C to achieve a final concentration of 20 and 10 mg/kg gluten. The final calculated values from the balance readings were within ±0.05 mg/kg, so we are confident to claim these as 10 and 20 mg/kg nominally. The largest source of error for these estimates is in the estimation of gluten as per the

Table 4. Comparison of wet chemical method to 6 h extraction

Sample/extraction	Sample weight, g	Dumas nitrogen, g/100 g	Mean, g/100 g	SD, g/100 g	Fraction gluten
Barley, no extraction	0.1833	1.83			
Barley, no extraction	0.1163	1.84			
Barley, no extraction	0.1934	1.83	1.83	0.0073	NA[a]
Barley, wet chem, 6 × 15 min[b]	0.15	1.30			
Barley, wet chem, 6 × 15 min	0.1503	1.33			
Barley, wet chem, 6 × 15 min	0.1502	1.31	1.31	0.0126	0.716
Barley, wet chem, 6 h	0.1504	1.26			
Barley, wet chem, 6 h	0.1503	1.31			
Barley, wet chem, 6 h	0.1495	1.35	1.31	0.0481	0.713

[a] NA = Not applicable.

[b] wet chem = Wet chemical method.

Table 5. Precision data for wet chemical method

Grain	*n* Samples[a]	*N* replicates[b]	Mean, g/100 g	Pooled SD, g/100 g	Pooled RSD[c], %
Wheat	10	23	8.75	0.169	1.94
Rye	9	21	3.50	0.0962	2.75
Barley	10	23	7.36	0.1033	1.40
Overall	29	67	6.63	0.128	1.93

[a] *n* Samples = Number of unique cultivar samples analyzed.

[b] *N* replicates = Total number of replicates analyzed (generally 2–5 replicates per sample).

[c] RSD = Relative SD.

Table 6. Spike amounts required per kilogram oat flour

Grain	Concn of gluten in spike blend, g/100 g	Amt of blend (mg) required to make 1 kg at 10 mg/kg gluten	Amt of blend (mg) required to make 1 kg at 20 mg/kg gluten
Rye	4.0	250	500
Barley	7.9	197.5	253.2
Wheat	9.21	108.6	217.2

Codex definition and in the use of the wet chemical method to determine gluten levels. Any other method for defining gluten will give different reference values for the samples.

Testing of Samples with the R5 Antibody Kit

The seven test samples were analyzed for gluten by the R5 ELISA test kit 18 times at 5 g test portion extraction (Appendix E). Table 8 summarizes the statistics of the results of the experiment.

Average values of gluten results for the kit were plotted against the nominal gluten value for the samples, and three regression lines were drawn to estimate the slope of the response, which is indicative of the gluten recovery of the kit. Figure 1 shows the plot of the responses of the kit to each grain spiked sample. Table 9 summarizes the slope and recovery of each grain, with associated confidence intervals.

Discussion

SDs observed on the gluten analysis of the spiked materials by R5 ELISA are much lower than those commonly seen on incurred oat flour samples. This is likely due to the fact that the contaminant grains were ground separately before spiking, resulting in much smaller particle sizes of contaminant flours than are obtained when the grains pass through a commercial oat mill. The spiked samples are intended to be used to compare recoveries of the three grains independently and should not be used to estimate precision statistics for incurred samples.

The recovery criterion listed in SMPR 2017.021 (10) for a prospective new kit is that the estimated recovery must be between 50 and 200%. In this case, the R5 kit would pass the criterion for wheat response but would not pass for rye or barley. The 95% confidence intervals for the recovery estimate are based on regression statistics of all individual responses regressed versus the spike levels. The size of these confidence intervals is dependent on the number of replicates taken and the test portion size used.

There are many ways to estimate recovery from a data set such as this. An alternative method would be to produce three recovery estimates for each grain, one from the 10 versus the 0 mg/kg sample, one from the 20 versus the 0 mg/kg sample, and finally, a third recovery from the 20 versus the 10 mg/kg sample. In the application, when comparing recovery to a single acceptance criterion, there may be a conflict within a given grain for which only one or two of the three recovery estimates pass acceptance. We prefer the least squares regression approach with all levels simultaneously because it provides a single unbiased estimate of recovery that can be compared to the criterion. Also, the regression statistics provide confidence intervals for the slope estimates, which are useful for evaluating the reliability of the estimates.

Conclusions

In support of AOAC SMPR 2017.021, we have developed and packaged a series of spiked oat flour samples to be used

Table 7. Target concentrations and calculated values for mixes and blends (mg/kg gluten)

Blend name	Target concn, mg/kg gluten	Actual concn by weight, mg/kg gluten	Nominal value for package
Wheat Master A	10000	10000.89	
Wheat Mix B	2000	2000.205	
Wheat Mix C	400	400.1036	
Wheat 20	20	20.00519	20 mg/kg
Wheat 10	10	10.00264	10 mg/kg
Barley Master A	10000	10000.02813	
Barley Mix B	2000	2000.8878	
Barley Mix C	400	400.240	
Barley 20	20	20.0125	20 mg/kg
Barley 10	10	10.00625	10 mg/kg
Rye Master A	5000	4999.89	
Rye Mix B	1000	1000.154	
Rye Mix C	200	200.0282	
Rye 20	20	20.00342	20 mg/kg
Rye 10	10	10.00164	10 mg/kg

Table 8. Statistical summary of replicate analyses of reference samples by R5 ELISA kit

	Blank	R10	R20	W10	W20	B10	B20
Spike level, mg/kg	0	10.0	20.0	10.0	20.0	10.0	20.0
N	18	18	18	18	18	18	18
Test portion, grams	5	5	5	5	5	5	5
Mean, mg/kg	0.8192	48.17	85.89	14.58	25.13	38.01	70.62
SD, mg/kg	0.463	3.134	4.933	5.196	2.683	4.010	4.702
LCL, mg/kg[a]	0.61	46.73	83.61	12.18	23.89	36.15	68.45
UCL, mg/kg[b]	1.03	49.62	88.17	16.98	26.37	39.86	72.79

[a] LCL = Lower limit of 95% confidence intervals on the mean, based on normal distribution.

[b] UCL = Upper limit of 95% confidence intervals on the mean, based on normal distribution.

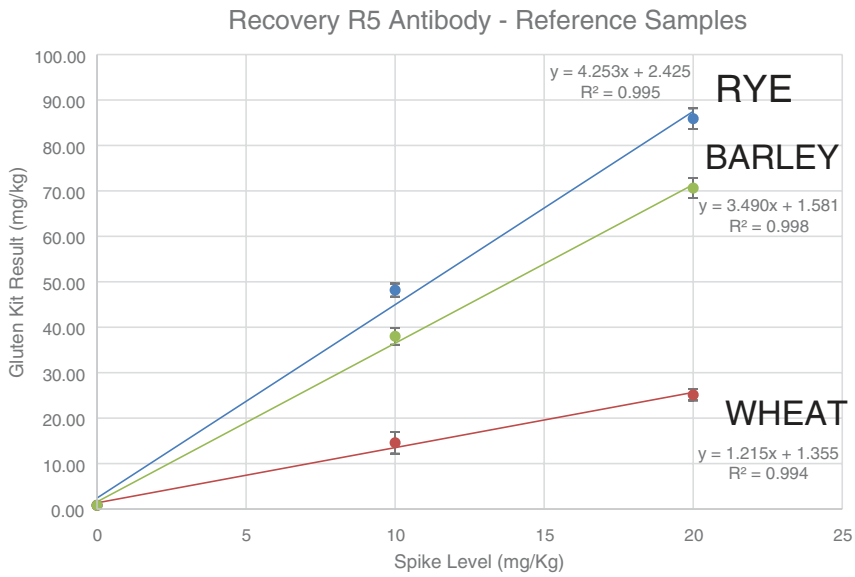

Figure 1. Plot of response for R5 kit to spiked recovery samples.

Table 9. Statistical summary of R5 ELISA kit response plot

Grain	Slope	Intercept, mg/kg	Rec., %	95% CI on rec.[a]
Wheat	1.22	1.36	122	110–133
Rye	4.25	2.42	425	412–439
Barley	3.49	1.58	349	337–361

[a] CI = Confidence interval.

to estimate gluten kit responses to wheat, rye, and barley. We have established a wet chemical method based on the Codex definition of gluten to estimate the level of gluten in the spiked samples. The series of samples can be tested by a prospective kit, and gluten recoveries can be estimated for each grain species independently. We have demonstrated the utility of this system by application to the R5 antibody kit. Reference materials are available for purchase from United States Pharmacopeia (Cat. No. 1294839; Rockville, MD).

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