Technical note: A modified method to quantify prolamin proteins in dry and high-moisture corn

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ABSTRACT

Numerous studies have evaluated laboratory methods to quantify prolamin proteins in dry corn; however, the utility of methods to quantify functional prolamins, which impede starch digestibility, in high-moisture corn (HMC) is less defined. As a result, a common rapid turbidimetric (rTM) laboratory procedure was modified (rapid Bradford method, rBM), extracting buffer-soluble proteins before prolamin solubilization in an effort to better quantify functional prolamins in HMC. Twenty samples of dry and HMC were evaluated by rTM and rBM procedures. Prolamin concentration in dry corn, as estimated by rTM or rBM methods, ranged from 6.12 to 2.20 g/100 g of DM or 5.46 to 2.41 g/100 g of DM, respectively. Dry corn mean prolamin concentrations, as estimated by rTM or rBM methods, were similar at 3.65 or 3.66 g/100 g of DM. Prolamin concentration in HMC, as estimated by rTM, ranged from 4.99 to 3.24 g/100 g of DM, with a mean prolamin concentration of 4.19 g/100 g of DM, but estimation of prolamins in HMC by the rBM method resulted in lower mean (4.19 vs. 3.24 g/100 g of DM) prolamin concentration. Prolamin concentration in dry and HMC measured by rTM was negatively related to peak absolute rates (PAR; mL/0.1 g of DM) of in vitro gas production. However, relationships between rTM prolamin concentration and PAR were not homogeneous and were different between dry and HMC. Prolamin proteins as determined by rBM were likewise negatively related to PAR, but corn type did not influence rBM prolamin concentration by PAR relationships. Data suggest that the rBM method defined more similar functional prolamin proteins, which impede starch degradability, in dry and HMC.

Key words: prolamin, high-moisture corn, starch degradability

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Corns with greater percentages of prolamin proteins have been shown to reduce starch degradability or digestibility, both in vitro (Hoffman et al., 2012) and in vivo (Lopes et al., 2009). Recently, Larson and Hoffman (2008) defined a low-cost, rapid turbidity method (**rTM**) to quantify prolamin proteins (zein) in dry corns of varying endosperm type. Since publication, the utility of the rTM has been evaluated in numerous studies. Giuberti et al. (2011) found the rTM to yield similar prolamin values for dried corn compared with the intensive methods of Landry et al. (2000) but yielded lower prolamin values compared with the method of Hamaker et al. (1995). Based on the work of Landry et al. (2002), Giuberti et al. (2012) modified the rTM, replacing isopropanol with *tert*-butanol to extract greater percentages of prolamins from dry and high-moisture corn (**HMC**).

The utility of the rTM in ruminant nutrition has also been evaluated in numerous studies. Lopes et al. (2009) quantified prolamin proteins using the rTM in near-isogenic variants of an Oh43x W64A normal dent endosperm hybrid carrying *floury-2* or *opaque-2* alleles. Hybrids carrying *floury-2* or *opaque-2* alleles contained significantly less prolamin protein, resulting in improved in vivo starch digestibility in lactating dairy cows. Hoffman et al. (2012) and Masoero et al. (2011) demonstrated negative relationships between rTM prolamin contents of dry corn and in vitro starch degradability.

The biological utility of the rTM in evaluating prolamins in HMC or corn silage is less defined because relationships between rTM prolamins in HMC or corn silage and starch degradability are not well established. Using HPLC methods, Hoffman et al. (2011) demonstrated that fermentation of HMC reduced all α , β , γ , and δ prolamin-zein subunits of the starch-protein matrix from 10 to 40%, but rTM did not detect changes in prolamin concentration induced by fermentation. Likewise, Hoffman et al. (2012) could not illicit relationships between prolamin protein in HMC and peak absolute rate (**PAR**) of in vitro gas production when prolamin was determined by rTM. These data suggest

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that the rTM as defined by Larson and Hoffman, (2008) may improperly define the biological functionality of prolamins proteins, as related to starch digestibility, in HMC. It was the objective of the current study to explore whether the rTM could be modified to quantify prolamins proteins in HMC, which are biologically more relevant to starch degradability in ruminants.

The rTM of Larson and Hoffman, (2008) can be generalized as follows: dried ground corn is defatted using acetone (100%), filtered, and dried, and acetone-insoluble DM is retained. Prolamins in acetone-insoluble DM are solubilized with 55.0% aqueous isopropyl alcohol containing 0.6% 2-mercaptoethanol. Turbidity of prolamins protein is achieved by incorporation of aqueous alcohol-solubilized prolamins with 0.15 M TCA. The degree of turbidity is measured by the logarithm of absorbance of the sample at 440 nm on a spectrophotometer and prolamins concentration is quantified using a standard absorbance curve developed from purified zein.

Intuitive shortcomings of the rTM exist with regard to measuring prolamins in HMC. First, the acetone rinse is designed to remove fat, with minimal loss of total proteins in the sample, because only prolamins proteins are assumed to become turbid in 0.15 M TCA (Larson and Hoffman, 2008). This assumption may be invalid for HMC, as proteolytic activity during fermentation may hydrolyze prolamins proteins into dysfunctional peptides, which may express turbidity in 0.15 M TCA. Second, turbidity in chloroacetic acid is rapid but not purely quantitative because turbidity is affected differently by differences in the tertiary structure of proteins (Ebina and Nagai, 1979).

Due to potential shortcomings and new information regarding efficiency of *tert*-butanol to extract prolamins (Giuberti et al., 2012), the following modifications were made to the rTM in an effort to improve its biological utility in determining functional prolamins, as related to starch digestibility, in HMC: (1) to facilitate removal of nonfunctional endosperm proteins (albumins, globulins, and hydrolyzed zein) in corn, defatting the sample with acetone in the rTM was replaced with a borate phosphate buffer extraction to remove buffer-soluble proteins as defined by Krishnamoorthy et al. (1983); (2) use of isopropanol to extract prolamins was abandoned and replaced with *tert*-butanol to more efficiently extract prolamins (Giuberti et al., 2012); and (3) because extraction of prolamins with *tert*-butanol without prior defatting of the sample with acetone would influence turbidity measurements, turbidity in 0.15 M TCA was abandoned and replaced with a Bradford protein assay (Bradford, 1976) to quantify prolamins proteins solubilized in *tert*-butanol. The changes were coalesced and

defined as a rapid Bradford method (**rBM**) to quantify prolamins proteins in dry and HMC.

Buffer-soluble proteins in dried ground corn are extracted using a borate phosphate buffer as defined by Krishnamoorthy et al. (1983), and buffer-insoluble DM (**biDM**) is retained. Prolamins in biDM are solubilized with 60.0% aqueous *tert*-butyl alcohol containing 0.6% 2-mercaptoethanol. Prolamins proteins are quantified using Coomassie Brilliant Blue dye (BP100-25 Brilliant Blue G-250; Fisher Scientific, Fair Lawn, NJ) in an acidic media, with the reddish dye turning blue when it binds to protein (Bradford, 1976). The colorimetric shift is measured on a spectrophotometer at a 595-nm wavelength and compared with a prolamins standard. The apparatus include a spectrophotometer set to 595 nm, forced-air ovens set to 55 and 105°C, balance accurate to 1 mg, centrifuge capable of $2,250 \times g$; vortex mixer, a cyclone mill fitted with a 1-mm screen, orbital shaker capable of 240 rpm, funnels capable of holding 125-mm Whatman 541 filter paper, 40-mL centrifuge tubes, 5-mL plastic disposable cuvettes, pipettes capable of dispensing 50 μ L or 1 to 10 mL, and glassware including test tubes, 100- and 1,000-mL volumetric flasks, and 125-mL Erlenmeyer flasks with stoppers.

Reagents included borate phosphate buffer [12.2 g of sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and 8.91 g of sodium borate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) per liter of distilled water (**dH₂O**), stirred at medium speed until completely dissolved]; aqueous alcohol solution [600 mL of *tert*-butanol (CAS 75-65-0 Fisher Scientific), 6 mL of 2-mercaptoethanol (Fisher Scientific O34461-100), brought to volume in a 1,000-mL volumetric flask with dH₂O]; Bradford reagent [100 mg of Coomassie Brilliant Blue powder dissolved in 5 mL of reagent alcohol (Fisher scientific A962P-4) and 100 mL of 85% (wt/vol) phosphoric acid (Fisher Scientific UN1805) and brought to volume in a 1,000-mL volumetric flask with dH₂O]; and finally, a prolamins standard prepared in a 100-mL volumetric flask: 94 mg of purified zein (CAS 9010-66-6; Acros Organics, Fair Lawn, NJ), 6 mg of albumin standard (23209; Thermo Scientific, Waltham, MA), brought to volume with aqueous alcohol solution and stirred for 1 h. The 94:6-mg ratio of purified zein to albumin was used as the standard to increase the absorbance per unit of CP (N) upon Bradford reagent addition because a prestudy evaluation (data not shown) of absorbance at 595 nm per unit of true N concentration (Lachat Instruments, 1995) in *tert*-butanol, 2-mercaptoethanol extracts of unknown corns was greater per unit of N compared with absorbance per unit of N of a pure zein standard.

Corn samples were dried in a 55°C forced-air oven for 24 to 48 h. Samples were ground in a cyclone mill

(Udy Corp., Boulder, CO) fitted with a 1-mm screen. The DM of the samples was determined by drying at 105°C for 3 h.

Filter papers (Whatman 541) were placed in a 105°C oven for 2 h, hot weights were recorded, and filters were folded to fit funnels. A 500-mg sample of dried, ground corn was placed into a 125-mL Erlenmeyer flask, 50 mL of borate-phosphate buffer was added, and the flask was covered and incubated in a 39°C water bath for 1 h. The contents were filtered through the funnel previously fitted with filter paper and the flask was rinsed with buffer to remove any remaining sample. The residue was then rinsed with an additional 150 mL of buffer. The filtrate was discarded and the biDM and filter paper were left to air dry for 1 h and then dried in a 55°C forced-air oven for 24 h. The dry weight of the biDM and filter paper was then recorded.

A 200-mg subsample of biDM was weighed into 40-mL centrifuge tubes, 20 mL of the aqueous alcohol solution was added, and the tube was covered and shaken on an orbital shaker at 240 rpm for 4 h. Tubes were then centrifuged at $2,250 \times g$ for 20 min. The Bradford solution was readied by inverting the container 2 to 3 times to mix, and then the solution was filtered through 541 Whatman filter paper fitted in a funnel placed over a 125-mL Erlenmeyer flask and was brought to room temperature.

Prolamin standards were prepared containing 0, 212.5, 425, 637.5, and 850 $\mu\text{g/mL}$. In 5 test tubes, 0 mL of prolamin standard solution was added to the first tube, and then 1, 2, 3, and 4 mL, respectively. Next, 4 mL of aqueous alcohol solution was added to the first tube, and then 3, 2, 1, and 0 mL, respectively. Each tube was vortexed gently for 2 to 3 s, 50 μL of each standard solution was pipetted into cuvettes, 2.5 mL of Bradford reagent was added, and then samples were incubated at room temperature for 5 min. After a blank standard (0 μg of prolamin/mL) was used to set the spectrophotometer (595 nm) to zero, the absorbance of each standard was read and a standard curve was fit using the known prolamin concentrations ($\mu\text{g/mL}$) as the dependent variable and the absorbance as the independent variable.

After centrifuging, 50 μL of supernatant from each unknown sample was pipetted into cuvettes, 2.5 mL of Bradford reagent was added, and samples were incubated at room temperature for 5 min. Then, the absorbance of each sample was measured at 595 nm.

Calculations were as follows:

$$\begin{aligned} &\text{Buffer-insoluble DM recovery (biDMr), g/100 g of DM} \\ &= [(\text{filter paper} + \text{biDM residue, g} \times 0.994) \\ &- (\text{filter paper weight, g})]/(\text{sample weight, g of DM}); \end{aligned}$$

$$\begin{aligned} &\text{Prolamin concentration, g/100 g of DM} = \\ &[(\text{prolamin, } \mu\text{g/mL} \times 0.02)/(\text{biDM sample weight,} \\ &\text{mg} \times \text{biDMr})] \times 100. \end{aligned}$$

To evaluate repeatability and utility of the proposed rBM, 20 samples of dry corn with varying CP content and 20 samples of HMC with varying DM content were procured from the University of Wisconsin Soil and Forage Testing Laboratory (Marshfield, WI). Dry corn samples (whole kernel), previously dried at 55°C for 24 h, and HMC after drying for 24 h at 55°C were divided into 2 subsamples, with one subsample ground through a cyclone mill fitted with a 1-mm screen and analyzed for DM, CP (AOAC, 1990), and soluble CP (**SOLCP**; Krishnamoorthy et al., 1983). The starch content of samples was estimated using near-infrared reflectance spectroscopy (Soil and Forage Analysis Laboratory). Prolamin contents of dry and HMC corns were then determined by rTM (Larson and Hoffman, 2008) and rBM methods as defined above. Prolamin concentration was determined in duplicate in 2 separate runs to provide inference of method precision. Estimates of rTM and rBM precision were calculated by the methods defined by Bland and Altman, (2010). The repeatability standard deviation (S_r) was estimated as the mean of the absolute differences between duplicates for each method (Bland and Altman, 2010) and S_r was alternatively expressed as relative S_r [$\text{RS}_r = (S_r/\text{mean value}) \times 100$], expressing repeatability as a percentage of the mean.

To evaluate the biological utility of rTM or rBM for prolamin, the remaining subsamples of dry and HMC were ground through a cutter mill (Arthur H. Thomas Co., Philadelphia, PA) fitted with a 2-mm screen and saved for in vitro gas production determinations. Detailed in vitro gas production methods have been previously published in Hoffman et al. (2012). General conditions of in vitro gas production determinations were as follows: in vitro gas production was measured on 2-mm ground dry and HMC samples using the Ankom-RFS system (Ankom Technology Corp., Macedon, NY) fitted with wireless transponders (Leibovich et al., 2009) using the in vitro technique of Goering and Van Soest (1970). In vitro gas production was measured in triplicate utilizing 3 separate in vitro runs with all samples of dry or HMC included in each run. In vitro gas production was determined on 0.5-g samples incubated for 24 h in a water bath maintained at 39°C. Rumen fluid was obtained from 2 lactating dairy cows fitted with ruminal cannula, fed a diet containing approximately 30% alfalfa-grass silage, 40% corn silage, and 25% ground dry shelled corn, with the remainder of the diet containing protein, vitamin, and mineral

Table 1. Nutrient composition of dry and high-moisture corns used in the evaluation of the rapid turbidity or rapid Bradford method to determine prolamin content¹

Item	Dry corn				HMC ²			
	Mean	Minimum	Maximum	SD	Mean	Minimum	Maximum	SD
DM, % as is	91.9	90.3	94.2	0.72	72.8	64.5	78.7	4.07
CP, % of DM	8.6	6.9	15.4	1.90	9.2	7.7	11.1	0.89
Soluble CP, % of DM	1.8	0.9	3.2	0.45	1.7	0.4	4.7	1.06
Starch, % of DM	70.8	53.4	73.7	4.31	70.7	67.0	74.1	2.06
PAR, ³ mL/0.1 g of DM per hour	2.6	2.1	3.2	0.28	2.8	2.3	4.0	0.45

¹All values are expressed as a percentage of DM, unless otherwise specified.

²High-moisture corn.

³Peak absolute rate of in vitro gas production.

supplements. After collection, rumen fluid was held at 39°C in CO₂-gassed vessels, mixed in a CO₂-gassed blender, and strained through 3 layers of cheesecloth before injection into the gas incubation flask. A blank (rumen fluid only) was included within each in vitro gas production run.

Cumulative in vitro gas production pressure of all samples was recorded every 0.25 h, with adjustment for the gas production of a blank sample within run. Gas production pressure readings were converted to gas volume and expressed as milliliters per 0.1 g of DM, resulting in expression of gas production volumes between 0 and 50 mL (Parissi et al., 2005). The PAR of gas production (mL/0.1 g of DM per hour), defined as the peak of the first derivative (Lanzas et al., 2007) of the smoothed cumulative gas production curves, was selected to represent ruminal degradation potential of dry and HMC as defined by Hoffman et al. (2012). Because all dry or HMC samples were represented in each run, the run average PAR for all corns was used as an internal standard. For dry and HMC, differences between the 3-run average PAR of the internal standard and the individual run average PAR were added to each test sample observation to minimize between-run variation.

Differences between mean prolamin concentrations, S_r , and RS_r of the rTM and rBM were conducted using the PROC ANOVA procedures of SAS (SAS Institute, 2001), with assay method, corn type, and their interaction in the model. For S_r and RS_r , the absolute or relative (% of mean) absolute differences between duplicates of the same corn were used as population variables for statistical analysis. Relationship differences between in vitro gas production (PAR) by corn type and prolamin concentrations as determined by the rTM were evaluated using the heterogeneity of slopes test (Littell et al., 1993). Because relationships between rBM prolamin concentrations were second order, heterogeneity of slopes tests were conducted on natural logarithmic transformations of PAR values.

Nutrient compositions of dry and HMC used in the evaluation of the rTM and rBM are presented in Table 1. For dry corns, DM, CP, SOLCP, and starch concentrations had ranges of 3.9, 8.5, 2.3, and 20.3 percentage units, respectively. Similar nutrient composition variance was observed within selected HMC, with DM, CP, SOLCP, and starch concentrations with ranges of 14.2, 3.4, 4.3, and 7.1 percentage units, respectively. Likewise, PAR had ranges of 1.1 and 1.7 mL/0.1 g of DM per hour for dry and HMC, respectively, indicating wide ranges of starch degradability potential (Hoffman et al., 2012).

Prolamin concentrations of dry and HMC, as determined by the rTM and rBM, are presented in Table 2. Prolamin concentrations were not different between corn types ($P = 0.71$) but were influenced by assay method ($P < 0.01$) and a corn type by assay interaction ($P < 0.01$) was observed. Because a corn type by assay interaction was observed ($P < 0.01$), summary statistics are presented independently for dry and HMC. Dry corn prolamin as estimated by the rTM ranged from 6.12 to 2.20 g/100 g of DM, with a mean prolamin content of 3.65 g/100 g of DM. The mean dry corn prolamin concentration as estimated by the rBM was almost identical at 3.66 g/100 g of DM. The dry corn S_r for the rTM prolamin determination was 0.38 g/100 g of DM and the RS_r was 11.5% of the mean. The S_r and RS_r indexes of method precision (Bland and Altman, 2010) are almost identical to the S_r and RS_r values of the rTM as originally published by Larson and Hoffman (2008). Corn type did not influence ($P = 0.12$) S_r or RS_r but S_r and RS_r were influenced by assay method ($P < 0.03$) and a corn type by assay interaction ($P = 0.03$) was observed for S_r but not for RS_r ($P = 0.23$).

Statistical differences between the rTM or rBM determination of mean prolamin concentration were primarily related to differences in determining prolamin concentrations in HMC. Mean prolamin concentration in HMC, as estimated by the rTM, was 4.19 g/100 g of DM compared with 3.24 g/100 g of DM when

Table 2. Statistical evaluation of the rapid turbidity or rapid Bradford method to determine prolamins content of dry and high-moisture corn^{1,2}

Item ³	Dry corn		HMC		SE	P-value		
	rTM	rBM	rTM	rBM		Corn	Assay	Corn × assay
Mean	3.65	3.66	4.19	3.24	0.170	0.71	0.01	0.01
S _r	0.38	0.71	0.42	0.43	0.076	0.12	0.03	0.03
RS _r , %	11.5	19.4	10.7	13.3	2.17	0.12	0.02	0.23
Minimum	2.20	2.41	3.10	1.92	—	—	—	—
Maximum	6.12	5.62	4.99	4.53	—	—	—	—
SD	0.94	0.84	0.50	0.69	—	—	—	—

¹All values are expressed as a percentage of DM, unless otherwise specified.

²HMC = high-moisture corn; rTM = rapid turbidity method; rBM = rapid Bradford method.

³S_r = repeatability standard deviation; RS_r = relative S_r.

determined by the rBM. In contrast, the precision (S_r or RS_r) of determining prolamins concentration by the rTM or rBM was primarily related to decreases in precision when measuring prolamins concentrations in dry corn by the rBM.

The biological implications of measuring prolamins proteins in dry or HMC using the rTM is represented in the relationship between in vitro gas production PAR and rTM prolamins concentration (Figure 1). The heterogeneity of slope test (Littell et al., 1993) revealed that corn type ($P = 0.01$) and rTM prolamins concentration ($P < 0.001$) influenced in vitro gas production PAR, with a corn type by prolamins concentration interaction ($P = 0.05$) observed. The prolamins concentration by corn type interaction suggests that measurement of prolamins proteins in dry and HMC using the rTM resulted in independent biological relationships between PAR and prolamins concentration. At greater rTM prolamins concentrations (>5.0 g/100 g of DM), dry and HMC had similar in vitro degradability as indexed by PAR. However, at lesser rTM prolamins concentrations (<3.5 g/100 g of DM), PAR indexes of in vitro degradability of HMC were greater than dry corns.

Modification of the rTM to the rBM appeared to mitigate the independent relationships between PAR and prolamins concentration in dry and HMC (Figure 2). Similar to rTM-determined prolamins, the rBM prolamins concentration ($P < 0.001$) negatively influenced in vitro gas production PAR, but the heterogeneity of slopes test (Littell et al., 1993) revealed that corn type ($P = 0.24$) had no influence on PAR and no rBM prolamins concentration by corn type interaction ($P = 0.38$) was observed. These data indicate that the rBM defined biologically similar prolamins proteins in dry and HMC, which were negatively related to in vitro degradability as indexed by PAR.

In conclusion, the rTM of Larson and Hoffman, (2008) was found to be feasible in quantifying prolamins proteins (zein) in dry corns and data from this inves-

tigation confirm that prolamins proteins are negatively related to in vitro degradation. However, this investigation suggest that the rTM of Larson and Hoffman, (2008) may over estimate functional prolamins that impede starch degradability in fermented feeds such as HMC. Removal of borate phosphate-soluble proteins before solubilization of prolamins proteins by aqueous *tert*-butanol appeared to largely resolve inconsistencies in biological relationships between prolamins protein and in vitro DM degradation potentials of dry and HMC. Finally, quantification of solubilized prolamins proteins using Coomassie brilliant blue powder (Bradford, 1976) was labor efficient and is a well-recognized alternative to turbidity to rapidly and economically quantify complex proteins.

Figure 1. Relationships between peak absolute rate (PAR) of gas production and prolamins as determined by the rapid turbidity method (rTM) for dry corn (—●—; $R^2 = 0.54$, $SE = 0.194$) and high-moisture corn (—□—; $R^2 = 0.34$, $SE = 0.374$).

Figure 2. Relationships between peak absolute rate (PAR) of gas production and prolamin as determined by the rapid Bradford method (rBM) for dry corn (—●—; $R^2 = 0.47$, $SE = 0.239$) and high-moisture corn (—□—; $R^2 = 0.48$, $SE = 0.342$).

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