



## A comparison of methods to quantify prolamin contents in cereals

Gianluca Giuberti, Antonio Gallo & Francesco Masoero

To cite this article: Gianluca Giuberti, Antonio Gallo & Francesco Masoero (2011) A comparison of methods to quantify prolamin contents in cereals, Italian Journal of Animal Science, 10:1, e2, DOI: [10.4081/ijas.2011.e2](https://doi.org/10.4081/ijas.2011.e2)

To link to this article: <http://dx.doi.org/10.4081/ijas.2011.e2>



©Copyright G. Giuberti et al., 2011



Published online: 18 Feb 2016.



Submit your article to this journal [↗](#)



Article views: 143



View related articles [↗](#)



Citing articles: 1 View citing articles [↗](#)

## PAPER

## A comparison of methods to quantify prolamin contents in cereals

Gianluca Giuberti, Antonio Gallo,  
Francesco Masoero

Istituto di Scienze degli Alimenti e della  
Nutrizione, Università Cattolica del Sacro  
Cuore, Piacenza, Italy

### Abstract

Hydrophobic prolamins are endosperm storage proteins accounting for about 40% of the total protein in most cereal seeds. Despite the absence of a reference method, several procedures have been periodically published to quantify prolamins in cereals. The aim of this study was to compare a conventional fractionation assay (LND) *vs* three other methods: one based on sequential extractions (HAM) and two rapid turbidimetric procedures (L&H and DRO). Prolamins were extracted in duplicate on barley, corn and wheat samples. For the turbidimetric prolamin evaluation in barley and wheat, a universally available purified gliadin, as alternative to purified zein, was also tested as standard reference material (SRM). The extraction prolamin values were different among grain types ( $P < 0.01$ ) and methods ( $P < 0.01$ ) without interaction ( $P > 0.05$ ). LND agreed sufficiently well both with HAM and with L&H methods ( $R^2 = 0.664$  and  $R^2 = 0.703$ , respectively,  $P < 0.01$ ). On all tested cereals, LND and L&H gave similar prolamin extraction values ( $P > 0.05$ ), whereas a higher prolamin quantification was obtained using HAM ( $P < 0.05$ ). Overall, DRO did not provide similar comparison and performance parameters with respect to other method comparisons. The effect of changing purified zein with purified gliadin was noteworthy only for L&H, both for wheat and barley samples ( $P < 0.01$ ). Considering the increasing attention of animal nutritionists on prolamins, our results could get useful information for routine laboratory analysis.

### Introduction

Prolamins are the major storage proteins in most cereal seeds. In barley, corn and wheat

they are called hordeins, zeins and gliadins, respectively. In particular, hordeins and gliadins are divided into three main groups based on their molecular weight and sulphur content (Shewry *et al.*, 1999), whereas zeins are classified into four groups in according to their amino acid composition (Buchanan *et al.*, 2000).

Moreover, this proline rich protein class, involved in the starch granule encapsulation (Lasztity, 1984; Buchanan *et al.*, 2000), is able to develop tertiary hydrophobic structures that are soluble in aqueous alcohol mixtures (Momany *et al.*, 2006).

Previous researches usually compared corn from different endosperm texture, founding negative correlations between vitreousness and *in situ* ruminal starch or dry matter (DM) degradability (Philippeau *et al.*, 1997, 1999; Correa *et al.*, 2002; Ngonyamo-Majee *et al.*, 2008b).

Some authors (Hamaker *et al.*, 1995; Larson and Hoffman, 2008; Lopes *et al.*, 2009) reported that vitreous corn types contain higher concentration of prolamins compared with floury or opaque corn types. However, this relationship was not reported by Pratt *et al.* (1995) and Landry *et al.* (2004). These discrepancies could be due to the extraction method used (Philippeau *et al.*, 2000). Currently, the assay of Landry and Moureaux (1970) is recognized to quantify zein as a conventional fractionation method (Hamaker *et al.*, 1995), but is seldom used by labs because it is considered laborious and time-consuming (Landry *et al.*, 2000).

Modifications of the extraction procedure of Landry and Moureaux method (1970) have been periodically evaluated (Wallace *et al.*, 1990; Hamaker *et al.*, 1995; Landry *et al.*, 2000).

Recently, turbidimetric methods have been proposed for a rapid quantification of prolamins in cereals (Drochioiu *et al.*, 2002; Aboubacar *et al.*, 2003; Larson and Hoffman, 2008) and in particular for screening prolamin in large cereals breeding programs.

For these reasons, it is important to verify if the results of less laborious procedures are consistent with those obtained by exhaustive sequential fractionation methods.

The aim of this study was to compare the method of Landry *et al.* (2000) (LND) against the procedures of Hamaker *et al.* (1995) (HAM), Larson and Hoffman (2008) (L&H) and Drochioiu *et al.* (2002) (DRO) on barley, corn and wheat samples.

In addition, since gliadins and hordeins are specific and without homology with zeins (Shewry and Tatham, 1990), a purified gliadin

Corresponding author: Prof. Francesco Masoero, Istituto di Scienze degli Alimenti e della Nutrizione, Università Cattolica del Sacro Cuore, via Emilia Parmense 84, 29100 Piacenza, Italy. Tel. +39.0523.599261 - Fax: +39.0523.599259 E-mail: francesco.masoero@unicatt.it

Key words: Extraction Method, Comparison, Prolamin, Cereals.

Acknowledgments: this project was supported by the AGROSCENARI project "Scenarios for climate change adaptation of Italian agriculture" of the Ministry of Agricultural, Food and Forestry Policies (MIPAAF, Italy).

Received for publication: 2 September 2010.

Revision received: 10 November 2010.

Accepted for publication: 24 November 2010

This work is licensed under a Creative Commons Attribution 3.0 License (by-nc 3.0).

©Copyright G. Giuberti *et al.*, 2011  
Licensee PAGEPress, Italy  
Italian Journal of Animal Science 2011; 10:e2  
doi:10.4081/ijas.2011.e2

was tested as an alternative standard reference material (SRM) for the evaluation of the prolamin concentration for L&H and DRO turbidimetric procedures on wheat and barley.

### Materials and methods

#### Sample preparation

Grain samples of commercially available cereals, eight yellow corn (*Zea mays*, L.), eight barley (*Hordeum vulgare*, L.) and eight wheat (*Triticum aestivum*, L.) grown in the 2009 season in the north of Italy were included in this study. Dried kernels were ground through a 1-mm screen using a laboratory mill (Thomas-Wiley, Arthur H. Thomas Co., Philadelphia, PA, USA) and stored for analysis. Samples were then assayed in duplicate according to the AOAC (2000) for dry matter (DM), crude protein (CP), ash and crude lipids content by methods 930.15, 976.05, 942.05 and 954.02, respectively. Starch content was determined using an enzymatic method (Blasel *et al.*, 2006) modified according to the procedure described by Masoero *et al.* (2010).

#### Reagents and chemicals

The reagents used were acetone, ethanol, hexane, isopropyl alcohol, petroleum ether and 2-mercaptoethanol (2-ME). The solvents were

HPLC grade (the purity of all reagents was higher than 98%) from Carlo Erba (Carlo Erba Reagenti SpA, Rodano, Milano, Italy). The chemical reagents were trichloroacetic acid (TCA), sodium chloride, sodium acetate, sodium borate, and sodium dodecyl sulphate (SDS). The compounds were analytical grade (purity:  $\geq 98\%$ ) from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Purified water was obtained from a Milli-Q Gradient A10 water purification device (Millipore, Bedford, MA, USA).

### Extraction procedures

Prolamins were extracted using four procedures. In particular, LND can divide cereal proteins into distinct fractions (i.e., non-protein nitrogen (NPN), albumins, globulins, prolamins and glutelins), whereas HAM can separate prolamins from non prolamins.

The remaining two turbidimetric methods (L&H and DRO) are prolamin specific. L&H uses a commercial available purified zein to prepare standard curves for the prolamin quantification on whole dry or high moisture corn, while DRO was developed on corn, wheat, barley and rye using no reference materials, but natural standards derived from selected cereal samples. Due to the physico-chemical differences of zeins with respect to barley and wheat prolamins, a purified gliadin (Sigma-Aldrich Co.) was used as an alternative SRM for hordeins and gliadins turbidimetric quantification.

Table 1 summarizes the sequential isolation of multiple protein fractions using the four aforementioned methods. All samples were analysed in duplicate.

### Sequential extraction methods

#### LND method

Samples (100 mg) were defatted by shaking twice with hexane at room temperature (RT) and vacuum dried. The albumin + globulin fraction ( $E_1$  extract) was obtained by removing supernatants isolated from particles (centrifugation at  $12000 \times g$  for 5 min) after two washes with 1 mL of 0.5 M NaCl at  $4^\circ\text{C}$  for 30 min. This initial extraction step was followed by two 15 min washes with water ( $E_2$  extract). The first extract of  $E_2$  was combined with  $E_1$ , while the second was discarded. Prolamins were obtained by a three times extraction using 1 mL of a solution containing 55% (w/w) 2-propanol + 0.6% (v/v) 2-ME ( $E_3$  extract) followed by a two times extraction with 1 mL of 0.5 M NaCl and 0.6% (v/v) 2-ME buffered at pH 10.0 (0.0125 M  $\text{Na}_2\text{B}_4\text{O}_7$ , 12  $\text{H}_2\text{O}$  and 0.02 M NaOH) ( $E_4$  extract).

Consequently, true-glutelin fraction was obtained from the remaining pellet by an extraction repeated three times with the similar sodium borate buffer described above except that NaCl was replaced with 0.5% (w/v) SDS ( $E_5$  extract). Insoluble proteins in the sample residue ( $E_6$  extract) were combined with  $E_5$  to give the evaluation of nitrogen for the glutelin content.

Furthermore, NPN ( $E_0$  extract) was isolated by treating samples with 1 mL of a water TCA solution (100 g/L) (30 min) at  $4^\circ\text{C}$  twice. After each extraction, samples were centrifuged, supernatants were pooled and total nitrogen content was determined by the microKjeldahl method 960.52 (AOAC, 2000) and converted to CP (percent N  $\times 6.25$  for corn and percent N  $\times 5.83$  for barley and wheat samples) in accordance to Drochioiu et al. (2002).

#### HAM method

Samples were initially defatted with petroleum ether solvent. To determine true prolamin content, NPN and albumin + globulin fractions were removed by suspending 200 mg of sample in 2 mL of 0.5M NaCl solution at  $4^\circ\text{C}$ . On following, the solution was centrifuged ( $5000 \times g$  for 10 min) and the supernatant discarded. The operation was repeated three times in NaCl solution and twice in water. The resulting pellet was gently shaken (300 rpm for 1 h; Universal table Shaker 709, ASAL srl, Milano, Italy) with 2 mL of a solution containing 0.0125 M sodium borate, 10 g/L SDS and 2% (v/v) 2-ME (pH 10.0). The solution was centrifuged and the recovered supernatants were pooled. The procedure was repeated twice. Then ethanol was added to the supernatants to a final concentration of 70% (v/v); the mixture was centrifuged after a 2 h suspension step.

The obtained supernatant, containing the prolamin fraction, was dried and the total nitrogen was determined by the microKjeldahl method described above (AOAC, 2000; Drochioiu et al., 2002).

#### Turbidimetric methods

##### L&H method

Samples were defatted using acetone, filtered and dried. Acetone-insoluble DM (aiDM) was retained. A 200 mg of aiDM sample was solubilised using 20 mL of a solution containing 55% (w/w) 2-propanol + 0.6% (v/v) 2-ME on a magnetic stir plate for 4 h.

After mixing, the solution was centrifuged ( $4500 \times g$  for 20 min) and 0.5 mL of the supernatant was added to a spectrophotometer tube containing 5.5 mL of 0.15 M TCA solution and mixed through a vortex. The turbidity was

**Table 1. Sequential isolation of multiple protein fractions, by using LND (Landry et al., 2000), HAM (Hamaker et al., 1995), L&H (Larson and Hoffman, 2008) and DRO (Drochioiu et al., 2002) methods.**

Fraction	LND <sup>o</sup>			HAM <sup>o</sup>			L&H <sup>o</sup>			DRO <sup>o</sup>		
	Solvent		Time <sup>#</sup>	Solvent		Time <sup>#</sup>	Solvent		Time <sup>#</sup>	Solvent		Time <sup>#</sup>
NPN	10% (w/v) TCA,	$4^\circ\text{C}$	30, 30									
Alb/Glo	0.5 M NaCl,	$4^\circ\text{C}$	30, 30	0.5 M NaCl,	$4^\circ\text{C}$	30, 30, 30						
	Water,	$4^\circ\text{C}$	15, 15	Water,	$4^\circ\text{C}$	15, 15						
Pro	55% (w/w) 2-propanol +		30, 30, 15	1% (w/v) SDS, 2%		60, 60	55% (w/w) 2-propanol +	240	70% ethanol + 0.5% (w/v)		60	
	0.6% (v/v) 2-ME, RT			(v/v) 2-ME, pH 10.0 <sup>s</sup> , RT			0.6% (v/v) 2-ME, RT		sodium acetate, RT			
Glu	0.5 M NaCl, 0.6% (v/v)		15, 15	Ethanol <sup>^</sup>		120						
	2-ME, pH 10.0 <sup>s</sup> , RT											
Residue	0.5% (w/v) SDS, 0.6%		30, 30, 15									
	(v/v) 2-ME, pH 10.0 <sup>s</sup> , RT											

NPN, non-protein Nitrogen; Alb/Glo, albumin + globulin; Glu, glutelin; RT room temperature <sup>o</sup>Preliminary defatting step with: hexane (LND), petroleum ether (HAM and DRO), acetone (L&H); <sup>#</sup>Extraction time (min); <sup>s</sup>Sodium borate buffer (0.0125M  $\text{Na}_2\text{B}_4\text{O}_7$ , 12  $\text{H}_2\text{O}$  and 0.02M NaOH); <sup>^</sup>To a final concentration of 70%.

allowed to equilibrate for 45 min.

Prolamin proteins in all samples were quantified by using a standard absorbance curve developed from purified zein (Acros Organics, Thermo Fisher Scientific, Waltman, MA, USA).

Moreover, purified gliadin (Sigma-Aldrich Co.) was employed as an alternative SRM for wheat and barley samples. The absorbance was read at 440 nm on a double beam spectrophotometer (PerkinElmer, San Jose, CA, USA) for both samples and SRMs.

#### DRO method

Samples were defatted with petroleum ether. A 200 mg defatted sample was weighed into a 50 mL Erlenmeyer flask, 20 mL of ethanol added (containing 5 g/L sodium acetate), shaken for 1 h and filtered. After filtration, 1 mL of the solution was transferred into a colorimeter tube containing 5 mL of 0.15 M TCA solution and shaken vigorously. After 60 min, the absorbance was read at 440 nm on a double beam spectrophotometer using standard absorbance curves as described above.

#### Statistical analyses

The data of chemical composition and protein fraction characterisation were tested for normality using the Shapiro-Wilk test. A one-way analysis of variance (ANOVA) was analysed by the general linear model (GLM) procedure of SAS (2003) according to the model:

$$Y_{ij} = \mu + M_i + \varepsilon_{ij};$$

where:  $Y_{ij}$  = observation,  $\mu$  = overall mean,  $M_i$  = fixed effect grain type ( $i = 1-3$ ), and  $\varepsilon_{ij}$  = residual error.

Prolamin content determined by using the tested methods was evaluated with the GLM procedure (SAS, 2003) according to a factorial model:

$$Y_{ijk} = \mu + M_i + T_j + (M \times T)_{ij} + \varepsilon_{ijk};$$

where:  $Y_{ijk}$  = observation,  $\mu$  = overall mean,  $M_i$  = fixed effect grain type ( $i = 1-3$ ),  $T_j$  = fixed effect of used method ( $j = 1-4$ ),  $(M \times T)_{ij}$  = first order interaction, and  $\varepsilon_{ijk}$  = residual error. Significance was declared at  $P \leq 0.05$  and  $P \leq 0.01$ .

The method comparisons were performed in agreement with Bland and Altman (1995): the prolamin content obtained using LND was compared with the results of HAM, L&H and DRO. LND was chosen as the reference method, because of its marked influence in developing corn protein fractionation methods. The first measurement by each method (replicate 1) was used to calculate the method comparison parameters, i.e., mean of differences (MD), standard deviation of differences, standard error of differences (SR), 95% limits of

agreement, while both measurements (replicates 1 and 2) were used to calculate the performance parameters of each method, i.e., coefficient of repeatability (CR) and relative repeatability standard deviation (RSr). The MD and the SR between measurements were calculated for the conventional method (i.e., LND) vs each method (i.e., HAM, L&H and DRO) and were used as indicators of mean bias (relative to LND). The 95% limits of agreement were calculated as the mean difference  $\pm 1.96$  times the standard deviation of differences. Thus, the observed value of a given method should be greater than the LND value minus the lower limit and less than the LND value plus the upper limit.

The CR and the RSr were calculated in agreement with Bland and Altman (1995) and Theander *et al.* (1995), respectively. Both CR and RSr were used as indicators of the precision of methods. Linear regression analyses were conducted both on all data and on each cereal type (i.e., barley, corn and wheat) between the reference method and each tested method using the PROC REG (SAS, 2003). The bias of each method (i.e., HAM, L&H and DRO) compared with the reference method (LND) was determined by the regression of the mean of differences between the observed values and LND values against the mean of observed and LND values.

## Results

### Chemical composition of grains

The chemical composition of cereals is shown in Table 2. As expected, the starch con-

tent varied being 68.61, 61.34 and 55.92 g/100 g of DM in corn, wheat and barley ( $P < 0.01$ ), respectively. Differences among cereals were also measured in lipid and ash contents ( $P < 0.01$ ). The CP content was lowest in corn (8.36 g/100 g of DM,  $P < 0.01$ ) compared with barley and wheat (13.51 g/100 g and 13.62 g/100 g of DM, respectively).

The cereals multiple protein fractions performed by LND are also presented in Table 2. Wheat had the lowest NPN ( $E_0$ ) and the highest albumin + globulin ( $E_{1,2} - E_0$ ) contents ( $P < 0.01$ ). Moreover gliadins content ( $E_{3,4}$ ) was about 27% and 45% higher ( $P < 0.05$ ) than hordeins and zeins, respectively. Besides, glutelin content ( $E_{5,6}$ ) was lower in corn than in wheat and barley (3.66 vs 5.16 and 7.61 g/100 g of DM, respectively,  $P < 0.01$ ).

The method protein extraction yields (calculated as percentage of  $(NPN, g/100g \text{ DM} + Alb+Glob, g/100g \text{ DM}) + Prolamin, g/100g \text{ DM} + Glutelin, g/100g \text{ DM} / Crude \text{ protein}, g/100g \text{ DM}$ ) were similar among grain types ( $P > 0.05$ ) with values ranging from 101.32 to 105.11%.

### Effect of purified gliadin as standard reference material on turbidimetric quantification in barley and wheat

Utilizing purified gliadin for the L&H's standard curves, the prolamin extraction values were about 2-fold higher than that obtained with purified zein (4.14 vs 2.25 g/100 g of DM for barley and 5.20 vs 2.66 g/100 g of DM for wheat, respectively,  $P < 0.01$ ) (Figure 1, section A). A not significant increase ( $P > 0.05$ ) in prolamin quantification values was observed for DRO with the alternative SRM (Figure 1, section B).

**Table 2. Chemical composition and multiple protein fraction characterization of barley, corn and wheat samples.**

	Barley	Corn	Wheat	SEM
Chemical composition, g/100 g DM				
Starch	55.92 <sup>A</sup>	68.61 <sup>C</sup>	61.34 <sup>B</sup>	0.962
Crude lipid	1.46 <sup>A</sup>	3.83 <sup>C</sup>	1.64 <sup>B</sup>	0.004
Ash	2.74 <sup>C</sup>	1.37 <sup>A</sup>	1.85 <sup>B</sup>	0.150
Crude protein <sup>o</sup>	13.51 <sup>B</sup>	8.36 <sup>A</sup>	13.62 <sup>B</sup>	0.445
Protein fraction <sup>#</sup> , g/100 g DM				
NPN	0.67 <sup>B</sup>	0.68 <sup>B</sup>	0.29 <sup>A</sup>	0.046
Alb+Glob	2.05 <sup>B</sup>	0.83 <sup>A</sup>	3.78 <sup>C</sup>	0.136
Prolamin <sup>s</sup>	3.87 <sup>a</sup>	3.30 <sup>a</sup>	4.92 <sup>b</sup>	0.266
Glutelin <sup>^</sup>	7.61 <sup>C</sup>	3.66 <sup>A</sup>	5.16 <sup>B</sup>	0.220
Extraction yield <sup>\$</sup> , %	105.11	101.32	103.89	1.045

NPN, non-protein nitrogen ( $E_0$ , Landry *et al.*, 2000); Alb+Glob, albumin + globulin ( $E_{1,2} - E_0$ , Landry *et al.*, 2000); <sup>a,b,c,A,B,C</sup> Means with different superscripts are significantly different ( $P < 0.05$  and  $P < 0.01$ ); <sup>o</sup> content for each fraction was obtained by multiplying total amino nitrogen x 6.25 for corn and x 5.83 for barley and wheat samples (Drochioiu *et al.*, 2002); <sup>#</sup> Extracted with LND (Landry *et al.*, 2000); <sup>s</sup>  $E_{3,4}$  (Landry *et al.*, 2000); <sup>^</sup>  $E_{5,6}$  (Landry *et al.*, 2000); <sup>\$</sup>  $(NPN, g/100g \text{ DM} + (Alb+Glob, g/100g \text{ DM}) + Prolamin, g/100g \text{ DM} + Glutelin, g/100g \text{ DM}) / Crude \text{ protein}, g/100g \text{ DM} \times 100$ .



### Prolamin extraction methods

The prolamin contents of barley, corn and wheat samples estimated by the four methods are presented in Table 3. For the two tested turbidimetric methods (i.e., L&H and DRO), the extraction data were calculated utilizing purified zein for corn and purified gliadin for barley and wheat samples as SRMs.

The values obtained by using the four methods significantly differed ( $P < 0.01$ ) and the

prolamin contents were different among grain types ( $P < 0.01$ ). No significant effect was due to grain type x method interaction ( $P = 0.335$ ).

Compared to the reference method (i.e., LND), although there were not significant differences ( $P > 0.05$ ), L&H gave lower zein and higher hordein and gliadin values (-17%, +7% and +6%, respectively). Overall, for all tested cereals, the highest ( $P < 0.05$ ) and the lowest ( $P < 0.01$ ) prolamin quantifications were

obtained with HAM and DRO, respectively.

The lowest CR and RSr values were measured for LND (0.27 and 3.40% of the mean, respectively).

The comparison results between LND vs HAM, L&H and DRO are presented in Table 4. Comparing LND with HAM or L&H, low MD (-0.51 and -0.07, respectively) and similar SR (0.134 and 0.135, respectively) were obtained. In contrast, when LND was compared with

**Table 3. Prolamin content (g/100 g DM) in barley, corn and wheat performed by LND (Landry et al., 2000), HAM (Hamaker et al., 1995), L&H (Larson and Hoffman, 2008) and DRO (Drochioiu et al., 2002) methods.**

	Method <sup>o</sup>											
	LND <sup>#</sup>			HAM <sup>†</sup>			L&H <sup>§</sup>			DRO <sup>§</sup>		
	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
<b>Barley</b>												
1	3.66	3.62	3.64	3.36	3.62	3.49	3.60	3.70	3.65	2.80	2.31	2.56
2	3.52	3.60	3.56	3.69	4.26	3.98	4.49	4.27	4.38	3.33	3.07	3.20
3	3.52	3.49	3.51	5.02	4.93	4.98	4.54	4.52	4.53	3.89	3.67	3.78
4	3.87	3.77	3.82	5.31	5.14	5.23	4.04	4.13	4.08	3.12	3.64	3.38
5	2.95	2.99	2.97	4.01	4.09	4.05	3.10	3.27	3.18	2.40	2.55	2.47
6	3.54	3.53	3.54	3.94	3.82	3.88	3.51	3.54	3.52	2.46	3.38	2.92
7	5.41	5.44	5.43	6.38	6.61	6.49	5.34	5.02	5.18	5.04	5.26	5.15
8	4.43	4.65	4.54	4.90	4.82	4.86	4.93	4.23	4.58	3.21	3.71	3.46
		3.87				4.62			4.14			3.37
<b>Corn</b>												
1	2.56	2.50	2.53	3.68	3.71	3.70	2.12	2.27	2.20	0.93	1.07	1.00
2	2.72	2.59	2.66	3.70	3.66	3.68	2.33	2.15	2.24	1.05	1.06	1.05
3	3.44	3.38	3.41	3.82	3.85	3.83	2.76	2.85	2.81	2.06	1.96	2.01
4	4.78	4.86	4.82	4.24	4.22	4.23	3.49	3.10	3.29	1.74	1.68	1.71
5	4.31	4.22	4.26	3.69	3.59	3.64	2.79	2.46	2.63	3.25	3.46	3.35
6	2.53	2.70	2.62	2.78	2.82	2.80	2.86	2.74	2.80	1.96	1.73	1.85
7	3.47	3.44	3.46	2.56	2.58	2.57	2.86	2.63	2.75	0.96	0.95	0.96
8	2.65	2.67	2.66	3.30	3.24	3.27	3.41	3.15	3.28	1.90	1.87	1.89
	3.30				3.46				2.75		1.73	
<b>Wheat</b>												
1	4.75	4.66	4.71	4.80	4.44	4.62	4.61	4.55	4.58	2.82	3.14	2.98
2	4.02	3.99	4.00	4.99	4.45	4.72	4.22	4.34	4.28	2.45	3.19	2.82
3	5.43	5.71	5.57	6.69	6.37	6.53	6.26	6.04	6.15	4.37	4.49	4.43
4	4.46	4.88	4.67	4.93	4.80	4.86	5.04	4.98	5.01	3.91	3.33	3.62
5	5.41	5.40	5.41	5.54	5.47	5.50	5.42	5.36	5.39	4.39	3.98	4.19
6	5.14	5.25	5.19	5.72	5.50	5.61	5.95	5.83	5.89	3.46	3.99	3.72
7	5.58	5.47	5.53	6.12	6.12	6.12	6.16	6.02	6.09	4.61	4.12	4.36
8	4.20	4.38	4.29	5.38	5.37	5.37	4.21	4.25	4.23	3.38	2.70	3.04
		4.92				5.42				5.20		3.65
CR		0.27				0.43				0.45		0.81
RSr, % of the mean		3.40				4.81				5.13		14.39
	DF	SS	MS	P								
<b>Factorial analysis</b>												
Grain type (G)	2	63.98	31.99	< 0.01								
Method (M)	3	32.66	10.92	< 0.01								
G x M interaction	6	3.69	0.62	0.335								
Error	84											

CR, coefficient of repeatability (Bland and Altman, 1995); RSr, relative repeatability standard deviation (Theander et al., 1995); <sup>o</sup>Values 1 and 2 indicate individual prolamin (replicates 1 and 2, respectively) determinations by the four methods; <sup>†</sup>Reported values calculated by multiplying total amino nitrogen x 6.25 for corn and x 5.83 for barley and wheat (Drochioiu et al., 2002); <sup>§</sup>Reported values calculated by using purified zein for corn and purified gliadin for barley and wheat samples as standard reference material.

DRO, higher MD and SR values (+1.12 and 0.151, respectively) were calculated.

Consequently, for this method comparison (i.e., LND vs DRO), a numeric wide range between the lower and the upper limits of agreement was calculated (being -0.33 and +2.57, respectively).

The regression analysis, conducted on the complete dataset, confirmed different agreement between methods (Table 4). The lowest  $R^2$  was measured for LND vs DRO ( $R^2=0.595$ ,  $P<0.01$ ) when compared with LND vs HAM and LND vs L&H, measuring  $R^2=0.664$  and  $R^2=0.703$ , respectively ( $P<0.01$ ).

By plotting the mean of individual differences of prolamin content measured with the reference method (i.e. LND) vs HAM, L&H or DRO against the mean of the prolamin values (g/100 g of DM) measured by the reference method and each tested method, no marked difference was shown in the variability between measurements (Figure 2, sections A, B and C, respectively). However, the lowest relationship was measured for LND versus HAM ( $R^2=0.060$ ,  $P=0.248$ , Figure 2, section A).

## Discussion

To measure cereal protein fractions, several laboratory methods based on different extraction solvents and procedures have been periodically published (Landry and Moureaux, 1970; Paulis *et al.*, 1974; Wallace *et al.*, 1990; Hamaker *et al.*, 1995; Landry *et al.*, 2000; Drochioiu *et al.*, 2002; Aboubacar *et al.*, 2003; Larson and Hoffman, 2008). In particular, the assay of Landry and Moureaux (1970) is historically considered as the most prominent method, especially for corn (Hamaker *et al.*, 1995; Landry *et al.*, 2000). Unfortunately, the lack of a reference method could result in uncertain prolamin quantification especially for routine lab analysis. Moreover, the increased attention in analysis of cereal grains for prolamin content (Philippeau *et al.*, 2000; Correa *et al.*, 2002; Lanzas *et al.*, 2007; Olakojo *et al.*, 2007; Ngonyamo-Majee *et al.*, 2008b; Lopes *et al.*, 2009), promote the interest both on the comparison of several matrices and procedure extraction results and on the applicability of the more rapid methods for screening prolamin in large cereals breeding programs.

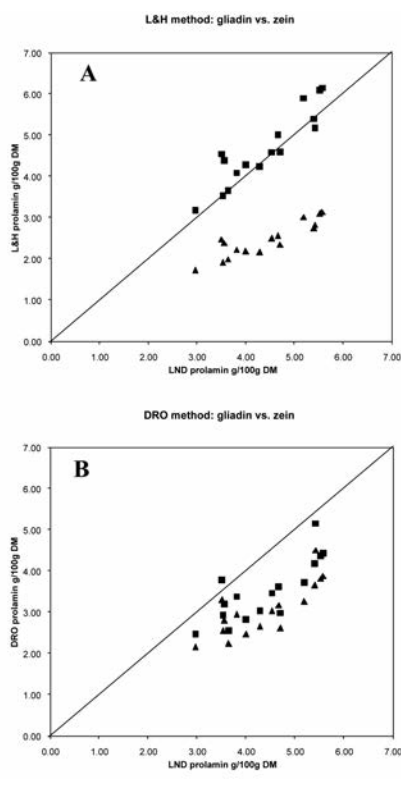
For these reasons, 24 commercially available cereals (i.e., barley, yellow corn and wheat) were tested. The high variability in prolamin content among and within cereals found in the present work could be due to several factors, such as genotype differences, stage of

**Table 4. Comparison between LND (Landry *et al.*, 2000) vs HAM (Hamaker *et al.*, 1995), L&H (Larson and Hoffman, 2008) and DRO (Drochioiu *et al.*, 2002) methods on barley, corn and wheat samples (n=24).**

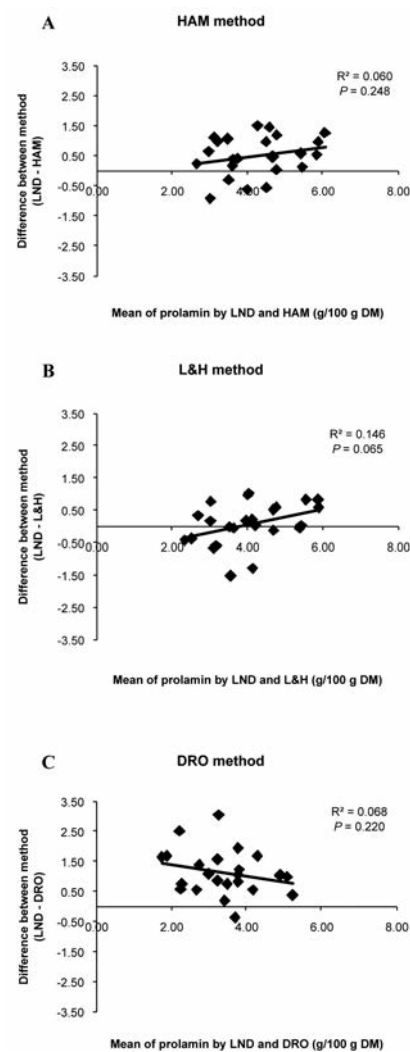
	Comparison between methods <sup>o</sup>							
	95% Limits of agreement				Slope estimate <sup>#</sup>	SE	P <sup>§</sup>	R <sup>2</sup>
	MD	SR	Lower limit	Upper limit				
LND vs HAM	-0.51	0.134	-1.79	+0.77	0.943	0.666	0.01	0.664
LND vs L&H	-0.07	0.135	-1.37	+1.23	1.048	0.676	0.01	0.703
LND vs DRO	+1.12	0.151	-0.33	+2.57	0.914	0.750	0.01	0.595

MD, mean of differences; SR, standard error of differences; <sup>o</sup>performed with Bland and Altman (1995); <sup>#</sup>intercept did not differ from 0 ( $P>0.05$ ); <sup>§</sup>P of the model of regression analysis.

maturity and nutritional status of the plant (Lasztity, 1984; Landry *et al.*, 2002; Larson and Hoffman, 2008). However, the coefficients of variability obtained for each tested method within grains resulted similar or lower than those reported by original papers (Hamaker *et al.*, 1995; Drochioiu *et al.*, 2002; Larson and Hoffman, 2008).



**Figure 1. Plot of prolamin content determined by L&H (Larson and Hoffman, 2008) (A) and by DRO (Drochioiu *et al.*, 2002) (B) methods in wheat and barley by using purified gliadin or purified zein as standard reference material vs reference values obtained by LND (Landry *et al.*, 2000).**



**Figure 2. Mean of individual differences of prolamin content measured using the reference method (LND, Landry *et al.*, 2000) vs HAM (Hamaker *et al.*, 1995), L&H (Larson and Hoffman, 2008), and DRO (Drochioiu *et al.*, 2002) methods (A, B and C, respectively) against the mean of the prolamin values (g/100g of DM) measured by reference method and each tested method.**

## Effect of the standard reference material, zein or gliadin, on turbidimetric methods performances

Recently, less laborious methods for quantifying prolamin in cereals were published (Drochioiu *et al.*, 2002; Larson and Hoffman, 2008).

Moreover, Larson and Hoffman (2008) suggested that improvements should be carried out. When the rapid L&H method was used to evaluate prolamin content in barley and wheat by using purified zein as SRM, lower values were measured with respect both to LND ( $P < 0.01$ ) and to literature values (Lasztity, 1984; Drochioiu *et al.*, 2002), which reported hordeins and gliadins content of about 40 and 45 g/100 g of CP, respectively.

Those observations suggest that using purified zein as SRM for prolamin quantification in barley and wheat may be ill advised: hordeins and gliadins are essentially similar with no structural homology with zeins (Shewry and Tatham, 1990; Shewry and Halford, 2002). This justifies the use of purified gliadin instead of purified zein as SRM to quantify hordeins and gliadins in rapid turbidimetric methods.

Moreover the utilization of a universally available prolamin source (purified zein or gliadin) for developing standard curves would be useful to facilitate procedural uniformity among labs (Larson and Hoffman, 2008).

In particular, when L&H was applied on wheat and barley using purified zein as SRM, the mean prolamin values of the unknown samples were 2.66 and 2.25 g/100 g of DM, respectively. On the contrary, higher values ( $P < 0.01$ ) were predicted using a linear standard curve developed with purified gliadin (5.20 and 4.14 g/100 g of DM, respectively).

A not significant improvement ( $P > 0.05$ ) in prolamin quantification due to the alternatively use of purified gliadin was obtained for DRO. This seems to suggest that an incomplete solubilisation of purified gliadin was obtained by using ethanol and sodium acetate in the DRO standard solution. Moreover, a lower quantitative precipitation of prolamin upon addition of TCA (Bensadoun and Weinstein, 1976) could further explain these turbidity differences.

## Comparison between prolamin extraction procedures

Usually, the original LND and HAM methods expressed their results as a percentage of the total protein recovered (Landry *et al.*, 2000; Hamaker *et al.*, 1995), whereas DRO and L&H used a DM, a CP or a starch basis (Drochioiu *et al.*, 2002; Larson and Hoffman, 2008). In this

trail, in order to have an uniform and a prompt view of all comparison data, the authors preferred to express data with a common DM basis. Moreover, a multiplier of 6.25 for corn and of 5.83 for barley and wheat was employed to convert N to CP for the microKjeldahl determination (Drochioiu *et al.*, 2002). Taking into account the differences in experimental conditions, when data reported in literature are recalculated (from the graphed and tabular values) on a DM basis, similar prolamin values were observed (Lasztity, 1984; Landry *et al.*, 2000; Drochioiu *et al.*, 2002).

Based on literature comparison (Lasztity, 1984), the LND procedure was found to be feasible in differentiating and quantifying the protein fractions in all tested cereals. Moreover, according to the authors (Landry *et al.*, 2000), the method seems to give exhaustive protein extractions not only for corn (where it was initially developed), but also for barley and wheat samples, confirming its validity.

Focussing on the comparison between procedures (i.e., LND, HAM, L&H and DRO), they gave different prolamin quantification ( $P < 0.01$ ). In any case, the grain type  $\times$  method interaction was not significant ( $P = 0.335$ ), suggesting that the discrepancies in the prolamin quantification measured with the four tested methods should be considered constant among cereals and not influenced by the matrix effect.

By the comparison between LND *vs* HAM on all the data set, a good agreement was calculated. In particular, a low standard error of difference (i.e., 0.134) and a moderate correlation ( $R^2 = 0.664$ ,  $P < 0.01$ ) were measured. Despite these considerations, prolamin extraction values obtained with HAM were higher ( $P < 0.05$ ) than those determined with LND.

Similarly, higher zein and kafirin values ( $P < 0.05$ ) were reported by Hamaker *et al.* (1995), when their method was compared to the Landry and Moureaux (1970) assay. The authors suggested that prolamins were more extractable by their method because protein fractions are first extracted in a common buffer and then separated from non-prolamin proteins. Instead in LND, using extraction solvents and procedures similar to Landry and Moureaux (1970), some prolamins could be retained in the fractions E<sub>5,6</sub> (glutelins fraction), causing a underestimated prolamins quantification (Hamaker *et al.*, 1995).

When LND was compared to L&H, the lowest mean of difference (-0.07) and the highest correlation ( $R^2 = 0.703$ ,  $P < 0.01$ ) were found. Even if similar prolamin extraction values were reported ( $P > 0.05$ ), L&H seems to be more efficient in the quantification of prolamin in barley or wheat (using the appropriate SRM) rather

than in corn samples with respect to LND, where a numeric reduction of about 20% was obtained. A possible explanation for these observations is that aqueous isopropyl alcohol (without and with a reducing agent) was unable to extract all of the zeins exhaustively (Paulis *et al.*, 1991; Dombrink-Kurtzman and Bietz, 1993). Overall, the differences between LND and L&H resulted not influenced ( $R^2 = 0.146$ ,  $P = 0.095$ ) by average prolamin values, even if the turbidimetric method tended to quantify less prolamin proteins under the value of 4 g/100 g DM. In spite of these considerations, when zeins were quantified ( $n = 8$ ), the agreement between LND *vs* HAM and L&H resulted not significant ( $R^2 = 0.203$ ,  $P = 0.263$  and  $R^2 = 0.199$ ,  $P = 0.261$ , respectively) and lower than those observed for hordeins ( $R^2 = 0.633$  and  $R^2 = 0.673$ , respectively,  $P < 0.01$ ) and gliadins ( $R^2 = 0.629$  and  $R^2 = 0.875$ , respectively,  $P < 0.01$ ). To our knowledge, no published works reported data about the agreement between LND and HAM on corn. On the contrary Larson and Hoffman (2008), by comparing their method against LND, reported a good agreement ( $R^2 = 0.88$ ) and a limited bias (0.13 g/100 g DM) between alcohol-soluble protein of aiDM (Landry *et al.*, 2000) and zein content estimated by their turbidimetric procedure. The different results obtained by Larson and Hoffman (2008) could be explained both by the quantification used during the development of LND, where a Bradford assay (Pierce, Rockford, IL, USA) (Bradford, 1976) was employed, and by the choice of analyzed corn. Specifically, some corn types used by these authors (i.e., floury and opaque) are not typical yellow dent or flint hybrids commercially available to livestock producers (Larson and Hoffman, 2008). Anyway, the three methods (i.e., LND, HAM and L&H) resulted highly repeatable, being their RSR lower than 6% of the mean. Larson and Hoffman (2008) reported a higher RSR (12.8% of the mean) for the zein determination in different corn endosperm varieties. In all cases, with respect to LND, the prolamin extraction values reported with DRO were lower ( $MD = +1.12$ ;  $P < 0.01$ ) and, as seen before for HAM and L&H, not significantly correlated on zein values ( $R^2 = 0.228$ ,  $P = 0.221$ ). Olakojo *et al.* (2007), by using the DRO method, tended to obtain low zein values too (Larson and Hoffman, 2008). Moreover, DRO resulted in lower method repeatability ( $RSr = 14.39\%$  of the mean) and weaker relationship with LND ( $R^2 = 0.595$ ,  $P < 0.01$ ) considering all the tested cereals. This could be due to limitations in prolamin extraction by using aqueous ethanol in combination with sodium acetate as a reducing agent in lieu of aqueous propanol and 2-ME.



## Conclusions

The study shows that the conventional LND agree sufficiently well with two methods (i.e., HAM and L&H) for prolamins determination. In particular HAM was able to improve the prolamins extraction from all tested cereals and could be considered less laborious than LND because it allows prolamins to be extracted in one group. Also L&H, by employing the correct SRM (purified zein or gliadin), could represent a good "ranking method", even if this procedure, in the original paper, lacks in the electrophoretic step for characterizing the purity of its protein extracts. The quantification of prolamin proteins could be useful, together with *in vitro*, *in situ* or *in vivo* digestion trials, to increase understanding of factors hypothetical related to starch digestibility and ruminant animal performance.

## References

- Aboubacar, A., Axtell, J.D., Nduulu, L., Hamaker, B.R., 2003. Turbidity assay for rapid and efficient identification of high protein digestibility sorghum lines. *Cereal Chem.* 80:40-44.
- AOAC, 2000. Official Methods of Analysis. 17th ed. AOAC, Gaithersburg, MD, USA.
- Bensadoun, A., Weinstein, D., 1976. Assay of proteins in the presence of interfering materials. *Anal. Biochem.* 70:241-250.
- Bland, M.J., Altman, D.G., 1995. Comparing methods of measurements: why plotting difference against a standard method is misleading. *Lancet* 346:1085-1087.
- Blasel, H.M., Hoffman, P.C., Shaver, R.D., 2006. Degree of starch access: an enzymatic method to determine starch degradation potential of corn grain and corn silage. *Anim. Feed Sci. Tech.* 128:96-107.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Buchanan, B.B., Gruissmen, W., Jones, R.L., 2000. Biochemistry and molecular biology of plants. Am. Soc. of Plant Physiol. Publ., Rockville, MD, USA.
- Correa, C.E.S., Shaver, R.D., Pereira, M.N., Lauer, J.G., Kohn, K., 2002. Relationship between corn vitreousness and ruminal *in situ* starch degradability. *J. Dairy Sci.* 85:3008-3012.
- Dombrink-Kurtzman, M.A., Bietz, J.A., 1993. Zein composition in hard and soft endosperm of maize. *Cereal Chem.* 70:105-108.
- Drochioiu, G., Druta, I., Petrovanu, M.N., Strajeru, S., 2002. A rapid method used at the Suceava Genebank to evaluate protein quality of some cereal grains. *PGR Newsletter* 129: 47-51.
- Hamaker, B.R., Mohamed, A.A., Habben, J.E., Huang, C.P., Larkins, B.A., 1995. Efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamin contents than the conventional method. *Cereal Chem.* 72:583-588.
- Landry, J., Delhaye, S., Damerval, C., 2002. Comparative efficiencies of isopropyl and tert-butyl alcohols for extracting zeins from maize endosperm. *J. Agr. Food Chem.* 50:4131-4134.
- Landry, J., Delhaye, S., Damerval, C., 2000. Improved method for isolating and quantitating  $\alpha$ -amino nitrogen as non-protein, true protein, salt-soluble proteins, zeins, and true glutelins in maize endosperm. *Cereal Chem.* 77:620-626.
- Landry, J., Delhaye, S., Damerval, C., 2004. Protein distribution pattern in flourey and vitreous endosperm of maize grain. *Cereal Chem.* 81:153-158.
- Landry, J., Moureaux, T., 1970. Heterogenicity of the glutelins of the grain core. Selective extraction and composition in amino acids of the three isolated fractions. *B. Soc. Chim. Biol.* 52: 1021-1037.
- Lanzas, C., Fox, D.G., Pell, A.N., 2007. Digestion kinetics of dried cereal grains. *Anim. Feed Sci. Tech.* 136:265-280.
- Larson, J., Hoffman, P.C., 2008. Technical note: a method to quantify prolamin proteins in corn that are negatively related to starch digestibility in ruminants. *J. Dairy Sci.* 91:4834-4839.
- Lasztity, R., 1984. The Chemistry of Cereal Proteins. 2nd edition. CRC Press, Inc., Boca Raton, FL, USA.
- Lopes, J.C., Shaver, R.D., Hoffman, P.C., Akins, M.S., Bertics, S.J., Gencoglu, H., Coors, J.G., 2009. Type of corn endosperm influences nutrient digestibility in lactating dairy cows. *J. Dairy Sci.* 92:4541-4548.
- Masoero, F., Gallo, A., Zanfi, C., Giuberti, G., Spanghero, M., 2010. Chemical composition and rumen degradability of three corn hybrids treated with insecticides against the European corn borer (*Ostrinia nubilalis*). *Anim. Feed Sci. Tech.* 155:25-32.
- Momany, F.A., Sessa, D.J., Lawton, J.W., Selling, G.W., Hamaker, S.A., Willet, J.L., 2006. Structural characterization of alpha-zein. *J. Agr. Food Chem.* 54:543-547.
- Ngonyamo-Majee, D., Shaver, R.D., Coors, J.G., Sapienza, D., Lauer, J.G., 2008. Relationship between kernel vitreousness and dry matter degradability for diverse corn germplasm: II. Ruminant and post-ruminal degradabilities. *Anim. Feed Sci. Tech.* 142:259-274.
- Olakojo, S.A., Omueti, O., Ajomale, K., Ogumbodede, B.A., 2007. Development of quality protein maize: biochemical and agronomic evaluation. *Tropical and Subtropical Agroecosystems* 7:97-104.
- Paulis, J.W., Bietz, J.A., Lambert, R.J., Villegas, E.M., 1991. Identification of modified high-lysine maize genotypes by reversed-phase high-performance liquid chromatography. *Cereal Chem.* 68:361-365.
- Paulis, J.W., Wall, J.S., Kwolek, W.F., 1974. A rapid turbidimetric analysis for zein in corn and its correlation with lysine content. *J. Agr. Food Chem.* 22: 313-315.
- Philippeau, C., Landry, J., Michalet-Doreau, B., 2000. Influence of the protein distribution of maize endosperm on ruminal starch degradability. *J. Sci. Food Agr.* 80:404-408.
- Philippeau, C., Le Deschault de Monredon, F., Michalet-Doreau, B., 1999. Relationship between ruminal starch degradation and the physical characteristics of corn grain. *J. Anim. Sci.* 77:238-243.
- Philippeau, C., Michalet-Doreau, B., 1997. Influence of genotype and stage of maturity of maize on rate of ruminal starch degradation. *Anim. Feed Sci. Tech.* 68: 25-35.
- Pratt, R.C., Paulis, J.W., Miller, K., Nelsen, T., Bietz, J.A., 1995. Association of zein classes with maize kernel hardness. *Cereal Chem.* 72:162-167.
- SAS, 2003. Guide for Personal Computers, Version 9.13. SAS Inst. Inc., Cary, NC, USA.
- Shewry, P.R., Halford, N.G., 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. *J. Exp. Bot.* 53:947-958.
- Shewry, P.R., Tatham, A.S., 1990. The prolamin storage proteins of cereals seeds: structure and evolution. *Biochem. J.* 267:1-12.
- Shewry, P.R., Tatham, A.S., Halford, N.G., 1999. The prolamins of Triticeae. In: P.R. Shewry and R. Casey (eds.) *Seed Proteins*. Kluwer Academic Publ., Dordrecht, The Netherlands.
- Theander, O., Aman, P., Westerlund, E., Anderson, R., Petterson, D., 1995. Total dietary fiber determined as neutral sugar residues, uronic acid residues, and klason lignin (The Uppsala Method): collaborative study. *J. AOAC Int.* 78:1030-1044.
- Wallace, J.C., Lopes, M.A., Paiva, E., Larkins, B.A., 1990. New methods for extraction and quantitation of zeins reveal a high content of  $\gamma$ -zein in modified opaque-2 maize. *Plant Physiol.* 92:191-196.