

Improved Method for Isolating and Quantitating α -Amino Nitrogen as Nonprotein, True Protein, Salt-Soluble Proteins, Zeins, and True Glutelins in Maize Endosperm

J. Landry,^{1,2} S. Delhaye,¹ and C. Damerval³

ABSTRACT

Cereal Chem. 77(5):620–626

The conventional Landry-Moureaux method for selective extraction of maize proteins was modified by reducing the contact time of meal with extractants and by removing 55% 2-propanol as extractant. The new procedure, coupled with a method for quantitating protein at microgram level, was used for assessing the nitrogen distribution of four soluble protein fractions present in 100-mg samples of endosperm originating from six maize inbreds and *opaque-2* versions. Proteins extracted with 55% 2-propanol plus reductant were made up of α -, β -, γ -, and δ -zeins. Proteins extracted subsequently with salt plus reductant were minor and poor in lysine (1 mol%). They were associated with zeins. Comparison of present

data with those available in the literature showed a close similarity for a given genotype between the percentage of total α -amino nitrogen extracted by 2-propanol plus reductant than by salt plus reductant under conditions of the modified procedure and that of total Kjeldhal nitrogen extracted by 2-propanol with and without reductant, and by salt plus reductant, using the conventional procedure. A simplified protocol was described and tested for isolating and quantitating α -amino nitrogen as nonprotein, true protein, salt-soluble proteins, zeins, and true glutelins in any sample of maize endosperm.

Proteins of maize grains were divided by Osborne into albumins, globulins, zeins, and glutelins extractable with water, salt, alcohol, and alkali solutions, respectively. Moureaux and Landry (1968), then Landry and Moureaux (1970) succeeded in solubilizing the bulk of glutelins and in separating them into three fractions using 2-mercaptoethanol successively with alcohol, salt, and detergent. As developed initially, the procedure led to five protein subsets to be isolated. It was laborious and time-consuming because it required 16 extractions over 8.5 hr and 16 centrifugations of 15 min (the actual time was longer if handling time before and after centrifugation was taken into account). Many workers using this extraction scheme have altered the procedure for practicality. Comparing their results suggested that relatively slight modifications of experimental conditions resulted in marked changes in protein distribution.

The present work was undertaken to reinvestigate the Landry-Moureaux (LM) method by solving the handicap of time length and extra handling for selective extraction of maize proteins. To this end, a reliable method of protein quantitation sensitive at microgram level was used that allowed quantitation of protein content of extracts isolated from meal samples as low as 100 mg placed in 2-mL Eppendorf tubes and subjected to shaking with 1 mL of extractant. Also, the number of steps were reduced from six to five by extracting zeins with 55% 2-propanol plus 0.6% 2-mercaptoethanol (2ME) because the use of 55% 2-propanol alone (step 3 of initial procedure) was unable to release α -zeins exhaustively. Similarly, the number of extractions at each step (except steps 3 and 5) was limited to two and the time length was also reduced. Also, starting was made up of endosperms from genotypes among the most studied and the *opaque-2* (*o2*) counterparts that allowed comparisons with data available in the literature.

MATERIALS AND METHODS

Whole maize grains used for endosperm isolation were from the wild (+) and *opaque-2* (*o2*) versions of six inbred lines: W64A, W22, W23, Oh43, F₂, and B37. All genotypes but F₂ and F_{2o2}

came from Maize Genetics Cooperation Stock Center (University of Illinois, Urbana-Champaign, IL). They were harvested in 1994 or 1995 (W23+ and *o2*). The *o2* mutation was introduced into F₂ line by eight backcross generations. Endosperms were isolated from grains previously soaked in water for \approx 30 min by peeling off the outer integuments and dissecting the germ with a scalpel. After lyophilization, they were ground for 2–3 min using a ball mill. The meals were stored at -20°C . Before any protein extraction, they were defatted by shaking 100 mg of meal twice with 1 mL of hexane (15 min) at room temperature and by removing supernatant isolated from particles after centrifugation of suspensions at $12,000 \times g$ for 5 min.

Extraction of Proteins and Nonprotein Nitrogen

Two procedures based on the initial LM procedure were examined to gradually solubilize nitrogen of defatted endosperm samples into four fractions.

The first procedure was the modified LM procedure used under conditions outlined in Table I. Nonprotein nitrogen (NPN) was isolated by precipitating salt-soluble proteins with trichloroacetic acid (TCA) in salt extracts to a final concentration of 10%. After standing overnight at 4°C , supernatant and precipitate were isolated by centrifugation at $12,000 \times g$ for 5 min.

The second procedure as a simplified LM procedure involving the same conditions as those described for the modified procedure for isolation of extracts E_{1,2} and E₃. Differences include the lack of second extraction with water, the combination of E₃ with E₄ isolated from a single extraction to yield extract E_{3,4}, and the presence of proteins of E₅ together with insoluble proteins in meal residue (equivalent to extract E_{5,6} by extension). Furthermore, NPN (E₀ by extension) was isolated by treating 100 mg of meal 2 \times with 1 mL of 10% (w/v) TCA (30 min) at 4°C .

Nitrogen Quantitation

Proteins of extracts were quantitated by ninhydrin assay of α -amino nitrogen released after alkaline hydrolysis (3M NaOH, 130°C , 45 min) according to Landry and Delhaye (1996), using an equimolecular mixture of 17 amino acids and ammonium chloride (Pierce) for calibration, and a conversion factor of 1.06 μg of protein for 10 nmol of amino acids. For alcoholic extracts, the presence of volatile compounds resulted in excessive overpressure with leak risks when tubes were gripped while removing from the heating block. This difficulty was eliminated by stopping the heating after 45 min and letting the block cool down to 100°C before the tubes were withdrawn.

¹ INRA, Laboratoire de Chimie Biologique, INA - PG, F78850 Thiverval-Grignon.
² Corresponding author. E-mail: landry@platon.grignon.inra.fr Fax: 033 1 30 81 53 73.
³ INRA, INA-PG, UPS, Station de Génétique Végétale, Le Moulon, F91190 Gif-sur-Yvette.

Free, and free plus peptide-bound amino acids were quantitated from TCA solution isolated from salt extract by ninhydrin assay with and without preliminary alkaline hydrolysis, respectively. Only free amino acids could be evaluated when salt extracts were treated with ninhydrin directly.

Protein present in 100 mg of meal or in residues coming from the treatment of 100 mg of meal were quantitated by ninhydrin analysis after acid hydrolysis (1 mL of constant boiling HCl, 115°C, 18 hr).

Protein Characterization

SDS-PAGE was performed according to Laemmli (1970) using precast 4–20% polyacrylamide gradient gels.

Amino acid compositions were determined from protein hydrolysates (24 hr with constant-boiling HCl under vacuum). Phenylthio-carbamyl (PTC) derivatives of released amino acids were separated using reverse-phase HPLC and quantitated from absorbance at 254 nm (Bidlingmeyer 1984).

Statistical Analysis

Analysis of variance was done using the SAS PROC GLM procedure (SAS Institute, Cary, NC). The two factors were the background (six levels) and genotype (wild vs. mutant).

RESULTS

Distribution of Protein Fractions in Endosperm Using Modified Procedure

The percentages of the total α -amino nitrogen extracted for 12 genotypes with each of the solvents is shown in Table II. They correspond to mean values obtained for each genotype from two endosperm samples isolated independently of each other and subjected to one analysis.

The salt-extract E_1 combined with the first wash with water E_2 , mixture designated as $E_{1,2}$, was made up of free and peptide-bound amino acids, albumins, and globulins, and corresponded to the fraction F_1 of the initial LM procedure. In a preliminary trial of 25 endosperm samples of wild genotypes, the first wash with water was contained $\approx 10\%$ of α -amino nitrogen extracted by salt, while minute amounts of nitrogen were released in the second wash with water. A statistically significant effect of the $o2$ mutation was observed for each fraction reported in Table II. Extracts $E_{1,2}$ averaged 4.9 and 16.2% of the total for wild (+) and $o2$ genotypes, respectively. The alcohol extracts E_3 predominated, averaging 72.1 and 42.9% for + and $o2$ versions of inbreds, respectively. The extracts E_4 were consistently low, accounting for 3.4 (+) and 4.9 ($o2$) % of the total. The extract E_5 constituted 16.1 (+) and 30.4 ($o2$) % of the total, whereas insoluble proteins E_6 accounted for 3.6 (+) and 5.7 ($o2$) %, indicating a near exhaustive extraction of proteins. Differences between inbred lines, whether + or $o2$ were also apparent for the $E_{1,2}$, E_3 , and E_6 fractions.

Distribution of Free, Peptide-Bound, and Protein-Bound Amino Acids in Salt Extracts

The percentages of total α -amino nitrogen present as free, free plus peptide-bound, and (salt-soluble proteins) bound amino acids are shown in Table III. They correspond to mean values obtained with the same samples used for isolating protein fractions. Irrespective of genotype, duplicate analysis of a given sample yielded very close, if not identical, values. But for some genotypes, analyses of two samples belonging to the same inbred showed marked variability. It is noteworthy that the level of free amino acid in grain is dependent on several factors such as synthesis and breakdown of specific amino acid, protein synthesis, and translocation into and out of the grain.

TABLE I
Sequential Isolation of Proteins from Endosperm^a

Extract	Solvent	Time (min) ^b
E_1	0.5M NaCl, 4°C	30, 30
E_2	Water, 4°C (2×)	15
—	Water, 4°C (2×)	15
E_3	55% (w/w) 2-propanol + 0.6% (v/v) 2-mercaptoethanol (2ME), RT ^c	30, 30, 15
E_4	0.5M NaCl, pH 10, ^d 0.6% (v/v) 2ME, RT	15, 15
E_5	0.5% (w/v) SDS, pH 10, 0.6% 2ME, RT	30, 30, 15
E_6	Residue	

^a Samples (100 mg) extracted by shaking with 1 mL of solvent in 2-mL Eppendorf tubes. Supernatants isolated from particles after centrifugation of suspensions at $12,000 \times g$ for 5 min were combined to give extracts for a given medium. Before NaCl extraction, samples were extracted for lipids by shaking twice with 1 mL of hexane (15 min) at room temperature and vacuum dried. After NaCl extraction, meal was extracted with water. The first extract (E_2) was combined with salt extract, yielding extract $E_{1,2}$ and the second was discarded.

^b Extraction time.

^c Room temperature.

^d Sodium borate buffer (0.0125M $Na_2B_4O_7$, 12 H₂O, and 0.02M NaOH).

TABLE II
Protein Distribution in Endosperm of Various Genotypes (% protein recovered)

Genotype	$E_{1,2}$ ^a	E_3 ^b	E_4 ^c	E_5 ^d	E_6 ^e
W64A+	5.1de ^f	72.3a	3.2c	16.8b	2.7c
W22+	4.9de	71.1a	3.2c	16.8b	4.1a–c
W23+	5.0de	69.6a	3.5bc	18.1b	3.8bc
Oh43+	4.8e	73.8a	3.6bc	14.0b	3.8bc
F_2 +	3.8e	73.5a	3.5bc	15.5b	3.8bc
B37+	5.5de	70.8a	3.5bc	16.4b	3.9a–c
Mean +	4.9	72.1	3.4	16.1	3.6
W64A $o2$	19.6a	38.8cd	4.9a	32.1a	4.6a–c
W22 $o2$	17.9ac	37.5d	4.7ab	33.1a	7.0a
W23 $o2$	13.5bc	46.6bc	4.8a	29.1a	6.1ab
Oh43 $o2$	17.8ac	42.1b–d	5.0a	30.0a	5.2a–c
F_2 $o2$	10.3bd	49.3b	5.2a	29.3a	5.9ab
B37 $o2$	18.1ac	42.8b–d	4.9a	29.1a	5.2a–c
Mean $o2$	16.2	42.9	4.9	30.4	5.7

^a Salt plus water.

^b Alcohol plus reductant.

^c Salt plus reductant.

^d Detergent plus reductant.

^e Residue.

^f Values followed by the same letter are not significantly different ($P < 0.05$).

TABLE III
Distribution of Nonprotein Nitrogen (NPN) and Albumins + Globulins in Endosperm (% protein recovered)

Genotype	NPN ₁ ^a	NPN ₂ ^b	NPN ₃ ^c	Alb + Glob ^d
W64A+	1.1b ^e	1.1b	1.8b	3.7bc
W22+	1.2b	1.2b	2.3b	2.8bc
W23+	1.1b	1.1b	2.4b	3.2bc
Oh43+	0.7b	0.8b	1.7b	3.2bc
F_2 +	0.6b	0.7b	1.4b	2.8c
B37+	1.4b	1.2b	2.2b	3.8bc
Mean+	0.99	1.00	1.94	3.22
W64A $o2$	8.5ab	8.2ab	11.4a	8.2a
W22 $o2$	6.5ab	6.3ab	9.1ab	8.8a
W23 $o2$	5.2ab	5.2ab	7.7ab	5.8ab
Oh43 $o2$	10.1a	9.6a	12.1a	5.7a
F_2 $o2$	1.7b	1.6ab	2.5b	7.8a
B37 $o2$	6.4ab	6.1ab	9.0ab	9.1a
Mean $o2$	6.36	6.13	8.6	7.6

^a Nonprotein nitrogen (α -amino) assayed directly from salt extract ($E_{1,2}$).

^b Nonprotein nitrogen (α -amino) assayed directly from trichloroacetic acid (TCA) supernatant.

^c Nonprotein nitrogen (α -amino) assayed from TCA supernatant after alkaline hydrolysis.

^d Albumins + globulins corresponding to $E_{1,2} - NPN_3$. Slight differences observed between the sum $NPN_2 + (Alb + Glo)$ and $E_{1,2}$ reported in Table II were related to a different number of analyses.

^e Values followed by the same letter are not significantly different ($P < 0.05$).

The free amino acids accounted for ≈ 1.0 (+) and 6.36 or 6.13 (*o2*) % of the total α -amino nitrogen whether they were quantified from salt extracts directly or after protein precipitation with TCA. This pointed out the insensitivity of assay toward proteins and reproducibility. Here again, the effect of the mutation was highly significant. The peptide-bound amino acids constituted ≈ 0.9 (+) and 2.5 (*o2*) %. These values are more theoretical than actual because nucleic acids and some free amino acids such as asparagine, glutamine, and arginine gave additional ammonia on alkaline hydrolysis. The sum of free and peptide-bound amino acids as assessed by the quantitation method used in this study can be considered a suitable estimate of NPN. Subtracting NPN₃ from salt-soluble nitrogen led to the quantitation of albumins plus globulins that averaged 3.2 (+) and 7.6 (*o2*) % of the total.

SDS-PAGE

Extracts E₃ and E₄ were subjected to SDS-PAGE to further determine the quality of proteins. The protein profiles of E₃ (Fig. 1) showed subunits corresponding to 22 and 19 kDa α -zeins, 14 kDa β -zein, 27 and 16 kDa γ -zeins, and 10 kDa δ -zeins together with minor polypeptides of 12 kDa and 30–50 kDa, the latter corre-

sponding to dimers (Wilson 1991). The occurrence of 27 kDa γ -zeins was unexpected because this protein was customarily extracted in the fraction F_{IV} when conditions of the initial LM protocol were rigidly adhered to. It involved the presence of salt, a prerequisite for solubilization in aqueous or alcoholic medium. This may be attributable to incomplete removal of salt because of a too short contact time of meal with water or to the lack of extraction with 2-propanol without reductant.

The protein profiles of E₄ (not shown) were characterized by streaking with faint bands at 60 and 27 kDa that confirmed the previous observation of E₃ profiles for 27 kDa γ -zein.

Amino Acid Analysis

Amino acid analysis was performed on extracts E₄ originating from W64A and W64A_{o2} to get a clearer idea of the quality of the proteins ill-defined by SDS-PAGE. The amino acid composition (Table IV) appear to be independent of genotype in agreement with data reported by Misra et al(1975, 1976) and by Landry and Moureaux (1982). They pointed out high levels of glutamic acid and proline together with low levels of aspartic acid, tyrosine, phenylalanine, and lysine, features of proteins belonging to F_{IV}, but medium levels of histidine together with high levels of isoleucine and arginine suggested the presence of other proteins that would be different from proteins belonging to F_V (G₃ glutelins). However, the proteins constituting E₄ can be considered as closer to prolamins by very low levels of lysine than to G₃ glutelins.

TABLE IV
Amino Acid Compositions of Extract E₄ and Landry-Moureaux (LM) Method Fractions F_{IV} and F_V (mol%)

	E ₄ W64A+	E ₄ W64A _{o2}	F _{IV} ^a	F _V ^a
Lysine	0.9	1.0	0.9	5.8
Histidine	3.3	3.4	6.2	2.3
Arginine	3.9	4.3	2.5	4.7
Aspartic acid	1.8	2.0	2.1	8.6
Glutamic acid	23.7	20.4	21.0	11.2
Serine	5.1	4.9	5.8	6.2
Threonine	3.7	4.1	5.6	5.0
Proline	20.1	21.3	20.5	5.9
Glycine	6.9	7.4	8.3	8.6
Alanine	5.6	6.2	6.2	10.8
Valine	7.1	7.7	5.9	7.2
Methionine	1.2	1.2	1.2	1.2
Isoleucine	5.2	4.1	2.0	4.6
Leucine	7.6	8.1	9.4	9.7
Tyrosine	2.2	2.0	1.3	4.2
Phenylalanine	1.7	1.8	0.9	5.8

^a Data from Landry and Moureaux (1980).

TABLE V
Evaluation of Total Protein, Nonprotein, Protein Fractions Using a Simplified Procedure of Sequential Extraction (Protein % Dry Matter)^a

	W64A+	W64A _{o2}
NT	14.59 (0.20)	11.35 (0.50)
N ₀	0.25 (0.02)	0.95 (0.01)
N _{A+G}	0.46 (0.04)	0.87 (0.40)
N _Z	10.64 (0.11)	5.17 (0.37)
N _{GLU}	2.72 (0.02)	4.34 (0.06)
N _{NZ}	3.18 (0.04)	5.21 (0.12)

^a Mean and standard deviation (in parentheses) from three replicates.

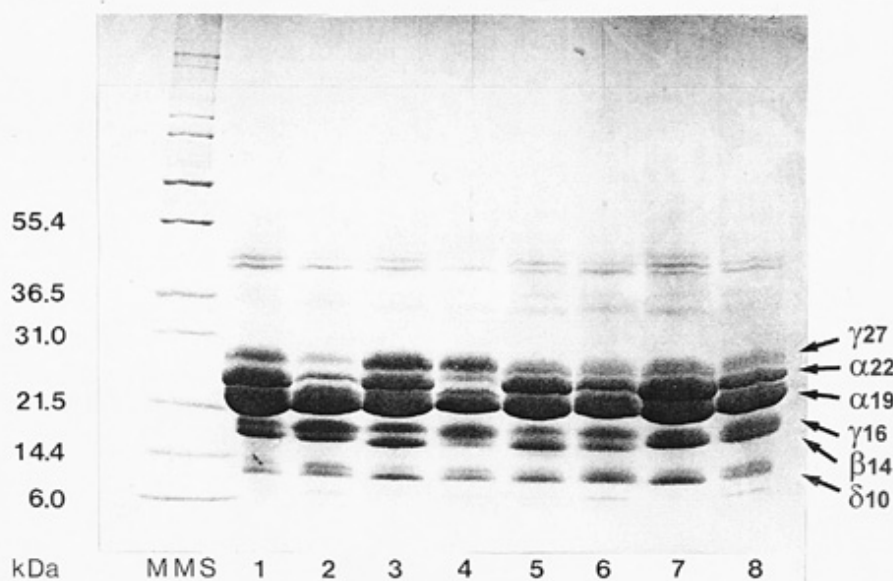


Fig. 1. SDS-PAGE of proteins extracted with 55% 2-propanol plus 0.6% 2-mercaptoethanol (E₃) from endosperms of W64A+, W64A_{o2}, W22+, W22_{o2}, Oh43+, Oh43_{o2}, B37+, B37_{o2} (lanes 1–8, respectively). Molecular mass standards (kDa) indicated on left.

Distribution of Protein Fractions Using the Simplified Procedure

These results allowed us to devise a simplified procedure for selectively isolating and quantitating nitrogen from maize endosperm. This may be looked on as making of NPN (N_0) and proteins (N_p) that are a mixture of albumins + globulins (N_{A+G}), zeins (N_Z), and true glutelins (N_{GLU}). From data reported in Table III, NPN₃ constitutes a low proportion of total nitrogen present in endosperm of wild maize and can be neglected as a first approximation. The same is not true of most *o2* mutants where it can reach up to 20% of total nitrogen (Morro et al 1996). In this context, quantitation of NPN (N_0) is a prerequisite to accurate evaluation of the zeins and nonzeins (N_{NZ}).

Using the fast procedure led to the isolation of extracts E_0 , $E_{1,2}$, $E_{3,4}$, and $E_{5,6}$. The evaluation of nitrogen contents led to the equalities: $N_T = N_0 + N_p$; $N_{A+G} = N_{1,2} - N_0$; $N_Z = N_{3,4}$; $N_{GLU} = N_{5,6}$; and $N_{NZ} = N_p - N_Z$. The results of this procedure tested on endosperms of W64A and W64A*o2* are given in Table V.

DISCUSSION

Comparison of Protocols for Quantitating Protein Distribution of Maize Endosperm

Table VI reports data concerning protein distribution of diverse genotypes allowing comparison of results from the present study with those available in the literature obtained by using the original LM protocol for extracting proteins and the Kjeldahl method for quantitation. Taking into account the differences in experimental conditions, there was good similarity, even identity (for Oh43 + sample), between protein distributions for a given genotype when the proportions of $E_{1,2}$, $E_3 + E_4$, E_5 , and E_6 were compared with those of F_I , $F_{II} + F_{III} + F_{IV}$, F_V , and F_{VI} , respectively. This identity suggested that the differences recorded between E_3 and $F_{II} + F_{III}$, and between E_4 and F_{IV} were not related to the quantitation procedure. Irrespective of genotype, the percentages of E_3 compared with those of $F_{II} + F_{III}$ were higher, whereas it was the reverse when E_4 was compared with F_{IV} . This is consistent with the observations made about the electrophoretic pattern of E_3 from quantitation. Therefore, reducing contact time of meal with any solvent medium did alter protein distribution provided that the sum amounts of nitrogen solubilized with alcohol plus reductant (E_3) and with salt plus reductant (E_4) was taken into account, but the lack of extraction with alcohol alone (F_{II}) was responsible for the cosolubilization of 27 kDa γ -zein with the other zein subunits. These comparisons confirmed the observations made previously for maize gluten as to the validity of experimental conditions used for assessing protein distribution (Landry et al 1999).

Regarding NPN, the values listed in Table III were expressed as a percentage of total nitrogen, which was not the case for data available in the literature expressed as μ mol of leucine per grain, or as relative value for a given genotype taken as reference, which makes comparisons approximate. Note that the percentage of total nitrogen as nonprotein found for W22+ was close to that reported by DiFonzo et al (1979) for the same inbred but using the Kjeldahl method for nitrogen evaluation. This similarity could be coincidental in view of the marked quantitative variability seen for NPN.

As for albumins plus globulins, our results concerning W64A+ were in agreement with those for the same inbred by Hansel et al (1973), who used the Kjeldahl analysis. Comparing the data of Table III with those of Dierks-Ventling (1981) exemplifies the influence of experimental conditions on the quantitation of these proteins. This author extracted albumins then globulins with water then with 0.5M NaCl, both in the presence of reductant, at room temperature, isolated them by dialysis and quantitated them according to Bradford (1976). The sum averaged 21.7 (+) and 38.8 (*o2*) % of total protein for four genotypes. These surprisingly high values probably originated in proteins of F_{IV} caused by the reductant in the extractant, as can be deduced from data of Landry and Moureaux (1970) and from SDS-PAGE patterns given by the author, and probably in the method used for protein quantitation.

Effect of *o2* Gene on Protein Distribution in Endosperm

The present results confirmed the observations reported by many others for the effect of the *o2* gene on protein distribution, excluding the quantitative increase of NPN, salt-soluble proteins ($E_{1,2}$), true glutelins (E_5 or F_V or G_3 glutelins), together with a decrease of zeins (E_3), 27 kDa γ -zein (E_4). They extended them by pointing out the shortcomings of a screening based on the quantitative importance of NPN to detect lysine-rich mutants. Indeed, F_2 *o2* had proportions of α -amino nitrogen as free amino acids (NPN₂ or NPN₃) similar to those found for several wild genotypes (Table III for W22, W23, B37 wild type) but very different proportion of zeins ($E_3 + E_4$). The same was noticed by Morro et al (1996). On this account, a screening based on the relative content of salt-soluble proteins or zeins seemed more adequate. But it is worth remembering that salt-soluble proteins are more susceptible to insolubilization partly under the influence of certain treatments related to drying (Wall et al 1975), age of the grain, or to isolation conditions (Landry and Moureaux 1994).

Comparison of Isolation Methods for Evaluating Zein Content of Endosperm

Wallace et al (1990) evolved a procedure for isolation of zeins entailing the extraction of the majority of protein endosperm in 1% SDS and 2% ME buffered at pH 10, and the subsequent precipi-

TABLE VI
Distribution of Protein Fractions in Endosperm of Four Genotypes as Assessed Using Modified and Original Landry-Moureaux (LM) Procedure^a

Protein Fractions		Genotypes							
		W64A+		W64A <i>o2</i>		Oh43+		Oh43 <i>o2</i>	
		Modified ^b	LM ^c	Modified	LM	Modified	LM	Modified	LM
$E_{1,2}$	F_I	5.1	5.7	19.6	15.5	4.8	5.8	17.8	13.6
E_3	$F_{II} + F_{III}$	72.3	60.6	38.8	35.2	73.8	64.8	42.1	35.3
E_4	F_{IV}	3.2	8.3	4.9	14.1	3.6	12.7	5.0	14.0
$E_3 + E_4$	$F_{II} + F_{III} + F_{IV}$	75.5	68.9	43.7	49.3	77.4	77.5	47.1	49.3
E_5	F_V	16.8	16.7	32.1	33.7	14.0	13.8	29.5	29.2
E_6^d	F_{VI}^d	2.7	8.6	4.6	4.2	3.8	2.9	5.2	7.9
Protein % dry matter		13.4	13.3	12.3	11.0	10.6	11.8	9.6	10.1
References ^e		PS	1	PS	1	PS	2	PS	2

^a Percent of total nitrogen recovered.

^b Fractions isolated using the modified procedure (present study, Table I).

^c Fractions isolated using the original procedure (Landry and Moureaux 1970).

^d Residue.

^e References: PS = present study, 1 = Paiva et al (1991), 2 = Misra et al (1972).

tation of nonzein proteins by adding ethanol to a final concentration of 70% (v/v). They asserted that it resulted in a higher yield of zeins when compared with that obtained using the LM method. The same was concluded by Hamaker et al (1995).

Table VII compares some results of the present study with data from Hamaker et al (1995) and Morro et al (1996) relative to the contents of: alcohol-soluble nitrogen (zein plus NPN) as determined by the original procedure developed by Wallace et al (1990); alcohol-soluble proteins (true zein as evaluated by applying Wallace procedure following the removal of salt-soluble nitrogen by three extractions with salt and two washes with water) or F_{II} + F_{III} as appraised using the initial LM procedure slightly modified or E₃ proteins by the LM procedure used in the present study; NPN as determined by the difference between zein plus NPN and true zein contents or as estimated by the present study (NPN); extraction yield (percentages of nitrogen solubilized).

The LM method, as applied in two studies (Hamaker et al 1995 and the present work) using near-identical operating conditions, yielded different values for true zein content (alcohol-soluble proteins) of a given inbred. The values reported by Hamaker et al (1995) were lower than those found in the present work, indicating the lack of F_{IV} proteins in alcoholic extracts, as suggested previously by Landry (1997). An explanation for the difference can be found in the efficiency of salt removal during washes with water before zein extraction. Indeed, the time of contact of meal with water was doubled in the LM procedure used by Hamaker et al (1995). Such an observation showed that factors considered as minor, like those governing wash with water, can have a marked effect on the protein content of alcoholic extract. This provides a plausible explanation to the quantitative and qualitative differences recorded for this extract by others who have applied the LM procedure but altered some parameters more or less arbitrarily.

The percentages of alcohol-soluble proteins isolated in E₃ under conditions used in the present study, or as true proteins by the modified Wallace method can be considered as identical for any wild genotype. The same comparison made with W64A_{o2} inbred emphasized a lower level of proteins in E₃. This was also seen with the data reported in Table III, suggesting an incomplete extraction of alcohol-soluble proteins that would originate from a rapid aging of grains used in the present study (Jones et al 1942). The same holds true for explaining the discrepancy between the levels of zeins isolated from W64A and W64A_{o2} maize by Paulis and Bietz (1986). From their chromatographic data, it can be calculated that zeins of mutant (originating from a 1967 crop) amounted to only 40% of those of a wild inbred.

The levels of NPN appeared to be dependent of estimation procedure. Those NPN₃ evaluated in the present study could have been underestimated because all nitrogenous compounds did not give the same response with ninhydrin as with the Kjeldahl procedure. But those reported by Hamaker et al (1995) seem to be overestimated. If this is true, we could conclude a virtual absence of salt-soluble proteins.

The modified Wallace and LM methods led to similar extraction yields that were higher than those obtained using unmodified Wallace procedure.

A detailed examination of the data reported by Morro et al (1996) emphasized the limits of Wallace method. These authors have used it for quantitating the magnitude of zeins and nonzeins in endosperms of 31 wild inbreds and the *o2* versions. Solubilized zeins (in reality alcohol-soluble nitrogen) accounted for 72.0 and 56.5% of the total nitrogen in the wild and *o2* genotypes, respectively. From the data reported in Tables II and III, the sum of nitrogen present in E₃ + E₄ + NPN₃ averaged 76.4 (+) and 56.4 (*o2*) % of the total nitrogen, suggesting a good agreement between both

TABLE VII
Comparison of Features of Wallace (W) and Landry-Moureaux (LM) Procedures^a

Genotypes	Alcohol-Soluble N ^b		Alcohol-Soluble Proteins ^c		Nonprotein N		Extraction Yield			
	uW ^d		mW ^e	LM	LM	W	LM	uW	mW	LM
Oh43+	79.7	nd	73.9	65.0	73.8	5.8	1.7	nd	96.4	96.2
W64A+	74.7	74.9	69.0	65.0	72.3	4.4	1.8	88.6	92.3	97.3
Mean +	74.7	72.0	68.6	61.3	72.1	6.1	1.8	90.2	94.1	96.3
(n) ^f	(4)	(31)	(4)	(4)	(6)	(4)	(4)	(31)	(4)	(6)
W64A _{o2}	62.4	49.3	47.9	37.5	38.8	14.5	11.4	83.1	93.4	94.4
Mean <i>o2</i>	nd	56.5	nd	nd	42.9	nd	9.7	88.1	nd	94.4
(n)		(31)			(6)		(6)	(31)		(6)
References ^g	H	M	H	H	PS	H	PS	M	H	PS

^a Data expressed as a percent of total nitrogen.

^b Alcohol-soluble nitrogen: true zeins plus nonprotein nitrogen.

^c Alcohol-soluble proteins: true zeins or proteins of E₃.

^d Unmodified Wallace procedure.

^e Modified Wallace procedure: the procedure was applied on meal freed from salt-soluble nitrogen and salt.

^f Number of genotypes.

^g References: H = Hamaker et al (1995), M = Morro et al (1996), PS = present study.

TABLE VIII
Distribution of Protein Fractions in Endosperms of W64A+ and W64_{o2} Maize Assessed by Simplified (S) and Modified (M) Procedures (P)^{a,b} (% protein recovered)

Protein Fractions		W64A+		W64A _{o2}	
SP	MP	SP	MP	SP	MP
E _{1,2}	E _{1,2}	5.03 (0.26)	5.08 5.13	16.07 (0.60)	15.29 19.63
E _{3,4}	E ₃ + E ₄	75.65 (0.20)	76.61 75.44	45.57 (1.68)	46.81 43.70
E _{5,6}	E ₅ + E ₆	19.32 (0.10)	18.41 19.42	38.37 (1.09)	37.90 36.67

^a Simplified procedure: mean and standard deviation (in parentheses) of triplicate extractions; modified procedure: data of first column originated from one extraction performed on the same endosperm sample used for simplified procedure; data of second column corresponded to mean values reported in Table II.

^b Value of Student *t*-test for calculating confidence interval at *P* < 0.05 is 4.303. Values calculated from sum of fractions isolated using modified procedure fall within the confidence interval of the corresponding fraction obtained using simplified procedure.

LITERATURE CITED

procedures. However, Morro et al (1996) considered, without providing evidence, that the residual proteins left after the extraction with SDS plus 2 ME at pH 10 were made up mainly of zeins, which overestimated zeins (averages of 80 [+] and 65 [o2] % were stated), underestimated nonzeins, and overestimated lysine contents. The latter, as deduced from the ratio of endosperm lysine to the amount of nonzeins were 7.5–15.4 g of lysine/16 g of nitrogen. Such values and the great variability were inconsistent with the fact, evidenced by Misra et al (1975 and 1976), that LM fraction F_V (true glutelins or membrane proteins), main constituents of nonzeins, have amino acid composition nearly independent of genotype and lysine content averaging $6.8 \pm g/16 g$ of nitrogen by excluding the too low value found for Oh43_{nz}. Recalculating lysine content of nonzeins by including residual proteins in this fraction led to values averaging $6.5 \pm I.I (+)$ and $7.9 \pm 1.0 (o2) g/16 g$ of N, therefore closer to the experimental values obtained by Misra et al (1975 and 1976).

Simplified Procedure for Selective Isolation and Quantitation of Nitrogen from Maize Endosperm

In Table VIII are indicated the nitrogen percentages isolated in E_{1,2}, E_{3,4}, and E_{5,6} using the simplified procedure with those obtained for E_{1,2}, E₃ + E₄, and E₅ + E₆ by applying the modified procedure on sample identical to that used for simplified procedure or on two different samples (Table II). No significant differences were found between E_{3,4} and E₃ + E₄ or between E_{5,6} and E₅ + E₆ irrespective of samples. On the other hand, significant difference (19.63 instead 16.07%) was evidenced for E_{1,2} from W64A_{o2}, although the isolation conditions were the same for both procedures. This is related to the difference in the magnitude of NPN (11.4 instead of 8.3).

It is noteworthy that the fractionation of endosperm proteins using fast procedure corresponds to that developed by Osborne and Mendel (1914) in which the greatest part of proteins solubilized by dilute alkali, considered as glutelins by these authors, are now regarded as zeins, that is prolamins, and insoluble proteins as true glutelins. Furthermore, the accurate quantitation of zeins, proteins deprived of lysine allows that of nonzeins and indirectly that of lysine content in true protein because there is a strict proportionality between lysine and nonzein contents, the coefficient being the mean content of lysine in nonzeins.

CONCLUSIONS

The data presented in this study regarding the distribution of nitrogen fractions as nonprotein, salt-soluble proteins, zeins, and true glutelins in endosperm of maize inbreds and *op-2* versions were in keeping with those available in the literature in a scattered form. Such comparisons point out that the LM method, even when applied with operating conditions different from those developed initially, gives consistent data provided that the proteins extracted by 2-propanol, with and without reductant, and by salt with reductant. The sum of F_{II} or zein or zein-1 (Sodek and Wilson 1971) plus F_{III} or G₁ glutelins or zein-2 (Sodek and Wilson 1971) or zein-like (Misra et al 1975) plus F_{IV} or G₂ glutelins or glutelin-like (Misra et al 1975) can be viewed as a whole. This set of endosperm-specific proteins (Landry and Moureaux 1976 and 1980) because of the similarity of their kinetic of accumulation in developing grain and their localization in endosperm only, are now considered zeins (Wilson 1991) following the work of Esen (1987). Note that the term of glutelin-like used to refer to G₂ glutelins (F_{IV}) is confusing because some authors have put these proteins together with true glutelins, which results in a loss of information.

ACKNOWLEDGMENTS

We are indebted to Michel Laurière and Isabelle Bouchez-Mahiout for electrophoreses.

- Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. 1984. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* 336:93-104.
- 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.
- Dierks-Ventling, C. 1981. Storage proteins in *Zea mays* (L.): Interrelationship of albumins, globulins and zeins in the *Opaque-2* mutation. *Eur. J. Biochem.* 120:177-182.
- Di Fonzo, N., Gentinetta, E., Salamini, F., and Soave, C. 1979. Action of the opaque-7 mutation on the accumulation of storage products in maize endosperm. *Plant Sci. Lett.* 14:345-354.
- Esen, A. 1987. A proposed nomenclature for the alcohol-soluble proteins (zeins) of maize (*Zea mays* L.). *J. Cereal Sci.* 5:117-128.
- Hamaker, B. R., Mohamed, A. A., Habben, J. E., Huang, C. P., and Larkins, B. A. 1995. Efficient procedure for extracting maize and sorghum kernel proteins reveals higher prolamins contents than conventional method. *Cereal Chem.* 72:583-588.
- Hansel, L. W., Tsai, C. Y., and Nelson, O. E. 1973. The effect of the *floury-2* gene on the distribution of protein fractions and methionine in maize endosperm. *Cereal Chem.* 50:383-394.
- Jones, D. B., Divine, J. P., and Gersdorff, C. E. F. 1942. Effect of storage of corn on the chemical properties of its proteins and on its growth-promoting value. *Cereal Chem.* 19: 819-830.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Landry, J. 1997. Comparison of extraction methods for evaluating zein content of maize grain. *Cereal Chem.* 74:188-189.
- Landry, J., and Delhay, S. 1996. A simple and rapid procedure for hydrolyzing minute amounts of proteins with alkali. *Anal. Biochem.* 243:191-194.
- Landry, J., Delhay, S., and Di Gioia, L. 1999. Protein distribution in gluten products isolated during and after wet-milling of maize grains. *Cereal Chem.* 76:503-505.
- Landry, J., and Moureaux, T. 1970. Hétérogénéité des glutelins du grain de maïs: Extraction sélective et composition en acides aminés des trois fractions isolées. *Bull. Soc. Chim. Biol.* 52:1021-1037.
- Landry, J., and Moureaux, T. 1976. Quantitative estimation of accumulation rate of protein fractions in unripe and ripe maize grain. *Qual. Plant. Plant Foods Hum. Nutr.* 25:343-360.
- Landry, J., and Moureaux, T. 1980. Distribution and amino acid composition of protein groups located in different histological parts of maize grain. *J. Agric. Food Chem.* 28:1186-1191.
- Landry, J., and Moureaux, T. 1982. Distribution and amino acid composition of protein fractions in *opaque-2* maize grain. *Phytochemistry* 21:1865-1869.
- Landry, J., and Moureaux, T. 1994. Albumins and globulins in maize grains. Pages 536-554 in: *Biotechnology in Agriculture and Forestry*, Vol. 25. Maize. Y. P. S. Bajaj, ed. Springer-Verlag: Berlin.
- Misra, P. S., Jambunathan, R., Mertz, E. T., Glover, D. V., Barbosa, H. M., and McWhirther, K. S. 1972. Endosperm protein synthesis in maize mutants with increased lysine content. *Science* 176:1425-1426.
- Misra, P. S., Mertz, E. T., and Glover, D. V. 1975. Characteristics of proteins in single and double endosperm mutants of maize. Pages 291-305 in: *High Quality Protein Maize*. CIMMYT-Purdue University, eds. Dowden, Hutchinson and Ross: Stroudsburg, PA.
- Misra, P. S., Mertz, E. T., and Glover, D. V. 1976. Studies on corn proteins. IX. Comparison of the amino acid composition of Landry-Moureaux and Paulis-Wall endosperm fractions. *Cereal Chem.* 53:699-704.
- Morro, G. L., Habben, J. E., Hamaker, B. R., and Larkins, B. A. 1996. Characterization of the variability in lysine content for normal and opaque-2 maize endosperm. *Crop Sci.* 36:1651-1659.
- Moureaux, T., and Landry, J. 1968. Extraction sélective des protéines du grain de maïs et en particulier de la fraction "glutélins". *C. R. Acad. Sci. (Paris)* 262 (Série D):2302-2305.
- Osborne, T. B., and Mendel, L. B. 1914. Nutritive properties of proteins of the maize kernel. *J. Biol. Chem.* 18:1-16.
- Paiva E., Kriz, A. L., Peixoto, M. J. V. D., Wallace, J. C., and Larkins, B. A. 1991. Quantitation and distribution of γ -zein in the endosperm of maize kernels. *Cereal Chem.* 68:276-279.
- Paulis, J. W., and Bietz, J. A. 1986. Separation of alcohol-soluble maize proteins by reversed-phase high performance liquid chromatography. *J. Cereal Sci.* 4:205-216.
- Sodek, L., and Wilson, C. M. 1971. Amino acid composition of proteins isolated from normal, opaque-2, and floury-2 corn endosperm by a

- modified Osborne procedure. *J. Agric. Food Chem.* 19:1144-1150.
- Wall, J. S., James C., and Donaldson, G. L. 1975. Corn proteins: Chemical and physical changes during drying of grain. *Cereal Chem.* 52:779-790.
- Wallace, J. C., Lopes, M. A., Paiva, E., and Larkins, B. A. 1990. New methods for extraction and quantitation of zeins reveals a high content of γ -zein in modified opaque-2 maize. *Plant Physiol.* 92:192-196.
- Wilson, C. M. 1991. Multiple zeins from maize endosperms characterized by reversed-phase high performance liquid chromatography. *Plant Physiol.* 95:777-786.

[Received December 15, 1999. Accepted May 29, 2000.]