Identification of Modified High-Lysine Maize Genotypes by Reversed-Phase High-Performance Liquid Chromatography[1]

J. W. PAULIS,[2] J. A. BIETZ,[2] R. J. LAMBERT,[3] and E. M. VILLEGAS[4]

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ABSTRACT

High-lysine *opaque*-2 (*o*2) lines of maize have floury endosperm textures. Maize breeders are developing modified *o*2 hybrids with hard, vitreous endosperm. Modified *o*2 kernels are similar in appearance to normal kernels. This article presents a rapid reversed-phase high-performance liquid chromatography (RP-HPLC) method to differentiate normal, *o*2, and modified *o*2 maize genotypes based on alcohol-soluble protein composition. Modified *o*2 endosperms contained more early eluting, high-proline, water-soluble, alcohol-soluble glutelin and less late-eluting zein than did nonmodified *o*2 or normal endosperms. The RP-HPLC method is potentially useful in maize breeding programs and commerce for determining modified *o*2 genotypes with increased lysine contents and hard endosperm texture.

The first high-lysine maize endosperm mutant was *opaque*-2 (*o*2) (Mertz et al 1964). This genotype has soft, opaque kernels rather than the hard, transparent ones typical of most maize, but its amino acid composition made it more nutritious than normal maize. The *o*2 maize has twice the level of lysine and contains more tryptophan than does normal maize, but it yields less and has more ear rot. To increase the hardness of high-lysine *o*2 maize genotypes, breeders at Centro Internacional de Mejoramiento de Maize y Trigo (CIMMYT) in Mexico and the University of Illinois (IL) combined the *o*2 gene with genetic endosperm modifiers to produce modified (mod.) *o*2 maize (also called "quality-protein maize" or QPM) (Vasal et al 1980, Wessel-Beaver and Lambert 1982). These modifier genes may regulate expression of major genes, make the endosperm more vitreous, eliminate ear rot, and enhance other traits (Vasal et al 1980). The endosperm phenotype of mod. *o*2 maize is similar to that of normal maize.

Mod. *o*2 genotypes can retain the improved nutritional quality of *o*2 maize lines. Alcohol-soluble prolamins have no lysine and are low (34–42% of total protein) in mod. *o*2 (or QPM) maize (Robutti et al 1974, Gentinetta et al 1975, Ortega and Bates 1983), similar to *o*2 maize, with a high level of remaining high-lysine proteins. This allows for the turbidimetric estimation of zein, which relates inversely to grain lysine content (Paulis et al 1974).

Using the protein isolation scheme of Landry and Moureaux (1970), Gentinetta et al (1975) showed that mod. *o*2 maize contains a higher percentage of fraction III (G_1, or zeinlike proteins extracted with 70% ethanol containing 0.6% mercaptoethanol) than do normal or *o*2 genotypes. Ortega and Bates (1983) also found that mod. *o*2 maize (hard-endosperm *o*2) contained more of this zeinlike or G_1 fraction, which increased in concentration as endosperm hardness of a population increased with selection. Earlier studies by electrophoresis and amino acid analysis showed that the solubility fraction G_1 contained water-soluble alcohol-soluble glutelin (wsASG) and water-insoluble alcohol-soluble glutelin (wiASG) components. Amounts of wsASG (mainly *M*r

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[2]Food Quality and Safety Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604.

[3]University of Illinois, Urbana, IL 61801.

[4]Centro International de Mejoramiento de Maize y Trigo, Mexico DF.

26 kDa), also called G_2 (Landry and Moureaux 1970), γ-zein (Thompson and Larkins 1989), and reduced-soluble protein (Wilson 1985), increase when sodium acetate is added to the extractant (Landry et al 1983).

Alcohol-soluble maize proteins can be extracted from normal and mutant maize lines with 70% ethanol + 0.5% sodium acetate + 5% β-mercaptoethanol and analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) (Paulis and Bietz 1986, Paulis et al 1990). Sugary-1 (su_1), *o2*, floury-2 (*fl2*), and normal genotypes can be distinguished by their RP-HPLC patterns of alcohol-soluble protein. We compared alcohol-soluble proteins of mod. *o2*, *o2*, *fl2* (all high lysine), and normal maize genotypes by RP-HPLC to determine whether specific amounts of alcohol-soluble glutelin or zein polypeptides could differentiate these genotypes.

MATERIALS AND METHODS

Maize Genotypes

Sixteen mod. *o2* maize samples were supplied by R. J. Lambert (IL), and six by E. M. Villegas (CIMMYT). The IL genotypes consisted of the inbreds R802 *o2 o2*, R802 *O2 O2* (normal), and an S4 R802 mod. *o2* line. The other 15 mod. *o2* IL lines were various endosperm hardness modifications of B73 mod. *o2*, Ms315 mod. *o2*, T/T mod. *o2*, TT mod. *o2*, E/I mod. *o2*, and Ia 36 mod. *o2*. CIMMYT genotypes Tuxpeño-1 (normal), Poza Rica, and Blanco Dentado-1 QPM (population 63) were of common genetic background. All six CIMMYT mod. *o2* QPM populations (63, 64, and 65) and pools (17, 23, and 26) were selected over about 11 cycles of selection for endosperm modification. Seeds came from open pollinated ears. All seed was grown in 1984 and 1985 in Mexico and Illinois, respectively. Illinois seed was produced by hand pollination to eliminate xenia effects. We also used grain from normal maize inbred B37 *O2 O2* and its mutants B37 *fl2 fl2* and B37 *o2 o2*.

Protein Extraction

Ten kernels of each maize line were soaked in water for 10 min, and endosperms were dissected from the seeds. Samples of this size were adequately reproducible on RP-HPLC. Air-dried endosperms were ground in a Udy cyclone mill through a 0.5-mm screen. Alcohol-soluble proteins were quantitatively extracted at room temperature by shaking with a vortex evaporator (Buchler) for 2 hr with 70% (v/v) ethanol containing 0.5% (w/v) sodium acetate and 5% (v/v) β-mercaptoethanol using 0.1 g of meal per 2 ml of solvent (Paulis and Bietz 1986).

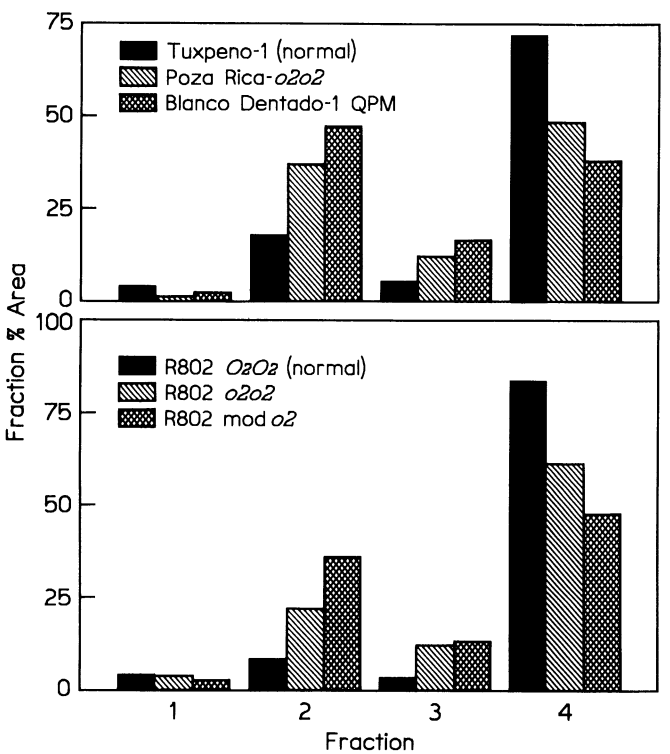

Fig. 1. Reversed-phase high-performance liquid chromatography fractions 1–4 (see Fig. 2) from normal (R802 *O2 O2*, Tuxpeño-1), *o2* (R802 *o2 o2*, Poza Rica *o2 o2*), and mod. *o2* (R802 mod. *o2*, Blanco Dentado-1 QPM) maize genotypes. *o2* = *opaque*-2.

Analytical Methods

Nitrogen in dried materials was determined by a semi-micro-Kjeldahl method. For amino acid analysis, samples of protein or endosperm meal (equivalent to 1–10 mg of protein) were hydrolyzed by refluxing with 6*M* HCl (2–10 ml per mg of sample) for 24 hr. Liberated amino acids were determined on an amino acid analyzer (Dionex D-300) using a physiological fluids analysis column with lithium buffers. Reported levels of methionine and cysteine included methionine sulfone and cysteic acid, respectively.

RP-HPLC

The RP-HPLC methods used were similar to those described previously (Paulis and Bietz 1986), except that in the present study, we used a Waters model 660 solvent programmer and model 450 variable wavelength detector at 210 nm (full scale, 0.4 absorbance units). Duplicate analyses were performed of all samples on separate 20-μl aliquots from two extracts of 10 combined endosperms from each genotype. Chromatograms were integrated using a computer program (MANCPC) in which the operator defines peak start and stop times and baseline positions (Butterfield et al 1978). Peak areas determined at 210 nm are assumed to be proportional to the number of peptide bonds and therefore to the amounts of total protein (Scopes 1974, Biemond et al 1979). RP-HPLC of all genotypes showed good quantitative reproducibility. Standard errors of duplicate determinations of relative peak areas for individual genotypes were generally less than 1%.

Relative percentages of proteins in other near-isogenic normal, *fl2*, and *o2* genotypes, as reported previously (Paulis and Bietz 1986), were combined and averaged with respective values for three normal genotypes (Tuxpeño-1, R802 *O2 O2*, and B37 *O2 O2*), three *o2* genotypes (Poza Rica *o2 o2*, B37 *o2 o2*, and R802 *o2 o2*), and one *fl2* (B37 *fl2 fl2*) maize.

RESULTS AND DISCUSSION

Extraction of R802 endosperms with ethanol + sodium acetate + β-mercaptoethanol as described above removed 62, 38, and 34% of total protein from normal, mod. *o2*, and *o2* lines, respectively. These data agree with reported percentages of alcohol-soluble proteins plus G_2 from defatted and nondefatted endosperms of the same strains (Gentinetta et al 1975, Ortega and Bates 1983). Alcohol-soluble protein content in CIMMYT Blanco Dentado-1 QPM was higher (36 mg of protein per gram of endosperm meal) than in R802 mod. *o2* (31 mg of protein per gram of endosperm meal), possibly because of the higher relative content of fraction 2 in Blanco Dentado-1 QPM (Fig. 1).

Figure 2 shows RP-HPLC patterns of alcohol-soluble proteins from R802 normal, *o2*, and mod. *o2* lines. Three early eluting peaks plus a major late-eluting group of peaks were evident on RP-HPLC of alcohol-soluble maize proteins (Fig. 2) (Paulis and Bietz 1986). Amino acid analyses and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Paulis and Bietz 1986) showed that fraction 1 contained mainly a high-methionine wiASG subunit of *M*r 15 kDa. Fraction 2 (wsASG, 26 kDa) was previously isolated by preparative RP-HPLC, eluting at 47 min (Paulis and Bietz 1986). Amino acid analyses (Paulis and Bietz 1986) (Table I) of two QPM lines verified that fraction 2 contained high-proline, high-histidine wsASG. Fraction 3 contained an *M*r 15.5 kDa high-proline wiASG subunit, and fraction 4 contained primarily zein (22 + 24 kDa), plus a minor *M*r 10 kDa high-methionine wiASG

subunit. The main components of peaks 1–3, wsASG and wiASG, were first described by Paulis and Wall (1977). Apparent molecular weights and amino acid compositions reveal that these proteins correspond to those called β, γ, α, and δ by Thompson and Larkins (1989). R802 mod. *o2* maize contained more early eluting wsASG (fraction 2) and less zein than did normal and *o2* lines (Fig. 2).

Relative amounts of the four fractions of alcohol-soluble maize proteins in normal, *o2*, and mod. *o2* genotypes are shown in Figure 1. Results for both the IL (R802) and CIMMYT samples were similar. In all genotypes, the amounts of fractions 1 and 3 were similar and slightly increased, respectively. Amounts of fractions 2 (wsASG) and 4 (zein) were considerably higher and lower, respectively, in mod. *o2* (QPM) than in normal or *o2* lines.

Amounts of RP-HPLC fractions 1-4 (Fig. 2) in IL mod. *o2*, CIMMYT mod. *o2* (QPM) maize, *o2*, *fl2*, and normal genotypes are shown in Figure 3. Duncan's multiple range test was used to test for significant differences between means of the four fractions between genotypes. Statistical analyses showed that all mod. *o2* lines had similar protein distributions. CIMMYT mod. *o2* lines had more fraction 2 than did IL mod. *o2* lines; this may be related to the harder endosperm and increased amount of wsASG in the G_1 fraction of CIMMYT lines (Ortega and Bates 1983). All mod. *o2* lines differed significantly from normal genotypes and from *o2* and *fl2* lines. In mod. *o2* lines, fraction 2 was considerably higher and fraction 4 was lower than in all other genotypes.

To verify that fraction 2 in mod. *o2* maize was wsASG, as in other genotypes (Paulis and Bietz 1986), amino acid compositions of fraction 2 from two CIMMYT QPM lines were determined and compared with isolated wsASG and fraction 2 from W64A (Paulis and Bietz 1986) (Table I). Fraction 2 from Blanco Dentado-1 QPM and Pool 23 QPM had high proline and histidine contents, characteristic of wsASG, also called proline-rich ASG (Esen et al 1985), G_2-Ac (Landry et al 1983), or reduced-soluble protein (Wilson 1985). Compositions of fraction 2 from these QPM genotypes were also similar to W64A fraction 2 and to wsASG (Paulis and Bietz 1986).

The wsASG and fraction 2 have lower apparent cysteine content (1.3–2.9 mol %) (Table I) than that reported by Esen et al (1985) for the same protein, proline-rich ASG (6.3 mol %). This is probably because we hydrolyzed these proteins directly with 6*M* HCl, whereas Esen et al stabilized cysteine by alkylation (Friedman et al 1970).

Although QPM contains more fraction 2 (which contains no lysine) than does normal maize, it has overall fewer alcohol-soluble proteins that lack lysine and more lysine-containing proteins. Thus QPM genotypes have better nutritional value and can be identified by relative amounts of alcohol-soluble proteins.

Robutti et al (1974) and Gentinetta et al (1975) reported that the *o2* mutation decreased zeins (fraction 4). In Poza Rica *o2 o2*, Blanco Dentado-1 QPM, R802 *o2 o2*, and R802 mod. *o2* genotypes, zeins decreased 70–76% compared with corresponding normal lines. The *fl2* mutation also reduced zeins from levels in normal genotypes, though not as much as did *o2* (Paulis et al 1990), when expressed as percentage of endosperm weight but not when expressed as percentage of total protein. In the current studies, to simplify identification, we reported amounts of fractions as relative percentages rather than on a weight basis. This emphasizes major differences in fractions 2 and 4 among normal, *o2*, and mod. *o2* genotypes. Differences expressed as

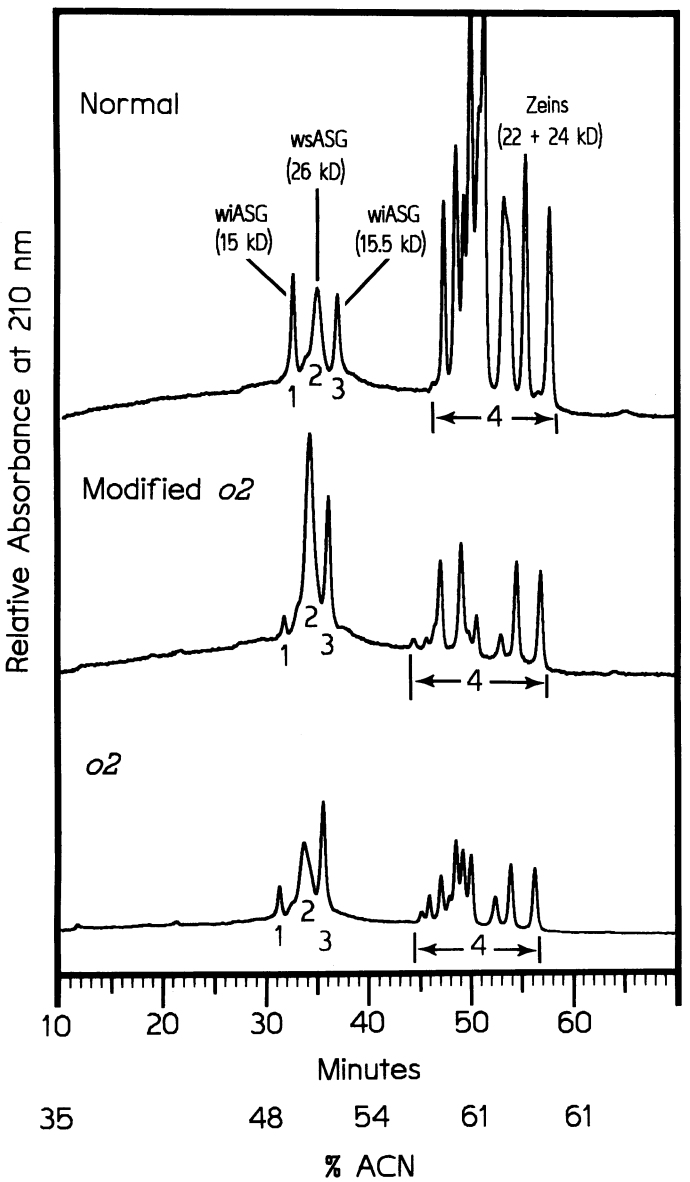

Fig. 2. Reversed-phase high-performance liquid chromatography of prolamins extracted from R802 normal, modified *o2*, and *o2* maize endosperms. Each 20-μl sample represents 1 mg of endosperm. *o2* = *opaque*-2.

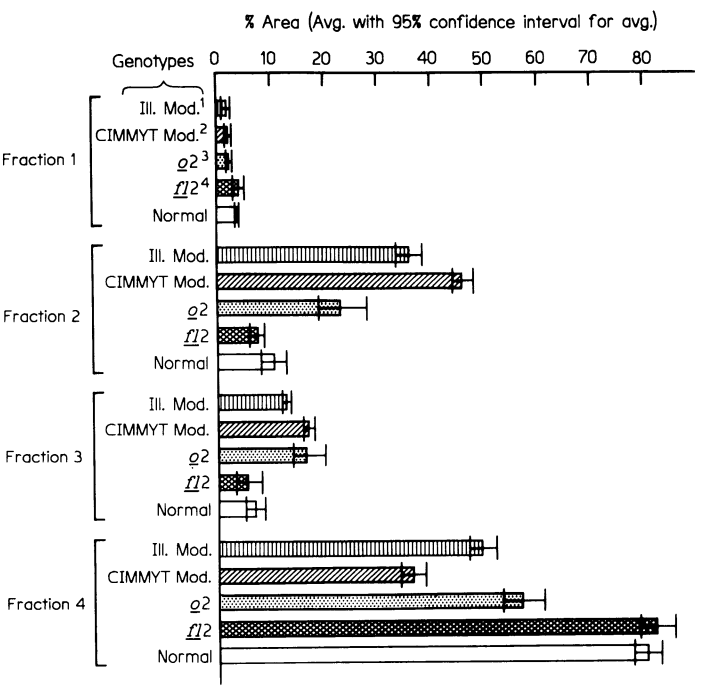

Fig. 3. Distribution of alcohol-soluble proteins in several genotypes of normal, *o2*, and modified (Mod.) *o2* endosperm. Ill. = University of Illinois; CIMMYT = Centro Internacional de Mejoramiento de Maize y Trigo, Mexico; *o2* = *opaque*-2; *fl2* = *floury*-2.

TABLE I
Amino Acid Compositions (mol %) of Water-Soluble, Alcohol-Soluble Glutelin (wsASG) and Reversed-Phase High Performance Liquid Chromatography Fraction 2 of Maize Genotypes

Amino Acid	Isolated wsASG[a]	Genotype W64A[a]	BD[b]	Pool 23[c]
Aspartic acid[d]	0.8	0.6	0.5	0.5
Threonine	4.2	4.1	4.5	4.7
Serine	4.5	4.2	4.2	4.4
Glutamic acid[d]	17.9	18.3	17.7	17.8
Proline	27.0	22.9	21.8	20.8
Glycine	7.6	7.5	7.3	7.5
Alanine	5.8	6.1	6.4	6.8
Cysteine	1.3	2.9	2.7	2.5
Valine	7.2	7.2	8.1	8.7
Methionine	ND[e]	ND	ND	ND
Isoleucine	1.9	2.1	2.1	2.3
Leucine	9.8	10.0	10.4	10.8
Tyrosine	1.5	2.3	2.6	2.8
Phenylalanine	1.3	1.6	1.6	1.6
Lysine	ND[e]	0.4	ND[e]	ND[e]
Histidine	6.5	6.9	6.8	6.7
Arginine	2.5	2.9	3.1	3.3

[a] Paulis and Bietz 1986.
[b] Blanco Dentado-1 QPM (Centro International de Mejoramiento de Maize y Trigo, Mexico), present study.
[c] Pool 23 QPM, present study.
[d] Aspartic and glutamic acids include amides.
[e] Not detected; negligible.

TABLE II
Relative Percentages of Alcohol-Soluble Maize Proteins in Normal, *Opaque*-2 (*o2*), and Modified (mod.) *o2* Genotypes

R802 Genotype	Reversed-Phase High-Performance Liquid Chromatography Fraction[a] 1	2	3	4
O2 O2 (normal)	4.1 a	8.5 c	3.5 b	83.9 a
o2 o2	4.0 a	22.1 b	12.4 a	61.5 b
Mod. *o2*	2.9 b	36.2 a	13.2 a	47.7 c

[a] Values represent means of two determinations. Means in a column followed by a different letter are significantly different ($P < 0.05$) as determined by Duncan's multiple range test. Standard errors of the treatment means were 0.14, 0.66, 1.69, and 1.24 for the four respective fractions.

TABLE III
Relative Percentages of Alcohol-Soluble Maize Proteins in Three CIMMYT[a] Genotypes

Genotype	Reversed-Phase High-Performance Liquid Chromatography Fraction[b] 1	2	3	4
Tuxpeño-1 (normal)	4.1 a	18.1 c	5.6 c	72.2 c
Poza Rica (*o2 o2*)	1.4 b	37.2 b	12.6 b	48.9 b
Blanco Dentado-1 QPM	2.2 ab	47.5 a	17.0 a	33.3 a

[a] From the Centro International de Mejoramiento de Maize y Trigo, Mexico.
[b] Values represent means of two determinations. Means in a column followed by a different letter are significantly different ($P < 0.05$) as determined by Duncan's multiple range test. Standard errors of treatment means were 0.31, 0.67, 0.53, and 0.56 for the four respective fractions.

percentage of endosperm weight are less because *o2* and mod. *o2* have less total alcohol-soluble protein than does normal maize (Fig. 2).

Table II summarizes the distribution of alcohol-soluble maize proteins of R802 mod. *o2*, *o2*, and normal inbred maize lines. In R802 *O2 O2* (normal), relative amounts of each fraction differed from mod. *o2*. The *o2* genotype was similar to that of the normal line in amount of fraction 1, was similar to mod. *o2* in relative amount of fraction 3, and was between normal and mod. *o2* for fractions 2 and 4. Fraction 2 was significantly higher in mod. *o2*, and fraction 4 was significantly lower.

TABLE IV
Relative Percentages of Alcohol-Soluble Maize Proteins in 16 IL[a] and Six CIMMYT Modified (mod.) *o2*, Six *o2*, Four *fl2*, and Seven Normal Genotypes

Genotype	Reversed-Phase High-Performance Liquid Chromatography Fraction[b] 1	2	3	4
IL mod. *o2*	1.8 b	35.8 b	12.9 b	49.5 c
CIMMYT mod. *o2* (QPM)	0.4 c	46.2 a	17.0 a	36.5 d
o2	2.5 b	23.4 c	17.0 a	57.2 b
fl2	4.4 a	7.3 d	6.1 c	82.3 a
Normal	4.0 a	11.2 d	6.7 c	80.4 a

[a] IL = University of Illinois, CIMMYT = Centro International de Mejoramiento de Maize y Trigo, Mexico.
[b] Values represent means of two determinations for each sample. Means in a column followed by a different letter are significantly different ($P < 0.05$) as determined by Duncan's multiple range test. Standard errors of treatment means were 0.71, 3.98, 1.90, and 4.08 for the four respective fractions.

Among CIMMYT genotypes (Table III), Tuxpeño-1 (normal) differed from Blanco Dentado-1 QPM in fractions 2–4, and Poza Rica (*o2 o2*) was intermediate in fractions 2–4. Blanco Dentado-1 QPM and R802 mod. *o2* had more fraction 2 and less fraction 4 than did their corresponding *o2* and normal genotypes.

Table IV shows mean values of protein fractions for all genotypes tested. Significant differences exist among means of different endosperm types for each fraction. IL and CIMMYT mod. *o2* lines were significantly different from other genotypes yet were similar to each other as shown by higher and lower fractions 2 and 4, respectively. Fractions 2 and 4 differed the most, with 2 being the highest and 4 the lowest in the mod. *o2* lines.

Wallace et al (1990) recently reported similar observations of elevated γ-zein (mainly RP-HPLC fractions 2 and 3) in modified *o2* maize kernels. However, the methods used in that study are more complex than those described here, and they require specific antibodies to maize zein fractions.

CONCLUSION

Since mod. *o2* kernels have phenotypes similar to those of normal kernels, we developed an RP-HPLC method of prolamin analysis to identify and differentiate mod. *o2* (QPM) maize from normal and *o2* genotypes. Four fractions characteristically separated on a C_{18} column when an aqueous acetonitrile-trifluoroacetic acid solvent gradient was used. Mod. *o2* genotypes from two sources contained significantly more fraction 2 (a wsASG component, mainly 26 kDa) and significantly less zein (fraction 4) than did normal, *o2*, or *fl2* genotypes (Paulis and Bietz 1987). These differences correlate with the harder endosperm of mod. *o2* compared with *o2* lines. Assays for these proteins during inbred development may be useful in identifying and breeding for mod. *o2*, high-lysine maize genotypes with superior physical and utilization properties.

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