Theor Appl Genet (1988) 76:244–252

Comparisons of zein profiles from inbred, F_1, and F_2 generations of maize as revealed by reversed-phase high-performance liquid chromatography

J. S. C. Smith and O. S. Smith

Department of Research Specialists and Data Management, respectively, Pioneer Hi-Bred International, Inc., 7301 NW 62nd Avenue, Johnston, IA 50131, USA

Received October 20, 1987; Accepted January 23, 1988
Communicated by A. R. Hallauer

Summary. Chi-square analyses were performed on zein chromatographic profiles of inbred lines, F_1, F_2, and reciprocal F_1 seed for 10 hybrids of maize (*Zea mays* L.). The objective was to test the goodness of fit of observed profiles with those expected on the basis that the F_1 and F_2 generations represent a 2:1 and 1:1 addition of female:male parents of the F_1, respectively. From 40 available comparisons, 39 showed no difference between the observed chromatograms and those that were expected on the basis of four models that were tested. The one exception was due to closely eluting peaks that were revealed as shoulders and not recorded as separate entities. Chromatographic profiles of inbreds, F_1, and bulk F_2 seed sources can be accurately simulated. Even though the chromatographic profile of the F_1 closely resembled that of the female parent, profiles of hybrids with common female but different male parents were distinguishable. The lack of novel peaks in both F_1 and F_2 generations compared with the inbred line thereby revealed no unpredictable interaction among zein loci. Zein protein data can be useful in registration, certification, and in the checking of hybrid pedigree especially when used in concert with isozymic data.

Key words: Genotype – Pedigree – Plant Variety Protection – Allozyme – Electrophoresis – Chi Square Analysis – Germplasm Security – Genetic Diversity

Introduction

The ability of data to provide genotypic identification of inbred lines and hybrids of maize (*Zea mays* L.) means that these data could be useful for registration, certification, patenting, and for the checking of pedigrees. Electrophoresis of isozymes and isoelectric focusing or reversed-phase high-performance liquid chromatography (RP-HPLC) of zein endosperm proteins are techniques that individually afford the unique characterization of numerous inbred lines and hybrids of maize (Stuber and Goodman 1983; Wilson 1984; Bietz 1985; Smith and Smith 1986; Smith 1988). Isozymic data have been used in patent application for a maize inbred line (Troyer 1986). Chromatographic and isozymic data persuaded a U.S. federal court that seed of a proprietary line of maize had been misappropriated and used (United States District Court 1987).

The presence of zein endosperm proteins in an unequal biparental dosage of 2 (female):1 (male) suggests that these data could be of additional and complimentary usage to isozymic data in the identification of hybrid pedigrees. First, visual inspection of the chromatographic profile of one hybrid N28 × Mp490 showed close agreement to a profile that was computer simulated using a 2:1 addition of the female:male parental profiles (Bietz 1985). Thus, zein chromatographic data could be used to indicate female parents of hybrids. Isozymic data do not permit the separate identification of parent lines of F_1 hybrids provided seed lots have been produced under strict standards of pollen control. Second, F_2 bulk field samples should contain an equal contribution of zein alleles from the parent lines of a single cross hybrid. Thus, Bietz (1985) postulated that a chromatographic profile from a bulk F_2 seed lot should be equivalent to a 1:1 addition of the profiles of the two parents of the original single-cross hybrid. Isozymic data have reduced utility to define pedigrees when only F_2 seed produced on F_1 plants is available. Segregation of isozyme alleles removes the possibility that the independent contributions of the single cross parents can be distinguished and outcrossing can provide additional isozyme alleles to those present in the single cross parents. However, F_2 zein chromatographic profiles could readily be simulated

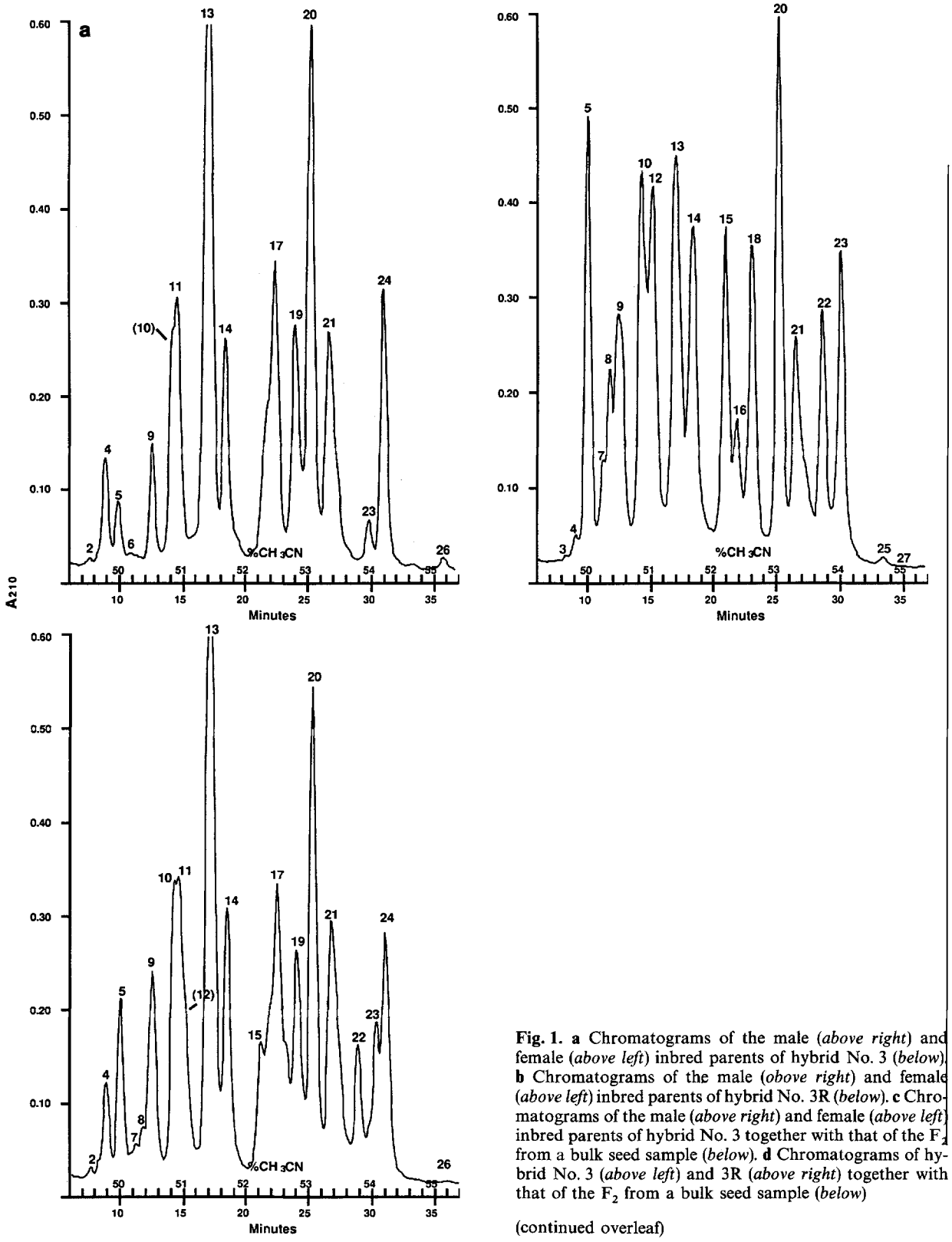


Fig. 1. **a** Chromatograms of the male (*above right*) and female (*above left*) inbred parents of hybrid No. 3 (*below*). **b** Chromatograms of the male (*obove right*) and female (*above left*) inbred parents of hybrid No. 3R (*below*). **c** Chromatograms of the male (*above right*) and female (*above left*) inbred parents of hybrid No. 3 together with that of the F_2 from a bulk seed sample (*below*). **d** Chromatograms of hybrid No. 3 (*above left*) and 3R (*above right*) together with that of the F_2 from a bulk seed sample (*below*)

(continued overleaf)

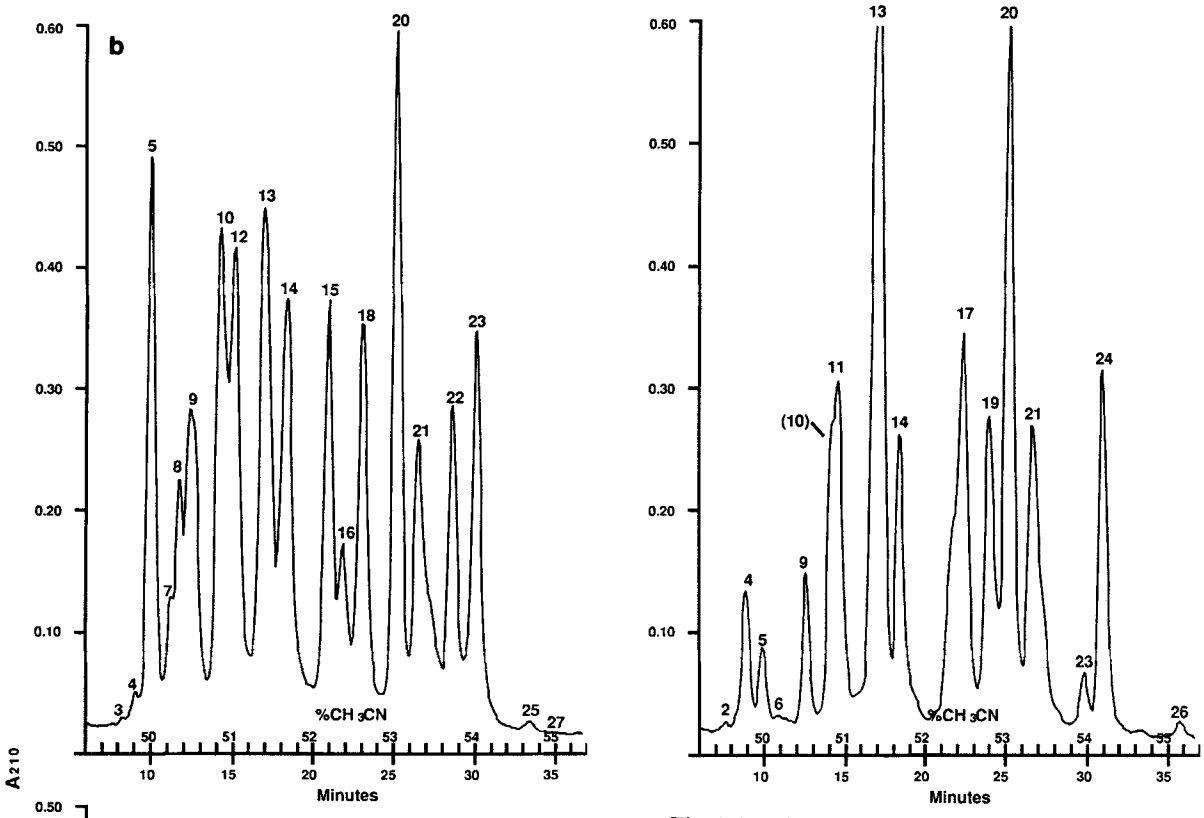
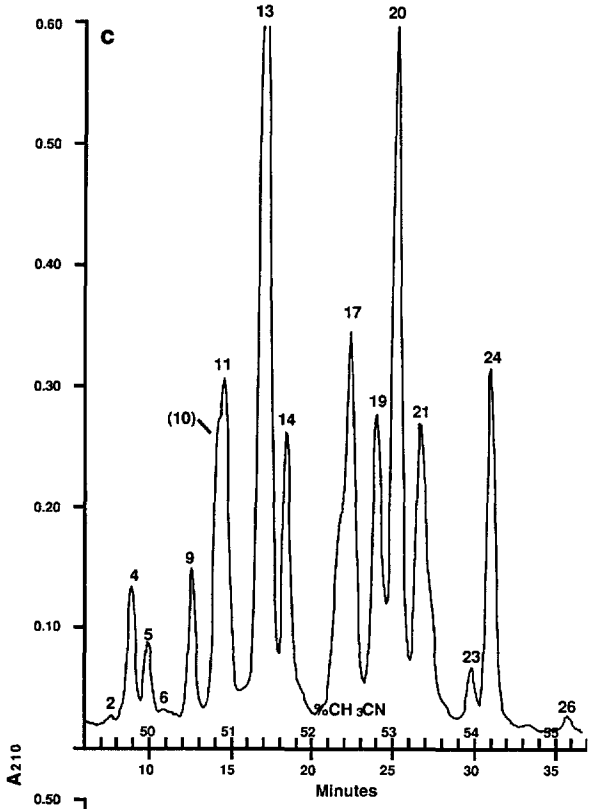


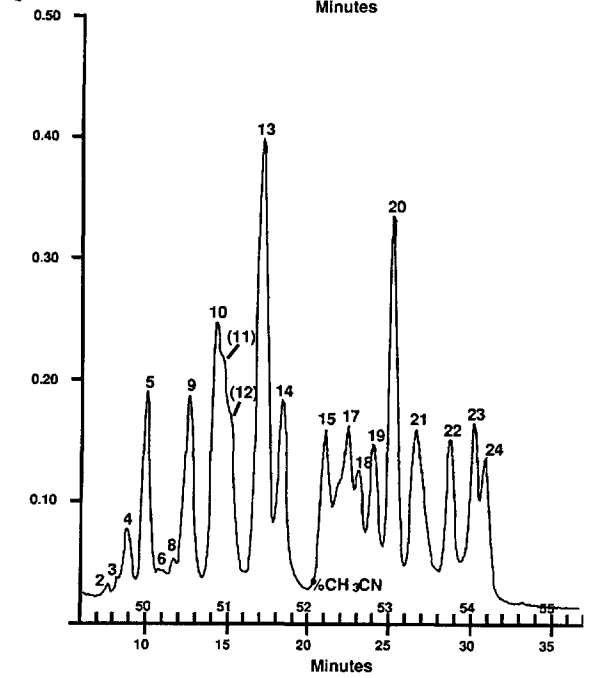
Fig. 1 (continued)

from available inbred profiles and then compared with the observed F_2 profile. The observed F_2 zein chromatographic profile would also be buffered against expression of any foreign alleles contributed by outcrossing. All zein alleles carried by pollen would be contributed by the male and, therefore, constitute an upper maximum bound of 33% of the observed profile. It is inconceivable that this level of contamination would normally occur.

Verification that chromatographic profiles of F_1 hybrids do routinely represent a 2:1 addition of female:male parental chromatograms and that extracts from F_2 bulk seed samples do give chromatograms equivalent to a 1:1 addition of the single-cross parents is advisable. RP-HPLC has a very high power of resolution (Bietz 1985) and zeins may exhibit unpredictable interactions mediated through structural and regulatory genes. Zein is coded by a multigenic set containing as many as 15 families of genes with possibly up to 100 alleles per haploid genome (Viotti et al. 1979). Zein structural genes are located as loosely linked clusters on chromosome arms 4S, 4L, and 7S (Soave and Salamini 1983). A two-dimensional gel electrophoretic profile of zein proteins showed the inbred line Mo17 to have some 25 zein proteins that had counterparts in one or both parent lines, C103 and CI.187-2. However, one spot did not have a counterpart in either parent. Wall et al. (1984), therefore, suggested that mutation or recombination could have affected the zein profile of Mo17.

The objective of this study was, therefore, to statistically test the fit of observed and expected chromatograms for several inbred lines, F_1's, reciprocal F_1's, and one set of F_2 generations using a broad base of germ-





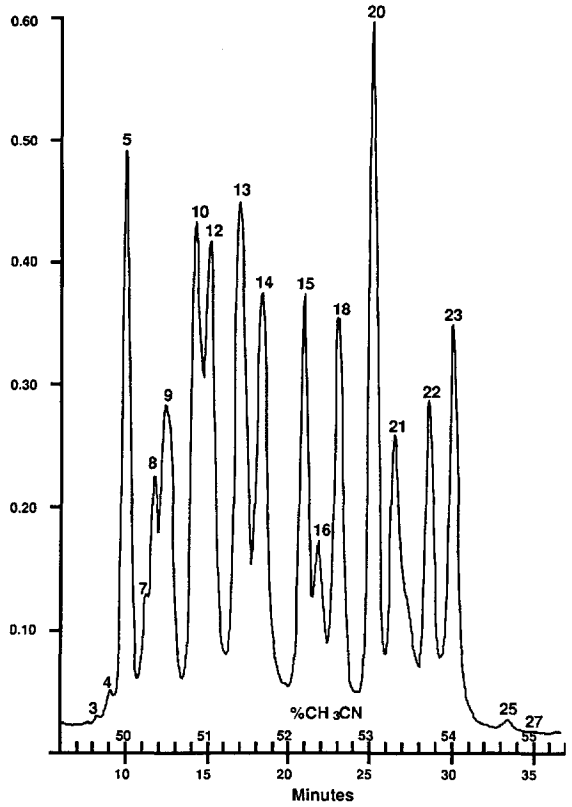
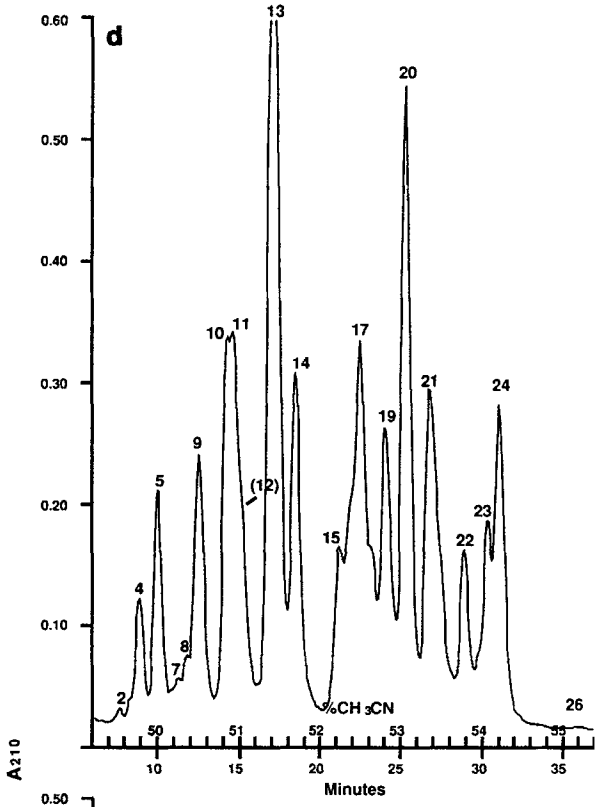


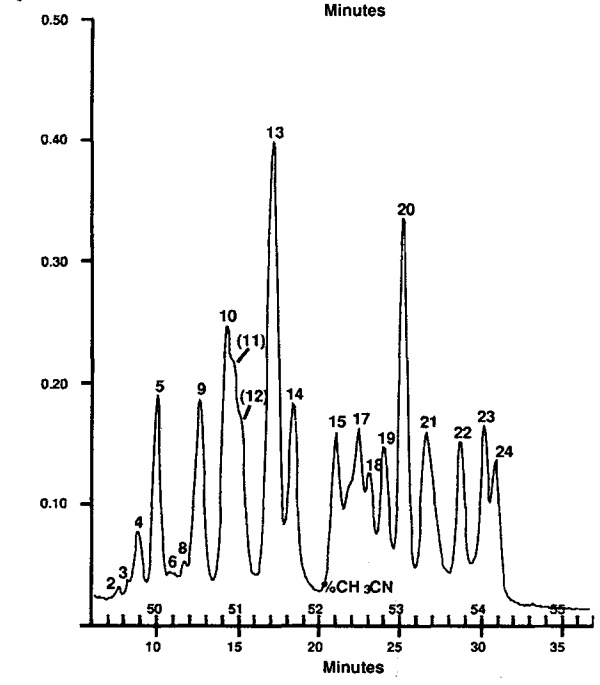
Fig. 1 (continued overleaf)

plasm which is representative of that used across the Corn Belt of the United States. Statistical analysis of data from several inbred lines and hybrids was deemed necessary because of the aforementioned complexities of zein inheritance, the detail of experimental resolution, and the potential practical import of zein and isozymic data in cultivar identification and pedigree analysis.

Materials and methods

Seed of 10 commercially available Pioneer brand single-cross hybrids (coded 1–10) and their reciprocals were produced at Johnston, Iowa in the summer of 1985. These hybrids are currently widely grown in the U.S. Corn Belt and encompass a range of maturities from 99 to 131 days from emergence to black layer. The 10 single-crosses were comprised of 11 inbred lines that included "Iowa Stiff Stalk Synthetic", "Lancaster Sure Crop", and "Iodent" backgrounds in their pedigrees. At the same time the F_1 and reciprocal crosses were made, a minimum of five plants of each F_1 in a separate planting was sib-pollinated from commercially available F_1 seed to produce F_2 seed. Thus, for each F_1, five seed samples were available for laboratory analysis: 1) female inbred; 2) F_1 female × male (commercially available hybrid seed); 3) male inbred; 4) F_1R (reciprocal of commercially available hybrid seed); and 5) F_2 (commercially available F_1 that was sib-pollinated). Seed sources of inbred lines used to produce both F_1 and subsequent F_2 seed traced back, within two generations of seed increase, to original hand-pollinated sources. Inbred seed increases were not made in the same season that other generations of seed were produced. However, increases made subsequent to hand-pollination were performed using strict isolation, planting, roguing, harvesting, and conditioning standards to protect against contamination. Thus, although source differences between inbred and other generations of seed could contribute to differences between expected and observed chromatograms, these differences have been found to be small and relatively insignificant (Smith and Smith 1986).





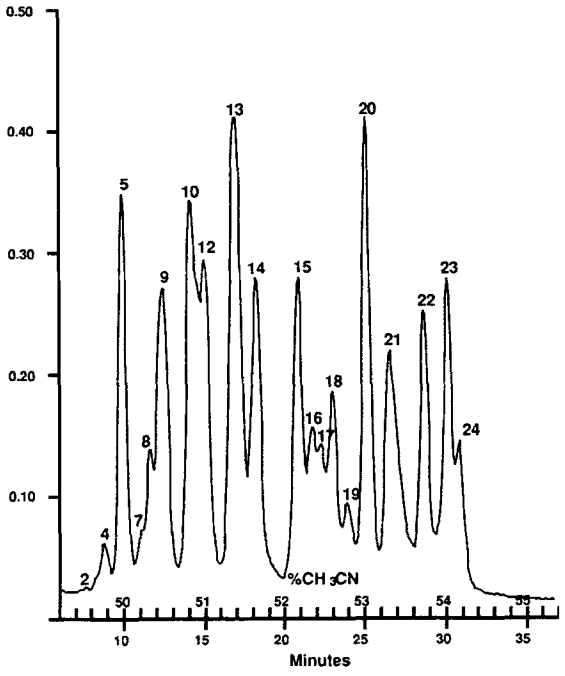


Fig. 1 (continued)

Within each inbred, F_1, and F_2 generation, five ears were bulked. Twenty to 25 kernels of each generation were bulked and ground to flour (Smith and Smith 1986). Zeins and alcohol soluble glutelins were extracted at 25 °C with vigorous shaking in 55% 2-propanol, 1% 2-mercaptoethanol (1:5; w/v) for 2.25 h, centrifuged for 12 min at 12,000 g and the supernatant filtered through a 0.5 µm membrane. Ten microliter of each sample were analyzed within 24 h of extraction. Samples were injected using a WISP 710B injector (Waters Assoc.) and proteins were eluted at 1 ml/min during a linear gradient from 48% to 55% acetonitrile plus 0.1% trifluoroacetic acid using a Brownlee aquapore RP-300 column at 70 °C with 15 min equilibration between runs. Eluent was monitored at 210 nm and spectrophotometric data were collected each 0.5 s. Data were stored and integrated using a chromatography software package supplied by Nelson Analytical (version 3.5) that was run on an IBM AT personal computer.

Peak areas were normalized by disregarding all peaks eluting prior to 5 min (solvent peaks) and expressing individual peak areas as a percentage of the remaining chromatogram. Peak numbers were assigned to the peaks for each sample such that the same peaks from different samples of the control genotype (B73), a publicly available and widely used line in the U.S., which was periodically injected, had the same peak numbers. Additional tests of the repeatability of the resolving power of the column were available since some of the hybrids had one inbred parent in common and thus replicate injections of other inbred lines, in addition to those provided by the control genotype, could be visually compared to monitor the activity of the column. All comparisons showed the column to be maintaining repeatable and detailed separations of zeins and alcohol soluble glutelins. A linear interpolation was used to adjust the sample peaks when times for common peaks of the controls differed by more than 15 s. Peaks from two samples were considered to be the same if they eluted, after adjustment, within 15 s of each other.

Chi-square values for were computed from the observed and expected values based upon four models. These were 1) $F_1 = \frac{2}{3} F + \frac{1}{3} M$; 2) $F_2 = \frac{1}{2} F + \frac{1}{2} M$; 3) $F_1R = \frac{2}{3} M + \frac{1}{3} F$; and 4) $F_2 = (F_1 + F_1R)/2$ where 'F' and 'M' relate to the assignations of female and male parents in the commercially available Pioneer brand hybrids, respectively. Models 1 and 3 test the goodness of fit of a chromatogram from an F_1 with that of a 2:1 addition of its female:male parents. Model 2 tests the fit of a chromatogram from an F_2 with that of a 1:1 addition of the inbred

Table 1. Probabilities of a greater chi-square for each of four tests of observed against expected chromatograms for each hybrid

Hybrid[a]	Model			
	$F_1=\frac{2}{3}F+\frac{1}{3}M$	$F_2=\frac{1}{2}F+\frac{1}{2}M$	$F_1R=\frac{2}{3}M+\frac{1}{3}F$	$F_2=(F_1+F_1R)/2$
1	0.83	0.57	0.98	0.88
2	0.99	0.90	0.35	0.65
3	1.00	0.01 *	0.98	0.54
4	1.00	0.99	1.00	1.00
5	1.00	0.94	0.74	1.00
6	0.99	0.82	0.73	1.00
7	1.00	1.00	0.99	1.00
8	0.87	1.00	0.99	1.00
9	0.99	0.27	0.59	0.92
10	1.00	0.78	0.67	0.93

[a] Pioneer brand hybrids coded 1–10
* Significantly different from expected

parents of the F_1 single-cross. Model 4 tests the fit of a chromatogram from an F_2 with that of a 1:1 addition of chromatograms from the F_1 and F_1 reciprocal generations.

Results

The probabilities of a greater chi-square value for each test of observed versus expected chromatograms are presented in Table 1. Among the 40 comparisons, 39 showed no difference between the observed chromatograms and those that were expected based on the four models. In one instance (for hybrid 3), a difference between observed and expected chromatograms was shown for the fit of $F_2 = ½\ F + ½\ M$ (Table 1).

Individual peak areas, chi-square values for these peaks, and chromatograms of inbreds, hybrids, and F_2 sources for hybrid 3 are presented in Table 2 and Fig. 1. (Elution times shown in the figures have not been standardized to elution times of the control genotype.) Peaks 10, 11, and 12 contributed more to the significant difference between observed and expected chromatograms. For peak 10, the male was over-represented in the F_1, for peak 11 the female was over-represented in the F_1, and for peak 12, the male was under-represented in the F_1. These peaks eluted closely together and overlapped (Fig. 1). Peak 10 was not identified as a separate entity in the female inbred but was present as a shoulder of peak 11. Similarly, peak 12 was not detected in the F_1 but was present as a shoulder. The F_2 chromatograms for hybrid 3 clearly showed that peaks 11 and 12 were only shoulders (Fig. 1).

Quantitative and qualitative similarities were exhibited by hybrids 5, 4R (pollinated in the reverse direction to the commercially available hybrid), and 7R (Fig. 2) that had common female parents. However, qualitative differences for peaks eluting between 12 and 16 min dis-

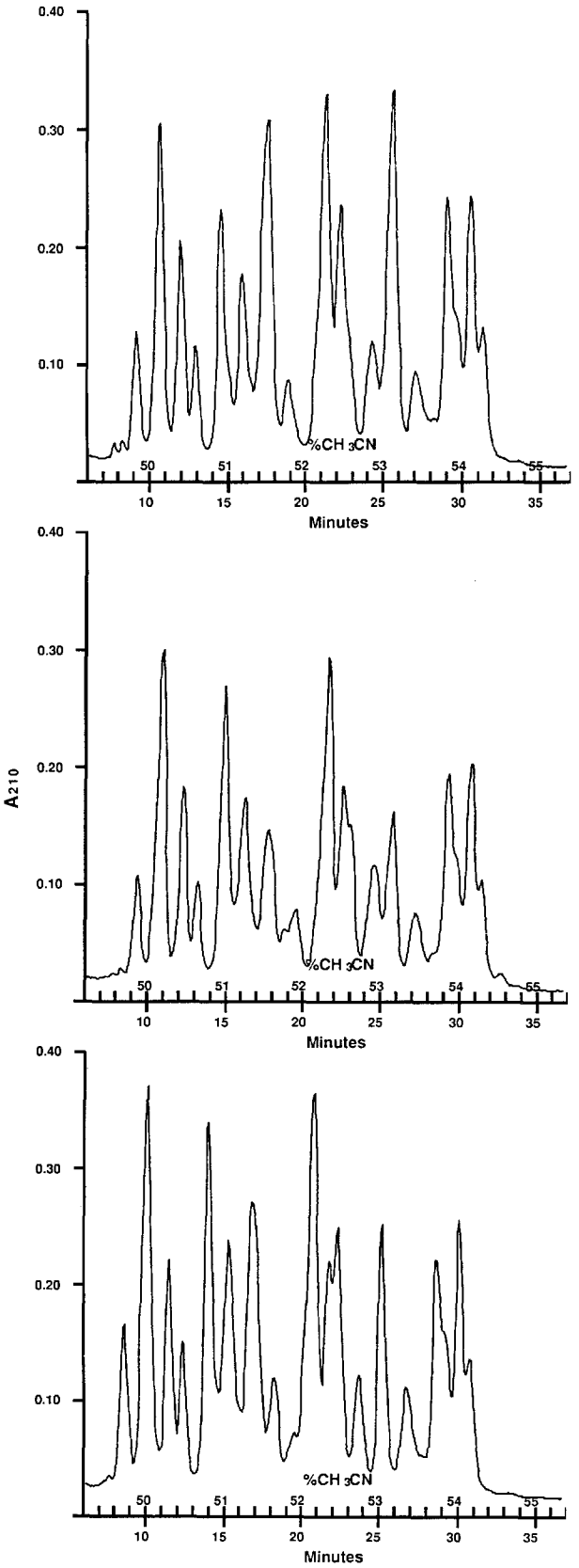


Fig. 2. Chromatograms of hybrids 5, 4R, and 7R (*top to bottom*, respectively) each with a common female inbred parent

Table 2a. Individual peak areas for inbred lines, F_1, F_1R, and F_2 sources for hybrid No. 3

Peak no.	Peak area (%)				
	Female inbred	F_2	F_1	Male inbred	F_1R
2	0.1224	0.1210	0.1349	0.0000	0.0164
3	0.0000	0.1860	0.0000	0.0503	0.0000
4	2.6374	1.5812	1.9728	0.3640	0.8757
5	1.5474	4.7216	3.4926	7.2996	6.4872
6	0.1608	0.2571	0.0000	0.0000	0.0000
7	0.0000	0.0000	0.5277	1.3033	0.7039
8	0.0000	0.6634	0.6961	2.7267	1.9204
9	2.7136	6.0586	4.9554	6.6030	7.3750
10	0.0000	14.6294	4.4326	8.3012	9.1877
11	10.7201	0.0000	8.3813	0.0000	0.0000
12	0.0000	0.0000	0.0000	8.1225	6.1726
13	21.6642	15.0372	17.4258	10.1048	11.8631
14	6.5371	6.1741	6.3096	9.1032	7.2699
15	0.0000	4.8735	2.8680	6.6047	6.2384
16	0.0000	0.0000	0.0000	2.8248	2.7796
17	13.4487	6.8951	11.6393	0.0000	2.2069
18	0.0000	3.1821	0.0000	7.0373	3.9308
19	7.0874	4.3706	5.5093	0.0000	1.8739
20	14.7996	10.1326	10.7267	10.4545	8.9808
21	9.7851	7.8060	8.8437	6.9071	7.7229
22	0.0000	4.5936	2.9610	5.0224	5.5080
23	1.2787	4.9525	3.5603	6.9972	6.2269
24	7.1989	3.7644	5.5628	0.0000	2.6599
25	0.0000	0.0000	0.0000	0.1641	0.0000
26	0.2988	0.0000	0.0000	0.0000	0.0000
27	0.0000	0.0000	0.0000	0.0092	0.0000

Table 2b. Chi-square values[a] for individual peaks for hybrid No. 3

Peak no.	Model			
	$F_1=\frac{2}{3}M+\frac{1}{3}F$	$F_2=\frac{1}{2}M+\frac{1}{2}F$	$F_1R=\frac{2}{3}M+\frac{1}{3}F$	$F_2=(F_1+F_1R)/2$
2	0.035	0.059	0.015	0.027
3	0.017	1.030	0.034	
4	0.005	0.004	0.054	0.017
5	0.000	0.020	0.227	0.014
6	0.107	0.388	0.054	
7	0.020	0.652	0.031	0.616
8	0.050	0.359	0.006	0.318
9	0.223	0.421	0.806	0.002
10	1.003	26.455*	2.412	8.978*
11	0.213	5.360*	3.573	4.191*
12	2.708	4.061	0.106	3.086
13	0.008	0.045	0.314	0.011
14	0.159	0.346	0.116	0.056
15	0.202	0.747	0.765	0.023
16	0.942	1.412	0.427	1.390
17	0.797	0.004	1.155	0.000
18	2.346	0.032	0.123	0.753
19	0.130	0.193	0.101	0.125
20	0.516	0.403	0.717	0.008
21	0.000	0.035	0.003	0.028
22	0.989	1.727	1.393	0.030
23	0.044	0.160	0.253	0.001
24	0.121	0.008	0.028	0.029
25	0.055	0.082	0.109	
26	0.199	0.149	0.100	
27	0.003	0.005	0.006	

[a] Values asterisked are ≥ 3.84 and are singificant at the 0.05 level

tinguished hybrid 4R from hybrids 5 and 7R. Hybrids 5 and 7R differed in their elution profiles at approx 21–22 min. Similarly, although hybrids 6, 1, 2, and 7 have common female parents and shared many qualitative and quantitative characteristics, each chromatogram could be uniquely identified (Fig. 3).

Discussion

Closely eluting proteins present within the F_1 or F_2 generation may not always be revealed as separate peaks, at least under routine operating conditions of the chromatograph or data integrator. It is, therefore, important to view not only peak areas and percentages in tabular format, but also to observe the chromatograms themselves so that presence of peak shoulders can be verified.

The results from this statistical comparison of inbred lines and single cross F_1, including reciprocal F_1 (F_1R), hybrid chromatograms provided additional support for the evidence that the F_1 chromatographic profile is a close fit to a 2:1 addition of the female:male parental chromatographic profiles (Bietz 1985). This had also been found previously, at least by visual inspection, for 2-dimensional electrophoretic profiles of zeins (Wall et al. 1984). These data, therefore, fully support the finding that chromatographic profiles of inbred lines and hybrids can be accurately simulated (Bietz 1985). Furthermore, the data presented herein support the contention that chromatographic profiles of F_2 field-grown samples can be accurately simulated (Bietz 1985). This is because RP-HPLC detects the specific contributions of the male and female gametes in both F_1 and F_2 generations. There was no example of a peak appearing in an F_2 source that was not also present in one or both parent lines that were used to make the F_1 from which seed of that F_2 was produced. Even though inbred seed lots were not produced in the same environment as other generations of seed, the results of this study substantiate that source effects are relatively insignificant (Smith and Smith 1986). Thus, chromatographic profiles can be accurately simulated without the necessity for a preplanned program of inbred seed increase coordinated with F_1 or F_2 seed production. It is imperative, however, that inbred seed sources be representative of those used in the production of F_1 seed. Isozyme electrophoresis could be

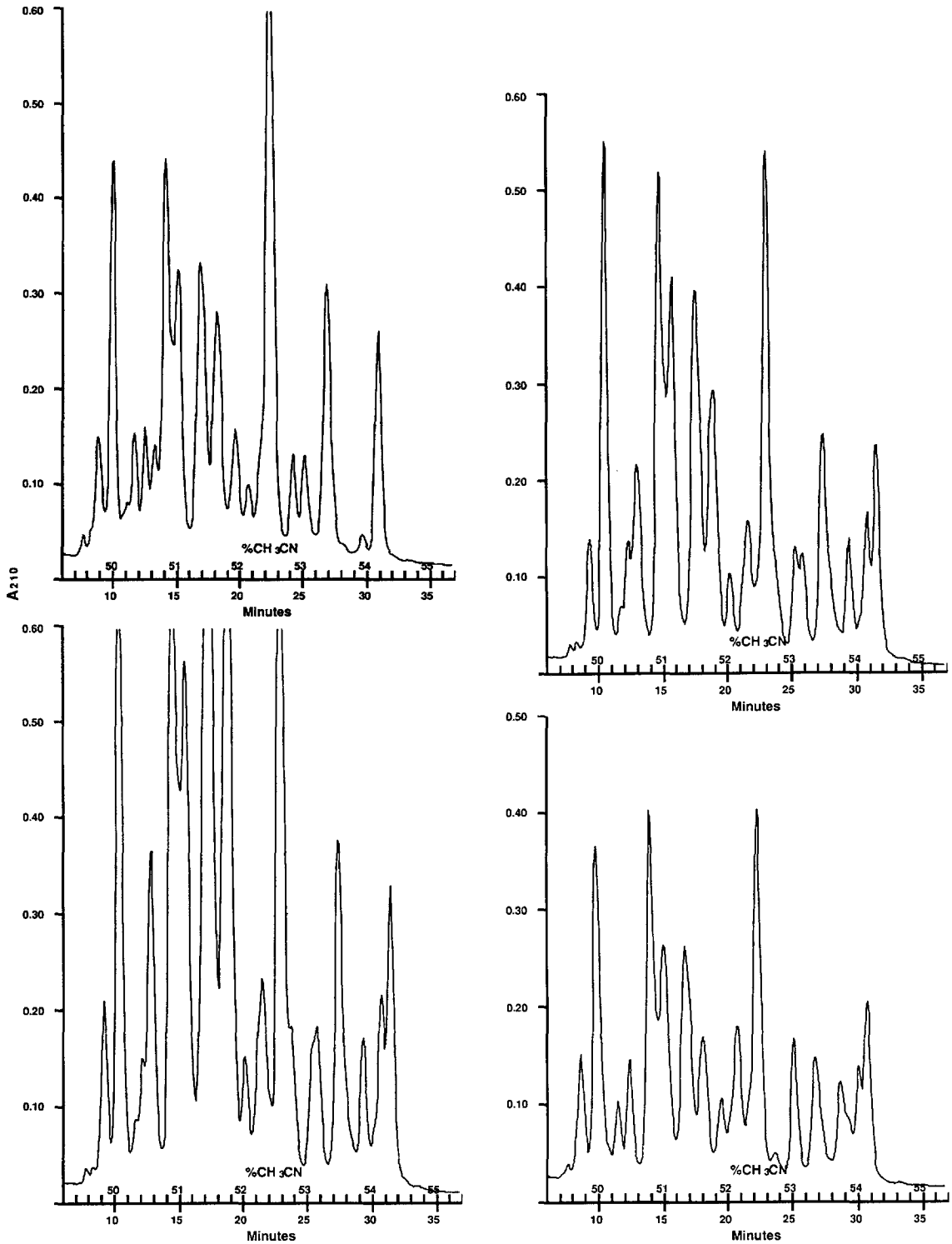


Fig. 3. Chromatograms of hybrids 6, 1, 2, and 7 (*top to bottom*, respectively) each with a common female inbred parent

used to check the genotypes of inbred seed lots available for analysis with those that were used to produce the F_1 and F_2 generations.

Previously, two-dimensional electrophoretic profiles of zein proteins (Wall et al. 1984) showed unexpectedly that one protein spot, out of some 25 detected in Mo17, did not have a counterpart in either parent. An additional explanation for that isolated phenomenon to those of mutation and recombination (Wall et al. 1984) or unexpected interaction among genes could be that at least

one of the seed sources used in electrophoresis was different from the original parental source either through outcrossing during line maintenance or because of a lack of fixation in one or both of the original parent lines, C103 and CI.187-2. Mo17 was released in 1964 and its parent lines were released prior to 1950 (Henderson 1976). It is, therefore, likely that one or more cycles of seed increase had occurred for these lines prior to the 2-dimensional electrophoretic study (Wall et al. 1984).

RP-HPLC could be useful in registration, certification, and in the checking of hybrid pedigrees. Even in those cases where hybrids shared a common female parent, the zein chromatographic profiles of the hybrids were distinguishable. Bietz (1985) has shown, as have the results of this study, that the chromatographic profile of a single cross hybrid closely resembles both parental lines and shows the predominant influence of the female inbred parent. Thus, the hypothetical female inbred of a single cross hybrid can be partially identified through comparisons of inbred and F_1 chromatograms. Isozymic data could then be used as a check of the pedigree indicated by zein chromatographic data. Chromatography of F_2 field grown samples could also assist in the identification of hybrid pedigree.

RP-HPLC data can, therefore, compliment isozymic data in studies designed to show hybrid pedigree. Isozymic data can uniquely identify 85% of U.S. public inbred lines (Stuber and Goodman 1983). This level of unique identification will most likely be raised with the introduction of additional polymorphic loci (Wendel et al. 1985, 1986) compared to those reported upon by Stuber and Goodman (1983). Chromatographic data can uniquely identify similar percentages of widely used inbred lines so that the complimentary use of isozymic and zein chromatographic data should provide unique characterization for nearly all widely used inbred lines, including lines that are closely related by pedigree (Smith and Smith 1987, 1988a). Concordant identification of the parents of a hybrid by complimentary techniques that assay different gene products minimizes the possibility that such identifications will be in error. If necessary, further analyses could be conducted using DNA descriptors (Restriction Fragment Length Polymorphisms or RFLP's), 2-dimensional gel electrophoresis, or tests of combining ability from measures of heterosis between hybrids (Troyer et al. 1983; Paszkiewicz et al. 1986) or between inbred lines (United States District Court 1987; Smith and Smith 1988b). Isozymic and chromatographic data (Smith 1988) would be usefully performed prior to these more expensive and time consuming genetic screening methods. An ability to precisely show the genotypes of inbreds and hybrids is important because it reveals the identity and level of genetic diversity that is available in production and in breeding.

Acknowledgements. S. Wall provided assistance in the statistical computations. Useful suggestions on a preliminary draft were provided by Drs. M. Albertsen, R. McConnell, R. Fincher, and A. Cavalieri.

References

Bietz JA (1985) High performance liquid chromatography: How proteins look in cereals. Cereal Chem 62:201–212

Henderson CB (1976) Maize research and breeders manual. 8. Illinois Foundation Seeds Inc, Champaign, Ill

Paszkiewicz SR, Schaffer JA, Darrah LL (1986) Genetic diversity among widely grown maize hybrids in Missouri. Agron Soc (Abstr), p 77

Smith JSC (1988) Genetic diversity in U.S. hybrid maize germplasm: allozymic and chromatographic evidence. Crop Sci 28:63–69

Smith JSC, Smith OS (1986) Environmental effects on zein chromatograms of maize inbred lines revealed by reversed-phase high-performance liquid chromatography. Theor Appl Genet 71:607–612

Smith JSC, Smith OS (1987) Associations among inbred lines of maize using electrophoretic, chromatographic, and pedigree data. 1. Multivariate and cluster analysis of data from Lancaster Sure Crop derived lines. Theor Appl Genet 73:654–664

Smith JSC, Smith OS (1988a) Associations among inbred lines of maize using electrophoretic, chromatographic, and pedigree data. 2. Multivariate and cluster analysis of data from Iowa Stiff Stalk Synthetic derived lines. Theor Appl Genet (in press) 76:39–44

Smith JSC, Smith OS (1988b) The description and assessment of distances between inbred lines of maize. II. The utility of morphological, biochemical, and genetic descriptors and a scheme for the testing of distinctiveness between inbred lines. Maydica (in review)

Soave C, Salamini F (1983) Genetic organization and regulation of maize storage proteins. In: Dussant J, Mosse J, Vaughan J (eds) Seed proteins. Academic Press, London, pp 205–218

Stuber CW, Goodman MM (1983) Allozyme genotypes for popular and historically important inbred lines of corn, *Zea mays* L. USDA-ARS ARR-S-16 USDA, New Orleans, La

Troyer AF (1986) United States Patent. Inbred Corn Line. Patent No 4,594,810, Appl No 703,589, Filed Feb 21, 1985

Troyer AF, Openshaw SJ, Knittle KH (1983) Measurement of genetic diversity among commercial corn hybrids: method. Am Agron Soc Meeting 1983 (Abstr), p 83

United States District Court (1987) Pioneer Hi-Bred International, Inc, an Iowa corporation, Plaintiff versus Holden Foundation Seed Inc, Defendants. US District Court, Southern District of Iowa. Case No 81-60-E, 108 pp

Viotti A, Sala E, Marotta R, Alberi P, Balducci C, Soave C (1979) Genes and mRNAs coding for zein polypeptides in *Zea mays*. Eur J Biochem 102:211–222

Wall JS, Fey DA, Paulis JW (1984) Improved two-dimensional electrophoretic separation of zein proteins: application to study of zein inheritance in corn genotypes. Cereal Chem 61:141–146

Wendel JF, Goodman MM, Stuber CW (1985) Mapping data for 34 isozyme loci currently being studied. Maize Genet Coop Newslett 59:90

Wendel JF, Goodman MM, Stuber CW (1986) Additional mapping of isozyme loci: localization of *Acp4, Dia2, Adk1, Tpi3* and *Sad1*. Maize Genet Coop Newslett 60:109–110

Wilson CM (1984) Isoelectric focusing of zein in agarase. Cereal Chem 61:198–200