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Analysis of European and North American Maize Inbred and Hybrid Lines by Monolithic and Perfusion Reversed-Phase High-Performance Chromatography and Multivariate Analysis

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Monolithic and perfusion RP-HPLC methods have been employed for the separation of maize proteins from several European and North American inbred and hybrid lines in analysis times close to 4 min for the perfusion column and close to 8 min for the monolithic column. A study of the repeatability of the protein extraction conditions and of the perfusion and monolithic RP-HPLC methods was performed, indicating low values of variance for the relative peak areas and for the retention times. On the other hand, a low inter-kernel variability of these chromatographic parameters was also found, confirming the possibility of a variety identification and classification of maize inbred and hybrid lines by using a RP-HPLC analysis of the maize proteins. A multivariate analysis of the chromatographic data was carried out in order to characterize and identify the inbred and hybrid maize lines. Cluster analysis of the data showed how far or closely related were the maize lines. Principal component analysis showed that protein chromatographic data had enough information to distinguish between the different groups of maize lines. Finally, a linear discriminant analysis enabled the correct classification of the inbred and hybrid lines according to their geographical origin showing the best percentage of cases correctly classified for monolithic RP-HPLC.

KEYWORDS: Maize; inbred; hybrid; monolithic RP-HPLC; proteins

INTRODUCTION

There is much interest in the characterization of genetic diversity within and among elite breeding materials and cultivars of maize (*1*, *2*). Knowledge of the genetic relationships among breeding materials is fundamental for the optimal designing of breeding programs. Moreover, this information could help to prevent the great risk of increasing uniformity in the elite germoplasm and could also ensure long-term selection grains. Different approaches are available to investigate the genetic diversity and relationships among maize lines (*3*). These include morphological traits, pedigree data, molecular techniques based on the analysis of DNA (*4*−*6*), and biochemical traits such as isoenzymes (*7*−*9*) and zein storage proteins (*1*, *2*, *8*).

By other hand, RP-HPLC has been applied to the selection, identification, and marketing of different plant genotypes (*10*). Varietal identification is possible since storage proteins expression is nearly invariant with genotypes, and "fingerprints" vary little with environment or even upon germination (*10*). In fact, zein storage proteins have usually been analyzed by RP-HPLC (*1*, *2*, *8*, *10*−*13*). In some cases, this has been applied to the identification of maize cultivars. In fact, Smith and Smith (*1*, *2*) and Smith (*8*) developed RP-HPLC methods for the separation of proteins in maize lines that was applied to the characterization of Lancaster Sure Corp and Iowa Stiff Stalk Synthetic derived maize inbred lines among others. Furthermore, Robutti et al. (*12*) found associations among Argentine maize races by using multivariate analysis of RP-HPLC data. Rodriguez-Nogales et al. (*14*) and Lookhart and Juliano (*1*) have reviewed several studies focused on the comparison of maize inbreeds and hybrids and genotype identification of maize lines using conventional RP-HPLC methods

RP-HPLC for the screening of maize lines has resulted in high analysis times (40 to 90 min) (*10*−*14*). This fact is related with the low diffusivity of proteins (*15*). The development of perfusion and monolithic stationary phases has enabled a major reduction of the analysis times in the separation of proteins by RP-HPLC. Perfusion supports overcome the mass transfer problems associated with conventional chromatography by using packing materials consisting of a cross-linked polystyrene−divinylbenzene matrix having large "throughpores" with diameter of 6000−8000 Å that allow sample molecules to "perfuse" rapidly through the interior of the particles. In addition, this material possesses very short "diffusive pores" with diameter of 500−1500 Å that branch off from the throughpores. On the other hand, monolithic columns are made of a single piece of a highly porous material (silica or organic polymers) with a bimodal pore structure of throughpores and mesopores enabling

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the separation of large molecules in low analysis times (*16*−*20*). In both cases, the combination of large and diffusive pores accelerates the mass transfer of large molecules such as proteins (*15*, *21*−*22*).

Recently, our research group separated maize proteins by perfusion (*23*) and monolithic (*24*) RP-HPLC observing a significant reduction of analysis times (<4 min in the perfusion column and <8 min in the monolithic column). These methods have been applied to the characterization of commercial maize products (*23*, *24*). Since these high-velocity stationary phases have never been applied to varietal identification, the aim of this work has been the characterization (identification and classification) of European and North American inbred and hybrid maize lines using perfusion and monolithic RP-HPLC and multivariate statistical techniques.

MATERIALS AND METHODS

Chemicals and Samples. 2-Mercaptoethanol (Merck, Darmstadt, Germany) and ammonium acetate (Panreac, Barcelona, Spain) were employed for the preparation of maize solutions. HPLC grade acetonitrile (ACN) (Merck), HPLC grade water (Milli-Q system, Millipore, Bradford, MA), and trifluoroacetic acid (TFA) (Sigma, St. Louis, MO) were used for the preparation of mobile phases.

Maize inbred and hybrid lines, 25 lines of each type, were kindly provided by Maize Germplasm Bank (Experimental Station of Aula Dei, CSIC, Zaragoza, Spain). The origin of these maize inbred lines was as follows: A239, A251, A427, A556, A619, A632, A635, A638, and A639 from Minnesota (USA); B14, B73, and B84 from Iowa (USA); Mo17 from Missouri (USA); Va26 from Virginia (USA); W64A from Wisconsin (USA); CM105 and CM109 from Morden (Canada); EZ1, EZ16A3, EZ18, EZ19, EZ6, EZ7, and EZ8 from Spain; and F212 from France. The hybrid lines studied were: EZ18 × EZ19, EZ19 × EZ27, EZ18 × EZ8, A239 × A251, A639 × EZ7, A632 × Mo17, EZ6 × B73, A632 × EZ16A3, EZ19 × EZ8, B73 × Mo17, A639 × A64, B73 × A632, EZ1 × EZ18, A556 × A427, A632 × EZ8, B73 × B84, A632 × EZ72, A632 × EZ6, A639 × F212, A641 × CM105, A632 × EZ19, A619 × A632, EZ18 × E27, EZ18 × E27, and A632 × EZ1.

Maize Protein Extraction. For every maize line, 30 kernels were ground using an analytical mill (IKA Labortechnik, Staufen, Germany) during 3 min at ambient temperature. Afterward, 60 mg of pulverized maize were weighed and dissolved in 2 mL of the extraction solution (0.5% (v/v) 2-mercaptoethanol, 0.5% (w/v) ammonium acetate in 45% ACN (v/v) and 55% Milli-Q water (v/v)), sonicated for 5 min in a bath sonicator (150 W, 50 Hz, FS-30, Fisher Scientific, Pittsburgh, PA), and centrifuged at 3362*g* (Avanti J-25 centrifuge, Beckman Coulter, USA) for 10 min at 25 °C (*23*, *24*). The supernatant was removed and injected into the chromatographic system.

High-Performance Liquid Chromatography. A Hewlett-Packard 1100 series liquid chromatograph (Hewlett-Packard, Pittsburgh, PA) with a degassing system, a binary pump, a thermostated compartment for the column, an injection system, and a diode-array detector was employed to carry out the separations. The chromatographic separations were accomplished with two different reversed-phase supports: a POROS R2/H perfusion column (4.6 × 50 mm; 10 μm particle size) (Perseptive Biosystems, Framingham, MA) and a monolith silica column Chromolith Performance RP-18e (4.6 × 100 mm) (Merck). The separation conditions for the perfusion column were optimized previously by our research team (*23*): injection volume, 20 μL; flow-rate, 3 mL/min; temperature, 25 °C; mobile phase A, 0.1% (v/v) TFA in Milli-Q water; mobile phase B, 0.1% (v/v) TFA in ACN; linear binary gradient, 5.0−50.2% B in 2.40 min, 50.2−65.4% B in 0.98 min, and 65.4−5.0% in 1 min; UV detection, 280 nm. The separation conditions for the monolithic column were those optimized previously by the authors (*24*): temperature, 35 °C; linear binary gradient, 5.0−26.4% B in 5.15 min, 26.4−87.5% B in 2.16 min, and 87.5−5.0% in 1 min. The injection volume, flow-rate, mobile phase composition, and wavelength detection were as in perfusion chromatography. HP-Chemstation software was used for data acquisition and processing.

Data Treatment. The area percentage for every peak was calculated as the average of two replicates (injected by triplicate). The integration was performed by setting the baseline from valley to valley. As the variables have the same units of measurement, the samples were not autoscaled. Cluster analysis, principal component analysis, and linear discriminant analysis were done with the computer program Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Rockville, MD).

RESULTS AND DISCUSSION

The chromatogram profiles obtained by perfusion and monolithic RP-HPLC for different kinds of commercial maize products enabled their application for the characterization of these products. These results encouraged us to test the applicability of these methods for the first time for the characterization of maize cultivars.

Figure 1 shows, as an example, the chromatographic profiles obtained for three related lines, the inbred maize lines A632 and EZ18 and their hybrid line A632 × EZ18, obtained with perfusion and monolithic RP-HPLC. Peak assignment and numbering were done after collecting and comparing all the protein patterns corresponding to all the maize samples. Chromatograms obtained with the perfusion column showed a main peak at about 1.2 min (peak D) together with a last peak at 3 min and some minor peaks between them. In some maize lines, as example EZ18, the peak at 3 min was partially resolved in two peaks (peak I and J). In a previous work of our team (*23*), the peaks I and J were identified as α-zein and the peaks D−G as (β- and γ-) zeins. The phenotypic expression of the two inbred lines was very similar but a detailed study showed differences in the content of (β- and γ-) zeins, while a notable reduction of synthesis of α-zein was observed for the hybrid line A632 × EZ18.

Chromatograms observed for monolithic RP-HPLC were totally different to those obtained with perfusion RP-HPLC. Maize proteins separated in a higher number of peaks with the monolithic column and, in principle, this column presented a higher potential for cultivar identification than the perfusion column. Unlike perfusion chromatography, the monolithic chromatograms yielded four groups of peaks with retention times ranging from 2.0 to 3.5 min (first group), 3.6 to 4.8 min (second group), 5.0 to 5.8 (third group), and 5.9 to 7.5 min (last group). The last peak (peak 24) was identified as α-zein and the peaks 19, 20, and 22 as (β- and γ-) zeins (*24*). Note that the triplet of peaks 16−18 was expressed with higher intensity for the A632 line than for the EZ18 one, while for the hybrid line A632 × EZ18, the expression of the triplet was more moderate than for its parent A632 line.

Mean values of relative peak areas obtained by monolithic RP-HPLC analysis of maize proteins for each group of inbred and hybrid lines grouped according to their geographical origin are summarized in **Table 1**. A total of 26 protein peaks were found but only the peaks with relative area higher than 1% are shown. The analysis of variance (ANOVA) performed on inbred and hybrid lines disclosed significant differences for all variables selected except for peaks 4, 6, and 19. A Tukey test (*25*) ($\alpha = 0.05$) was carried out to investigate the existence of statistically significant differences between samples on the significant peaks. For that purpose, this test performs a pairwise comparison of the means to see where the significant differences are. Mean values of relative areas for the same peak with different superscripted numbers (every number different) differ significantly among the different maize lines at 5% significance level. Therefore, two means indicated with a superscripted number containing one or more common number do not present any statistically significant difference (e.g., $19.2^{1,2}$ is statistically

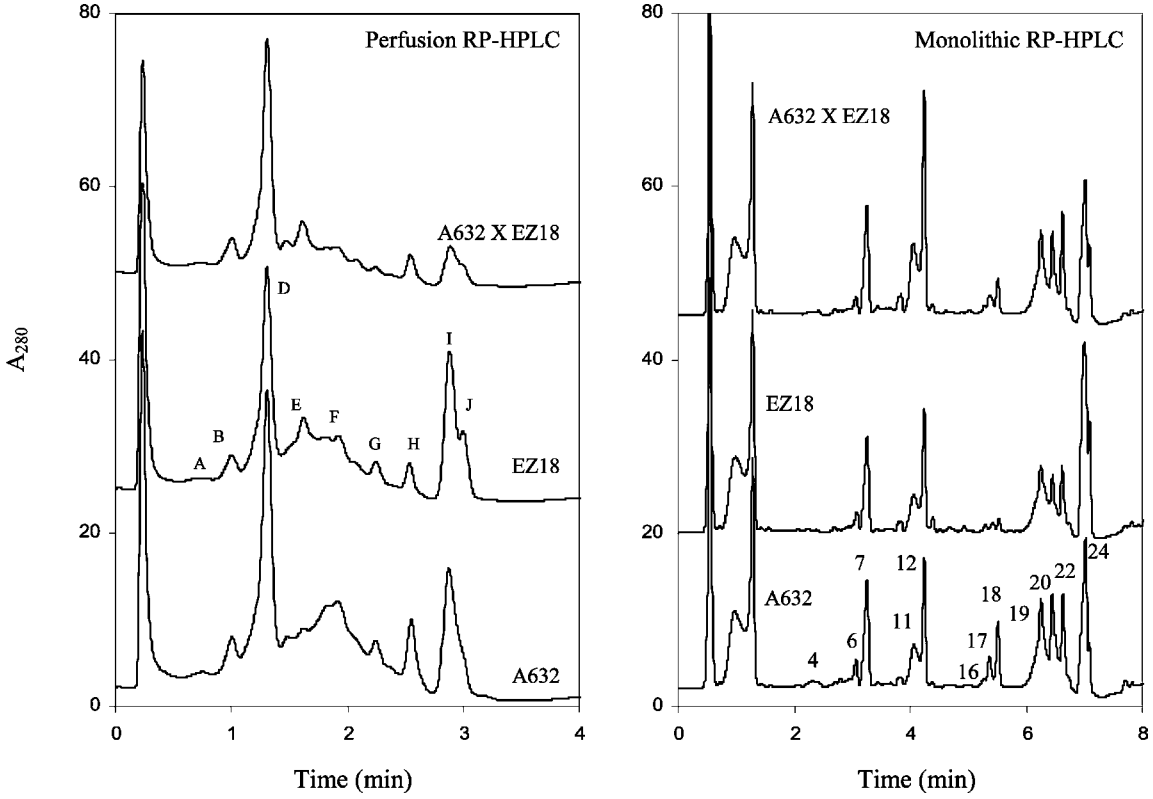

Figure 1. Perfusion and monolithic RP-HPLC chromatograms of proteins extract from several maize inbred and hybrid lines.

Table 1. Peak Area Percentages Corresponding to the Analysis of Protein Extracts from Different Inbred and Hybrid Maize Lines by Monolithic RP-HPLC

	peak area percentages (%)[a,b]											
peaks:	4	6	7	11	12	16	17	18	19	20	22	24
retention time (t_R, min):	2.44	3.06	3.26	4.06	4.24	5.26	5.37	5.51	6.25	6.45	6.62	6.99
origin of maize lines												
Minnesota	0.4 ^{1}	1.6 ^{1}	19.2 ^{1,2}	4.0 ^{1,2}	9.8 ^{1}	2.7 ^{1}	7.0 ^{1,2}	13.7 ^{1,2}	5.1 ^{1}	8.4 ^{4}	4.6 ^{1,2}	18.5 ^{3,4,5}
Iowa	0.4 ^{1}	1.5 ^{1}	22.2 ^{2}	5.5 ^{1,2}	14.7 ^{1,2,3}	1.1 ^{1}	0.6 ^{1}	19.1 ^{2}	6.3 ^{1}	7.2 ^{4}	9.0 ^{1,2}	2.8 ^{1,2}
Morden	0.0 ^{1}	2.0 ^{1}	21.4 ^{1,2}	5.4 ^{1,2}	13.5 ^{1,2,3}	1.2 ^{1}	3.9 ^{1,2}	11.5 ^{1,2}	5.4 ^{1}	10.1 ^{5}	2.8 ^{1,2}	11.5 ^{1,2,3,4,5}
Spain	0.2 ^{1}	1.3 ^{1}	14.2 ^{1}	6.3 ^{2}	15.6 ^{1,2,3}	2.5 ^{1}	11.3 ^{2}	12.5 ^{1,2}	3.7 ^{1}	6.5 ^{4}	8.6 ^{2}	5.1 ^{1,2}
France	0.0 ^{1}	2.2 ^{1}	23.5 ^{1,2}	5.6 ^{1,2}	12.5 ^{1,2,3}	1.9 ^{1}	11.9 ^{1,2}	7.2 ^{1}	5.3 ^{1}	8.0 ^{4}	3.5 ^{1,2}	10.6 ^{1,2,3,4,5}
Virginia	1.0 ^{1}	2.3 ^{1}	16.1 ^{1,2}	1.6 ^{1,2}	3.7 ^{1,2}	3.4 ^{1}	8.0 ^{1,2}	14.8 ^{1,2}	5.1 ^{1}	3.2 ^{1,2,3}	0.0 ^{1,2}	36.1 ^{5}
Wisconsin	0.8 ^{1}	1.5 ^{1}	15.3 ^{1,2}	5.8 ^{1,2}	14.2 ^{1,2,3}	1.3 ^{1}	3.9 ^{1,2}	14.9 ^{1,2}	9.1 ^{1}	9.9 ^{4}	2.0 ^{1,2}	6.4 ^{1,2,3,4}
Missouri	0.0 ^{1}	1.8 ^{1}	14.5 ^{1,2}	4.5 ^{1,2}	12.1 ^{1,2,3}	1.3 ^{1}	1.0 ^{1,2}	12.4 ^{1,2}	5.2 ^{1}	5.6 ^{2,3,4}	33.8 ^{3}	0.0 ^{1,2,3}
Iowa × Iowa	0.4 ^{1}	2.8 ^{1}	18.0 ^{1,2}	3.8 ^{1,2}	9.6 ^{1,2,3}	1.2 ^{1}	2.1 ^{1,2}	14.8 ^{1,2}	4.2 ^{1}	0.0 ^{1}	0.6 ^{1,2}	32.5 ^{4,5}
Minnesota × France	1.1 ^{1}	3.6 ^{1}	23.5 ^{1,2}	3.1 ^{1,2}	8.2 ^{1,2,3}	3.1 ^{1}	13.6 ^{1,2}	16.6 ^{1,2}	3.1 ^{1}	0.4 ^{1,2}	0.5 ^{1,2}	1.6 ^{1,2,3}
Minnesota × Minnesota	0.3 ^{1}	1.7 ^{1}	14.8 ^{1,2}	2.9 ^{1,2}	22.1 ^{2,3}	3.1 ^{1}	8.3 ^{1,2}	10.6 ^{1}	4.2 ^{1}	0.3 ^{1}	2.0 ^{1,2}	0.2 ^{1,2}
Minnesota × Missouri	0.5 ^{1}	2.0 ^{1}	19.7 ^{1,2}	5.4 ^{1,2}	13.5 ^{1,2,3}	1.2 ^{1}	2.5 ^{1,2}	16.7 ^{2}	5.3 ^{1}	0.0 ^{1}	0.7 ^{1,2}	0.0 ^{1,2,3}
Minnesota × Morden	0.7 ^{1}	1.2 ^{1}	17.1 ^{1,2}	3.1 ^{1,2}	7.8 ^{1,2,3}	17.8 ^{2}	0.0 ^{1,2}	11.0 ^{1,2}	4.9 ^{1}	0.0 ^{1}	0.5 ^{1,2}	0.3 ^{1,2,3}
Minnesota × Spain	0.6 ^{1}	1.9 ^{1}	16.8 ^{1,2}	4.9 ^{1,2}	16.5 ^{1,2,3}	1.8 ^{1}	5.8 ^{1,2}	14.3 ^{1,2}	4.9 ^{1}	0.3 ^{1}	1.0 ^{1}	2.6 ^{1}
Minnesota × Iowa	0.0 ^{1}	3.2 ^{1}	24.9 ^{1,2}	5.2 ^{1,2}	12.9 ^{1,2,3}	1.5 ^{1}	2.7 ^{1,2}	22.1 ^{1,2}	7.4 ^{1}	0.0 ^{1}	0.6 ^{1,2}	2.7 ^{1,2,3}
Spain × Iowa	0.3 ^{1}	3.8 ^{1}	14.7 ^{1,2}	0.0 ^{1}	29.5 ^{3}	3.9 ^{1}	17.2 ^{1,2}	12.6 ^{1,2}	1.1 ^{1}	0.6 ^{1,2}	0.5 ^{1,2}	3.3 ^{1,2,3}
Spain × Spain	0.2 ^{1}	2.2 ^{1}	16.4 ^{1,2}	6.0 ^{1,2}	15.1 ^{1,2,3}	1.6 ^{1}	6.0 ^{1,2}	12.8 ^{1,2}	5.8 ^{1}	0.1 ^{1}	1.0 ^{1,2}	13.9 ^{1,2,3}

[a] Means were calculated as the average of *n* individual samples (each individual sample was prepared by duplicate and each replicate was injected by triplicate). [b] Means within a column followed by a different superscripted number are significantly different at the 0.05 level as determined by the Tukey test.

similar to 22.2^{2} and 14.2^{1} while 22.2^{2} is statistically different from 14.2^{1}). The data grouped in **Table 1** show the existence of differences among maize lines but also demonstrates the need of superior tools enabling to properly manage the data obtained in order to extract conclusive results.

Results obtained by perfusion RP-HPLC analysis for inbred and hybrid lines are shown in **Table 2**. In this case, from the 12 peaks found in all maize lines analyzed, only those with relative peak areas higher than 0.2% were considered. An ANOVA disclosed no differences in the relative areas of the peaks B and F among the geographical groups. Like previously, a Tukey test was carried out to check the significant differences among the means of the significant peaks, and it was observed that there are clear differences among the chromatographic profiles obtained from maize lines of different origins but also show the difficulty for studying this complex variability of data.

In order to ensure that the observed differences could be attributed to the differences among cultivars and, thus, applicable to the characterization of maize cultivars, two previous studies on the precision of these chromatographic methods and the

Table 2. Peak Area Percentages Corresponding to the Analysis of Protein Extracts from Different Inbred and Hybrid Maize Lines by Perfusion RP-HPLC

	peak area percentages (%)[a,b]							
peaks:	A	B	D	E	F	G	H	I
retention time (t_R, min):	0.73	0.97	1.30	1.49	1.62	1.93	2.25	2.54
origin of maize lines								
Minnesota	3.5 ^{2}	14.3 ^{1}	46.1 ^{2}	2.5 ^{1}	4.9 ^{1}	12.3 ^{1,2}	3.8 ^{1}	4.8 ^{1}
Iowa	0.5 ^{1,2}	9.7 ^{1}	49.8 ^{2}	8.1 ^{2}	2.6 ^{1}	8.8 ^{1}	10.1 ^{2}	9.4 ^{1,2,3}
Morden	0.4 ^{1,2}	16.8 ^{1}	30.0 ^{1,2}	4.7 ^{1,2}	0.3 ^{1}	8.1 ^{1}	1.6 ^{1}	6.3 ^{1,2}
Spain	0.7 ^{1}	11.9 ^{1}	56.0 ^{2}	3.9 ^{1,2}	2.3 ^{1}	12.6 ^{1,2}	4.9 ^{1}	6.0 ^{1}
France	1.6 ^{1,2}	10.0 ^{1}	0.0 ^{1}	2.2 ^{1,2}	0.0 ^{1}	24.2 ^{2}	9.1 ^{1,2}	1.1 ^{1}
Virginia	0.0 ^{1,2}	11.3 ^{1}	0.0 ^{1}	1.0 ^{1,2}	1.7 ^{1}	7.5 ^{1,2}	0.0 ^{1}	7.5 ^{1,2}
Wisconsin	1.8 ^{1,2}	21.6 ^{1}	0.0 ^{1}	0.0 ^{1,2}	10.5 ^{1}	15.3 ^{1,2}	0.4 ^{1}	5.2 ^{1,2,3}
Missouri	0.0 ^{1,2}	15.0 ^{1}	49.2 ^{1,2}	5.1 ^{1,2}	0.0 ^{1}	16.8 ^{1,2}	0.6 ^{1}	7.0 ^{1,2,3}
Iowa × Iowa	2.6 ^{1,2}	22.2 ^{1}	36.5 ^{1,2}	0.0 ^{1,2}	8.3 ^{1}	14.0 ^{1,2}	3.4 ^{1,2}	10.7 ^{1,2,3}
Minnesota × France	2.4 ^{1,2}	25.0 ^{1}	34.1 ^{1,2}	3.0 ^{1,2}	4.0 ^{1}	11.4 ^{1,2}	4.0 ^{1,2}	13.9 ^{1,2,3}
Minnesota × Minnesota	2.2 ^{1,2}	12.6 ^{1}	53.4 ^{2}	1.9 ^{1,2}	5.7 ^{1}	4.9 ^{1}	6.0 ^{1,2}	10.5 ^{1,2,3}
Minnesota × Missouri	1.5 ^{1,2}	14.1 ^{1}	48.6 ^{1,2}	3.0 ^{1,2}	0.8 ^{1}	9.6 ^{1,2}	7.0 ^{1,2}	13.2 ^{1,2,3}
Minnesota × Morden	1.2 ^{1,2}	17.0 ^{1}	32.7 ^{1,2}	4.4 ^{1,2}	5.3 ^{1}	11.4 ^{1,2}	4.5 ^{1,2}	21.2 ^{3}
Minnesota × Spain	1.5 ^{1,2}	14.8 ^{1}	46.3 ^{2}	1.8 ^{1}	3.4 ^{1}	10.1 ^{1}	4.1 ^{1}	12.4 ^{2,3}
Minnesota × Iowa	2.1 ^{1,2}	11.3 ^{1}	58.8 ^{2}	2.8 ^{1,2}	0.0 ^{1}	4.5 ^{1}	6.7 ^{1,2}	9.0 ^{1,2,3}
Spain × Iowa	1.8 ^{1,2}	15.9 ^{1}	43.6 ^{2}	3.7 ^{1,2}	0.1 ^{1}	10.4	8.7 ^{1}	13.8 ^{1,2,3}
Spain × Spain	3.5 ^{1,2}	14.3 ^{1}	46.1 ^{2}	2.5 ^{1}	4.9 ^{1}	12.3 ^{1}	3.8 ^{1,2}	4.8 ^{1,2}

[a] Means were calculated as the average of *n* individual samples (each individual sample was prepared by duplicate and each replicate was injected by triplicate). [b] Means within a column followed by a different superscripted number are significantly different at the 0.05 level as determined by the Tukey test.

variability within cultivars were carried out. Precision of the chromatographic methods was evaluated by determining repeatability, intermediate precision, and between-day reproducibility.

Repeatability was obtained by injecting one maize solution ten consecutive times in the same day. The mean of the coefficient of variation (CV) for the relative peak areas (calculated as the average of the ratio between the standard deviation and the mean of relative area for each one of the peaks selected given in percentage) obtained with the perfusion and the monolithic RP-HPLC were 4.0% and 2.9%, respectively. Between-days reproducibility was evaluated by the injection of a maize solution in two consecutive days (ten consecutive times each day). The mean of the CV for the area percentage obtained with the perfusion and the monolithic RP-HPLC were 4.1% and 2.9%, respectively. The repeatability and between-days reproducibility of both methods concerning to retention times were less than 0.2%. Intermediate precision was determined by injecting ten independently extracted solutions obtained from the same maize cultivar. As for the repeatability and between-days reproducibility, the mean of the CV for the area percentage was lower with the monolithic column than with the perfusion column. Precision in terms of retention times was excellent in both cases with a mean of the CV very close to zero.

Inter-kernel variability was determined by the application of both RP-HPLC methods to the analysis of six kernels of a maize inbred line (EZ1) and a synthetic maize population (Spanish Rastrojero). Each kernel was individually ground and extracted, and injected by triplicate. Retention time variability assayed with the monolithic column was very good for the inbred line and the synthetic population with a mean for the CV of 0.1% and 0.9%, respectively. Similar results were found for the perfusion column. The variabilities of the relative peak area for the inbred line (4.2% and 5.6% for the monolithic and the perfusion columns, respectively) were lower than those found for the synthetic population (36.8% and 40.8% for the monolithic and perfusion column, respectively). This highest inter-kernel variability observed for the synthetic maize population is due to the lack of phenotypic uniformity among kernels derived from an open pollination. In order to develop an inbred line, a controlled self-pollination of the flowers is needed during several generations to obtain phenotypically stable kernels.

The low variability observed for the maize inbred line on the area percentages and retention times could enable, a priori, a variety identification of maize lines by using a RP-HPLC analysis of the maize proteins. Since variabilities observed in all cases were low, differences observed among cultivars could be attributed to genotype differences and not to the unreproducibility of the analytical methods.

Despite visual inspection of chromatographic data can reveal some interesting features enabling the differentiation among maize lines; the amount and complexity of these data make necessary the use of chemometric tools in order to fully extract the wealth of this information. Different multivariate methods (cluster analysis, principal component analysis, and discriminant analysis) were applied to the chromatographic data obtained by both perfusion and monolithic RP-HPLC.

Cluster analysis was used for searching natural grouping among the studied maize lines. This analysis will allow an association of samples based on their similarities in protein profiles. Hierarchical agglomerative clustering was performed by means of the Ward method on raw data using squared Euclidean distances as a measure of similarity. In short, this method attempts to optimize the minimum variance within clusters that can be formed at each step (*26*). In general, this method is regarded as very efficient and tends to create clusters of small size. In the case of monolithic RP-HPLC, relative area of 26 peaks with retention times ranging from 2.04 to 7.14 min were selected for this study. When the perfusion column was used, area percentages of 12 peaks with retention times ranging from 0.75 to 2.84 min were evaluated.

First, a cluster analysis of the chromatographic data was applied to the inbred lines. The inbred lines contained in the subclusters obtained through the analysis of the perfusion and monolithic RP-HPLC are shown in **Table 3**. In the case of monolithic RP-HPLC, two clusters and six subclusters could be distinguished. The first cluster contained four subclusters (M1, M2, M3, and M4) and the second one contained two subclusters (M5 and M6). The data obtained with the perfusion chromatography showed two clusters and seven subclusters. The

Table 3. Cluster Analysis of the Chromatographic Data Obtained by RP-HPLC with the Monolithic and Perfusion Columns

monolithic RP-HPLC		perfusion RP-HPLC	
subcluster	maize inbred line	subcluster	maize inbred line
M1	Mo17	P1	Mo17
	EZ18		EZ18
	A556		A556
	B73		B73
M2	CM109	P6	CM109
	A239		A239
	F212		F212
M3	CM105		CM105
	B14		B14
	B84		B84
	A251		A251
	EZ1		EZ1
	EZ19		EZ19
	W64A	P3	W64A
M4	EZ16A3	P2	A632
	EZ6		EZ16A3
	EZ7		EZ6
			EZ7
M5	A635	P7	A635
	A619		A619
	Va26		
M6	A639	P4	**Va26**
	EZ8		A639
	A427		EZ8
	A632		A427
	A638	P5	A638

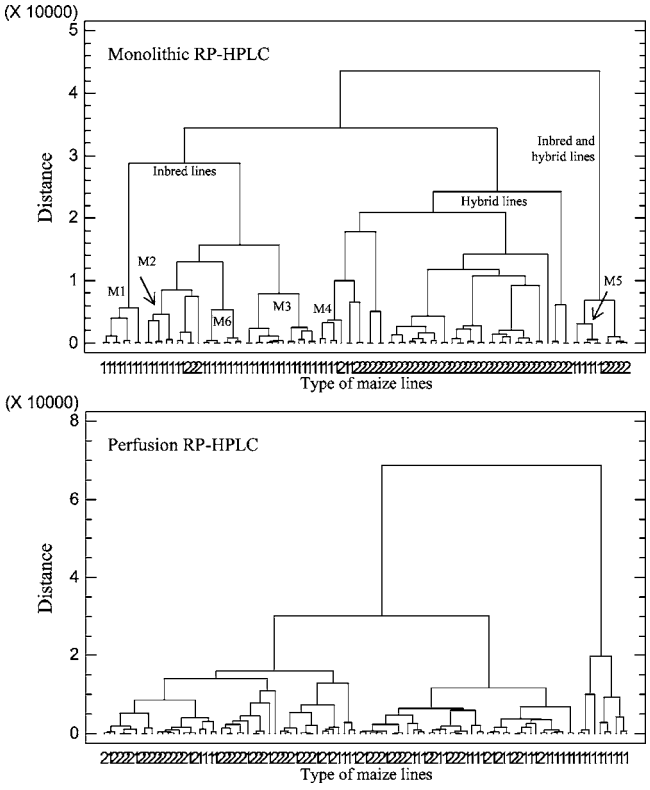

Figure 2. Dendograms obtained through Ward's method applied to inbred and hybrid maize lines analyzed by monolithic and perfusion RP-HPLC. Sample codes (1, inbred lines; 2, hybrid lines).

first clusters consisted of the subclusters P1, P2, P3, P4, and P5 and the second one of the subclusters P6 and P7. Similarities in the agglomeration of the inbred lines were found for both types of columns. Subclusters M1 and P1 consisted of four inbred lines (Mo17, EZ18, A556, and B73). The inbred lines grouped in subclusters M2 and M3 (monolithic column) were

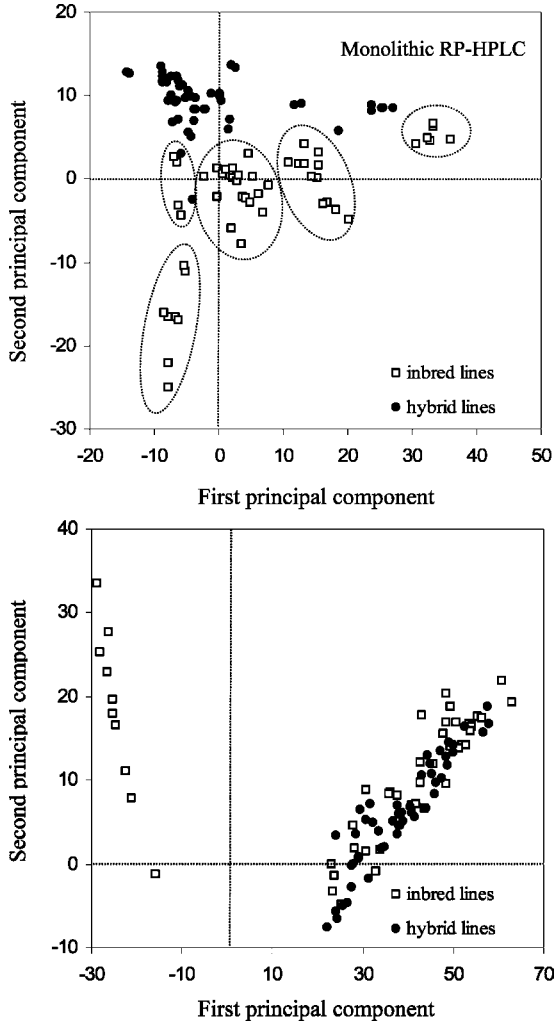

Figure 3. Scores of the samples on the two first principal components.

located in subclusters P6 and P3 (perfusion column). All the samples from subcluster M4 and M5 (monolithic column) were found in subclusters P2 and P7, respectively, although inbred line Va26 (subcluster M5) was grouped into subcluster P4. Finally, the inbred lines from subcluster M6 were grouped into two subclusters (P4 and P5) at exception of inbred line A632 located in subcluster P2.

The cluster analysis was also applied to the complete set of maize lines. The results of the cluster analysis for monolithic and perfusion RP-HPLC are shown as dendograms in **Figure 2**. Three main clusters were found in the dendogram of the samples analyzed by monolithic RP-HPLC. The first cluster, from left, contained only inbred lines (except three samples from B73 × A632 and EZ18 × EZ18 hybrid lines). The inbred lines coincided with the lines grouped into four of the subclusters found in the previous cluster analysis (M1, M2, M3, and M6). The second cluster was the group of hybrid lines which also included the inbred lines of the subcluster M4. The third cluster was subdivided into two clusters (subcluster M5 and a group of five samples of hybrid lines). These results display that the monolithic RP-HPLC data possess enough information to distinguish between inbred and hybrid maize lines, however, when cluster analysis was applied to the perfusion RP-HPLC data, no clear grouping of the samples according to the established categories was achieved.

The results of principal component analysis for monolithic RP-HPLC gave fifteen significant components with eigenvalues greater than unity, accounting for 73% of the total variance the

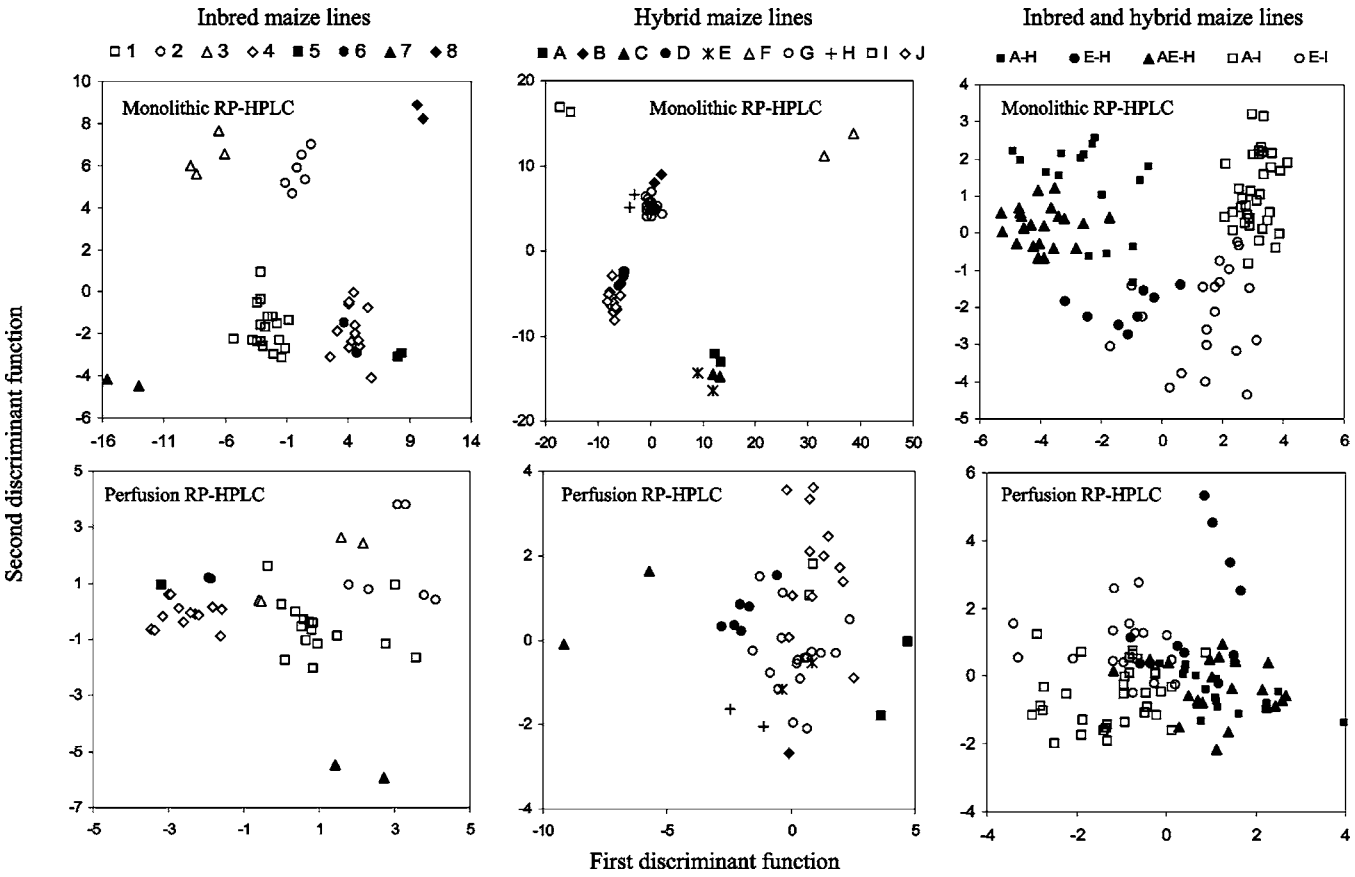

Figure 4. Distribution of inbred lines, hybrid lines and all maize lines analyzed by monolithic and perfusion RP-HPLC in the plane defined by the two first discriminant functions. Inbred line codes: 1, Minnesota; 2, Iowa; 3, Morden; 4, Spain; 5, France; 6, Missouri; 7, Virginia; 8, Wisconsin. Hybrid line codes: A, Iowa × Iowa; B, Minnesota × France; C, Minnesota × Iowa; D, Minnesota × Minnesota; E, Minnesota × Missouri; F, Minnesota × Morden; G, Minnesota × Spain; H, Minnesota × Iowa; I, Spain × Iowa; J, Spain × Spain. Maize line codes: A-H, American hybrid; E-H, European hybrid; AE-H, American-European hybrid; A-I, American inbred; E-I, European inbred.

four first components. From the loadings of the variables, the most influential variables on the first principal component (PC1) were the peaks 24 ($t_R = 6.99$) and 12 ($t_R = 4.24$) with loading values of 0.89 and −0.27, respectively, and for the second principal component (PC2) were the peaks 23 ($t_R = 6.73$), 21 ($t_R = 6.50$), 22 ($t_R = 6.63$), and 25 ($t_R = 7.04$) with loading values of −0.74, −0.36, 0.35, and 0.28, respectively.

The graphical representation (**Figure 3**) of the maize lines in the space defined by the two first components shows a clear separation between the inbred and hybrid lines. The group of hybrid lines presented positive values of PC2 and, was characterized by low values for the peaks 23 and 21, and high values for the peaks 12, 22, and 25. At the same time, the hybrid samples showed negative values of PC1, what means low levels for the peak 24. An exception is the group of seven hybrid samples which were found in the area of positive PC1 and PC2 corresponding to the samples from the third cluster obtained in the cluster analysis of all maize lines. On the other hand, the samples of inbred lines were grouped into different sectors of the plot. An inbred group presented negative values of PC1 and PC2 (inbred samples from cluster M1) pointing high relative area of peak 23. Samples from cluster M5 were grouped in the sector of positive PC1 and PC2 showing very high values of peak 24. Finally, it can be observed that the other inbred samples were along PC1, with negative values of this component for the samples from cluster M4, values near zero for the samples from cluster M1 and M2, and positive values for the samples grouped into cluster M6.

Regarding the results obtained by the principal component analysis for the perfusion RP-HPLC data, nine significant components with eigenvalues greater than unity were found, accounting for almost 80% of variance the two first PC's. PC1 was positively contributed by peak D ($t_R = 1.30$) and negatively by peak C ($t_R = 1.21$) with loading values of 0.84 and −0.47, respectively, while PC2 was positively participated by peaks C and D (loading values of 0.70 and 0.34, respectively) and negatively by peak B (loading value of −0.43). **Figure 3** shows an overlapping among the inbred and hybrid samples analyzed by perfusion RP-HPLC, although 10 inbred samples were independently grouped in the sector of negative PC1. These scores correspond to samples from the inbred lines Mo17, CM109, Va26, A638, and B73, which were not grouped into any previous cluster.

These results are in agreement with those found in the cluster analysis, although, methodologically there is no relation between cluster analysis and principal component analysis. This fact enables us to confirm that the chromatographic data contains enough information to classify the different groups of maize lines.

To understand the relationships between the maize lines and their geographical origin, a linear discriminant analysis (LDA) of the chromatographic data obtained with monolithic and perfusion RP-HPLC was performed. The inbred lines were classified into eight groups according to their geographical origin: North American inbred lines from USA (Minnesota, Iowa, Missouri, Virginia, and Wisconsin) and Canada (Morden);

European inbred lines from Spain and France. In a similar way, the hybrid lines were classified into nine groups according to the geographical origin of their parent lines: North American hybrid lines (Iowa × Iowa, Minnesota × Iowa, Minnesota × Minnesota, Minnesota × Morden, and Minnesota × Missouri); American-European hybrid lines (Minnesota × France, Minnesota × Spain; Spain × Iowa); and European hybrid lines (Spain × Spain).

Initially, the discriminant analysis was applied to the group of inbred samples. The best results of classification were obtained for monolithic RP-HPLC (with a 96% of classification ability) where all the samples were correctly classified with exception of the two samples of the inbred line Mo17 from Missouri, which were non-correctly classified as Spanish inbred lines. These results are plotted in **Figure 4**. Good results were also found for perfusion RP-HPLC where a 92% of the samples were correctly classified. All the samples from Minnesota, Iowa, France, Missouri, Virginia, and Wisconsin lines were correctly classified. Two of the four samples from Morden were incorrectly grouped as inbred lines from Iowa, and two of the 14 samples from Spain were misclassified as inbred lines from Missouri. The prediction capability of the models was evaluated by cross validation in four steps, excluding 25% of the samples in each step, so that all maize lines have been predicted once. A global percentage of correct classification of 92.1% and 82.3% using cross-validation was achieved with monolithic and perfusion RP-HPLC.

On the other hand, the hybrid lines were discriminated in ten groups according to the geographical origin of their initial parent lines (**Figure 4**). For monolithic RP-HPLC, the hybrid lines were correctly classified using the projection plane determined by the first and the second discriminant functions (100% of correct classification and 98.3% using cross-validation). The hybrid samples from the groups of Minnesota × Minnesota (D) and Spain × Spain (J) were perfectly separated, however the distance between centroids was not very large. Similar behaviors could be observed for Iowa × Iowa (A), Minnesota × Missouri (E), and Minesota × Iowa (C) and for Minnesota × France (B), Minnesota × Spain (G), and Minnesota × Iowa (H). The chromatographic data obtained by perfusion RP-HPLC had lower discriminating power successfully separating 84.0% of the hybrid samples (68.3% using cross-validation). Several maize samples of group G were incorrectly grouped as hybrid lines from group E (three samples) and from group F (one sample), and four samples of group J were misclassified as hybrid lines from group E (two samples) and from group G (two samples).

Finally, a new discriminant analysis was applied to all maize samples. For this analysis, the maize lines were grouped into five categories (American hybrid lines, European hybrid lines, American-European hybrid lines, American inbred lines, and European hybrid lines). The scatterplot of samples analyzed by monolithic RP-HPLC in the plane defined by the first two discriminant functions showed a good discrimination between the maize lines (94% of samples correctly classified and an 88.3% using cross-validation). The first discriminant function was critical in order to discriminate between inbred and hybrid maize samples while the second function achieved a notable discriminating power between American and European lines. The inbred lines were situated on the right of the origin of the first discriminant function while the hybrid lines were on the left. On the other hand, American lines appeared at positive values of the second discriminant function and European lines at negative values. Moreover, American-European hybrid lines were situated between the groups of American and European hybrid lines near the origin of the second discriminant function. Regarding perfusion RP-HPLC data, the close proximity of these five groups of maize lines analyzed by perfusion chromatography reflected the poor discriminating power of the model toward these groups (62.6% of samples were correctly classified and 53.9% using cross-validation). However, some tendencies were observed. Inbred lines had negative values of factor one, while hybrid lines presented positive values for this factor. Furthermore, European and American lines seemed to take the highest and the lowest values of factor 2, respectively.

In conclusion, monolithic and perfusion RP-HPLC methods were successfully employed for the differentiation of maize cultivars. The results obtained in this study allow us to conclude that the protein profiles obtained by monolithic and perfusion RP-HPLC, jointly with several chemometric techniques, were appropriate for differentiating between inbred and hybrid maize lines according to their geographical origin. Cluster analysis and principal component analysis revealed the natural relationships among the maize inbred lines and between the categories of inbred and hybrid lines. From the discriminant analysis, the models built for each category were highly sensitive (they recognized the samples within their own class) and specific (they did not accept lines of other categories in their category). It was demonstrated that the best results for the differentiation of maize cultivars were obtained for monolithic RP-HPLC, possibly, due to the fact that monolithic RP-HPLC enabled the separation of maize proteins in a large number of peaks.

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