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Monolithic Supports for the Characterization of Commercial Maize Products Based on Their Chromatographic Profile. Application of Experimental Design and Classification Techniques

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Modern analytical techniques based on the use of RP-HPLC with monolithic stationary phases and the application of experimental design and classification tools have been applied to the analysis of maize proteins. Solubilization conditions of maize proteins and separation conditions (temperature, detection wavelength, type and concentration of ion-pairing agent, and gradient) were optimized. The elution gradient was optimized by the application of experimental design techniques. The optimized method consisted of a linear binary gradient of water/acetonitrile/0.1% trifluoroacetic acid in three steps at a flow rate of 3 mL/min with a column temperature of 35 °C and UV detection at 280 nm. The developed method enabled the separation of maize proteins in an analysis time close to 8 min. Moreover, this is the first time that commercial maize products have been characterized by the use of multivariate classification techniques.

KEYWORDS: Maize; monolithic support; RP-HPLC; proteins

INTRODUCTION

High-performance liquid chromatography (HPLC) has become one of the most frequently used analytical techniques for the separation and characterization of proteins, reversed-phase (RP) chromatography being the most popular chromatographic mode for this purpose (*1*−*7*). This mode involves the use of organic modifier gradients of an organic solvent, frequently acetonitrile, in aqueous solutions of ion-pairing agents in conjunction with hydrophobic stationary phases. Generally, RP-HPLC systems operate with conventional silica columns composed by macroporous butyl- or octadecyl-silica particles having 10−30 nm pores (*1*). The use of this kind of column results in very high analysis time in the separation of high molecules such as proteins. This is due to the difficulty in the diffusion through the inner part of the stationary phase particles observed for proteins (*8*).

An alternative to conventional columns enabling the reduction of analysis times is the use of monolithic columns. Monolithic columns are characterized by their excellent mass transfer properties and a low-pressure drop, making these supports excellent stationary phases for protein chromatography (*9*). Monolithic columns are made of a single piece of a highly porous material (*10*). They present a bimodal pore structure consisting of macropores or through-pores and mesopores in the silica skeleton, where the transport of the solute to the surface is solely by convection instead of diffusion as in conventional media (*11*). As a consequence, the separation of large molecules such as proteins can be achieved with a lower analysis time (from seconds to a few minutes) (*12*) and with a lower backpressure (*9*) than in conventional supports. Monolithic columns are prepared by different processes from either organic polymers, such as polymethacrylates, or inorganic polymers, such as silica (*10*). Methacrylate-based monolithic columns are prepared via bulk polymerization (*13*), whereas silica-based monolithic columns are prepared using a sol−gel process (*10*).

Maize (*Zea mays*) is, after wheat and rice, the most important cereal in the world, providing nutrients for humans and animals (*14*). Proteins are the second most abundant chemical component of the maize kernel (*15*). Protein content determines the technological importance of the different varieties of maize (*16*). Thus, knowledge of maize protein fractions using high-resolution separation techniques has permitted maize cultivar differentiation (*4*, *17*, *18*), the characterization of genetically modified maize (*6*, *7*), and the study of the relationships between protein maize and end-use quality (*10*, *20*, *21*). However, these studies were performed using conventional columns, and analysis times ranged from 60 to 80 min.

Despite the fact that monolithic columns are being used as stationary phases in the rapid separation of peptides and proteins with low analysis time (*22*−*27*), no papers have been found dealing with the application of monolithic columns to the separation of maize proteins. Thus, the aim of this work was the development of a rapid and suitable method for the separation of maize proteins using either silica-based or methacrylate-based monolithic columns. The applicability of the

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method was tested by means of the analysis and characterization of different commercial maize products applying a multivariate analysis of the chromatographic data.

EXPERIMENTAL PROCEDURES

Chemicals and Samples. HPLC grade acetonitrile (ACN) (Merck, Darmstadt, Germany) and HPLC grade water (Milli-Q system, Millipore, Bredford, MA) were used for the preparation of mobile phases. Trifluoroacetic acid (TFA) (Sigma, St. Louis, MO), formic acid (Merck), morpholine (Sigma), and acetic acid (Merck) were used as ion-pairing agents. Urea, 2-mercaptoethanol, and sodium dodecyl sulfate (SDS) (all from Merck), ethanol and 2-propanol (both from Scharlab, Barcelona, Spain), dithiothreitol (ICN, Aurora, OH), and sodium acetate and ammonium acetate (both from Panreac, Barcelona, Spain) were used for the extraction of maize proteins.

Corn gluten meal (CGM) (purity 60%) from Sigma and zein F4000 (purity 92%) from Freeman Industries LLC (Tuckahoe, NY) were used as standards of maize proteins. Seeds from the EZ6 maize line were kindly supplied by the Maize Germplasm Bank (Experimental Station of Aula Dei, CSIC, Zaragoza, Spain). Several maize products (flours, precooked flours, fried snacks, and extruded snacks) were purchased from local markets in Alcalá de Henares (Madrid, Spain). Thirty kernels (for the EZ6 maize line) or 5 g (for commercial products) was ground during 3 min using an analytical mill A10 (IKA labortechnik, Staufen, Germany).

Maize Protein Extraction and Fractionation of Zein. Maize proteins were extracted from maize kernels and maize products by weighing 0.15 g of ground sample, dissolving this in 5 mL of the extraction solution (0.5% 2-mercaptoethanol and 0.5% ammonium acetate in 45% acetonitrile), and sonicating the mixture for 5 min in a bath sonicator (150 W, 50 Hz, FS-30, Fisher Scientific). The solution was centrifuged at 3362*g* (Avanti J-25 centrifuge, Beckman Coulter) for 10 min at 25 °C, and the supernatant containing the maize proteins was analyzed by RP-HPLC. Zein proteins (prolamine protein) from CGM were extracted and fractionated using a procedure described previously (*28*).

High-Performance Liquid Chromatography. Separations were performed using a Hewlett-Packard 1100 series liquid chromatograph (Hewlett-Packard, Pittsburgh, PA) equipped with a diode array detector, an injection system, a degassing system, a binary pump, and a thermostated compartment for the column. HP Chemstation software was used for instrument control and data acquisition. Two different monolithic supports were used for RP-HPLC separation: a monolith silica column Chromolith Performance RP-18e (4.6 × 100 mm) (Merck KGaA, Darmstadt, Germany) and a methacrylate monolithic disk CIM RP-SDVB (diameter = 16 mm, thickness = 3 mm, active bed volume = 0.34 mL) (BIA Separations, Ljubljana, Slovenia). The injection volume was 20 μL, and the detection was carried out at 280 nm. After optimization of the experimental conditions, a suitable chromatographic separation of maize proteins was achieved with the monolith silica column at a flow of 3 mL/min at 35 °C using the following 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 optimized composition of the mobile phases was 0.1% (v/v) TFA in Milli-Q water (mobile phase A) and 0.1% (v/v) TFA in ACN (mobile phase B).

Experimental Design for the Optimization of the Elution Gradient Program. The optimization of the elution gradient program of the RP-HPLC method using the monolithic silica stationary phase was performed using a central composite design (2^4 + star). For this purpose, a binary linear gradient elution program in three steps was chosen with the following four variables: ΔACN_1, the variation of ACN percentage for the first step; ΔACN_2, the variation of ACN percentage for the second step; Δt_1, the time required to pass from an ACN percentage of 5% to the final percentage of the first step; Δt_2, the time required to pass from the initial ACN percentage to the final ACN percentage of the second step.

A total of 10 responses divided into two groups were chosen for this study. The first group of responses was the peak plates calculated for three peaks (peaks E, H, and K). The second group of responses was the resolution between different adjacent peaks (peaks B and C, D and E, F and G, G and H, I and J, J and K, and K and L). HP Chemstation software was employed to determine the number of plates or efficiency and the resolution between adjacent peaks. Finally, the time required for the separation of maize proteins was included as response in this study. The Statgraphics software package (Statgraphics Plus for Windows 4.0, Statistical Graphics Corp., Rockville, MD) was used to fit the second-order model to the independent variables using the equation

$$y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ii} x_i^2 + \sum_{i}^{i<j} \sum_{j} b_{ij} x_i x_j \qquad (1)$$

where y is the dependent variable (response variable) to be modeled, x_i and x_j are the independent variables (factors), and b_0, b_i, b_{ii}, and b_{ij} are the regression coefficients of the model.

Data Treatment. Peak areas from maize proteins were integrated by setting the baseline from valley to valley. The area percentage for every peak was calculated as the average of two replicates (injected by triplicate), and an analysis of variance (ANOVA) ($\alpha = 0.05$) for the data was applied. The application of multivariate analysis was performed using the computer program Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp.).

RESULTS AND DISCUSSION

To carry out a suitable analysis of maize proteins, a study on the best conditions enabling the solubilization of maize proteins was performed. For that purpose, two different monolithic stationary phases were employed: a silica-based column and a methacrylate-based disk. Several binary gradients were tried for the separation of maize proteins with the methacrylate-based disk, although none of them enabled a suitable separation of maize proteins. In the case of the silica column, different gradients were tried at 3 mL/min and 40 °C. UV detection were performed at 210, 254, and 280 nm, and the mobile phases consisted of 0.1% (v/v) TFA in Milli-Q water (mobile phase A) and 0.1% (v/v) TFA in ACN (mobile phase B). Suitable separation was achieved with the use of a linear binary gradient from 5.0 to 30.0% B in 5 min, from 30.0 to 50.0% B in 1 min, from 50.0 to 95.0% B in 2.5 min, and finally, from 95.0 to 5.0% B in 1 min, employing this gradient as a first approach for the separation of maize proteins.

A review of the literature concerning the composition of the solutions for cereal protein extraction showed that in most cases solutions prepared by mixing an organic solvent with a denaturing agent and a saline solution were used for this purpose (*1*, *6*, *29*, *30*). Thus, to study the effect of the conditions of solubilization of maize proteins on the variation of total peak area percentages and the number of peaks with the monolithic column, different denaturing agents (dithiothreitol, 2-mercaptoethanol, urea, and SDS) and saline solutions of sodium and ammonium acetate in the presence of 45% ACN [amount optimized in an earlier study (*31*)] were tried. In all of these experiments the extraction time was 5 min. The relative responses (percent) obtained for maize proteins when using these media for protein solubilization are shown in **Table 1**. Relative response was expressed as percentage relative to the maximum value of the ratio between the total peak area obtained and the concentration injected.

In comparison with 45% ACN without denaturing agents or salts, the addition of different concentrations of denaturing agents provided an increase in the solubility of maize proteins. The best results were found for 8 M urea and for 5% 2-mercaptoethanol, but no statistically significant differences were found between both denaturing agents. Although urea was an effective protein solubilization agent, it was discarded in favor

Table 1. Effect of Different Extraction Solutions Employed for the Solubilization of Proteins from EZ6 Maize Line with the Monolithic Column[a]

extraction solution[b]	no. of peaks[c]	relative response[d,e] (%)
45% ACN	12	56.1 ± 2.3 a
45% ACN + 1% DTT	12	65.8 ± 1.0 b
45% ACN + 2% DTT	12	66.3 ± 4.3 b
45% ACN + 2% 2-ME	12	75.3 ± 3.8 cd
45% ACN + 5% 2-ME	12	86.0 ± 2.9 g
45% ACN + 6 M urea	12	84.3 ± 2.7 f
45% ACN + 8 M urea	12	87.3 ± 3.3 g
45% ACN + 4% SDS	12	64.3 ± 3.3 b
45% ACN + 8% SDS	12	77.4 ± 1.9 de
45% ACN + 5.0% 2-ME + 1.0% NaAc	12	99.7 ± 2.4 h
45% ACN + 5.0% 2-ME + 0.5% NaAc	12	98.9 ± 2.1 h
45% ACN + 5.0% 2-ME + 1.0% NH_4Ac	12	98.7 ± 1.9 h
45% ACN + 5.0% 2-ME + 0.5% NH_4Ac	12	99.1 ± 0.9 h
45% ACN + 4.0% 2-ME + 0.5% NH_4Ac	12	99.3 ± 0.7 h
45% ACN + 2.0% 2-ME + 0.5% NH_4Ac	12	99.6 ± 2.1 h
45% ACN + 1.0% 2-ME + 0.5% NH_4Ac	12	100.0 ± 0.9 h
45% ACN + 0.5% 2-ME + 0.5% NH_4Ac	12	99.1 ± 2.3 h
70% ethanol + 5% 2-ME + 0.5% NaAc[f]	9	80.4 ± 3.3 ef
55% 2-propanol + 5% 2-ME + 0.5% NaAc[g]	9	72.6 ± 0.9 c

[a] Experimental conditions: 30 mg/mL of sample; flow rate, 3 mL/min; temperature, 40 °C; injection volume, 20 μL; gradient, 5.0–30.0% B in 5 min, 30.0–50.0% B in 1 min, 50.0–95.0% B in 2.5 min, and 95.0–5.0% B in 1 min; mobile phases, (A) 0.1% (v/v) TFA in water, (B) 0.1% (v/v) TFA in ACN; detection, 280 nm. [b] Extraction solutions in Milli-Q water. ACN, acetonitrile; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; NH_4Ac, ammonium acetate; NaAc, sodium acetate. [c] Number of peaks found on the chromatograms with a relative peak area >1%. [d] Determined as percentage relative to the maximum value of the ratio between the total peak area obtained and the concentration of sample injected. [e] Mean ± standard deviation. Means were calculated as the average of two replicates (injected by triplicate). Means within a column followed by a different letter are significantly different at the 0.05 level as determined by the Tukey test. [f] From ref *29*. [g] From ref *30*.

of 2-mercaptoethanol because high concentrations of urea are problematic because it readily crystallizes and has substantial absorbance in the low UV (32). Other experiments using combinations of these denaturing agents were tried, although no increase of the total peak area was observed (results not shown).

Once the type of denaturing agent was selected, the effect of ammonium and sodium acetate on the solubilization of maize proteins was investigated. For this study, ammonium and sodium acetate (dissolved in 45% ACN plus 5% 2-mercaptoethanol) were tried at concentrations ranging from 0.5 to 1.0% (w/v). The presence of these salts in the media increased noticeably the solubilization of maize protein. It was also observed that an increase on the salt concentration did not enable a significant improvement in the total peak area, and no statistically significant differences were found for both salts regardless of the concentrations assayed. Moreover, the concentration of 2-mercaptoethanol was modified from 0.5 to 5.0% (v/v), but it was observed that concentrations >0.5% did not improve the total peak area of the chromatograms. From these results, we selected a medium consisting of 45% ACN in water containing 0.5% 2-mercaptoethanol and 0.5% ammonium acetate.

Finally, a comparison of our optimized extraction solution with two established media usually employed for the extraction of cereal proteins [70% ethanol plus 5% 2-mercaptoethanol and 0.5% sodium acetate (*29*) and 55% 2-propanol plus 5% 2-mercaptoethanol and 0.5% sodium acetate (*30*)] was performed. Statistically significant differences in the extraction levels were observed among the three media. The media prepared with 45% ACN (plus 0.5% 2-mercaptoethanol and 0.5% ammonium acetate) clearly extracted more maize proteins than either 70% ethanol or 55% 2-propanol. Furthermore, a reduction in the number of peaks from 12 to 9 was also observed when ethanol and 2-propanol were employed.

The effect of varying the extraction time ranging from 5 min to 12 h was also tested, and no statistically significant differences in extraction levels were observed (results not shown). On the basis of the results, an extraction time of 5 min was selected.

Optimization of the Chromatographic Conditions. The optimization of the chromatographic conditions was performed by evaluating the effect of UV detection wavelength, column temperature, composition of the mobile phase, and gradient on the separation of maize proteins. All of these studies were performed with the protein extract from the EZ6 maize line obtained by the previously optimized procedure.

Three different wavelengths (210 nm corresponding with the absorbance of peptide bonds, 254 nm corresponding with the absorbance of phenylalanine residues, and 280 nm corresponding with the absorbance of tryptophan and tyrosine residues) were studied. The chromatogram monitored at 210 nm presented the highest sensitivity, but the number of peaks detected was smaller than those detected at 254 and 280 nm. Because the numbers of peaks for the chromatograms detected at 254 and 280 nm were similar and the wavelength at 280 nm gave a higher signal than that at 254 nm, 280 nm was the wavelength selected for the detection of maize proteins.

The influence of the column temperature on the separation of maize proteins was studied in the range from 15 to 45 °C because higher column temperatures could compromise column lifetime. Protein retention decreased when the temperature was increased to 45 °C, except for the most retained peaks. This behavior could be explained by the fact that an increase in the temperature results in a decrease of the viscosity of the mobile phase facilitating the mobility of the solutes, and especially, of the less retained ones. Regarding the number of peaks, maize proteins from the EZ6 maize line were separated into 12 peaks at 30−35 °C and into 11 peaks at 40−45 °C. On the basis of these results and the fact that a decrease in the backpressure was observed at temperatures >30°C, the column temperature was fixed at 35 °C.

The separation of maize proteins was studied in the presence of three acidic reagents [0.1% TFA (v/v), 0.3% acetic acid (v/v), and 0.2% formic acid (v/v)] and one basic reagent (10 mM morpholine) as ion-pairing agents at concentrations usually employed for protein separation. These ion-pairing agents were dissolved in Milli-Q water (mobile phase A) and ACN (mobile phase B) at the same concentration. Chromatograms obtained for the EZ6 maize line when using these ion-pairing agents are shown in **Figure 1**. The best separation was achieved using TFA, where the maize proteins eluted in 12 separate peaks (peaks A−L). When using morpholine, the maize proteins coeluted as only a very broad peak. To study the influence of the variation of the concentration of TFA, the concentration of this additive was varied from 0.01 to 0.1% (v/v). A reduction in the concentration of the mobile phase additive impaired the chromatographic profiles observed with 0.1% TFA.

Finally, a fine optimization of the gradient elution was performed using a central composite design (2^4 + star). For that purpose, ΔACN_1 was varied from 5.0 to 29.0%, and Δt_1 ranged from 3.4 to 6.0 min; ΔACN_2 was also varied from 26.4 to 94.0%, and Δt_2 ranged from 0.9 to 3.5 min. All of the experiments were ended with a linear gradient to 5% ACN in

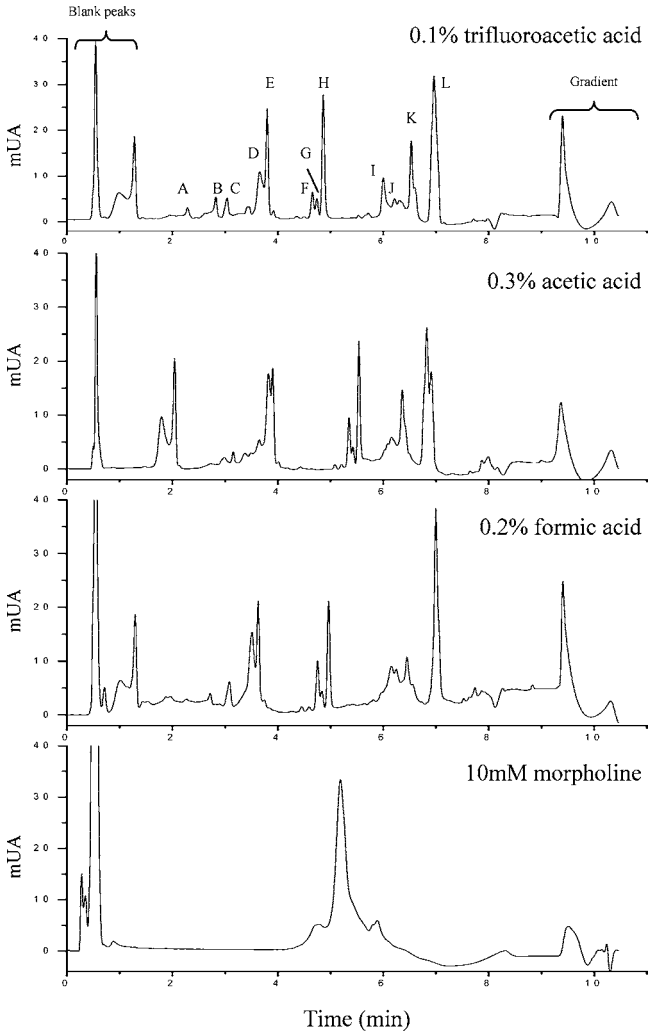

Figure 1. Chromatograms corresponding to the separation of proteins from the EZ6 maize line using different ion-pairing agents. Experimental conditions: 30 mg/mL of sample; flow rate, 3 mL/min; temperature, 35 °C; injection volume, 20 μL; gradient, 5.0–30.0% B in 5 min, 30.0–50.0% B in 1 min, 50.0–95.0% B in 2.5 min, 95.0–5.0% B in 1 min; mobile phases, (A) ion-pairing agent in water, (B) ion-pairing agent in ACN.

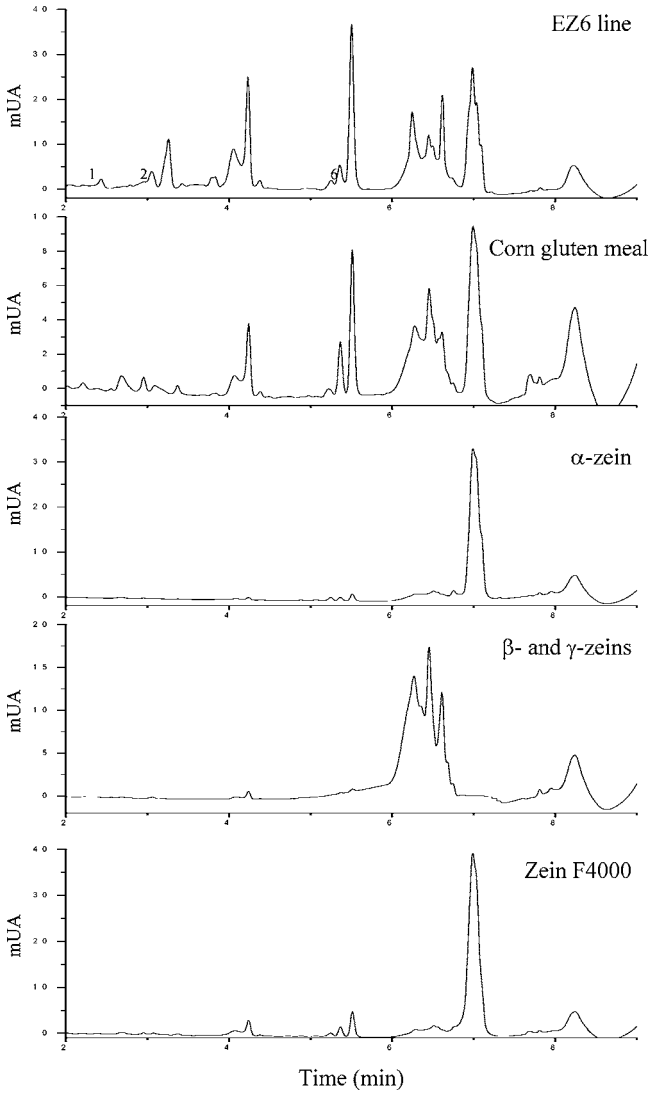

Figure 2. Chromatograms corresponding to separation of maize protein solutions of the EZ6 maize line (30 mg/mL) and corn gluten meal (30 mg/mL), α-zein, (β- and γ-) zeins, and zein F4000 (2 mg/mL) under the optimized conditions.

1.0 min to equilibrate the column to initial conditions between runs. This experimental design required 28 runs, which were carried out in random order and by triplicate. The number of plates (measured as the width of a peak relative to its retention), the resolution between adjacent peaks (a resolution of 1.5 implied a complete separation of two peaks), and the analysis time allowed the comparison of the efficiency of the different separation conditions tried (*33*).

According to eq 1, mathematical models were built through regression based on the response results. The models were simplified by dropping the terms that were not statically significant ($P > 0.05$). The statistical analysis of the models showed that the coefficients of determination (R^2) for the resolution between peaks were >0.99, except for the resolution between peaks D and E ($R^2 = 0.84$), and for the number of plates >0.93, indicating the good predictability of the models. All of the models obtained, with the exception of the model built with the resolution between peaks D and E, were used for the determination of conditions maximizing simultaneously the responses. For this purpose, a multioptimization stage was carried out using the desirability function calculated through the Statgraphics Plus software. This function permits us to identify the combination of factor settings that jointly optimize a set of responses (*34*). In this particular study, a desirability function was built on the premises that the resolution between peaks and the number of plates for each peak were maximized, whereas the time of analysis would be minimized. The best conditions were calculated by maximizing the desirability function and were found at the following values: ΔACN_1, 21.4%; ΔACN_2, 61.1%; Δt_1, 5.15 min; Δt_2, 2.16 min. These values correspond with the following linear binary gradient in three steps: 5.0−26.4% B in 5.15 min, 26.4−87.5% B in 2.16 min, and finally, 95.0−5.0% B in 1 min to equilibrate the column. In comparison with the previous elution gradient (5.0−30.0% B in 5 min, 30.0−50.0% B in 1 min, 50.0−95.0% B in 2.5 min, and 95.0−5.0% B in 1 min), the optimized conditions enable a reduction of the number of steps of the gradient and a decrease in the analysis time of 1.2 min, with a higher efficiency and a better resolution between adjacent peaks.

The chromatogram obtained for the protein extract from the EZ6 maize line under the optimized conditions is shown in **Figure 2**. Maize proteins eluted in 12 majority peaks (with peak areas >1%) separated in three groups: one at the beginning of the chromatogram (retention times between 2.0 and 4.0 min), a

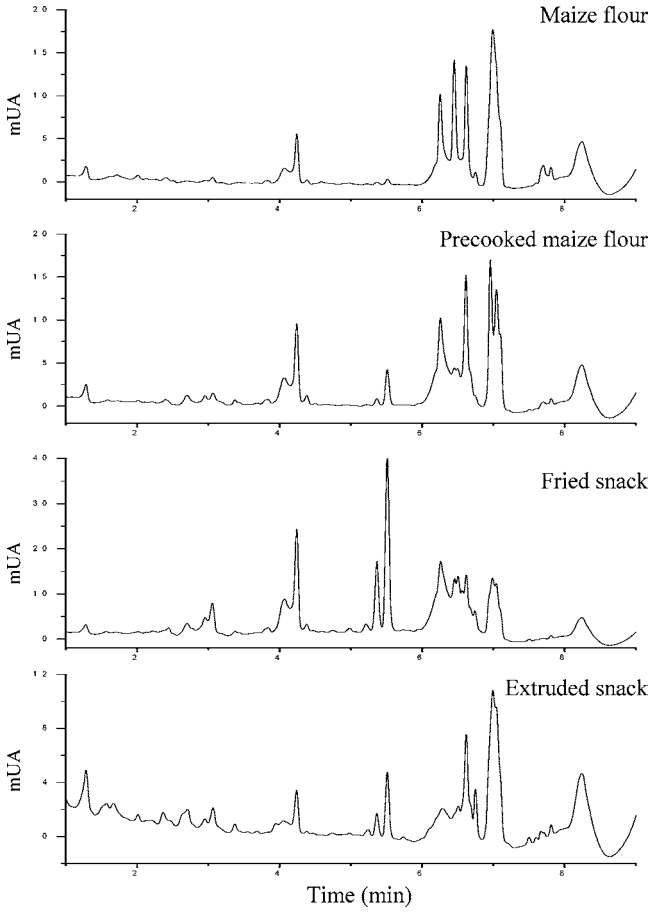

Figure 3. Chromatograms corresponding to the separation of maize protein solutions (30 mg/mL) of four maize products under the optimized conditions.

second group at the middle of the chromatogram (retention times between 4.7 and 5.0 min), and the other at the end of the chromatogram (retention times between 5.9 and 7.1 min). To study the chromatographic profiles of maize proteins, solutions of the CGM, its fractionated zeins (α, *β*, and *γ*), and a commercial zein (zein F4000) were injected (**Figure 2**). The chromatographic profile obtained for CGM was similar to that corresponding to the EZ6 line, where the last group of peaks corresponded with the zein fraction. Moreover, it was possible to affirm that the last peak observed on the CGM and EZ6 maize line chromatograms corresponded with the α-zein fraction and that the three previous peaks of the same group corresponded with *β*- and *γ*-zeins.

Characterization of Commercial Products Prepared with Maize. The optimized method was applied for the first time to the characterization of several products for human consumption derived from maize and commercially available. Twenty-five maize products were classified into four groups: six flours, four precooked flours, six fried snacks, and nine extruded snacks. **Figure 3** illustrates, as an example, the chromatograms obtained from a maize flour, a precooked yellow maize flour, tortilla chips (fried snack), and an extruded snack. Significant differences between these chromatograms and that corresponding to the EZ6 line were observed. Moreover, differences in the chromatographic profiles corresponding to the four different kinds of products were also found. These differences could be useful for the characterization of these products. To make easier this task, univariate and multivariate techniques were used.

Univariate Data Analysis. A total of 26 peaks (P1−P26) with relative peak areas >0.5% (retention times ranging from 2.03 to 7.09 min) were selected. Peaks P7, P19, and P24 in maize flours and in precooked maize flours, peaks P7, P18, and P19 in fried snacks, and peaks P7, P19, and P24 in extruded snacks presented the highest relative peak areas. An ANOVA disclosed no differences in the relative areas of the peaks P3, P6, P8−P13, P15−16, P21, P22, and P26 ($\alpha = 0.05$) among the four groups of the maize products. To check the significant differences among the means of the significant peaks, a Tukey test was carried out, and it was observed that the means of the peaks P5 and P20 from the maize flours were significantly different from those calculated from the other three groups. In fact, a graphic representation of relative areas of the peak P5 as a function of the peak P20 permitted the clear separation of the samples of maize flours from the other samples. A similar behavior could be found for the precooked maize flour when a graphic representation of the relative area of the peak P2 as a function of peak P25 was chosen. Nevertheless, despite the differences observed in the means of the relative peak areas (P1−P2, P4−P5, P7, P14, P17−P20, and P23−P25) among the groups, it was not possible to find a suitable association of the samples into the four groups by plotting the relative area of any peak in an $X-Y$ diagram. Therefore, it was necessary to move on to a multivariate data analysis.

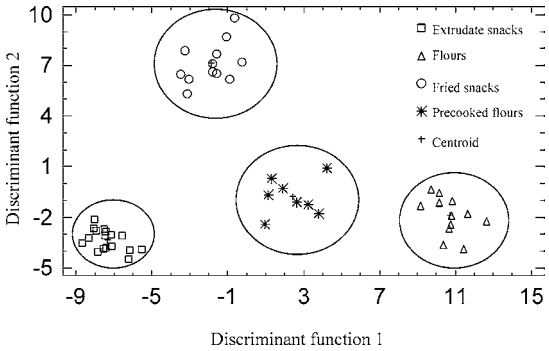

Figure 4. Classification results of linear discriminant analysis using a representation of the two first discriminant functions found from relative peak areas obtained by RP-HPLC for the maize products studied.

Multivariate Data Analysis. Principal component analysis (PCA) was applied to get an overall view of the correlation among variables; no classification was observed when using either peak area (26 variables) or kind of maize product (4 variables) as variable. Because classification based on PCA was unfeasible, linear discriminant analysis (LDA) was performed. Three discriminant functions were selected based on the $P < 0.05$ criterion, the two first discriminant functions accounting for >78% of the total system variability. From the discriminant coefficients, the most influential peak area percentages were those from peaks P7, P24, and P25, with positive coefficients on the first discriminant function and with negative coefficients on the second discriminant function. The representation of the two first discriminant functions enabled the classification of every maize product studied into one of the four previously established groups (**Figure 4**). To test the stability of the built model for the prediction and evaluation process, the sensitivity of the model (percentage of objects belonging to the category which are correctly identified by the mathematical model) and specificity (percentage of objects foreign to the category which are classified as foreign) were calculated. The sensitivity and specificity results obtained for each category were very high (100% for both parameters and for all categories). The reliability of the prediction based on the discriminant functions was further

tested by classifying four new maize products (one sample from each group previously established), and the results showed that all new samples were correctly classified. On the other hand, the validation of this model was evaluated by cross-validation in four steps, excluding 25% of the samples in each class to make up an evaluation set; while the remaindering 75% formed the training set. This process was repeated four times. A prediction rate of 92% was achieved, suggesting that the presented model may be a potential choice for checking the type of commercial maize products.

Conclusions. The use of a monolithic column has enabled the drastic reduction of the separation time (to 8.3 min) usually employed to separate maize proteins with conventional columns (60−80 min). The best chromatographic conditions for the separation of maize proteins with the monolithic column were a linear binary gradient of water/acetonitrile/0.1% trifluoracetic acid at a flow rate of 3 mL/min, a column temperature of 35 °C, and UV detection at 280 nm. The use of an extracting solution consisting of 0.5% 2-mercaptoethanol and 0.5% ammonium acetate in 45% acetonitrile significantly improved the solubilization ratio of maize proteins from maize commercial products in comparison with the conditions employed in the literature. The optimized method enabled the separation of maize proteins into three groups, the last one being the zein fraction. The application of multivariate analysis techniques to the chromatographic data obtained with the proposed method enabled the characterization of commercial maize products and their classification according to the technological process to which these products were subjected.

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