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Ultrarapid Quantitation of Maize Proteins by Perfusion and Monolithic Reversed-Phase High-Performance Liquid Chromatography

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The main objective of this study was to develop a new methodology alternative to the classical Kjeldahl analysis for determining maize proteins in maize products and seeds. For that purpose, two different chromatographic methodologies using perfusion and monolithic stationary phases, both enabling rapid separations of maize proteins, were investigated. Due to the difficulty to find suitable standards for this type of analysis, three different maize products were initially tested as proteins standards: zein F4000, corn gluten meal, and maize flour. Different figures of merit (i.e., linearity, correlation coefficient, precision, limits of detection and quantitation), as well as the presence of matrix inferences, were investigated. The results obtained for the different chromatographic stationary phases and protein standards were compared in order to select the most suitable analytical conditions. Despite both perfusion and monolithic methodologies resulting, in general, as appropriate for the quantitation of maize proteins, the highest reduction of analysis time and lowest detection and determination limits provided by perfusion methodology enabled to select this one as the method of choice for the quantitation of maize proteins. Regarding the different protein standards studied in this work, in general the best results were obtained using the zein standard. Compared to Kjeldahl methodology, perfusion chromatography yields total protein contents in shorter analysis time while enabling the separation of the different kinds of proteins. Due to the high diversity and complexity of industrial maize products, the proposed chromatographic method could be a very useful tool for their routine analysis.

KEYWORDS: Maize proteins; perfusion chromatography; monolithic chromatographic; quantitation

INTRODUCTION

Maize (*Zea mays*) is one of the most important cereal crops worldwide with a production of 784 million metric tons in 2007 (*1*). Nowadays, maize production provides feed, food, and a resource of many unique products (*2*). Proteins are the second large chemical component of the maize kernel (*3*) accounting for 8−10%. These proteins are classified into albumins (water-soluble proteins), globulins (proteins soluble in saline solutions), prolamins or zeins (proteins soluble in relatively strong alcohols), and glutelins (alkali-soluble proteins) (*4*). The technological value of the maize kernel and the maize flour is determined by the quantity and the quality of the proteins it contains (*5*).

HPLC has been applied to study maize protein fractions with different purposes: maize cultivar differentiation (*6*−*8*), characterization of genetically modified maize (*9, 10*), cereal protein measurement (*11*), and assessment of the relationships between protein maize and end-used quality (*12*−*15*). Generally, HPLC systems designed for the separation of proteins use conventional silica columns composed by macroporous butyl- or octadecyl-silica particles (*16*). This type of stationary phases results in very high analysis times due to the difficulty in the diffusion through the inner part of the stationary phase particles experienced by proteins (*17*).

Replacement of conventional columns by perfusion and monolithic stationary phases is an alternative in order to

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overcome the consequences derived from the low diffusivity of proteins. These new phases constitute a great advance in the separation of proteins, permitting their separation in very short analysis times without impairing resolution (*18*). Perfusion chromatography uses packing materials of cross-linked polystyrene-divinylbenzene having a bidisperse porous structure constituted by a macroporous region with 6000−8000 Å transecting pores (through pores) and a connected network of smaller size diffusive pores (800−1500 Å) that provide a large adsorption surface area (*19*). On the other hand, monolithic columns are made of a single piece of a highly porous material prepared by different processes from either organic polymers, such as polymethacrylates, or inorganic polymers, such as silica. They present a bimodal pore structure consisting of macro- or through-pores and mesopores where the transport of the solute to the surface is by convection instead of diffusion as in conventional media (*20*).

Recently, perfusion and monolithic reversed-phase HPLC (RP-HPLC) was applied, for the first time, by our research group to the separation of maize proteins obtaining very short analysis times (<4 min with the perfusion column and <8 min with the monolithic column) in comparison with the high analysis times (40 to 90 min) reported with conventional reversed-phased columns (*21*−*25*). These methods have successfully been applied to the characterization of commercial maize products (*26*, *27*), the identification and classification of European and North American inbred and hybrid maize lines (*28*), and the characterization of albumins, globulins, and storage proteins (prolamins and glutelins) from Bt-11 maize lines (*29*).

To our knowledge, no study has been published so far on the application of perfusion and monolithic RP-HPLC for the quantitation of maize proteins. These rapid methods would constitute an alternative to other reference methods such as Kjeldahl and Dumas methodologies that yield total protein contents in high analysis times and that are based on the determination of the nitrogen content. These kinds of methodologies are not really safe since the adulteration of foods with other products containing nitrogen cannot be detected. Consequently, the existence of methodologies enabling not only the determination of the protein content but also the identification of the kinds of proteins present in such a rapid way that it can be applied for routine analysis are needed. Moreover the use of other nonofficial methodologies based on UV-spectrophotometry techniques such as the Bradford method or the direct absorbance measurement at 280 nm are of limited use, are nonspecific (as the Kjeldahl and Dumas methods) and present limited sensitivity.

Therefore, the aim of this work was to quantify the content of maize protein of commercial products based on their protein profiles obtained by perfusion and monolithic RP-HPLC and to ascertain the reliability of both methods following a standardized validation procedure for food chemistry laboratories (*30*).

EXPERIMENTAL PROCEDURES

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

Zein F4000 (ZF) (Freeman Industries LLC, Tuckahoe, NY), corn gluten meal (CGM) (Sigma), and maize flour (MF) (El Granero, Madrid, Spain) were used as standards of maize proteins. Their protein contents, determined by Kjeldahl analysis (AOAC method 979.09, three replicates) (*31*), were 85.1 ± 0.7%, 68.3 ± 0.5%, and 5.8 ± 0.2% (mean ± relative standard deviation), respectively. Nineteen commercial maize products (flours, precoocked flours, fried snacks and extruded snacks) purchased in local markets in Madrid (Spain), and different transgenic Bt-11 maize seeds were also employed in this study. Namely, transgenic maize seeds (PR33P67, DKC6575, and Aristis Bt) and their non-Bt isogenic varieties (PR33P66, Tietar, and Aristis, respectively) were employed. Conventional and transgenic maize cultivars were obtained from a field assay carried out in Estación Experimental Agrícola Mas Badía in Tallada d'Empordá (Girona, Spain) using commercial varieties. In order to skip any influence from the growing conditions, Aristis maize (wild type and its Bt transgenic variety), Tietar maize (wild type and its Bt transgenic variety DKC6575), and PR33P66 maize (wild type and its Bt transgenic variety PR33P67) were grown under the same field conditions and investigated in this work. The transgenic or non-transgenic nature of all these maize samples was confirmed based on their DNA using an analytical procedure described elsewhere (*32*−*36*).

When necessary, samples were ground with an automatic miller (IKA Labortechnik, Staufen, Germany). Before analysis by HPLC, dry matter content of maize samples was determined by drying at 130 °C to constant weight (AOAC method 925.10) (*37*). Protein contents were also determined by Kjeldahl analysis (AOAC method 979.09) (*31*).

The protocol for preparing sample and standard solutions was the following (*26*, *27*): pulverized maize samples were dissolved in 1 mL of the extracting solution consisting of 0.5% (v/v) 2-mercaptoethanol, 0.5% (w/v) ammonium acetate, and 45% ACN (v/v) in water, sonicated for 5 min in a bath sonicator (150 W, 50 Hz, FS-30, Fisher Scientific), and centrifuged for another 5 min at 3400*g* to remove the supernatant that was injected in the chromatographic system.

High-Performance Liquid Chromatography. Chromatography was carried out with a Hewlett-Packard 1100 series liquid chromatograph (Hewlett-Packard, Pittsburgh, PA) consisting of a degassing system, a binary pump, a thermostatted compartment for the column, an injection system, and a diode-array detector. The separation of maize proteins was accomplished with a Poros R2/H perfusion column (4.6 × 50 mm; 10 μm particle size) (Perseptive Biosystems, Framingham, MA) and with a monolithic silica column Chromolith Performance RP-18e (4.6 × 100 mm) (Merck). Chromatographic conditions for the perfusion column were optimized previously by our research team (*26*): 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; injection volume, 20 μL; flow-rate, 3 mL/min; temperature, 25 °C; UV detection, 280 nm. Separation conditions for the monolithic column were also optimized previously (*27*): 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; temperature, 35 °C. The injection volume, flow-rate, mobile-phase composition, and wavelength detection were as in perfusion chromatography. Data were recorded and processed with the HP-Chemstation software.

Calibration and Validation. Calibration was performed by the external standard and by the standard additions calibration methods. For that purpose, ZF, CGM, and MF were assayed as standards of maize proteins due to the lack of commercial certified standard of maize protein suitable for quantitative purposes.

Calibration by the external standard method was carried out injecting maize protein standard solutions prepared by duplicate and injected in the chromatographic system by duplicate. Peak areas corresponding to maize proteins were integrated by setting the baseline from valley to valley, and the total area (calculated by addition of the individually integrated peak areas) was plotted against the injected concentration of ZF, CGM, or MF for calculating the linear calibration models. Content in protein of each standard solution was determined taking into account its purity and moisture. The linearity domain of the calibration plot, coefficients of correlation (r) and determination (R^2), and confidence limits for the slope and intercept of the regression lines were calculated. The linearity domain of the calibration plot of each standard was defined by using least-squares regression analysis and validated by means of the analysis of residuals and variance. The limits of detection (LOD) and quantitation (LOQ) were determined from the calibration plot as the concentration corresponding to a signal equal to the intercept plus three or ten times the standard deviation of the regression lines, respectively. Calibration by the standard additions

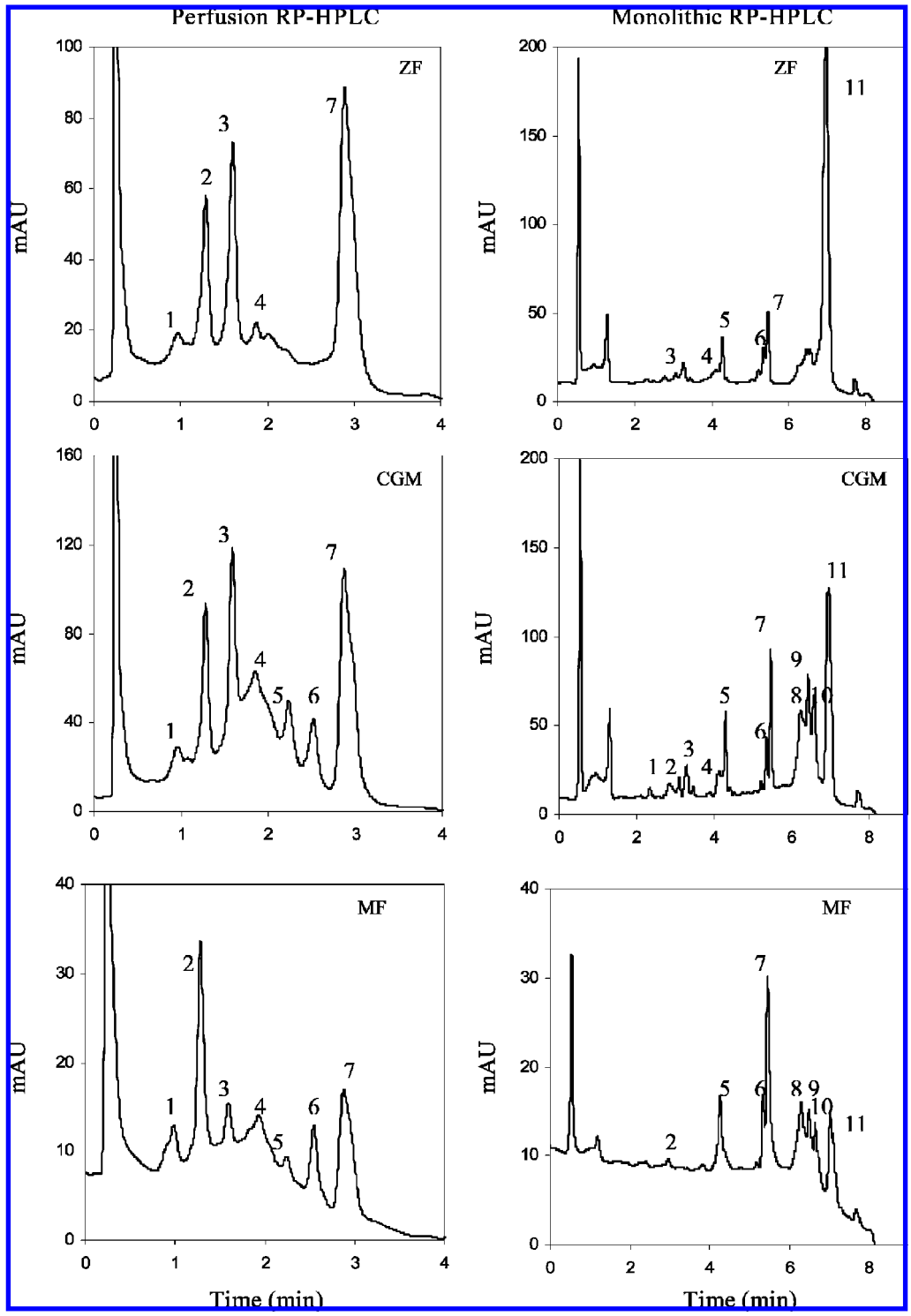

Figure 1. Perfusion and monolithic RP-HPLC chromatograms of protein extracts from ZF, CGM, and MF.

method was performed by injecting (by duplicate) extracts of maize products (a known amount of maize product was taken as the blank) spiked with five known and increasing amounts of ZF, CGM or MF (two replicates of each).

Precision was assayed calculating the repeatability, interday reproducibility, and interanalyst reproducibility. Repeatability was performed by injecting ten consecutive times a solution of 8 mg/mL (corresponding to 6.3 mg/mL of maize proteins), 15 mg/mL (corresponding to 9.5 mg/mL of maize proteins), and 30 mg/mL (corresponding to 1.6 mg /mL of maize proteins) of ZF, CGM, and MF, respectively. The reproducibility was obtained injecting (by duplicate) two solutions (each one prepared by duplicate) of ZF (1.6 and 6.3 mg protein/mL), CGM (3.2 and 9.5 mg protein/mL), and MF (0.8 and 1.6 mg protein/mL) in seven different days. The interanalyst reproducibility was verified by injecting (by duplicate) a standard solution of ZF (1.6 mg protein/mL), CGM (3.2 mg protein/mL) and MF (0.8 mg protein/ mL) prepared by two different analysts and by triplicate.

Accuracy was determined calculating the recoveries obtained for maize proteins when different amounts of ZF were added to a known amount of pulverized maize products. The specificity was verified by adjusting a straight line between added and recovered concentrations. The study of existence of matrix interferences was performed by comparing the slopes of the calibration plots obtained by the external and the standard additions calibration methods.

Robustness test of perfusion chromatographic method was carried out to search for sources that have an evident effect on the response

Table 1. Characteristics of the Calibration Straight Lines Obtained by Perfusion and Monolithic Methods Using Different Potential Standards (Zein (ZF) Corn Gluten Meal (CGM), and Maize Flour (MF))

					slope (mAU/(mg/mL protein))[c]		intercept (mAU)[d]			
std	linear concn range (mg/mL)	regression	r^a	R^b (%)	low lim	high lim	low lim	high lim	LOD (mg/mL)[e]	LOQ (mg/mL)[f]
				Perfusion Column						
ZF	0.427 −8.639	$y = 201.280x + 8.233$	0.999	99.948	168.324	212.327	6.123	10.333	0.120	0.538
CGM	0.752−13.885	$y = 120.258x + 34.872$	0.998	99.622	103.928	136.588	16.222	53.524	0.719	2.398
MF	0.291−8.73	$y = 78.156x + 3.878$	0.996	99.379	68.369	88.233	1.233	5.238	0.577	1.920
				Monolithic Column						
ZF	0.485 −14.114	$y = 197.588x + 208.468$	0.999	99.910	181.991	213.184	190.957	225.98	0.384	1.281
CGM	0.342−9.188	$y = 106.831x + 18.073$	0.997	99.546	93.397	120.265	2.049	34.097	0.579	1.931
MF	0.873−8.730	$y = 83.789x + 3.004$	0.995	99.079	70.263	98.372	2.032	5.013	0.733	2.440

[a] Squared correlation coefficient. [b] Squared correlation coefficient as percentage. [c] Errors in the slope of the regression line expressed at 95% confidence level. [d] Errors in the intercept of the regression line expressed at 95% confidence level. [e] Detection limit. [f] Quantitation limit.

Table 2. Precision Expressed as Average of RSD (%) of Each Peak Area and Retention Time Corresponding to the Analysis of Each Standard (Zein (ZF), Corn Gluten Meal (CGM), and Maize Flour (MF)) at the Indicated Concentrations

	repeatability ($n = 10$)[a]			interday reproducibility ($n = 7$)[b]	
	peak area	retention time		peak area	retention time
			Perfusion Column		
ZF (8 mg/mL)[c]	3.259	0.111	ZF (2 mg/mL)	8.968	0.457
			ZF (8 mg/mL)	8.431	0.597
CGM (15 mg/mL)	2.827	0.082	CGM (5 mg/mL)	7.870	0.553
			CGM (15 mg/mL)	8.381	0.662
MF (30 mg/mL)	9.075	0.114	MF (15 mg/mL)	9.172	0.383
			MF (30 mg/mL)	9.272	0.389
			Monolithic Column		
ZF (8 mg/mL)	7.128	0.039	ZF (2 mg/mL)	9.250	0.390
			ZF (8 mg/mL)	9.041	0.350
CGM (15 mg/mL)	4.581	0.024	CGM (5 mg/mL)	9.259	1.160
			CGM (15 mg/mL)	8.741	0.546
MF (30 mg/mL)	5.869	0.110	MF (15 mg/mL)	8.306	0.371
			MF (30 mg/mL)	8.780	0.350

[a] Number of injections of two independent solutions with the same concentration. [b] Analysis performed in seven different days using two independent solutions; each solution was injected by duplicate. [c] Expressed as mg of product/mL.

when small changes occur in its operating conditions (*38*). Four factors were studied at two levels: percentage of TFA in mobile-phase A (Milli-Q water) and B (acetonitrile) (0.08% and 0.12% were assayed), column temperature (23 and 27 °C), and flow-rate (2.8 and 3.2 mL/min). A fractional factorial design with eight runs (2^{4-1}) was used taking peak area and resolution between peaks as responses of the robustness test. A sample of ZF of 6.3 mg protein/mL, injected by triplicate, was used in this test.

All statistical analyses were performed using the computer program Statgraphics Plus for Windows 4.0 (Statistical Graphics Corp., Rockville, MD).

RESULTS AND DISCUSSION

In this work, we propose the use of RP-HPLC for the quantitative analysis of maize proteins in maize products testing the possibilities of perfusion and monolithic stationary phases. To do this, three different potential maize protein standards were also investigated. In fact, one difficulty found in the development of a method for the quantitation of maize proteins is the selection of a suitable protein standard. To our knowledge, there is not any certified reference material that could be used as a standard of maize proteins for their quantitation in commercial products and seeds. Therefore, three different commercial products from maize were tested for this purpose: zein F4000 (ZF), corn gluten meal (CGM), and maize flour (MF).

Perfusion and monolithic chromatograms obtained for ZF, CGM, and MF are shown in **Figure 1**. Peak assignment and numbering were done after collecting and comparing all protein patterns corresponding to all samples. Seven and eleven different peaks were observed, respectively, in perfusion and monolithic profiles. The perfusion chromatogram from ZF showed five peaks in only 3 min, peaks 2, 3, and 7 being those presenting the highest peak areas (more than 85% of the total peak area). CGM showed seven peaks (peak 1−7) with the maximum signal for peaks 2, 3, and 7. A similar protein profile was also found for MF showing a main peak at the beginning of the chromatogram (peak 2) and other one at the end of the chromatogram (peak 7). According to a previous paper (*26*), peak 7 corresponds to the α-zein fraction, while peaks 3−6 correspond to β- and γ-zeins. Chromatograms observed for monolithic RP-HPLC were different from those obtained with perfusion RP-HPLC. Maize proteins were separated in a higher number of peaks although in a longer time (8 min). Unlike perfusion chromatography, the monolithic chromatograms presented three groups of peaks: one at the beginning of the chromatogram with retention times ranging from 2.0 to 4.5 min (peaks 1−5), a second group at the middle of the chromatograms (retention times between 5.0 and 6.0 min, peaks 6 and 7), and a third group at the end (retention times between 6.0 and 7.1 min, peaks 8−11). The chromatogram obtained for MF was similar to that corresponding to CGM, although peaks in the first and third groups were less intense for MF. Regarding the ZF chromatogram, the maximum signal was observed for peak 11. This last peak corresponds with α-zein, while the three previous peaks (peaks 8−10) correspond with β- and γ-zeins (*27*).

Calibration curves were linear over the concentration range 0.29−13.89 mg protein/mL for ZF, CGM, and MF and the perfusion column and in the range 0.34−14.12 mg protein/mL for ZF, CGM, and MF and the monolithic column (see **Table 1**). A good linear correlation ($r > 0.995$) between the total peak area measured for ZF, CGM, and MF and the concentration using both perfusion and monolithic columns was always observed. The detection and quantitation limits (LOD and LOQ) observed for ZF and MF with the perfusion column resulted lower than that obtained with the monolithic column while the opposite was observed for the LOD and LOQ obtained for CGM. As a result, the highest sensitivity would be obtained using ZF as standard and the perfusion methodology.

The precision of the methods was determined by evaluating repeatability, interday reproducibility, and interanalyst reproducibility. **Table 2** shows the RSD values obtained for the repeatability and intermediate precision. Best repeatability was observed for CGM using both perfusion and monolithic

Table 3. Maize Protein Contents Determined in Maize Products and Seeds by the External Standard and the Standard Additions Calibration Methods Using Perfusion RP-HPLC and Monolithic RP-HPLC

(**a**) Perfusion RP-HPLC: Protein Concentration (mg/100 mg sample)[a,b]

		using external standard[c]			using standard additions[d]		
maize product	Kjeldahl method[e]	ZF	CGM	MF	ZF	CGM	MF
flour 1	6.82(0.20)	6.92(0.23)	7.32(0.33)	11.23(2.20)	6.88(0.12)	7.52(0.22)	11.53(1.23)
flour 1	6.82(0.20)	6.92(0.23)	7.39(0.25)	11.23(1.11)	6.90(0.14)	7.57(0.60)	11.99(0.23)
flour 2	8.23(0.04)	8.21(0.29)	8.99(0.30)	13.22(1.63)	8.42(0.23)	9.01(0.51)	13.13(0.39)
flour 3	6.85(0.23)	6.92(0.23)	7.85(0.19)	12.28(1.99)	6.90(0.12)	7.55(0.23)	12.67(1.23)
flour 4	7.23(0.21)	7.22(0.30)	8.01(0.55)	11.01(1.32)	7.22(0.22)	8.23(0.13)	11.32(1.62)
precooked flour 1	7.54(0.32)	7.64(0.12)	8.21(0.31)	15.01(1.33)	7.66(0.32)	8.53(0.33)	14.09(1.54)
precooked flour 2	8.23(0.12)	8.19(0.42)	9.99(0.58)	13.25(1.89)	8.25(0.23)	9.95(0.22)	13.28(1.77)
precooked flour 3	7.85(0.09)	7.79(0.23)	8.99(0.33)	14.01(1.23)	7.52(0.09)	8.79(0.21)	14.23(1.33)
precooked flour 4	6.89(0.23)	6.77(0.15)	7.54(0.37)	10.99(0.69)	6.52(0.42)	7.60(0.57)	10.19(0.59)
fried snack 1	5.01(0.12)	5.09(0.12)	5.97(0.22)	10.12(0.33)	5.20(0.33)	5.88(0.16)	10.55(0.33)
fried snack 2	5.63(0.09)	5.59(0.23)	6.59(0.16)	10.23(0.36)	5.55(0.12)	6.99(0.57)	10.01(0.52)
fried snack 3	6.23(0.01)	6.12(0.59)	7.09(0.18)	11.28(1.98)	6.85(0.13)	7.12(0.36)	11.33(1.36)
fried snack 4	5.12(0.12)	5.09(0.13)	5.99(0.56)	10.16(1.25)	5.00(0.69)	5.79(0.33)	10.25(1.12)
extruded snack 1	5.80(0.23)	5.98(0.19)	7.01(0.17)	10.89(1.36)	7.56(0.11)	7.14(0.58)	10.77(1.55)
extruded snack 2	7.01(0.05)	6.99(0.12)	7.89(0.36)	12.07(0.13)	7.23(0.23)	7.88(0.12)	11.97(0.23)
extruded snack 3	8.50(0.17)	8.55(0.32)	9.36(0.41)	13.30(1.98)	8.23(0.33)	9.66(0.31)	13.50(1.78)
extruded snack 4	6.23(0.11)	6.36(0.63)	7.32(0.23)	12.23(0.26)	5.39(0.19)	7.38(0.13)	12.99(0.56)
extruded snack 5	5.23(0.16)	5.33(0.22)	5.23(0.69)	10.36(0.36)	5.66(0.17)	5.23(0.69)	10.66(0.56)
extruded snack 6	6.98(0.16)	7.00(0.33)	7.91(0.37)	12.98(0.39)	7.03(0.28)	7.91(0.37)	12.88(0.53)
Aristis	7.69(0.13)	7.50(0.59)	9.93(0.23)	17.10(1.61)	7.74(0.10)	6.69(0.08)	14.46(0.08)
Aristis Bt11	7.64(0.16)	6.79(0.60)	6.61(1.73)	12.07(1.60)	7.44(0.01)	7.47(0.22)	13.29(0.23)
PR33P66	8.01(0.02)	7.61(0.60)	7.80(1.03)	13.87(1.60)	7.69(0.09)	8.20(0.30)	13.94(0.07)
PR33P67 (Bt11)	7.80(0.08)	7.11(0.59)	8.25(0.85)	14.54(1.59)	7.66(0.95)	8.64(0.28)	10.46(0.68)
Tietar	7.89(0.08)	7.32(0.59)	8.60(1.71)	15.07(1.59)	6.85(0.09)	9.10(0.32)	13.87(0.77)
DKC6575 (Bt11)	7.81(0.08)	7.19(0.59)	8.01(1.72)	14.19(1.60)	6.13(0.22)	9.38(0.42)	13.42(0.09)

(**b**) Monolithic RP-HPLC: Protein Concentration (mg/100 mg sample)[a,b]

		using external standard[c]			using standard additions[d]		
maize product	Kjeldahl method[e]	ZF	CGM	MF	ZF	CGM	MF
flour 1	6.82(0.20)	5.23(0.33)	7.98(0.42)	11.26(0.35)	5.21(0.12)	7.85(0.33)	11.69(1.23)
flour 1	6.82(0.20)	5.69(0.88)	7.95(0.14)	11.26(0.25)	5.96(0.14)	8.05(1.60)	11.02(0.88)
flour 2	8.23(0.04)	7.23(0.79)	9.23(0.55)	13.99(0.99)	7.25(0.23)	9.55(0.89)	13.89(0.39)
flour 3	6.85(0.23)	5.99(0.73)	7.85(1.01)	12.89(0.89)	6.00(0.12)	8.08(0.39)	12.21(1.04)
flour 4	7.23(0.21)	6.23(0.50)	9.01(1.32)	12.01(0.99)	6.52(0.22)	9.30(0.70)	11.98(1.36)
precooked flour 1	7.54(0.32)	6.23(0.52)	8.55(0.88	13.23(1.22)	6.58(0.32)	8.99(0.69)	13.99(1.77)
precooked flour 2	8.23(0.12)	7.28(0.88)	9.82(0.98)	14.25(1.99)	7.35(0.23)	10.08(0.77)	14.28(0.33)
precooked flour 3	7.85(0.09)	6.58(0.95)	9.03(0.87)	15.03(1.03)	7.02(0.09)	9.28(0.87)	14.77(1.25)
precooked flour 4	6.89(0.23)	5.98(0.49)	9.31(0.69)	11.63(1.36)	6.02(0.42)	9.85(0.39)	11.98(0.99)
fried snack 1	5.01(0.12)	4.21(0.72)	6.23(0.87)	11.03(1.22)	4.20(0.33)	6.80(0.32)	11.35(1.98)
fried snack 2	5.63(0.09)	4.25(0.85)	6.99(0.45)	12.36(1.69)	4.55(0.12)	6.99(0.84)	12.85(0.96)
fried snack 3	6.23(0.01)	5.36(0.99)	7.55(0.66)	13.69(2.01)	5.85(0.13)	7.42(0.20)	13.43(1.13)
fried snack 4	5.12(0.12)	4.89(0.75)	6.98(0.78)	11.98(0.98)	5.00(0.69)	6.55(0.85)	11.65(1.14)
extruded snack 1	5.80(0.23)	4.23(0.33)	7.23(0.83)	11.23(0.69)	4.56(0.22)	7.33(0.58)	10.99(1.11)
extruded snack 2	7.01(0.05)	6.22(0.33)	8.23(0.39)	13.06(1.12)	6.23(0.45)	8.25(0.28)	12.98(0.74)
extruded snack 3	8.50(0.17)	7.28(0.75)	9.52(0.38)	14.22(1.09)	7.23(0.23)	9.16(0.87)	14.33(1.25)
extruded snack 4	6.23(0.11)	5.26(0.89)	7.26(0.87)	12.68(1.89)	5.39(0.18)	7.98(0.69)	12.68(0.36)
extruded snack 5	5.23(0.16)	4.33(0.15)	6.98(0.99)	11.36(0.77)	4.66(0.17)	6.83(0.23)	11.60(0.22)
extruded snack 6	6.98(0.16)	5.23(0.23)	7.88(1.02)	11.98(0.98)	5.03(0.18)	8.01(0.37)	12.03(0.28)
Aristis	7.69(0.13)	5.81(0.92)	10.21(0.23)	15.33(1.56)	7.29(0.99)	9.96(0.95)	10.78(0.24)
Aristis Bt11	7.64(0.16)	4.73(0.93)	8.22(1.38)	12.86(1.57)	7.10(2.82)	9.03(0.43)	12.08(0.28)
PR33P66	8.01(0.02)	5.39(0.92)	9.44(1.38)	14.38(1.57)	7.57(0.31)	9.16(0.23)	12.20(0.29)
PR33P67 (Bt11)	7.80(0.08)	5.14(0.92)	8.98(1.37)	13.75(1.55)	7.39(0.23)	7.66(0.95)	11.02(0.23)
Tietar	7.89(0.08)	6.02(0.92)	10.61(1.37)	15.81(1.55)	5.48(0.23)	9.76(1.88)	11.21(0.67)
DKC6575 (Bt11)	7.81(0.08)	5.56(0.92)	9.76(1.37)	14.75(1.56)	7.83(2.03)	8.98(1.72)	11.50(1.30)

[a] Standard deviation given in parentheses. [b] Protein concentrations determined using ZF, CGM, and MF as standards. [c] Mean of two individual determinations (every solution injected by duplicate). [d] Calibration by the standard additions method was performed by injecting an extract of maize product spiked with five known and increasing amounts of standard (ZF, CGM or MF). [e] Mean of three individual determinations.

methodologies. Interday reproducibility was determined by injecting different standard solutions in seven different days using the perfusion and monolithic methods. The RSD values observed were good, below 9.3% in peak area and below 1.2% in retention time. Best results for peak area and retention time variability were observed for CGM (<8.5%) and MF (<0.4%), respectively, using the perfusion methodology. For the monolithic method, a value of RSD <8.8% was obtained in peak area for MF while the best RSD in retention time was always for ZF and MF standard solutions (<0.5%). Interanalyst reproducibility was determined by injecting standard solutions prepared by two different analysts. The best results were obtained with the monolithic column (RSD <3.3% in peak area and RSD <0.04% in retention time).

Table 4. Investigation of the Existence of Matrix Interference in the Determination of Maize Protein Contents Using Perfusion and Monolithic RP-HPLC

	ZF		CGM		MF	
	slope[a]	*p*-value[b]	slope[a]	*p*-value[b]	slope[a]	*p*-value[b]
Perfusion RP-HPLC						
using external standard	203.16		120.26		79.14	
using standard additions	203.51	0.951	156.73	0.000	96.14	0.000
	195.34	0.065	129.25	0.050	84.76	0.041
	202.72	0.941	121.04	0.917	96.48	0.000
Monolithic RP-HPLC						
using external standard	197.58		106.83		86.12	
using standard additions	184.73	0.056	124.96	0.000	91.99	0.000
	196.58	0.862	141.92	0.000	94.26	0.002
	190.74	0.298	120.74	0.547	98.78	0.000

[a] Slope expressed in mAU/(mg/mL protein). [b] *p*-Value of the hypothesis test to compare the external standard and standard additions calibration slopes with ZF, CGM or MF as standards.

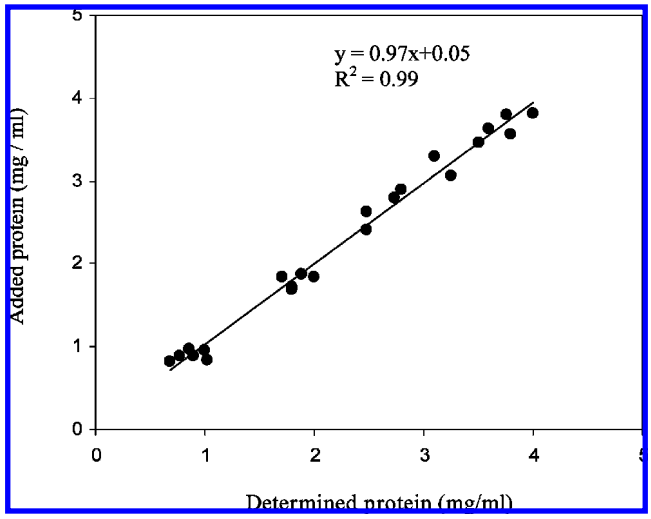

Figure 2. Variation of the theoretical concentration of ZF added to the samples versus concentration determined of ZF using perfusion RP-HPLC.

Table 5. Study of the Robustness of the Perfusion RP-HPLC Method Using Zein (ZF) as Standard

	modified variables[a]			
	% of TFA in			
responses	mobile phase A (Milli-Q water)	mobile phase B (ACN)	*T* (°C)	flow-rate (mL/min)
peak area				
peak 1	0.913	0.058	0.600	0.934
peak 2	0.174	0.147	0.384	0.936
peak 3	0.369	0.133	0.668	0.708
peak 7	0.082	0.133	0.668	0.708
total area ZF	0.634	0.118	0.625	0.744
resolution				
res 12	0.013	0.027	0.023	0.007
res 23	0.165	0.112	0.951	0.097
av peak resolution	0.189	0.169	0.487	0.197

[a] *p*-Values.

The quantitative results obtained using the perfusion and monolithic methodologies were compared with the results obtained using the classical Kjeldahl analysis. **Table 3a** and **Table 3b** show the protein contents obtained for different commercial products (four maize flours, four precooked flours, four fried snacks, and six extruded snacks) and for several transgenic and nontransgenic Bt-11 maize lines using the external standard and the standard additions methods, the perfusion RP-HPLC (**Table 3a**) and the monolithic RP-HPLC (**Table 3b**) methodologies and ZF, CGM, and MF as standards. A good agreement between the values of protein content given by Kjeldahl analysis and those obtained by RP-HPLC was observed with the ZF standard using both the monolithic and the perfusion columns. The protein contents obtained when using CGM and MF as standards were higher than those estimated with ZF. When plotting the content determined by the perfusion method or the monolithic method using ZF as standard versus that obtained by the Kjeldahl method, regression lines obtained presented slopes and intercepts that were statistically similar to 1 and 0, respectively (*t* test, $P > 0.05$). The results obtained indicated no differences between the protein contents obtained using the official method and both chromatographic methods with ZF as standard. Interestingly, the total time required for the chromatographic methods (including proteins extraction) was shorter than 20 min, while the Kjeldahl method required 3−4 h (*39*−*41*).

The study of the existence of matrix interferences was performed by comparing the slopes of the calibration plots obtained by the external and the standard additions calibration methods. **Table 4** shows the slopes and *p*-values obtained in the hypothesis test to compare the external standard and the standard additions calibrations using ZF, CGM, and MF as standards. The slopes of the regression lines obtained by both methods of calibration did not differ significantly (*F*-test to compare variances and *t* test to compare slopes, $p > 0.05$), when ZF was used as standard demonstrating that the quantitative method was not affected by matrix interferences neither using the perfusion or the monolithic methods. Nevertheless, the methods seemed to be affected by matrix interferences when CGM or MF were employed as standards. This behavior could be due to the degree of purity of the commercial maize products assayed as standards, because ZF presents the highest protein content (85.1%) in relation to CGM (68.3%) and MF (5.8%). Taking into account the good results found for both chromatographic methods using ZF as standard and that the analysis of maize proteins is faster using perfusion RP-HPLC, the accuracy and robustness were estimated only for the perfusion method using ZF as standard.

Accuracy was determined by adding known quantities of ZF to maize products. **Figure 2** shows the variation of the theoretical concentration of ZF added to the samples as a function of the ZF concentration determined using perfusion RP-HPLC. As it can be observed, a good linear correlation ($R^2 > 0.99$) was found.

Finally, a robustness test was performed in order to identify possible sources of error when changes occur in the specified method conditions (*42*). Critical chemical and instrumental chromatographic parameters such as the composition of mobile-phases, flow-rate, and temperature were deliberately varied in the range ± 20.0% compared to their optimal values. A fractional factorial design (2^{4-1}) with eight runs was used for this purpose. The effect of the variation of each factor level on peak area and resolution between peaks are grouped in **Table 5** as *p*-values. The significance of an effect was estimated by a *t* test, comparing the effect with the experimental error (*43*). No significant variance in the peak area or in the retention time was observed when deliberately varying the mobile phase composition, the flow-rate or the separation temperature of the perfusion RP-HPLC methods (*p*-values >0.05).

This is the first time a quantitative methodology for maize proteins in commercial maize products and seeds based on

perfusion and monolithic RP-HPLC methodology has been proposed. These methods enable a drastic reduction of analysis time needed in relation to the classical Kjeldahl method (<20 min for chromatography vs 3−4 h for Kjeldahl method, per analysis). Moreover, unlike other rapid methodologies used for the quantification of proteins, such as combustion and UV-spectrophotometry techniques, perfusion and monolithic chromatographies enable a simultaneous separation and quantification of the different fractions of maize proteins. Due to the absence of certified reference standards for the quantitative determination of maize proteins, different commercial maize products were tested: zein F4000, corn gluten meal, and maize flour. In general, best results regarding rapidity, sensitivity, and precision were obtained with the perfusion column using zein F4000 as standard. The protein analysis for commercial products and for several transgenic and nontransgenic Bt-11 maizes showed a good agreement between the values of protein content given by Kjeldahl analysis and those obtained by perfusion RP-HPLC using zein F4000 as standard. Moreover, the comparison of the slopes of the calibration lines obtained by the external standard and by the standard additions calibration methods enabled us to conclude that the proposed methodology was not subjected to matrix interference. Accuracy and robustness were also estimated in the perfusion methodology proposed observing both were adequate. All these results suggested that the proposed method provided a solid alternative to the classical Kjeldahl analysis and for the quantitative estimation of maize proteins in maize products being of great interest for routine analysis.

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