Food Chemistry 111 (2008) 483–489

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Estimation of the percentage of transgenic Bt maize in maize flour mixtures using perfusion and monolithic reversed-phase high-performance liquid chromatography and chemometric tools

J.M. Rodríguez-Nogales [a], A. Cifuentes [b], M.C. García [c], M.L. Marina [c,*]

[a] Área de Tecnología de los Alimentos, Departamento de Ing. Agraria y Forestal, ETS de Ingenierías Agrarias, Universidad de Valladolid, 34004 Palencia, Spain
[b] Departamento de Análisis de Alimentos, Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain
[c] Departamento de Química Analítica e Ingeniería Química, Facultad de Química, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain

ARTICLE INFO

Article history:
Received 1 March 2007
Received in revised form 22 February 2008
Accepted 30 March 2008

Keywords:
Transgenic Bt maize
Perfusion RP-HPLC
Monolithic RP-HPLC
Maize proteins
Chemometric analysis

ABSTRACT

The estimation of the percentage of transgenic Bt maize in maize flour mixtures has been achieved in this work by high-performance liquid chromatography using perfusion and monolithic columns and chemometric analysis. Principal component analysis allowed a preliminary study of the data structure. Then, linear discriminant analysis was used to develop decision rules to classify samples in the established categories (percentages of transgenic Bt maize). Finally, linear regression (LR) and multivariate regression models (namely, principal component analysis regression (PCR), partial least squares regression (PLS-1), and multiple linear regression (MLR)) were assayed for the prediction of the percentages of transgenic Bt maize present in a maize flour mixture. Using the relative areas of the protein peaks, MLR provided the best models and was able to predict the percentage of transgenic Bt maize in flour mixtures with an error of ±5.3%, ±2.3%, and ±3.8% in the predictions of Aristis Bt, DKC6575, and PR33P67, respectively.

1. Introduction

Genetically modified maize is one of the most extensively cultivated genetically modified organism (GMO), being the modifications introduced in these cultivars, basically, aimed to enable resistance to herbicides or to increase tolerance to insects and pests (Hernández, Esteve, Prat, & Pla, 2004). Three different events of transgenic maize (*Zea mays*) containing different sequences of a synthetic CryIA(b) gene have been authorized for commercialization in Europe: Event 176, MON 810, and Bt11 (Margarit, Reggiardo, Vallejos, & Permingeat, 2006). The transgenic maize Event 176, MON 810, and Bt11 were genetically engineered to resist the European corn borer (*Ostrinia nubilalis*) which is the most damaging insect pest of maize (Agriculture and Biotechnology Strategies, 2005). In Spain, several maize lines with the event Bt-176 and MON 810 have been inscribed in the Spanish Commercial Varieties's Register (7052/1998, BOE 26th March; APA/520/2003, BOE 11th March; APA/314/2004, BOE 16th February; APA/3059/2009, BOE 6th October).

The need to verify GMO levels in foods has created a new demand for analytical testing (Erickson, 2000). Many governments have already implemented regulations for the use and labeling of genetically modified organism (GMO)-derived ingredients (Cardarelli, Branquinho, Ferreira, da Cruz, & Gemal, 2005). Verification that non-GMO food products really do not contain GMO will likely continue driving the demand for GMO testing (Erickson, 2000). On the other hand, several companies are developing products, in which composition GMOs have been included so they need specific methods for testing their final products. Consequently, reliable and accurate methods for the identification and quantification of transgenic maize are required.

The approaches developed for the identification of transgenic cultivars are based on the detection of those targets making different the transgenic and the non-transgenic cultivar: the recombinant DNA fragment inserted or the expressed protein. The two most common approaches for GMO detection are polymerase chain reaction (PCR)-based methods (Cankar, Stebih, Dreo, Zel, & Gruden, 2006; Deisingh & Badrie, 2005; García-Cañas, Cifuentes, & González, 2004; García-Cañas, González, & Cifuentes, 2002a, 2002b; García-Cañas, González, & Cifuentes, 2004; Hubner, Studer, & Luthy, 1999; Papazova et al., 2006; Trapmann & Emons, 2005), which detect genetically modified DNA sequences and immuno-assay (Head, Surber, Watson, Martin, & Duan, 2002; Luthy, 1999; Margarit et al., 2006; Markoulatos et al., 2004; Stave, 1999; Van Duijn, van Biert, Bleeker-Marcelis, Peppelman, & Hessing, 1999; Volpe, Ammid, Moscone, Occhigrossi, & Palleschi, 2006), which measures levels of proteins expressed by transgenic genes. Despite these methodologies are well established, the result may be significantly affected by the processing procedure followed by the food

* Corresponding author.
E-mail address: mluisa.marina@uah.es (M.L. Marina).

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doi:10.1016/j.foodchem.2008.03.079

containing the transgenic cultivar, the DNA extraction procedure employed, etc. Moreover, these methods are very tedious and time consuming and if for each modification a different assay has to be set up, the analysis will soon become too costly and, thus, not suitable for routine analysis (Engel, Moreano, Ehlert, & Busch, 2006; Margarit et al., 2006; Walschus, Witt, & Wittmann, 2002). As a consequence, scientific community is trying to improve the weakness of the existing proposals.

Reversed-phased high-performance liquid chromatography (RP-HPLC) has been applied to the characterization of different plant genotypes (Lookhart, Bean, & Bietz, 2003). To our knowledge, no study has been published so far on the application of RP-HPLC to the estimation of transgenic Bt maize in maize flour mixtures. Recently, rapid perfusion and monolithic RP-HPLC methods were designed for the separation of maize proteins (Rodríguez-Nogales, García, & Marina, 2006a, 2006b). These methods were successfully applied to the characterization of inbred and hybrid maize lines (Rodríguez-Nogales, García, & Marina, 2006c). Moreover, we have characterized, for the first time, the protein fractions from Bt-transgenic and non-transgenic maize varieties using perfusion and monolithic RP-HPLC in very short analysis times (<4 min with the perfusion column and <8 min with the monolithic column) (Rodríguez-Nogales, Cifuentes, García, & Marina, 2007). A comparison of the chromatograms of protein fractions relative to transgenic and non-transgenic varieties evidenced quantitative differences on the percentages of area. These results encouraged us to test the applicability of these chromatographic methods for the quantitative determination of transgenic Bt-maize in maize flour mixtures.

Principal component regression (PCR), partial least squares (PLS) regression, and multiple linear regression (MLR) are multivariate statistical techniques that have been applied in different sciences to obtain calibration models as an alternative to linear regressions. These statistical methods have provided good predictive models for the simultaneous analysis of multi-mixtures in foods (Moreno, Merkoçi, Alegret, Hernández-Cassou, & Saurina, 2004; Poveda, García, Martín-Alvarez, & Cabezas, 2004; Rodriguez-Nogales, 2006). Multivariate regressions have also been used to distinguish transgenic products from conventional ones. Roussel, Hardy, Hurburgh, and Rippke (2001) detected and segregated transgenic Roundup Ready™ soybeans from conventional soybeans using PLS models by NIR spectroscopy. Recently, Xie, Ying, Yinga, Yua, and Fua (2007) have also used PLS regressions for the discrimination of transgenic tomatoes based on visible/near-infrared spectra.

Therefore, the goal of this work was to provide a procedure for the estimation of the percentage of transgenic Bt maize in maize flour mixtures based on perfusion and monolithic RP-HPLC analysis of their protein extracts. Principal component analysis (PCA) and linear discriminant analysis (LDA) were used to explore the data matrix. Finally, linear regression (LR) and multivariate regressions (principal component analysis regression (PCR), partial least squares regression (PLS-1), and multiple linear regression (MLR)) were compared to develop models for the prediction of percentages of transgenic Bt maize in maize flour mixtures.

2. Materials and methods

2.1. Chemicals

HPLC grade acetonitrile (ACN) (Merck, Darmstadt, Germany), HPLC grade water (Milli-Q system, Millipore, Bedford, MA, USA), and trifluoroacetic acid (TFA) (Sigma, St. Louis, MO, USA) were used in the preparation of mobile phases. 2-mercaptoethanol (Merck) and ammonium acetate (Panreac, Barcelona, Spain) were employed for the extraction of maize proteins.

2.2. Samples

Transgenic Bt maize seeds with the even MON 810 (PR33P67 (from Pioneer Hi-Bred International, Inc.), DKC6575 (from Monsanto Company), and Aristis Bt (from Nickerson Ltd.)) and its non-Bt transgenic control maize varieties (PR33P66, Tietar, and Aristis, respectively) were employed for this study. Flours of these maize lines were produced by grinding with an analytical mill (IKA Labortechnik, Staufen, Germany) thirty kernels during three minutes at ambient temperature.

For each one of the three mentioned Bt maize cultivars, twenty flour mixtures with different percentages of transgenic Bt maize (calibration samples: 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, and 100% (w/w)) were prepared by appropriate mixing of each transgenic and non-transgenic lines. Dry matter content of the maize flours was determined by drying at 130 °C to constant weight. For the validation of the prediction models, five different flour mixtures (4, 15, 20, 60, and 80% (w/w) of transgenic Bt maize) were also prepared by duplicate.

The protocol for preparing the protein extracts from each maize flour mixtures was the following: 30 mg of maize flour mixture were weighed and dissolved in 1 mL of 0.5% (v/v) 2-mercaptoethanol, 0.5% (w/v) ammonium acetate, and 45% ACN (v/v) in water. The mixture was sonicated (150 W, 50 Hz, FS-30, Fisher Scientific, Pittsburgh, PA) for 5 min and centrifuged (Avanti™ J-25 centrifuge, Beckman Coulter, USA) for another 5 min at 3362*g* to remove the supernatants that were injected in the chromatographic system (Rodríguez-Nogales et al., 2006a; Rodríguez-Nogales et al., 2006b).

2.3. High-Performance liquid chromatography

A Hewlett–Packard 1100 Series liquid chromatograph (Hewlett–Packard, Pittsburgh, PA, USA) equipped with a degassing system, a binary pump, a thermostated compartment for the column, an injection system, and a diode-array detector was used. Data were recorded and processed with the HP-Chemstation software. 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, USA) and a monolith silica column Chromolith™ Performance RP-18e (4.6 × 100 mm) (Merck).

Maize proteins were eluted using a previously optimized perfusion RP-HPLC method (Rodríguez-Nogales et al., 2006a): 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. The separation conditions for the monolithic column were those optimized previously (Rodríguez-Nogales et al., 2006b): 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.

2.4. Data treatment

The area percentage for every peak was calculated as the average of two replicates (injected by duplicate). The integration was performed by setting the baseline from valley to valley. The original data of the peak areas were normalized before the statistical analysis by subtracting the average from each variable and then dividing by the standard deviation. Chromatographic data of the calibration samples of maize flours were analyzed by one-way ANOVA, principal component analysis (PCA), and linear discriminant analysis (LDA). One-way ANOVA was performed (with a significant

level of 0.05) for each type of maize flour mixture and for each peak, using as factor the percentage of transgenic Bt-maize on the flour mixture. PCA was applied in order to study the association of samples. Stepwise LDA was performed for the classification of the samples according to the percentage of transgenic Bt maize in the maize flours. For that purpose, the "forward" procedure, which begins with no variables in the model and adds the variables with the greatest discriminating power, was selected. This analysis selects the variables that allow differentiating among flours with different percentages of Bt-transgenic maize. The statistical *F*-function was used as criterion for variable selection. The prediction capacity of the discriminant models was studied by "cross-validation". The cross-validation is done by treating $n-1$ out of n observations as the training dataset to determine the discrimination rule and using the rule to classify the one observation left out (Webb, 1999).

Principal component analysis regression (PCR), partial least squares regression (PLS-1), multiple linear regression (MLR), and linear regression (LR) were used for the prediction of the percentage of transgenic Bt-maize in flour mixtures (X-variable) based on the parameters analyzed (relative peak areas from perfusion and monolithic RP-HPLC analysis of the protein extracts, Y-variables). The errors associated with the calibration and prediction were obtained by calculating the standard error of calibration (SEC) and prediction (SEP), respectively (CAMO, 1996). SEC was defined as:

$$\mathrm{SEC} = \sqrt{\sum_{i=1}^{m} \frac{(y_{Ci} - y_i)^2}{n}}$$

where y_{Ci} is the predicted percentage of transgenic sample in calibration sample i, y_i is the real percentage in the calibration samples i, and n is the number of calibration samples. SEP (error of prediction) was chosen as an optimized criterion to validate the built calibration, which was defined as:

$$\mathrm{SEP} = \sqrt{\sum_{i=1}^{m} \frac{(y_{Pi} - y_i)^2}{m}}$$

where y_{Pi} is based on the previously developed calibration models, y_i is the real percentage in the calibration samples i, and m is the number of evaluation samples.

The detection limit (DL, 3·SEC/b) of the linear regression can be calculated from the standard error of calibration (SEC) and the slope of the calibration lines (b) (Miller & Miller, 2000).

ANOVA, PCA, LDA, and linear regressions (LR) were done with the computer program Statgraphics® Plus for Windows 4.0 (Statistical Graphics Corp., Rockville, MD 20852-4999 USA). Unscrambler program v. 7.01 (Camo, ASA, Trondeheim, Norway) was used for the application of PCR, PLS-1, and MLR.

3. Results and discussion

3.1. Analysis of maize flour mixtures with different percentages of transgenic Bt maize

Fig. 1 shows the perfusion chromatographic profiles obtained for the flour mixtures with a 0%, 50%, and 100% of transgenic Bt maize from the three cultivars (Aristis Bt, DKC6575, and PR33P67). The chromatograms with a 0% of transgenic Bt maize correspond to the non-transgenic maize lines (Aristis, Tietar, and PR33P66, respectively). Maize proteins eluted in eight peaks in about three min. In previous works performed by our research team, these eight peaks could be assigned to proteins belonging to albumin, globulin, prolamin, and glutelin fractions of proteins from maize (Rodríguez-Nogales et al., 2006a, 2007). For Aristis Bt, the signal for peak 3 was higher than that found for peak 2. Furthermore, the ratio between the signals of peaks 3 and 2 was smaller for the transgenic Aristis Bt line (100% in the flour mixture) than for its non-transgenic Aristis line (0% in the flour mixture). The chromatograms obtained for the other transgenic and non-transgenic Bt maize lines showed that the signal for peak 3 was always smaller than that for peak 2, that is the signal for peak 2 was more intense for the lines PR33P67 and PR33P66 than for the lines Tietar and DKC6575. The results of the one-way ANOVA showed that significant differences were not found for peaks 5 and 6 in Aristis Bt flours, for peaks 1, and 4–7, in DKC6575 flours, and for peaks 1 and 6 in PR33P67 flours.

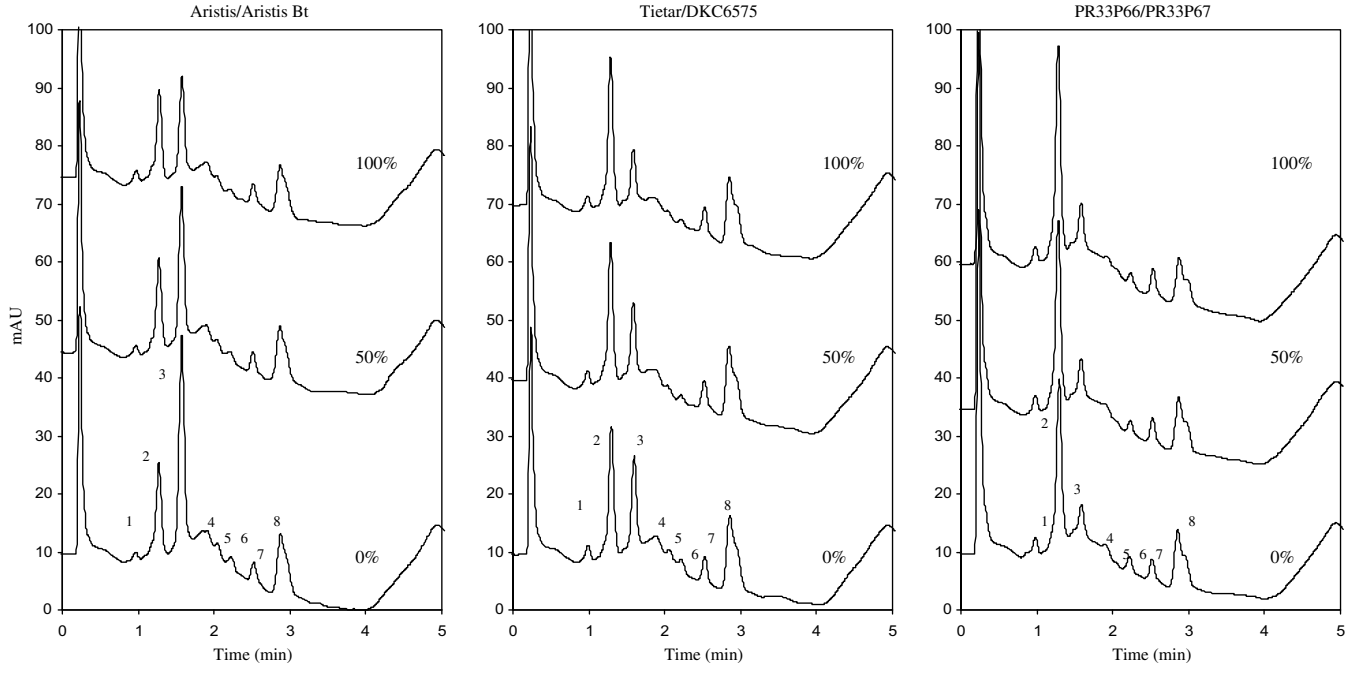

Fig. 1. Perfusion RP-HPLC chromatograms of maize proteins from flour mixtures of different cultivars with different percentage of transgenic Bt maize.

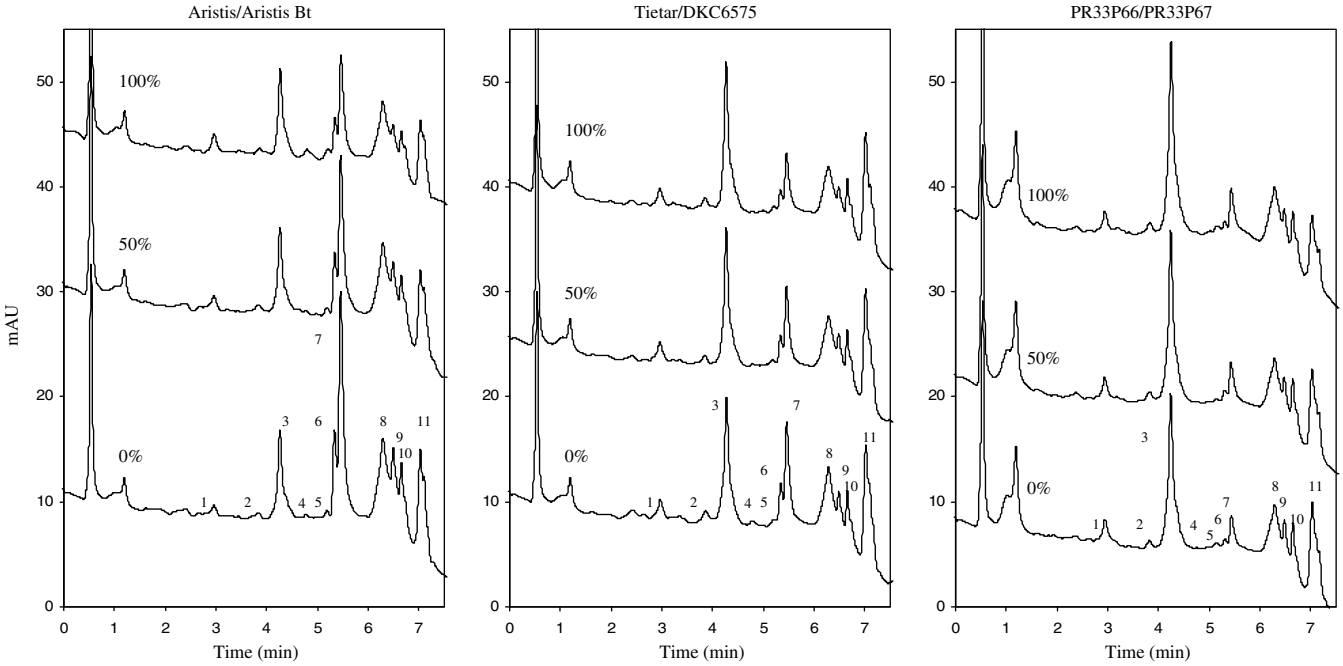

Fig. 2. Monolithic RP-HPLC chromatograms of maize proteins from flour mixtures of different cultivars with different percentage of transgenic Bt maize.

The chromatograms obtained for the monolithic RP-HPLC analysis of control and transgenic Bt maize lines plus a mixture of control and transgenic Bt maize (50%) of each one of the lines studied are shown in Fig. 2. Maize proteins were separated in eleven peaks and were characterized by the presence of a big peak (peak 3) at a retention time of 4.3 min, a duplet of peaks partially resolved (peaks 6 and 7) in the middle of the chromatograms, and a triplet of peaks (peaks 8, 9 and 10) together with peak 11 at the end of the chromatograms. The signals for peaks 6 and 7 decreased at increasing the percentage of transgenic line in the flour mixtures containing Aristis Bt and DKC6575. On the other hand, the signal for peak 3 was also smaller than that found for peak 7 in Aristis and Aristis Bt. However, in Tietar (the non-Bt transgenic maize variety of DKC6575) and DKC6575 and in PR33P66 (the non-Bt transgenic maize variety of PR33P67) and PR33P67 maize lines, the signal for peak 3 was always higher than that observed for peak 7. The results of the one-way ANOVA revealed significant differences between the different calibration flour mixtures of transgenic and non-transgenic maize lines, specifically, for peaks 1, and 3–11 in Aristis and Aristis Bt, for peaks 3, 5–7, and 9 in Tietar and DKC6575, and for peaks 3, 5, 7–9, and 11 in PR33P66 and PR33P67. Good reproducibility was achieved in both columns. For duplicate injection, the RSD was better than 1% in peak area and in retention times, whereas for three replication samples, the RSD values were below 1% in retention time and 3–4% in peak area (Rodríguez-Nogales et al., 2007).

The total protein content for the transgenic cultivars PR33P67 (7.86%), DKC6575 (7.81%), and Aristis Bt (7.64%) was equivalent to their control lines (PR33P66, 8.01%; Tietar, 7.89%; Aristis, 7.69%, respectively). However, we observed differences on protein profiles analyzed by perfusion and monolithic RP-HPLC among Bt-maize and control lines. Since both cultivars (transgenic and control lines) were grown under similar agronomic conditions, the differences observed on chromatographic profiles could be attributed to the genetic modifications. Indeed, different examples have shown that genetic modifications in a food not detected by standard nutritional analyses (protein, fat, ash, carbohydrates, calories and amino acid) could be observed using other analytical methodologies (FAO/WHO, 2000).

Although the differences observed in the means of some variables among Bt-maize flour mixtures may reveal some interesting features and enabling the differentiation among the groups of samples with different percentages of Bt-maize, the amount and complexity of these data make the use of PCA and LDA necessary to fully extract the wealth of this information.

Data for relative peak areas from perfusion RP-HPLC analysis of the flour mixtures were analyzed using principal component analysis (PCA). The PCA extracted two components (with eigenvalues greater than one) for Aristis Bt and DKC6575, and only one component for PR33P67. These components accounted for 76.2%, 66.9% and 69.5% of the variability of the original data for Aristis Bt, DKC6575, and PR33P67, respectively. The first component (PC1) discriminated samples from the relative areas of peaks 2 and 3 for Aristis Bt and DKC6575 and peaks 2 and 7 for PR33P67. Relative area for peaks 5 and 4 and 1 and 7 were the variables that showed the highest weight on the second component (PC2) for Aristis Bt and DKC6575, respectively.

Fig. 3 shows a PCA bi-plot of PC1 versus PC2 with the factors (relative area of the eight peaks) and the samples of flour blends of Aristis and Aristis Bt analyzed by perfusion and monolithic RP-HPLC. These plots provide information regarding patterns in the sample set. For perfusion RP-HPLC, peaks 2 and 3 were the variables with more importance for PC1 with eigenvectors of 0.39 and −0.38, respectively. The group of the flours with the lowest percentages of transgenic Bt maize were situated on the left of the origin (negative values of PC1) presenting high values of the relative area for peak 3. The samples of flours with the highest percentages of Aristis Bt were situated to the right of the origin (positive values of PC1) and they were characterized by presenting high area percentages for peaks 2 and 1. These results suggest that peaks 2 and 3 are very important to differentiate among samples on the basis of their percentage in transgenic Bt maize.

When PCA was applied to the monolithic RP-HPLC data, four PC's were extracted for each line accounting for 78.5% of the total variability in Aristis Bt, 78.9% in DKC6575, and 71.1% in PR33P67. For PC1, relative area for peaks 6 and 7, 3 and 9, and 3 and 7 presented the highest eigenvectors in Aristis Bt, DKC6575, and PR33P67, respectively. Fig. 3 shows the distribution of the samples

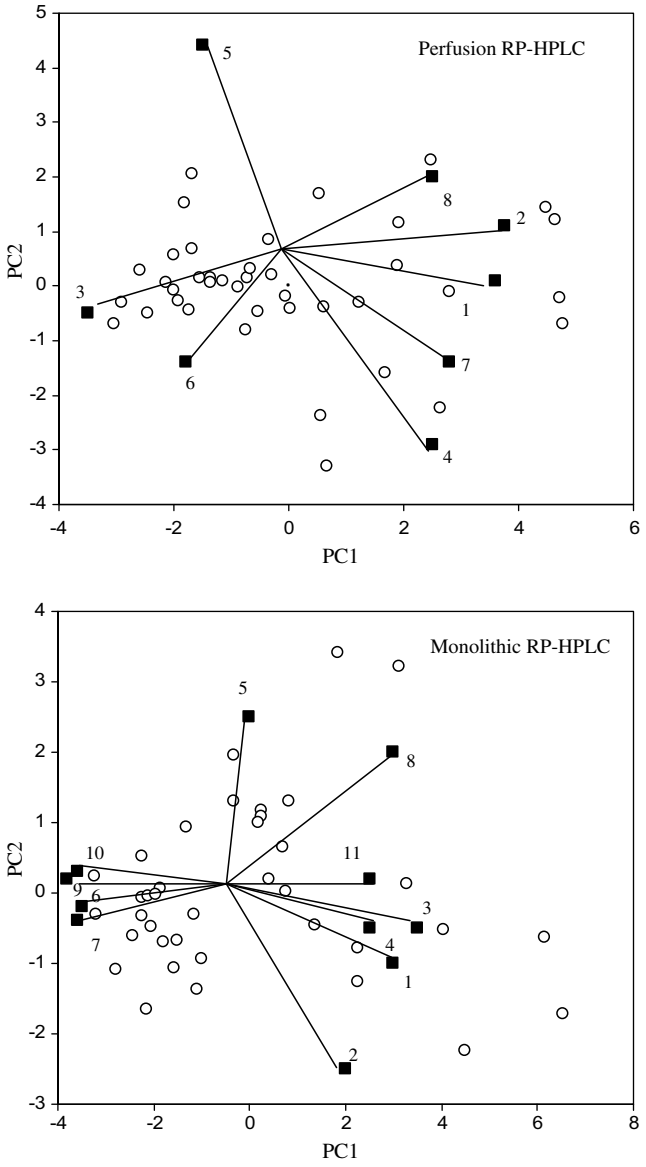

Fig. 3. Biplot of the scores and loadings for the Aristis Bt in the plane defined by the two first component principal functions for perfusion and monolithic RP-HPLC. The numbers in the plots correspond with the peak number.

of flour mixtures of Aristis and Aristis Bt in the space of the PC1 and PC2. Samples were distributed along PC1 on the basis of the content in Bt maize. The samples with the least percentages of transgenic maize presented negative values for PC1 (high values of relative area for peaks 6, 7, 9, and 10). On the other hand, the mixtures with the highest percentages of Bt maize were located on the positive side of PC1 (high values of relative area for peaks 3, 1, 4, and 11). The factors are positively correlated if they are situated on the same side of the origin and negatively correlated if they lie on the opposite side, thus, relative area for peaks 6, 7, 9, and 10 for Aristis and Aristis Bt were located very close to each other in the plot indicating that they are closely related. Similar results were found for the PCA of the other two Bt-maize varieties. Showing the PCA results, it was expected that the perfusion and monolithic RP-HPLC chromatographic profiles could be useful to discriminate the flour mixtures according to the content in Bt maize.

Then, a stepwise discriminant analysis was applied to perfusion and monolithic RP-HPLC data using the forward procedure. Fig. 4 shows, as example, the plot of the flour mixtures with different concentrations of Aristis Bt analyzed by perfusion and monolithic RP-HPLC and defined by the two first discriminant functions. Very good and clear separation among the samples according to their percentages in transgenic Bt maize was observed. The relative area for peaks 2, 3 and 7 for perfusion RP-HPLC, and peaks 6, 7, and 11 for monolithic RP-HPLC were the most important variables for the differentiation among Aristis Bt flour mixtures. In both chromatographic methods, the maize flours with the highest and the lowest

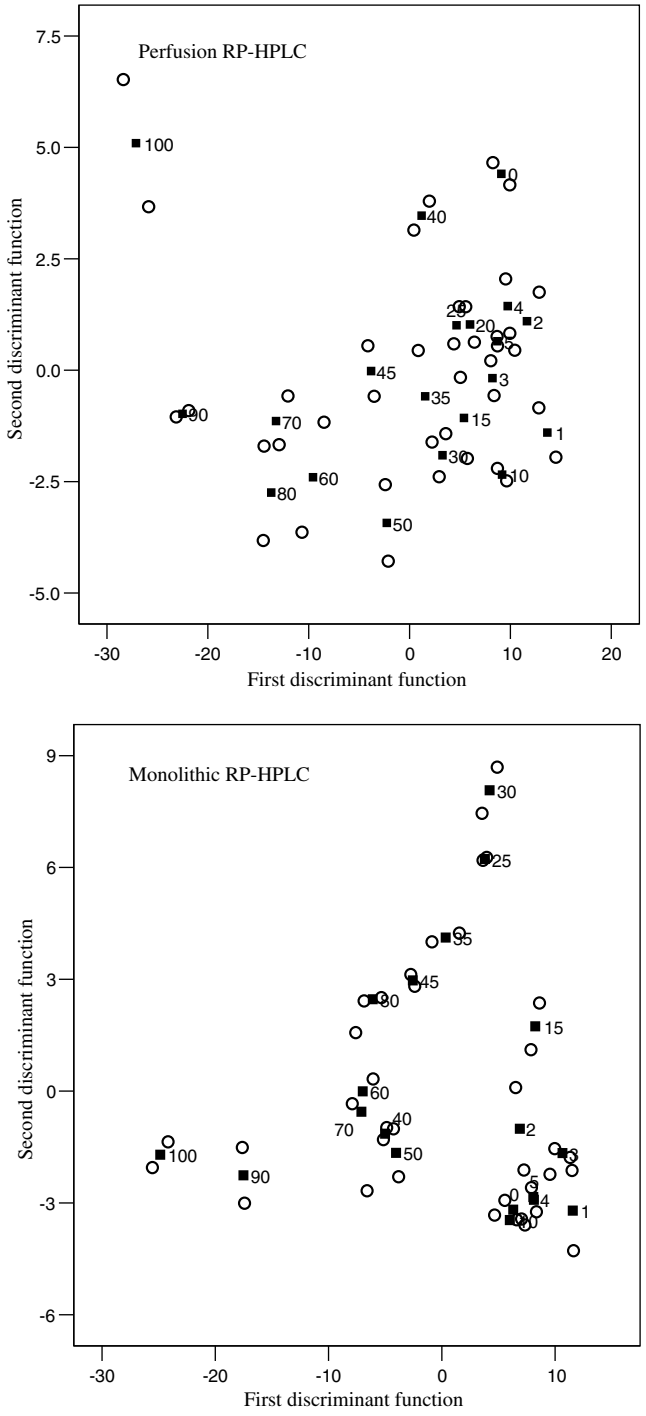

Fig. 4. Distribution of flours with different percentage of Aristis Bt analyzed by perfusion and monolithic RP-HPLC in the plane defined by the two first discriminant functions. The numbers in the plots correspond with the percentage of transgenic Bt maize in the maize flour mixture.

concentrations of Bt maize were located in the negative and positive side of the first discriminant function, respectively. A percentage of correct classification for perfusion and monolithic data of 100% was achieved. The prediction capability of the model was evaluated by cross-validation achieving a percentage of prediction of 92.0% and 90.1%, for perfusion and monolithic RP-HPLC, respectively. For DKC6575 and PR33P67 Bt maize cultivars, a 100% of correct classification was also achieved by both chromatographic methods. Percentages of prediction of 90.2% and 87.3% (for perfusion RP-HPLC), and 86.3% and 85.2% (for monolithic RP-HPLC), were obtained, for DKC6575 and PR33P67, respectively.

3.2. Estimation of the percentage of transgenic Bt maize in maize flour mixtures

With the aim of predicting the percentage of transgenic Bt maize in flour mixtures, several chemometric approaches (LR, PLS, PCR, and MLR) were evaluated. The error of calibration (SEC) and the square correlation coefficient (R^2) were used to compare models for accuracy and robustness. SEC is an indicator of the average error in the analysis for each component and how well the model fits to the data. The preferred model was the one that produced the lowest SEC and the R^2 value closest to one.

Table 1 summarizes the results of the models developed with the perfusion RP-HPLC data. Linear regressions were applied to the calibration samples, using as Y-calibration variables relative areas for peaks 2 and 3 for Aristis Bt and DKC6575, and relative areas for peaks 2 and 7 for PR33P67. The best results were found with the ratio of relative areas for peaks 3 and 2, for Aristis Bt and DKC6575, and for the relative area of peak 2 for PR33P67, achieving R^2 values higher than 0.98 and SEC values lower than 5.14%. Similar results were found for the detection limit, showing the lowest values using as Y-variables the ratio of relative areas for peaks 3 and 2, for Aristis Bt and DKC6575, and for the relative area of peak 7 for PR33P67. Multivariate regressions (PLS, PCR, and MLR) were also tested achieving, in general, higher values for R^2 and lower values for SEC than those found for the linear regressions. The best calibrations (with the lowest values of SEC) were found when applying PCR to the Aristis Bt and DKC6575 data set and MLR regression to the PR33P67 data set. These models were used to predict the percentage of transgenic maize in different flour mixtures prepared with known quantities of Bt maize (Table 1). In this case, error of prediction (SEP) was chosen as an optimizing criterion to validate the built calibrations. Better results were achieved for multivariate regressions (with SEP ranging from 2.58% to 6.12%) especially with MLR regressions achieving SEP values of 5.25%, 2.34%, and 4.68% for Aristis Bt, DKC6575, and PR33P67, respectively.

The results of the linear and multivariate models applied to the prediction of the percentage of transgenic Bt maize in flour mixtures analyzed by monolithic RP-HPLC are summarized in Table 2. Regarding the linear regressions, peaks 6 and 7 for Aristis Bt, peaks 6, 3, and 7 for DKC6575, and peaks 3 and 9 for PR33P67 were employed as calibration variables. With exception of the model built with the ratio between peak 6 and 7 for Aristis Bt, the R^2 values were higher than 0.91 and yielded good correlations between the observed and calculated percentage of Bt maize. The values of SEC were worse than those found in perfusion RP-HPLC.

Additionally, PLS, PCR and MLR regressions were applied to the monolithic RP-HPC data set. For Aristis Bt, the best calibration model was obtained using PCR achieving a SEC value of 6.84% and a R^2 value of 0.9735. The values of SEC and R^2 were similar in DKC6575 data set, independently of the multivariate regression assayed, oscillating between 7.67% (MLR regression) and 7.83% (PCR regression) for SEC, and between 0.9553 (MLR regression) and 0.9725 (PCR regression) for R^2. In the case of PR33P67, the best results were found for PLS1 and PCR with the same value of R^2 (0.9930) and similar values of SEC (4.37% for PLS1, and 4.43% for PCR). The limits of detection of the models were higher than those obtained with the perfusion RP-HPLC data. According to the values of SEP, for the three Bt maize cultivars, and similarly to perfusion, MLR regressions were superior at modeling percentages of Bt maize.

Table 1
Statistical parameters of validation and prediction of the regression models for the transgenic maize lines analyzed by perfusion RP-HPLC

Regression models	Aristis Bt						DKC6575						PR33P67					
	P2	P3	P3/P2	PLS-1	PCR	MLR	P2	P3	P3/P2	PLS-1	PCR	MLR	P2	P7	P7/P2	PLS-1	PCR	MLR
R^2 [a]	0.968	0.985	0.987	0.987	0.987	0.973	0.979	0.988	0.988	0.955	0.955	0.990	0.981	0.943	0.967	0.985	0.985	0.986
SEC [b]	7.78	5.41	4.96	4.27	4.25	4.66	6.43	4.83	4.80	3.46	3.20	3.50	5.14	7.88	5.95	4.93	4.91	4.06
DL [c]	0.70	0.85	0.63				0.57	0.39	0.17				0.48	0.30	0.68			
SEP [d]	5.93	5.33	6.02	5.27	6.12	5.25	3.86	3.84	3.46	2.58	3.20	2.34	5.09	11.98	10.71	4.93	4.92	4.68

[a] Coefficient of determination of the model.
[b] Standard error of calibration (in %).
[c] Detection limit calculated as 3 SEC/m where m is the slope of the model (in g of transgenic maize/100 g of flour).
[d] Standard error of prediction (in%).

Table 2
Statistical parameters of validation and prediction of the regression models for the transgenic maize lines analyzed by monolithic RP-HPLC

Regression models	Aristis Bt						DKC6575						PR33P67					
	P6	P7	P6/P7	PLS-1	PCR	MLR	P6	P6/P3	P7/P3	PLS-1	PCR	MLR	P3	P9	P3/P9	PLS-1	PCR	MLR
R^2 [a]	0.914	0.932	0.257	0.973	0.974	0.961	0.932	0.942	0.931	0.971	0.973	0.955	0.934	0.972	0.963	0.993	0.993	0.978
SEC [b]	13.17	11.78	31.37	7.29	6.84	7.86	10.56	9.73	10.59	7.73	7.83	7.67	11.59	7.58	8.76	4.37	4.43	5.49
DL [c]	1.29	1.03	2.23				1.27	1.41	1.63				1.95	1.81	1.35			
SEP [d]	14.06	6.73	34.45	7.80	6.84	5.71	7.96	7.68	7.10	7.73	7.84	5.71	6.43	5.49	5.40	4.38	4.43	3.84

[a] Coefficient of determination of the model.
[b] Standard error of calibration (in %).
[c] Detection limit calculated as 3 (SEC/m where m is the slope of the model (in g of transgenic maize/100 g of flour).
[d] Standard error of prediction (in %).

In conclusion, perfusion and monolithic RP-HPLC maize proteins presented appropriate information to classify maize flour mixtures containing Bt maize based on the results found with the principal component and linear discriminant analysis. Furthermore, perfusion and monolithic RP-HPLC coupled with univariate and multivariate regressions were successfully used to develop calibration models to predict the percentage of transgenic Bt maize in flours achieving better calibration and prediction power using multivariate regressions. According to the SEP values, the best results were found with the application of MLR regression models to the perfusion RP-HPLC data in Aristis Bt and DKC6575 flour mixtures, and to the monolithic RP-HPLC data in PR33P67 flour mixtures with SEP values of 5.27, 2.34, and 3.84%, respectively. Despite these methodologies constitute a first approach and further investigations are needed, they enabled significant reductions in analysis time, price and complexity in comparison with the established (PCR)-based methods and immuno-assay.

Acknowledgements

The authors thank the Ministerio de Educación y Ciencia (Spain) for project AGL2005-05320-C02-01. M.C. García thanks the Comunidad Autónoma de Madrid for project CAM-UAH2005/010.

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