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Analysis of Chiral Amino Acids in Conventional and Transgenic Maize

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In this work, a new chiral micellar electrokinetic chromatography with laser-induced fluorescence detection (chiral-MEKC-LIF) method is proposed to identify and quantify D- and L-amino acids in three lines of transgenic maize and their corresponding nontransgenic parental lines grown under identical conditions. The optimized procedure includes amino acids extraction, derivatization with FITC and chiral-MEKC-LIF separation in a background electrolyte composed of 100 mM sodium tetraborate, 80 mM SDS, and 20 mM β-CD at pH 10.0. The D- and L-forms of Arg, Ser, Ala, Glu, and Asp, corresponding to the majority amino acids usually found in maize, are separated in less than 25 min with efficiencies up to 890 000 plates/m and high sensitivity (i.e., LODs as low as 160 nM were obtained for D-Arg for a signal-to-noise ratio of three), allowing the detection of 1% D-Arg in the presence of 99% of its opposite enantiomer. Using this method, different D-amino acids are detected in all investigated maize samples providing the reproducible quantification of the D-enantiomeric excess (% D-aa) for each amino acid calculated as % D-aa = 100D-aa/(D-aa + L-aa). Thus, significant differences were observed among the % D-aa values for the different conventional varieties (Aristis, Tietar, and PR33P66 maize) as could be expected from their natural variability. More interestingly, comparing each conventional maize with its corresponding transgenic line, very similar % D-aa values were obtained for one of the studied maize couples (Tietar vs Tietar-Bt) what could be presented as a new proof of their substantial equivalence. However, significant differences in the % D-aa values were observed for the other lines of maize studied. It is concluded that enantioselective procedures can open new perspectives in the study of transgenic organisms in order to corroborate (or not) the equivalence with their conventional counterparts.

The use of genetically modified crops has seen a great increase in the agriculture and food industry. Thus, genetic engineering is used to improve some characteristic of the original crop such as its resistance to plagues, pesticides, and extreme environmental conditions, to provide better nutritional properties.[1,2]

In spite of the aforementioned advantages, the use of genetically modified organisms (GMOs) in agriculture and food science is not commonly accepted in many countries.[3] Thus, different ecologist groups support international campaigns against the use of this kind of products, claiming their negative impact on human health, on the environment, or both.[4] On the other side, biotechnology companies point out the potential benefits for the agricultural and food industries and for the general public as well as the lack of scientific evidence of their threat for the human health.[5] This situation has led to the implementation in some countries of different regulations regarding the development, growing, and commercialization of genetically modified products.[6,7]

As a consequence, at present, research on how the different genetic modifications can impact on the chemical composition of these products is of great interest. In this regard, a mixed strategy is usually carried out by the biotechnology companies and regulatory laboratories to assess the safety equivalence between transgenic and parental nontransgenic organisms (maize or soy, for instance) including field investigations, animal nutrition, and basic chemical composition studies. However, it has repeatedly been demonstrated that these strategies are not very useful to detect unexpected modifications in GMOs.[8] Moreover, the mentioned strategies devised to study the nutritional, safety assessment, and chemical composition of the first GMOs generation will be much more difficult to apply to the coming new generation of GMOs in which significant changes in other constituents have been deliberately introduced (e.g., increased fatty acids or amino acid content, polyphenols, vitamins, and reduced undesirable constituents), requiring the development of more powerful and informative analytical procedures.[9−11]

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(1) Koziel, M. G.; Beland, G. L.; Bowman, C.; Carozzi, N. B.; Crenshaw, R.; Crossland, L.; Dawson, J.; Desai, N.; Hill, M.; Kadwell, S.; Launis, K.; Lewis, K.; Maddox, D.; McPherson, K.; Meghji, M. R.; Merlin, E.; Rhodes, R.; Warren, G. W.; Wright, M.; Evola, S. V. *Biotechnology* **1993**, *11*, 194−200.
(2) Gao, A. G.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, H.; Stark, D. H.; Shah, D. H.; Liang, J.; Tommens, C. M. *Nat. Biotechnol.* **2000**, *18*, 1307−1310.
(3) García-Cañas, V.; Cifuentes, A.; González, R. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 425−436.
(4) http://www.greenpeace.org/international/campaigns/genetic-engineering Last access, 03-05-2007.
(5) http://www.monsanto.com Last access, 03-05-2007.
(6) Regulation (EC) 1829/2003 of the European Parliament and of the Council of September 22, 2003.
(7) Regulation (EC) 1830/2003 of the European Parliament and of the Council of September 22, 2003.).
(8) National Research Council and Institute of Medicine. *Safety of Genetically Engineered Foods*; The National Academies Press; Washington DC, 2004.
(9) Flachowsky, G.; Chesson, A.; Aulrich, K. *Arch. Anim. Nutr.* **2005**, *1*, 1−40.
(10) Simó, C.; Rizzi, A.; Barbas, C.; Cifuentes, A. *Electrophoresis* **2005**, *26*, 1432−1441.
(11) Simó, C.; Barbas, C.; Cifuentes, A. *Electrophoresis* **2005**, *26*, 1306−1318.

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In this work, an original analytical strategy is proposed able to provide new information on the composition of transgenic crops based on the quantification of chiral amino acids in several transgenic maize varieties and their corresponding nontransgenic parental lines grown under identical conditions. It is well-known that chiral analysis of amino acids is a remarkable methodology that can provide important information allowing a better understanding of the chemistry, nutrition, safety, microbiology, metabolic pathways, etc., of the organisms in which these molecules are found.[12−13] Thus, it has been demonstrated that the profile of D/L-amino acids (D/L-aa) can be an useful tool for detailed characterization of different samples, not only to evaluate differences in their composition but also to assess food adulteration,[12,14−16] food quality,[17] or digestibility and nutritional value of foods.[12] For instance, D-amino acids seem to be involved in aging and disease in humans.[18,19] On the other hand, as indicated by Friedman,[12] racemization impairs digestibility and nutritional quality, with some D-amino acids both beneficial and deleterious. For instance, although D-phenylalanine in an all amino acid diet is utilized as a nutritional source of L-phenylalanine, high concentrations of D-tyrosine in such diets inhibit the growth of mice. The wide variation in the utilization of D-amino acids is illustrated by the fact that whereas D-methionine is largely utilized as a nutritional source of the L-isomer, D-lysine is totally devoid of any nutritional value.[12]

However, in spite of this interest, to our knowledge, no method has been developed so far to separate and identify the main L- and D-amino acids found in transgenic crops.

HPLC and GC are the separation techniques usually selected to analyze chiral amino acids in many different matrixes due to the remarkable results that they can provide.[20−23] However, these techniques present some drawbacks since they may need expensive chiral columns, laborious sample pretreatments, relatively lengthy analysis time (usually ∼50 min),[24−25] complicated derivatizing procedures, or impossibility to detect some nonderivatizable amino acids.[24,25] For instance, the purification and derivatization of amino acids prior to GC analysis can require more than 10 different steps,[24,25] and usually the procedure does not work for some basic amino acids such as arginine.[24,25] These disadvantages have promoted the development of new analytical procedures that could overcome these limitations. In this sense, capillary electrophoresis (CE) has already demonstrated to be well suited for the analysis of chiral compounds providing fast, efficient, reproducible, and low-cost separations.[26−31]

The goal of this work is, therefore, to carry out the profiling of the main D- and L-amino acids that can be found in transgenic and nontransgenic maize. To do this, a new analytical CE method is developed that combines micellar electrokinetic chromatography (MEKC) with a chiral selector and laser-induced fluorescence (LIF) to analyze a group of L- and D-aa in maize. The developed chiral-MEKC-LIF method is fast and reproducible and allows the quantification of the enantiomers with good efficiency and sensitivity.

EXPERIMENTAL SECTION

Chemicals. All chemicals were of analytical reagent grade and used as received. β-cyclodextrin (β-CD) from Fluka Buchs (St. Louis, MO) was used as chiral selector for the MEKC running buffer together with sodium dodecyl sulfate (SDS) from Acros Organics and boric acid from Riedel-De Häen (Seelze, Germany). A water solution containing 5 mol/L sodium hydroxide from Panreac Quimica S.A. (Barcelona, Spain) was used to adjust the pH of the buffers, and 0.1 mol/L NaOH was used to rinse the capillary. The buffer was stored at 4 °C and warmed at room temperature before use. Water was purified by using a Milli-Q system (Millipore, Bedford, MA). Fluorescein isothiocyanate (FITC; from Fluka Buchs, St. Louis, MO) was dissolved in acetone analytical grade (Merck, Darmstadt, Germany). Standard L- and D-amino acids were from Sigma (St. Louis MO). Trichloroacetic acid (TCA; from Merck) and sodium deoxycholate (minimum 97%, from Sigma, Madrid, Spain) were used for amino acids extraction.

Maize Samples: Obtention and Characterization. The investigated varieties of conventional and transgenic maize were obtained from a field assay carried out in Estación Experimental Agrícola Mas Badia in Tallada d'Empordà (Girona, Spain) using commercial varieties. Namely, 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), and PR33P66 maize (wild type and its Bt transgenic variety) were grown under the same field conditions and investigated in this work. As mentioned, in all cases, the maize genetic modification consisted of introducing a new Cry-type gene able to synthesize a new protein (protein Bt) that acts as insecticide obtaining a maize resistant to some worm plagues.

The transgenic and nontransgenic nature of all these maize samples was confirmed based on their DNA using an analytical procedure developed in our laboratory and described elsewhere.[32−36]

(12) Friedman, M. *J. Agric. Food Chem.* **1999**, *47*, 3457−3479.
(13) Armstrong, D. W.; Chang, C. D.; Li, W. L. *J. Agric. Food Chem.* **1990**, *38*, 1674−1677.
(14) Simó, C.; Barbas, C.; Cifuentes, A. *Electrophoresis* **2003**, *24*, 2431−2441.
(15) Simó, C.; Barbas, C.; Cifuentes, A. *J. Agric. Food Chem.* **2002**, *50*, 5288−5293.
(16) Simó, C.; Martín-Álvarez, P. J.; Barbas, C.; Cifuentes, A. *Electrophoresis* **2004**, *25*, 2885−2891.
(17) Bednar, P.; Aturki, Z.; Stransky, Z.; Fanali, S. *Electrophoresis* **2001**, *22*, 2129−2135.
(18) Helfman, P. M.; Bada, J. L.; Zigler, J. L. *Nature* **1977**, *268*, 71−73.
(19) Mann, E. H.; Sandhouse, M. E.; Burg, J.; Fisher, G. H. *Science* **1983**, *220*, 1407−1408.14,15.
(20) Rubio-Barroso, S.; Santos-Delgado, M. J.; Martin-Olivar, C.; Palo-Diez, L. M. *J. Dairy Sci.* **2006**, *89*, 82−89.
(21) Bruckner, H.; Westhauser, T. *Amino Acids* **2003**, *24*, 43−55.
(22) Yokoyama, T.; Kan-No, N.; Ogata, T.; Kotaki, Y.; Sato, M.; Nagahisa, E. *Biosci. Bitechnol. Biochem.* **2003**, *67*, 388−392.
(23) Yokoyama, T.; Tanaka, Y.; Sato, M.; Kan-No, N.; Nakano, T.; Yamaguchi, T.; Nagahisa, E. *Fish. Sci.* **2005**, *71*, 924−930.
(24) Erbe, T.; Brückner, H. *Z. Lebensm. Unters. Forsch. A* **1998**, *207*, 400−409.
(25) Erbe, T.; Brückner, H. *Eur. Food Res. Technol.* **2000**, *211*, 6−12.
(26) Chankvetadze, B. In: *Capillary Electrophoresis in Chiral Analysis*; John Wiley & Sons: Chichester, England, 1997.
(27) Hernández-Borges, J.; Rodríguez-Delgado, M. A.; García-Montelongo, F. J.; Cifuentes, A. *Electrophoresis* **2005**, *26*, 3799−3813.
(28) Cifuentes, A. *Electrophoresis* **2006**, *27*, 283−303.
(29) Gassman, E.; Kuo, J. E.; Zare, R. N. *Science* **1985**, *230*, 813−815.
(30) Fanali, S. *J. Chromatogr.* **1989**, *474*, 441−446.
(31) Yoshinaga, M.; Asano, S.; Tanaka, M.; Shono, T. *Anal. Sci.* **1991**, *7*, 257−260.
(32) García-Cañas, V.; González, R.; Cifuentes, A. *J. Agric. Food Chem.* **2002**, *50*, 4497−4502.
(33) García-Cañas, V.; González, R.; Cifuentes, A. *J. Agric. Food Chem.* **2002**, *50*, 1016−1021.
(34) García-Cañas, V.; González, R.; Cifuentes, A. *J. Sep. Sci.* **2002**, *25*, 577−583.

Extraction of Amino Acids from Maize. For the extraction of free amino acids, the maize kernels were ground and the obtained flour was treated as follows:[37] 1 mL of 0.37 M TCA was added to 100 mg of maize flour. The mixture was vortexed for 2 min. Then, 0.2 mL of 3.6 mM sodium deoxycholate was added to make possible the protein precipitation. The mixture was left to stand for 10 min, and then, a 15-min centrifugation at 3000*g* was carried out. The supernatant was separated and submitted to another 1-h centrifugation process at 4500*g*. Again, the supernatant was collected and employed for the derivatization procedure described below.

Derivatization Procedure. The FITC derivatization procedure was optimized as described below. The selected procedure consisted of mixing an aliquot of 625 μL of the maize extract with 675 μL of water and 1.5 mL of 355 mM sodium tetraborate buffer at pH 10. This mixture was adjusted to pH 10 by adding 1 M sodium hydroxide. For amino acid standards derivatization, a 625-μL aliquot was mixed with 1375 μL of water and 7 mL of 355 mM sodium tetraborate buffer at pH 10. Again, this mixture was adjusted to pH 10 by adding 1 M sodium hydroxide, and water was added until a final volume equal to 10 mL; 250 μL of these final solutions were mixed with 50 μL of a 3.75 mM FITC solution in acetone. The reaction took place overnight in darkness at room temperature. After derivatization, samples were diluted with water prior to their injection in the MEKC-LIF as described below.

MEKC-LIF Conditions. All analyses of L- and D-amino acids were carried out in triplicate using a P/ACE 2100 CE apparatus from Beckman Instruments (Fullerton, CA) equipped with an Ar^+ laser at 488 (excitation wavelength) and 520 nm (emission wavelength), also from Beckman Instruments, to detect FITC-amino acids. Bare fused-silica capillary was purchased from Composite Metal Services (Worcester, England). The capillary dimensions were 50 cm of detection length, 57 cm of total length, and 50-μm i.d. and was thermostated at 30.0 °C. Injections were made at the anodic end using N_2 at 0.5 psi (3.45 kPa) for 3 s (approximate hydrodynamic injection of 3.48 nL), and the applied voltage was +20 kV. The P/ACE 2100 CE instrument was controlled by a PC running the System GOLD software from Beckman.

Before first use, new capillaries were preconditioned by rinsing with 0.1 M NaOH for 30 min. The washing protocol between runs was optimized to obtain adequate repeatability, selecting the following conditions: at the beginning of each run, the capillary was rinsed with 0.1 M NaOH for 1 min, followed by 2 min with Milli-Q water, and then equilibrated for 5 min with the optimized running buffer (100 mM sodium tetraborate, 80 mM SDS, and 20 mM β-CD at pH 10.0, vide infra). At the end of the day, the capillary was rinsed with Milli-Q water for 10 min, and then nitrogen was passed for 2 min.

Statistical Data Analysis. Several statistical methods were used for data analysis. Namely, regression analysis was used for the calibration curves; one-way analysis of variance (ANOVA) was used to determine if there were significant differences between maize samples; and the least significant differences (LSD) test was applied for means comparisons. A two-sample *t*-test was applied to determine if there were significant differences between the different couples of maize samples (conventional vs transgenic). STATISTICA program for Windows (version 7.1, Statsoft, Inc., 2005, www.statsoft.com) run on a personal computer was used for data processing.

Table 1. Volumes of Sample (Maize Extract) and Derivatizing FITC Solution Tested To Optimize the Derivatization Yield Determined as Amino Acid Signal (S_{AA})/FITC Signal (S_{FITC}) Ratio

exp	sample (μL)[a]	FITC solution (μL)[b]	(S_{AA}/S_{FITC})[c]
1	100	200	0.093
2	150	150	0.106
3	200	100	0.145
4	250	50	0.281
5	400	100	0.183
6	400	50	0.192

[a] Aristis sample, 2.1 mg/mL. [b] FITC concentration, 3.75 mM FITC in acetone. [c] Ratio of amino acid signal (S_{AA})/FITC signal (S_{FITC}).

RESULTS AND DISCUSSION

Derivatization Procedure. Previous to the optimization of the chiral-MEKC-LIF method, the derivatization procedure was studied in order to achieve the maximum amino acid signal with the lowest number of interferences from the derivatizing reagent FITC, which is known to produce a high number of interfering fluorescent compounds.[15] To do so, different volumes of the conventional Aristis sample (obtained using the amino acids extraction protocol described above) and FITC solutions were mixed at five different ratios to carry out the derivatization reaction as indicated in Table 1. To monitor the results of this initial optimization, a published chiral-MECK-LIF method employed to analyze amino acids in different microalgae samples was used.[37] The derivatization conditions that gave higher amino acid signals (S_{AA}) together with lower FITC signal (S_{FITC}), that is, better derivatization yields, were selected. As can be observed in Table 1, the higher the proportion of sample in the mixture, while maintaining the total reaction volume at 300 μL, the higher the S_{AA}/S_{FITC} ratio. Higher sample volume/FITC volume ratios for this reaction did not provide better yields, as can be deduced from experiment 6 in Table 1. Thus, the reaction mixture containing 250 μL of sample and 50 μL of FITC solution was finally selected. The repeatability of the whole extraction−derivatization process was then checked. Results from four replicates confirmed the high repeatability of the procedure since no significant variation in the electropherogram profile was observed.

MEKC-LIF Optimization and Figures of Merit. Once the derivatization procedure was optimized, the MEKC-LIF separation conditions were further improved. To perform this study, a mixture of 20 different chiral amino acids, namely, D/L-Arg, D/L-Leu, D/L-Asn, D/L-Pro, D/L-Phe, D/L-Ser, D/L-Ala, D/L-His, D/L-Glu, and D/L-Asp, was used. This selection was done considering the impossibility to find ab initio information on amino acid content of transgenic maize in the literature and, therefore, trying to cover a profile of amino acids as large as possible (i.e., including positively charged, neutral, and negatively charged amino acids).

(35) García-Cañas, V.; Cifuentes, A.; González, R. *Anal. Chem.* **2004**, *76*, 2306−2313.
(36) García-Cañas, V.; González, R.; Cifuentes, A. *Electrophoresis* **2004**, *25*, 2219−2226.
(37) Herrero, M.; Ibáñez, E.; Fanali, S.; Cifuentes, A. *Electrophoresis*. In press.

Under these conditions, the published background electrolyte (BGE) developed to carry out the separation of chiral amino acids from algae was tested.[37] Namely, a BGE composed of 100 mM sodium tetraborate buffer with 30 mM SDS and 20 mM β-CD at pH 9.70 was used, providing the electropherogram shown in Figure 1A. As can be seen, under these conditions, no separation of the neutral amino acids migrating between 12.5 and 15.0 min was obtained. Next, different sodium tetraborate concentrations (60, 80, 100, and 120 mM), SDS concentrations (20, 30, 40, 60, 80, and 100 mM), pHs (9.4, 9.7, 10.0, and 10.3), and β-CD concentrations (10, 15, and 20 mM) were tested. Although the running buffer containing lower sodium tetraborate concentrations produced faster separations, the resolution obtained using the original concentration (i.e., 100 mM) was still better, although at the expense of a higher analysis time. Using higher sodium tetraborate concentrations (120 mM), no improvement of the initial resolution was observed while longer analysis times were obtained. Therefore, the initial sodium tetraborate concentration was maintained (100 mM). Next, different SDS concentrations were assayed (20, 40, 60, 80, and 100 mM) while maintaining the rest of conditions (100 mM sodium tetraborate, 20 mM β-CD, pH 9.7) constant. From the results obtained, it could be observed that an increase of the SDS concentration produced improvements in the resolution of the different peaks up to 80 mM SDS. Concentrations above this value did not provide any further improvement in the central region of the separation, while a lower resolution was observed for the first two migrating peaks (i.e., D- and L-Arg). Besides, the analysis time increased with the SDS concentration. Therefore, 80 mM SDS was chosen as the optimum value to carry out the rest of the experiments. Once the background electrolyte composition was selected, different pH values were tested (namely, 9.4, 9.7, 10.0, and 10.3) while keeping the rest of conditions constant, that is, 100 mM sodium tetraborate, 80 mM SDS, and 20 mM β-CD. Since an improvement on the separation resolution was observed at pH 10.0, compared to the other three pHs, at reasonable analysis time, this value was finally selected. Finally, different β-CD concentrations (10, 15, and 20 mM) were assayed showing that, in general, the lower the concentration of β-CD the worse the separation resolution between the enantiomers, and therefore, 20 mM was selected.

Thus, the selected background electrolyte composition was 100 mM sodium tetraborate, 80 mM SDS, and 20 mM β-CD at pH 10.0. A typical electropherogram obtained using these conditions for the amino acids mixture is shown in Figure 1B. As can be seen, using this new BGEm it is possible to achieve the complete chiral-MEKC-LIF separation of D- and L-Arg, D- and L-Leu, L-Asn, D- and L-Ser, D- and L-Ala, D- and L-Glu, and D- and L-Asp in less than 25 min. Besides, these new MEKC-LIF conditions bring about a noticeable improvement of the resolution, as can be observed in Figure 1C. This improvement can be clearly appreciated considering the less separated compounds (among those that could be quantified), i.e., D-Ala and L-Ser. The resolution (R_s) (calculated as $(t_{L-Ser} - t_{D-Ala})/w_{L-Ser}$, where t_{L-Ser} and t_{D-Ala} are the migration times for L-Ser and D-Ala and w_{L-Ser} is the baseline peak width for the last migrating peak, i.e., L-Ser in this case) increased from 0.70 in the initial separation conditions (Figure 1A) to 0.90 in the final selected conditions (Figure 1C). Likewise, the resolution observed between the first migrating amino acids,

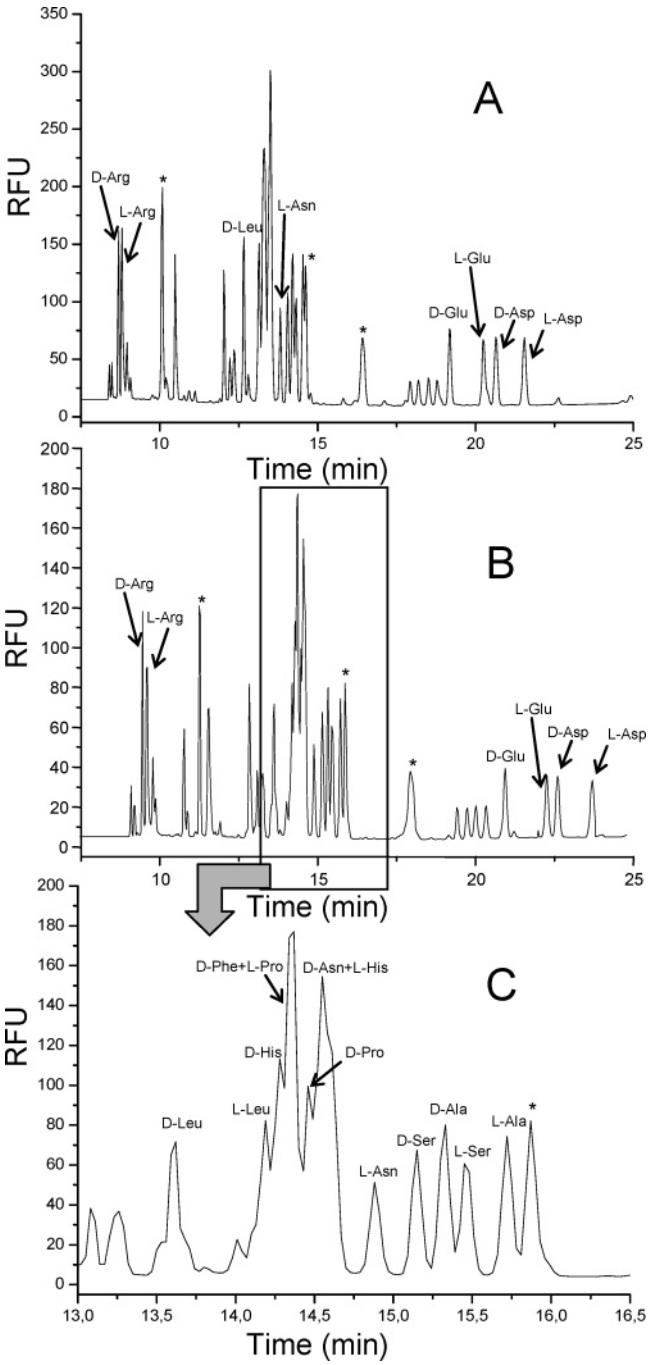

Figure 1. MEKC-LIF electropherograms of a standard mixture of 20 D/L-amino acids (D-, L-Arg; D-, L-Leu; D-, L-Asn; D-, L-Pro; D-, L-Phe; D-, L-Ser; D-, L-Ala; D-, L-His; D-, L-Glu; D-, L-Asp) obtained using the initial (A) and optimized (B) MEKC-LIF conditions. (C) Enlargement of the marked area of electropherogram B. Peaks marked with an asterisk correspond to FITC. Background electrolytes: (A) 100 mM sodium tetraborate, 30 mM SDS, 20 mM β-CD at pH 9.7; (B) 100 mM sodium tetraborate, 80 mM SDS, 20 mM β-CD at pH 10.0. Rest of conditions: sample, standard mixture of FITC-derivatized amino acids injected for 3 s at 0.5 psi; bare fused-silica capillary, 57-cm total length, 50-cm detection length, and 50-μm i.d.; running voltage, 20 kV; LIF detection, Ar^+ laser at 488 (excitation wavelength) and 520 nm (emission wavelength).

D- and L-Arg, improved changing from 0.80 in the initial conditions to 1.10 in the final conditions as well as between the peak corresponding to L-Ala and one of the FITC impurities marked with an asterisk (from 0.70 to 0.90). Moreover, efficiencies ranging

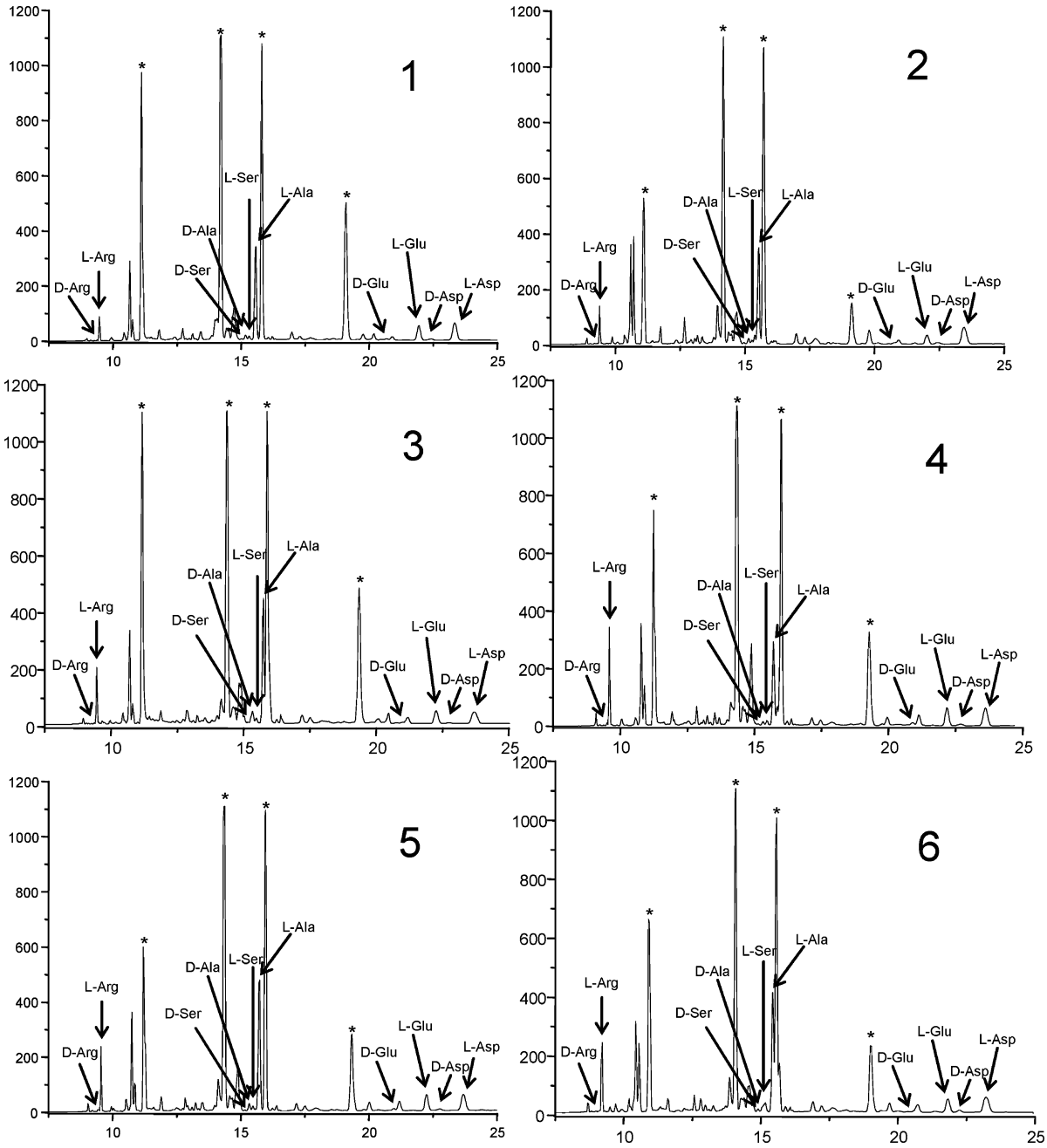

Figure 2. Chiral-MEKC-LIF electrophoregrams of free D/L-amino acids from the maize varieties: (1) Aristis; (2) Aristis-Bt; (3) Tietar; (4) Tietar-Bt; (5) PR33P66; (6) PR33P66-Bt. Peaks marked with an asterisk correspond to FITC. All the conditions as in Figure 1B.

from 330 000 plates/m for L-Asp to 890 000 plates/m for D-Arg were achieved, corroborating the usefulness of this procedure. Also, the limits of detection, calculated by considering a signal-to-noise ratio equal to three, ranged from 160 nM for D-Arg to 790 nM for L-Asp, providing limits of quantitation ranging from 530 nM for D-Arg to 2.6 μM for L-Asp. By injecting (in triplicate) five different concentrations of the standard solutions containing L- and D-amino acids, the method was determined to be linear in the concentration ranges indicated in Table 2. Thus, R^2 values better than 0.996 were obtained in all cases for the equations shown in Table 2 obtained plotting corrected peak area (i.e., area/time) versus amino acid concentration (expressed as μg/mL). Moreover, $RSD_{n=3}$ values obtained for intraday repeatability were better than 5.8 and 1.2% for peak areas and migration time, respectively. On the other hand, RSD values obtained for three different days were better than 8.2 and 1.9% for peak areas and migration time, respectively, assuring an adequate quantitation of the analytes.

In spite of these good results, the present chiral-MEKC-LIF separation of amino acids can still be improved as can be deduced from the comigration of several enantiomers shown in Figure 1C between 14 and 14.7 min. However, given the good figures of merit in terms of analysis speed, efficiency, and sensitivity of this chiral-MEKC-LIF procedure, this method was used to carry out the chiral amino acids profiling in transgenic and their parental nontransgenic maize varieties.

Identification and Quantification of D/L-Amino Acids in Transgenic and Nontransgenic Maize Samples. Amino acids

Table 2. Linear Range and Calibration Curves for the Quantitative Determination of the Ten D- and L- Amino Acids Used in This Work

amino acid	concentration range[a] (μg/mL)	equation[b]	R^2 [c]
D-Arg	10.4−0.28	$y = 18696x + 149$	0.999
L-Arg	10.4−0.28	$y = 16856x + 1015$	0.999
D-Ala	10.4−0.28	$y = 13568x - 436$	0.999
L-Ala	20.8 − 0.42	$y = 13975x + 118$	0.998
D-Ser	10.4 − 0.21	$y = 12535x + 491$	0.999
L-Ser	20.8 − 0.42	$y = 14041x + 876$	0.998
D-Glu	20.8 − 0.46	$y = 11063x - 2428$	0.998
L-Glu	20.8 − 0.46	$y = 11604x - 2934$	0.996
D-Asp	20.8 − 0.46	$y = 11074x - 915$	0.997
L-Asp	20.8 − 0.46	$y = 10763x - 3746$	0.999

[a] five different concentrations were used for each calibration curve, and each concentration was injected in triplicate. [b] y = amino acid peak area; x = amino acid concentration given as μg/mL. [c] R^2, coefficient of determination.

from six different maize samples (i.e., three transgenic varieties plus their respective parental nontransgenic lines grown under identical conditions) were extracted, derivatized, and injected using the selected chiral-MEKC-LIF conditions described above, giving rise to the electropherograms shown in Figure 2. In order to identify the different enantiomers, a coinjection procedure as shown in Figure 3 was applied allowing the accurate identification of 10 free L/D-aa in all the maize samples. Namely, L/D-Arg, L/D-Ala, L/D-Ser, L/D-Glu, and L/D-Asp were identified in all the investigated maize varieties. Quantification was next carried out using the equations shown in Table 2.

Table 3 shows the amount of L- and D-aa determined by this chiral-MEKC-LIF method in the six real maize samples investigated, i.e., Aristis, Aristis-Bt, Tietar, Tietar-Bt, PR33P66 and PR33P66-Bt, as well as the % D (calculated as the amount of D-aa divided by (L-aa + D-aa) and multiplied by 100) including their relative standard deviation values. As can be deduced from the Arg column in Table 3, the chiral-MEKC-LIF method allows the determination of less than 1% D-Arg in the presence of more than 99% of its corresponding L- opposite enantiomer, corroborating the high analytical capability of this procedure.

Next, a comparison among the different samples of Table 3 was done using the % D values for each amino acid, since by using these values any possible unexpected experimental variation (e.g., induced by different injection volumes) will affect equally TO both enantiomers, reducing in this way the variability and increasing the confidence of the comparison. It can be observed from the results given in Table 3 that the different conventional varieties (Aristis, Tietar, PR33P66) show different profiles of L- and D-aa, what seems to corroborate the natural variability expected.[38] This result was corroborated with the one-way ANOVA and posterior LSD test indicating that the three samples were statistically different ($P < 0.05$) in all cases except for the % D-Glu for which no significant differences were observed. Next, a comparison was established between the different couples of samples (i.e., conventional maize vs its corresponding Bt variety) using the two-sample *t*-test. Thus, for Tietar and Tietar-Bt it could be observed

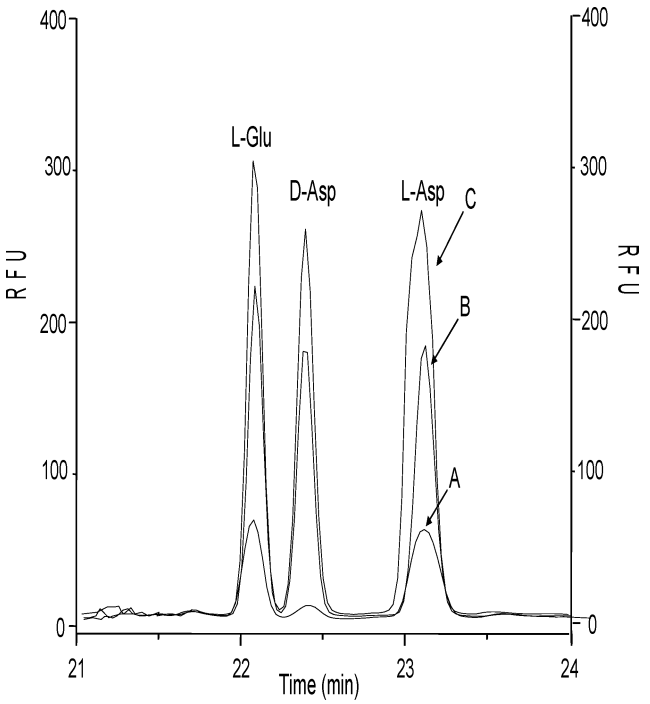

Figure 3. Chiral-MEKC-LIF electrophoregram from Tietar-Bt maize sample (A); the same sample injected in (A) plus 10 μg/mL D-Asp, L-Asp, and D-Glu (B); and the same sample injected in (B) plus 10 μg/mL D-Asp, L-Asp, and D-Glu (C). All the conditions as in Figure 1B.

that their % D values were equivalent in all cases; i.e., nonsignificant differences were observed from the *t*-test ($P > 0.05$). This result seems to indicate that, in these maize samples, the new inserted Cry1Ab transgene has not modified any metabolic pathway linked to the detected amino acids, which seems to add a further proof about the safety equivalence of these samples. However, in the case of Arsitis/Aristis-Bt, significant differences ($P < 0.05$) were observed in the % D content of Arg, Ser, and Asp between the conventional and transgenic line. Similar results were observed for the couple PR33P66/PR33P66-Bt, for which significant differences ($P < 0.05$) were observed for the % D content of Arg, Ser, and Ala. Although it has already been demonstrated, using metabolomic techniques, that genetic modifications in the grains of *Zea mays* introducing Cry1Ab gene expression induces unexpected metabolic variations involving the primary nitrogen pathway and thus affecting osmolytes and branched amino acids,[39] at the moment we are not able to find a suitable explanation for the differences detected in the amino acids enantiomeric composition of these maizes. These results demonstrate for the first time that the use of enantioselective procedures can provide some new light on the investigations of GMOs including their chemical composition and the detection of unexpected modifications.

CONCLUSIONS

The analysis of chiral amino acids in transgenic foods demonstrated for the first time in the present work, apart from having interesting nutritional and safety implications, can be used as an additional indicator for assessing the existence (or not) of

(38) Reynolds, T. L.; Nemeth, M. A.; Glenn, K. C.; Ridley, W. P.; Astwood, T. D. *J. Agric. Food Chem.* **2005**, *53*, 10061−10067.

(39) Manetti, C.; Bianchetti, C.; Casciani, L.; Castro, C.; Di Cocco, M. E.; Miccheli, A.; Motto, M.; Conti, F. *J. Exp. Bot.* **2006**, *57*, 2613−2625.

Table 3. Free L- and D-Amino Acid Content (in μg/g)[a] in the Six Maize Samples Studied

	Arg			Ser			Ala			Glu			Asp		
sample	L-[b]	D-[b]	% D[c](RSD)[d]	L-	D-	% D(RSD)	L-	D-	% D(RSD)	L-	D-	% D(RSD)	L-	D-	% D(RSD)
Aristis	146.1	1.2	0.84(5.3)	19.9	4.5	18.6(8.6)	753.7	46.8	5.9(6.6)	210.8	42.1	16.7(4.5)	314.3	31.7	9.2(4.0)
Aristis-Bt	209.1	1.41	0.67(2.6)	20.7	6.2	23.2(6.8)	819.1	51.8	5.9(5.5)	142.3	26.1	15.5(8.7)	336.2	39.3	10.5(0.4)
Tietar	346.0	3.4	1.0(8.0)	32.5	2.9	8.2(4.4)	954.9	70.0	6.8(3.7)	199.6	43.0	17.7(5.4)	269.9	33.2	9.9(5.6)
Tietar-Bt	547.8	6.0	1.1(13.9)	26.6	2.4	8.4(3.0)	656.6	46.7	6.6(9.5)	239.6	55.7	18.9(6.2)	309.3	39.4	11.3(0.7)
PR33P66	398.2	7.5	1.9(5.9)	41.8	3.7	8.1(4.8)	1138.9	71.5	5.9(4.9)	251.1	49.2	16.4(7.2)	317.5	42.7	11.9(0.3)
PR33P66-Bt	468.3	4.67	1.0(5.3)	26.3	6.4	19.7(7.6)	843.1	32.8	3.7(0.7)	203.4	33.4	14.1(4.1)	323.1	42.4	11.6(2.2)

[a] μg of D- or L-aa per g of maize. When necessary, samples were conveniently diluted to fit within the linear ranges given in Table 2. [b] Average values from three replicates given as μg/g. [c] Relative percentage of D-aa calculated as, % D = 100·D-aa/(D-aa + L-aa). [d] Relative standard deviation (% RSD, $n = 3$).

unexpected modifications in other metabolic pathways linked to the amino acids profile within a GMO. Thus, this methodology can highlight details about the changes induced (if any) on some parts of the metabolic network by the presence of significant modifications in the L/D content of amino acids that could be linked to the new transgene inserted (or deleted) in the original genome of the GMO. From our results, it can be concluded that the use of enantioselective procedures can open new perspectives in the study of the chemical composition, unexpected modifications, or both of GMOs.

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