**Sensitive and simultaneous analysis of five transgenic maizes
using multiplex PCR, capillary gel electrophoresis
and laser induced fluorescence.**

Virginia García-Cañas, Ramón González*, Alejandro Cifuentes*

Institute of Industrial Fermentations (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain.

Running title: Multiplex PCR-CGE-LIF of transgenic maizes

* corresponding authors

rgonzalez@ifi.csic.es and acifuentes@ifi.csic.es Fax# 34-91-5644853

Abbreviations: AGE (agarose gel electrophoresis), CGE-LIF (capillary gel electrophoresis with laser induced fluorescence), GM (genetically modified), GMO (genetically modified organism), PCR (polymerase chain reaction).

Keywords: Capillary electrophoresis, CE, genetically modified organisms, GMOs, multiple detection, PCR optimization, food, transgenic food, transgenic corn.

SUMMARY

In this work, the benefits of using multiplex PCR followed by CGE-LIF for the simultaneous detection of five transgenic maizes (Bt11, T25, MON810, GA21 and Bt176) are demonstrated. The method uses a hexaplex PCR protocol to amplify the five mentioned transgenic amplicons plus the zein gene used as reference, followed by a CGE-LIF method to analyze the six DNA fragments. CGE-LIF was demonstrated very useful and informative for optimizing multiplex PCR parameters such as time extension, PCR buffer concentration and primers concentration. The method developed is highly sensitive and allows the simultaneous detection in a single run of percentages of transgenic maize as low as 0.054% of Bt11, 0.057% of T25, 0.036% of MON810, 0.064% of GA21 and 0.018% of Bt176 in flour obtaining signals still far from the detection limit (namely, the signal/noise ratios for the corresponding DNA peaks were 41, 124, 98, 250, 252 and 473, respectively). These percentages are well below the minimum threshold marked by the European Regulation for transgenic food labeling (i.e., 0.5-0.9%). A study on the reproducibility of the multiplex PCR-CGE-LIF procedure was also performed. Thus, values of RSD lower than 0.67% and 6.80% were obtained for migration times and corrected peak areas, respectively, for the same sample and three different days (n=12). On the other hand, the reproducibility of the whole procedure, including four different multiplex PCR amplifications, was determined to be better than 0.66 and 23.3% for migration times and corrected peak areas, respectively. Agarose gel electrophoresis (AGE) and CGE-LIF were compared in terms of resolution and sensitivity for detecting PCR products, demonstrating that CGE-LIF can solve false positives induced by artifacts from the multiplex PCR reaction that could not be addressed by AGE. Moreover, CGE-LIF provides better resolution and sensitivity. To

our knowledge, these results demonstrate for the first time that multiplex PCR-CGE-LIF is a solid alternative to determine multiple genetically modified organisms in maize flours in a single run.

1. INTRODUCTION

In the last decade, the agricultural and food industry have experienced innovative changes by means of recombinant-DNA technology. Genetically modified (GM) products have already been introduced in the European Union market, and numerous notifications concerning genetically modified organisms (GMOs) release and placing on the market are waiting for being approved [1]. Also, new EU regulations regarding GM food and feed authorization, traceability and labeling have recently been approved [2,3]. Applicability of these new regulations will imply mandatory labeling of products containing approved GMOs in a proportion higher than 0.9% of the food ingredients considered individually, and the prohibition of placing on the market products that contain pending GMOs in a proportion higher than 0.5%. Therefore, in order to verify whether food products are in compliance with legislation, suitable GMO qualitative and quantitative detection methods are demanded.

Screening and identification methods play an important role in GMO analysis in foods. These methods are based on the detection of two types of macromolecules, specific for the genetic modification, to reveal the presence of GMOs (or a derivative) in foods: proteins and DNA. However, the higher thermal stability of DNA and the fact that it is present in most biological tissues makes it a more suitable target for GMO detection. Most DNA-based detection methods for GMOs rely on the use of polymerase chain reaction (PCR) [4,5]. An interesting feature of PCR technique is the possibility of multiplexing different DNA sequences used as targets, allowing the simultaneous amplification of several transgenic sequences in a single reaction. Last challenges in

GMO screening and identification methods trend towards multiplex PCR approaches due to its benefits in terms of speed of analysis, labor and cost reduction.

Several studies have recently described the use of multiplex PCR for the detection of GMOs. Identification of transgenic lines has been achieved by amplifying transgenic constructs-specific sequences (named junction fragments) [6], or a combination of widespread transgenic sequences used in GMOs, e.g. *NOS*, *pat*, *cryIA(b)*, etc [7]. Multiplex PCR methods have been reported for the simultaneous detection of numerous GMOs including five genetically modified maize lines [8] and four canola transgenic lines [9]. Multiplex PCR involves more complex reaction system than the simplex procedure and, therefore, yields of amplification are more difficult to predict because of the presence of multiple primer pairs in the reaction, reducing the robustness of PCR [10]. Henegariu *et al.* [11] have reported a systematic step-by-step protocol for optimization of experimental parameters (type and concentration of reagents, thermocycling parameters, etc) in order to obtain adequate amplification yields for all the PCR products.

Generally, the final step of multiplex PCR analysis consists in the separation and detection of PCR products. This is usually achieved by agarose gel electrophoresis (AGE) combined with ethidium bromide staining. The main advantages of this technology are its simplicity and cost effectiveness, and the fact that it is implemented in almost every Molecular Biology laboratory worldwide. Nevertheless, the limited sensitivity and resolution, and the difficulties for automation, have encouraged the development of innovative procedures for multiplex analysis. In this sense, membrane hybridization techniques (combining biotinylated primer pairs followed by the

hybridization of PCR products with a membrane-bound oligonucleotide probe and subsequent colorimetric detection) have been employed to avoid false positives [12]. Similar procedure using surface plasmon resonance-based biosensors technology has been applied to detect simultaneously two PCR products. Hence, biotinylated amplicons are produced by means of multiplex PCR, and PCR products are captured onto a streptavidin-coated surface where hybridization with specific oligonucleotide probes takes place. Detection of amplified sequences are carried out measuring changes in refractive index in the surrounding area of a sensor chip's surface to which probes have been immobilized [13]. Both detection methods require the use of specific oligonucleotide probes, and subsequent steps of manipulation of PCR products. On the other hand, two genetically modified maize lines (GA21 and Bt176 maize) have been detected simultaneously at the level of 1% GMO in a multiplex assay based on melting temperature differences between PCR products using a real-time instrument and SYBR Green I dye [14]. The main advantages of this technique are high processing capability and the minimal risk of cross-contamination, due to the use of a single tube for both amplification and detection. However, resolution is based on melting temperatures and it has only been possible to distinguish PCR products showing T_m differences higher than 1.5°C [14]. Recently, commercial microfluidic electrophoresis system (LabChipTM) has been used for the analysis of triplex PCR assay to detect genetically modified soy [15].

Recently, we have demonstrated the benefit of using capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) in terms of speed, resolution and sensitivity for the qualitative and quantitative detection of Bt maize in food [16-19]. Based on these results, it can be expected that CGE-LIF can provide interesting advantages for the

analysis of multiplex amplification products compared with traditional analytical procedures based on AGE. Besides, CGE-LIF meets a number of interesting properties that can make it very suitable for monitoring the effect of PCR parameters during development and optimization of any new multiplex reaction [20].

The goals of this work were: to investigate the capabilities of CGE-LIF as a monitoring tool during the systematic optimization of a hexaplex PCR method and to study the possibilities of CGE-LIF combined with multiplex PCR for the simultaneous analysis of five different GM maize lines. To our knowledge, this is the first work dealing with CGE-LIF for both optimizing multiplex PCR parameters and separating multiple transgenic foods in a single run.

2. MATERIALS AND METHODS

2.1 Chemicals.

All chemicals were of analytical reagent grade and used as received. Tris[hydroxymethyl]aminomethano (TRIS), sodium dodecyl sulfate (SDS), ethidium bromide, ethylenediaminetetraacetic acid (EDTA) and Lambda DNA *HindIII* Digest were from Sigma (St. Louis, MO). 100 bp ladder molecular marker was from Biotools (Madrid, Spain). 2-hydroxyethylcellulose (HEC) (Mw 90000) and poly(vinyl alcohol) (PVA) (Mw 50000) were from Aldrich (Milwaukee, WI). MS4 agarose was from Pronadisa (Madrid, Spain). Proteinase K and RNase A from Roche (Barcelona, Spain), chloroform from Scharlau (Barcelona, Spain), isoamyl alcohol and N-cetyl-N,N,N-trimethylammonium bromide (CTAB) from Merck (Darmstadt, Germany), and phenol

from LabClinics (Madrid, Spain) were used. YOPRO1 and SYBR Green-I were from Molecular Probes (Leiden, The Netherlands). Buffers were stored at 4°C and warmed at room temperature before use. Oligonucleotides were synthesized at Genotek (Sabadell, Spain). AmpliTaq Gold DNA polymerase, including GeneAmp PCR buffer II and MgCl₂, was from Perkin-Elmer (Madrid, Spain). Deoxynucleotides were from Amersham Pharmacia Biotech Europe GmbH (Barcelona, Spain). Uracil DNA glycosylase was purchased from New England Biolabs (Beverly, MA) and distilled water was deionised by using a Milli-Q system (Millipore, Bedford, MA).

2.2 Samples.

Certified reference maize powder MZ0 (conventional, i.e., containing 0% transgenic maize) produced by the Institute of Reference Materials and Measurements (IRMM) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Transgenic maize flour was obtained from USDA/GIPSA Proficiency Program. DNA samples containing transgenic maize DNA (T25, MON810, GA21, E176 and Bt11) were prepared mixing DNA extracts from maize flours with T25, MON810, GA21, E176 and Bt11 maize content ranging from 0 to 5.0%.

2.3 DNA extraction.

DNA purification was carried out by the *SDS/proteinase K method* modified from reference [21]. Homogenized samples (100 mg) were incubated at 37 °C overnight in 1 mL extraction buffer (1% SDS, 100 µg/mL proteinase K, 50 mM TRIS-HCl (pH 8), 20 mM EDTA). The suspension was centrifuged for 10 min at 5000 rpm. The supernatant

was extracted with 1 volume of phenol and subsequently with 1 volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was transferred to a new tube and 30 µg RNase A were added and incubated at 37 °C for 15 min. The extract was mixed with 0.1 volume of 3 M sodium acetate at pH 4.8. Then, the mix was overlaid with 2.5 volumes of 100% ethanol. The two phases were mixed carefully by gentle agitation and then centrifuged for 10 min at 12000 x g. The supernatant was discarded and the pellet washed with 70% ethanol. Finally, DNA was dissolved in 50 µL of TE buffer (10 mM TRIS-HCl (pH 8.0); 1 mM EDTA). Total dsDNA was quantified in a RF1501 spectrofluorimeter (Schimadzu, Spain) at $\lambda_{exc}=480$ nm and $\lambda_{emi}=520$ nm, using a 1:2000 SYBRGreen-I dilution from commercial stock solution and a calibration curve made with Lambda DNA *HindIII* Digest.

2.4 PCR conditions.

The oligonucleotides used in this multiplex system were described by Matsuoka *et al.* [8], and their sequences are listed in Table 1. Initially, PCR was carried out under the conditions reported by Matsuoka *et al.* with some modifications (*vide infra*). Each PCR reaction (50 µL total volume) contained 50 ng genomic DNA, 2 mM MgCl₂, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.4 mM dUTP, 0.2 µM each primer except 0.6 µM cryIA 1-3' primer, 0.25 U of Uracil-DNA Glycosilase (UDG) and 1.25 U of AmpliTaq Gold DNA polymerase in GeneAmp PCR buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl). Amplification reactions were run in a Mastercycler gradient thermocycler (Eppendorf, Spain). PCR reaction was preceded by an incubation period of 10 min at 37°C for UDG activity. The thermal parameters used for the amplifications were as follows: 10 min at 95°C; followed by 10 cycles of 30 sec at 95°C, 1 min at

63°C, 1 min at 72°C; 30 cycles consisting of 30 sec at 95°C, 1 min at 60°C, 1 min at 72°C; and a final elongation step of 7 min at 72°C. Various parameters were tested to obtain optimal conditions for multiplex PCR. These parameters included time extension (1 and 2 min), GeneAmp PCR buffer II concentration (0.5X, 0.7X and 1X), and *Zel* 1-5'/*Zel* 1-3' and *M810* 1-5'/*cryIA* 1-3' primer pair concentration (0.2, 0.5 and 0.8 µM *Zel* 1-5'/*Zel* 1-3'; 0.2 µM *M810* 1-5' and 0.5 µM *cryIA* 1-3'; 0.5 µM *M810* 1-5' and 0.9 µM *cryIA* 1-3'). The rest of primer concentrations were the same as is described above.

2.5 Capillary gel electrophoresis.

Analyses of PCR samples were carried out in a PACE-MDQ (Beckman Instruments, Fullerton, CA, USA) equipped with an Ar⁺ laser working at 488 nm (excitation wavelength) and 520 nm (emission wavelength). Bare fused-silica capillaries with 75 µm I.D. were purchased from Composite Metal Services (Worcester, England). Injections were made at the cathodic end using N₂ pressure of 1 p.s.i. for 12 sec (1 p.s.i.=6894.76 Pa). Data acquisition and integration were performed with 32 Karat Software (Beckman Instruments, Fullerton, CA, USA).

Before first use, uncoated capillaries were preconditioned by rinsing with 0.1 M HCl for 30 min. Between injections, capillaries were physically coated using 0.1 M HCl for 4 min, 1% PVA (Mw 50000; Aldrich, Spain) for 2 min and separation buffer for 4 min. At the end of the day, the capillary was rinsed with deionized water for 5 min and stored overnight with water inside. The following conditions were used for PCR product separation: Separation buffer (20 mM Tris, 10 mM phosphoric acid, 2 mM EDTA, 1.5

M urea, 500 nM YOPRO1 (Molecular Probes, The Netherlands) and 4.5 % HEC (Mw 90000; Aldrich, Spain) at pH 7.3); temperature of separation: 45 °C; running electric field: -217 V/cm.

2.6 Slab gel electrophoresis.

Fifteen microliters of each PCR mixture were electrophoresed in slab gel containing 3% MS4 agarose in TAE buffer (40 mM Tris-acetate, at pH 8, 1 mM EDTA). Gels were run at 3 V/cm during 2.5 hours, stained with 0.5 µg/mL ethidium bromide and photographed using a Kodak DC265 Digital camera (Kodak, Spain). Image processing was carried out using Adobe Photodeluxe software (Adobe Systems Inc.).

3. RESULTS AND DISCUSSION

3.1 Optimization of multiplex PCR conditions using CGE-LIF.

The multiplex reaction under study was initially developed by Matsuoka *et. al.* [8]. Primers were designed for targeting specific genomic DNA sequences. The hexaplex PCR system was developed to amplify the zein gene and five transgenic constructs at the junction fragment level (i.e. sequences containing transgenic elements of diverse origins in a particular arrangement).

Initially, simplex PCR amplifications were performed using thee conditions described under Materials and Methods. The same maize DNA extract containing five transgenic maize lines (2.5% Bt11, 2.75% T25, 0.25% MON810, 2.55% GA21 and 0.75% Bt176)

was amplified by PCR in six independent reactions. Each amplification reaction containing a primer pair (i.e. *Ze1* 1-5'/*Ze1* 1-3'; *E176* 1-5'/*cryIA* 1-3'; *M810* 1-5'/*cryIA* 1-3'; *Bt11* 1-5'/*cryIA* 1-3'; *T25* 1-5'/*T25* 1-3'; *GA21* 1-5'/*GA21* 1-3') was next analyzed by CGE-LIF. Length of DNA fragments were verified comparing theoretical and calculated sizes. Migration times (t_m) of the peaks were used to calculate the length of the PCR amplicons. To do this, an equation was obtained plotting logarithm of length of DNA fragments (bp) ranging from 80 to 700 bp *versus* logarithm of inverse t_m data of corresponding DNA fragments obtained from the separation of a 100 bp DNA ladder molecular marker sample co-injected with a PCR reaction ($\log(\text{bp}) = 3.95 - 35.54/t_m$ ($r^2 = 0.997$, $n=8$)). By interpolation of migration times from the peaks displayed in electropherograms of DNA separations, the calculated sizes values obtained for PCR products were 109, 148, 199, 271, 359 and 528 bp (for Bt11, T25, MON810, GA21, Bt176 and zein peak, respectively). The experimentally calculated DNA sizes were in good agreement with the theoretically expected mostly for the four small fragments (110, 149, 199, 270 bp, respectively), demonstrating that CGE-LIF provides accurate determination of the sizes of these amplified fragments. However, the sizing accuracy for Bt176 and zein PCR was slightly worse (theoretical sizes: 343 and 508 bp, respectively) as a consequence of a resolution decrease in this region. This detrimental effect on resolution might be explained because the mobilities of the larger PCR products are closer when separation buffer containing low-molecular weight HEC polymers are used [22]. From these experiments it could be deduced that every simplex reaction yielded the DNA fragment with a length similar to that expected according to the values reported in literature [8], demonstrating the usefulness of the PCR primers selected.

The same DNA sample as above was next amplified in one tube with a mixture of ten primers by multiplex PCR method. **Figure 1A** shows the CGE-LIF electropherogram obtained for the multiplex PCR amplification of DNA sample containing the five GM maize lines according to the conditions given in reference [8]. As can be seen in **Figure 1A**, under the conditions reported in the original protocol by Matsuoka *et. al.* [8], no amplification was detected for the longest DNA fragments (i.e. 343 bp Bt176 and 508 bp zein DNA sequences). This behavior can be due to instrumental differences in the thermocyclers used (a Mastercycler gradient in our case and a GeneAmp PCR System 9700 in reference [8]). Therefore, a further optimization of the multiplex PCR reaction parameters had to be carried out in order to obtain optimal amplification for all the DNA sequences under study. In this sense, since CGE-LIF provides accurate quantitative information, high sensitivity and high resolution [19,20], this technique can be conveniently used to adjust PCR parameters with confidence.

Concentration of PCR reagents as well as different thermocycling parameters were adjusted in order to improve co-amplification of the longest PCR fragments. Hence, initially the time extension influence on the hexaplex PCR system was tested. Increasing from 1 to 2 min extension time did not alter the amplification efficiency of PCR products (data not shown). The effect of GeneAmp PCR buffer II concentration on PCR product yield was also studied. Amplifications were performed with 1X, 0.7X and 0.5X PCR buffer concentrations and then injected in the CGE-LIF instrument. No amplification was detected when a 0.5X PCR buffer was used. On the other hand, when PCR buffer concentration was diminished from 1X (**Figure 1A**) to 0.7X (**Figure 1B**), the signal/noise ratio for the GA21 peak increased five times (from 860 to 4520) while the ratio for the Bt11 peak, corresponding to the shortest DNA fragment, decreased

twice (from 690 to 390). Using the 0.7X buffer, peaks corresponding to Bt176 and zein DNA fragments were detected with signal/noise ratios of 140 and 120, respectively. This PCR buffer concentration effect on longer product yield is in good agreement with that reported in the literature [11].

Although the signal/noise ratios values obtained for all the DNA peaks were high enough to detect the amplification PCR products, the yield of PCR products was significantly unbalanced. Mainly, amplification efficiency of zein PCR product showed to be low in comparison with other amplicons which are supposed to be less abundant than zein genomic sequence in the DNA sample tested. Since zein amplification reveals the quality and amplificability of the extracted DNA when maize genome sequences are present in the sample, it would be convenient to improve the amplification efficiency of zein PCR product. For this reason, the effect of zein primer pair (*Zel 1-5'*/*Zel 1-3'*) concentration on the multiplex PCR was studied. Multiplex PCR amplifications were then performed using higher zein primer concentrations (0.5 and 0.8 μM). CGE-LIF analysis of PCR reactions showed the optimal yield of zein amplicon when 0.5 μM primer concentration was used (**Figure 1C**). Under these experimental conditions zein peak signal/noise ratio was more equilibrate than with the previous conditions, but MON810 peak signal/noise ratio decreased (from 83 to 24). Therefore, *M810 1-5'* and *cryIA 1-3'* primer concentration was increased up to 0.5 μM and 0.9 μM , respectively, in order to improve Mon810 amplicon yield. Under these conditions, signal/noise ratio for MON810 peak improved three times (from 24 to 76) while GA21 peak signal/noise ratio decreased three times as well (from 1704 to 668) and the other peaks presented similar values to the previous optimization step. **Figure 1D** shows the electropherogram obtained after optimum hexaplex PCR amplification conditions (namely, 0.7X PCR

buffer; 0.5 μ M *Zel* 1-5', 0.5 μ M *Zel* 1-3', 0.5 μ M *M810* 1-5' and 0.9 μ M *cryIA* 1-3' primer). These results clearly demonstrate that CGE-LIF is a highly informative technique for multiplex-PCR optimization.

In order to demonstrate that the DNA fragments obtained under these optimum multiplex PCR conditions are the expected ones, the length of DNA fragments were determined as above. The same calculated sizes were obtained, namely, 109, 148, 199, 271, 359 and 528 bp for Bt11, T25, MON810, GA21, Bt176 and zein peak, respectively, what demonstrates the absence of any deleterious interference during the multiplex amplification reaction.

A reproducibility study of the whole procedure was next performed (**Table 2**). First, reproducibility of migration times and corrected peak areas was studied injecting the same PCR reaction products from a given multiplex PCR. Reproducibility of the CGE-LIF procedure was good, as can be deduced from the results of **Table 2**. As an example, the %RSD for migration time for the MON810 peak was 0.49% for the same day (n=5) and 0.62% for three different days (n=12). Moreover, %RSD values for corrected peak areas for MON810 peak range from 3.50 to 6.30% for the same day (n=5) and three different days (n=12), respectively. Each multiplex PCR reaction was repeated four times and each sample was injected in the capillary electrophoresis instrument. In this case, the highest %RSD value obtained for reproducibility of corrected peak areas (n=4) was 23.3% for the MON810 peak (**Table 2**). In addition, the efficiency achieved was up to $1.0 \cdot 10^6$ plates/m calculated for the GA21 peak in the CGE-LIF electropherogram of **Figure 2**.

3.2 AGE vs. CGE-LIF for multiple analysis of transgenic maize lines.

Agarose gel electrophoresis (AGE) for routine analysis of multiplex PCR products might lead to false positive results if an artifact of similar size as a given target sequence is amplified [4]. This limitation can be overcome using a highly resolving analytical technique, as e.g. CGE-LIF, for the analysis of multiplex PCR amplifications. A typical example of this effect is next discussed. **Figure 2** displays the analysis of the hexaplex PCR amplification of maize DNA containing 0.67% Bt11, 1.94% T25, 0.94% MON810, 0.91% GA21 and 0.24% Bt176 maize DNA. As can be seen in the CGE-LIF electropherogram (**Figure 2-II**), an extra peak named T25' can be detected at 24.3 min (corresponding to a DNA size of 294 bp), which is close to the GA21 peak (271 bp, migrating at 23.8 min). This T25' peak was systematically detected under our conditions by PCR-CGE-LIF in amplifications of DNA containing T25 maize by both multiplex and simplex PCR reactions. The analysis of peak corrected areas from the CGE-LIF electropherograms showed that the non-specific T25' fragment constituted of a 5-10% of the total T25 PCR product. These fragments of 271 bp (GA21) and 294 bp (T25') comigrate in AGE due to its lower resolution power and, as a consequence (Lane A in **Figure 2-I**), high percentage of T25 maize in a sample without GA21 maize would lead to a GA21 false positive result. However, this false positive would be correctly assigned by CGE-LIF.

The improvement in resolution is also demonstrated with the appearance of the Bt176' peak in the CGE-LIF separations of the multiplex PCR amplifications of maize DNA extracts containing Bt176 maize line (**Figure 2-II**) while a single band can be observed in agarose gel (Lane A in **Figure 2-I**). Bt176' peak was always detected as a non-

specific PCR product in amplifications of DNA from maize flours containing Bt176 maize using the *E176 1-5'* / *cryIA 1-3'* primer pair.

Figure 2 also allows establishing an initial and rough comparison in terms of analysis sensitivity provided by AGE and CGE-LIF. As can be seen, the sensitivity provided by CGE-LIF was much better than that obtained with AGE for the same sample (i.e., the transgenic BT11, MON810 and GA21 maize could only be detected by CGE-LIF).

As indicated above, the EU regulation regarding GM labeling [3] implies mandatory labeling of products containing approved GMOs in a proportion higher than 0.9% of the food ingredients considered individually, and the prohibition of placing on the market products that contain pending GMOs in a proportion higher than 0.5%. Therefore, our multiplex PCR-CGE-LIF method must also fulfill this requirement, i.e. to prove that it is sensitive enough. In order to demonstrate this point, samples of maize DNA containing different percentages of GM maize were prepared and analyzed. First, negative control of amplification and control in the absence of transgenic sequences (conventional maize DNA) were confirmed by both AGE and CGE-LIF analysis (see line G and electropherogram G in **Figure 3**). **Figure 3** also shows the AGE and CGE-LIF separations of several maize DNA extracts containing different types and percentages of transgenic maize DNA after multiplex PCR amplification. Thus, electropherogram of **Figure 3E** corresponds to the simultaneous PCR-CGE-LIF analysis in a single run of maize DNA containing 0.054% Bt11, 0.057% T25, 0.036% MON810, 0.064% GA21 and 0.018% Bt176. As can be seen, only bands corresponding to GA21 and Bt176 could be visualized for this sample on the corresponding ethidium bromide-stained gel (line E in **Figure 3I**). Moreover, the DNA peaks detected in the

case of the CGE-LIF electropherogram of **Figure 3E** have signal/noise ratios equal to 41, 124, 98, 250, 252 and 473, for the 0.054% Bt11, 0.057% T25, 0.036% MON810, 0.064% GA21 and 0.018% Bt176, respectively. These results demonstrate that the sensitivity provided by the multiplex PCR-CGE-LIF procedure developed in this work was good enough to fulfill the requirements of the EU regulation (i.e., a threshold of 0.5-0.9% for GMOs in foods has to be accomplished).

4. CONCLUDING REMARKS

A new multiplex PCR-CGE-LIF separation method is proposed for the simultaneous detection of five GM maize lines in a single run. It was demonstrated that CGE-LIF meets a number of advantages over AGE including sensitivity, resolution, efficiency and reproducibility for multiplex PCR analysis. It was also proven that these advantages make of CGE-LIF a very suitable technique for monitoring optimization of multiplex PCR reactions. Moreover, the results obtained demonstrate that the proposed method is reproducible and efficient, and therefore can be used with confidence for analyzing multiplex PCR products in order to detect several transgenic maize lines (Bt11, T25, MON810, GA21 and Bt176) simultaneously in a single run at levels below the minimum threshold currently marked by the European Regulations.

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Table 1. Primer sequences used in this work to perform multiplex PCR reactions [8].

Primer	Sequence (5'-3')	Event	Target
Zel 1-5'	CCTCAGTCGCACATATCTACTATACT	maize	<i>Zel</i> /sense
Zel 1-3'	CTAGAATGCAGCACCAACAAA		<i>Zel</i> /antisense
E176 1-5'	GTAGCAGACACCCCTCTCCACA	Event 176	<i>PEPC pro</i> /sense
M810 1-5'	GAGTTTCCTTTTTGTTGCTCTC	MON810	<i>hsp70 int.</i> /sense
Bt11 1-5'	CCATTTTTCAGCTAGGAAGTTC	Bt11	<i>adh1-IS IVS6</i> /sense
cryIA 1-3'	TCGTTGATGTT(GT)GGGTTGTTGTCC	Event 176, MON810, Bt11	<i>cryIA(b)</i> /antisense
T25 1-5'	GCCAGTTAGGCCAGTTACCCA	T25	<i>pat</i> /sense
T25 1-3'	TGAGCGAAACCCTATAAGAACCCT		<i>35S terminator</i> /antisense
GA21 1-5'	ACGGTGGAAGAGTTCAATGTATG	GA21	<i>OTP</i> /sense
GA21 1-3'	TCTCCTTGATGGGCTGCA		<i>m-epsps</i> /antisense

Table 2. Reproducibility of migration times (t_m) and corrected peak areas (A) for the same day, three different days and four different PCR reactions. All conditions as in **Figure 3**.

	Bt11	T25	Mon810	GA21	Bt176	Zein
Same day (n=5), same sample						
t_m (min)	18,70	20,10	21,70	23,80	25,80	29,30
%RSD_t	0,47	0,49	0,49	0,50	0,54	0,55
A (arb. units)	21000	4180	18200	117000	53100	125000
%RSD_A	3,20	3,50	3,50	3,40	3,50	3,10
Three different days (n=12), same sample						
t_m (min)	18,80	20,20	21,80	23,90	25,90	29,50
%RSD_t	0,59	0,58	0,62	0,63	0,66	0,67
A (arb. units)	21800	43600	18500	121000	55900	125000
%RSD_A	6,70	6,80	6,30	6,80	5,20	6,60
Four different PCR reactions, same day						
t_m (min)	18,80	20,20	21,90	23,90	25,90	29,60
%RSD_t	0,61	0,51	0,53	0,66	0,57	0,65
A (arb. units)	27300	12100	32200	160000	58800	117000
%RSD_A	21,0	19,4	23,3	21,7	17,1	19,5

FIGURE LEGENDS

Figure 1. Sequence of electrophoregrams showing the step-by-step optimization procedure of multiplex PCR using 50 ng of DNA containing 2.5% Bt11, 2.75% T25, 0.25% MON810, 2.55% GA21 and 0.75% Bt176. PCR conditions were: A) 1X PCR buffer, 0.2 μ M Ze1 1-5', Ze1 1-3', M810 1-5' and 0.6 μ M cryIA 1-3' primer; B) 0.7X PCR buffer, 0.2 μ M Ze1 1-5', Ze1 1-3', M810 1-5' and 0.6 μ M cryIA 1-3' primer; C) 0.7X PCR buffer, 0.5 μ M Ze1 1-5', Ze1 1-3', 0.2 μ M M810 1-5' and 0.6 μ M cryIA 1-3' primer; D) 0.7 X PCR buffer, 0.5 μ M Ze1 1-5', Ze1 1-3', M810 1-5' and 0.9 μ M cryIA 1-3' primer. CGE-LIF separation conditions: uncoated fused silica capillary with 60 cm of total length, 50 cm of effective length and 75 μ m i.d. Separation voltage: -13 kV. Running temperature: 45°C. Injection for 12 s using N₂ pressure (1 psi).

Figure 2. Electrophoretic analysis of a multiplex PCR amplification of maize DNA containing 0.67% Bt11, 1.94% T25, 0.94% MON810, 0.91% GA21 and 0.24% Bt176 maize DNA (electrophoregram and lane A). The analysis was performed by I) agarose gel electrophoresis or II) CGE-LIF. Lane M, 100 bp ladder molecular marker. (Bt11) 110 bp Bt11 maize DNA, (T25) 149 bp T25 maize DNA, (Mon810) 199 bp MON810 maize DNA, (GA21) 270 bp GA21 maize DNA, (Bt176) 343 bp Bt176 maize DNA and (zein) 508 bp zein maize DNA fragments. For other separation conditions see text.

Figure 3. Electrophoretic analysis of a series of multiplex PCR amplification of maize DNA samples (A-G). The analysis was performed by I) agarose gel electrophoresis or II) CGE-LIF. Samples: M, 100 bp ladder molecular marker; A, 0.867% Bt11, 0.917% T25, 0.583% MON810, 1.017% GA21 and 0.283% Bt176 maize; B, 0.433% Bt11, 0.458% T25, 0.292% MON810, 0.508% GA21 and 0.142% Bt176 maize; C, 0.217%

Bt11, 0.229% T25, 0.146% MON810, 0.254% GA21 and 0.071% Bt176 maize; **D**, 0.108% Bt11, 0.115% T25, 0.073% MON810, 0.127% GA21 and 0.035% Bt176 maize; **E**, 0.054% Bt11, 0.057% T25, 0.036% MON810, 0.064% GA21 and 0.018% Bt176 maize; **F**, 0.027% Bt11, 0.029% T25, 0.018% MON810, 0.032% GA21 and 0.009% Bt176 maize; **G**, 0% GM maize; **N**, non template control. (Bt11) 110 bp Bt11 maize DNA, (T25) 149 bp T25 maize DNA, (Mon810) 199 bp MON810 maize DNA, (GA21) 270 bp GA21 maize DNA, (Bt176) 343 bp Bt176 maize DNA and (zein) 508 bp zein maize DNA fragments. For other separation conditions see text.