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Analytical Methods

Detection of genetically modified soya and maize: Impact of heat processing

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ABSTRACT

The analysis of processed foods entails a number of complications, which negatively affect the performance of DNA based detection methods. Heat-processing methods viz. autoclaving and micro-waving, that mimic processing and manufacturing, as model unit operation systems were used to study their effect on the detection of genetically modified organisms (GMOs). This study confirms the premise that high temperature and/or pressure significantly reduce the level of detectable DNA. PCR methods were developed and adapted to target varying amplicon sizes of the trait, construct and event specific gene sequences that occur in MON-810 maize and Roundup Ready® soybean. Integrity of DNA, recovery and PCR amplicon size (<200 bp) are major factors that direct the successful detection of GMOs in processed foods. The model systems used provide a platform to devise better strategies in developing detection protocols, especially for processed foods containing GMOs.

1. Introduction

A tremendous and rapid increase in the global area dedicated to transgenic crops has occurred in recent years. Herbicide tolerant soybean is the principal genetically modified (GM) crop occupying 71% of global biotech area. USA, Argentina, Brazil, Canada and China currently share 98% of the worldwide area planted with GM crops whereas India accounts for 1% of the total global area (James, 2005). Since the approval of *Bt* cotton in 2002, to date 62 *Bt* cotton hybrids have been granted market authorisation in India. Consumer's awareness and concerns of the potential risks associated with GM crops on environment, biological diversity, human health and safety have burgeoned. A number of countries have therefore adopted, or are in the process of developing legislation related to the approval of GM-products. Regulatory provisions for labelling of products derived from genetically modified organisms (GMOs) have been introduced in the European Union, Japan, Korea and other countries with different threshold levels (EC 1829/2003; Notification No. 1775, 2000; Notification No. 2000-31, 2000). India too has a robust regulatory framework and an elaborate review process, which is backed by well-developed bio-safety guidelines (Warrier, 2006). Rule 37E and 48F of the recently enacted Food and Safety Standards Act (Food Safety and Standards Act, 2006) states that all imports of GMOs for the purpose of food, feed, industrial processing, research and development for environment release or commercialisation will be governed by and allowed only with the certification of the Genetic Engineering Approval Committee (GEAC). Moreover, imported consignments containing genetically modified products must carry a declaration stating that the product is genetically modified (Notification No. 2 RE-2006/2004–209, 2006). A process-based rather than a product-based labelling approach is under consideration so that any product derived from gene technology must be compulsorily labelled, even if the novel DNA and/or protein are absent (Draft notification on labelling of GM food, 2006).

Polymerase chain reaction (PCR) is a widely used technique considered as the method of first choice in GMO detection to comply with a robust labelling system. Several DNA based screening methods have been developed to detect GMOs in raw food materials (James, Schmidt, Wall, Green, & Marsi, 2003; Lau, Collins, Yiu, Xing, & Yu, 2004; Lipp et al., 2001). Food manufacturing entails a number of complex processing steps that are harsh and might negatively influence detection of GMOs. These include cooking, heating (dry as well as in presence of moisture), high pressure, pH treatments, physically shearing, extrusion at high temperatures and high torque settings. Each food and each food-processing step contribute to a unique environment in which, DNA undergoes deterioration and proteins are denatured. Hupfer, Hotzel, Sachse, and Engel (1998) reported that temperature and pH influenced degradation of a *cry*1A(b) sequence in *Bt*-maize during preparation of polenta. Thermal stress in combination with pH affects DNA integrity (Bauer, Weller, Hammes, & Hertel, 2003). Murray, Butler, Hardacre, and Vaughan (2007) demonstrate a substantial degradation of endogenous maize DNA at high temperature and torque settings. The degree of DNA degradation adversely affects the limit of detection by qualitative PCR, which is dependent on the effective concentrations of amplifiable DNA sequences (Terry, Harris, & Parkes, 2002).

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Heat transfer, an operation that occurs repeatedly in food manufacture namely cooking, baking, drying, sterilizing or freezing is part of the processing of almost every food. It is therefore important to understand the effect of heat transfer on the target molecules viz. DNA and protein. The investigations on how various food-processing parameters affect the level and quality of DNA are limited.

MON-810 maize and Roundup Ready® soybean (RR-soy) are currently the major GM crops. The qualitative detection of these two transgenic events are based on PCR amplification using primers that recognise regulatory regions of the promoter derived from Cauliflower mosaic virus (CaMV35S) and *Agrobacterium tumefaciens* nopaline synthase (TNOS) terminator, both of which have a significant risk of obtaining false positives. These two elements are natural DNA sequences occurring in the growing environment (Brodmann et al., 1997). Furthermore, the trait specific transgenic gene, the enoyl pyruvyl shikimate phosphate synthase (EPSPS) or *cry*1A(b) can be incorporated in various independent transformation events of various species. The detection of the transgene cannot distinguish between an authorised and unauthorised GMO. In the present study, PCR methods were developed to detect the event: the junction between the newly introduced genes and the integration into the host genome, referred to as event specific methods (Burns, Shanahan, Valdivia, & Harris, 2003). In addition, PCR methods to amplify the cross border sequence of the transgenic construct were also developed. The specific genetic elements of RR-soy and MON-810 maize were targeted and their capability to direct PCR in unit heat operations (a) microwave treatment (MWO) at 540 W and 900 W, (b) autoclaving, (c) baking (180 °C) and (d) freezing (−80 °C) were evaluated. Microwave treatment simulates dry heating, a common method of processing used in post-baking, drying and moisture control of biscuits, crackers, bread and other bakery products. Autoclaving simulates moist heating under pressure, used to manufacture soy drink, textured vegetable protein, soybean meal, maize instant tortilla, etc. The key questions to be answered were: (1) can the DNA be detected when GMO containing grain/seed is subjected to heat processing and (2) what are the implications on PCR based detection of such GMOs? The PCR detection methods described are based on amplifying much smaller segments (200 bp) of the target DNA in processed foods.

2. Materials and methods

2.1. Materials

Standard flours (Certified reference materials, IRMM-410S 5% Roundup Ready® soy and IRMM-413 5% MON-810) containing defined percentages of GMO material prepared and certified by the Institute of Reference Materials and Measurements (Geel, Belgium) were purchased from Fluka, (Riedel-deHaën, Germany). Crushed seed powder of Roundup Ready® (RR) soybeans and Yieldgard® (MON-810) were a gift from Monsanto Co. (St Louis, MO, USA) obtained with the approval of the Research Committee for Genetic Manipulation, Department of Biotechnology, Government of India (India). For simplicity sake, the term RR-soy and MON-810 will be used hereafter to refer Roundup Ready® soy and MON-810 maize, respectively. A conventional non-GM-soy and maize were procured from the pilot plant, Department of Protein Chemistry and Technology, Central Food Technological Research Institute (Mysore, Karnataka, India). Wizard™ magnetic DNA purification kits for food were purchased from Promega Corporation (Madison, USA). *Taq* PCR core kit was obtained from Qiagen Gmbh (Hilden, Germany). Agarose was from Amresco (Solon, Ohio, USA). Ethidium bromide was purchased from Sigma–Aldrich Chemicals Private Limited (Bangalore, India). Mass loading dye (6×) and 100 bp ladder size marker were obtained from Fermentas Inc., (Maryland, USA).

2.2. Oligonucleotide primers

Oligonucleotide primers EPSPS-101F (5′-AAGTCGATCTCCCACCGGTC-3′), EPSPS-101R (5′-TTGCCCGTATTGATGACGTC-3′), EPSPS-210F (5′-ATCGAACTCTCCGATACGAAG-3′), NOS-210R (5′-CCCATCTCATAAATAACGTCAT-3′), GTS-181F (5′-GCATGCTTTAATTTGTTTCTAT-3′), GTS-181R (5′-ATCTTGAACGATAGCCTTTCCT-3′), GTS-121F (5′-TAGCGCGCAAACTAGGATAAA-3′), GTS-121R (5′-CGGTGATGCGCGTTTCA-3′), hsp-95F (5′-AGCACCTCGACCTCAGGGTT-3′) and cry1A(b)-95R (5′-TTGTAATGCAGATACCAAGCG-3′) employed in this study were designed using Primer Express™ 2.0 software (Applied Biosystems, Foster City, CA, USA), to amplify distinctly different sized products of the host genome-junction and cross border junction DNA sequences unique to either RR-soy or MON-810. The primers chosen from literature, target gene, primer sequence and expected amplicon lengths are listed in Table S1 (Supplementary data). The single-copy lectin gene (*Le*1) of soy and zein (*Ze*1) of maize were selected as internal controls. The primers synthesised by Sigma–Genosys (Sigma–Aldrich Chemical Private Limited, Bangalore, India) were diluted to a final concentration of 1 nmol μL^{-1} with nuclease free water and stored at −20 °C.

2.3. Preparation of raw material

The RR-soy and MON-810 crushed seed powders were further ground to a fine powder and sieved (Aperture size of 450 μm). One% RR-soy and MON-810 flour were prepared in-house by spiking the defatted conventional soy or maize flour with an appropriate quantity of RR-soy or MON-810 flour, respectively. Adequate care was taken to avoid contamination during spiking and the subsequent sieving step. The proportion of GM-ingredient in the spiked samples was deliberately set at 1% (w/w) to assess the degree of the expected effects at a level that would be of practical interest for threshold labelling.

2.4. Heat processing operations

All of these experiments were bench top experiments. Utmost care was taken to avoid the intermixing of transgenic samples during heat processing. All the processing steps were carried out individually and in triplicate.

2.4.1. Autoclaving

One gram of flour was directly autoclaved using an automated high-pressure steam steriliser (HA-240MIV/300 mV, Hirayama Manufacturing Corporation, Japan) at 121 °C and 15 lbs pressure for 20 min. The time did not include raising and lowering of the temperature.

2.4.2. Microwave treatment

One gram of flour was spread uniformly and heated in a microwave oven [(MWO) LG, India] at 540 and 900 W for 2 min. The MWO exposure was restricted to 2 min as charring occurred when subjected to 3 min.

2.4.3. Baking

Biscuits containing 0.67 wt.% level of RR-soy and MON-810 were prepared by conventional baking. The dough containing GMOs was sheeted to about 3.5 mm thickness and cut into biscuit shapes and placed on a tray. Biscuits were baked at 180 °C for 15 min and cooled to room temperature.

2.4.4. Freezing

One gram of the flour in an air tight container was kept at −80 °C for 48 h.

2.5. Extraction of genomic DNA

DNA was extracted from finely ground individual powders (0.2–0.5 g) using Wizard™ Magnetic DNA purification kits following the manufacturers protocol, with some modifications. The RNase (10 mg ml^{-1}) added to the autoclaved and MWO flours suspended in lysis buffer was increased from 10 to 25 μL and incubated at 25 ± 2 °C for 10 min. The suspensions were centrifuged at 13,000*g* for 10 min. Fifty microlitres of paramagnetic particle (PMP) solution were added to the supernatant, washed thoroughly with 70% ethanol and air dried. Bound DNA was eluted in 100 μL of nuclease free water and PMPs removed by magnetic separation. The DNA concentration was checked by spectrophotometry (UV-601, Shimadzu, Japan) after dilution. The DNA concentration and impurity factor (A_{260}/A_{280} ratio) were recorded. DNA integrity was evaluated by 1% agarose gel electrophoresis in 0.5× TBE (5× TBE: 450 mM Tris–Borate, 10 mM EDTA) at 100 V. The Gel Doc 2000™ documentation system with Quantity One software (Bio-Rad Laboratories Inc., CA, USA) was used to visualise the ethidium bromide stained DNA.

2.6. Qualitative PCR for detection of RR-soy and MON-810 DNA fragments

The PCR reactions performed in a total volume of 50 μL contained 1× PCR buffer containing 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 μM dNTP and 1.5 U of *Taq* polymerase. The concentration of template DNA was varied depending on the percentage of GMO and the degree of heat processing (unprocessed: 80 ng; autoclaved: 1000 ng; MWO: 500 ng and baking: 850 ng). The PCR thermal profile included an initial 10 min denaturation step at 95 °C, followed by 40 cycles of denaturation at 94 °C (20 s), annealing at either 60 °C (lectin inner, lectin outer, CaMV35S-CTP, EPSPS-TNOS) or 61 °C (TNOS-soy genome, EPSPS, *hsp*70 intron1-*cry*1A(b)-95) or 59 °C (soy genome-CaMV35S) (60 s) and extension at 72 °C (30 s). A final extension step was performed at 72 °C for 3 min. The thermal cycling program used for Zein (*Ze*1) (Hohne, Santisi, & Meyer, 2002), CaMV35S promoter-*hsp*70 intron1 and *hsp*70 exon1 (Zimmermann, Hemmer, Liniger, Luthy, & Pauli, 1998), maize genome-CaMV35S promoter (ISO/FDIS 21569: 2005) and *hsp*70–*cry*1A(b) (Yamaguchi, Sasaki, Umetsu, & Kamada, 2003) were as reported. The PCR was carried out using a Gene Amp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplified DNA fragments were separated by electrophoresis in a 2% agarose gel (supplemented with 0.5 μg ml^{-1} ethidium bromide) in 0.5× TBE buffer at 150 V and visualised using The Gel Doc 2000™ documentation system (Bio-Rad Laboratories Inc., CA, USA). Samples were analysed in triplicate.

2.7. Direct sequencing analysis of PCR products

The PCR products were purified using a PCR product purification kit (Promega, Madison, USA) following the manufacturer's instructions and subjected to direct dideoxy sequencing on an automated DNA sequencer (ABI 310, Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Influence of processing on DNA quantity and integrity

The DNA extracted from 200 mg of MWO and baked products were quantifiable. In contrast, the extracted DNA from autoclaved flours was not quantifiable. Increasing the initial sample size to 500 mg yielded sufficient DNA (30 ng/mg flour). The quantity of extractable DNA is therefore affected by heat-processing (Fig. 1). Yoshimura et al. (2005a) reported that increasing the initial weight of cornstarch from 2 to 5 g resulted in the extraction of sufficient DNA. Statistical analyses performed for DNA extracted reveals a significant impact of the processing method applied (Fig. 1). The quantity of extracted DNA varied with the severity of the processing method used. The highest yields were obtained with unprocessed flours, followed by baking, MWO and autoclaved. Within the MWO model system, flours microwaved at 900 W yielded less DNA than the 540 W. The most notable effects occurred during autoclaving, wherein the yield of DNA was <70% of unprocessed

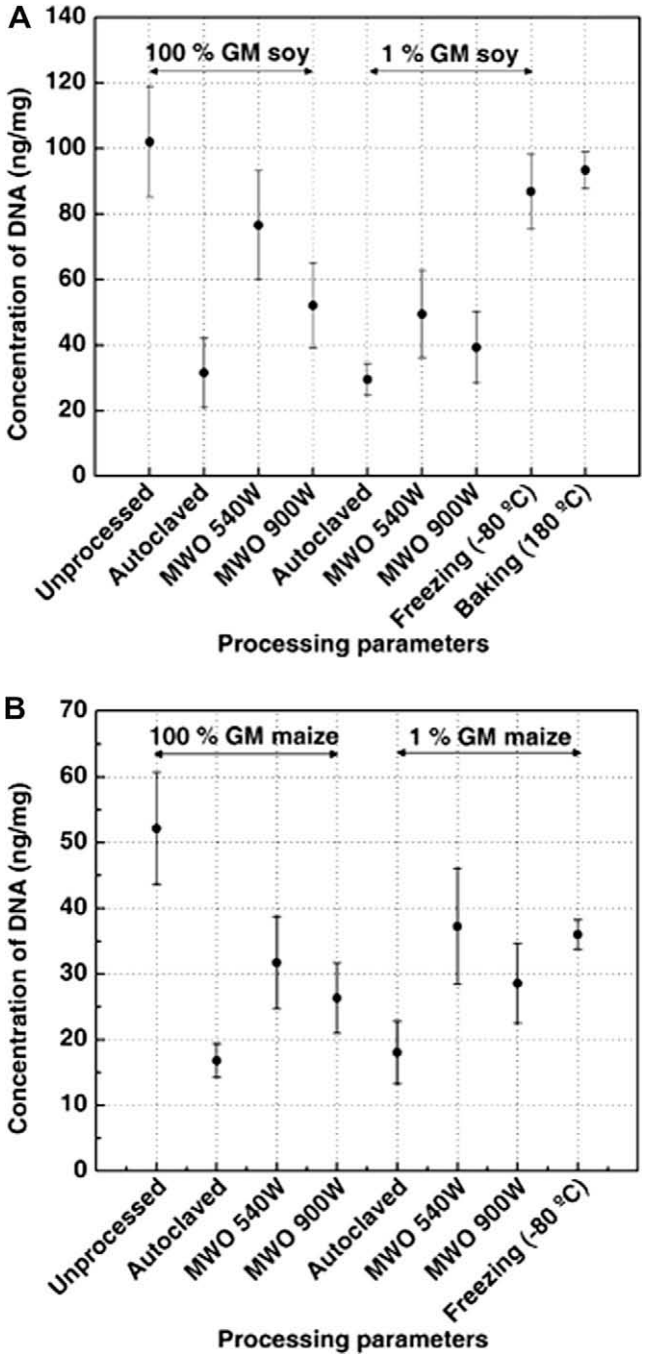

Fig. 1. Concentration of DNA extracted from model heat processed samples. (A) RR-soy and (B) MON-810. Vertical bars denote 95% confidence intervals. The mean values are derived from five extractions for each model system.

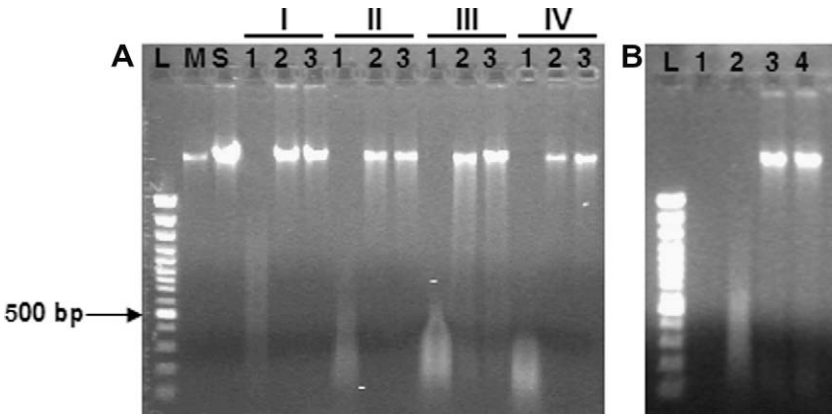

Fig. 2. Degradation of DNA induced by (A) autoclaving and microwave treatment. I: 100% RR-soy; II: 1% RR-soy; III: 100% MON-810; IV: 1% MON-810. Lane L, 100 bp ladder size marker; Lane M, unprocessed MON-810; Lane S, unprocessed RR-soy; Lane 1, autoclaved; Lane 2, MWO 540 W; Lane 3, MWO 900 W, (B) baking and freezing. Lane 1, control biscuit; Lane 2, 0.67 wt.% GM-biscuit; Lane 3, 1% MON-810 and Lane 4, 1% RR-soy. DNA loaded was 250 ng for all samples except autoclaved and baking where 1000 ng was loaded.

flours. Freezing at −80 °C also yielded less DNA. In addition, it is also evident that the DNA yield from soy was higher when compared to maize, either unprocessed or heat processed (Fig. 1). This lower yield in maize can be ascribed to the albuminous nature of the grain. Differences in the DNA size, a parameter of DNA integrity is dependent on the degree of processing the sample has been subjected to. DNA extracted from unprocessed RR-soy and MON-810 appear as clear distinct bands of high intensity >3000 bp (the highest band of the DNA marker 3000 bp) (Fig. 2). The DNA from autoclaved flours shows the complete absence of the longer DNA with the concomitant appearance of a smear smaller in size indicating DNA deterioration (Fig. 2). In contrast, a major portion of the DNA from the MWO treated is still intact with minimal smearing (<500 bp) when compared with DNA ladder accounting for some sheared DNA. These results reckon that the shearing of DNA during MWO though not severe, yet occurs. The fragmentation of DNA was most extensive in the autoclaved flours with the long DNA fragment being totally absent (Fig. 2, Lane 1). This is probably because of the intensity of the heat and the presence of moisture during autoclaving. Exposure of DNA to high temperature and pressure is known to cause fragmentation of DNA and breaks in strands thus reducing the average fragment size. Nevertheless DNA fragments <500 bp are present reckoning that amplification of DNA through PCR is still possible. The quantity of DNA loaded for detection was approximately five times greater for the autoclaved in comparison to the MWO. These results reckon that heat processing not only affects the yield of DNA but also has a significant impact on DNA deterioration. Low temperatures (−80 °C) had no effect on the DNA. The impurity factor defined by the A_{260}/A_{280} ratio showed values ranging from 1.6 to 1.8, which indicate that the DNA extracts are sufficiently pure for PCR amplification.

3.2. Specificity of the oligonucleotide primers

On the basis of published recombinant DNA sequences of RR-soy and MON-810 maize and data obtained by sequencing, primer pairs were designed to distinctly amplify specific DNA sequences for RR-soy and MON-810 (Table S1 (Supplementary data)). DNA degradation and amplicon length are crucial factors in the success of a PCR designed to identify GMOs in highly processed foods (Van Hoef, Kok, Bouw, Kuiper, & Keijer, 1998). The primers used in this study were designed to generate shorter amplicons, ~200 bp, targeting the trait (EPSPS gene), construct (EPSPS-NOS) and event (integration site: soy genome-CaMV35S promoter and NOS-soy genome) specific gene sequences of RR-soy. The primer pair *hsp*–95F/*cry*1A(b)-95R amplifies a 95 bp fragment unique to MON-810, which incidentally has a high GC content. Specificities of the primers designed were individually assessed and validated using DNA extracts of IRMM-410S (5% RR-soy) and IRMM-413 (5% MON-810). Compact and distinct bands of the expected length are visible (Fig. 3). These amplicons are visibly absent in the non-GM-soy and -maize (Fig. 3A and B). Specificity of the PCR, was

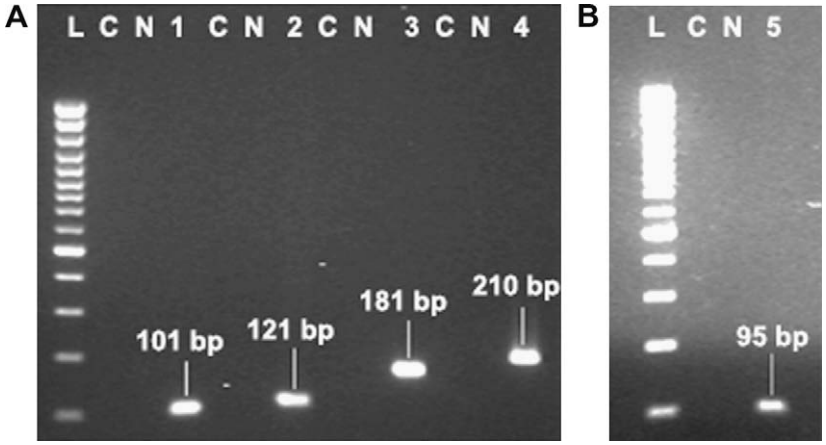

Fig. 3. Agarose gel electrophoresis of PCR products amplified from genomic DNA of (A) RR-soy (IRMM-410S). Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, non-GM-soy; Lane 1, EPSPS (101 bp); Lane 2, TNOS-soy genome (121 bp); Lane 3, Soy genome-CaMV35S promoter (181 bp); Lane 4, EPSPS-TNOS (210 bp) and (B) MON-810 (IRMM-413). Lane N, non-GM maize; Lane 5, *hsp*70 intron1–*cry*1A(b) (95 bp).

further evidenced when DNA isolated from rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) showed no amplification when used as template (data not shown).

3.3. Effect of microwave treatment and autoclaving on detection

The detection of a species-specific single copy gene is often used to assess the amplifiability of DNA from soybean-derived products. The primer pairs GMO1/GMO2 and GMO3/GMO4 directed to amplify a 413 bp and an internal 118 bp fragment, respectively, specific for soy lectin *Le*1 gene (Meyer & Jaccaud, 1997) were used. The 413 bp amplicon was discernable in the MWO but absent in the autoclaved flours (Fig. 4A). This result is not unexpected considering the highly fragmented DNA of <500 bp in the autoclaved samples (Fig. 2). In contrast, a smaller internal fragment (118 bp) was amplifiable in all the heat processed samples (Fig. 4B). This result emphasises the suitability of primers that generate shorter PCR products when analysing processed foods. This observation was the premise for designing the other primers listed in Table S1 (Supplementary data).

The primer pair EPSPS-101F/EPSPS-101R was designed to amplify a 101 bp region of EPSPS gene. The amplicon size was minimised to obtain the highest possible sensitivity without the interferences of primer–dimer formation. A 101 bp fragment was observed in both the autoclaved and MWO processed RR-soy (Fig. 4C). The EPSPS gene of *A. tumefaciens* has been used in developing several herbicide tolerant GMOs, therefore the specificity is restricted to this trait and not the GM-crop. Construct specific primer pair EPSPS-210F/NOS-210R attaches to the EPSPS sequence and the adjacent nopaline synthase gene which is used to end the expression of EPSPS in RR-soy. Positive amplification of the 210 bp fragment was observed in both the MWO samples (Fig. 4D). The 210 bp fragment was amplified in the autoclaved 100% but not in 1% RR-soy (Fig. 4D). The primer pair 35SF-2/petuR-1 amplifies a 172 bp junction sequence between the CaMV35S promoter and the petunia hybrid chloroplast transit signal sequence (ISO/FDIS 21569: 2005). The 172 bp product is observed in both the autoclaved and MWO processed flours (Fig. 4E). It is feasible that this construct is present in several GMOs; therefore two independent GMO events cannot be distinguished by this method. Ultimate specificity for GMO detection is achieved by the use of an event- or line-primers that identifies a DNA sequence that spans the junction between the newly introduced genes and the host plant DNA. This site of integration in the plant genome will be unique to a GMO and therefore will specifically identify only the GMO in question. For RR-soy event specific detection, primer pairs GTS-181F/GTS-181R and GTS-121F/GTS-121R that encompass the sequence of the integration site of the transgene and soy plant genome were designed to amplify 181 and 121 bp, respectively. The pair GTS-181F/GTS-181R amplifies a 181 bp region between the soy plant genome and CaMV35S promoter whereas GTS-121F/GTS-121R pair detects the NOS terminator and soy plant genome integration site at the other end. A 181 bp product was visible in both autoclaved and MWO samples (Fig. 4F). A 121 bp fragment was amplified in the autoclaved and MWO RR-soy flours (Fig. 4G). The results reckon that these intergenic DNA sequences were stable to the processing deterioration. The lower limit of detection for the amplification of these RR-soy line-specific genes under this simulated model study was 1% (Fig. 4F and G).

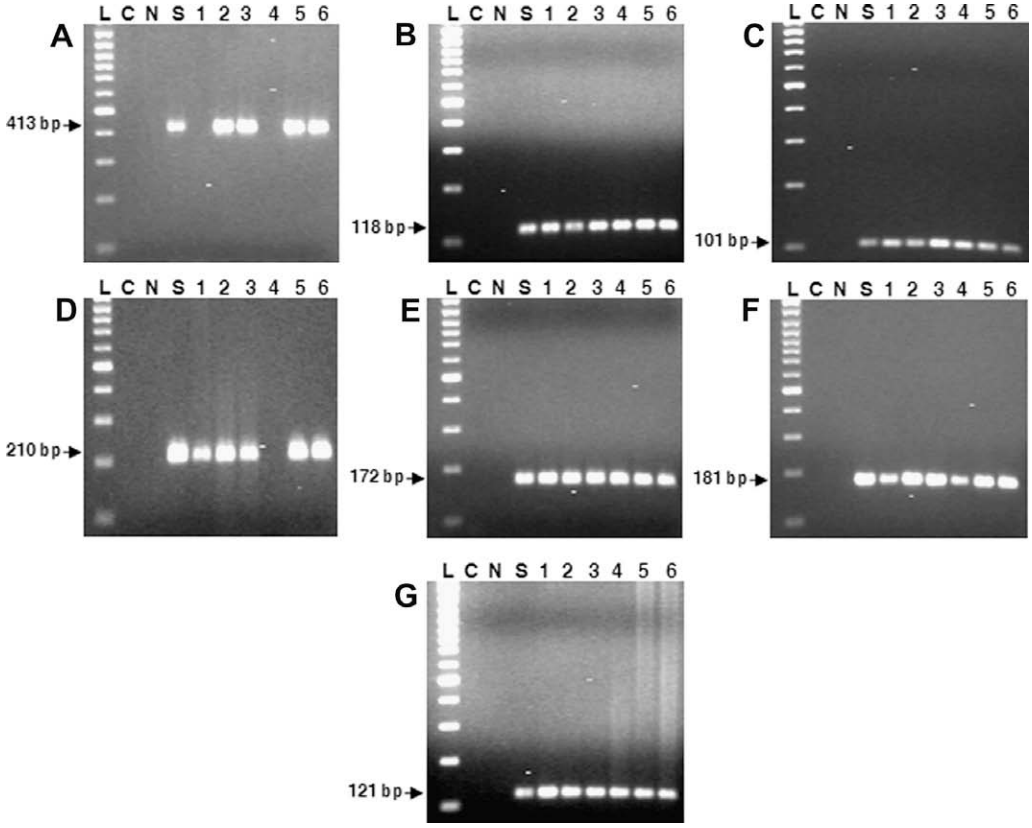

Fig. 4. Agarose gel electrophoresis of PCR products amplified from genomic DNA of heat processed RR-soy. The arrows indicate the expected PCR amplification products. Primer pairs (Table S1 in the Supplementary data section) were used for the detection of lectin outer (A), lectin inner (B), EPSPS (C), EPSPS-TNOS cross border (D), CaMV35S promoter-Petunia-CTP cross border (E), soy genome-CaMV35S promoter junction region (F) and TNOS-soy genome junction region (G). Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, non-GM-maize in (A) and (B) non-GM-soy in (C)–(G); Lane S, unprocessed 100% RR-soy; Lane 1, autoclaved (100% RR-soy); Lane 2, MWO 540 W (100% RR-soy); Lane 3, MWO 900 W (100% RR-soy); Lane 4, autoclaved (1% RR-soy); Lane 5, MWO 540 W (1% RR-soy) and Lane 6, MWO 900 W (1% RR-soy).

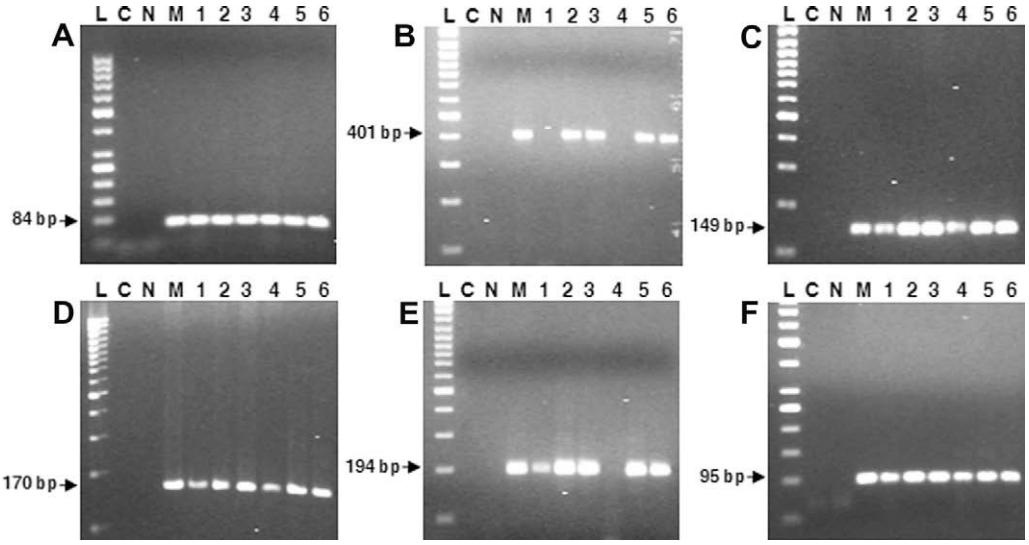

Fig. 5. Agarose gel electrophoresis of PCR products amplified from genomic DNA of heat processed MON-810. The arrows indicate the expected PCR amplification products. Primer pairs (Table S1 in the Supplementary data section) were used for the detection of Zein gene (A), CaMV35S promoter–*hsp*70 intron1 cross border (B), CaMV35S promoter-*hsp*70 exon1 cross border (C), maize genome-CaMV35S promoter junction region (D), *hsp*70 intron1–*cry*1A(b) cross border (E) and *hsp*70 intron1–*cry*1A(b) smaller fragment (F). Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, non-GM-soy in (A) non-GM-maize in (B)–(F); Lane M, Unprocessed 100% MON-810; Lane 1, Autoclaved (100% GM); Lane 2, MWO 540 W (100% GM); Lane 3, MWO 900 W (100% GM); Lane 4, Autoclaved (1% GM); Lane 5, MWO 540 W (1% GM) and Lane 6, MWO 900 W (1% GM). A 50 bp ladder size marker was used for A and F.

The amplifiability of DNA extracted from the processed GM-maize (MON-810) was confirmed using a primer pair ZeinF/ZeinR for the endogenous reference gene Zein (*Ze*1) (Hohne et al., 2002). An 84 bp amplicon was detected in both the MWO and autoclaved MON-810 flours (Fig. 5A). The gene stretch of CaMV35S promoter, followed *hsp*70 intron1 and the *hsp*70 exon1 is unique and specific to only MON-810. The primer pairs mg1/mg2 and mg3/mg4, which amplify parts of the CaMV35S promoter, *hsp*70 intron1 and *hsp*70 exon1 have been previously used for the specific detection of MON-810 (Zimmermann et al., 1998). The outer primers (mg1 and mg2) yield an amplicon of 401 bp spanning the sequence between the CaMV35S promoter and *hsp*70 intron1 whereas the inner primers (mg3/mg4) amplify a 149 bp fragment comprising the CaMV35S promoter and *hsp*70 exon1. When the primer pair mg1/mg2 was used positive amplification was observed only with the MWO processed samples. The signal for 401 bp was not discernable in the autoclaved flours (Fig. 5B). In contrast, the smaller 149 bp inner fragment was observed in both MWO and autoclaved maize samples (Fig. 5C), clearly demonstrating that primers targeting shorter fragments are more effective in detection of GMOs in processed foods.

The primer pair VW01F/VW03R (ISO/FDIS 21569: 2005) is MON-810 specific detecting the integration site between the maize genome and CaMV35S promoter. Using this primer pair a 170 bp fragment was observed for both autoclaved and MWO samples. The fluorescence intensity for autoclaved samples was visibly lower than that observed for the MWO (Fig. 5D). These PCR results are concurrent with the observed extensive fragmentation of the extracted DNA following autoclaving (Fig. 2). The primer pair MonF/MonR has often been used to amplify a 194 bp fragment, by hybridizing with the cross border region between *hsp*70 and *cry*1A(b) of MON-810 maize (Yamaguchi et al., 2003). Though the signal for the 194 bp amplicon was visible in the autoclaved 100% MON-810, it was not discernable in the 1% MON-810 (Fig. 5E). This can be explained by the fact that the copy number of the inserted sequence is much lower. Moreover, with increased processing, the size of the genomic DNA fragments available for detection also decreases. In consequence a primer pair hsp-95F/

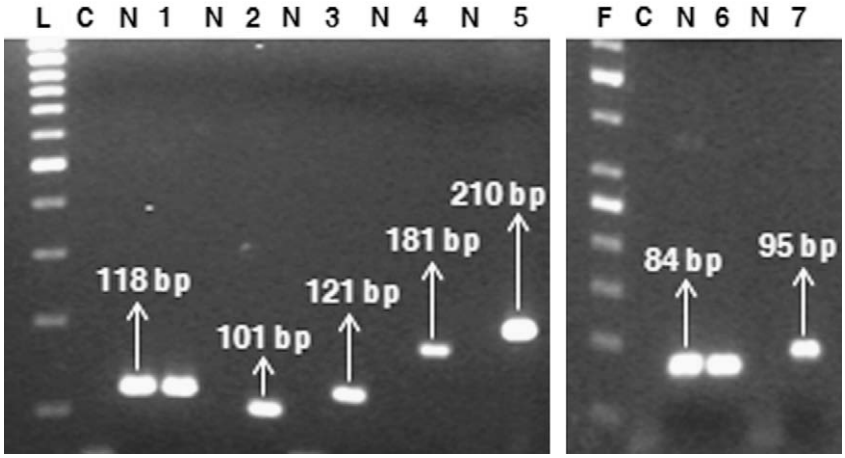

Fig. 6. Agarose gel electrophoresis of PCR products amplified from genomic DNA of baked product. Lane L, 100 bp ladder size marker; Lane C, premise control; Lane N, control biscuit; Lane 1, lectin inner (118 bp); Lane 2, EPSPS (101 bp); Lane 3, TNOS-soy genome (121 bp); Lane 4, Soy genome-CaMV35S promoter (181 bp); Lane 5, EPSPS-TNOS (210 bp); Lane F, 50 bp ladder size marker; Lane 6, Zein (84 bp) and Lane 7, *hsp*70 intron1–*cry*1A(b) (95 bp).

cry1A(b)-95 R was designed to amplify a much smaller inner fragment of the 194 bp sequence. A 95 bp fragment was amplified in both the autoclaved and MWO treated flours (Fig. 5F). These results further advocate that amplification of smaller fragments facilitate detection of GMOs in highly processed foods. The sequences of all the PCR products shown in Figs. 3–5 were verified by direct sequencing.

In the present study, the concentration of template DNA was varied (80–1000 ng) depending on the percentage of GMO and the degree of heat processing. The DNA required for amplification of the transgenes from MWO heated 100% MON-810 and RR-soy was ~4–5 folds greater than that of the unprocessed flours. MON-810 and RR-soy flours subjected to autoclaving required more template DNA as compared to MWO treated. A 9–13 fold increase of the autoclaved processed GMO-DNA was required for positive amplification over that of the raw material. It is clearly evident that the PCR assays of heat processed samples require a higher concentration of template DNA. It is reckoned that as the defined percentage of GMO decreases, a higher amount of template DNA is required.

In all the above PCR assays, appropriate controls were used. PCR set up without template DNA served as premise control labelled as Lane 'C' (Figs. 3–5). PCR set up with template DNA from the conventional non-GM-soy or non-GM-maize served as a negative control (Figs. 3–6, Lane N). PCR set up with unprocessed 100% RR-soy and MON-810, served as positive controls (Lanes S and M, respectively). The absence of any amplification product in the premise control indicates the absence of contaminating DNA from the environment, buffers or reagents used.

3.4. Detection of RR-soy and MON-810 under practical conditions: effect of baking

Baking was chosen as a practical food processing method for monitoring DNA degradation and detection of GMOs. Biscuits containing 0.67 wt.% each of RR-soy and MON-810 were prepared. Control biscuits contained exactly the same amount of conventional soya and maize flours. The DNA of longer length (>3000 bp) was visibly absent in the baked products (Fig. 2B). The extracted DNA showed a smear of short DNA fragments <500 bp with a total loss of the long DNA fragment as observed in the processed flours (Fig. 2B). These results are in close agreement with the DNA smearing reported for bread and cookies (Gryson, Dewettinck, & Messens, 2007; Straub, Hertel, & Hammes, 1999). Although the extracted DNA was fragmented this difference was not observed in the impurity factor (A_{260}/A_{280} = 1.7). The quantity of DNA extracted was greater than that extracted from the same amount of autoclaved or MWO processed flours. Although the extracted DNA was <500 bp in length, the sequences required for the construct and line specific event detection were intact as evidenced by the positive PCR amplification of these sequences (Fig. 6). These results further affirm that the newly designed PCR systems dovetailed to detect RR-soya and MON-810 specific sequences exhibit sufficient specificity for qualitative PCR analyses of a practical unit operation of food processing. The near to identical signal intensity for both the plant specific (lectin/zein) and line-specific sequences of RR-soy/MON-810 (Fig. 6A and B) suggest the usefulness of these assays in the quantitation of GMOs in processed foods.

4. Discussion

It is generally considered difficult to accurately determine the GMO content of processed foods. The suitability of isolated DNA as an analyte for PCR based detection or characterisation technique depends on the concentration, purity, and integrity, each of which, may be influenced by the sample matrix and extraction technique (Terry et al., 2002). Although several factors are important in achieving successful amplification, the template DNA concentration that is often overlooked was also found to be a critical factor in the present study. Despite the genomic DNA of autoclaved RR-soy and MON-810, not being visible by agarose gel electrophoresis (Fig. 2), the PCR detection of smaller fragments suggests that the extracted DNA was capable of directing amplification in the subsequent PCR (Figs. 4 and 5). The copy number of the transgene decreases with considerable physical damage and fragmentation as a result of high temperature and pressure associated with food processing. It can be raised above the detection limit by increasing the amount of template DNA used in PCR (Yoshimura et al. 2005a, 2005b). The results of this study reconfirm that the amount of the DNA present in autoclaved GM-foods is several orders of magnitude lower than the DNA found in the corresponding unprocessed samples (Fig. 1). The difficulty in recovering amplifiable DNA target sequences increases proportionately to the severity of processing. The higher the temperature and/or pressure, and longer the processing time, greater the damage to DNA (Fig. 2). Degradation of genomic DNA is primarily linked to processes carried out at low pH and increases dramatically with thermal stress. DNA fragmentation has been observed as a result of enzymatic hydrolysis (Klein, Altenbuchner, & Mattes, 1998) and to a certain extent on milling and grinding owing to the impact of both shear forces and mechanical stress (Moreano, Busch, & Engel, 2005). The quantity of DNA and DNA degradation in complex matrices, differences in DNA recovery and presence of PCR inhibitors are factors that direct a successful amplification. Baking served not only as a practical food processing condition, but also the effect of the food matrix could be evaluated. In the baking process although a significant deterioration of intact DNA was visible, amplifiable sequences still existed. These results indicate that the complex food matrix is not a protective factor. A pH-dependent fragmentation of DNA has also been reported during baking (Straub et al., 1999). Degradation of both transgene and endogenous reference gene sequences during the processing of soybean to soy powder, tofu and soymilk have been reported by Chen, Wang, Ge, and Xu (2005). Murray et al. (2007) using maize nuclear sequences encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase and cell wall invertase show that maize DNA was degraded by a number of heat processing procedures including extrusion at high temperatures and/or high torque settings.

Sufficient amounts of DNA may be extractable following heat processing, yet it is the extent of degradation, which is a critical factor in determining whether a specific sequence can be amplified or not. Our results also demonstrate that amplification of <200 bp fragments are most suited for the detection of GMOs in highly processed foods. Our results are in agreement with those of Lin, Wei, Lin, and Shih (2006) who showed that the effect of DNA degradation from heating could be reduced by targeting shorter genes. Hupfer et al. (1998) showed that DNA fragments of 1914 bp were no longer detectable after boiling *Bt*-176 maize flour for 5 min at pH 2 whereas boiling for 60 min at pH 8.5–9.5 a shorter 211 bp fragments remained detectable. Therefore, it was recommended that the primers used in PCR detection of GMOs in processed foods, yield amplification products of 150–300 bp (Hupfer, Hotzel, Sachse, & Engel, 1999). This is further validated from this study as all the primers designed by us amplify fragments of ~200 bp. It is also likely that the degradability of the PCR target sequences also depends on the GC content. DNA regions containing high GC content are generally considered to be more heat stable. As observed all the target sequences with 40% GC content or higher were amplifiable (Table S1 (Supplementary data)). Yoshimura et al. (2005b) demonstrated that the construct specific region of MON-

810 and CaMV35S was more strongly degraded than the SSIIb1–4 as the GC content was lower.

Yoshimura et al. (2005) based on their heat processing studies conclude that the amplification regions used to quantitate recombinant and taxon-specific DNA should be closely similar in terms of size. To this end the primers designed in this study to amplify 101 bp (EPSPS-101F/EPSPS-101R) and 121 bp (GTS-121F/GTS-121R) meet this criterion and therefore would be very useful to quantify RR-soy in processed foods owing to their closeness in size to the 118 bp *Le*1 taxon specific gene of soya. In addition, the primer pair hsp-95F/cry1A(b)-95R amplifies a 95 bp fragment of MON-810, which once again in similar in size to the 84 bp product of *Ze*1 the maize taxon specific product.

5. Conclusion

The models of simulated food processes revealed a strong correlation between the degree of processing and the recovery of target DNA. Such simulation studies can be used to predict the availability of target DNA sequences for GMO detection. We believe that they provide an appropriate and a reliable basis that can be extrapolated to commonly used food technological processes such as retorting and rendering, which combine heat and high pressure to sterilise, and cook food and animal feed, respectively. When analysing products that have undergone processing, one can expect the DNA to be highly fragmented and hence can choose an appropriate method to meet the testing requirement, preferably one which targets smaller fragments. The effects of processing steps on DNA degradation must be clearly understood for each processed food on a case to case basis before any DNA based method can be applied to detect GMOs. The present study therefore contributes to the accumulation of basic data necessary to consider the impact food processing has on DNA based detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.04.028.

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