Combined Effects of Temperature, Pressure and Low pH on the Amplification of DNA of Plant Derived Foods

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Abstract

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The effect of food processing on the DNA integrity was studied by means of PCR amplification of soybean, transgenic MON 810 and non-transgenic maize, bean, and pea. The degree of DNA degradation was checked by PCR and visualised by agarose gel electrophoresis. The conditions of technological treatment such as temperature, pH, pressure, and their combination may negatively influence the integrity of DNA in processed foods and hence PCR detection of food components. The DNA over 300 bp was amplifiable when mild processing parameters up to 100°C were performed at approximately neutral or low acidic pH. The autoclaving (12°C; 0.1 MPa) significantly reduced the size of amplifiable DNA in the time dependant manner and that was intensified by acidic pH. The maximum amplicons length achieved for highly processed matrices was 300 bp. The major impact on the DNA integrity was exerted by the combination of pressure, temperature, and low pH.

Keywords: GMO; food processing; DNA degradation; DNA amplification

Safety and quality of food is the primary focus of the competent authorities responsible for food control. Processed food often contains material with allergenic potential that is obligatory listed on the labelling (Directive 2007/68/EC – European Commission 2007). Apart from that, several other potential food allergens can represent a hazard for susceptible individuals.

Soybean (*Glycine max* L. Merrill) crop belongs to the main foodstuffs responsible for allergic reactions worldwide, but the products from it are widespread in a variety of processed foods due to its well-documented health benefits (Brouns 2002). Soybean major allergens are vicilin and legumin (Batista *et al*. 2007). Other legumes also display allergenic properties that are not yet included in the list of allergens (Directive 2007/68/EC – European Commission 2007). Non-specific lipid transfer protein (nsLTPs) is the key allergen of the green bean (*Phaseolus vulgaris*) (Zoccatelli *et al*. 2010). Potential main allergens from pea seeds (*Pisum sativum*) are vicilin and convicilin occasionally displaying cross-reactivity to the peanut allergen (Wensing *et al*. 2003; Sanchez-Monge *et al*. 2004). Genetically modified crops may, potentially, also cause allergy (Taylor 1997). Maize and soybean represent the majority of the genetically modified food crops (James 2009). Maize MON 810 (*Zea mays* L.) is the only transgenic cultivar grown in the EU, while transgenic soybean is only imported. An additional aspect of the food quality is the adulteration of valuable components with

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cheaper ones. Beans (*Phaseolus vulgaris*) are traditionally used for chestnut adulteration in cakes and chestnut pureé as their organoleptic feature is not significantly different (Krahulcová *et al*. 2003). Soybean and pea proteins are used as inexpensive substitutes for milk and meat protein in many foodstuffs including meat products such as ham, sausage, etc. (Alli & Abdolgader 2000; Štefanovičová *et al*. 2000).

The biological methods for the detection of food components are generally protein or DNA based. The method of choice for DNA detection and quantification is PCR (Michelini *et al*. 2008).

DNA-based methods using PCR are widely used for food analysis. Their efficacy strongly depends on the DNA stability during food processing and on the efficiency of DNA recovery. The thermal treatment with temperatures over 200°C considerably reduces the size of the extracted DNA (Hrnčírová *et al.* 2008). The food processing elicits DNA degradation to such an extent that food analysis based on DNA may be affected (Meyer *et al*. 1996; Straub *et al*. 1999; Gryson *et al*. 2002; Bergerová *et al*. 2010). DNA extractability depends on the particle size and increases with the time of boiling, reaching a maximum after 30 minutes. The reason for better extractability might be the different degradation of high-molecular-weight DNA at the beginning of the heat treatment and better extractability of smaller food matrix particles (Hrnčírová *et al.* 2008).

Food processing associated DNA degradation may affect the quality of PCR analytical results based on DNA in cereal and plant derived foods (Meyer *et al*. 1996; Gryson *et al*. 2002). Thus, the PCR detection of soybeans in baked bread was affected (Straub *et al*. 1999).

On the other hand it was also shown that DNA degradation caused by food processing demonstrated no effect on the relative quantification of transgenic content (Debode *et al*. 2007; Bergerová *et al*. 2010). Hird *et al.* (2006) also presented successful quantification of extremely processed meat products by real-time PCR when amplicons up to 351 base pairs were amplifiable with better specificity than the smaller ones.

In this study, the effect of food preservation on the integrity of DNA with subsequent plant species detection by PCR is presented. The combination of temperature (100°C, 120°C), low pH, and pressure represents the technology generally used for vegetable preservation by sterilisation. A set of primers was designed to obtain different sizes of amplicons capable of monitoring the degradation of DNA in soybean, maize, bean, pea, and their products.

MATERIAL AND METHODS

Plant material. The soybean (*Glycine max* L.), maize (*Zea mays* L.), beans (*Phaseolus vulgaris*), and pea (*Pisum sativum*) seeds were purchased from local markets in Bratislava, Slovak Republic. The modified samples of MON 810 maize were obtained from Agrokomplex Kunovice, Czech Republic.

Conditions of sterilisation and autoclaving. The dry soybean, maize, and pea seeds were soaked in distilled water for 24 h and than together with fresh common bean pods, 75 g each, were pickled in three different brines. The volume of the glass container with metal lid was 150 ml, that of brine was 75 ml. The control brine was salty (pH 7.6; 20 g table salt per 1 l of drinking water). The first brine was sweet and sour (pH 2.25) containing 20 g table salt, 100 g saccharose, 250 ml 8% vinegar, and 1 l of drinking water. The second sweet and sour brine (pH 4.25) was the same as the first one except that only 3 ml vinegar was used. The samples of soybean, maize seeds, bean, and pea pods were processed by autoclaving (120°C; 2 min, 5 min and 10 min, 0.1 MPa) and/or sterilised in the water-bath (100°C; 10 min, 20 min, and 30 min). The samples were left in these brines for 3 weeks, than dried and 5 g of each was used for DNA extraction.

DNA extraction. The sterilised samples were homogenised with a mixer AY47R1 (Moulinex, Barcelona, Spain) providing fine powder. The powder was subsequently sieved to obtain particle size in the range of 0.2–0.4 mm. DNA was then extracted. 200 mg of each of the powder fractions were extracted in triplicate using cetyl trimethyl ammonium bromide (CTAB) method (ISO 2005), or GeneSpin kit (GeneScan, Teltow, Germany) and also by Wizard Genomic DNA Purification Kit (Promega, Madison, USA) or DNeasy® Plant Mini Kit (Qiagen, Valencia, USA) for use as positive control. CTAB methods was modified in such a way that to the amount of 200 mg of dried powder dissolved in 600 µl of deionised water 800 µl of extraction buffer, 30 µl of proteinase K (20 mg/ml; Sigma-Aldrich, Steinheim, Germany) and 30 µl of RNAse (10 mg/ml; Serva, Heidelberg, Germany)

Table 1. Qualitative PCR conditions

Step	Temperature (°C)	Time
Initial denaturation	95	5 min
Denaturation	95	30 s
Annealing	65	30 s
Extension 40 cycles	72	1 min
Final extension	72	10 min
Cooling	4	

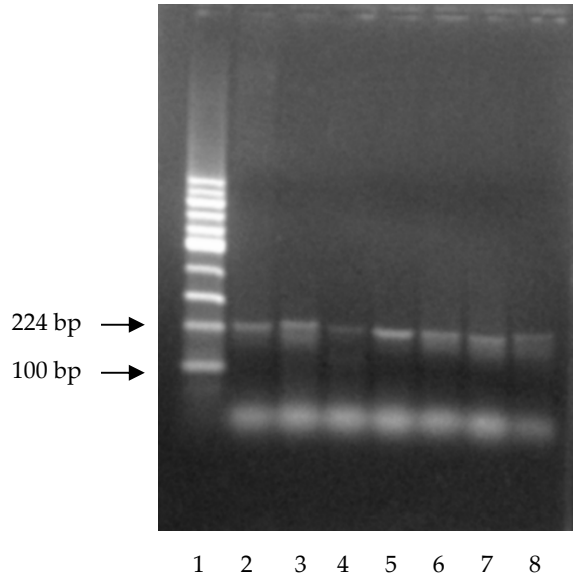
were added and the mixture was placed on ice for 10 minutes. Than 2 fold amount of 70% ethanol was added and the resulting mixture was centrifuged at 0°C at high speed setting. The pellet was dissolved in 60 µl of TE buffer, pH 8.0. DNA concentration was determined spectrophotometrically (SmartSpec™ Plus spectrophotometer, BioRad, Hercules, California, USA), the final volume of DNA solution having been set to 60 µl. The integrity of DNA was documented electrophoretically by visualisation on agarose gel (1.0%) and also documented by means of a digital camera Canon Power Shot S30 (Canon, Tokyo, Japan).

Monitoring of DNA degradation. PCR in qualitative setting was used to monitor DNA degradation. It was performed in 25 µl volumes using GeneAmp® PCR System 7900 (Applied Biosystems, Foster City, USA) and BioRad iCycler (Thermal Cycler, Sergate, Italy). The protocols for PCR are summarised in Table 1. The reaction mixture consisted of 1× concentrated PCR buffer (Qiagen, Hieden, Germany); 2.5 mmol/l $MgCl_2$; 200 µmol/l dNTP (Invitrogen, Carlsbad, USA); 0.3 µmol/l primers as presented (Table 2); 1 U HotStar Taq polymerase (Qiagen); and 2.5 µl of DNA. The sequences in GeneBank (National Center for Biotechnology Information, Bethesda, USA) for maize HMG (high mobility group), *cry* (Cry protein), and invertase gene, for bean phaseoline gene and for pea and soybean lectine genes were used for primers design, which was performed by the program Primer 3 (Whitehead Institute Nine Cambridge Center, Cambridge, USA). Amplicons were analysed by electrophoresis in 1.5% agarose gel.

RESULTS AND DISCUSSION

In order to determine the degree of DNA degradation of the technologically treated plant-derived food samples, the PCR method for DNA amplification was used. The content and quality of the extracted DNA depend on the degree of technological processing and extraction method used (Grison *et al*. 2002; Trifa *et al.* 2004; Hird *et al*. 2006; Bergerová *et al*. 2010).

In our experiments, the food samples were exposed to different processing conditions such as temperature, pressure, pH, and were collected at different time intervals. Than the samples were homogenised and DNA was extracted by three different methods: CTAB (precipitation DNA using by CTAB solution), GeneSpin (binding DNA to silica membrane), and Wizard (binding DNA to magnetic beads). In addition to that, the DNAeasy Plant Mini Kit was used for DNA extraction from leaves and used as a positive control. The validated methods for soybean DNA extraction of the Community reference laboratory for GM food and feed, Biotechnology and GMO unit of the Join Research Centre, Ispra, Italy, were CTAB (ISO 21571:2005) or CTAB precipitation/WIZARD extraction methods. Generally, the extraction method for the plant derived materials was CTAB method (ISO 21571:2005). The DNA of both unprocessed and processed food samples were extracted with different efficiency depending on the processing conditions and the extraction method used. A modified CTAB procedure al-



1 – standard 100 bp; 2 – pH 2.25, 10 min; 3 – pH 2.25, 20 min; 4 – pH 2.25, 30 min; 5 – raw sample; 6 – pH 4.25, 10 min; 7 – pH 4.25, 20 min; 8 – pH 4.25, 30 min

Figure 1. Amplification of the 224-bp amplicon of the maize MON 810 invertase gene after sterilisation at 100°C

Table 2. Primers used

Primer	Sequence (5'→3')	Size of PCR products (bp)	Organisms/Source
HMG-F HMG-R	ttggactagaaatctcgtgctga gctacatagggagccttgtcct	79	maize MON 810/Aguilera *et al.* (2008)
ADH2-F ADH2-R	ccagcctcatggccaaag ccttcttggcggcttatctg	70	maize NK 603/this paper
MON810-F MON810-R	tcgaaggacgaaggactctaacgt gccaccttccttttccactatctt	92	maize MON 810/Aguilera *et al.* (2008)
IVR1F-I IVR1R-B	tcctccactggctgcacctagcg ggagcccgtgtagagcatgacgatc	124	maize/Hrnčírová *et al.* (2008)
IVR1F-A IVR1R-B	ccgctgtatcacaagggctggtacc ggagcccgtgtagagcatgacgatc	224	maize/Hrnčírová *et al.* (2008)
MON810-F MON810-R	caaacatgcgaagcgactta ctgtttcgtgtttggcattg	311	maize/this paper
HMG-F HMG-R	atggaagaagggcaaggact aatccgcgtcttgttattcg	313	maize/this paper
IVR1F-E IVR1R-C	agtgggtcaagtcggacgccaacc cgtaggtgccgatcgcgtagtagtc	401	maize/Hrnčírová *et al.* (2008)
IVR1F-A IVR1R-C	ccgctgtatcacaagggctggtacc cgtaggtgccgatcgcgtagtagtc	696	maize/Hrnčírová *et al.* (2008)
IVR1F-A IVR1R-D	ccgctgtatcacaagggctggtacc aggatcggggcctctctgcttgaac	1339	maize/Hrnčírová *et al.* (2008)
LEC-F3 LEC-R1	ttgtcataaatgcacccaacagttacaacg catactctgcgctattgaaaactccgag	122	pea/Hrnčírová *et al.* (2008)
LEC-F2 LEC-R1	atggcttctcttcaaacccaaatgatctcg catactctgcgctattgaaaactccgag	417	pea/Hrnčírová *et al.* (2008)
LEC-F2 LEC-R2	atggcttctcttcaaacccaaatgatctcg gcatattctgctcctgtggtagctgag	748	pea/Hrnčírová *et al.* (2008)
LEC-F3 LEC-R3	ttgtcataaatgcacccaacagttacaacg ccaaaatgttgagaggtgcacatgaacc	1129	pea/Hrnčírová *et al.* (2008
FAZ-F2 FAZ-R2	cagtagacctgaagagcgttcttcc cggagagcttggaagcaaaagacc	116	bean/Hrnčírová *et al.* (2008)
FAZ-F1 FAZ-R1	cctcttccttgtgcttctcaccc tgatggagttcacgtcgatgcc	469	bean/Hrnčírová *et al.* (2008)
FAZ-F1 FAZ-R2	cctcttccttgtgcttctcaccc cggagagcttggaagcaaaagacc	724	bean/Hrnčírová *et al.* (2008)
FAZ-F3 FAZ-R3	gacgacgatgggcaaaagttgcatg cattgtgtactaataacgcgtgtaaactc	1339	bean/this paper
Lec-F Lec-R	ccagcttcgccgcttccttc gaaggcaagcccatctgcaagcc	78	soybean/European Commission (2009)
SLe-1F SLe-2R	tgggacaaagaaaccggtag gtcaaactcaacagcgacga	201	soybean/European Commission (2005)
SLe-1F SLe-3R	tgggacaaagaaaccggtag aaatcgaccacatcggagag	410	soybean/European Commission (2005)

Table 3. The efficiency of methods used for DNA extraction of maize boiled at 100°C

Time of treatment (min)	GENESPIN		CTAB		WIZARD	
	[*c*] DNA (ng/µl)	A_{260}/A_{280}	[*c*] DNA (ng/µl)	A_{260}/A_{280}	[*c*] DNA (ng/µl)	A_{260}/A_{280}
0	1328.31	1.91	254.55	2.01	165.23	1.34
10	1581.12	2.08	187.15	2.02	88.55	1.56
30	532.73	2.11	265.36	1.95	64.36	1.15
45	730.27	2.15	187.29	2.01	20.39	1.38
60	671.36	2.11	189.97	2.01	112.34	1.28
120	285.71	1.73	298.85	1.95	652.44	1.18
180	1940.25	2.02	258.12	2.01	71.67	1.21
225	–	–	287.61	1.94	172.51	1.24

[*c*] – concentration of DNA

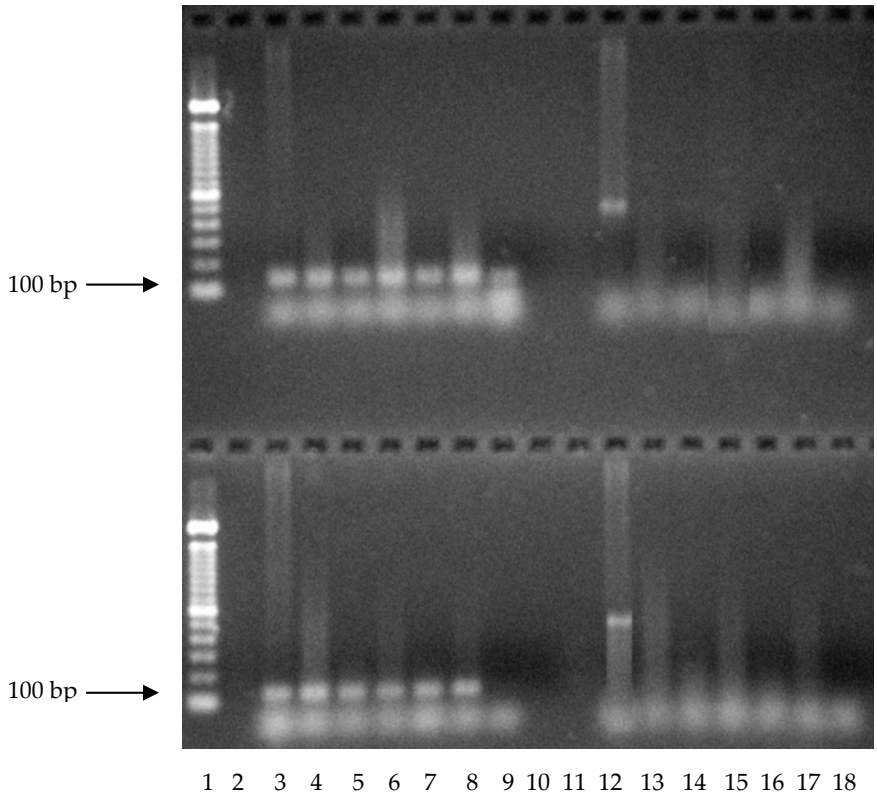
lowed us to obtain sufficient quality and yield of nucleic acids for PCR reactions (Tables 4 and 5). The CTAB method was equally effective for DNA extraction from both raw and processed materials (concentration 71–229 µg/ml, A_{260}/A_{280}: 1.7–2.00, Tables 3 and 4). When GeneSpin kit was used, the DNA concentration was high but purity was not optimal. The use of the commercially available Wizard kit produced DNA of lower quality and quantity (Table 3).

The degree of DNA degradation was demonstrated electrophoretically by visualisation on agarose gel (1.0%) after PCR (Figures 1–3).

The food processing triggers off DNA degradation and may affect DNA-based food analysis (Bauer *et al*. 2003, 2004; Tilley 2004; Yioshimura *et al*.

Table 4. Quality of extracted pea DNA and MON 810 DNA after autoclaving and sterilization using CTAB

Sample treatment		[*c*] DNA (µg/ml)	A_{260}/A_{280}	Sample treatment time (min)	[*c*] DNA (µg/ml)	A_{260}/A_{280}
pH	time (min)					
Pea at 120°C				**Pea at 100°C**		
2.25	2	71.61	1.93	10	90.63	1.87
	5	73.89	1.93	20	150.83	1.92
	10	72.76	1.97	30	90.38	1.83
4.25	2	165.43	1.75	10	225.45	1.86
	5	158.34	1.86	20	221.17	1.77
	10	170.2	1.88	30	97.96	1.74
7.6	2	177.94	1.87	10	208.97	1.98
	5	98.72	1.88	20	164.83	1.84
	10	211.80	2.08	30	182.57	1.80
MON 810 at 120°C				**MON 810 at 100°C**		
2.25	2	87.83	1.89	10	103.87	1.91
	5	110.21	1.94	20	79.37	1.7
	10	74.07	1.97	30	92.02	1.99
4.25	2	99.71	1.76	10	85.82	1.86
	5	70.23	1.87	20	79.48	2.00
	10	59.46	2.10	30	137.03	1.81
7.6	2	192.41	1.97	10	147.34	1.78
	5	10072	1.87	20	94.64	1.79
	10	229.80	2.01	30	164.82	1.82



Upper: 1 – standard 100 bp; 2 – negative control (116 bp); 3 – raw sample; 4 – pH 7.6, 2 min; 5 – pH 2.25, 2 min; 6 – pH 7.6, 5 min; 7 – pH 2.25, 5 min; 8 – pH 7.6, 10 min; 9 – pH 2.25, 10 min; 10–11 – negative control (469 bp); 12 – raw sample; 13 – pH 7.6, 2 min; 14 – pH 2.25, 2 min; 15 – pH 7.6, 5 min; 16 – pH 2.25, 5 min; 17 – pH 7.6, 10 min; 18 – pH 2.25, 10 min
Lower: 1 – standard 100 bp; 2 – negative control (116 bp); 3 – raw sample; 4 – pH 7.6, 10 min; 5 – pH 2.25, 10 min; 6 – pH 7.6, 20 min; 7 – pH 2.25, 20 min; 8 – pH 7.6, 30 min; 9 – sample of preserved (Commercial Products); 10–11 – negative control (469 bp); 12 – raw sample; 13 – pH 7.6, 10 min; 14 – pH 2.25, 10 min; 15 – pH 7.6, 20 min; 16 – pH 2.25, 20 min; 17 – pH 7.6, 30 min; 18 – pH 2.25. 30 min

Figure 2. DNA amplification of bean genes after autoclaving at 120°C (upper gel) and sterilisation at 100°C (lower gel) – Amplicons: 116 bp phaseoline gene (left): line 3–10 and 469 bp phaseoline gene (right): line 12–18

2004; Moreano *et al*. 2005; Gryson *et al*. 2008; ISO 2005) of GM and non-GM plant samples. PCR analysis of the sterilised (100°C) and autoclaved (120°C) samples revealed the reduction of the extracted DNA size in the time dependent manner under different pH and pressure conditions which is in good agreement with the previous findings (Kollárovič *et al*. 2005; Moreano *et al*. 2005; Hrnčírová *et al*. 2008). PCR primers for different sizes of amplicons were applied for the monitoring of DNA degradation. Amplicons above 400 bp of the autoclaved (120°C) and sterilised (100°C) samples were not amplifiable in either matrix (non-GM and GM) while DNA fragments of the lower size (around and bellow 300 bp) were amplifiable under all experimental conditions (Figures 1–3, Table 5). A clear influence of the temperature and pressure combination during autoclaving (120°C; 0.1 MPa) on DNA fragmentation was observed for the amplicon length around 300 bp. Larger maize gene amplicons as are those of 311 bp of the *cry* gene and 313 bp of the high mobility gene (HMG) were not amplifiable (Figure 3, Table 5). It was also evident that the degradation of DNA under highly acidic pH (Figure 3) in autoclaving at 120°C was so massive that no amplicons of all analysed genes of pea, bean, maize and MON 810 above 300 bp were present (Table 6).

The effect of different pH (2.25, 4.25, and 7.6 at 100°C or 120°C) was studied. The amplification of small amplicons of up to 313 bp occurred with all samples except those with pH lower then 4.2. A significant degradation of DNA was observed in the samples exposed to pH 2.25. Larger amplicons

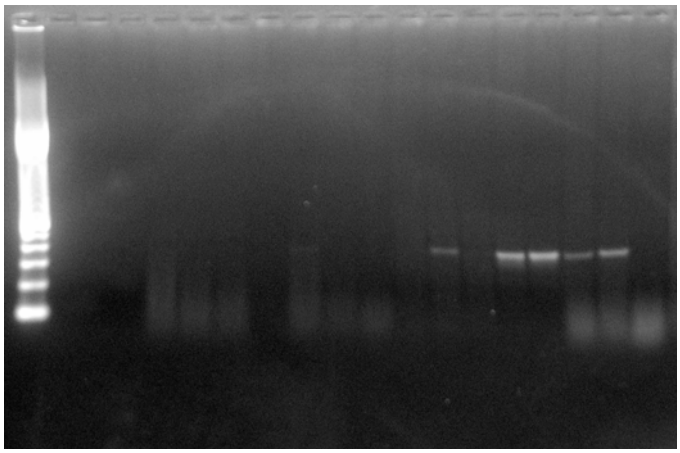


Figure 3. Amplification of the 311 bp amplicon of the MON 810 high mobility gene: sterilization 100°C – line 9–14 and autoclaving 120°C – line 2–7

1 – standard 100 bp; 2 – pH 2.25, 2 min; 3 – pH 2.25, 5 min; 4 – pH 2.25, 10 min; 5 – pH 4.25, 10 min; 6 – pH 4.25, 20 min; 7 – pH 4.25, 30 min; 8–9 – pH 2.25, 10 min; 10 – pH 2.25, 20 min; 11 – pH 2.25, 30 min;. 12 – pH 4.25, 20 min; 13 – pH 4.25, 10 min; 14 – pH 2.25, 30 min; 15 – raw sample; 16. bread (mix); 17 – bread (top); 18 – bread (bottom); 19 – negative control (311 bp); 16–18 – control: raw sample, only heat treated bread of maize

(400–1339 bp) from all plant food matrices tested were not amplifiable at all (Figure 2). The most prominent influence on the DNA fragmentation was revealed by the combination of pressure and temperature that was shown by PCR amplification. Fragments of 70–300 bp were amplified under all conditions (Figures 1–3) at both 100°C and 120°C. Similar results were obtained for amplicons of pea and bean genes.

In order to detect the border line of the DNA degradation of the most processed food matrices, we designed a new set of primers for the amplicons size over 300 bp for the maize MON 810 *cry* gene (311 bp) and HMG gene (313 bp). The amplicons of the samples exposed to sterilisation at 100°C were amplifiable at pH 7.6 and pH 4.25 when the time of sterilisation was short (Table 5). A similar result was obtained for cry transgene of maize MON 810. No amplification of the maize

Table 5. Limits for the DNA amplification after processing of used plant food matrices

Conditions	pH	Time (min)	Amplicons (bp)						
			< 100	< 200	< 300	> 300	> 400	> 700	> 1300
Sterilisation at 100°C	2.25	10	+	+	+	–	–	–	–
		20	+	+	+	–	–	–	–
		30	+	+	+	–	–	–	–
	4.25	10	+	+	+	+	–	–	–
		20	+	+	+	–	–	–	–
		30	+	+	+	–	–	–	–
	7.60	10	+	+	+	+	–	–	–
		20	+	+	+	+	–	–	–
		30	+	+	+	+	–	–	–
Autoclaving at 120°C 0.1 MPa	2.25	2	+	+	+	–	–	–	–
		5	+	+	+	–	–	–	–
		10	+	+	+	–	–	–	–
	4.25	2	+	+	+	–	–	–	–
		5	+	+	+	–	–	–	–
		10	+	+	+	–	–	–	–
	7.60	2	+	+	+	–	–	–	–
		5	+	+	+	–	–	–	–
		10	+	+	+	–	–	–	–

+ = DNA amplification yes; – = DNA amplification no

MON 810 *cry* gene and HMG gene was observed at 120°C; 0.1 MPa.

It was shown that all genes used in PCR analysis in all the matrices examined of either experimental or commercial food samples displayed similar characteristics as regards amplification. We therefore believe that the DNA degradation in highly processed matrices, both non-genetically modified and genetically modified, is so massive that amplicons shorter than 300 bp should be used for PCR analysis as documented in Table 5.

CONCLUSION

The results of these experiments confirm that DNA is highly resistant to various physical processes. Maximum size of PCR products, that were achievable, reflected the level of DNA degradation due to processing (Hrnčírová *et al.* 2008; Bergerová *et al*. 2010). DNA over 300 bp was amplifiable when processing at 100°C was performed at neutral pH. The autoclaving (120°C; 0.1 MPa) significantly reduced the size of DNA (in the time dependant manner) of the samples and that was intensified by acidic pH. The maximum amplicons length for highly processed matrices was around 300 bp (Table 5).

The amplicon size, degrese, and duration of technological treatment may negatively influence the detection of food components. The temperature, pressure, and pH influence the degradation of DNA in processed foods. The extent of DNA degradation in a specific food product can be predicted if the processing parameters are available.

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