Effect of High Temperature and Pressure on Quantification of MON 810 Maize

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Abstract

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Maize MON 810 (*Zea mays* L.) is the only transgenic cultivar grown in the European Union countries and food products with its content higher than 0.9% must be labelled. Processing such as high temperature (121°C), elevated pressure (0.1 MPa), and low pH 2.25 fragmented DNA. A two order difference in the species specific gene content compared to the transgenic DNA content in plant materials used has led to false negative results in the quantification of transgenic DNA. The maize containing 4.2% of the transgene after processing appeared to be as low as 3.0% (100°C) and 1.9% (121°C, 0.1 MPa). The 2.1% amount of the transgene dropped at 100°C to 1.0% and at 121°C, 0.1 MPa to 0.6%. Determination of GMO (Genetically Modified Organism) content in processed foods may lead to incorrect statement and labelling could mislead consumers in these cases.

Keywords*:* DNA degradation; PCR; highly processed foods

Food inspection bodies in the EU countries focus on food quality and safety. Genetically modified foods are of specific interest due to a long-term controversy accompanying their cultivation and consumption (Klintman 2002; Frewer *et al*. 2004). Maize and soybean represent the majority of genetically modified food crops cultivated until now (James 2010). Maize MON 810 (*Zea mays* L.) is the only transgenic cultivar grown in the EU countries. Determination of the precise content of a transgenic constituent in food is an obligatory requirement of the EU legislative (Regulation EU 1829/2003; Regulation EU 1830/2003). The European legislation requires labelling of the GMO (Genetically Modified Organism) content in foods; however, the analytical methods were developed for raw plant materials only (Mazzara *et al.* 2011). Food processing, and DNA degradation associated with it, may affect the quality of analytical results. It was found that the degree of technological treatment of foods affects the quantity assessment of the transgenic ingredient. Then, the declared GM content of the processed food may be under- or overestimated (Berdal & Holst-Jensen 2001), which may mislead consumers. Labelling of the GMO content is obligatory except the amount lower than 0.9% of the accidentally and technically unavoidable admixture.

The biological methods for detection of food components are generally protein- or DNA-based. The method widely used for DNA detection and quantification is PCR (Michelini *et al*. 2008). The qualitative and quantitative methods, primarily DNA-based polymerase chain reaction (PCR), were developed and validated by JRC of EU for raw plant materials, not for processed foods (http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm).

The efficacy of PCR strongly depends on the DNA stability during food processing and on the efficiency of DNA recovery from food samples (Meyer *et al*. 1996; Straub *et al*. 1999; Gryson *et al*. 2008; Bergerová *et al*. 2010, 2011). The quantification of food components can be nega-

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tively influenced by processing (Gryson *et al*. 2008; Hrnčírová *et al.* 2008; Bergerová *et al.* 2010, 2011).

Moderate food processing has shown no significant effect on relative quantification of transgenic content (Debode *et al*. 2007; Bergerová *et al*. 2010). A successful quantification of extremely processed meat products by the real-time PCR was achieved when amplicons up to 351 base pairs were used (Hird *et al*. 2006). Several authors found out that food processing associated DNA degradation may affect the quality of PCR analytical results (Meyer *et al*. 1996; Straub *et al*. 1999; Gryson *et al*. 2008). Significant differences between the raw materials and the trial-produced processed foods were shown later (Yoshimura *et al*. 2005).

Thermal sterilisation is a basic procedure of food preservation. Temperature up to 121°C is used in the canning industry to inactivate toxicogenic microflora. Pickles, including maize in sweet and sour vinegar brine, are usually offered as side meals or as appetisers.

The aim of this study was to determine how technological processing such as high temperature, elevated pressure and low pH may affect DNA degradation and quantification in the transgene content of the plant matrix using PCR.

MATERIAL AND METHODS

Plant material. Raw and canned maize (*Zea mays* L.) grains were purchased from local markets. The modified samples of maize MON 810 were obtained from Agrokomplex Kunovice, Czech Republic.

Processing methods. Maize seeds were pickled in three different brines. The control brine was salty (pH 7.6; 20 g table salt/1 l of drinking water). The 1st experimental brine was sweet and sour (pH 2.25; 20 g table salt, 100 g table sugar, 250 ml 8% vinegar and 1 l of drinking water). The 2nd sweet and sour brine (pH 4.25) was the same as the first one with the exception that only 3 ml of vinegar were used. Samples of maize seeds were processed by two different sterilisation procedures – the first one (100°C; 10, 20, and 30 min) in a water bath, and the second one (121°C; 2, 5, and 10 min; 0.1 MPa) – autoclaving. The samples were left in these brines for 3 weeks and afterwards DNA was extracted.

DNA extraction. Sterilised samples were homogenised by the AY47R1 mixer (Moulinex, Barcelona, Spain). Each of the flour fractions was extracted in triplicate by the cetyl trimethyl ammonium bromide (CTAB) method (Trifa & Zhang 2004), as this method appeared to be the most suitable in our experiments due to a high yield of good quality DNA and was used in all extraction procedures. DNA concentration was determined spectrophotometrically (SmartSpec Plus spectrophotometer; BioRad, Hercules, USA), the final volume of DNA solution was set to 60 µl.

Monitoring of DNA degradation. PCR in qualitative setting was used to monitor DNA degradation. It was performed in 25 ml volumes using the Thermal Cycler (BioRad iCycler; Bio-Rad Laboratories, Sergate, Italy). The protocols for PCR involved 40 cycles of initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 60°C for 30 s, polymerisation at 72°C for 1 min and final extension at 72°C for 10 minutes. The sequences in the GeneBank (National Center for Biotechnology Information, Bethesda, USA) for maize *hmg*, *cry1Ab,* and *inv* genes (Table 1) were used for primer design, which was performed by the Primer 3 program (Whitehead Institute Nine Cambridge Center, Cambridge, USA). Negative control samples consisted of the master mix only with no DNA added.

Quantitative analysis of transgene DNA and plant specific DNA. The cyclers GeneAmp PCR System 7900 (Applied Biosystems, Foster City, USA) and the BioRad iCycler for quantitative analysis of transgene DNA of MON 810 were used. The reaction mixture consisted of 1× concentrated PCR buffer (Qiagen, Hilden, Germany); 2.5 mmol/l $MgCl_2$; 200 µmol/l dNTP (Invitrogen, Carlsbad, USA); 0.3 µmol/l primers (Table 1); 10 mmol/l probe (Table 1); 1 U Hot Star Taq polymerase (Qiagen); and 2.5 µl of DNA. The real-time PCR was performed in 96-well reaction plates covered by optical caps or optical films (Applied Biosystems, Foster City, USA). The protocols for PCR involved 45 cycles of initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 s and annealing at 60°C for 30 seconds.

The amplification of maize high mobility group gene (79 bp) and *cry1Ab* gene (92 bp) was used for transgenic DNA quantification (Table 1). The standard curve calculated from the reactions had a correlation coefficient of 0.98–1.0 corresponding to PCR efficiency of 99.1–99.8% in both cases. The reference materials were used to construct a calibration curve. All results were statistically evaluated using the Microsoft Office Excel 2007 Program.

Table 1. Primers and probes used

Primer	Sequence (5'→3')	Size of PCR products (bp)	Organism/source
HMG-F	ttggactagaaatctcgtgctga	79	maize
HMG-R	gctacatagggagccttgtcct		Aguilera *et al.* 2008
HMG-P	caatccacacaaacgcacgcgta		
MON 810-F	tcgaaggacgaaggactctaacgt	92	maize, MON 810
MON 810-R	gccaccttccttttccactatctt		Aguilera *et al.* 2008
MON 810-P	aacatcctttgccattgcccagc		
IVR1F-A	accaccgtccaaactgaatc	78	maize
IVR1F-C	cacacctgtacacgtccctg		this paper
IVR1F-P	attgttcaagcagagaggcc		

HMG – primers for high mobility gene (reference gene); MON 810 – primers for the maize MON 810 (transgene); IVR – primers for invertase gene (reference gene); F – forward primer; R – reverse primer; P – probe; bp – basic pair

RESULTS AND DISCUSSION

The influence of technological processes on the measurement of transgenic DNA content in genetically modified food samples was studied by the real-time PCR. This study proved that harsh food processing affects the quantification of transgenic content of maize products. Maize was processed under various technological conditions (temperature, pressure, pH) and collected at different time intervals.

Quantitative analyses of experimentally processed foods containing maize MON 810 at two percentage levels of the transgenic content (4.2 and 2.1%) were studied in this publication (Table 2). The results showed that DNA degradation is affected considerably by the type of processing used. The type of plant matrix had a smaller impact (Table 2). After processing (100°C) of the maize samples containing 4.2 and 2.1% of MON 810 the transgenic content was 3.13 and 1.13%, respectively (Table 2). The most pronounced effect on DNA integrity was exerted by the combination of pressure, pH, and temperature (121°C and 0.1 MPa). Transgenic content after such processing showed a lower percentage (0.7 and 2.26%) than was the initial one (2.1 and 4.2%) at the beginning of the experiment (Table 2). We assume that the determination of the lower content in our processed samples was on the account of a two order difference in the species specific gene content compared to the transgenic DNA content in plant materials used. Under such experimental make up the DNA degradation of transgenic content showed a 2 or 3 times higher decrease as a consequence of the unequal gene presence when the transgene is present in one copy per cell, while housekeeping gene is present in two copies. Moreover, this inequality is accentuated by the above-mentioned two order differences in the percentage content of the compared genes when the species specific genes are present as 100% and the content of transgenic genes is 2.1% or 4.2%. Such disparity is expressed as a considerable decrease in the transgenic content while the decrease in species specific gene content remains unnoticed. In such a case labelling of the GMO content in foods may be underestimated and incorrect as such.

When moderate processing conditions were used, no effect on DNA quantification was observed in our previous experiments. We recommended the application of amplicons shorter than 300 bp if amplification was done in highly processed food (Bergerová *et al.* 2011). Contrary to our earlier findings the discrepancy in the quantification of MON 810 transgenic content in maize pickled in vinegar brine and preserved by sterilisation is shown in this paper.

Statistically significant differences were found in the quantification of transgenic content between raw and trial-produced processed foods, when maize starch, maize meal, maize puffs, maize chips, tofu, soybean milk, and boiled beans, containing the genetically modified maize MON 810 and Roundup Ready soybean were assessed (Yoshimura *et al.* 2005). Other results revealed that the physical degradation of DNA demonstrated no effect on detection (Hurst *et al.* 1999) and on relative quantification of the transgenic content (Debode *et al.* 2007). Within this context more experiments that would show that neither food composition nor processing influenced

Table 2. Effect of technological treatment on the quantification DNA in MON 810 – 2.1% and 4.2% of transgenic content

Sample/conditions		% GM	C_T		ΔC_T	SD		ΔSD
			transgen	*hmg*		transgen	*hmg*	
2.1% of transgenic content								
Raw sample of MON810		2.1 ± 0.2	30.98	23.70	7.28	0.098	0.311	0.224
MON 810 (100°C) sterilisation	pH 2.25 10 min	1.2 ± 0.2	32.85	23.56	9.29	0.107	0.189	0.091
	pH 2.25 20 min	1.2 ± 0.1	32.95	23.89	9.06	0.318	0.141	0.152
	pH 2.25 30 min	1.0 ± 0.3	33.10	24.02	9.08	0.123	0.041	0.086
	pH 4.25 10 min	1.3 ± 0.3	31.55	23.41	8.14	0.021	0.234	0.203
	pH 4.25 20 min	1.3 ± 0.2	31.72	23.76	7.96	0.156	0.321	0.147
	pH 4.25 30 min	1.2 ± 0.2	32.41	24.15	8.26	0.387	0.225	0.153
	pH 7.6 10 min	2.0 ± 0.1	30.80	23.87	6.93	0.154	0.071	0.081
	pH 7.6 20 min	1.9 ± 0.2	30.96	24.01	6.95	0.102	0.089	0.019
	pH 7.6 30 min	1.7 ± 0.1	31.32	24.62	6.70	0.099	0.305	0.201
Raw sample of MON 810		2.1 ± 0.3	31.00	23.68	7.32	0.145	0.297	0.149
MON 810 (121°C) 0.1MPa autoclaving	pH 2.25 2 min	0.8 ± 0.4	33.17	24.10	9.07	0.286	0.183	0.092
	pH 2.25 5 min	0.7 ± 0.2	33.87	24.79	9.08	0.321	0.144	0.168
	pH 2.25 10 min	0.6 ± 0.1	34.12	25.25	8.87	0.204	0.142	0.053
	pH 4.25 2 min	1.3 ± 0.3	31.98	23.50	8.48	0.081	0.035	0.044
	pH 4.25 5 min	1.3 ± 0.1	32.04	23.68	8.36	0.212	0.367	0.147
	pH 4.25 10 min	1.2 ± 0.2	32.49	24.19	8.30	0.098	0.076	0.022
	pH 7.6 2 min	1.8 ± 0.2	31.43	24.48	6.95	0.133	0.058	0.077
	pH 7.6 5 min	1.8 ± 0.1	31.52	24.70	6.82	0.111	0.159	0.052
	pH 7.6 10 min	1.6 ± 0.2	32.10	24.91	7.19	0.134	0.171	0.029
4.2% of transgenic content								
Raw sample of MON 810		4.2 ± 0.2	27.00	21.70	5.30	0.121	0.456	0.448
MON 810 (100°C) sterilization	pH 2.25 10 min	3.2 ± 0.3	28.85	22.85	6	0.081	0.167	0.102
	pH 2.25 20 min	3.2 ± 0.2	28.91	22.90	6.01	0.334	0.165	0.151
	pH 2.25 30 min	3.0 ± 0.3	29.10	23.10	5.9	0.189	0.054	0.112
	pH 4.25 10 min	3.8 ± 0.3	28.32	21.25	7.07	0.045	0.267	0.197
	pH 4.25 20 min	3.5 ± 0.2	28.39	21.53	6.86	0.182	0.453	0.371
	pH 4.25 30 min	3.3 ± 0.2	28.05	21.59	6.91	0. 415	0.245	0.171
	pH 7.6 10 min	3.9 ± 0.3	28.57	21.22	6.35	0.188	0.076	0.115
	pH 7.6 20 min	3.9 ± 0.2	27.89	21.06	6.83	0.045	0.076	0.029
	pH 7.6 30 min	3.8 ± 0.1	27.91	21.12	6.79	0.121	0.345	0.209
Raw sample of MON 810		4.2 ± 0.4	27.02	21.55	5.47	0.112	0.312	0.198
MON 810 (121°C) 0.1MPa autoclaving	pH 2.25 2 min	2.5 ± 0.4	30.14	22.1	8.04	0.211	0. 289	0.056
	pH 2.25 5 min	2.4 ± 0.2	31.45	22.78	8.67	0.456	0.179	0.248
	pH 2.25 10 min	1.9 ± 0.3	34.96	23	11.96	0.215	0.098	0.126
	pH 4.25 2 min	3.0 ± 0.3	28.29	21.97	6.32	0.187	0.225	0.033
	pH 4.25 5 min	2.9 ± 0.1	28.95	23.17	5.78	0.276	0.464	0.187
	pH 4.25 10 min	2.8 ± 0.2	29	23.48	5.52	0.107	0.056	0.051
	pH 7.6 2 min	3.7 ± 0.2	28.2	22.3	5.9	0.234	0.076	0.142
	pH 7.6 5 min	3.6 ± 0.1	29.01	22.64	6.37	0.088	0. 105	0.023
	pH 7.6 10 min	3.6 ± 0.3	29.10	22.85	6.25	0.121	0.167	0.047

% GM – percentage of genetic modification; C_T – value of treshold cycle by PCR ; ΔC_T – value of delta the treshold cycle by PCR; SD – value of standard deviation; ΔSD – value of delta the standard deviation; *hmg* – high mobility gene (reference gene)

the trueness of relative quantification of genetically modified foods were demanded (Engel *et al.* 2006). In our previous experiments we also showed that moderate food processing conditions resulted in no differences of the GMO content when compared to the untreated control (Hrnčírová *et al.* 2008; Bergerová *et al.* 2010).

Food processing degrades DNA and may affect the DNA-based food analyses (Meyer *et al*. 1996; Straub *et al*. 1999; Gryson *et al*. 2008; Bergerová *et al*. 2010). PCR analysis of the sterilised (100 and 121°C) samples revealed a reduction of the extracted DNA size in a time dependent manner and different conditions such as pH and increased pressure (Kollárovič *et al.* 2005; Moreano *et al.* 2005; Hrnčírová *et al.* 2008). A similar effect of baking on the DNA integrity was also described previously (Kollárovič et al. 2005; Moreano *et al*. 2005; Gryson *et al.* 2008; Hrnčírová *et al.* 2008).

Small or no differences in amplification rates of boiled or baked meat samples compared to raw samples for amplicons ranging from 81 bp up to 240 bp were proved (Hird *et al*. 2006). However, the more harsh treatment, such as canning, significantly increased C_T (Cycle Threshold) values for all amplicons, where the highest values were for the larger ones (Hird *et al.* 2006). Similarly, we observed increased C_T values for transgenic content after processing. These values increased with the harsh conditions expressed as ΔC_T (delta Cycle Threshold). This was not the case of the two plant specific genes used as a control to each other.

CONCLUSION

The effect of technological processing parameters on the quantification of GM content in foods was monitored by the real time-PCR. We concluded that heat processing *per se* has no practical consequences for the quantification of transgene content in foods. In a model setting where high temperature processing was combined with higher pressure and low pH a pronounced effect on the integrity of plant DNA was demonstrated and thus the quantification of transgenic content in foods was influenced. The two- and three-fold drop of the transgenic content was shown. We assume that the lower transgenic content in processed samples compared to that before processing was observed due to uneven gene copy numbers of the involved genes. The two orders of magnitude of the higher content of species specific genes compared to the transgenes combined with the harshness of processing were involved. As a consequence of this disparity the lower copy number gene degradation was seen as a drop of the GM content. The other reason for the discrepancies in GMO content is a difference in the size of the control and transgenic amplicon. It appears that the transgene is more affected and less stable after technological treatment than the reference gene. So in practical/routine analysis, the decrease in GMO content of processed food might be shown, as we observed.

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